AN EXAMINATION OF THE MICROBIOME OF BROILER CHICKENS IN VARYING STATES OF STRESS AND NECROTIC ENTERITIS CHALLENGE

By

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Abstract

The gut influences and is in turn shaped by its microbial communities, with the host's environment and its history molding those populations in ways unique to the specific location and stimulus received. For domestic chickens these influential factors can vary with the minutest changes ranging from what the bird has been fed to how its grandparents were raised or even how its egg was stored and handled before hatching.

To further explore these influences three trials were conducted to collect gut samples at various days post hatch and observe changes in composition of the microbial communities therein. The experiments consisted of three separate trials:

An exploratory study establishing the influence of different flocks of origin by placing birds from 2 separate hatcheries (hatchery A, hatchery B) either in homogenous or mixed groups on floor pens or in battery cages. Midgut (defined as the area between the duodenal loop and Meckel's diverticulum) and ceca samples were collected via necropsy on day 0, 5, and 14 for battery cage birds, and day 0,5,14, 16, 21, 28, and 48 for floor raised birds.

A trial observing the influence of environmental stressors by placing birds in normal and less than optimal lighting and/or temperatures during rearing in floor pens or battery cages. Samples of the crop, midgut and the ceca were taken via necropsy at day 0,5,14, 28 and 36.

A final study was conducted to record differences in gut communities during times of disruptive stressors and sickness in challenged vs unchallenged birds in normal/low lighting in floor pen/battery cages. Challenge was achieved by dosing challenge birds with 1 ml coccidiosis vaccine at 10x the recommended dose then inoculating with 10^7 CFU/ml *C. perfringens* at days 18, 19, and 20. Samples of the crop, midgut, and the ceca were taken at day 0 and 21.

All samples were extracted of DNA and Illumina sequenced. Raw sequence reads were run through the bioinformatic pipeline program QIIME, and the resulting data was used to generate alpha and beta analyses as well as general classification.

Overall birds from different origins were characterized both by distinctly different microbiomes when raised homogenously, and by exerting influence over each other when raised together. Birds raised together showed characteristics of the group possessing more diverse day 0

microbiome, with midgut more closely resembling hatchery B and ceca resembling hatchery A . Possible influence of parental microbiome was observed, with hatchery A being known to have fed all vegetarian diets to breeders. Chicks from that location were slower to adopt *Bacteroides* a classification of bacterium that are known to be prevalent in diets high in animal protein and fat, when fed a diet containing animal protein compared to hatchery B chicks.

Birds raised in less than optimal temperature and lighting conditions were not significantly different in diversity, however midgut samples from birds in high temperature/low light and low temperature/low light pens had a higher proportional prevalence of *Lactobacillus* when compared to high temperature/high light and low temperature/high light pens, with low temperature/high lighting level pens having the lowest *Lactobacillus* prevalence.

Due to sample contamination it is unclear to what extent bacterial challenge influences the populations in low light vs normal lighting reared birds. However the continued trend of higher proportional *Lactobacillus* prevalence in low light pens was observed.

Based on observations, mixing day old chicks from a flock with known superior microbial communities with chicks from an flock with weak or non-beneficial microbial communities may have a net positive effect on the new flock overall. Lighting seems to have some influence on microbes generally considered to be beneficial, with *Lactobacillus* being consistently more prevalent in low light situations.

The effects of mixed flock brooding has many implications to the ability to somewhat correct for poor quality chicks or chicks from low production flocks. Further exploration as to whether this effect is only observed between distinct hatchery locations or is observable between individual flocks is recommended. Investigating a potential link between lighting and *Lactobacillus* presence is highly recommended, as little research was found as to the effect of light on *Lactobacillus* in poultry or even in general.

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Please note that the formatting for this thesis is done in the style of *Animal Microbiome.*

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Chapter 1 -Review of Literature

Introduction

The Veterinary Feed Directive banned usage of all subtherapeutic antibiotic drugs [1] for production animals in the United States in October of 2015. Many of these products were used prophylactically to inhibit gastrointestinal diseases[2, 3]. Subsequently interest in the gut and its impact on the life cycle of broilers has increased due to the potential for alternatives to the former prophylactic practices[4]. The general mechanisms of the triploblastic gut have become increasingly better studied, however the amount of knowledge on the individual idiosyncrasies of distinct species' gut functions remains small with many newer studies claiming to examine aspects of an organism's microbiome for the first time on record [5, 6, 7, 8,9]. With molecular biology techniques becoming more and more the norm, closer inspection of these mechanisms and communities allows for higher quality quantitative and qualitative insights[6,7].

Overall, the hierarchy of available knowledge and research on the microbiome and its functions for a specific species is dependent on its importance (economic or otherwise) to humans [10, 11, 12, 13]. This is best represented by a hypothetical negative exponential slope. Not surprisingly at the top of the slope, humans and human model species, along with the associated diseases and maladies, are the most well defined by microbiome research; these models are important to humans as a whole, and therefore they amass the most interest and funding [11, 13]. Zebra fish, mice and rats, and drosophila are common human models and are used for their quick generation turnover that allows for rapid data collection and study, resulting in a deep and well-defined pool of data to reference [14, 15, 16, 17, 18, 19].

The next step down is filled by economically important species of both plant and animal. Widely produced foods sources such as grains, livestock and commercial fish species, as well as the associated pests and diseases have enough economic gravity to attract research funding with relative ease [15, 20,21]. As molecular methods improve the depth of available data will increase as well, allowing for more precise and targeted approaches to industry problems.

As the hypothetical slope increases however, the data on a particular species decreases and further removed from humans in relative importance a species is the less is known. Domestic

animals are next, followed by vertebrates in general with the amount available information rapidly decreasing. Following this trend, other than the aforementioned, economically important organisms, many invertebrates are virtually unknown, putting researchers looking to study them at a disadvantage [5, 6, 7, 22, 23]. Exploratory studies serve to deepen already available knowledge and confirm trends unearthed by previous studies.

Review of Literature

Microbiome-General definition

A microbiome, or microbiota, is generally accepted to be a community of microorganisms within an environment with the microbial communities consisting of complex populations of bacterium, protists, archaea, fungi, and viruses [24, 25]. The environment inhabited can be as varied and diverse as the microbes that live within it. Microbial communities occur ubiquitously in the natural world exploiting any acceptable niche and can be observed in a plethora of environments, from the depths of the ocean to the gut of an organism slightly larger than the microbiota themselves [24, 25, 26]. Generally, a microbiome will form in close proximity to a nutrient source, the most common being the nutrient absorption, waste disposal, and reproductive systems maintained by the host organism; in many cases these communities have been selected through host pressures and environmental factors to be mutually beneficial, allowing the host the advantages of an additional layer of security from harmful outside factors such as invasion by disease causing microbes, anti-nutritional factors, or general instability of the aforementioned systems [24]. In many cases the makeup of a microbiome of various systems can highly influence host behaviors, immunity, growth, development and a multiplicity of health factors, making it one of the most vital systems of an organism has. Incidentally the microbiome is not of the host itself and its formation is completely dependent on the environment, sex, parentage, diet, and other circumstances outside of control of the host [27, 28, 29, 30, 31, 32]

The Gut -A General Overview

The gastrointestinal tract, or gut, in its most basic definition is a tube that begins at the mouth and ends at the anus/cloaca [33].

Nutrients are ingested entering through the mouth, becoming “ingesta” and often subjected to mechanical breakdown, normally with a form of dentition or with a specialized organ. Many species also secrete specialized enzymes during ingestion to speed up the breakdown process [34]. The ingesta is then passed on to a stomach for chemical breakdown by acid secretion. In some organisms bacterial fermentation is performed in a pseudo-stomach prior to being passed to the true stomach for chemical breakdown [35]. After exiting the stomach and being subject to a neutralizing treatment to remove any remaining acid the ingesta, now termed “digesta”, passes through the intestines for absorption of nutrients. While moving through the intestines digesta is subject to bacterial and enzymatic breakdown [36, 37]. Most nutrients are absorbed here and excess materials are coated in mucin, a secreted mucus, to help with breakdown and passage. With a majority of usable nutrients extracted the ingesta moves to the colon where in some species bacterial hind gut fermentation occurs to take advantage of the nondigestible nutrients. The digesta finally moves to the anus where it is ejected as feces [38].

A fully functioning healthy gut is vital to the growth and general wellbeing an organism, making it conceivably one of the most important of the organism’s systems, yet much less is known about the gut’s health and the microbiota it hosts than would be preferred [39, 40, 41].

The Gut’s Roles in Health

Simply put, an organism without a functioning gut is an organism that will most likely soon die. Outside of every other system, the gut is most important to producers due to its roll in preserving the life of an organism and ensuring proper growth [42, 43, 44, 45]; a healthy gut leads to a healthy animal and a healthy animal is a profitable one. Though generally the term health is meant to define absence of disease, besides the obvious implications of the impact of a complete overgrowth of foreign organisms inside the gut, the gastrointestinal tract and its microorganisms also impacts the overall health of its organism in multiple ways. Bischoff [46] defines gut health as “a state of physical and mental well-being in the absence of GI complaints that require the consultation of a doctor, in the absence of indications of or risks for bowel disease and in the absence of confirmed bowel disease.” Bischoff goes on to define five major

criteria for gut health as: 1) effective digestion and absorption of food, 2) absence of gastrointestinal illness, 3) normal and stable intestinal microbiome, 4) effective immune status, and 5) status of well-being. Though these factors are specifically meant to apply to human medicine, we can extend them to animals as well allowing the formation of a general guideline for gut health [47, 48, 49]

These criteria for a healthy gut and its ability to function can be further broken into three factors that influence the above: diet, mucosa, and microbiota composition [50].

Diet

Diet is known to have a substantial shaping effect on the microbiota composition in the gut, suppressing or promoting certain microbes and often heavily contributing to severity of disease or lack thereof. In pigs the addition of dried distillers grains (DDGS) to a diet is associated with a reduction in incidence and severity of certain bacterial challenges [51], however when fed to broiler chickens DDGS is associated with increased incidence of gastric disease, especially microbe driven Necrotic Enteritis [52]. Diet can also directly impact gut epithelium and the production of cytokines, which in turn affects immune response [53]. When comparing fecal microbiomes of European and West African children, De Filippo et al [54] reported that the west African children whose diets were rich in whole unprocessed grains were found to contain large populations of short-chain fatty acid (SCFA) producing microbes such as *Xylanibacter*, *Prevotella*, *Butyrivibrio*, and *Treponema* spp. along with a notable decrease in infectious gastrointestinal microbes present. It was inferred that the aforementioned microbes took advantage of available xylane, xylose, and carboxymethylcellulose to produce SCFAs, thus making the gut environment inhospitable to other transient organisms. These microbes were completely absent in the samples from European children, though both groups possessed *Bacteroides* and *Faecalibacterium* spp. which also produce some SCFAs. It was further noted that non-infectious colonic disease is rare in regions with high fiber diets [56, 57], perhaps due to the highly increased production of SCFAs by these microbes taking in large amounts of the plant polysaccharides. The SCFAs produced: butyrate, acetate, lactate, and propionate, form part of the normal energy supply used by the gut epithelia and encourage healthy growth and mucin production. This promotes more prolific epithelial cilia, which in turn provide greater

surface area for the bacteria to proliferate and produce more SCFAs. Butyrate is especially singled out as an energy source, being the preferred luminal substrate for the epithelial cells. Furthermore, butyrate has a differentiating and antiproliferative effect on certain colon cancer cell lines by inducing apoptosis at the appropriate times [58].

Mucosa

Mucosa, the inner lining of the gut formed by a layer of mucus over gut-associated lymphoid tissue (GALT) and the gut epithelium, plays a role in absorption of nutrients as well as providing a habitat and energy source for microbiota. Epithelial cells form a mechanical barrier in addition to the mucin layer produced by goblet cells for prevention of pathogen invasion, as well as acting as a communication link to the mucosal immune cells. Goblet cells secret mucin, a glycoprotein, and in doing so create barrier over the unstirred water layer to preserve the enzymes and nutrients within from microbial attack [59, 60, 61]. Slowing of gastrointestinal motility and increases in mucosa viscosity can allow opportunistic microbes to proliferate and degrade the mucosal barrier, often resulting in a fully realized bacterial infection [36]. Disruption of this layer can trigger severe growth delay, increased incidence and severity of disease, and general overall negative effects on the wellbeing of the organism. In many relevant species inflammatory disorders, such as inflammatory bowel disease in humans and other mammals, impair these functions and are often expressed by extreme intolerance to certain food items [62].

Microbiota

Though microbiota colonize sites throughout the entire body of an organism, places with exposure to large amounts of nutrients are most heavily populated; namely, the digestive tract and, more specifically in larger organisms, the colon. Because of the influx of excess and indigestible nutrients that the host cannot utilize, microbiota have evolved to take advantage of this valuable niche and incentivize the continued tolerance of the host. A key function of microbiota in the gut is carbohydrate degradation, specifically through fermentation of indigestible dietary components such as large polysaccharides in the form of cellulose, hemicellulose, other resistant starches, gums and pectens, as well as digestion of intestinal mucosa produced be epithelial cells and other carbohydrates that escaped initial digestion [63].

The digestion and fermentation of these various carbohydrates by the gut microbiota results in the production of volatile fatty acids (VFAs). These VFAs, specifically short chain fatty acids(SFCAs) (such as acetate, propionate, lactate, and butyrate), have been known to have a net positive impact on gut health by both suppressing hostile microbes and promoting epithelial health [54, 55, 64]. Degradation and metabolization of peptides by anaerobic microbes results in SCFAs as well; however, another byproduct of this metabolization are potential cytotoxic compounds that include ammonia, amines, phenols, and alkaloids, resulting in a oftentimes net negative effect [65]. Microbiota also aid in vitamin synthesis and the absorption of essential minerals, often generating a product that the host organism can create very little of or cannot produce at all. Because SCFAs sizable influence of host gut physiology and health, the high level of SCFA production by gut microbiota has a profound impact on the growth and proliferation of lumen epithelial cells. This is especially apparent in animals raised in sterile “germ-free” environments, with some studies showing distinct differences in size and number of luminal crypt cells, densities of lymphoid cells, and levels of immunoglobin in circulation between germ-free and conventional counterparts. These animals also showed a more severe immune reaction when introduced to an antigen, a response that was partially reversable in young animals by reintroduction of normal flora underlining the importance of early exposure and establishment of a healthy gut microbiota community [17, 18, 66]. In adults, repeat exposure to various microbiota or persistent diet changes can drastically change the community composition within a relatively short period of time by changing the gut environment to favor one particular type of microbiota over another and allowing for newcomers to take hold [67, 68]. Massive disruptions such as antibiotic use, starvation, or inoculations with extremely high dosages of specific microbiota can have long term sweeping effects on host physiology [69]. Reports of allergy and food intolerance development are quite common following heavy antibiotic use or severe gastrointestinal illness in humans indicating a link between gut immunoresponce and memory, and microbiota composition [70, 71]. Besides obvious physiological effects many studies indicate a deep and little understood influence of microbiota on host psychology as well. Recent research involving brain-gut axis interactions suggests the influences of gut health on mental state and behavior, especially in

humans, is much stronger than previous investigation has indicated, falling into the "status of well-being" criteria [72]. The third constituent of a healthy gut, microbiota, is highly variable in terms of composition and the definitions of "healthy" and "optimal". Though a large amount of research has been done with human and research model organism gut microbiota and its optimal compositions, other organisms such as economically important plants and animals lack the same depth of research, adding an additional layer of complexity to questions involving their microbiota's upkeep and community modification for production purposes [59]. The given definition of "optimal" for humans is geared towards overall health and longevity, while production organisms are often more optimized towards production output, nutritional efficiency, and maximized growth. Besides being influenced by diet and mucosa, microbiota is also often shaped by multiple other factors as well, including sex and age of the host, the hosts environment, the host's exposure to other organisms, and exposure to antimicrobial agents both accidentally and intentionally [73, 74, 75] . A review of available research by Ikeda-Ohtsubo et al. [29] found that, generally, a healthy gut microbiome is often dominated by three phyla, Proteobacteria, Firmicutes, and Bacteroidetes, all in differing proportions.
Insufficient or intentionally reduced exposure of the gut to microbiota in developing organisms can often result in a dysbiosis in later life and has been associated with the pathogenesis of the previously quoted serious gastrointestinal disorders and inflammatory diseases such as irritable bowel syndrome and celiac disease; however, immunodeficiency and immune system mediated related disorders like obesity and chronic kidney disease also become more common in both humans and animal models [61, 76, 77]. Furthermore, there has been some evidence that gut-microbiota interactions influence mental states through production of neurotransmitters and neurotrophins [78] and have been associated with various psychological, neurological, and cognitive disorders including dementia, autism spectrum disorder (ASD), and depression [79, 80, 81, 82].

Importance of the Gut in Agriculture

In animals reduced gut health is associated with a downturn in growth rate, disease morbidity, feed efficiency and over all livability [10, 19]; since the objective of production agriculture is to maximize production output from each individual, this makes the gut quite possibly the most

important body system producers will encounter on a daily basis when managing their animals. In pigs entering the weaning stage and beyond, gut health often is equal to the overall health of the animal and regularly determines how well the animal will perform in terms of growth and efficiency, making it imperative for producers to regulate and influence these systems. Even in the absence of disease-causing microbes young pigs often experience compromised gastrointestinal tracts and reduced production due to failure to nurture and develop a healthy gut, accomplishing many of the same deleterious effects as an infectious disease would [83]. With aquaculture production, management of gut health takes on an additional dimension as the animals are incessantly exposed to other organisms through the water they are raised in. Emerging research suggests that certain fish species are able to use their host factors such as age and sex as an ecological filter to shape gut microbiome development and thus gut health, though these can be overwhelmed fairly easily by exposure to other microbes through host to host transmission [16]. In cattle and dairy cattle in particular, due to industry practices of removing calves from their dams immediately after delivery, proper establishment of a healthy gut soon after birth will most likely determine if the calf will survive [84]. According to data collected by the National Animal Health Monitoring System in 2007 approximately 25% of U.S pre-weaned dairy heifers suffered from some form of digestive upset, and of the 7.8% of animals that died during the preweaning period 57% succumbed to scours or other digestive problems [85].

Gut Health in Poultry

As more research sheds light on the function of the gut in production agriculture, in an industry already focused on maximizing efficiency, poultry gut health and management has come to the forefront as a subject to study in recent years. With production animals becoming as genetically optimized as physically possible, other options for further improvement in growth and feed efficiency are sought. Because of the gut's influence on almost all other parts of the body, specific emphasis on management of this system has become a top priority for most commercial poultry producers [86, 87].

The Gastrointestinal System in Poultry

Outside the normal configuration of the monogastric gut, the gastrointestinal system in poultry contains three additional major structures: the crop, gizzard, and ceca.

The crop functions as a storage area for excess ingesta before digestion, as the proventriculus and gizzard can only hold a small amount at a time. The crop also moistens ingesta in preparation for mechanical and chemical breakdown. The pH can vary widely in the crop, being partially dependent on the pH of whatever food was ingested with reported ranges being between 4.0-7.8 [88,89]. The premoistening activity, along with variable pH, provides a favorable environment for niche microbiota, specifically lactic acid bacteria, some of which are thought to aid in production of phytase as well as beginning the process of breaking down starches. Though no digestion is initiated by the animal itself, it is believed the crop may be capable of absorbing glucose and some amino acids, allowing the bird to benefit from commensal bacteria byproducts. Besides fermentation of sugars these commensal bacteria also act as a barrier to the colonization of the crop by pathogenic bacteria both physically through competitive exclusion and chemically via the production of bactericidal compounds. Disruption of this environment by stress or sickness can result in an increase in pathogen colonization and subsequent infections. Depending on feeding regimen the volume of the crop is highly variable, with birds fed ad libitum possessing much lower storage capacity than birds on a daily feeding schedule. The perceived scarceness of food on a restricted schedule can cause birds to train the crop to hold up to 40% of the daily feed intake at once, which further impacts rate of passage through the rest of the gastrointestinal tract [90], ultimately restricting the amount of feed that is able to be processed and utilized day to day. After the holding time has elapsed and the ingested feed is premoistened, ingesta is subsequently passed to the proventriculus or true stomach to initiate chemical breakdown [91]. The proventriculus secretes hydrochloric acid and pepsinogen similarly to most other monogastrics, reportedly maintaining a pH between 1.9 and 4.5 depending on the buffering capacity of the ingested feed. The ingesta is passed into the gizzard through peristaltic contractions from the duodenum and processed in a grinding cycle up to four times a minute [92]. Due to the size of aforementioned contractions, ingesta may be

pushed in and out of the proventriculus multiple times, re-exposing it to stomach acids and further aiding break down.

The gizzard is an organ purely of mechanical breakdown, serving the same purpose as teeth in other organisms. Consisting mostly of muscle, the gizzard is a mass of highly myolinated tissue fibers incasing a thin sandpaper like layer of tissue known as the koilin layer which serves to reduce ingesta to a suitable size for passage to the small intestine. Ingesta is retained in the gizzard for grinding between 30 minutes and an hour, though factors such as feeding regimen, fiber content and structure of feed, and genetic predisposition can reduce or extend retention time considerably allowing for almost complete bypass of the gizzard or retention times upwards of 2 hours. Following mechanical breakdown ingesta is pushed into the duodenal loop of the small intestine where it is subjected to bile and pancreatic excretions, and the pH rapidly raised to around 6. As the main function of this segment is the neutralization of stomach acids, retention time of the duodenal loop is very short, reportedly around 5 minutes. Digesta is then passed on through the segments of small intestine: the jejunum, which is defined as the area starting at the end of the duodenal loop to the yolk sac residue known as Meckel's diverticulum and the ileum, which stretches from Meckel's diverticulum to the ileo-ceco-colic junction. The bulk of digestion of fats, proteins, and starches occur in the jejunum, with further absorption occurring in the ileum, along with some mineral and water absorption. After passage through the small intestine remaining small particulate matter is collected in the paired ceca via antiparastaltic movement of the colon, where the bulk of water and electrolytes are taken up. The ceca are also home to the largest and most diverse population of microbes in the gastrointestinal tract, which produce a variety of beneficial byproducts as they break down the remaining digesta and renal byproducts [93].

Poultry Gut Microbiome

In poultry, due to variances in pH and other environmental factors, each segment of the gut contains a distinct microbial community. In the foregut specifically, the crop provides a favorable anaerobic environment for the growth of microbes both in terms of temperature and nutrients. Microbial communities in the gastrointestinal tract change frequently due to diet and other host factors; however, *Lactobacillus, Bifidobacterium* and occasionally the

Enterobacteriaceae family are the commonly dominant species in the crop [94, 95, 96, 97, 98, 99, 100, 101, 102, 103, 104]. Acting as fermenters and producers of volatile fatty acids (VFAs), these species produce bacterial amylase to break down starches into maltose, maltotriose and glucose, which are then absorbed into the lining of the crop or used as a substrate for VFAs [105].

Due to low pH the proventriculus and gizzard harbor comparatively few microbes; common phyla include Proteobacteria, Firmicutes, and Actinobacteria, though it is thought none contribute to any significant fermentation or ingesta breakdown. However, these conditions do favor lactic acid producers over other more harmful organisms such as *Salmonella enterica* and *Campylobacter jejuni*, so negligible fermentation could be present [105, 106, 107].

The lower digestive tract can be split into two distinct communities, the intestines and cecum. A further distinction can be made in the intestine into the duodenum, the jejunum, and the ileum. There is a limited amount of bacterial colonization in the duodenum due to fast passage times combined with the lower pH and dilution factors of pancreatic and bile secretions [108]. In the intestines Firmicutes, Proteobacteria, Actinobacteria, Cyanobacteria and Bacteroidetes are most commonly dominant, though the exact composition and ratio is dependent on the age, sex, health, and other factors within the hosts sphere of influence [109, 110, 111, 112]. Because of its comparatively slow passage time, the ceca contains its own unique populations and is most commonly dominated by Bacteroidetes and Firmicutes with a large proportion of Proteobacteria and Archaea. The abundance of partially digested metabolic byproducts makes the ceca an ideal location for microbial fermentation and VFA production. Furthermore, because of these factors, competition is fierce and invasive pathogens are often competitively excluded [106, 107, 113, 114, 115].

Recent research has begun to delve deeper into these gut communities, examining their impact on overall efficiency and production ability of various poultry species at different life stages [116]. Recent studies such as Cox [117] serve to emphasize the malleability of gut microbiota by demonstrating the effect a probiotic treatment has on colonization of the gut in ducklings, even when applied prehatch at approximately day 25 (duck incubation duration equals ~28 days) . When applied prehatch, these probiotics containing lactic acid producing bacteria were shown

to affect the establishment of the gut microbiota, significantly reducing overall gram-negative bacteria presence.

Both parental environment and post hatch factors also continue to weigh heavily on how the gut microbiota is established, even when birds of different genetic backgrounds are raised together [59, 118]. Because of the effect of the history of the breed-strain, parental environment, incubation environment, rearing environment, etc. on microbe population composition there is not an absolute date at which certain microbes establish themselves and are seceded by newer or slower species. Because secession times and composition vary from breed to breed [118, 119, 120, 121, 122] it could be inferred that any probiotic treatment should be tailored to the known history of the parent flock in order to elicit a maximum positive response. Much as how antibiotics use has little positive effect on germfree animals in the absence of microbiota [123], the positive effect of probiotics lessens when applied to animals already exposed to beneficial microbes and the positive effect varies greatly from study to study [117, 124, 125, 126].

Effects of Stress and Stressors

Many situations and factors can result in a stressful environment for poultry; however heat stress and overcrowding is often cited as a major contributor to negative impacts on growth and production [127, 128, 129]. Heat stress in particular can contribute greatly to dysbiosis and negative microbiota growth [130, 131] leading to shifts in dominant genera resulting in the occurrence of gastrointestinal diseases such as necrotic enteritis [132, 133].

Overcrowding causes multiple stressors to occur over time including heat stress, higher litter moisture and other negative environmental factors, cannibalism, and feed limitation [134, 135]. Besides the aforementioned effects of heat stress, stressors such as higher moisture litter result in foot pad sores, higher ammonia production, and endurance of undesirable pathogens that would have otherwise been unable to gain a foothold in the environment [136, 137].

Molecular biology

Molecular Biology is a branch of life sciences dedicated to study and characterization of the macromolecules responsible for the biological processes of living things [138]. The central dogma of molecular biology, as stated by Crick [139] in paraphrase, is that once information is

encoded into protein it cannot be transferred back into DNA or RNA, describing the basic flow of genetic information. As microbiota composition becomes a baseline inquiry in most biological research, many "unculturable" bacteria go undetected by traditional means of observation. Biases due to lack of appropriate substrate, community influence, environmental effects, or a myriad of other factors can often come together to prevent growth and observation of under-represented or unknown microbes [140]. With the advent of simpler and faster molecular methods that allow for amplification and sequencing of microbial communities, these "unculturable" phyla can now be quantified. 16s rRNA amplification now allows for capture of snapshots into these communities unavailable before. With the advancement of technology, Illumina sequencing and other next gene sequencing methods have become cheaper and easier to access. A full genome sequencing using the sanger method in the early 2000's could cost up to $50,000 and take months to complete [141], today many labs offer prokaryote genome sequencing for less than a hundred U.S. dollars and promise a return within a few weeks [142]. With the decline in cost, molecular analysis has slowly become part of almost every field that deals with any sort of genetic material. Until the establishment of the Crick and Watson DNA model [143, 144] molecular biology was a minor field. It expanded exponentially, compounded upon the introduction of polymerase chain reaction (PCR), cloning, DNA sequencing, and other molecular techniques. A quick search on the PubMed Central database for "molecular biology" at the time of this writing turns up over one million results, with only about 600 predating the 1953 Crick and Watson paper, and approximately 40,000 that predate Mullis' PCR paper in 1987 [145].

Today molecular methods include the aforementioned PCR, DNA cloning, gel electrophoresis, blotting, molecular probes, and microarrays [146].

DNA Extraction

The first successful DNA extractions were devised by Friedrich Miescher in a paper published in 1871 as a result of his attempts to study the pus (and by extension white blood cells) collected from various infections collected from local hospitals. After subjecting samples to multiple purification treatments of various salt solutions Miescher observed a substance he dubbed "soluble and insoluble nuclein" [147], which was in fact nucleic acids. Though the process has

evolved greatly since Miecher's time, the basic concept remains the same; a sample is put through multiple steps of removing degradatory factors, lysing cells, and extracting nucleic acid for further use in downstream applications [148].

Though there are many subtypes the three main variations are organic extractions, Chelex extractions, and solid phase binding extraction that takes advantage of DNA's proclivity for binding to silica. The subtype being used depends greatly on what the end purpose of the extraction is and the project budget, as a protocol such as Chelex is faster and lower cost but does not produce a product suitable for anything but PCR applications [149]. The most commonly used industry standard is a microcentrifuge-based silica spin column kit with modifications based on sample type [150]. Samples with excess organic matter often have a higher yield when subjected to a bead beating lysis step before hand [151].

PCR and 16s

Polymerase Chain Reaction or PCR is a highly modifiable DNA amplification technology capable of quickly and accurately producing exponential amounts of DNA useful in a multitude of downstream applications. It also has the advantage of an overall lower investment of time, money, and energy compared to other molecular cloning methods such as plasmid vector cloning [149, 152, 153, 154]. After the addition of heat stable Taq polymerase, stabilizers, and primers, the sample is placed in a sealed canister and heated to 90^{o}C to allow for denaturation and unraveling of the molecular structure. The sample is then cooled to annealing temperature, which is normally between 40^{o}-60^{o}C depending on the sample and primer combination being used. This allows the primer to anneal to the 3^{o} end of the unraveled DNA . Subsequently the sample is heated to 72^{o}C to allow the polymerase to elongate along the 3^{o} end towards the 5^{o} end, duplicating the strand.

This process is repeated 20-30x in order to produce sufficient quantities for further analysis such as genomic sequencing and genetic testing. Other applications include determining the purity of food products, detecting the presence of pathogens, or determining the identity of pathogens in samples hundreds of years old [155, 156, 157].

Though PCR represents a vast improvement to the qualification of environmental samples containing "As yet to be cultured" organisms, it is much harder to quantify the populations that

the PCR reveals as be present. Because of inherent bias due to unequal extraction, inhibitory factors, or simply the wrong annealing temperature, a PCR sample does not accurately represent the numbers of an organism present in a sample. Using protocols and methods specific to quantification will help mitigate these biases if quantification is the end goal. However the most important factor is the care and consideration that must be placed on primer selection and PCR protocol in order to get the best possible data from individual samples [158, 159, 160, 161].

Illumina Sequencing

Illumina began as the sequencing by synthesis project at the University of Cambridge in the late 1990s [162]. It has since evolved to become the primary method of genetic sequencing. The basic concept is the use of DNA immobilized by chemical crosslink attached to previously available microarray technology to enable in real-time [163, 164], via bridge amplification or other similar amplification methods. Due to the multiple binding sites, Illumina has the capability to produce up to 8 gb of data using Miseq system and approximately 200-600 gb of data using the Hiseq system depending on what read length is desired. Miseq is preferred for microbiome research due to a longer read length that provides a higher resolution, approximately 250 bp [165, 166, 167] though it produces less data in total. Hiseq produces a shorter read length, between 100-150 bp; but yields a larger pool of data [168, 169]. With the rise in popularity of 16S genetic region analysis, Illumina has become the premier method of obtaining 16S data[170].

Bioinformatics

In this day and time data is often freely available in amounts larger than any one individual can use and analyze in a lifetime. On one hand this is an extraordinary opportunity for researchers as data put in public depositories can be used for multiple purposes other than the original intention and all one would have to do is look for it. However, it raises the problem of just how to process the sheer quantity available. As metagenomics becomes exponentially more common as an invaluable tool for a scientist working in any field remotely involving biology, programs for manipulating and processing the collected data have become stronger and faster, allowing for the processing of greater amounts of data with less computing power [171].

Because of its status as an emerging field there are a multitude of open source programs that specifically target various niche aspects of data manipulation. Recently there has been more concerted efforts to bench mark these programs in order to form some idea of performance standards. These benchmark efforts include the Critical Assessment of Metagenome Interpretation (CAMI), an open application benchmarking challenge now in its second iteration [172], and individual works, such as Vollmers *et al* [173], that all seek to provide a baseline for each program to be judged. CAMI attempts this by providing a mock microbiome to use for each competitor and providing framework for the setup of a digital docker container so that each program can be run without having to download the multiple dependences that virtually all open source software require to run [174]. Papers like Vollmers *et al* [174], or Awad *et al,* [175] used a specific known soil sample, and because they are benchmarking a much smaller sample size of programs it does not use a docker program.

When investigating a data set a researcher typically requires a minimum of an assembler to read the raw data, a contig binner to group like organisms, and a taxonomic binning program to shuffle the assembled contigs into a database of species composition and classification [171, 176]. Depending on the stage at which the researcher has access to the data, there may also be need for a quality control program to remove erroneous material before running through the assembler [172, 176].

Consistently the de novo assembler MEGAHIT tends to come to the forefront of most assessments as a (if not the) top contender in terms of accuracy and speed for general genomic assembly. Using a concept to that is also utilized by internet search engines during a query for keywords [177] and de Brujin graphs, MEGAHIT makes use of k-mers (monomers of the length "k") to parse string overlap in raw sequencing data in order to reassemble sequences. Because it does not require large reads and can be adjusted for desired k-mer length, MEGAHIT does not require impossible amounts of computing power and can conceivably be run (very slowly) on a suitably powerful desktop computer as opposed to a supercomputer cluster required for most other assemblers [178, 179, 180].

Popular binning programs include SourMASH, a taxonomic binner that utilizes MinHASH (**min**-wise independent permutations locality sensitive **hash**ing scheme) to create accurate

lightweight sketches of sequences enabling efficient containment queries in order to speed up and minimize computing time; and MAGpy, a pipeline conglomerate of multiple programs including SourMASH that creates and annotates phylogenetic trees as well as predicts the next closest relative for the sequenced genome by pulling from multiple databases simultaneously [171, 181].

Because converting between file formats and transferring large packets of data between programs isn't always feasible, more researchers have started to favor pipeline processing programs to streamline their processes. One such program that is readily available and possesses a large library of tutorials is QIIME or **Q**uantitative **I**nsights **I**nto **M**icrobial **E**cology (pronounced "chime"). QIIME is an open-source bioinformatics pipeline program that was first made available in 2010 [182] that functions as a start to finish modular pipeline capable of handling raw 16s or 18s RNA sequencing data in multiple FASTA and FASTQ input formats. QIIME handles demultiplexing, paired end joining, denoising/clustering, feature table and representative sequences generation, taxonomic classification, sequence alignment, phylogeny generation, diversity analysis, statistics, and plotting of genomic data.

A second version, QIIME 2, was released in 2018 [183] to replace the original. QIIME 2 was designed with plug in functionality to allow for improved accuracy and flexibility for users bring in raw data from different sequencing platforms by allowing third party tool plugins. QIIME 2 also has multiple interface choices to better serve a wider user base; these range from a graphical studio interface and online viewer for end users, to a command line interface wrapper for advanced users operating through high performance super computers.

Because QIIME is a complete pipeline, data can be completely processed within the program. This eliminates the extra steps of moving between programs and provides a streamlined process in comparison to similar programs such as Mothur [184] which produces data to be used with other programs requiring transfer of files, or Phyloseq [185] which uses already demultiplexed and quality controlled data necessitating the use of an upstream processor beforehand.

References

[1] Federal Registrar. Veterinary Feed Directive; Final Rule. Food and Drug Administration, Department of Health and Human Services. 2015;Jun3:80(106).

[2]Prescott JF, Sivendra R, Barnum DA. The use of bacitracin in the prevention and treatment of experimentally-induced necrotic enteritis in the chicken. The Canadian veterinary journal. 1978;19(7):181.

[3]Elwinger K, Berndtson E, Engström B, Fossum O, Waldenstedt L. Effect of antibiotic growth promoters and anticoccidials on growth of Clostridium perfringens in the caeca and on performance of broiler chickens. Acta Veterinaria Scandinavica. 1998;39(4):433-441.

[4] Van Immerseel F, Rood JI, Moore RJ, Titball RW. Rethinking our understanding of the pathogenesis of necrotic enteritis in chickens. Trends in microbiology. 2009;17(1):32-36.

[5] Tian J, Du J, Lu Z, Han J, Wang Z, Li D, Guan X, Wang Z. Distribution of microbiota across different intestinal tract segments of a stranded dwarf minke whale, Balaenoptera acutorostrata. *MicrobiologyOpen.* 2020;9(10):e1108.

[6] Wang W, Zheng S, Li L, Yang Y, Liu Y, Wang A, Sharshov K, Li Y. Comparative metagenomics of the gut microbiota in wild greylag geese (Anser anser) and ruddy shelducks (Tadorna ferruginea). MicrobiologyOpen. 2019;8(5):e00725.

[7] Li H, Li T, Beasley DE, Heděnec P, Xiao Z, Zhang S, Li J, Lin Q, Li X. Diet diversity is associated with beta but not alpha diversity of pika gut microbiota. Frontiers in microbiology. 2016;7:1169.

[8] Ferguson RMW, Merrifield D L, Harper G M, Rawling MD, Mustafa S, Picchietti S, Balcàzar JL, Davies SJ. The effect of Pediococcus acidilactici on the gut microbiota and immune status of on-growing red tilapia (Oreochromis niloticus). Journal of applied microbiology. 2010;109(3):851-862.

[9] Little T J, Hultmark D, Read A F. Invertebrate immunity and the limits of mechanistic immunology. Nature immunology. 2005;6(7):651-654.

[10] Brugman S, Ikeda-Ohtsubo W, Braber S, Folkerts G, Pieterse CM, Bakker PA. A Comparative Review on Microbiota Manipulation: Lessons From Fish, Plants, Livestock, and Human Research. Frontiers in Nutrition. 2018;5:80.

[11] Segata N, Waldron L, Ballarini A, Narasimhan V, Jousson O, Huttenhower C. Metagenomic microbial community profiling using unique clade-specific marker genes. Nature methods. 2012;9(8):811-814.
[12] Huttenhower C, Gevers D, Knight R, Abubucker S, Badger JH, Chinwalla AT, Creasy HH, Earl AM, FitzGerald MG, Fulton RS, Giglio MG. Structure, function and diversity of the healthy human microbiome. Nature. 2012;486(7402):207
[13] Langille MG, Zaneveld J, Caporaso JG, McDonald D, Knights D, Reyes JA, Clemente JC, Burkepile DE, Thurber RL, Knight R, Beiko RG. Predictive functional profiling of microbial communities using 16S rRNA marker gene sequences. Nature biotechnology. 2013;31(9):814-821.
[14] Douglas AE. Simple animal models for microbiome research. Nature Reviews Microbiology. 2019;1-12.
[15] Nagpal R, Wang S, Solberg Woods LC, Seshie O, Chung ST, Shively CA, Register TC, Craft S, McClain DA, Yadav H. Comparative microbiome signatures and short-chain fatty acids in mouse, rat, non-human primate, and human feces. Frontiers in microbiology. 2018;9:2897.
[16] Burns AR, Miller E, Agarwal M, Rolig AS, Milligan-Myhre K, Seredick S, (2017) . Interhost dispersal alters microbiome assembly and can overwhelm host innate immunity in an experimental zebrafish model. Proc Natl Acad Sci USA. 114 (42).
[17] Klaasen HL, Van der Heijden PJ, Stok W, et al. Apathogenic, intestinal, segmented, filamentous bacteria stimulate the mucosal immune system of mice. Infect Immun. 1993; 61: 303–06.
[18] Moreau MC, Gaboriau-Routhiau V. The absence of gut flora, the doses of antigen ingested and aging affect the long-term peripheral tolerance induced by ovalbumin feeding in mice. Research in immunology. 1996;147(1):49-59.
[19] Bauer H, Horowitz RE, Levenson SM, Popper H. The response of the lymphatic tissue to the microbial flora. Studies on germfree mice. The American journal of pathology. 1963;42(4):471.
[20] Kostic AD, Howitt MR, Garrett WS. Exploring host–microbiota interactions in animal models and humans. Genes & development. 2013;27(7):701-18.

[21] Gootenberg DB, Turnbaugh PJ. Companion animals symposium: humanized animal models of the microbiome. Journal of animal science. 2011;89(5):1531-7.
[22] Alzubaidy H, Essack M, Malas TB, Bokhari A, Motwalli O, Kamanu FK, Jamhor SA, Mokhtar NA, Antunes A, Simões MF, Alam I. Rhizosphere microbiome metagenomics of gray mangroves (Avicennia marina) in the Red Sea. Gene. 2016;576(2):626-36.
[23] Vandenkoornhuyse P, Quaiser A, Duhamel M, Le Van A, Dufresne A. The importance of the microbiome of the plant holobiont. New Phytologist. 2015;206(4):1196-206.
[24] Berg G, Rybakova D, Fischer D, Cernava T, Vergès MC, Charles T, Chen X, Cocolin L, Eversole K, Corral GH, Kazou M. Microbiome definition re-visited: old concepts and new challenges. Microbiome. 2020;8(1):1-22.
[25] Marchesi JR, Ravel J. The vocabulary of microbiome research: a proposal.
[26] Ainsworth TD, Krause L, Bridge T, Torda G, Raina JB, Zakrzewski M, Gates RD, Padilla-Gamiño JL, Spalding HL, Smith C, Woolsey ES. The coral core microbiome identifies rare bacterial taxa as ubiquitous endosymbionts. The ISME journal. 2015;9(10):2261-74.
[27] Bhat MI, Kapila R. Dietary metabolites derived from gut microbiota: critical modulators of epigenetic changes in mammals. Nutrition reviews. 2017;75(5):374-89.
[28] Galindo-Villegas J, García-Moreno D, De Oliveira S, Meseguer J, Mulero V. Regulation of immunity and disease resistance by commensal microbes and chromatin modifications during zebrafish development. Proceedings of the National Academy of Sciences. 2012;109(39):E2605-14.
[29] Ikeda-Ohtsubo W, Brugman S, Warden CH, Rebel JM, Folkerts G, Pieterse CM. How can we define “optimal microbiota?”: A comparative review of structure and functions of microbiota of animals, fish, and plants in agriculture. Frontiers in nutrition. 2018;5:90.
[30] Lauzon HL, Gudmundsdottir S, Petursdottir SK, Reynisson E, Steinarsson A, Oddgeirsson M, Bjornsdottir R, Gudmundsdottir BK. Microbiota of Atlantic cod (Gadus morhua L.) rearing systems at pre-and posthatch stages and the effect of different treatments. Journal of applied microbiology. 2010;109(5):1775-89.
[31] Ringo E. Intestinal microflora of fish larvae and fry. Aquacult. Res.. 1999;30:73-93.

[32] van Beers-Schreurs HM, Nabuurs MJ, Vellenga L, Valk HJ, Wensing T, Breukink HJ. Weaning and the weanling diet influence the villous height and crypt depth in the small intestine of pigs and alter the concentrations of short-chain fatty acids in the large intestine and blood. The Journal of nutrition. 1998;128(6):947-53.
[33] Denbow DM. Gastrointestinal anatomy and physiology. In Sturkie's avian physiology. Academic Press; 2015. p. 337-366.
[34] Beidler LM. Saliva: its functions and disorders. NEUROLOGICAL DISEASE AND THERAPY. 1994;32:503.
[35] Hofmann RR. 1. Morphophysiological Evolutionary Adaptations of the Ruminant Digestive System. In Aspects of digestive physiology in ruminants. Cornell University Press; 2019. p. 1-20.
[36] Moran Jr ET. Gastric digestion of protein through pancreozyme action optimizes intestinal forms for absorption, mucin formation and villus integrity. Animal Feed Science and Technology. 2016;221:284-303.
[37] Moran Jr ET. Digestion and absorption of carbohydrates in fowl and events through perinatal development. The Journal of nutrition. 1985;115(5):665-74.
[38] Sherwood L, Klandorf H, Yancey PH. The digestive system. Human physiology. From cells to systems. 3rd ed. Belmont: Wadworth Publ. Co. 1997:546-99.
[39] Burel C, Valat C. The effect of the feed on the host-microflora interactions in poultry: an overview. In Proceedings in International Symposium on Sustainable animal production-the challenges and potential developments for professional farming. 2009. p. 365-383.
[40] Snedeker SM, Hay AG. Do interactions between gut ecology and environmental chemicals contribute to obesity and diabetes?. Environmental health perspectives. 2012;120(3):332-9.
[41] Tuohy KM, Rouzaud GC, Bruck WM, Gibson GR. Modulation of the human gut microflora towards improved health using prebiotics-assessment of efficacy. Current pharmaceutical design. 2005;11(1):75-90.
[42] Barug D, de Jong J, Kies AK, Verstegen MW, editors. Antimicrobial growth promoters: where do we go from here?. Wageningen Academic Publishers; 2006 Mar 20.

[43] Gaucher ML, Quessy S, Letellier A, Arsenault J, Boulianne M. Impact of a drug-free program on broiler chicken growth performances, gut health, Clostridium perfringens and Campylobacter jejuni occurrences at the farm level. Poultry science. 2015;94(8):1791-801.
[43] Jayaraman B, Nyachoti CM. Husbandry practices and gut health outcomes in weaned piglets: A review. Animal Nutrition. 2017;3(3):205-11.
[44] De Verdal H, Narcy A, Bastianelli D, Chapuis H, Même N, Urvoix S, Le Bihan-Duval E, Mignon-Grasteau S. Improving the efficiency of feed utilization in poultry by selection. 1. Genetic parameters of anatomy of the gastro-intestinal tract and digestive efficiency. BMC genetics. 2011;12(1):1-9.
[46] Bischoff SC. 'Gut health': a new objective in medicine?. BMC medicine. 2011;9(1):1-4.
[47] Li E, Xu C, Wang X, Wang S, Zhao Q, Zhang M, Qin JG, Chen L. Gut microbiota and its modulation for healthy farming of Pacific white shrimp Litopenaeus vannamei. Reviews in Fisheries Science & Aquaculture. 2018;26(3):381-99.
[48] Niu Q, Li P, Hao S, Zhang Y, Kim SW, Li H, Ma X, Gao S, He L, Wu W, Huang X. Dynamic distribution of the gut microbiota and the relationship with apparent crude fiber digestibility and growth stages in pigs. Scientific reports. 2015;5(1):1-7.
[49] Hooper LV, Midtvedt T, Gordon JI. How host-microbial interactions shape the nutrient environment of the mammalian intestine. Annual review of nutrition. 2002;22(1):283-307.
[50] Conway PL. Function and regulation of the gastrointestinal microbiota of the pig. Publication-European Association For Animal Production. 1994;80:231.
[51] Whitney MH, Shurson GC, Guedes RC. Effect of including distillers dried grains with solubles in the diet, with or without antimicrobial regimen, on the ability of growing pigs to resist a Lawsonia intracellularis challenge. Journal of animal science. 2006;84(7):1870-9.
[52] Singh B: The Effects of Feeding Reduced-oil DDGS to Broilers when Challenged with C. perfringens and Eimeria spp. on Necrotic Enteritis and Intestinal Microbiome. Master's thesis. Auburn University, Poultry Science Department ; 2017.
[53] De Lange CF, Pluske J, Gong J, Nyachoti CM. Strategic use of feed ingredients and feed additives to stimulate gut health and development in young pigs. Livestock Science. 2010;134(1-3):124-34.

[54] De Filippo C, Cavalieri D, Di Paola M, Ramazzotti M, Poullet JB, Massart S, Collini S, Pieraccini G, Lionetti P. Impact of diet in shaping gut microbiota revealed by a comparative study in children from Europe and rural Africa. Proceedings of the National Academy of Sciences. 2010;107(33):14691-6.
[55] Scheppach W. Effects of short chain fatty acids on gut morphology and function. Gut. 1994;35 Suppl 1:S35-8.
[56] Burkitt DP. Epidemiology of large bowel disease: the role of fibre. Proceedings of the Nutrition Society. 1973;32(3):145-9.
[57] Wang T, Cai G, Qiu Y, Fei N, Zhang M, Pang X, Jia W, Cai S, Zhao L. Structural segregation of gut microbiota between colorectal cancer patients and healthy volunteers. The ISME journal. 2012;6(2):320-9.
[58] Orchel A, Dzierżewicz Z, Parfiniewicz B, Weglarz L, Wilczok T. Butyrate-induced differentiation of colon cancer cells is PKC and JNK dependent. Digestive diseases and sciences. 2005;50(3):490-8.
[59] Bedford MR, Partridge GG, editors. Enzymes in farm animal nutrition. Cabi; 2001.
[60] Gaboriau-Routhiau V, Rakotobe S, Lécuyer E, Mulder I, Lan A, Bridonneau C, Rochet V, Pisi A, De Paepe M, Brandi G, Eberl G. The key role of segmented filamentous bacteria in the coordinated maturation of gut helper T cell responses. Immunity. 2009;31(4):677-89.
[61] Rooks MG, Garrett WS. Gut microbiota, metabolites and host immunity. Nature reviews immunology. 2016;16(6):341-52.
[62] Roda G, Sartini A, Zambon E, Calafiore A, Marocchi M, Caponi A, Belluzzi A, Roda E. Intestinal epithelial cells in inflammatory bowel diseases. World journal of gastroenterology: WJG. 2010;16(34):4264.
[63] Cummings JH, Beatty ER, Kingman SM, Bingham SA, Englyst HN. Digestion and physiological properties of resistant starch in the human large bowel. British Journal of Nutrition. 1996;75(5):733-47.
[64] Cummings J, Pomare EW, Branch WJ, Naylor CP, MacFarlane G. Short chain fatty acids in human large intestine, portal, hepatic and venous blood. Gut. 1987;28(10):1221-7.

[65] Macfarlane GT, Cummings JH, Allison C. Protein degradation by human intestinal bacteria. Microbiology. 1986;132(6):1647-56.
[66] Alam M, Midtvedt T, Uribe A. Differential cell kinetics in the ileum and colon of germfree rats. Scandinavian journal of gastroenterology. 1994;29(5):445-51.
[67] Hills RD, Pontefract BA, Mishcon HR, Black CA, Sutton SC, Theberge CR. Gut microbiome: profound implications for diet and disease. Nutrients. 2019;11(7):1613.
[68] Bäckhed F, Ley RE, Sonnenburg JL, Peterson DA, Gordon JI. Host-bacterial mutualism in the human intestine. science. 2005;307(5717):1915-20.
[69] Xia JH, Lin G, Fu GH, Wan ZY, Lee M, Wang L, Liu XJ, Yue GH. The intestinal microbiome of fish under starvation. BMC genomics. 2014;15(1):1-1.
[70] Hanevik K, Dizdar V, Langeland N, Hausken T. Development of functional gastrointestinal disorders after Giardia lamblia infection. BMC gastroenterology. 2009;9(1):1-5.
[71] O'Keefe SJ, Ou J, Aufreiter S, O'Connor D, Sharma S, Sepulveda J, Fukuwatari T, Shibata K, Mawhinney T. Products of the colonic microbiota mediate the effects of diet on colon cancer risk. The Journal of nutrition. 2009;139(11):2044-8.
[72] Oriach CS, Robertson RC, Stanton C, Cryan JF, Dinan TG. Food for thought: The role of nutrition in the microbiota-gut–brain axis. Clinical Nutrition Experimental. 2016;6:25-38.
[73] Navarrete P, Mardones P, Opazo R, Espejo R, Romero J. Oxytetracycline treatment reduces bacterial diversity of intestinal microbiota of Atlantic salmon. Journal of Aquatic Animal Health. 2008;20(3):177-83.
[74] Papalini S, Michels F, Kohn N, Wegman J, van Hemert S, Roelofs K, Arias-Vasquez A, Aarts E. Stress matters: Randomized controlled trial on the effect of probiotics on neurocognition. Neurobiology of stress. 2019;10:100141.
[75] Wang Y, Lyu N, Liu F, Liu WJ, Bi Y, Zhang Z, Ma S, Cao J, Song X, Wang A, Zhang G. More diversified antibiotic resistance genes in chickens and workers of the live poultry markets. Environment International. 2021;153:106534.
[76] Guarner F, Malagelada JR. Gut flora in health and disease. The Lancet. 2003;361(9356):512-9.

[77] Gopalakrishnan V, Helmink BA, Spencer CN, Reuben A, Wargo JA. The influence of the gut microbiome on cancer, immunity, and cancer immunotherapy. Cancer cell. 2018;33(4):570-80.
[78] Gondalia S, Parkinson L, Stough C, Scholey A. Gut microbiota and bipolar disorder: a review of mechanisms and potential targets for adjunctive therapy. Psychopharmacology. 2019;236(5):1433-43.
[79] Griffiths JA, Mazmanian SK. Emerging evidence linking the gut microbiome to neurologic disorders. Genome medicine. 2018;10(1):1-3.
[80] Fowlie G, Cohen N, Ming X. The perturbance of microbiome and gut-brain axis in autism spectrum disorders. International journal of molecular sciences. 2018;19(8):2251.
[81] Kazemi A, Noorbala AA, Azam K, Eskandari MH, Djafarian K. Effect of probiotic and prebiotic vs placebo on psychological outcomes in patients with major depressive disorder: A randomized clinical trial. Clinical Nutrition. 2019;38(2):522-8.
[82] Liu RT, Walsh RF, Sheehan AE. Prebiotics and probiotics for depression and anxiety: A systematic review and meta-analysis of controlled clinical trials. Neuroscience & Biobehavioral Reviews. 2019;102:13-23.
[83] Pluske JR, Turpin DL, Kim JC. Gastrointestinal tract (gut) health in the young pig. Animal Nutrition. 2018;4(2):187-96.
[84] Malmuthuge N. Understanding the gut microbiome of dairy calves: Opportunities to improve early-life gut health. Journal of dairy science. 2017;100(7):5996-6005.
[85] NAHM. National animal health monitoring system. Heifer calf health and management practices on U.S. dairy operations in 2007. USDA:APHIS:VS, CEAH. Fort Collins, CO;2010.
[86] Celi P, Cowieson AJ, Fru-Nji F, Steinert RE, Kluenter AM, Verlhac V. Gastrointestinal functionality in animal nutrition and health: new opportunities for sustainable animal production. Animal Feed Science and Technology. 2017;234:88-100.
[87] Kogut MH. The effect of microbiome modulation on the intestinal health of poultry. Animal feed science and technology. 2019;250:32-40.

[88] Józefiak D, Sip A, Rawski M, Rutkowski A, Kaczmarek S, Hojberg O, Jensen BB, Engberg RM. Dietary divercin modifies gastrointestinal microbiota and improves growth performance in broiler chickens. British Poultry Science. 2011;52(4):492-9.
[89] Józefiak D, Kierończyk B, Rawski M, Hejdysz M, Rutkowski A, Engberg RM, Højberg O. Clostridium perfringens challenge and dietary fat type affect broiler chicken performance and fermentation in the gastrointestinal tract. animal. 2014;8(6):912-22.
[90] Kierończyk B, Rawski M, Długosz J, Świątkiewicz S, Józefiak D. Avian crop function–a review. Ann. Anim. Sci. 2016;16(3):653-78.
[91] Oviedo-Rondón EO. Holistic view of intestinal health in poultry. Animal Feed Science and Technology. 2019;250:1-8.
[92] SVIHUS B. The gizzard: function, influence of diet structure and effects on nutrient availability. World's Poultry Science Journal. 2011;67(2):207-24.
[93] Svihus B. Function of the digestive system. Journal of Applied Poultry Research. 2014;23(2):306-14.
[94] Oakley BB, Lillehoj HS, Kogut MH, Kim WK, Maurer JJ, Pedroso A, Lee MD, Collett SR, Johnson TJ, Cox NA. The chicken gastrointestinal microbiome. FEMS microbiology letters. 2014;360(2):100-12.
[95] Polansky O, Sekelova Z, Faldynova M, Sebkova A, Sisak F, Rychlik I. Important metabolic pathways and biological processes expressed by chicken cecal microbiota. Applied and environmental microbiology. 2016;82(5):1569-76.
[96] Pourabedin M, Zhao X. Prebiotics and gut microbiota in chickens. FEMS microbiology letters. 2015;362(15):fnv122.
[97] Rychlik I. Composition and function of chicken gut microbiota. Animals. 2020;10(1):103.
[98] Richards-Rios, P. Understanding the chicken intestinal microbiome: towards a rational approach to feed-based interventions. Doctoral dissertation. The University of Liverpool (United Kingdom);2020.
[99] Shang Y, Kumar S, Oakley B, Kim WK. Chicken gut microbiota: importance and detection technology. Frontiers in Veterinary Science. 2018;5:254.

[100] Siegerstetter SC, Schmitz-Esser S, Magowan E, Wetzels SU, Zebeli Q, Lawlor PG, O'Connell NE, Metzler-Zebeli BU. Intestinal microbiota profiles associated with low and high residual feed intake in chickens across two geographical locations. PloS one. 2017;12(11):e0187766.
[101] Sood U, Gupta V, Kumar R, Lal S, Fawcett D, Rattan S, Poinern GE, Lal R. Chicken gut microbiome and human health: past scenarios, current perspectives, and futuristic applications. Indian journal of microbiology. 2020;60(1):2-11.
[102] Waite DW, Taylor MW. Characterizing the avian gut microbiota: membership, driving influences, and potential function. Frontiers in microbiology. 2014;5:223.
[103] Xiao SS, Mi JD, Mei L, Liang J, Feng KX, Wu YB, Liao XD, Wang Y. Microbial Diversity and Community Variation in the Intestines of Layer Chickens. Animals. 2021;11(3):840.
[104] Wei S, Morrison M, Yu Z. Bacterial census of poultry intestinal microbiome. Poultry science. 2013;92(3):671-83.
[105] Borda-Molina D, Seifert J, Camarinha-Silva A. Current perspectives of the chicken gastrointestinal tract and its microbiome. Computational and structural biotechnology journal. 2018;16:131-9.
[106] Yeoman CJ, Chia N, Jeraldo P, Sipos M, Goldenfeld ND, White BA. The microbiome of the chicken gastrointestinal tract. Animal health research reviews. 2012;13(1):89-99.
[107] Feye KM, Baxter MF, Tellez-Isaias G, Kogut MH, Ricke SC. Influential factors on the composition of the conventionally raised broiler gastrointestinal microbiomes. Poultry science. 2020;99(2):653-9.
[108] Rehman HU, Vahjen W, Awad WA, Zentek J. Indigenous bacteria and bacterial metabolic products in the gastrointestinal tract of broiler chickens. Archives of animal nutrition. 2007;61(5):319-35.
[109] Xiao Y, Xiang Y, Zhou W, Chen J, Li K, Yang H. Microbial community mapping in intestinal tract of broiler chicken. Poultry science. 2017;96(5):1387-93.
[110] Jin LZ, Ho YW, Abdullah N, Kudo H, Jalaludin S. Studies on the intestinal microflora of chicken under tropical condition. Asian-Australasian Journal of Animal Sciences. 1997;10(5):495-504.

[111] Lu J, Idris U, Harmon B, Hofacre C, Maurer JJ, Lee MD. Diversity and succession of the intestinal bacterial community of the maturing broiler chicken. Applied and environmental microbiology. 2003;69(11):6816-24.
[112] Glendinning L, Stewart RD, Pallen MJ, Watson KA, Watson M. Assembly of hundreds of novel bacterial genomes from the chicken caecum. Genome biology. 2020;21(1):1-6.
[113] Mead GC. Microbes of the avian cecum: types present and substrates utilized. Journal of Experimental Zoology. 1989;252(S3):48-54.
[114] Cherrington CA, Hinton M, Mead GC, Chopra I. Organic acids: chemistry, antibacterial activity and practical applications. Advances in microbial physiology. 1991;32:87-108.
[115] Kimura N, Mimura F, Nishida S, Kobayashi A, Mitsuoka T. Studies on the relationship between intestinal flora and cecal coccidiosis in chicken. Poultry Science. 1976;55(4):1375-83.
[116] Diaz Carrasco JM, Casanova NA, Fernández Miyakawa ME. Microbiota, gut health and chicken productivity: what is the connection?. Microorganisms. 2019;7(10):374.
[117] Cox, J. Characterization of intestinal microbiota of newly hatched ducklings. Doctoral dissertation, The Ohio State University;2020.
[118] Richards P, Fothergill J, Bernardeau M, Wigley P. Development of the caecal microbiota in three broiler breeds. Frontiers in veterinary science. 2019;6:201.
[119] Metzler-Zebeli BU, Siegerstetter SC, Magowan E, Lawlor PG, Zebeli Q. Fecal microbiota transplant from highly feed efficient donors affects cecal physiology and microbiota in low- and high-feed efficient chickens. Frontiers in microbiology. 2019;10:1576.
[120] Gilroy R, Ravi A, Getino M, Pursley I, Horton DL, Alikhan NF, Baker D, Gharbi K, Hall N, Watson M, Adriaenssens EM. Extensive microbial diversity within the chicken gut microbiome revealed by metagenomics and culture. PeerJ. 2021;9.
[121] Glendinning L, Watson KA, Watson M. Development of the duodenal, ileal, jejunal and caecal microbiota in chickens. Animal microbiome. 2019;1(1):1-1.
[122] Mignon-Grasteau S, Narcy A, Rideau N, Chantry-Darmon C, Boscher MY, Sellier N, Chabault M, Konsak-Ilievski B, Le Bihan-Duval E, Gabriel I. Impact of selection for digestive efficiency on microbiota composition in the chicken. PLoS One. 2015;10(8):e0135488.

[123] Dibner JJ, Richards JD. Antibiotic growth promoters in agriculture: history and mode of action. Poultry science. 2005;84(4):634-43.
[124] O'dea EE, Fasenko GM, Allison GE, Korver DR, Tannock GW, Guan LL. Investigating the effects of commercial probiotics on broiler chick quality and production efficiency. Poultry Science. 2006;85(10):1855-63.
[125] O'dea EE, Fasenko GM, Allison GE, Korver DR, Tannock GW, Guan LL. Investigating the effects of commercial probiotics on broiler chick quality and production efficiency. Poultry Science. 2006;85(10):1855-63.
[126] Mercenier A, Pavan S, Pot B. Probiotics as biotherapeutic agents: present knowledge and future prospects. Current pharmaceutical design. 2003;9(2):175-91.
[127] Shi D, Bai L, Qu Q, Zhou S, Yang M, Guo S, Li Q, Liu C. Impact of gut microbiota structure in heat-stressed broilers. Poultry science. 2019;98(6):2405-13.
[128] Altan Ö, Altan AL, Cabuk M, Bayraktar H. Effects of heat stress on some blood parameters in broilers. Turkish journal of veterinary and animal sciences. 2000;24(2):145-8.
[129] Tankson JD, Vizzier-Thaxton Y, Thaxton JP, May JD, Cameron JA. Stress and nutritional quality of broilers. Poultry Science. 2001;80(9):1384-9.
[130] Crumeyrolle-Arias M, Jaglin M, Bruneau A, Vancassel S, Cardona A, Daugé V, Naudon L, Rabot S. Absence of the gut microbiota enhances anxiety-like behavior and neuroendocrine response to acute stress in rats. Psychoneuroendocrinology. 2014;42:207-17.
[131] Caekebeke N, Ringenier M, De Meyer F, Ducatelle R, Ongena N, Van Immerseel F, Dewulf J. A study on risk factors for macroscopic gut abnormalities in intensively reared broiler chickens. Avian Pathology. 2020;49(2):193-201.
[132] Tsiouris V, Georgopoulou I, Batzios C, Pappaioannou N, Ducatelle R, Fortomaris P. High stocking density as a predisposing factor for necrotic enteritis in broiler chicks. Avian Pathology. 2015;44(2):59-66.
[133] Kosiewicz MM, Zirnheld AL, Alard P. Gut microbiota, immunity, and disease: a complex relationship. Frontiers in microbiology. 2011;2:180.

[134] Gomes AV, Quinteiro-Filho WM, Ribeiro A, Ferraz-de-Paula V, Pinheiro ML, Baskeville E, Akamine AT, Astolfi-Ferreira CS, Ferreira AJ, Palermo-Neto J. Overcrowding stress decreases macrophage activity and increases Salmonella Enteritidis invasion in broiler chickens. Avian pathology. 2014;43(1):82-90.
[135] Kabir SL, Rahman MM, Rahman MB, Rahman MM, Ahmed SU. The dynamics of probiotics on growth performance and immune response in broilers. International Journal of Poultry Science. 2004;3(5):361-4.
[136] Pal A, Bailey MA, Talorico AA, Krehling JT, Macklin KS, Price SB, Buhr RJ, Bourassa DV. Impact of poultry litter Salmonella levels and moisture on transfer of Salmonella through associated in vitro generated dust. Poultry Science. 2021:101236.
[137] Bilgili SF, Hess JB, Blake JP, Macklin KS, Saenmahayak B, Sibley JL. Influence of bedding material on footpad dermatitis in broiler chickens. Journal of Applied Poultry Research. 2009;18(3):583-9.
[138] Schmitt FO. Contributions of Molecular Biology to Medicine. BULLETIN OF THE NEW YORK ACADEMY OF MEDICINE. 1960;36(11):725–749.
[139] Crick FH. The biological replication of macromolecules. In Symp. Soc. Exp. Biol. 1958;12. p. 138-163.
[140] Vartoukian SR, Palmer RM, Wade WG. Strategies for culture of ‘unculturable’bacteria. FEMS microbiology letters. 2010;309(1):1-7.
[141] Land M, Hauser L, Jun SR, Nookaew I, Leuze MR, Ahn TH, Karpinets T, Lund O, Kora G, Wassenaar T, Poudel S. Insights from 20 years of bacterial genome sequencing. Functional & integrative genomics. 2015;15(2):141-61.
[142] MR DNA (Molecular Research LP). (n.d.). Retrieved May 6, 2020, from http://www.mrdnalab.com/genome-sequencing-service.html
[143] Mullis KB. The unusual origin of the polymerase chain reaction. Scientific American. 1990;262(4):56-65.
[144] CRICK FHC, WATSON JD. Molecular Structure of Nucleic Acids: A Structure for Deoxyribose Nucleic Acid. Nature. 1953;171;737–738.

[145] National Center for Biotechnology Information(NCBI). molecular biology - PMC - NCBI. 2020. Retrieved May 7, 2020, from https://www.ncbi.nlm.nih.gov/pmc/?term=molecular+biology
[146] Tian J, editor. Molecular imaging: Fundamentals and applications. Springer Science & Business Media; 2013.
[147] Miescher-Rüsch F. Ueber die chemische Zusammensetzung der Eiterzellen. 1871.
[148] Dahm R. Discovering DNA: Friedrich Miescher and the early years of nucleic acid research. Human genetics. 2008;122(6):565-81.
[149] Tan SC, Yiap BC. DNA, RNA, and protein extraction: the past and the present. Journal of Biomedicine and Biotechnology. 2009.
[150] Yoshikawa H, Dogruman-Ai F, Turk S, Kustimur S, Balaban N, Sultan N. Evaluation of DNA extraction kits for molecular diagnosis of human Blastocystis subtypes from fecal samples. Parasitology research. 2011;109(4):1045-50.
[151] Miller DN, Bryant JE, Madsen EL, Ghiorse W. Evaluation and optimization of DNA extraction and purification procedures for soil and sediment samples. Applied and environmental microbiology. 1999;65(11):4715-24.
[152] Mullis KB, Erlich HA. Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. Science. 1988;239(4839):487-91.
[153] Mullis K, Faloona F, Scharf S, Saiki R, Horn G, Erlich H. Specific enzymatic amplification of DNA in vitro: the polymerase chain reaction. InCold Spring Harbor symposia on quantitative biology. Cold Spring Harbor Laboratory Press. 1986;51. p. 263-273.
[154] Sanger F, Coulson AR. A rapid method for determining sequences in DNA by primed synthesis with DNA polymerase. Journal of molecular biology. 1975;94(3):441-8.
[155] Raoult D, Aboudharam G, Crubézy E, Larrouy G, Ludes B, Drancourt M. Molecular identification by “suicide PCR” of Yersinia pestis as the agent of medieval black death. Proceedings of the National Academy of Sciences. 2000;97(23):12800-3.
[156] Kaltenboeck B, Wang C. Advances in real-time PCR: Application to clinical laboratory diagnostics. Advances in clinical chemistry. 2005;40:219.

[157] Amin MA, Hamid SB, Ali ME. A method for the detection of potential fraud of bringing feline meat in food chain. International Journal of Food Properties. 2016;19(7):1645-58.
[158] Bustin SA, editor. The PCR revolution: basic technologies and applications. Cambridge University Press; 2010.
[159] Costa E, Puhl NJ, Selinger LB, Inglis GD. Characterization of mucosa-associated bacterial communities of the mouse intestine by terminal restriction fragment length polymorphism: utility of sampling strategies and methods to reduce single-stranded DNA artifacts. Journal of microbiological methods. 2009;78(2):175-80.
[160] Freeman WM, Walker SJ, Vrana KE. Quantitative RT-PCR: pitfalls and potential. Biotechniques. 1999;26(1):112-25.
[161] Rychlik WJ, Spencer WJ, Rhoads RE. Optimization of the annealing temperature for DNA amplification in vitro. Nucleic acids research. 1990;18(21):6409-12.
[162] Balasubramanian S. Solexa sequencing: Decoding genomes on a population scale. Clinical chemistry. 2015;61(1):21-4.
[163] Furey WS, Joyce CM, Osborne MA, Klenerman D, Peliska JA, Balasubramanian S. Use of fluorescence resonance energy transfer to investigate the conformation of DNA substrates bound to the Klenow fragment. Biochemistry. 1998;37(9):2979-90.
[164] Meyer M, Kircher M. Illumina sequencing library preparation for highly multiplexed target capture and sequencing. Cold Spring Harbor Protocols. 2010;2010(6):pdb-rot5448.
[165] Yoo K, Lee TK, Choi EJ, Yang J, Shukla SK, Hwang SI, Park J. Molecular approaches for the detection and monitoring of microbial communities in bioaerosols: A review. Journal of Environmental Sciences. 2017;51:234-47.
[166] Degnan PH, Ochman H. Illumina-based analysis of microbial community diversity. The ISME journal. 2012;6(1):183-94.
[167] Metzker ML. Sequencing technologies—the next generation. Nature reviews genetics. 2010;11(1):31-46.
[168] Huang B, Jennison A, Whiley D, McMahon J, Hewitson G, Graham R, De Jong A, Warrilow D. Illumina sequencing of clinical samples for virus detection in a public health laboratory. Scientific reports. 2019;9(1):1-8.

[169] Tan G, Opitz L, Schlapbach R, Rehrauer H. Long fragments achieve lower base quality in Illumina paired-end sequencing. Scientific reports. 2019;9(1):1-7.
[170] Kozich JJ, Westcott SL, Baxter NT, Highlander SK, Schloss PD. Development of a dual-index sequencing strategy and curation pipeline for analyzing amplicon sequence data on the MiSeq Illumina sequencing platform. Applied and environmental microbiology. 2013;79(17):5112-20.
[171] Breitwieser FP, Lu J, Salzberg SL. A review of methods and databases for metagenomic classification and assembly. Briefings in bioinformatics. 2019;20(4):1125-36.
[172] Bremges A, McHardy AC. Critical assessment of metagenome interpretation enters the second round. MSystems. 2018;3(4).
[173] Vollmers J, Wiegand S, Kaster AK. Comparing and evaluating metagenome assembly tools from a microbiologist's perspective-not only size matters!. PloS one. 2017;12(1):e0169662.
[174] Sczyrba A, Hofmann P, Belmann P, Koslicki D, Janssen S, Dröge J, Gregor I, Majda S, Fiedler J, Dahms E, Bremges A. Critical assessment of metagenome interpretation—a benchmark of metagenomics software. Nature methods. 2017;14(11):1063-71.
[175] Awad S, Irber L, Brown CT. Evaluating metagenome assembly on a simple defined community with many strain variants. bioRxiv. 2017;155358.
[176] Forouzan E, Shariati P, Maleki MS, Karkhane AA, Yakhchali B. Practical evaluation of 11 de novo assemblers in metagenome assembly. Journal of microbiological methods. 2018;151:99-105.
[177] Broder AZ. On the resemblance and containment of documents. InProceedings. Compression and Complexity of SEQUENCES 1997 (Cat. No. 97TB100171). 1997. p. 21-29. IEEE.
[178] Ayling M, Clark MD, Leggett RM. New approaches for metagenome assembly with short reads. Briefings in bioinformatics. 2020;21(2):584-94.
[179] Li D, Luo R, Liu CM, Leung CM, Ting HF, Sadakane K, Yamashita H, Lam TW. MEGAHIT v1. 0: a fast and scalable metagenome assembler driven by advanced methodologies and community practices. Methods. 2016;102:3-11.

[180] Li D, Liu CM, Luo R, Sadakane K, Lam TW. MEGAHIT: an ultra-fast single-node solution for large and complex metagenomics assembly via succinct de Bruijn graph. Bioinformatics. 2015;31(10):1674-6.
[181] Stewart RD, Auffret MD, Snelling TJ, Roehe R, Watson M. MAGpy: a reproducible pipeline for the downstream analysis of metagenome-assembled genomes (MAGs). Bioinformatics. 2019;35(12):2150-2.
[182] Caporaso JG, Kuczynski J, Stombaugh J, Bittinger K, Bushman FD, Costello EK, Fierer N, Peña AG, Goodrich JK, Gordon JI, Huttley GA. QIIME allows analysis of high-throughput community sequencing data. Nature methods. 2010;7:335-6.
[183]Bolyen E, Rideout JR, Dillon MR, Bokulich NA, Abnet CC, Al-Ghalith GA, Alexander H, Alm EJ, Arumugam M, Asnicar F, Bai Y. Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. Nature biotechnology. 2019;37(8):852-7.
[184] Schloss PD, Westcott SL, Ryabin T, Hall JR, Hartmann M, Hollister EB, Lesniewski RA, Oakley BB, Parks DH, Robinson CJ, Sahl JW. Introducing mothur: open-source, platform-independent, community-supported software for describing and comparing microbial communities. Applied and environmental microbiology. 2009;75:7537-41.
[185] McMurdie PJ, Holmes S. phyloseq: an R package for reproducible interactive analysis and graphics of microbiome census data. PloS one. 2013;8:e61217.

Chapter 2 -Establishment of Base Interactions of Select Classifications Between Multiple Chick Sources in Differential Housing

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Abstract

Background: Microbial communities in the gut are influenced and shaped by both an individual's interactions with their environment and the interactions of their progenitors. In chickens, this includes the hatchery they originated from, as well as the environment in which the egg was laid. This study aimed to establish the extent to which the microbial communities of source hatcheries influenced the microbiome of broilers over time by placing birds from two separate source hatcheries (HA,HB) in to both a floor pen and battery cage system as three distinct populations HA, HB, and mixed population (MP). MP was made up of half HA individuals and half HB individuals. Samples of the midgut (defined as the area between the duodenal loop and Meckel's diverticulum) and the ceca were taken at day 0, 5, and 14 for battery cage birds, and day 0,5,14, 16, 21, 28, and 48 for floor raised birds. Samples were DNA extracted, amplified via PCR, and sequenced using 16S rRNA Illumina MiSeq protocol. Raw reads were then passed through the QIIME bioinformatics pipeline for analysis.

Results: Birds from HA and HB both displayed distinct microbial communities in day 0 samples and retained some of these characteristics through day 48. Furthermore MP samples seemed to be influenced more by the microbiome of the source hatchery that displayed a higher number of OTUs at day zero. More diverse day 0 microbiomes also seemed more resistant to larger shifts in community composition as time progressed. There is some indication that parental diet also shaped the microbiomes of the broiler chickens, with the genus *Bacteroides* appearing in HB (parent stock fed non-vegetarian diet) populations as early as day 5, MP day 14, and finally appearing in HA (parent stock feed vegetarian diet) at day 21.

Conclusions: The results suggest that there may be a net positive effect on the microbiome of a flock by placing birds with strong day 0 microbial communities in a mixed flock with those with weak or non-beneficial microbial communities.

Keywords: Broiler Chickens, Microbiota, Influence, Beneficial, Diversity

Background

As of October of 2015 the Veterinary Feed Directive banned usage of all subtherapeutic antibiotic drugs [1] in production animals, many of which were used to control diseases affecting the gut including necrotic enteritis in broiler chickens [2, 3]. The resulting upturn in incidence of these associated diseases has resulted in a greatly increased interest in the functions and influences of the gut in health and growth of broilers [4]. Because of the gut's influence on health and general wellbeing it has been subject to a great amount of study in other species and with more research becoming available every year it has become easier to discern the overarching mechanics of the triploblastic gut. However, the finer details involved in said functions within separate animal species remain opaque with omissions and exclusions being a rule more often than an exception; studies purporting to cover an under or never before examined aspect of a certain species' microbiome have become more and more common [5, 6, 7, 8, 9] With the adoption of molecular biological techniques, insight into the composition, interactions, and byproducts of microbial communities has become more widely available, allowing for closer inspection and observation of these previously inaccessible machinations [6, 7].

Overall, the hierarchy of available knowledge and research on the microbiome and its functions for a specific species is dependent on its importance (economic or otherwise) to humans [10, 11, 12, 13] . Economically important species of plant and animal are higher in the theoretical hierarchy of importance to humans. Commercial crops such as corn, wheat, or soybean and widely produced animals such as chickens, pigs, cattle, and commercial fish species, along with their associated pests and disease bearers, garner enough attention to attract research and funding to their areas [14, 15, 16]. Though broad, the available data and research for these species has little depth; but with the constant improvement of molecular methods and new studies being produced, greater depth will be achieved in time. The further removed from humans, the less is known about a particular species [5, 6, 7, 17, 18]. Exploratory studies serve to deepen already available knowledge and confirm trends unearthed by previous studies. This trial was conducted to establish a baseline for further exploration within the environments and organism populations available to the researchers. To this purpose day old broiler chicks were

acquired from two different hatcheries and raised in two pen types: traditional floor pens and battery cages.

Results

Day zero birds from both hatcheries exhibited distinct microbiomes visibly different from one another, with hatchery A having an overwhelming percentage presence of Escherichia coli at day zero while hatchery B exhibited a more diffuse spread of common microbes. An inquiry to both hatcheries confirmed that neither practiced any sort of post-hatch probiotics program at the time of the experiment. These differences continue through all days with the mixed population birds retaining midgut microbial population elements of both source hatcheries for both floor and battery raised birds. At the conclusion of this study, the midgut microbiome of day 48 floor raised birds more closely resembled the composition of a Hatchery B, while the cecal microbiome was virtually identical in top taxa with minor variations outside the top three most common genera.

Succession in the Cecal Microbiota

Though distinctly diverse at day zero, as birds matured cecal communities settled into a more standardized pattern as time increased. Though the top taxa varied from sample day to sample day the top three or four genera typically fell within +/- 5% of one another in terms of percent abundance. **See Fig. 2-8 for a visual representation of ratios of top classifications as well as Table 2-1 for a list of top percentages.**

Cecal samples in general tend to be the most diverse due to the nature of the cecal environment, this was confirmed by the Kruskal-Wallis (pairwise) significance of Faith's phylogenetic diversity (PD) test between body site sample types with ceca samples averaging a branch length of 10.972 at the deepest sampling depth, being extremely significant with ($p= 1.447x10^{-4}$)when compared to midgut samples which averaged only a branch length of 5.67 at the same depth. Ceca samples also exhibited a much deeper sequencing depth allowing for higher accuracy reads, with 20 out of 26 samples reaching the sampling depth of 24,000 vs midgut samples having only 6 out of 26 total samples reaching the same depth. Furthermore cecal samples presented a much higher average number of observed OTUs a with the average

cecal sample OTU count being 259.8 and the average midgut sample OTU count being 64.33 at the max sequencing depth of 24,000.

Ceca samples exhibited much higher and less variable evenness (Pielou's Evenness index) compared to midgut samples, with cecal samples displaying an average of 0.868 (high of 0.945, low 0.701 with one major outlier with day 0 at 0.425) vs midgut samples at an average of 0.686. When run with a Kruskal-Wallis (pairwise) significance test results were statistically significant with p = 1.30E-05. As previously mentioned, as time increased the communities within the ceca became more standardized with the top five genera (*Lachnospiraceae*, *Ruminococcaceae*, family *Lachnospiraceae* designation Torques group *Ruminococcus*, *Ruminococcaceae* (UCG-014), *Lactobacillus*), with the top two (*Lachnospiraceae*, *Ruminococcaceae*) switching in ranking but not by an extreme amount. The Jaccard coefficient PCOA (Fig. 2-6 (A-G)) demonstrates visually how after an initial heavy diversification over the first few weeks of life (D 0 to D 14), the different treatments fell into a similar community pattern by day 48. Shannon diversity score was very high over all for almost all samples, the average being 6.84 with the exception of the day 0 sample with a score of 2.16.

The principal investigator contacted hatchery B to confirm no probiotics were in use during the time period chicks were received for the trial, as it was a possibility that the diversity observed in hatchery B may have come from a commercial probiotics treatment.

Over the first two weeks of the trial hatchery B seemed to exhibit some resistance to extreme population shifts.

Succession in the Midgut Microbiota

Though *Lactobacillus* was the dominant genus for many sample days midgut samples seemed more prone to population disruption after feed changes with *Lactobacillus* percentage dropping greatly in the time period between day 14 and 16, and again two weeks after the transition to finisher at day 48. **See Fig. 2-9 for a visual representation of ratios of top classifications as well as Table 2-2 for a list of top percentages.**

Midgut samples averaged a PD score of 5.67 (high 8.86, low 3.27) at maximum sampling depth (24,000), however only 6 of 26 samples reached that depth compared to the ceca samples of which 20 of 26 reached max depth. Because of the small number of samples at max depth a

subsection of the PD scoring was taken at the depth of 2667 sequences to get a better picture of overall PD score, this sample depth yielded an average of 3.52 (high 8.7, low 2.27). Midgut samples were significantly less diverse than cecal samples with the Kruskal-Wallis (pairwise) significance of PD ($p= 1.447x10^{-4}$). Midgut samples presented a much lower average number of observed OTUs with the average midgut sample OTU count being 64.33 compared to the average cecal sample OTU count being 259.8 at the max sequencing depth of 24,000. Midgut samples exhibited much lower and more variable evenness compared to ceca samples, with midgut samples displaying an average evenness score of 0.686 (high of 0.91, low 0.49) vs ceca samples at an average of 0.868. A Kruskal-Wallis (pairwise) significance test was statistically significant with $p = 1.30x10^{-5}$. For midgut samples the top five genera varied greatly with the only real constant top genus being *Lactobacillus* and for a brief time later in grow out, *Clostridioides*.

After initial closeness on day zero, midgut samples plotted on a Jaccard coefficient PCOA (Fig. 2-6 (A-G)) trended towards differentiation. Unlike cecal samples which experienced a period of differentiation followed by a return to a similar population uniformity; midgut samples continue the trend of differentiation through day 48, spreading further and further apart. Shannon diversity score was moderate over samples at max sampling depth, the average being 3.22 (high 6.36, low 1.93). At retention sampling depth Shannon diversity was 3.55 (high 6.32, low 1.93).

Trends in Source Hatchery Microbiota

Source hatchery samples begin at day 0 with distinct but low diversity populations. As previously mentioned, HB was contacted to confirm no probiotic treatment was applied post hatch. No history of parent stock was disclosed; however it is noted that HA utilizes a plant-based all vegetarian diet for its birds. HA was less diverse than HB at day zero and as the trial progressed seemed to display a greater susceptibility to more extreme population shifts, especially in battery cage systems. HB also displayed a notably higher level of prevalence of unidentified bacterial reads (~44% ceca, ~26% midgut) versus HA (~2% ceca, ~0.3% midgut). Mixed population birds displayed elements from both source hatcheries, with cecal

communities more often mirroring HA in composition and mid-gut communities more closely resembling HB.

HA samples averaged a PD score of 8.85 (high 16.59, low 3.84) at maximum sampling depth (24,000); however, only 7 of 18 samples reached that depth. HB samples averaged a PD score of 9.52 (high 13.49, low 3.27) at maximum sampling depth, with 9 of 17 samples reaching max depth. MP samples averaged a PD score of 9.97 (high 15.03, low 3.84) at maximum sampling depth, with 11 of 17 samples reaching max depth. Because of the small number of samples at max depth a subsection of the PD scoring was taken at the depth of 2667 sequences to get a better picture of overall PD score at a shallower point, this sample depth yielded an average of 9.75 (high 15.23, low 3.78) for HA, an average of 7.31 (high 11.46, low 2.65) for HB, and an average of 7.82 (high 14.26, low 2.86) for MP. Samples were not significantly diverse from one another when analyzed using Kruskal-Wallis (pairwise) significance test ($p>0.05$).

HA samples presented an average 189 of observed OTUs, HB samples averaged an OTU count 222, and MP samples averaged an OTU count of 226 at the max sequencing depth of 24,000. Samples did not display significantly different evenness when compared with Kruskal-Wallis (pairwise) significance test ($p>0.05$).

For HA midgut samples top genera varied greatly between time points with top genus being *Lactobacillus* for 4 out of 9 points sampled (D5B, D5F, D14F, D21F. B= battery, F= floor, none= no pen) and a different top genus for the remaining 5 (D0 *Escherichia-Shigella*, D14B family Lachnospiraceae designation Torques group Ruminococcus, D16F *Clostridioides*, D28F *Lachnospiraceae*, D48F *Clostridiaceae*). HA ceca samples varied to some degree as well with *Lachnospiraceae* being the top genera for 5 of the 9 sample points (D5B, D5F, D14B, D16F, D48F) and a different top genus for the remaining 4 (D0 *Escherichia-Shigella*, D14F *Ruminococcaceae* UCG-014, D21F *Lactobacillus*, D28F *Faecalibacterium*).

Similar to HA, HB midgut top five genera varied greatly between time points with top genus being *Lactobacillus* for 4 out of 8 points sampled (D5B, D14B, D28F, D48F) and a different top genus for the remaining 4 (D0 unidentified bacteria, D14F *Candidatus Arthromitus*, D16F/D21F *Clostridioides*). HB ceca samples varied greatly with family Lachnospiraceae designation Torques group Ruminococcus (D5B, D14B, D16F), *Lachnospiraceae* (D5F, D21F), generic

Ruminococcaceae (D14F, D48F), *Ruminococcaceae* UCG-014 (D28F), and unidentified bacteria (D0) filling the top positions.

MP midgut samples top genera varied somewhat with *Lactobacillus* being most prevalent for 5 out of 8 points sampled (D5B, D5F, D14BF, D14F, D28F), *Clostridioides* for two sample days (D16F,D21F), and *Lachnospiraceae* for D48F. MP ceca samples were also similarly stratified with *Lachnospiraceae* being the top genera for 5 of the 8 sample points (D5B, D5F, D16F, D21F, D48F), *Faecalibacterium* for 2 sample points (D14F, D28F), and generic *Ruminococcaceae* on D14B. A few occurrences of individual genera were noted, first that *Escherichia-Shigella* dominated most of D0 for HA samples (ceca ~84.25, midgut ~88.01%), being present but not overwhelming in HB (ceca ~7.57, midgut ~11.55) on the same day. This trend continued at day 5 with HA diversifying but still mostly displaying a high proportion of *Escherichia-Shigella* (Battery- ceca ~15.09%, midgut ~1.97%. Floor- ceca ~0.91%, midgut ~0.07%.). Though *Escherichia-Shigella* is present in the cecal samples of other treatments it does not make up the same proportion of the population as it does in HA. Furthermore, population ratios vary but as a whole *Escherichia-Shigella* is present in all but one of the HA midgut samples. *Escherichia-Shigella* is present in all but one sample of HB as well however the proportion is smaller. In MP samples, *Escherichia-Shigella* is not detected in samples past day 14.

A second notable occurrence related to source hatchery is a pattern of *Bacteroides* occurring in HB cecal samples early on in the trial (D5B, D14B, D14F, D16F) as well as MP cecal sample (D14B, D14F, D16F), with the genus only being detected in HA at day 21 or later.

HA Shannon diversity score was moderate over samples at max sampling depth, the average being 5.66 (high 8.06, low 1.93). At retention sampling depth Shannon diversity was 5.63 (high 8.05, low 2.19). HB Shannon diversity score was moderate over samples at max sampling depth, the average being 6.41 (high 7.76, low 2.74). At retention sampling depth Shannon diversity was 5.24 (high 7.72, low 1.94). MP Shannon diversity score was moderate over samples at max sampling depth, the average being 6.14 (high 7.68, low 2.59). At retention sampling depth Shannon diversity was 5.10 (high 7.65, low 2.17).

Trends in Pen Types

Birds were only kept in battery to day 14 due to the difficulty of maintaining larger birds in the available battery cages designed to be used as brooders. Floor birds were placed on fresh pine shavings to limit outside influence.

Battery samples averaged a PD score of 8.32 (high 9.97, low 5.37) at the max sequencing depth of 24,000. Floor samples averaged a PD score of 10.63 (high 16.59, low 3.27). Samples were not significantly diverse from one another when analyzed using Kruskal-Wallis (pairwise) significance test. Battery samples presented an average 152 of observed OTUs (high 232, low 50) at the max sequencing depth of 24,000. Floor samples averaged 250 observed OTUs (high 431, low 27). Samples did not display significantly different evenness when compared with Kruskal-Wallis (pairwise) significance test ($p>0.05$).

For battery midgut samples, top genera was mostly consistent between time points with top genus being *Lactobacillus* for all sample points save for HA D14 which sported family Lachnospiraceae designation Torques group Ruminococcus as its top genus. For battery ceca samples top genera was *Lachnospiraceae* (HAD5, MPD5, HAD14), sported family Lachnospiraceae designation Torques group Ruminococcus (HBD5, MPD14), and generic *Ruminococcaceae* for HB day 14. For floor midgut samples 8 of the 17 samples retained were characterized with *Lactobacillus* (HAD5, MPD5, HAD14, MPD14, HAD21, HBD28, MPD28, HBD48), 5 of the 17 were characterized by *Clostridioides* (HAD16, HBD16, MPD16, HBD21, MPD21), 2 of the 17 were characterized by *Lachnospiraceae* (HAD28, MPD48), day HB14 was characterized by *Candidatus Arthromitus*, and day HA48 was characterized by group 1 *Clostridiaceae*. Day HB5 was omitted from the analysis due to very poor read counts.

Floor ceca samples were stratified similarly to floor midgut with 9 out of 18 samples with *Lachnospiraceae* (HAD5, HBD5, MPD5, HAD16, MPD16, HBD21, MPD21, HAD48, MPD48) holding the top genus spot, 3 out of 18 were characterized by *Faecalibacterium* (MPD14, HAD28, MPD28), 2 out of 18 were characterized by generic *Ruminococcaceae* (HBD14, HBD48), 2 out of 18 were characterized by *Ruminococcaceae* UCG-014 (HAD14, HBD28), HA D21was characterized by *Lactobacillus,* and HB D16 was characterized by family Lachnospiraceae designation Torques group Ruminococcus. Notably *Candidatus Arthromitus* genus occurs on

day 14 in all floor midgut samples (HA ~10%, HB ~47%, MP ~17%). It is *only* detected on day 14 with no trace observed before or after in any other samples.

Battery Shannon diversity score was moderate over samples at max sampling depth, the average being 5.83 (high 6.82, low 3.10). Floor Shannon diversity score was also moderate over samples at max sampling depth, the average being 6.44 (high 8.06, low 2.59).

Discussion

Though different source hatcheries and pen types influenced the overall composition, by day 48 all treatments were similarly diverse though not entirely identical. Early establishment of microbiota influenced composition later in life as well as diminished the effects of later colonization.

Bacterial Colonization of the Caecum and Midgut

For the ceca the most common genera were similar to previously reports and belonged mostly to the phylum *Firmicutes* [19, 20]; furthermore, the most common genera belonged to the order *Clostridiale*, not diverging until the next level down by family. Ceca samples started with very low diversity and underwent a period of succession with many genera appearing and fading before returning to a community similar to its starting community displaying a much greater and noticeable evenness by the last sample period compared to midgut samples. Cecal samples possessed the more diverse communities overall and when plotted with a Jaccard biplot in QIIME2 seemed most influenced by uncultured *Faecalibacterium* (Fig. 2-6 (A-G)). Midgut samples diversified quickly after day zero (Fig. 2-6 (A-G)) spreading out before contracting back to a composition that was similar to day 5 and 14, showing influence by *Lactobacillus* and *Clostridioides*.

Differences Between Source Hatcheries

For source hatcheries day zero diversity appeared to influence pen mates diversity and composition. Day 0 ceca HB possessed 8 unique identified reads whereas HA possessed 15; MP ceca trends more closely resembles HA ceca trends. Day 0 midgut HB possessed 17 identified reads and HA possessed 7; MP midgut composition more closely resembles HB composition.

The stronger early midgut community of HB seemed to exhibit greater resistance to larger fluctuations in community composition as time progressed and retained more of the original day 0 and day 5 community characteristics at day 48 than HA.

As previously mentioned, HA utilizes a plant-based all vegetarian diet for its birds and Auburn University did not at the time of the trial. It has been observed in humans that bacterial order Bacteroidales makes up a significant portion of gut microbiota and has been thought to be associated with many health disorders including obesity [21]. Additionally once viewed at the family level Bacteroidales primarily split into two main major families: *Bacteroides* associated with meat and heavily processed starch in western human diets , and *Prevotella* which is associated with fiber and plant matter heavy non-western human diets. *Prevotella* was not detected in any gut samples; however *Bacteroides* were present in many samples, specifically appearing in the non-vegetarian feed source flocks ceca samples first (HB and later MP). Bacteroides are known to utilize mucus glycopeptides as and energy source [22], with increased mucus production being a possible sign of gut irritation from either diet or microbial incursion [23]

Bacteroides only appeared in HA at day 21 or later (Fig. 2-5). This appearance of *Bacteroides* in later sampling days may suggest that the non-vegetarian diet provided by Auburn University influenced the spread and establishment of *Bacteroides* across all treatments. This implies at least a partial transfer between parental microbial communities and an ability to influence offspring despite the lack of contact between generations beyond laying of eggs. This aforementioned phenomenon taken with the apparent influence birds with stronger day 0 microbial communities have on less diverse pen mates implies the potential for improvement of individual grow out flocks when the production value and health of the parent stock is known (e.g. a healthy high production flock mixed with a low production or unhealthy flock to increase the quality and strength of the overall microbiome). Further exploration is needed to confirm these implications.

Differences Between Pen Types

Floor pens produced more diversity than battery cages; most likely influenced in the floor pens by the ability to access both the litter and the subjects' feces, serving as an amplifier for the available microbial communities. It could be speculated if the birds had been placed on old used litter instead of fresh shavings that a completely different succession pattern may have arisen due to the exposure to previous flocks microbiomes contained in said litter. Because the sample window for battery cages was far smaller than that of the floor pen treatments, it is not entirely assured that the trend of lower diversity and less visible community secessions would continue onward to later sample days. However, because of factors limiting the transfer of microbiota such as wire flooring and cleaning it could be speculated that the trend would continue similarly to that previously observed in earlier samplings.

A unique occurrence of *Candidatus* Arthromitus appeared on day 14 of the midgut floor pen treatments. *C.* Arthromitus did not appear on any day prior and was not detected on any day hence. A few possible hypotheses for this occurrence can be offered. The first is that it is merely contamination. However, *C.* Arthromitus is almost exclusively specific to the gut communities of terrestrial arthropods including beetle larva [24], an anaerobic environment not conducive to spreading in a lab setting. Furthermore, it occurred in three of twelve samples taken from the same sample day.

Logically, if contamination occurred during sample collection, the microbe would also be likely to have been found in other samples taken at the same time making the possibility of commination low. Because of this, the investigators formed a theory based around the timing of the bird placement and the life cycle of the darkling beetle as a possible answer to the unexpected presence. The darkling beetle lifecycle is approximately 48 days under ideal conditions, which a commercial broiler house provides. Assuming the beetles became more active and started laying eggs when the house was prepared for chicks at day 0, a large population of larva would begin to emerge around day 8. At day 5 broiler chicks are not strong or heavy enough to scratch away the layer of litter but at day 14 chicks are much larger yet are still small and agile enough to be active and practice foraging behaviors such as scratching and chasing potential food sources. Because the hypothetical beetle larva population increase

coincides very readily with a time point at which broilers are still willing to chase their food it would be possible that the broilers readily attacked this novel food source thus introducing *C.* Arthromitus into the gut as a transient microbe. Furthermore since darkling beetles and their larva actively avoid light sources and disturbances in their environment, broilers may have reduced the larval population sufficiently enough to drive the remainder deep into the litter thus preventing further occurrences of *C.* Arthromitus as a transient microbe.

Conclusion

Overall, though communities from various sources started out distinct from one another (HA, HB, MP) as time progressed these communities became more uniform and more closely resembled one another by day 48. Hatchery source and diet of parent stock may influence what microbiota the individual is predisposed to, birds with vegetarian fed parent stock picked up *Bacteroides* a full two weeks later than HB or MP. Day 0 communities influenced community composition as time progressed; with the stronger initial communities buffering the extent of further colonization by new microbes, while less diverse initial communities saw more extreme swings in composition as time progressed. Pen type influenced diversity, with birds living in floor pens displaying a more diverse and variable microbial community. Because of the variability, floor raised birds are exposed to more variable microbial populations with the greater possibility of non-beneficial microbial communities having a stronger presence and influence. Conversely floor raised birds may also have better access to the beneficial microbes from previous flocks if placed on used litter.

Methods

Bird and Farm Management

Unsexed and unvaccinated day-old broiler chicks were obtained from two separate commercial hatcheries and transported to the Auburn University Poultry Research Farm. Birds were split into three groups: **A** (hatchery A), **B** (hatchery B), and **MP** (equal numbers from both A and B). These groups were then randomly placed in two pen types: traditional floor, and battery cages. ~1.5m by ~1.5m floor pens received 40 birds each. ~0.6m by ~0.6m battery cages received 20. Chicks from hatchery B were wing-banded before being placed in treatments receiving mixed source birds to differentiate from source A birds during necropsy. Rooms were held at 35 ± 2°C

for the first 7 days and reduced 2-3°C weekly as per industry standard. Birds were fed a standard starter diet from day 0 to day 14, grower from day 15 to day 28, then switched to a finisher diet for the remainder of the trial. Due to space constraints birds in battery cages were only kept until day 21. Necropsies to acquire samples of the midgut (defined as the section between the duodenal loop and meckel's diverticulum) and entire ceca were conducted at days 0, 5, 14, 16, 21, 28, 30, 42, and 48 for floor housed birds; and days 0, 5, 14, 16, and 21 for battery cages. Four birds from each treatment were euthanized via CO_2 asphyxiation and sampled using aseptic technique. Samples were pooled by treatment and type then placed on ice after acquisition. After transport to the lab samples were placed in -80^{o}C freezer until DNA extraction.

DNA Extraction

After removal from the -80^{o}C freezer, ceca and midgut samples were thawed and extracted using the Omega Bio-tek E.Z.N.A. Stool DNA Extraction kit according to manufacturer's instructions, with one modification in that for step 4 DNA was incubated at 54^{o}C overnight followed by 10 minutes at 70^{o}C to insure cell breakdown as per recommendations from Omega Bio-tek trouble-shooting staff. Extracted DNA was tested for concentration and purity with a NanoDrop® ND-1000 Spectrophotometer, the desired concentration being a 260nm/280nm ratio between 1.8 - 2.0. Afterwards, DNA was place in a 2^{o}C refrigerator to await further processing.

Polymerase Chain Reaction (PCR) Amplification

DNA amplification via PCR was performed using a BioRad iQ5 thermocycler by touchdown protocol in order to maximize the amount of DNA amplified (Fig. 2-7). To that point universal target primers CS1/515F (5'-GTGYCAGCMGCCGCGGTAA-3') and CS2/926R (5'-CCGYCAATTYMTTTRAGTTT-3') were used to further expand potential targets. Amplification was confirmed with agarose gel electrophoresis on a 2% gel made with Tris-acetate-EDTA (TAE) buffer using Lonza® 100 bp Extended Range DNA Ladder. Gels were run for 75v for ~1 hour or until satisfactory visual conformation. Following confirmation, DNA PCR product was stored in a 2^{o}C refrigerator.

Illumina MiSeq Sequencing

PCR product was subsequently labeled and sent to University of Illinois Chicago DNA Services Facility (UIC DNAS) for under temperature-controlled conditions for 16S rRNA Illumina gene sequencing under an Illumina MiSeq protocol.

Data Analysis and Statistics

Following sequencing, raw FASTQ files were uploaded to the Illumina BaseSpace cloud database. Raw FASTQ files were downloaded from BaseSpace and uploaded to the Alabama Supercomputer (ASC) for more in-depth memory heavy analysis with QIIME2 pipeline [25]. Fastq files in Casava 1.8 paired end demultiplex format were read into QIIME2 to be joined and denoised using DADA2 [26]. Denoising and dereplication proceeded based on demultiplexing stats with forward reads being truncated at 220 base pairs and a max error rate of 4 (Fig. 2-1). The resulting feature table and representative sequences table were further filtered to exclude eukaryotic sequences and then used to determine optimum sampling depth in order to retain the most features without excluding a large number of samples. The highest feature frequency per sample being 50,684, the lowest being 361 and the mean frequency being 22,519 (Fig. 2-1). For diversity analysis a sampling depth of 900 was chosen to retain as many samples as possible. Reads with a frequency less than 5 were removed from sampling. A phylogenetic diversity analyses tree was generated using the q2-phylogeny plug-in. Taxonomic classification plug-in classify-sklearn was run using the Silva 132 99% full length classifier sequences set to identify Operational Taxonomic Units (OTUs). Using the classified data, filtering was preformed using the “filter table” function to remove sequences identified as belong to Eukaryotic organisms to eliminate host DNA. The QIIME2 diversity core-metrics-phylogenetic command was used to generate alpha and beta diversity analysis. QIIME2 “diversity alpha-rarefaction” command was used to generate an alpha rarefaction curve. A PCOA biplot was generated using the “diversity pcoa_biplot” command to establish a directional key for the prominent taxa responsible for some of the biggest shifts in the Jaccard graph. Taxonomic bar-plots were generated using the “barplot” command in R.

Figures

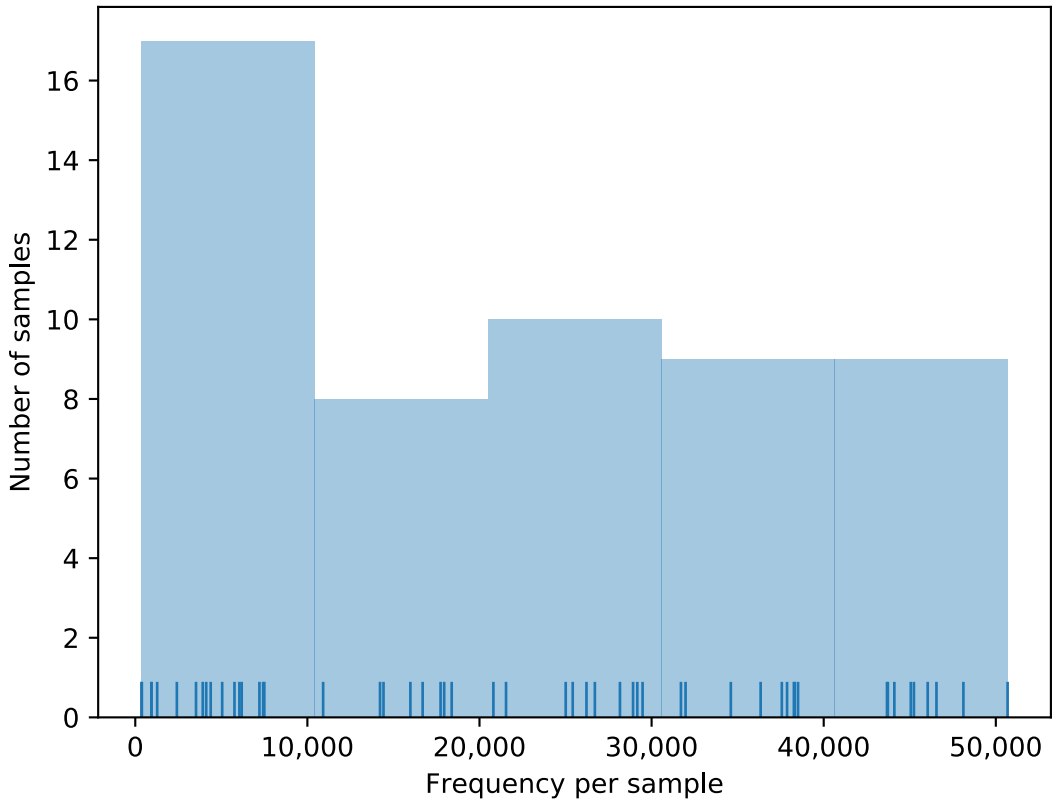


Figure 2-1-frequency of features per sample after denoising. Highest feature frequency per sample 50,684, lowest feature frequency 361, mean feature frequency 22,519.

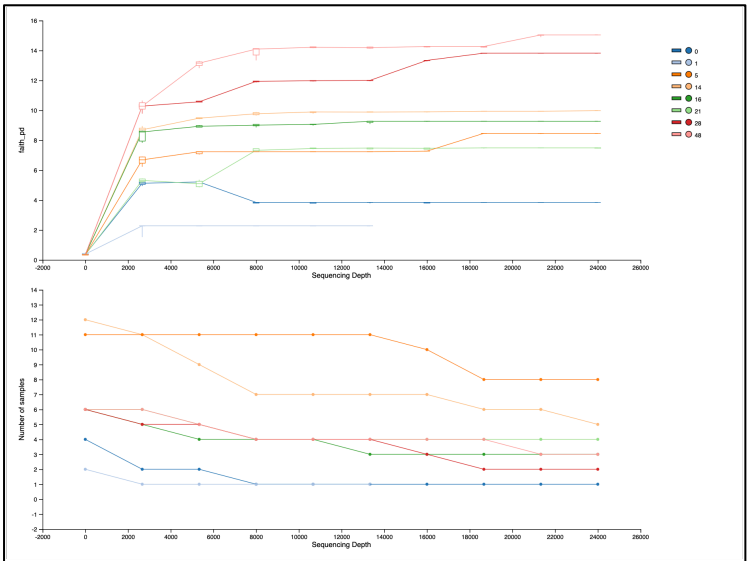


Figure 2-2-Alpha rarefaction curve of Faith's phylogenetic diversity by sample day.

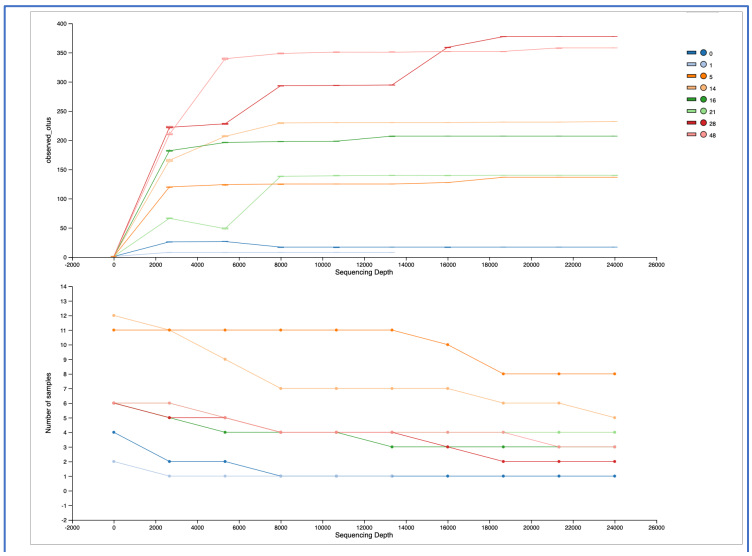


Figure 2-3-Alpha rarefaction curve of OTUs by sample day.

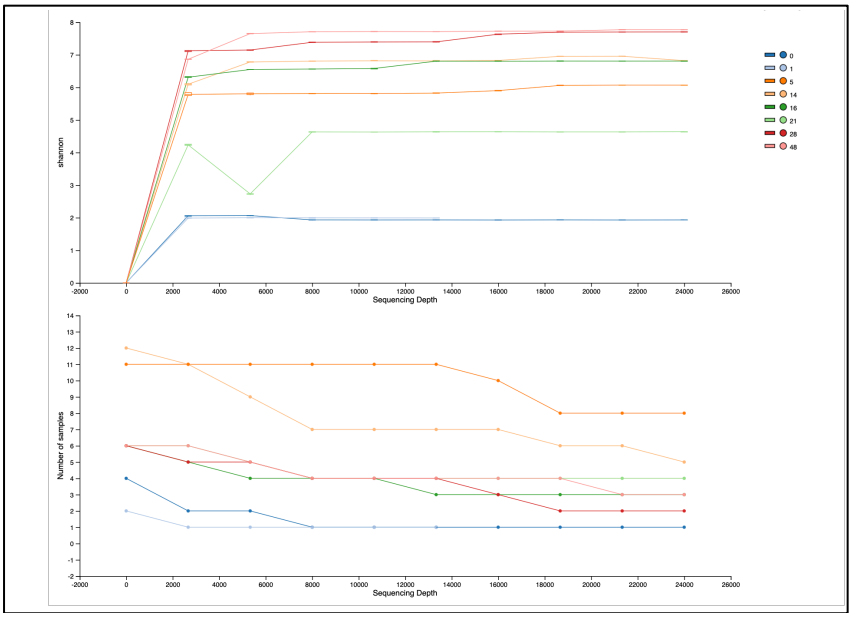


Figure 2-4- Alpha rarefaction curve of Shannon Diversity Index by sample day.

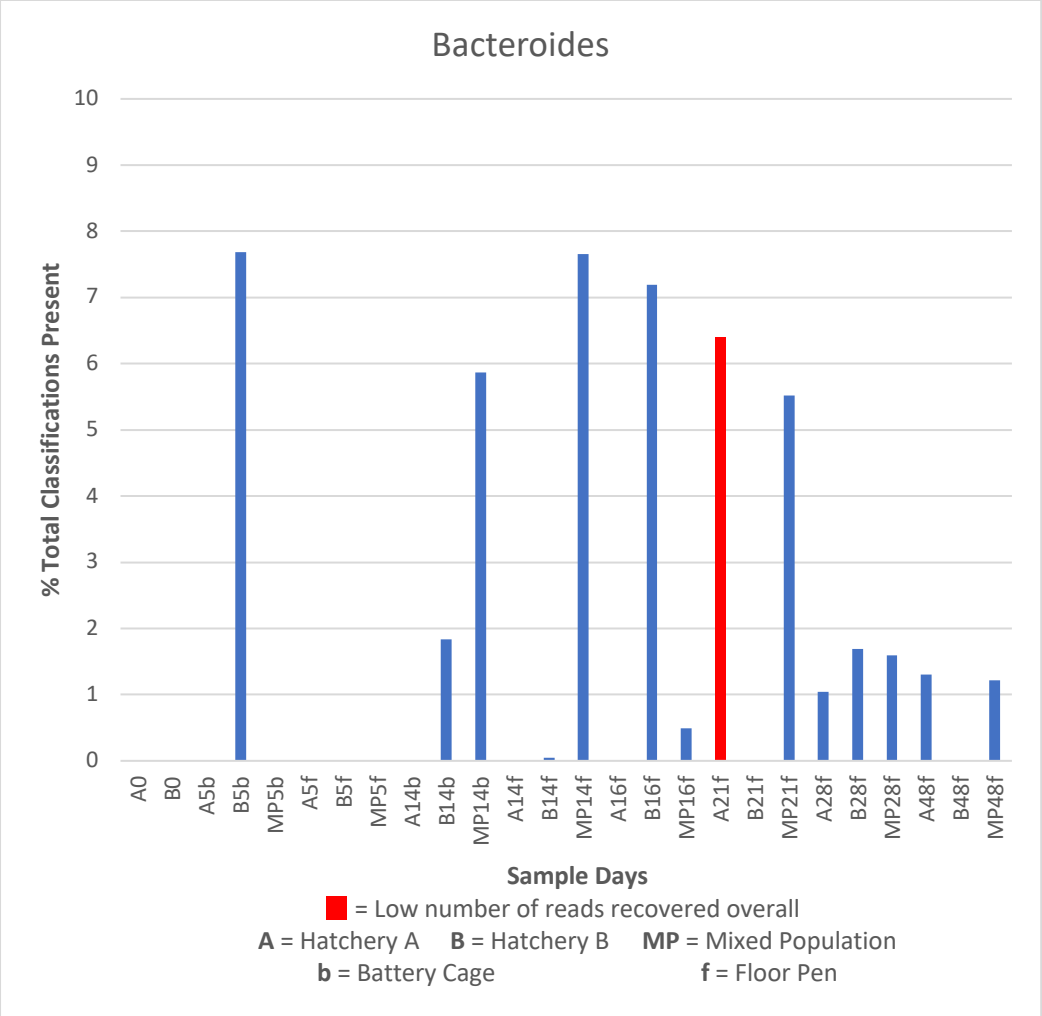


Figure 2-5-Bacteroides genus frequency over sample day, source hatchery, and pen type. Bacteroides are associated with meat and fat heavy diets, hatchery A parent stock were fed an all vegetarian diet. Bacteroides only appear in A at day 21 or later.

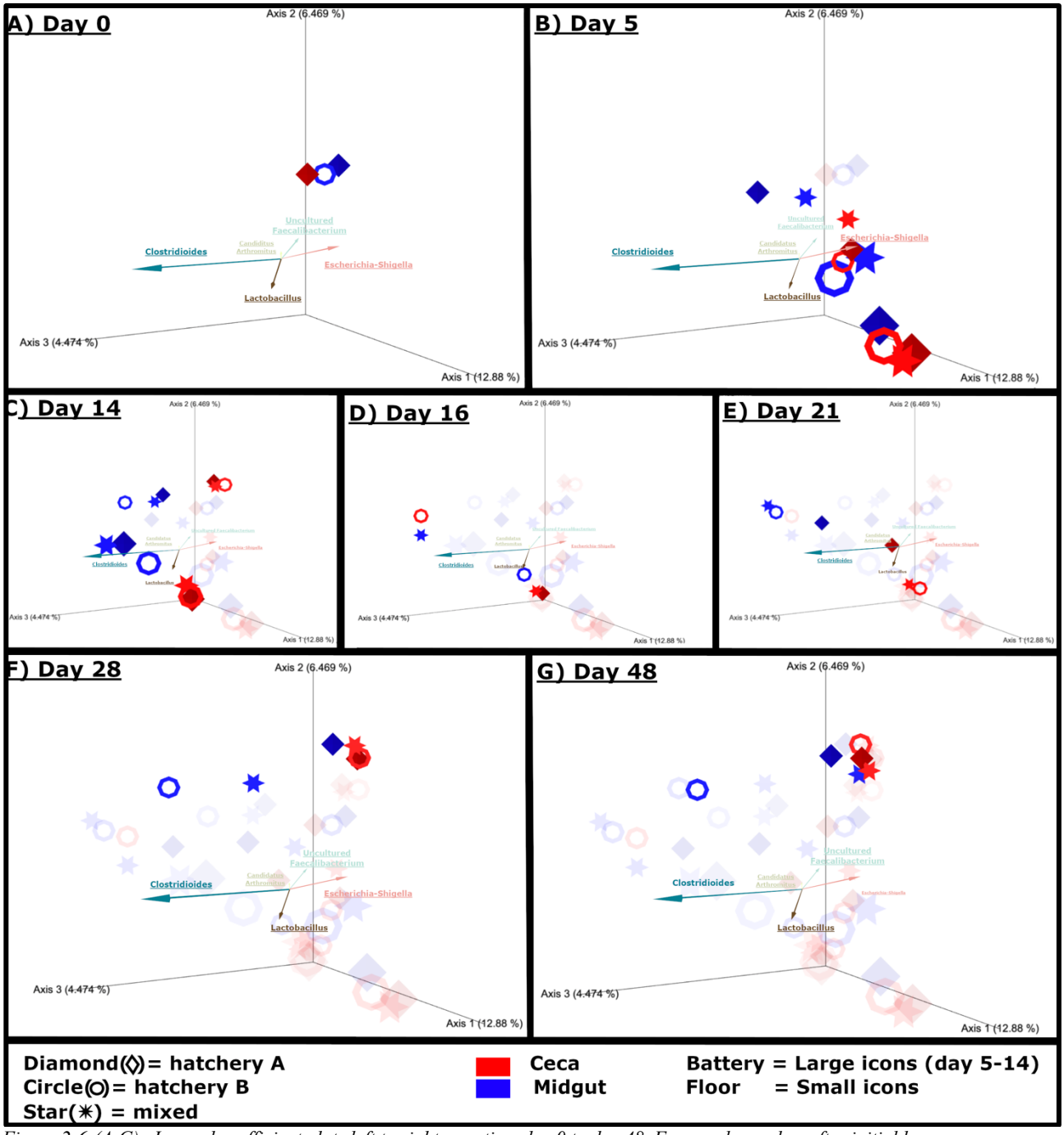


Figure 2-6-(A-G)- Jaccard coefficient plots left to right over time day 0 to day 48. For cecal samples, after initial heavy diversification over D 0 to D 14, different treatments fell into a similar community pattern by day 48. Midgut samples however continued to differentiate through day 48. When tied to a PCOA biplot using the "diversity pcoa_biplot"command in QIIME2 a directional key was generated using the prominent taxa responsible for some of the biggest shifts in the graph. These were Clostridioides, Candidatus Arthromitus, Escherichia-Shigella, Lactobacillus, and Uncultured Faecalibacterium.

Reaction conditions	1 x Rxn volume (µL)
Stage 1[1]	
Master Mix[2]	12.5
DdH_2O[3]	10.75
DNA Template[4]	1.0
Forward Primer[5]	0.5
Reverse Primer[6]	0.5
BSA[7]	0.25
Stage 2[8]	
Primers[9]	0.4
Stage 1 PCR Yield	1.0

[1]Performed at Auburn University Laboratory (Auburn, AL).

[2]Master Mix = Lucigen EconoTaq Plus 2x Master Mix (Middleton, WI): 0.1 units/µL of EconoTaq DNA Polymerase, Reaction Buffer (pH 9.0), 400 µM dATP, 400 µM dGTP, 400 µM dCTP, 400 µM dATP, 3 mM MgCl2, and a proprietary mix of PCR Enhancer/Stabilizer and blue and yellow tracking dyes.

[3]DdH_2O = double distilled H_2O.

[4]DNA Template = pooled jejunum tissue sample (5 birds/pen, each 2 cm in length, 15 cm proximal from Meckel's diverticulum) bacterial DNA extracted using Wizard Genomic Purification Kit (Madison, WI).

[5]Forward Primer = Eurofins Genomics CS1/515F Universal Target 16S rRNA Gene (Louisville, KY): 5'-GTGYCAGCMGCCGCGGTAA-3', expected amplicon size with CS1 456, annealing temperature 50°C.

[6]Reverse primer = Eurofins Genomics® CS2/926R Universal Target 16S rRNA Gene (Louisville, KY): 5'-CCGYCAATTYMTTTRAGTTT-3', expected amplicon size with CS2 456, annealing temperature 50°C.

[7]BSA (Bovine Serum Albumin) = Purified BSA 100x (Lawrenceville, GA).

[8]Performed at University of Illinois Chicago DNA Services Facility (Chicago, IL).

[9]Primers = Fluidigm's AccessArray Barcoded Primers (San Francisco, CA): Read 1: Standard CS1: A+CA+CTG+ACGACATGGTTCTACA; Index Read: CS2rc: A+GAC+CA+AGTCTCTGCTACCGTA; Read 2: Standard CS2: T+AC+GGT+AGCAGAGACTTGGTCT (A "+" preceding a base indicates a Locked Nucleic Acids(LNA)-base).

Cycle Step	Temperature	Time	Cycles
Stage 1[1]			
Initial Denaturation	94°C	5 min	1x
Variable Temperature Annealing			15x, reduce 1°C/cycle
Denaturation	94°C	30 sec	
Annealing	60°C	30 sec	
Extension	72°C	30 sec	
Constant Temperature Annealing	94°C		30x
Denaturation	94°C	30 sec	
Annealing	55°C	30 sec	
Extension	72°C	30 sec	
Final Extension	72°C	7 min	1x
Hold	4°C	∞	
Stage 2[2]			
Initial Denaturation	95°C	5 min	1x
Variable Temperature Annealing			28x
Denaturation	95°C	30 sec	
Annealing	60°C	30 sec	
Extension	72°C	30 sec	

[1]Performed at Auburn University Laboratory (Auburn, AL) using a Bio-Rad IQ5 thermocycler (Hercules, CA).

[2]Performed at University of Illinois Chicago DNA Services Facility (Chicago, IL) in preparation for Illumina MiSeq (San Diego, CA).

Figure 2-7- Reaction conditions and primers for amplification of bacterial populations by polymerase chain reaction (PCR) via touchdown protocol [19].

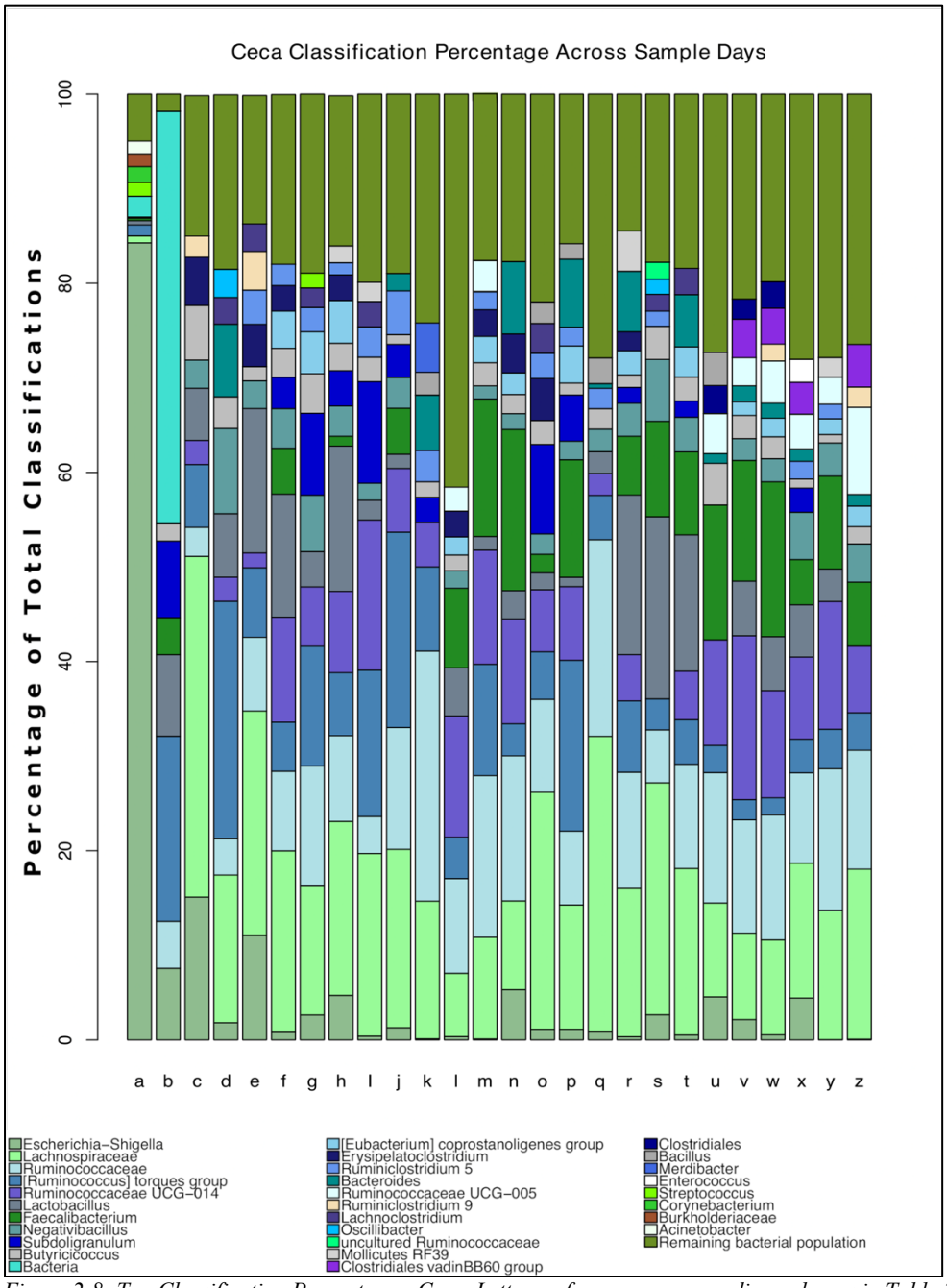


Figure 2-8- Top Classification Percentages, Ceca. Letters reference corresponding columns in Table 2-1.

Ref.	Day	Pen	Hatchery	Top Classifications Ceca
a	0	NA	HA	*Escherichia-Shigella* (~83%), unidentified bacterial reads (~2%), family Lachnospiraceae designation Torques group Ruminococcus (~1%)
b			HB	Unidentified bacterial reads (~26%), family Lachnospiraceae designation Torques group Ruminococcus (~20%), *Lactobacillus* (~9%), *Subdoligranulum* (~8%), *Escherichia-Shigella* (~8%), generic *Ruminococcaceae* (~5%), *Faecalibacterium* (~4%), *Butyricicoccus* (~2%),
c	5	Battery	HA	*Lachnospiraceae* (~36%), *Escherichia-Shigella* (~15%), *Lactobacillus* (~5%)
d			HB	Family Lachnospiraceae designation Torques group Ruminococcus (~ 25%), *Lachnospiraceae* (~16%), *Negativibacillus* (~9%), *Bacteroides* (~8%)
e			MP	*Lachnospiraceae* (~18%), *Lactobacillus* (~15%)
f		Floor	HA	*Lachnospiraceae* (~19%), *Lactobacillus* (~13%)
g			HB	*Lachnospiraceae* (~13%), Family Lachnospiraceae designation Torques group Ruminococcus (~12%), generic *Ruminococcaceae* (~12%),
h			MP	*Lachnospiraceae* (~23%), *Lactobacillus* (~15%)
i	14	Battery	HA	*Lachnospiraceae* (~19%), *Ruminococcaceae* UCG-014 (~16%), Torques group *Ruminococcus* (~16%), *Subdoligranulum* (~11%)
j			HB	Torques group *Ruminococcus* (~21%), *Lachnospiraceae* (~19%), generic *Ruminococcaceae* (~13%)
k			MP	Generic *Ruminococcaceae* (~26%), *Lachnospiraceae* (~16%), Torques group *Ruminococcus* (~9%), *Bacteroides* (~6%)
l		Floor	HA	*Ruminococcaceae* UCG-014 (~13%), generic *Ruminococcaceae* (~10%), *Faecalibacterium* (~8%), *Lachnospiraceae* (~7%)
m			HB	Generic *Ruminococcaceae* (~17%), *Faecalibacterium* (~15%), *Ruminococcaceae* UCG-014 (~12%), Torques group *Ruminococcus* (~12%), *Lachnospiraceae* (~11%)
n			MP	*Faecalibacterium* (~17%) closely followed by generic *Ruminococcaceae* (~15%), *Ruminococcaceae* UCG-014 (~11%), *Lachnospiraceae* (~9%), *Bacteroides* (~8%)
o	16	Floor	HA	*Lachnospiraceae* (~25%), generic *Ruminococcaceae* (~10%), *Subdoligranulum* (~9%), *Ruminococcaceae* UCG-014 (~7%), Torques group *Ruminococcus* (~5%)
p			HB	Torques group *Ruminococcus* (~18%), *Lachnospiraceae* (~13%), generic *Ruminococcaceae* (~8%), *Ruminococcaceae* UCG-014 (~8%), *Bacteroides* (~7%)
q			MP	*Lachnospiraceae* (~31%), followed by generic *Ruminococcaceae* (~20%), Torques group *Ruminococcus* (~5%)
r	21	Floor	HA	*Lactobacillus* (~17%), *Lachnospiraceae* (~16%), generic *Ruminococcaceae* (~12%), Torques group *Ruminococcus* (~8%), *Faecalibacterium* (~6%), *Bacteroides* (~6%)
s			HB	*Lachnospiraceae* (~25%), *Lactobacillus* (~19%), *Faecalibacterium* (~10%), *Negativibacillus* (~7%), generic *Ruminococcaceae* (~6%)
t			MP	*Lachnospiraceae* (~18%), *Lactobacillus* (~14%), generic *Ruminococcaceae* (~11%), *Faecalibacterium* (~9%), *Bacteroides* (~5%)
u	28	Floor	HA	*Faecalibacterium* (~14%), generic *Ruminococcaceae* (~14%), *Ruminococcaceae* UCG-014 (~11%), *Lachnospiraceae* (~10%), *Escherichia-Shigella* (~5%).
v			HB	*Ruminococcaceae* UCG-014 (~17%), *Faecalibacterium* (~13%), generic *Ruminococcaceae* (~12%), *Lachnospiraceae* (~9%), *Lactobacillus* (~6%)
w			MP	*Faecalibacterium* (~16%), generic *Ruminococcaceae* (~13%), *Ruminococcaceae* UCG-014 (~11%), *Lachnospiraceae* (~10%), *Lactobacillus* (~6%)
x	48	Floor	HA	*Lachnospiraceae* (~14%), generic *Ruminococcaceae* (~10%), *Ruminococcaceae* UCG-014 (~9%), *Lactobacillus* (~6%), Negativibacillus (~5%).

y			**HB**	Generic *Ruminococcaceae* (~15%), *Lachnospiraceae* (~14%), *Ruminococcaceae* UCG-014 (~14%), *Faecalibacterium* (~10%), torques group *Ruminococcus* (~4%).
z			**MP**	*Lachnospiraceae* (~18%), generic *Ruminococcaceae* (~13%), *Ruminococcaceae* UCG-005 (~9%), *Ruminococcaceae* UCG-014 (~7%), vadinBB60 group *Clostridiales* (~5%).

Table 2-1-Top Classifications, Ceca. Microbes from the phylum Firmicutes was the most common classification found, with Clostridiale being the most common order.

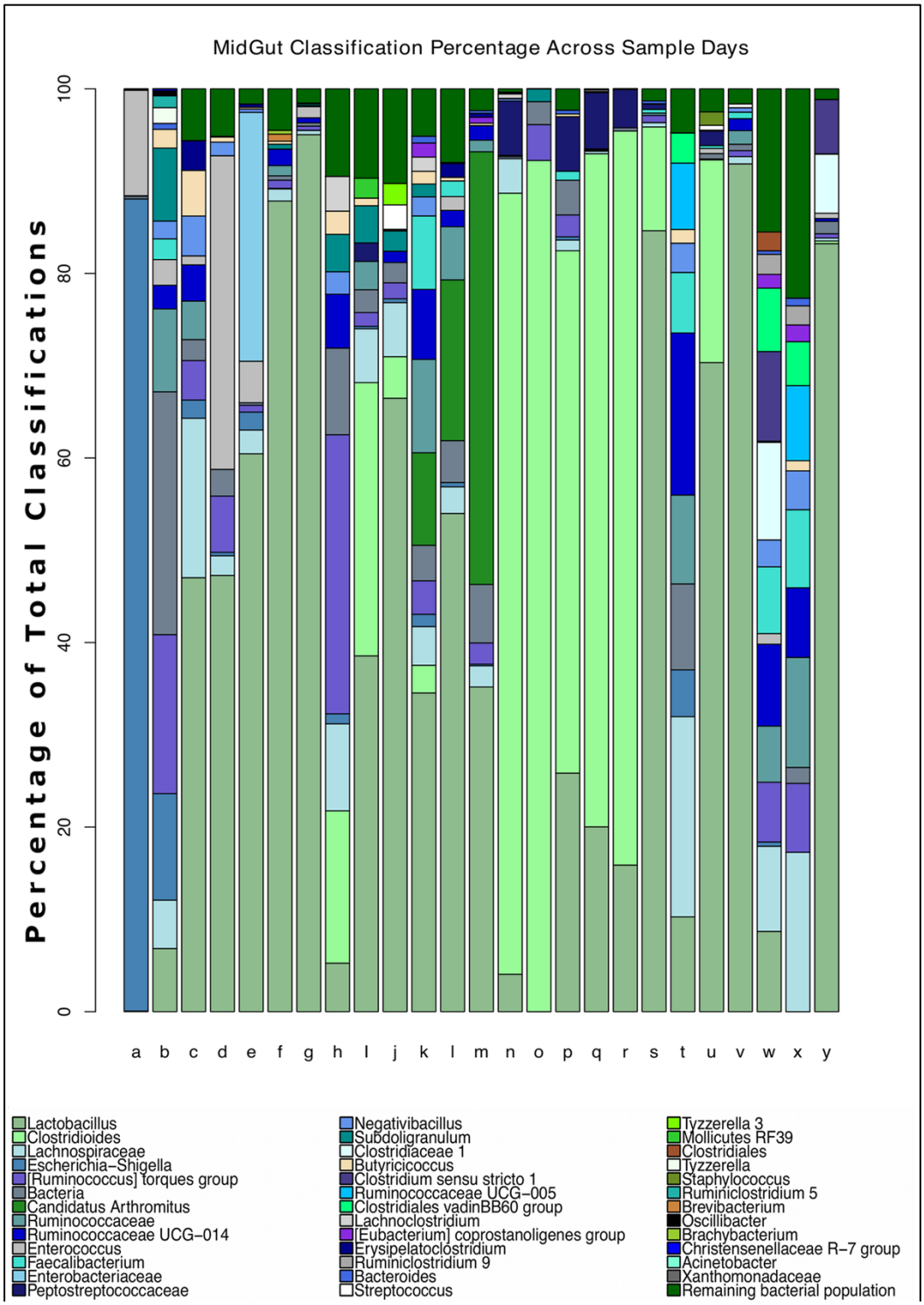


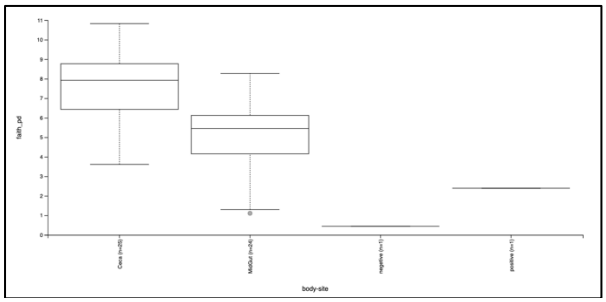
Figure 2-9-Top Classification Percentages, Midgut. Letters reference corresponding columns in Table 2-2.

REF.	Day	Pen	Hatchery	Top Classifications Midgut
a	0	NA	HA	*Escherichia-Shigella* (~88%), *Enterococcus* (~11%)
b			HB	Unidentified bacterial reads (~26%), Family Lachnospiraceae designation Torques group Ruminococcus (~17%), *Escherichia-Shigella* (~12%), generic *Ruminococcaceae* (~9%).
c	5	Battery	HA	*Lactobacillus* (~47%), *Lachnospiraceae* (~17%), *Butyricicoccus* (~5%), *Negativibacillus* (~4%), family Lachnospiraceae designation Torques group Ruminococcus (~4%), generic *Ruminococcaceae* (~4%)
d			HB	*Lactobacillus* (~47%), *Enterococcus* (~34%), family Lachnospiraceae designation Torques group Ruminococcus (~6%), unidentified bacterial reads (~3%)
e			MP	*Lactobacillus* (~61%), *Enterobacteriaceae* (~27%), *Enterococcus* (~5%), *Lachnospiraceae* (~3%)
f		Floor	HA	*Lactobacillus* (~88%), *Ruminococcaceae* UCG-014 (~2%), *Lachnospiraceae* (~1%), generic *Ruminococcaceae* (~1%)
N/A			HB	N/A
g			MP	*Lactobacillus* (~95%), *Enterococcus* (~1%)
h	14	Battery	HA	Family Lachnospiraceae designation Torques group Ruminococcus (~30%), *Clostridioides* (~17%), *Lachnospiraceae* (~9%), unidentified bacterial reads (~9%)
i			HB	*Lactobacillus* (~61%), *Lachnospiraceae* (~6%), *Clostridioides* (~5%), unidentified bacterial reads (~2%)
j			MP	*Lactobacillus* (~39%), *Clostridioides* (~30%), *Lachnospiraceae* (~6%), *Subdoligranulum* (~4%)
k		Floor	HA	*Lactobacillus* (~35%), *Candidatus Arthromitus* (~10%), generic *Ruminococcaceae* (~10%), *Faecalibacterium* (~8%).
l			HB	*Candidatus Arthromitus* (~46%), *Lactobacillus* (~35%), unidentified bacterial reads (~6%), *Lachnospiraceae* (~2%), family Lachnospiraceae designation Torques group Ruminococcus (~2%).
m			MP	*Lactobacillus* (~54%), *Candidatus Arthromitus* (~17%), generic *Ruminococcaceae* (~6%), unidentified bacterial reads (~5%)
n	16	Floor	HA	*Clostridioides* (~92%), family Lachnospiraceae designation Torques group Ruminococcus (~4%), unidentified bacterial reads (~3%), *Subdoligranulum* (~1%)
o			HB	*Clostridioides* (~57%), *Lactobacillus* (~26%), *Peptostreptococcaceae* (~6%), unidentified bacterial reads (~3%),family Lachnospiraceae designation Torques group Ruminococcus (~2%)
p			MP	*Clostridioides* (~85%), *Peptostreptococcaceae* (~6%), *Lactobacillus* (~4%), *Lachnospiraceae* (~4%).
q	21	Floor	HA	*Lactobacillus* (~85%), *Clostridioides* (~11%), family Lachnospiraceae designation Torques group Ruminococcus (~1%)
r			HB	*Clostridioides* (~73%), *Lactobacillus* (~20%), *Peptostreptococcaceae* (~6%)
s			MP	*Clostridioides* (~80%), *Lactobacillus* (~16%), *Peptostreptococcaceae* (~4%).
t	28	Floor	HA	*Lachnospiraceae* (~22%), *Ruminococcaceae* UCG-014 (~18%), *Lactobacillus* (~10%), generic *Ruminococcaceae* (~10%), unidentified bacterial reads (~9%)
u			HB	*Lactobacillus* (~70%), *Clostridioides* (~22%), *Peptostreptococcaceae* (~2%)
v			MP	*Lactobacillus* (~93%), generic *Ruminococcaceae* (~2%), *Ruminococcaceae* UCG-014 (~1%), *Lachnospiraceae* (~1%)
w	48	loor	HA	Group 1 *Clostridiaceae* (~11%), *Clostridium sensu stricto* (~10%), *Lachnospiraceae* (~9%), *Lactobacillus* (~9%), *Ruminococcaceae* UCG-014 (~9%), *Faecalibacterium* (~7%),

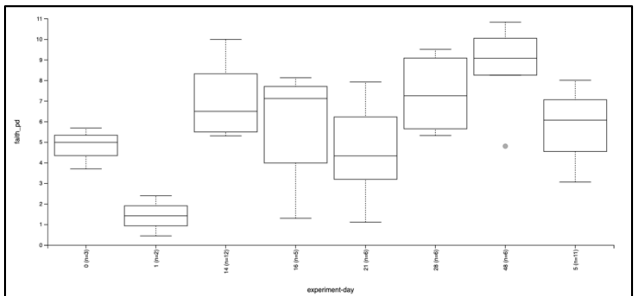
			vadinBB60 group *Clostridiales* (~7%), family Lachnospiraceae designation Torques group Ruminococcus (~6%), generic *Ruminococcaceae* (~6%)
x		**HB**	*Lactobacillus* (~83%), group 1 *Clostridiaceae* (~6%), *Clostridium sensu stricto* (~6%), unidentified bacterial reads (~1%)
y		**MP**	*Lachnospiraceae* (~17%), generic *Ruminococcaceae* (~12%), *Faecalibacterium* (~9%), *Ruminococcaceae* UCG-005 (~8%), *Ruminococcaceae* UCG-014 (~8%), family Lachnospiraceae designation Torques group Ruminococcus (~8%), vadinBB60 group *Clostridiales* (~5%), and Negativibacillus (~4%)

Table 2-2-Top Classifications, Midgut. Lactobacillus, Clostridioides, and Lachnospiraceae were the most common top classifications for midgut samples. Notably HA was dominated by E. coli at day 0 and seems to have less influence in the MP samples.

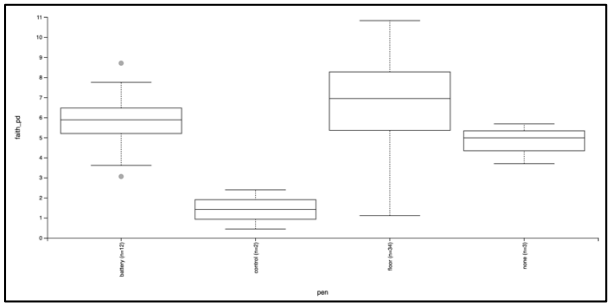
Supplementary Figures



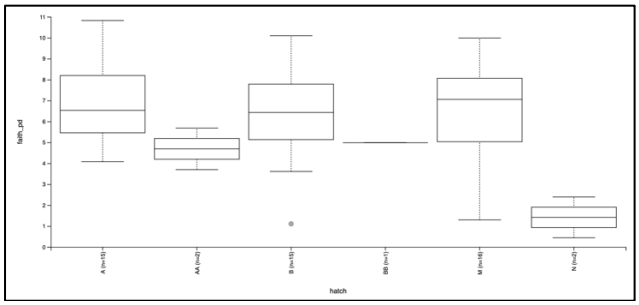
Supplement 1- Box plot of Faith's phylogenetic diversity over body site.



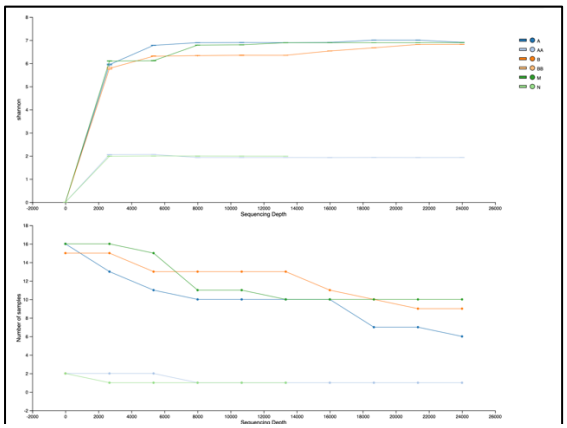
Supplement 2-Box plot of Faith's phylogenetic diversity over sample day.



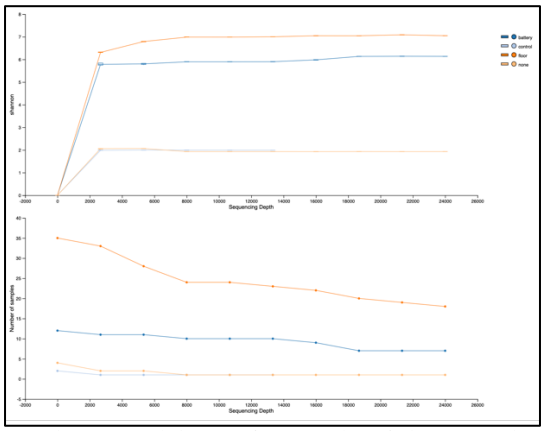
Supplement 3-Box plot of Faith's phylogenetic diversity over pen type.



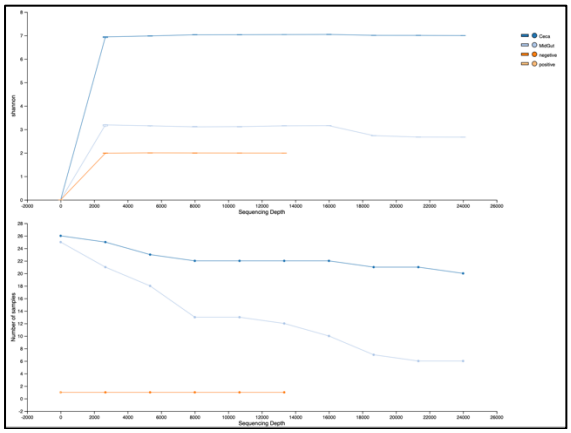
Supplement 4-Box plot of Faith's phylogenetic diversity over source hatchery.



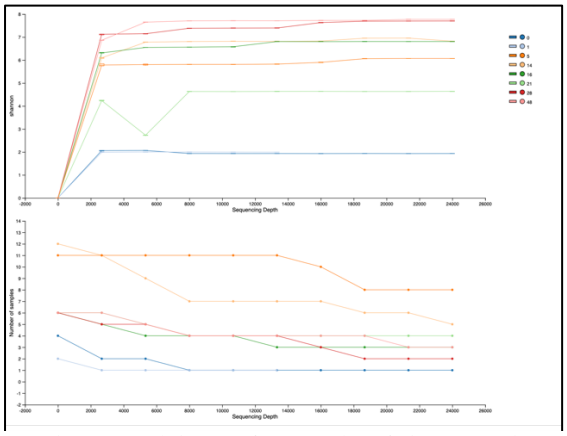
Supplement 5-Alpha rarefication plot of Shannon index over source hatchery



Supplement 6-Alpha rarefication plot of Shannon index over pen type.



Supplement 7-Alpha rarefication plot of Shannon index over body site.



Supplement 8-Alpha rarefication plot of Shannon index over sample day.

Definitions/abbreviations

Shannon index- (Shannon, 1948; Tuomisto, 2010)- Diversity index that predicts how diverse a community is. Originally proposed to compare differences between strings of text.

Faith's phylogenetic diversity-(Faith, 1992)-A phylogenetic generalization of species richness that measures average branch length of phylogenetic trees.

Jaccard coefficient-(Jaccard,1912)- The fraction of unique features in a sample set.

Pielou's evenness-(Pielou, 1966)- An index of diversity and species richness, on a scale of zero to one.

Operational taxonomic unit (OTU)-(Sokal & Sneath,1963)- an operational definition method used to classify groups of related entities by their similarity threshold.

References

[1] Food and Drug Administration. Veterinary feed directive. Federal Register. 2015;80(106):31708-35.

[2] Prescott JF, Sivendra R, Barnum DA. The use of bacitracin in the prevention and treatment of experimentally-induced necrotic enteritis in the chicken. The Canadian veterinary journal. 1978;19(7):181.

[3] Elwinger K, Berndtson E, Engström B, Fossum O, Waldenstedt L. Effect of antibiotic growth promoters and anticoccidials on growth of Clostridium perfringens in the caeca and on performance of broiler chickens. Acta Veterinaria Scandinavica. 1998;39(4):433-41.

[4] Van Immerseel F, Rood JI, Moore RJ, Titball RW. Rethinking our understanding of the pathogenesis of necrotic enteritis in chickens. Trends in microbiology. 2009;17(1):32-6.

[5] Tian J, Du J, Lu Z, Han J, Wang Z, Li D, Guan X, Wang Z. Distribution of microbiota across different intestinal tract segments of a stranded dwarf minke whale, Balaenoptera acutorostrata. MicrobiologyOpen. 2020;9(10):e1108.

[6] Wang W, Zheng S, Li L, Yang Y, Liu Y, Wang A, Sharshov K, Li Y. Comparative metagenomics of the gut microbiota in wild greylag geese (Anser anser) and ruddy shelducks (Tadorna ferruginea). Microbiologyopen. 2019;8(5):e00725.

[7] Li H, Li T, Beasley DE, Heděnec P, Xiao Z, Zhang S, Li J, Lin Q, Li X. Diet diversity is associated with beta but not alpha diversity of pika gut microbiota. Frontiers in microbiology. 2016;7:1169.

[8] Ferguson RM, Merrifield DL, Harper GM, Rawling MD, Mustafa S, Picchietti S, Balcàzar JL, Davies SJ. The effect of Pediococcus acidilactici on the gut microbiota and immune status of on-growing red tilapia (Oreochromis niloticus). Journal of applied microbiology. 2010;109(3):851-62.

[9] Little TJ, Hultmark D, Read AF. Invertebrate immunity and the limits of mechanistic immunology. Nature immunology. 2005;6(7):651-4.

[10] Brugman S, Ikeda-Ohtsubo W, Braber S, Folkerts G, Pieterse CM, Bakker PA. A comparative review on microbiota manipulation: lessons from fish, plants, livestock, and human research. Frontiers in nutrition. 2018;5:80.

[11] Segata N, Waldron L, Ballarini A, Narasimhan V, Jousson O, Huttenhower C. Metagenomic microbial community profiling using unique clade-specific marker genes. Nature methods. 2012;9(8):811-4.
[12] Human Microbiome Project Consortium. Structure, function and diversity of the healthy human microbiome. nature. 2012;486(7402):207.
[13] Langille MG, Zaneveld J, Caporaso JG, McDonald D, Knights D, Reyes JA, Clemente JC, Burkepile DE, Thurber RL, Knight R, Beiko RG. Predictive functional profiling of microbial communities using 16S rRNA marker gene sequences. Nature biotechnology. 2013;31(9):814-21.
[14] Nagpal R, Wang S, Solberg Woods LC, Seshie O, Chung ST, Shively CA, Register TC, Craft S, McClain DA, Yadav H. Comparative microbiome signatures and short-chain fatty acids in mouse, rat, non-human primate, and human feces. Frontiers in microbiology. 2018;9:2897.
[15] Kostic AD, Howitt MR, Garrett WS. Exploring host–microbiota interactions in animal models and humans. Genes & development. 2013;27(7):701-18.
[16] Gootenberg DB, Turnbaugh PJ. Companion animals symposium: humanized animal models of the microbiome. Journal of animal science. 2011 May 1;89(5):1531-7.
[17] Alzubaidy H, Essack M, Malas TB, Bokhari A, Motwalli O, Kamanu FK, Jamhor SA, Mokhtar NA, Antunes A, Simões MF, Alam I. Rhizosphere microbiome metagenomics of gray mangroves (Avicennia marina) in the Red Sea. Gene. 2016;576(2):626-36.
[18] Vandenkoornhuyse P, Quaiser A, Duhamel M, Le Van A, Dufresne A. The importance of the microbiome of the plant holobiont. New Phytologist. 2015;206(4):1196-206.
[19] Singh B: The Effects of Feeding Reduced-oil DDGS to Broilers when Challenged with C. perfringens and Eimeria spp. on Necrotic Enteritis and Intestinal Microbiome. Master's thesis. Auburn University, Poultry Science Department ; 2017.
[20] Wei S, Morrison M, Yu Z. Bacterial census of poultry intestinal microbiome. Poultry science. 2013 Mar 1;92(3):671-83.
[21] Wu GD, Chen J, Hoffmann C, Bittinger K, Chen YY, Keilbaugh SA, Bewtra M, Knights D, Walters WA, Knight R, Sinha R. Linking long-term dietary patterns with gut microbial enterotypes. Science. 2011;334:105-8.

[22] Wilkinson RK, Roberton AM. A novel glycosulphatase isolated from a mucus glycopeptide-degrading Bacteroides species. FEMS microbiology letters. 1988;50:195-9.
[23] Williams RB. Intercurrent coccidiosis and necrotic enteritis of chickens: rational, integrated disease management by maintenance of gut integrity. Avian pathology. 2005;34:159-80.
[24] Thompson CL, Vier R, Mikaelyan A, Wienemann T, Brune A. 'Candidatus Arthromitus' revised: segmented filamentous bacteria in arthropod guts are members of Lachnospiraceae. Environmental microbiology. 2012;14(6):1454-65.
[25] Bolyen E, Rideout JR, Dillon MR, Bokulich NA, Abnet CC, Al-Ghalith GA, Alexander H, Alm EJ, Arumugam M, Asnicar F, Bai Y. Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. Nature biotechnology. 2019;37(8):852-7.
[26] Callahan BJ, McMurdie PJ, Rosen MJ, Han AW, Johnson AJ, Holmes SP. DADA2: high-resolution sample inference from Illumina amplicon data. Nature methods. 2016;13(7):581-3.

Availability of data and materials

The amplicon sequencing data obtained in this study are available in the National Center for Biotechnology Information under accession number PRJNA759337.

Chapter 3 -The Effect of Lighting and Temperature as Stressors on the Broiler Microbiome in Floor Raised vs Cage Raised Birds

Abstract

Background: Microbial communities in the gut are influenced and shaped by an individual's interactions with their environment from the first day of life. This study aimed to observe any effects on gut microbial populations due to less-than-optimal starting conditions. Day old broilers were placed in both floor pens and battery cages in four different rooms. Room 1- normal starting lighting (3 foot candles [fc]), normal starting temperature (~33^{o}C). Room 2- half normal starting lighting (1.5 fc), normal starting temperature (~33^{o}C). Room 3- normal starting lighting (3 fc), 5 degree lower starting temperature (~28^{o}C). Room 4- half normal starting lighting (1.5 fc), 5 degree lower starting temperature (~28^{o}C). Lighting was adjusted down at day 7 (1 fc normal, .5 fc low) and day 12 (.3 fc normal, .15 fc low). Temperature was adjusted down weekly by ~5^{o}. Samples of the crop, midgut (defined as the area between the duodenal loop and Meckel's diverticulum) and the ceca were taken at day 0,5,14, 28. And 36. Samples were DNA extracted, amplified via PCR, and sequenced using 16S rRNA Illumina MiSeq protocol. Raw reads were then passed through the QIIME bioinformatics pipeline for analysis.

Results: Midgut samples from birds in high temperature/low light and low temperature/low light pens had a higher prevalence of *Lactobacillus* when compared to high temperature/high light and low temperature/high light pens. Low temperature pens displayed less differences in proportional representation between microbial community members. *Faecalibacterium* was present in all low temperature pen ceca samples at day 5, in contrast with no detectible levels in high temperature pens despite having been present in day 0 sampling.

Conclusions: Starting light and temperature both had impacts on proportional representation of members in microbial gut populations. Brighter lighting conditions may inhibit beneficial

bacteria. Lower starting temperatures may encourage less desirable populations but may also encourage greater diversity.

Keywords: Broiler Chickens, Microbiota, Influence, Beneficial, Diversity

Background

The October of 2015 enactment of the Veterinary Feed Directive that banned usage of all subtherapeutic antibiotics in production animals [1] including those used to control necrotic enteritis in broiler chickens [2, 3], caused the incidence diseases previously managed by these prophylactics to increase. Because the option of subtherapeutic level antibiotics is no longer available, the poultry industry has turned a renewed interest to the gut and the potential to perhaps replace some of the lost efficiencies by its manipulation and more precise management[4]. As the amount of bioinformatic studies increase, a clearer picture is formed on what precisely should be targeted to help both the wellbeing of the animal and the needs of the producer. The gut has been subject to many general studies, in-depth looks at individual factors are still relatively rare with new "first known examination of" research being regularly published [5, 6, 7, 8, 9]. Because species are often studied on a human centric basis, the microbiome data of many organisms is obscure simply because it doesn't have a large enough impact on the human biosphere; however it has become more evident that even relatively obscure organisms can have hidden aspects that could be of great interest and use [10, 11, 12, 13, 14, 15, 16, 17, 18].

Stress can have profound impact upon the physiology and health of the gut and its microbial inhabitants [3, 8, 10]. Access or lack thereof to essential nutrients or basic needs sets the baseline for the entire organ and the communities within it. Furthermore the environment in which the host resides can encourage or hinder the host's bacterial communities, to the extent at which hosts with more or less completely identical backgrounds placed in different environments possess notably different microbial communities[3, 8, 10, 19] and may be more or less vulnerable to large scale community shifts when compared to the other host. This trial was conducted to further explore the relationship between differentiation in day 1 starting environments and populations within the broiler chicken gut.

Results

General overall patterns

Succession in the Crop Microbiota

For crop samples of birds reared under standard temperatures microbial communities rapidly changed from day 0 to day 5 with the exception of high temperature/high lighting battery birds, whereas birds placed in less than optimal conditions retained more of the day 0 characterization through day 5. From day 14 onward all treatments returned to a similar composition to day 0 with few exceptions. **See Fig. 3-4 for a visual representation of ratios of top classifications as well as Table 3-1 for a list of top percentages.**

Crop samples in general trend towards lower diversity due to the swiftly changing environment within the crop. For the Kruskal-Wallis (pairwise) significance of Faith's phylogenetic diversity (PD) test between body site sample types crop samples averaged a branch length of 17.187 at the deepest sampling depth (7 samples) and 11.738 at the depth of 3333 (19 samples); being not significantly different with a p-value of 0.865 when compared to midgut samples which a branch length of 19.863 at the deepest sampling depth of 75,000 (5 samples) and 13.568 at the depth of 3333 (17 samples). Branch length was significantly shorter ($p= 9.07 \times 10^{-9}$) when compared to ceca samples at the deepest sampling depth 17.428 (13 samples) and 13.49 at the depth of 3333 (29 samples).

Crop samples exhibited shallow sequencing depth which may result in lower accuracy reads, with 7 out of 32 samples reaching the sampling depth of 75,000 similarly to mid gut samples 5 out of 32 total samples reaching the same depth. Comparatively, 13 of 32 cecal samples reached the maximum depth. Crop samples presented a lower average number of observed OTUs with the average crop sample OTU count being ~295, the average midgut sample OTU count being ~416, and the average ceca sample OTU count being ~472 at the max sequencing depth of 75,000. Crop samples displayed lower and less variable evenness (Pielou's Evenness index) compared to midgut samples and much lower evenness when compared to cecal samples; crop samples displayed an average of 0.643 (high of 0.914, low 0.484), cecal samples

displayed an average of 0.889 (high of 0. 936, low 0.658), and midgut samples at an average of 0.66 (high of 0. 905, low 0.443). When run with a Kruskal-Wallis (pairwise) significance test results were statistically significant with $p = 3.05x10^{-11}$. As time increased the communities within the crop became more standardized with the top classification across all days being *Lactobacillus*. The Jaccard coefficient PCOA (Fig.3-3(a-e)) demonstrates visually how after an initially close grouping over the first few weeks of life (D 0 to D 5), the different treatments became more and more distinct over time. Shannon diversity (SD) score was relatively high over all for all samples, the average being 4.87.

Succession in the Midgut Microbiota

For midgut samples of birds placed in normal temperatures microbial communities rapidly changed from day 0 to day 5 with the exception of high temperature/high lighting battery birds which retained most day 0 characteristics. Birds placed in less than optimal conditions retained more of the day 0 characterization through day 5 with the exception of low temperature/high lighting floor birds. From day 14 onward high temperature treatments retained similar compositions through day 36 with few exceptions, whereas low temperature treatments showed a great deal more variability in composition. Birds raised in low light were more likely to be characterized by *Lactobacillus* with 9 out of 16 sample days showing *Lactobacillus* as its top classification vs high light birds having *Lactobacillus* as top classification 4 sample days out of 16. High light birds were more often characterized by *Clostridioides,* representing the top classification of 8 out of 16 sample days in contrast to low light birds with 6 of 16. **See Fig. 3-5 for a visual representation of ratios of top classifications as well as Table 3-2 for a list of top percentages.**

Midgut samples in general trend towards moderate diversity due to the transient nature of the gut. For the Kruskal-Wallis (pairwise) significance of Faith's phylogenetic diversity (PD) test between body site sample types midgut samples averaged a branch length of 19.863 at the deepest sampling depth of 75,000 (5 samples) and 13.568 at the depth of 3333 (17 samples); being not significantly different with a p-value of 0.865 when compared to midgut samples which a branch length of 17.187 at the deepest sampling depth (7 samples) and 11.738 at the depth of 3333 (19 samples).

Branch length was significantly shorter ($p= 3.55 \times 10^{-6}$) when compared to ceca samples at the deepest sampling depth 17.428 (13 samples) and 13.49 at the depth of 3333 (29 samples). Midgut samples exhibited shallow sequencing depth which may result in lower accuracy reads, with 5 out of 32 samples reaching the sampling depth of 75,000. Midgut samples presented a median average number of observed OTUs with the average midgut sample OTU count being ~416 at the max sequencing depth of 75,000. Midgut samples displayed similar evenness (~0.66, high of 0. 905, low 0.443) compared to crop samples and much lower evenness when compared to cecal samples. When run with a Kruskal-Wallis (pairwise) significance test results were statistically significant with $p = 3.44 \times 10^{-10}$. Jaccard coefficient PCOA (Fig. 3-3 (a-e)) a rapid divergence from day 0 onward, becoming more divergent over time. Shannon diversity (SD) score was relatively high over all for all samples, the average being 5.5.

Succession in the Cecal Microbiota

Though distinctly diverse at day zero, as birds matured cecal communities settled into a more standardized pattern as time increased. Though the top taxa varied from sample day to sample day the top three or four genera typically fell within +/- 5% of one another in terms of percent abundance. **See Fig. 3-6 for a visual representation of ratios of top classifications as well as Table 3-3 for a list of top percentages.**

Cecal samples were the most diverse according to the Kruskal-Wallis (pairwise) significance of Faith's phylogenetic diversity (PD) test between body site sample types with ceca samples averaging a branch length of 17.428 (13 samples) at deepest sampling depth (75,000) and 13.49 at the depth of 3333 (29 samples); being extremely significant with a p-value of 9.07×10^{-9} when compared to crop samples and a p-value of 3.55×10^{-6}. Cecal samples presented a much higher average number of observed OTUs a with the average cecal sample OTU count being ~472 at the max sequencing depth of 75,000 and ~355 at the depth of 3,333.

Ceca samples exhibited much higher and less variable evenness compared to midgut or crop samples, with cecal samples displaying an average of 0.889 (high of 0.936, low 0.658). Kruskal-Wallis (pairwise) significance was statistically significant with a p-value of 3.43×10^{-10} when compared to crop samples and a p-value of 2.54×10^{-10} when compared to crop samples.

Communities within the ceca mostly shared the top five genera of *Lachnospiraceae*, generic *Ruminococcaceae*, *Ruminococcus* (torques group), and uncultured *Faecalibacterium*. The Jaccard coefficient PCOA (Fig. 3-3(a-e)) shows that as time progressed the cecal communities stayed much more similar up until day 36 when compared to crop and midgut samples, at which there was some visible diversification.

Shannon diversity (SD) score was high over almost all samples, the average being 7.54 at deepest sampling and 7.19 at the depth of 3,333.

Trends in Pen Types

Battery samples averaged a PD score of ~18.08 (high 22.33, low 16.01) at the max sequencing depth of 75,000. Floor samples averaged a PD score of ~17.6 (high 21.47, low 12.18). Samples were not significantly different from one another when analyzed using Kruskal-Wallis.

Battery samples presented an average ~417 of observed OTUs (high 644, low 175) at the max sequencing depth of 75,000. Floor samples averaged ~405 observed OTUs (high 605, low 139). Samples did not display significantly different evenness when compared with Kruskal-Wallis (pairwise) significance test with battery displaying an average evenness of 0.730 and floor pens an average of 0.735 .

Battery crop samples showed *Lactobacillus* as the top classification for all sample points. For battery midgut samples, top classifications were high variable between time points and pen conditions with top genus being *Lactobacillus* for 3 of 4 treatments (**high temperature/high light** [HTHL], **high temperature/low light**[HTLL], **low temperature/low light** [LTLL]) on day 14, 3 of 4 treatments (HTHL, HTLL, LTLL) on day 28, and 2 of 4 treatments (HTLL, **low temperature/high light** [LTHL]) on day 36. *Clostridioides* was top classification in 3 of 4 treatments (HTHL, LTHL, LTLL) on day 5, only LTHL treatment on day 14, and 3 of 4 treatments (HTHL, HTLL which shared with top spot with *Lactobacillus*, LTLL) on day 36 . The only day *Lachnospiraceae* was top classification for HTLL day 5. An uncultured *Faecalibacterium* represented the remaining two samples on day 28 (LTHL, LTLL).

Battery ceca samples top classifications were either shared or represented by *Lachnospiraceae* on all days and treatments save for day 14 LTHL, day 28 LTHL and LTLL, and day 36 LTLL.

An uncultured *Faecalibacterium* was top or shared top classification for day 14 HTLL and LTHL, all treatments on day 28, and 3 of 4 treatments on day 36 (HTLL, LTHL, LTLL). Generic *Ruminococcaceae* shared top classification in day 5 LTLL, day 14 LTHL, and day 28 HTHL. *Ruminococcaceae* UCG-014 shared top spot of day 36 HTHL and LTHL.

Floor crop samples showed *Lactobacillus* as the sole top classification for all sample points except for day 5 HLHT in which it shared top classification with *Weissella*.

Floor midgut samples, like battery midgut samples were high variable between time points and pen conditions. *Lactobacillus* filled top classification for 2 of 4 treatments (HTHL, HTLL) on day 5, 2 of 4 treatments (HTLL, LTLL) on day 14 , and HTLL on day 36. *Clostridioides* was top classification in LTLL and HTLL on day 5, HTHL treatment on day 14, HTHL, on day 28, and in all treatments on day 36 . The only day *Lachnospiraceae* was top classification for LTHL day 5. An uncultured *Faecalibacterium* represented LTHL on day 14 and two samples on day 28 (LTHL, LTLL). Top classifications for floor ceca samples were more varied than battery samples. *Lachnospiraceae* either shared or represented top classification in all treatments on day 5, 2 of 4 samples on day 14 (HTHL, LTHL), 2 of 4 samples on day 28 (HTHL, LTLL), and 2 of 4 samples on day 36 (HTHL, LTHL). An uncultured *Faecalibacterium* was top or shared top classification of 3 of 4 treatments for day 14 (HTHL, HTLL, LTLL), 3 of 4 treatments on day 28 (HTLL, LTHL, LTLL), and 2 of 4 treatments on day 36 (HTLL, LTHL). Generic *Ruminococcaceae* shared top classification in day 5 LTLL, and day 14 HTHL and LTHL. *Ruminococcaceae* UCG-014 shared top spot of day 14 and 28 for LTLL. Battery Shannon diversity (SD) score was moderate over samples at max sampling depth, the average being 6.456 (high 8.077, low 2.907). Floor Shannon diversity (SD) score was also moderate over samples at max sampling depth, the average being 6.308 (high 8.015, low 3.601). Battery was not statistically significant from the floor pen.

Trends in Normal Temperature and Low Temperature

High temperature (HT) samples averaged a PD score of ~17.85 (high 22.33, low 14.01) at the max sequencing depth of 75,000. Low temperature (LT) samples averaged a PD score of ~17.85 (high 21.78, low 12.18). Samples were not significantly different from one another when analyzed using Kruskal-Wallis. HT samples presented an average ~422 of observed OTUs (high 644, low 139) at the max sequencing depth of 75,000. Floor samples averaged ~403 observed

OTUs (high 589, low 245). Samples did not display significantly different evenness when compared with Kruskal-Wallis (pairwise) significance test with HT displaying an average evenness of 0.730 and LT pens an average of 0.735. HT crop samples showed *Lactobacillus* as the top classification for all sample points except for day 5 High Light Floor pen (HLF).
HT midgut samples displayed either *Lactobacillus* (9 of 16 samples- D5: High Light Battery (HLB) and HLF, Low Light Floor(LLF). D14: HLB, Low Light Battery (LLB) and LLF. D28: HLF, LL battery and floor. D36: LLB), *Clostridioides* (8 of 16 samples- D5: HLB, LLF. D14: HLF. D28: HLF. D36: HLB, HLF, LLB, LLF), or *Lachnospiraceae* (D5: LLB).
High temperature ceca samples top classifications were either shared or represented by *Lachnospiraceae* on all days and treatments with the exception of day 14, 28, and 36 in LLF samples. An uncultured *Faecalibacterium* was top or shared top classification for day 14 (LLB, LLF,HLF), day 28 (HLB, LLB, LLF), and day 36 (LLB, LLF). Generic *Ruminococcaceae* shared top classification in day 14 HLF and day 28 HLB. *Ruminococcaceae* UCG-014 shared top spot of day 36 HLB.
LT crop samples showed *Lactobacillus* as the top classification for all sample days. LT midgut samples displayed a highly variable selection of top genus across sample days.
Clostridioides represented top classification for 7 of 16 sample points: day 5 (HLB, LLB, LLF), day 14 (HLB), and day 36 (LLB, HLB, HLF). An uncultured *Faecalibacterium* was top classification for 6 of 16 samples: day 14 (HLF) and day 28 (HLB, LLB, HLF, LLF). *Lactobacillus* as the top classification for 4 of 16 samples: day 14 (LLB, LLF), day 28 (LLB), and day 36 (HLB). *Lachnospiraceae* was top classification for day 5 HLF.
LT ceca top classifications were more variable than HT ceca samples. *Lachnospiraceae* represented or shared top representation in 9 of 16 samples: day 5 (HLB, LLB, HLF, LLF), day 14 (LLB, HLF), day 28 (LLF), day 36 (HLB, LLF). An uncultured *Faecalibacterium* was top or shared top classification 9 of 16 samples: day 14 (HLB, LLF), day 28 (HLB, LLB, HLF, LLF), day 36 (HLB, LLB, HLF). Generic *Ruminococcaceae* shared top classification in day 5 LLB and LLF, and day 14 HLB and HLF. *Ruminococcaceae* UCG-014 shared top classification in day 14 and day 28 LLF, and day 36 LLB. HT Shannon diversity (SD) score was moderate over samples at max sampling depth, the average being 6.20 (high 10.96, low 2.60). LT Shannon diversity (SD) score was also

moderate over samples at max sampling depth, the average being 6.997 (high 11.98, low 2.05). HT was not statistically significant from LT.

Trends in Normal Lighting and Low Lighting

High Lighting (HL) samples averaged a PD score of ~18.02 (high 22.33, low 12.17) at the max sequencing depth of 75,000. Low Light (LL) samples averaged a PD score of ~17.67 (high 20.28, low 14.01). Samples were not significantly different from one another when analyzed using Kruskal-Wallis. HL samples presented an average ~408 of observed OTUs (high 644, low 175) at the max sequencing depth of 75,000. LL averaged ~415 observed OTUs (high 605, low 139). Samples did not display significantly different evenness when compared with Kruskal-Wallis (pairwise) significance test with HL displaying an average evenness of 0.732 and LL pens an average of 0.729.

HL crop samples showed *Lactobacillus* as the sole top classification for all sample points except for day 5 HTF in which it shared that classification with *Weissella*.

HL midgut samples displayed either *Clostridioides* (8 of 16 samples- D5: HTB, LTB. D14: HTF, LTB. D28: HTF.), *Lactobacillus* (4 of 16 samples- D5: HTF. D14:HTB. D28: HTB. D36: LTB), uncultured *Faecalibacterium* (D14: LTB, LTF. D28: LTF), and *Lachnospiraceae* (D5: LTF).

HL ceca samples top classifications were for the most part shared or represented by *Lachnospiraceae* (12 of 16 samples- all HTB and HTF samples on all days. D5: LTB, LTF. D14: LTF. D36: LTB). An uncultured *Faecalibacterium* was also present (7 of 16 samples- D14: HTF, LTB. D28: HTB, LTB, LTF. D36: LTB, LTF). Generic *Ruminococcaceae* shared top classification on day 14 (HTF, LTB, LTF) and day 28 (HTB). *Ruminococcaceae* UCG-014 shared top classification on day 36 HTB and LTB. LL crop samples showed *Lactobacillus* as the top classification for all sample days. LL midgut samples displayed moderate variability in top genus across sample days. *Lactobacillus* was top classification for 9 of 16 samples: day 5 (HTF), day 14 (HTB, HTF, LTB, LTF),day 28 (HTB, HTF,LTB), and day 36 (HTB). *Clostridioides* represented or shared top classification for 7 of 16 sample points: day 5 (HTF, LTB, LTF) and day 36 (HTB, HTF, LTB, LTF). An uncultured *Faecalibacterium* was top or shared top classification for day 28 LTB and LTF. *Lachnospiraceae* was top classification for day 5 HTB.

LL ceca top classifications were slightly more variable than HL ceca samples. *Lachnospiraceae* represented or shared top representation in 10 of 16 samples: day 5 (HTB, HTF, LTB, LTF), day 14 (HTB, LTB), day 28 (HTB, LTF), day 36 (HTB, LTF). An uncultured *Faecalibacterium* was top or shared top classification 10 of 16 samples: day 14 (HTB, HTF, LTF), day 28 (HTB, HTF, LTB, LTF), day 36 (HTB, HTF, LTB). Generic *Ruminococcaceae* shared top classification in day 5 LTB and LTF. *Ruminococcaceae* UCG-014 shared top classification in day 14 and day 28 LTF. HL Shannon diversity (SD) score was moderate over samples at max sampling depth, the average being 6.37 (high 8.08, low 2.91). LL Shannon diversity (SD) score was also moderate over samples at max sampling depth, the average being 6.4 (high 8.02, low 3.60). HL was not statistically significant from LL.

Discussion

Overall, all environmental changes enacted in this trial exerted some influence over the composition of the microbial communities present with some microbes having notable preferences for specific environments.

Bacterial Colonization of the Gastrointestinal Tract

Like previous trials [19] there was an initial day 5 diversification, especially in cecal and midgut samples. However unlike previous trials the cecal samples did not have as great of change in composition between sample days, staying close to the original plot of the PCOA (Fig. 3-3(a-e)). There was a visible difference in the proportions of microbial populations depending on the host birds rearing environment as well, with some classifications being much more prominent in certain situations.

Crop

Crop samples changed very little over all sample days. *Lactobacillus* was dominant as the top classification for all days except day 5 **high temperature/high light/floor** [HTHLF] which was shared with *Weissella*. *Weissella* was present in day 0 samples at a high level but fell quickly by day 5. By day 14 it was not detected at a notable level in most samples except the occasional battery sample. Interestingly, presence of non-chicken-related microbes and non-microbial DNA did fluctuate between sample points with one notable incursion on day 28 samples having contained bee or wasp DNA and related bee or wasp microbiome community members.

Midgut

Most midgut samples retained their day 0 characteristics of a strong *Clostridioides* presence through day 36 with only 8 samples showing either low or no presence. Similarly *Lactobacillus* remained present in all samples at varying levels over all time points. Inversely the uncultured *Faecalibacterium* was not present at day 0, beginning to appear on day 5 in small proportions (especially in lower temperature rooms) and becoming more prevalent as time progressed culminating on day 36 as being present in reasonably large proportions. Similarly *Lachnospiraceae* started as a low proportion or not present at all in most day 0 and 5 samples, becoming a notable proportion of the population by day 36.

Ceca

Similarly to previous observations [19], most ceca samples changed very little from day 0 to day 5 retaining *Lachnospiraceae* as the dominant classification in varying proportions. From day 14 onward *Lachnospiraceae* became less dominant though no less prevalent, continuing as a sizable presence through day 36. Comparable to midgut samples, uncultured *Faecalibacterium* was present in low proportion at day 0. The microbe was present in LT environments but not detectible in HT environments at day 5 and continued to be present through day 14 onward to day 36. Both generic *Ruminococcaceae* and *Ruminococcaceae* UCG-014 steadily increased in representation from day 0 to day 36. Similarly to trial 1 (*Chapter 2-Establishment of Base Interactions of Select Classifications Between Multiple Chick Sources in Differential Housing*) ceca samples remained comparably even through all sample days, with evenness increasing with time (Fig. 3-3(a-e)). When plotted with a Jaccard biplot in QIIME2 ceca samples seemed most influenced by uncultured *Faecalibacterium* and possibly *Clostridiaceae*.

Differences Between Pen types

There was very little difference in crop composition between housing types beyond the ratio of uncultured *Faecalibacterium* in floor samples being higher than that of battery samples as time progressed.

Housing type seemed to influence midgut sample composition the most of all sample types with *Clostridioides* being much more dominant and/or common in floor samples than battery samples. Inversely, battery samples contained equal or higher proportions of *Lactobacillus*

alongside a proportionally reduced *Clostridioides* population. In general, floor samples tended to display lower ratios between top classifications starting at day 5 while battery samples maintained greater differences in proportional representation through the first few sample days with these differences reducing in size as time went on.

Differences Between Lighting Levels

The most notable influence of lighting levels seemed to be on the presence of *Lactobacillus* in midgut samples, specifically in low light environments with 9 of 16 samples taken from low light pens having *Lactobacillus* as the top or shared top classification vs 4 of 16 samples taken from high light pens. This trend is evenly spread between both battery and floor pens, becoming less common in day 28 and 36. It is unclear whether this affinity is due to a sensitivity to available light, less competition with microbes that prefer higher light levels, or another unseen factor. Uncultured *Faecalibacterium* seemed also to prefer lower light conditions, becoming more prevalent in samples day 14 and onward, however since it also seems to be more prevalent in lower temperature settings there are most like tertiary factors affecting its proportional representation. These differences were not statically significant when compared using an ANOVA analysis ($p=0.178$ in normal vs low light, $p=0.0575$ low temperature/normal light vs. normal temperature/ low light, $p=0.0779$ when comparing sample days, all other p-values > 0.28), however a repeated trial with more than a single day sample per treatment may clarify the trend.

Differences Between Temperatures

A few prominent overall trends regarding high and low starting temperatures emerged. The first was that uncultured *Faecalibacterium* initially was only found in low temperature ceca samples on day 5, despite being present at low but detectible levels in day 0 chicks. Uncultured *Faecalibacterium* becomes more prevalent as time progresses seemingly without preference for pen type or light level; it became a top classification in a large portion of samples at day 14, a time at which high temperature pens would be set to the same temperature (~28^{o}C) as day 0 low temperature pens had been set two weeks prior. The uncultured *Faecalibacterium* became prevalent to the point that of the 16 combined day 28 and day 36 samples only 4 did not have the microbe as a top classification.

A second trend seems to be that *Clostridioides* seems to favor high temperature environments being more prevalent early on in higher temperature ceca and midgut samples. However since it also appears later on albeit with in lower proportion, the simpler conclusion may be while temperature was beneficial *Clostridioides* may just be highly opportunistic.

Conclusion

Starting light and temperature both had impacts on proportional representation of members in microbial gut populations. Brighter lighting conditions may inhibit beneficial bacteria. Lower starting temperatures may encourage less desirable populations but may also encourage greater diversity. Future studies may want to explore more extreme deviations in environmental settings such as extremely low light (1.5 ftc) from day zero or a 26.7°C starting temperature. Though it was expected that temperature would have an impact on proportional populations, lighting also seem to have a visible affect. Because of this, bacteria like lactobacillus that are widely considered to be beneficial seem to be present in higher proportion in a low light high temperature environment. However light intensity also seem to have an impact on lactobacillus presence in low temperature environments as well, being more common in a low light low temperature environment than a high light low temperature environment regardless of pen type.

Methods

Bird and Farm Management

Unsexed and unvaccinated day-old broiler chicks were obtained from a commercial hatchery and transported to the Auburn University Poultry Research Farm. Birds were split into eight groups:

A-Floor, normal starting lighting (3 foot candles [fc]), normal starting temperature (~35^{o}C).

B-Battery, normal starting lighting (3 fc), normal starting temperature (~35^{o}C).

C- Floor, half normal starting lighting (1.5 fc), normal starting temperature (~35^{o}C).

D- Battery, half normal starting lighting (1.5 fc), normal starting temperature (~35^{o}C).

E- Floor, normal starting lighting (3 fc), ~6-degree lower starting temperature (~28^{o}C).

F- Battery, normal starting lighting (3 fc), ~6-degree lower starting temperature (~28^{o}C).

G- Floor, half normal starting lighting (1.5 fc), ~6-degree lower starting temperature (~28^{o}C).
H- Battery, half normal starting lighting (1.5 fc), ~6-degree lower starting temperature (~28^{o}C).

These groups were randomly placed in two pen types in four rooms: traditional floor(1 pen per room), and battery cages (3 per room). Floor pens received 30 birds each. Batteries received 10 per cage. Rooms **1** and **2** were held at 35 ± 2°C for the first 7 days and reduced 2-4°C weekly as per industry standard. Rooms **3** and **4** were held at 28 ± 2°C for the first 7 days and reduced 2-4°C weekly. Rooms **1** and **3** lighting was started at a normal lighting of 3 foot candles as per industry standard, reduced to 1.5 fc at day 7 and again reduced to 0.3 at day 12. Rooms **2** and **4** lighting was started at a reduced 1.5 fc, being further reduced to 0.3 fc at day 7 and again to 0.15 fc at day 12.

Birds were fed a standard starter diet from day 0 to day 14, grower from day 15 to day 28, then switched to a finisher diet for the remainder of the trial. Necropsies to acquire samples of the crop, midgut (defined as the section between the duodenal loop and Meckel's diverticulum) and entire ceca were conducted at days 0, 5, 14, 28, 36.

Four birds per group were euthanized via CO_2 asphyxiation and sampled using aseptic technique. Samples were individual stored by treatment and type then placed on ice after acquisition. After transport to the lab samples were placed in -80^{o}C freezer until DNA extraction.

DNA Extraction

After removal from the -80^{o}C freezer, ceca and midgut samples were thawed and extracted using the Omega Bio-tek E.Z.N.A. Stool DNA Extraction kit according to manufacturer's instructions, with one modification in that for step 4 DNA was incubated at 54^{o}C overnight followed by 10 minutes at 70^{o}C to ensure cell breakdown due as per recommendations from Omega Bio-tek trouble-shooting staff. Extracted DNA was tested for concentration and purity with a NanoDrop® ND-1000 Spectrophotometer, the desired concentration being a 260nm/280nm ratio between 1.8 - 2.0. Afterwards, DNA was place in a 40^{o}C refrigerator to await further processing.

PCR Amplification

DNA amplification via PCR was performed at Auburn University using a BioRad iQ5 thermocycler by touchdown protocol in order to maximize the amount of DNA amplified (Fig 3-1). To that point universal target primers CS1/515F (5'-GTGYCAGCMGCCGCGGTAA-3') and CS2/926R (5'-CCGYCAATTYMTTTRAGTTT-3') were used to further expand potential targets. Amplification was confirmed with agarose gel electrophoresis on a 2% gel made with TAE buffer using Lonza® 100 bp Extended Range DNA Ladder. Gels were run for 75v for ~1 hour or until satisfactory visual conformation. Following confirmation, DNA PCR product was stored in a 40^{o}C refrigerator.

Illumina MiSeq Sequencing

PCR product was consequently pooled by type and treatment, labeled, and sent to University of Illinois Chicago DNA Services Facility (UIC DNAS) facility under temperature-controlled conditions for 16S rRNA Illumina gene sequencing.

Data Analysis and Statistics

Following sequencing, raw FASTQ files were uploaded to the Illumina BaseSpace cloud database. Raw FASTQ files were downloaded from BaseSpace and uploaded to the Alabama Supercomputer (ASC) for more in-depth memory heavy analysis with QIIME2 pipeline [20]. Fastq files in Casava 1.8 paired end demultiplex format were read into QIIME2 to be joined and denoised using DADA2 [21].

Denoising and dereplication proceeded based on demultiplexing stats with forward reads being truncated at 220 base pairs and a max error rate of 4. The resulting feature table and representative sequences table were further filtered to exclude eukaryotic sequences and then used to determine optimum sampling depth in order to retain the most features without excluding a large number of samples. The highest feature frequency per sample being 185,844 , the lowest being 642 and the mean frequency being 49215 (Fig. 3-2). For diversity analysis a sampling depth of 900 was chosen to retain as many samples as possible. Reads with a frequency less than 5 were removed from sampling. Taxonomic classification plug-in classify-sklearn was run using the Silva 132 99% full length classifier sequences set to identify OTUs. Using the classified data, filtering was preformed using the "filter table" function to remove

sequences identified as belong to Eukaryotic organisms to eliminate host DNA. A phylogenetic diversity analyses tree was generated using the q2-phylogeny plug-in. The QIIME2 diversity core-metrics-phylogenetic command was used to generate alpha and beta diversity analysis. QIIME2 diversity alpha-rarefaction command was used to generate an alpha rarefaction curve. A PCOA biplot was generated using the “diversity pcoa_biplot” command to establish a directional key for the prominent taxa responsible for some of the biggest shifts in the Jaccard graph. Taxonomic bar-plots were generated by loading taxonomic data into R Studio and generating barplots using the ggplots package.

Figures

Reaction conditions	1 x Rxn volume (µL)
Stage 1[1]	
Master Mix[2]	12.5
DdH_2O[3]	10.75
DNA Template[4]	1.0
Forward Primer[5]	0.5
Reverse Primer[6]	0.5
BSA[7]	0.25
Stage 2[8]	
Primers[9]	0.4
Stage 1 PCR Yield	1.0

[1]Performed at Auburn University Laboratory (Auburn, AL).

[2]Master Mix = Lucigen EconoTaq Plus 2x Master Mix (Middleton, WI): 0.1 units/µL of EconoTaq DNA Polymerase, Reaction Buffer (pH 9.0), 400 µM dATP, 400 µM dGTP, 400 µM dCTP, 400 µM dATP, 3 mM MgCl2, and a proprietary mix of PCR Enhancer/Stabilizer and blue and yellow tracking dyes.

[3]DdH_2O = double distilled H_2O.

[4]DNA Template = pooled jejunum tissue sample (5 birds/pen, each 2 cm in length, 15 cm proximal from Meckel's diverticulum) bacterial DNA extracted using Wizard Genomic Purification Kit (Madison, WI).

[5]Forward Primer = Eurofins Genomics CS1/515F Universal Target 16S rRNA Gene (Louisville, KY): 5'-GTGYCAGCMGCCGCGGTAA-3', expected amplicon size with CS1 456, annealing temperature 50°C.

[6]Reverse primer = Eurofins Genomics® CS2/926R Universal Target 16S rRNA Gene (Louisville, KY): 5'-CCGYCAATTYMTTTRAGTTT-3', expected amplicon size with CS2 456, annealing temperature 50°C.

[7]BSA (Bovine Serum Albumin) = Purified BSA 100x (Lawrenceville, GA).

[8]Performed at University of Illinois Chicago DNA Services Facility (Chicago, IL).

[9]Primers = Fluidigm's AccessArray Barcoded Primers (San Francisco, CA): Read 1: Standard CS1: A+CA+CTG+ACGACATGGTTCTACA; Index Read: CS2rc: A+GAC+CA+AGTCTCTGCTACCGTA; Read 2: Standard CS2: T+AC+GGT+AGCAGAGACTTGGTCT (A "+" preceding a base indicates a Locked Nucleic Acids(LNA)-base).

Cycle Step	Temperature	Time	Cycles
Stage 1[1]			
Initial Denaturation	94°C	5 min	1x
Variable Temperature Annealing			15x, reduce 1°C/cycle
Denaturation	94°C	30 sec	
Annealing	60°C	30 sec	
Extension	72°C	30 sec	
Constant Temperature Annealing	94°C		30x
Denaturation	94°C	30 sec	
Annealing	55°C	30 sec	
Extension	72°C	30 sec	
Final Extension	72°C	7 min	1x
Hold	4°C	∞	
Stage 2[2]			
Initial Denaturation	95°C	5 min	1x
Variable Temperature Annealing			28x
Denaturation	95°C	30 sec	
Annealing	60°C	30 sec	
Extension	72°C	30 sec	

[1]Performed at Auburn University Laboratory (Auburn, AL) using a Bio-Rad IQ5 thermocycler (Hercules, CA).

[2]Performed at University of Illinois Chicago DNA Services Facility (Chicago, IL) in preparation for Illumina MiSeq (San Diego, CA).

Figure 3-1- Reaction conditions and primers for amplification of bacterial populations by polymerase chain reaction (PCR) via touchdown protocol [22]

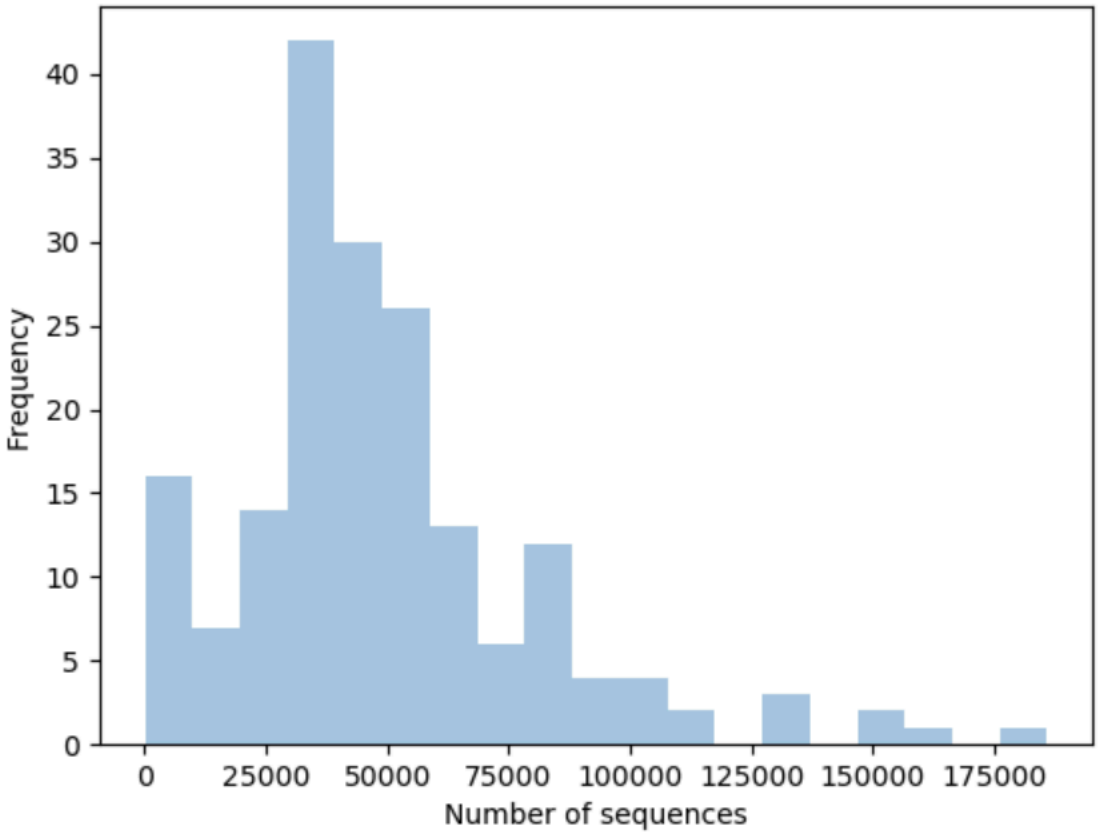


Figure 3-2-frequency of features per sample after denoising. Highest feature frequency per sample 185,844, lowest 642, mean frequency 49,215

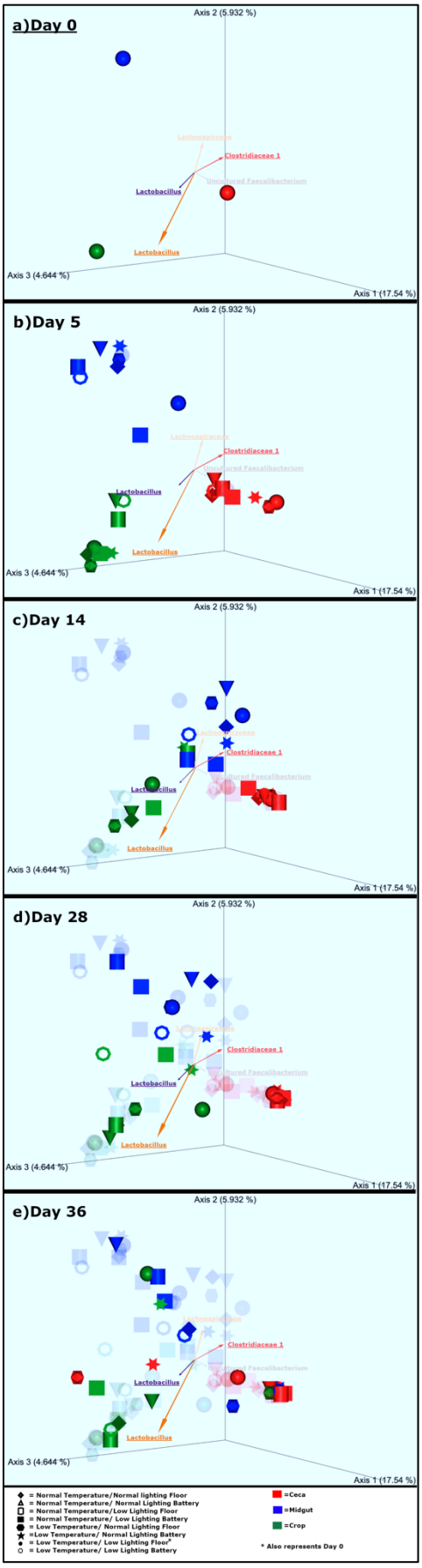


Figure 3-3(a-e)-Jaccard coefficient plots left to right over time day 0 to day 36. For crop samples differentiation was slight and less prominent in comparison to midgut samples which experienced differentiation through day 14 followed by a retraction of range. In contrast ceca samples stayed grouped closely with very little differentiation up until day 36 when some larger variation is seen. When tied to a PCOA biplot using the "diversity pcoa_biplot"command in QIIME2 a directional key was generated using the prominent taxa responsible for some of the biggest shifts in the graph. These were Clostridiaceae 1, Lachnospiraceae, Lactobacillus, and Uncultured Faecalibacterium.

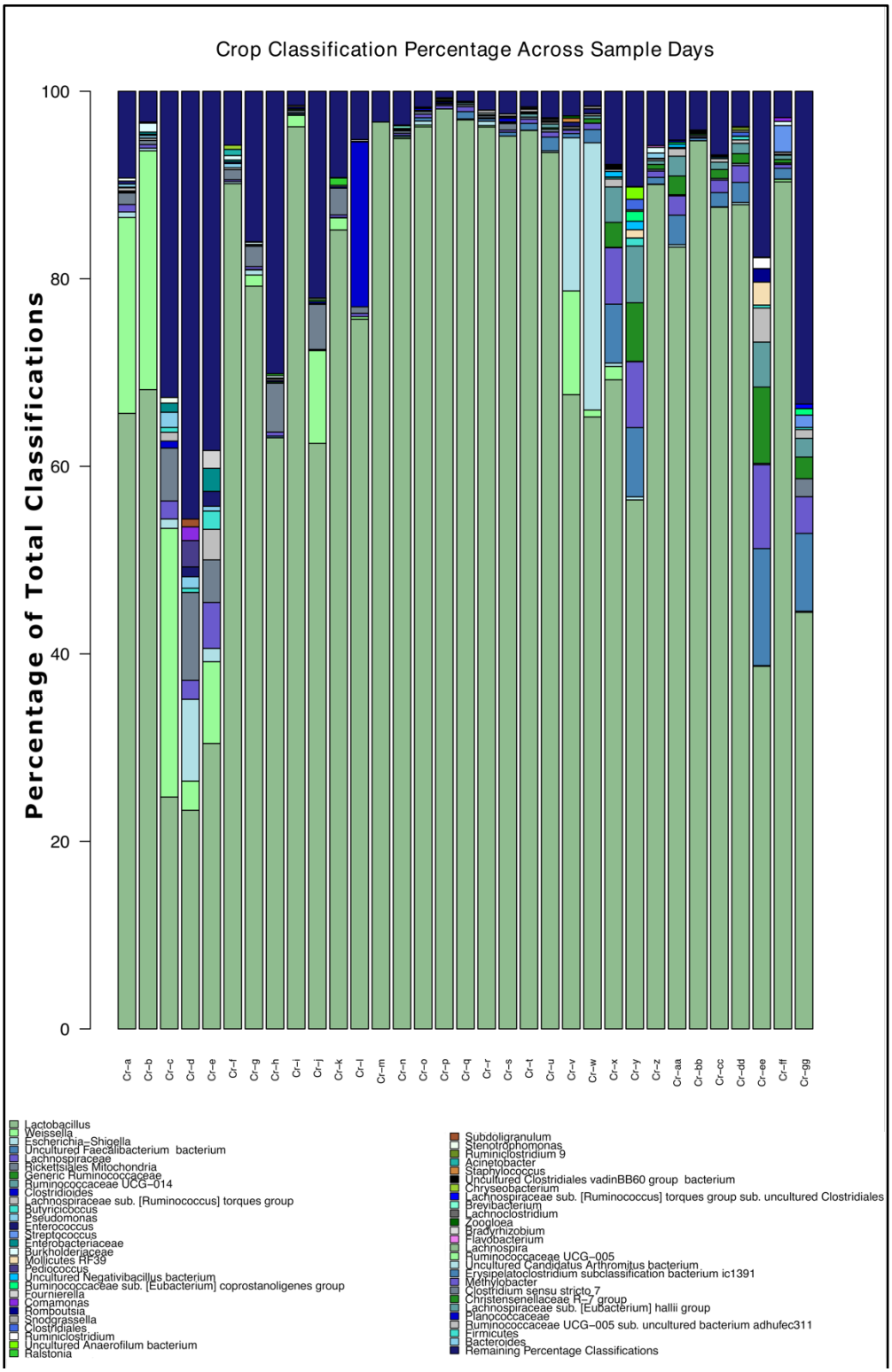


Figure 3-4-Top Classifications across all days, Crop. Letters reference corresponding columns in Table 3-1.

Ref.	Day	Pen	Light	Temp.	Top Classifications Crop
Cr-a	0	NA	NA	NA	*Lactobacillus*(~66%), *Weissella*(~21%), *Rickettsiales Mitochondria*(~1%)
Cr-b	5	B	H	H	*Lactobacillus*(~68%), *Weissell*(~26%), *Burkholderiaceae*(~1%)
Cr-c			H	L	*Lactobacillus* (~25%), *Weissella*(~29%), *Rickettsiales Mitochondria*(~6%), *Lachnospiraceae*(~2%), *Pseudomonas*(~2%), *Escherichia-Shigella*(~1%), *Enterobacteriaceae*(~1%), *Lachnospiraceae sub. [Ruminococcus] torques group*(~1%), *Clostridioides*(~1%), *Stenotrophomonas*(~1%), *Butyricicoccus* (~1%)
Cr-d			L	H	*Lactobacillus*(~23%), *Rickettsiales Mitochondria*(~9%), *Escherichia-Shigella*(~9%), *Weissella*(~3%), *Pediococcus*(~3%), *Lachnospiraceae*(~2%), *Comamonas*(~2%), *Pseudomonas*(~1%), *Enterococcus* (~1%)
Cr-e				L	*Lactobacillus*(~31%), *Weissella*(~9%), *Lachnospiraceae*(~5%), *Rickettsiales Mitochondria*(~5%), *Lachnospiraceae sub. [Ruminococcus] torques group*(~3%), *Enterobacteriaceae*(~3%), *Butyricicoccus*(~2%), *Fournierella*(~2%), *Enterococcus*(~2%), *Escherichia-Shigella*(~1%), *Pseudomonas*(~1%)
Cr-f		F	H	H	*Lactobacillus*(~90%), *Rickettsiales Mitochondria*(~1%), *Acinetobacter*(~1%), *Burkholderiaceae*(~1%), *Pseudomonas*(~1%)
Cr-g				L	*Lactobacillus*(~79%), *Rickettsiales Mitochondria*(~2%), *Weissella*(~1%), *Escherichia-Shigella*(~1%)
Cr-h			L	H	*Lactobacillus*(~63%), *Rickettsiales Mitochondria*(~5%)
Cr-i				L	*Lactobacillus* (~96%), *Weissella*(~1%)
Cr-j	14	B	H	H	*Lactobacillus* (~62%), *Weissella*(~10%), *Rickettsiales Mitochondria*(~5%)
Cr-k				L	*Lactobacillus* (~85%), *Weissella*(~3%), *Rickettsiales Mitochondria*(~1%), *Ralstonia*(~1%)
Cr-l			L	H	*Lactobacillus*(~76%), *Clostridioides*(~18%) , *Rickettsiales Mitochondria*(~1%)
Cr-m				L	*Lactobacillus*(~97%)
Cr-n		F	H	H	*Lactobacillus*(~95%)
Cr-o				L	*Lactobacillus*(~96%)
Cr-p			L	H	*Lactobacillus*(~98%)
Cr-q				L	*Lactobacillus(~97%), Uncultured Faecalibacterium bacterium*(~1%), *Lachnospiraceae*(~1%)
Cr-r	28	B	H	H	*Lactobacillus*(~96%)
Cr-s				L	*Lactobacillus*(~95%), *Rickettsiales Mitochondria*(~1%)
Cr-t			L	H	*Lactobacillus(~96%), Uncultured Faecalibacterium bacterium*(~1%), *Lachnospiraceae*(~1%)
Cr-u				L	*Lactobacillus(~94%), Uncultured Faecalibacterium bacterium*(~2%), *Lachnospiraceae*(~1%)
Cr-v		F	H	H	*Lactobacillus(~94%), Escherichia-Shigella*(~16%), *Weissella*(~11%), *Uncultured Faecalibacterium bacterium*(~1%), *Enterococcus*(~1%)
Cr-w				L	*Lactobacillus(~65%), Escherichia-Shigella*(~29%), *Uncultured Faecalibacterium bacterium*(~1%), *Weissella*(~1%), Lachnospiraceae(~1%), *Enterococcus*(~1%), *Generic Ruminococcaceae* (~1%)
Cr-x			L	H	*Lactobacillus(~69%), Uncultured Faecalibacterium bacterium*(~6%), Lachnospiraceae(~6%), *Ruminococcaceae UCG-014* (~3%), *Generic Ruminococcaceae* (~3%), *Lachnospiraceae sub. [Ruminococcus] torques group*(~1%), *Uncultured Negativibacillus bacterium* (~1%), *Weissella*(~1%), *Uncultured Clostridiales vadinBB60 group bacterium* (~1%)
Cr-y				L	*Lactobacillus(~56%), Uncultured Faecalibacterium bacterium*(~7%), Lachnospiraceae(~7%), *Ruminococcaceae UCG-014* (~6%), *Generic Ruminococcaceae* (~6%), *Uncultured Anaerofilum bacterium* (~1%), *Uncultured Negativibacillus bacterium* (~1%), *Clostridiales* (~1%), *Ruminococcaceae sub. [Eubacterium] coprostanoligenes group* (~1%), *Mollicutes RF39* (~1%), *Butyricicoccus*(~1%)
Cr-z	36	B	H	H	*Lactobacillus*(~90%), *Uncultured Faecalibacterium bacterium* (~1%), Lachnospiraceae (~1%), *Burkholderiaceae*(~1%), *Pseudomonas*(~1%), *Generic Ruminococcaceae* (~1%)
Cr-aa				L	*Lactobacillus(~83%), Uncultured Faecalibacterium bacterium*(~3%), Lachnospiraceae(~2%), *Ruminococcaceae UCG-014* (~2%), *Generic Ruminococcaceae* (~2%), *Lachnospiraceae sub. [Ruminococcus] torques group*(~1%)
Cr-bb			L	H	*Lactobacillus(~95%)*
Cr-cc				L	*Lactobacillus*(~88%), *Uncultured Faecalibacterium bacterium* (~2%), Lachnospiraceae (~1%), *Generic Ruminococcaceae* (~1%), *Ruminococcaceae UCG-014* (~1%)
Cr-dd		F	H	H	*Lactobacillus*(~88%), *Uncultured Faecalibacterium bacterium* (~2%), Lachnospiraceae (~2%), *Generic Ruminococcaceae* (~1%), *Ruminococcaceae UCG-014* (~1%)

Cr-ee			L	*Lactobacillus(~39%), Uncultured Faecalibacterium bacterium*(~13%), Lachnospiraceae(~9%), *Generic Ruminococcaceae* (~9%), *Ruminococcaceae UCG-014* (~5%), *Lachnospiraceae sub. [Ruminococcus] torques group*(~4%), *Mollicutes RF39* (~1%), *Romboutsia* (~1%), *Ruminiclostridium* (~1%)
Cr-ff		L	H	*Lactobacillus*(~90%), *Streptococcus* (~3%), *Uncultured Faecalibacterium bacterium* (~1%), *Ruminococcaceae UCG-014* (~1%)
Cr-gg			L	*Lactobacillus(~44%), Uncultured Faecalibacterium bacterium*(~9%), Lachnospiraceae(~4%), *Ruminococcaceae UCG-014* (~2%), *Generic Ruminococcaceae* (~2%), *Lachnospiraceae sub. [Ruminococcus] torques group*(~1%), *Lachnospiraceae sub. [Ruminococcus] torques group* uncultured Clostridiales bacterium (~1%), *U Ruminococcaceae sub. [Eubacterium] coprostanoligenes group* (~1%)

Table 3-1-Top Classifications through all days, Crop. Lactobacillus was dominant through all sample days and treatments.

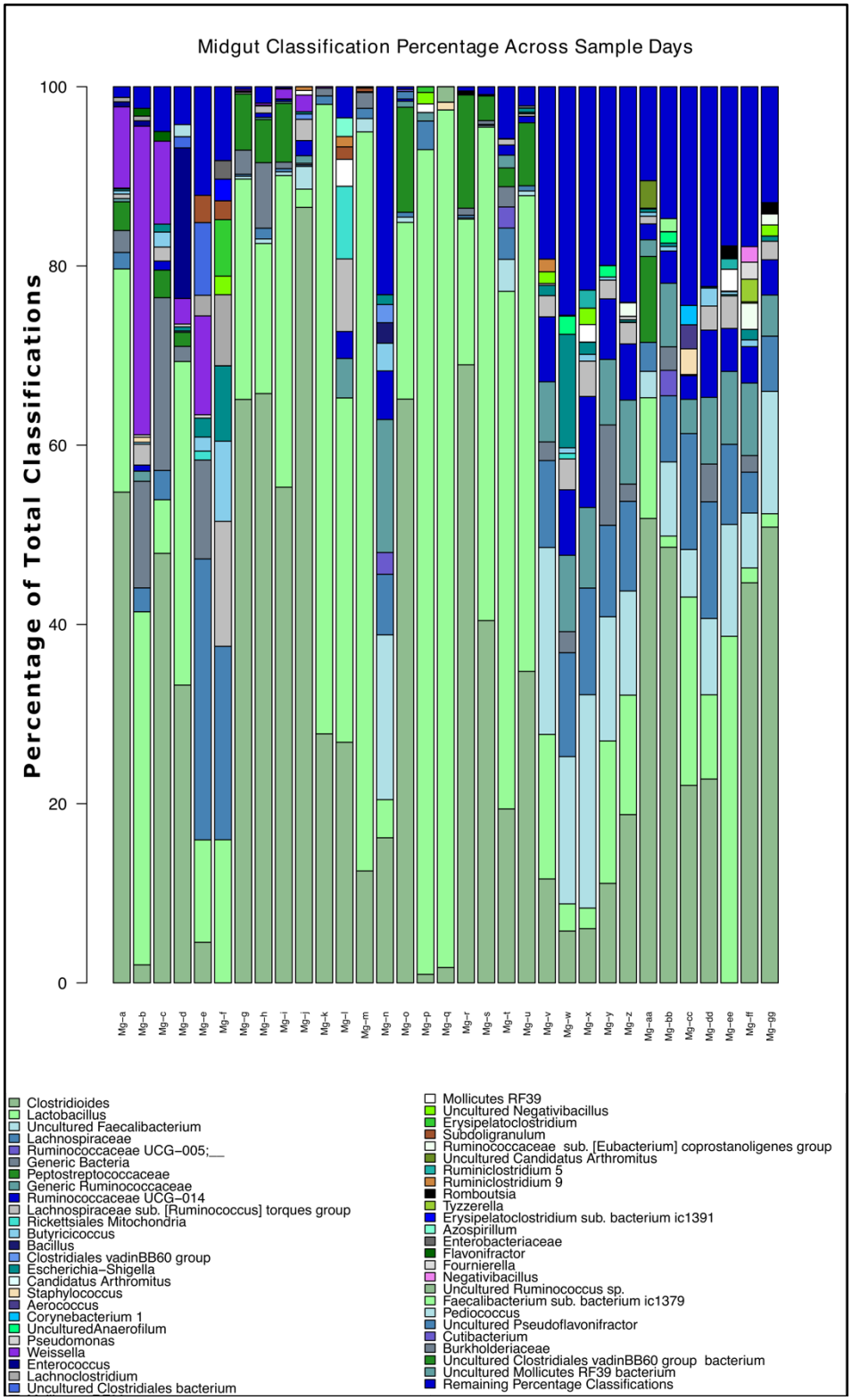


Figure 3-5-Top Classifications across all days, Midgut. Letters reference corresponding columns in Table 3- 2.

Ref.	Day	Pen	Light	Temp.	Top Classifications Midgut
Mg-a	0	NA	NA	NA	*Clostridioides*(~55%), *Lactobacillus* (~25%), *Weissella* (~9%), *Peptostreptococcaceae*(~2%), *Generic Bacteria*(~2%), *Lachnospiraceae*(~2%), *Enterococcus* (~1%), *Lachnospiraceae sub. [Ruminococcus] torques group* (~1%), *Lachnoclostridium* (~1%)
Mg-b	5	B	H	H	*Lactobacillus* (~39%), *Weissella*(~34%), *Generic Bacteria*(~12%), *Lachnospiraceae*(~3%), *Lachnospiraceae sub. [Ruminococcus] torques group*(~2%), *Clostridioides*(~2%), *Generic Ruminococcaceae* (~1%), *Flavonifractor*(~1%), *Ruminococcaceae UCG-014*(~1%), *Enterococcus* (~1%), *Staphylococcus* (~1%), *Lachnoclostridium* (~1%)
Mg-c				L	*Clostridioides*(~48%), *Generic Bacteria*(~19%), *Weissella* (~9%), *Lactobacillus* (~6%), *Lachnospiraceae*(~3%), *Peptostreptococcaceae*(~3%), *Butyricicoccus* (~2%), *Lachnospiraceae sub. [Ruminococcus] torques group* (~2%), *Flavonifractor* (~1%), *Ruminococcaceae UCG-014*(~1%), *Escherichia-Shigella*(~1%)
Mg-d			L	H	*Lactobacillus*(~36%), *Clostridioides*(~33%), *Enterococcus*(~17%), *Weissella*(~3%), *Generic Bacteria*(~2%), *Peptostreptococcaceae*(~2%), *Pediococcus*(~1%), *Escherichia-Shigella*(~1%), *Uncultured Clostridiales bacterium* (~1%)
Mg-e				L	*Lachnospiraceae*(~31%), *Lactobacillus*(~11%), *Weissella*(~11%), *Generic Bacteria*(~11%), *Uncultured Clostridiales bacterium* (~8%), *Clostridioides* (~5%), *Subdoligranulum*(~3%), *Lachnoclostridium*(~2%), *Butyricicoccus*(~2%), *Escherichia-Shigella*(~2%)
Mg-f		F	H	H	*Lachnospiraceae*(~22%), *Lactobacillus*(~16%), *Lachnospiraceae sub. [Ruminococcus] torques group*(~14%), *Butyricicoccus*(~9%), *Escherichia-Shigella*(~8%), *Lachnoclostridium*(~8%), *Erysipelatoclostridium* (~6%), *Erysipelatoclostridium sub. bacterium ic1391* (~2%), *Subdoligranulum*(~2%), *Uncultured Negativibacillus*(~1%), *Enterobacteriaceae*(~1%)
Mg-g				L	*Clostridioides*(~65%), *Lactobacillus* (~25%), *Peptostreptococcaceae*(~6%), *Generic Bacteria*(~3%)
Mg-h			L	H	*Clostridioides*(~66%), *Lactobacillus* (~17%), *Generic Bacteria*(~7%), *Peptostreptococcaceae*(~5%), *Lachnospiraceae*(~1%), *Lachnospiraceae sub. [Ruminococcus] torques group*(~1%), *Ruminococcaceae UCG-014* (~1%), *Uncultured Faecalibacterium* (~1%)
Mg-i				L	*Clostridioides*(~55%), *Lactobacillus* (~35%), *Peptostreptococcaceae*(~7%), *Weissella*(~1%), *Generic Bacteria*(~1%)
Mg-j			H	H	*Clostridioides*(~87%), *Uncultured Faecalibacterium* (~3%), *Lachnospiraceae sub. [Ruminococcus] torques group*(~2%), *Lactobacillus* (~2%), *Weissella*(~2%), *Ruminococcaceae UCG-014*(~2%), *Generic Ruminococcaceae*(~1%), *Clostridiales vadinBB60 group* (~7%), *Mollicutes RF39* (~1%)
Mg-k		B		L	*Lactobacillus* (~70%), *Clostridioides*(~28%), *Lachnospiraceae*(~1%), *Generic Bacteria*(~1%)
Mg-l			L	H	*Lactobacillus* (~38%), *Clostridioides*(~27%), *Lachnospiraceae sub. [Ruminococcus] torques group*(~8%), *Rickettsiales Mitochondria* (~8%), *Generic Ruminococcaceae*(~4%), *Ruminococcaceae UCG-014* (~3%), *Mollicutes RF39* (~3%), *Azospirillum* (~2%), *Subdoligranulum* (~1%), *Ruminiclostridium 9* (~1%)
Mg-m	14			L	*Lactobacillus* (~83%), *Clostridioides*(~13%), *Generic Bacteria*(~2%), *Lachnospiraceae*(~2%), *Uncultured Faecalibacterium* (~1%)
Mg-n			H	H	*Uncultured Faecalibacterium* (~18%), *Clostridioides*(~16%), *Generic Ruminococcaceae*(~15%), *Lachnospiraceae*(~7%), *Ruminococcaceae UCG-014* (~5%), *Lactobacillus* (~4%), *Butyricicoccus*(~3%), *Ruminococcaceae UCG-005* (~2%), *Bacillus* (~2%), *Clostridiales vadinBB60 group* (~2%), *Escherichia-Shigella*(~1%)
Mg-o		F		L	*Clostridioides*(~65%), *Lactobacillus* (~20%), *Peptostreptococcaceae*(~12%), *Uncultured Pseudoflavonifractor*(~1%), *Generic Ruminococcaceae*(~1%), *Uncultured Faecalibacterium* (~1%), *Lachnospiraceae*(~1%)
Mg-p			L	H	*Lactobacillus* (~92%), *Lachnospiraceae*(~3%), *Uncultured Negativibacillus* (~1%), *Clostridioides*(~1%), *Generic Ruminococcaceae*(~1%), *Mollicutes RF39* (~1%), *Erysipelatoclostridium*(~1%)
Mg-q				L	*Lactobacillus* (~96%), *Clostridioides*(~2%), *Uncultured Ruminococcus sp.* (~2%), *Staphylococcus* (~1%)
Mg-r			H	H	*Clostridioides*(~69%), *Lactobacillus* (~16%), *Peptostreptococcaceae*(~13%), *Generic Bacteria*(~1%)
Mg-s				L	*Lactobacillus* (~55%), *Clostridioides*(~40%), *Peptostreptococcaceae*(~3%)
Mg-t		B	L	H	*Lactobacillus* (~58%), *Clostridioides*(~19%), *Uncultured Faecalibacterium* (~4%), *Lachnospiraceae*(~4%), *Ruminococcaceae UCG-005* (~2%), *Generic Bacteria*(~2%), *Peptostreptococcaceae*(~2%), *Generic Ruminococcaceae*(~1%), *Ruminococcaceae UCG-014* (~1%), *Lachnospiraceae sub. [Ruminococcus] torques group*(~1%)
Mg-u	28			L	*Lactobacillus* (~53%), *Clostridioides*(~35%), *Peptostreptococcaceae*(~7%), *Ruminococcaceae UCG-014* (~1%), *Lachnospiraceae*(~1%), *Uncultured Faecalibacterium* (~1%), *Escherichia-Shigella*(~1%)
Mg-v		F	H	H	*Uncultured Faecalibacterium* (~21%), *Lactobacillus* (~16%), *Clostridioides*(~12%), *Lachnospiraceae*(~10%), *Ruminococcaceae UCG-014* (~7%), *Generic Ruminococcaceae*(~7%), *Lachnospiraceae sub. [Ruminococcus] torques group*(~2%), *Generic Bacteria*(~2%), *Ruminiclostridium 9* (~1%), *Uncultured Negativibacillus* (~1%), *Escherichia-Shigella*(~1%)
Mg-w				L	*Uncultured Faecalibacterium* (~16%), *Escherichia-Shigella*(~13%), *Lachnospiraceae*(~12%), *Generic Ruminococcaceae*(~9%), *Ruminococcaceae UCG-014* (~7%), *Clostridioides*(~6%), *Lachnospiraceae sub. [Ruminococcus] torques group*(~3%), *Lactobacillus* (~3%), *Generic Bacteria*(~2%), *Uncultured Anaerofilum* (~2%), *Rickettsiales Mitochondria* (~1%), *Butyricicoccus* (~1%)

Mg-x			L	H	*Uncultured Faecalibacterium* (~24%), *Ruminococcaceae UCG-014* (~12%), *Lachnospiraceae*(~12%), *Generic Ruminococcaceae*(~9%), *Clostridioides*(~6%), *Lachnospiraceae sub. [Ruminococcus] torques group*(~4%), *Lactobacillus* (~2%), *Ruminiclostridium 5* (~2%), *Mollicutes RF39* (~2%), *Uncultured Negativibacillus* (~2%), *Escherichia-Shigella*(~1%), *Butyricicoccus*(~1%)
Mg-y				L	*Lactobacillus* (~16%), *Uncultured Faecalibacterium* (~14%), *Generic Bacteria*(~11%), *Clostridioides*(~11%), *Lachnospiraceae*(~10%), *Generic Ruminococcaceae*(~7%), *Ruminococcaceae UCG-014* (~7%), *Lachnospiraceae sub. [Ruminococcus] torques group*(~2%), *Uncultured Anaerofilum* (~1%)
Mg-z			H	H	*Clostridioides*(~19%), *Lactobacillus* (~13%), *Uncultured Faecalibacterium* (~12%), *Lachnospiraceae*(~10%), *Generic Ruminococcaceae*(~9%), *Ruminococcaceae UCG-014* (~6%), *Lachnospiraceae sub. [Ruminococcus] torques group*(~2%), *Generic Bacteria*(~2%), *Ruminococcaceae sub. [Eubacterium] coprostanoligenes group* (~2%)
Mg-aa				L	*Clostridioides*(~52%), *Lactobacillus* (~14%), *Peptostreptococcaceae*(~10%), *Lachnospiraceae*(~3%), *Uncultured Candidatus Arthromitus* (~3%), *Uncultured Faecalibacterium* (~3%), *Generic Ruminococcaceae*(~2%), *Ruminococcaceae UCG-014* (~2%), *Lachnospiraceae sub. [Ruminococcus] torques group*(~1%)
Mg-bb		B	L	H	*Clostridioides*(~49%), *Uncultured Faecalibacterium* (~8%), *Lachnospiraceae*(~7%), *Generic Ruminococcaceae*(~7%), *Ruminococcaceae UCG-014* (~4%), *Ruminococcaceae UCG-005* (~3%), *Generic Bacteria*(~3%), *Faecalibacterium sub. bacterium ic1379*(~2%), *Uncultured Anaerofilum* (~1%), *Lactobacillus* (~1%), *Butyricicoccus* (~1%)
Mg-cc				L	*Clostridioides*(~22%), *Lactobacillus* (~21%), *Lachnospiraceae*(~13%), *Uncultured Faecalibacterium* (~5%), *Generic Ruminococcaceae*(~4%), *Staphylococcus*(~3%), *Aerococcus*(~3%), *Ruminococcaceae UCG-014* (~3%), *Corynebacterium 1*(~2%),
Mg-dd	36		H	H	*Clostridioides*(~23%), *Lachnospiraceae*(~13%), *Lactobacillus* (~9%), *Uncultured Faecalibacterium* (~9%), *Ruminococcaceae UCG-014* (~8%), *Generic Ruminococcaceae*(~7%), *Generic Bacteria*(~4%), *Lachnospiraceae sub. [Ruminococcus] torques group*(~3%), *Butyricicoccus* (~2%)
Mg-ee				L	*Lactobacillus* (~39%), *Uncultured Faecalibacterium* (~13%), *Lachnospiraceae*(~9%), *Generic Ruminococcaceae*(~8%), *Ruminococcaceae UCG-014* (~5%), *Lachnospiraceae sub. [Ruminococcus] torques group*(~4%), *Mollicutes RF39* (~2%), *Romboutsia*(~1%), *Ruminiclostridium 5*(~1%),
Mg-ff		F	L	H	*Clostridioides*(~45%), *Generic Ruminococcaceae*(~8%), *Uncultured Faecalibacterium* (~6%), *Lachnospiraceae*(~5%), *Ruminococcaceae UCG-014* (~4%), *Ruminococcaceae sub. [Eubacterium] coprostanoligenes group* (~3%), *Tyzzerella*(~3%), *Fournierella*(~2%), *Generic Bacteria*(~2%), *Negativibacillus*(~2%), *Lactobacillus* (~2%), *Escherichia-Shigella*(~1%), *Butyricicoccus* (~1%)
Mg-gg				L	*Clostridioides*(~51%), *Uncultured Faecalibacterium* (~14%), *Lachnospiraceae*(~6%), *Generic Ruminococcaceae*(~5%), *Ruminococcaceae UCG-014* (~4%), *Lachnospiraceae sub. [Ruminococcus] torques group*(~2%), *Lactobacillus* (~2%), *Uncultured Negativibacillus*(~1%), *Ruminococcaceae sub. [Eubacterium] coprostanoligenes group* (~1%), *Romboutsia*(~1%), *Escherichia-Shigella*(~1%)

Table 3-2-Top Classifications through all days, Midgut. Clostridioides, Lactobacillus, and an Uncultured Faecalibacterium were the most common top classifications for midgut samples.

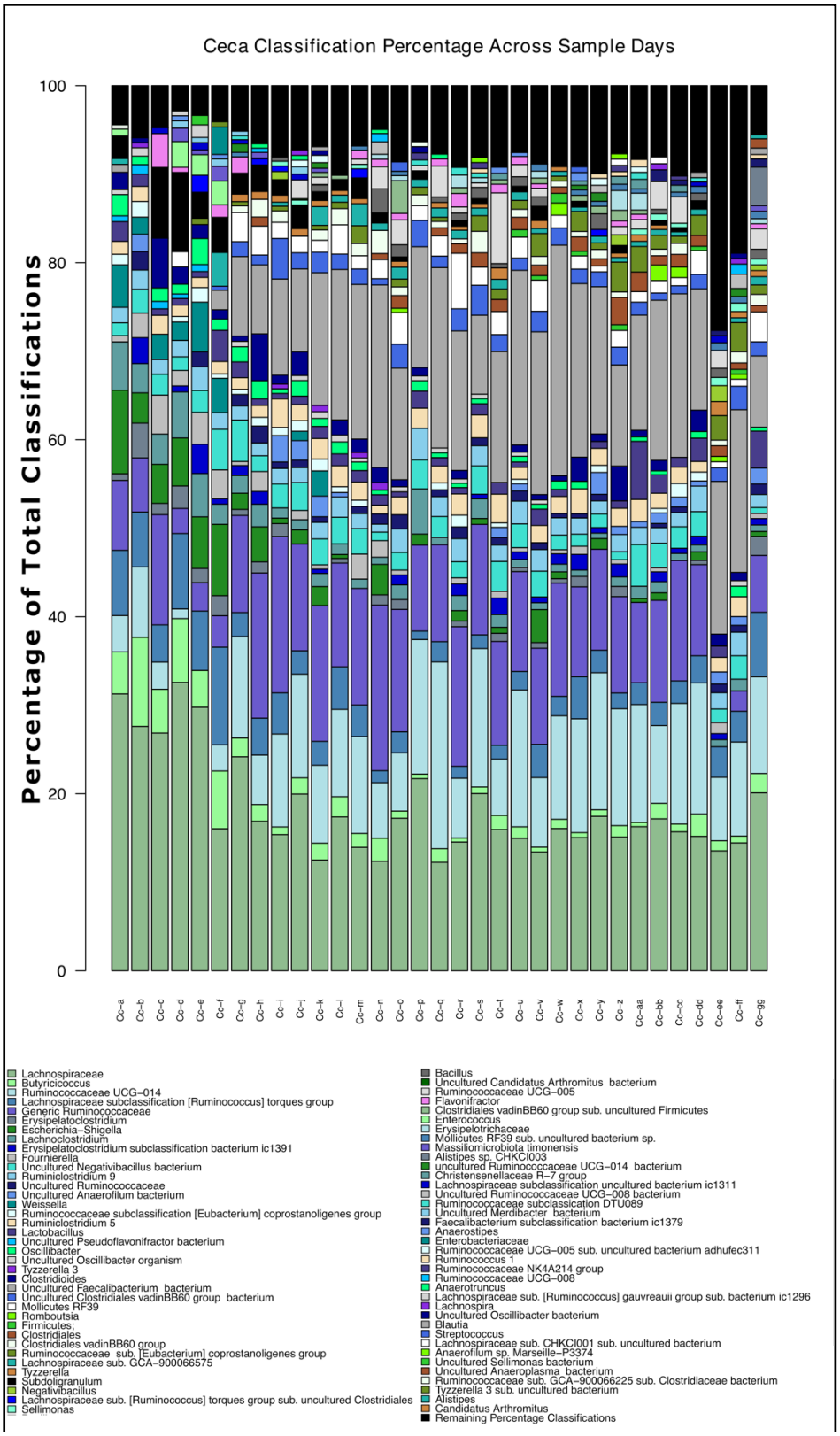


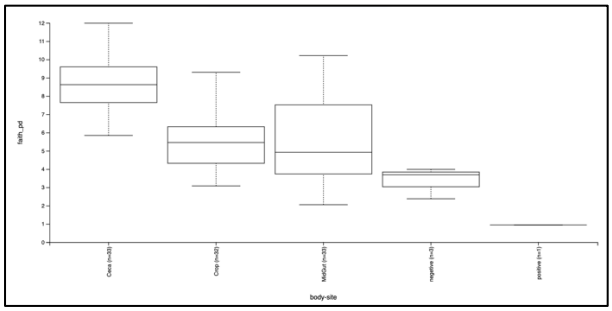
Figure 3-6-Top Classifications across all days, Ceca. Letters reference corresponding columns in Table 3-3.

Ref.	Day	Pen	Light	Temp.	Top Classifications Ceca
Cc-a	0	NA	NA	NA	*Lachnospiraceae*(~31%), *Escherichia-Shigella*(~9%), *Generic Ruminococcaceae*(~8%), *Lachnospiraceae sub. [Ruminococcus] torques group*(~7%), *Lachnoclostridium*(~6%), *Weissella*(~5%), *Butyricicoccus*(~5%), *Ruminococcaceae UCG-014*(~4%), *Subdoligranulum*(~3%), *Oscillibacter*(~2%)
Cc-b	5	B	H	H	*Lachnospiraceae*(~28%), *Butyricicoccus*(~10%), *Ruminococcaceae UCG-014*(~8%), *Lachnospiraceae sub. [Ruminococcus] torques group*(~6%), *Generic Ruminococcaceae*(~6%), *Erysipelatoclostridium*(~4%), *Escherichia-Shigella*(~3%), *Lachnoclostridium*(~3%), *Erysipelatoclostridium sub. bacterium ic1391* (~3%), *Fournierella*(~3%)
Cc-c				L	*Lachnospiraceae*(~27%), *Generic Ruminococcaceae*(~13%), *Subdoligranulum*(~8%), *Clostridioides*(~6%), *Butyricicoccus*(~5%), *Escherichia-Shigella*(~4%), *Fournierella*(~4%), *Lachnospiraceae sub. [Ruminococcus] torques group*(~4%), *Flavonifractor*(~4%), *Lachnoclostridium*(~4%)
Cc-d			L	H	*Lachnospiraceae*(~33%), *Subdoligranulum*(~9%), *Lachnospiraceae sub. [Ruminococcus] torques group*(~9%), *Butyricicoccus*(~7%), *Escherichia-Shigella*(~5%), *Lachnoclostridium*(~5%), *Enterococcus*(~3%), *Generic Ruminococcaceae*(~3%), *Erysipelatoclostridium*(~3%), *Weissella*(~2%)
Cc-e				L	*Lachnospiraceae*(~30%), *Lachnospiraceae sub. [Ruminococcus] torques group*(~7%), *Escherichia-Shigella*(~6%), *Weissella*(~6%), *Lachnoclostridium*(~5%), *Butyricicoccus*(~4%), *Fournierella*(~4%), *Erysipelatoclostridium sub. bacterium ic1391* (~3%), *Generic Ruminococcaceae*(~3%), *Oscillibacter*(~3%)
Cc-f			H	H	*Lachnospiraceae*(~16%), *Lachnospiraceae sub. [Ruminococcus] torques group*(~11%), *Escherichia-Shigella*(~8%), *Butyricicoccus*(~7%), *Uncultured Negativibacillus*(~5%), *Subdoligranulum*(~4%), *Weissella*(~4%), *Lachnospiraceae sub. GCA-900066575*(~4%), *Generic Ruminococcaceae*(~4%), *Lactobacillus* (~4%)
Cc-g				L	*Lachnospiraceae*(~24%), *Ruminococcaceae UCG-014*(~12%), *Generic Ruminococcaceae*(~11%), *Uncultured Faecalibacterium*(~9%), *Uncultured Negativibacillus*(~5%), *Mollicutes RF39*(~3%), *Lachnospiraceae sub. [Ruminococcus] torques group*(~3%), *Subdoligranulum*(~2%), *Butyricicoccus*(~2%), *Lachnoclostridium*(~2%)
Cc-h		F	L	H	*Lachnospiraceae*(~17%), *Generic Ruminococcaceae*(~16%), *Uncultured Faecalibacterium*(~8%), *Ruminococcaceae UCG-014*(~6%), *Clostridioides*(~5%), *Lachnospiraceae sub. [Ruminococcus] torques group*(~4%), *Escherichia-Shigella*(~4%), *Mollicutes RF39*(~3%), *Subdoligranulum*(~3%), *Lachnoclostridium*(~3%)
Cc-i				L	*Generic Ruminococcaceae*(~18%), *Lachnospiraceae*(~15%), *Uncultured Faecalibacterium*(~11%), *Ruminococcaceae UCG-014*(~11%), *Lachnospiraceae sub. [Ruminococcus] torques group*(~5%), *Uncultured Clostridiales vadinBB60 group*(~5%), *Ruminiclostridium 5*(~3%), *Uncultured Anaerofilum*(~3%), *Uncultured Negativibacillus*(~3%), *Mollicutes RF39*(~2%)
Cc-j			H	H	*Lachnospiraceae(~20%), Generic Ruminococcaceae(~12%), Ruminococcaceae UCG-014(~12%), Uncultured Faecalibacterium(~9%), Uncultured Negativibacillus(~3%), Clostridioides(~3%), Subdoligranulum(~3%), Lachnospiraceae sub. [Ruminococcus] torques group(~3%), Uncultured Anaerofilum(~3%),Ruminococcaceae UCG-005(~2%)*
Cc-k		B		L	*Generic Ruminococcaceae(~15%), Uncultured Faecalibacterium(~15%), Lachnospiraceae(~13%), Ruminococcaceae UCG-014(~9%), Uncultured Negativibacillus(~3%), Weissella(~3%), Lachnospiraceae sub. [Ruminococcus] torques group(~3%), Uncultured Anaerofilum(~2%), Uncultured Clostridiales vadinBB60 group(~2%), Ruminiclostridium 5(~2%)*
Cc-l			L	H	*Lachnospiraceae(~17%), Uncultured Faecalibacterium(~17%), Generic Ruminococcaceae(~12%), Ruminococcaceae UCG-014(~10%), Lachnospiraceae sub. [Ruminococcus] torques group(~5%), Mollicutes RF39(~3%), Uncultured Negativibacillus(32%), Ruminiclostridium 5(~2%), Ruminiclostridium 9(~2%), Butyricicoccus(~2%)*
Cc -m	14			L	*Uncultured Faecalibacterium(~18%), Lachnospiraceae(~14%), Generic Ruminococcaceae(~13%), Ruminococcaceae UCG-014(~11%), Lachnospiraceae sub. [Ruminococcus] torques group(~4%), Uncultured Negativibacillus(~3%), Fournierella(~3%), Lachnospiraceae sub. GCA-900066575(~3%), Subdoligranulum(~2%), Ruminiclostridium 5(~2%)*
Cc-n			H	H	*Uncultured Faecalibacterium(~21%), Generic Ruminococcaceae(~19%), Lachnospiraceae(~12%), Ruminococcaceae UCG-014(~6%), Escherichia-Shigella(~3%), Bacillus(~3%), Clostridiales vadinBB60 group(~3%), Butyricicoccus(~3%), Ruminococcaceae UCG-005(~3%), Mollicutes RF39(~2%)*
Cc -o		F	H	L	*Lachnospiraceae(~17%), Generic Ruminococcaceae(~14%), Uncultured Faecalibacterium(~13%), Ruminococcaceae UCG-014(~7%), Clostridiales vadinBB60 group sub. uncultured Firmicutes(~4%), Mollicutes RF39(~4%), Ruminococcaceae UCG-005(~3%), Uncultured Clostridiales vadinBB60 group(~3%), Ruminiclostridium 9(~3%), Lachnospiraceae sub. [Ruminococcus] torques group(~2%)*
Cc-p			L	H	*Lachnospiraceae(~22%), Ruminococcaceae UCG-014(~15%), Uncultured Faecalibacterium(~14%), Generic Ruminococcaceae(~10%), Lachnoclostridium(~5%), Ruminiclostridium 9(~4%), Uncultured Negativibacillus(~3%), Uncultured Clostridiales vadinBB60 group(~3%), Ruminiclostridium 5(~2%), Lactobacillus(~2%)*
Cc-q				L	*Ruminococcaceae UCG-014(~21%), Uncultured Faecalibacterium(~20%), Lachnospiraceae(~12%), Generic Ruminococcaceae(~11%), Ruminococcaceae UCG-005(~4%), Uncultured Negativibacillus(~2%), Lachnospiraceae sub. [Ruminococcus] torques group(~2%), Mollicutes RF39(~2%), Ruminiclostridium 9(~2%), Ruminiclostridium 5(~2%)*
Cc-r	28	B	H	H	*Generic Ruminococcaceae(~16%), Uncultured Faecalibacterium(~16%), Lachnospiraceae(~15%), Ruminococcaceae UCG-014(~7%), Mollicutes RF39(~6%), Ruminiclostridium 9(~3%), Uncultured Clostridiales vadinBB60 group(~3%), Ruminiclostridium 5(~2%), Uncultured Negativibacillus(~2%), Lachnoclostridium(~2%)*

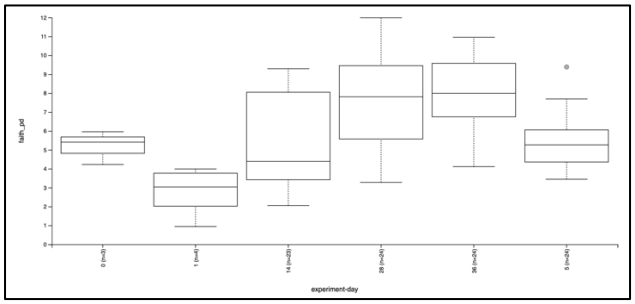
Cc-s			L	*Lachnospiraceae(~20%), Ruminococcaceae UCG-014(~16%), Generic Ruminococcaceae(13%), Uncultured Faecalibacterium(~9%), Uncultured Clostridiales vadinBB60 group(~3%), Uncultured Negativibacillus(~3%), Ruminiclostridium 5(~3%), Clostridiales vadinBB60 group(~2%), Ruminiclostridium 9(~2%), Lachnoclostridium(~2%)*
Cc-t		L	H	*Lachnospiraceae(~16%), Uncultured Faecalibacterium(~15%), Generic Ruminococcaceae(~12%), Ruminococcaceae UCG-005(~8%), Ruminococcaceae UCG-014(~6%), Uncultured Negativibacillus(~3%), Ruminiclostridium 5(~3%), Mollicutes RF39(~3%), Uncultured Clostridiales vadinBB60 group(~2%), Erysipelatoclostridium sub. bacterium ic1391(~2%)*
Cc-u			L	*Uncultured Faecalibacterium(~20%), Ruminococcaceae UCG-014(~16%), Lachnospiraceae(~15%), Generic Ruminococcaceae(~11%), Uncultured Negativibacillus(~3%), Ruminiclostridium 9(~3%), Mollicutes RF39(~2%), Lachnospiraceae sub. [Ruminococcus] torques group(~2%), Clostridiales(~1%), Uncultured Clostridiales vadinBB60 group(~1%)*
Cc-v		H	H	*Uncultured Faecalibacterium(~18%), Lachnospiraceae(~13%), Generic Ruminococcaceae(~11%), Ruminococcaceae UCG-014(~8%), Lachnospiraceae sub. [Ruminococcus] torques group(~4%), Escherichia-Shigella(~4%), Mollicutes RF39(~4%), Uncultured Negativibacillus(3%), Ruminococcaceae sub. [Eubacterium] coprostanoligenes group(~3%), Ruminiclostridium 9(~2%)*
Cc-w			L	*Uncultured Faecalibacterium(~26%), Lachnospiraceae(~16%), Generic Ruminococcaceae(~13%), Ruminococcaceae UCG-014(~12%), Lachnospiraceae sub. [Ruminococcus] torques group(~2%), Ruminiclostridium 9(~2%), Uncultured Clostridiales vadinBB60 group(~2%), Ruminiclostridium 5(~2%), Uncultured Negativibacillus(~2%), Mollicutes RF39(~2%)*
Cc-x	F	L	H	*Uncultured Faecalibacterium(~20%), Lachnospiraceae(~15%), Ruminococcaceae UCG-014(~13%), Generic Ruminococcaceae(~10%), Lachnospiraceae sub. [Ruminococcus] torques group(~5%), Ruminiclostridium 5(~3%), Clostridioides(~3%), Ruminococcaceae sub. [Eubacterium] coprostanoligenes group(~2%), Ruminiclostridium 9(~2%), Erysipelatoclostridium sub. bacterium ic1391(~2%)*
Cc-y			L	*Lachnospiraceae(~17%), Uncultured Faecalibacterium(~17%), Ruminococcaceae UCG-014(~16%), Generic Ruminococcaceae(~11%), Lachnospiraceae sub. [Ruminococcus] torques group(~3%), Uncultured Anaerofilum(~3%), Ruminiclostridium 5(~2%), Bacillus(~2%), Uncultured Clostridiales vadinBB60 group(~2%), Uncultured Negativibacillus(~2%)*
Cc-z		H	H	*Lachnospiraceae(~15%), Ruminococcaceae UCG-014(~13%), Uncultured Faecalibacterium(~11%), Generic Ruminococcaceae(~11%), Clostridioides(~4%), Ruminococcaceae sub. [Eubacterium] coprostanoligenes group(~3%), Clostridiales(~3%), Erysipelotrichaceae(~2%), Ruminiclostridium 5(~2%), Uncultured Clostridiales vadinBB60 group(~2%)*
Cc-aa	B		L	*Lachnospiraceae(~16%), Ruminococcaceae UCG-014(~13%), Uncultured Faecalibacterium(~13%), Generic Ruminococcaceae(~9%), Lactobacillus(~7%), Uncultured Negativibacillus(~5%), Ruminococcaceae sub. [Eubacterium] coprostanoligenes group(~3%), Ruminiclostridium 5(~3%), Lachnospiraceae sub. [Ruminococcus] torques group(~3%), Clostridiales(~2%)*
Cc-bb		L	H	*Uncultured Faecalibacterium(~18%), Lachnospiraceae(~17%), Generic Ruminococcaceae(~12%), Ruminococcaceae UCG-014(~9%), Ruminococcaceae UCG-005(~3%), Uncultured Negativibacillus(~3%), Lachnospiraceae sub. [Ruminococcus] torques group(~3%), Lactobacillus(~2%), Ruminiclostridium 9(~2%), Butyricicoccus(~2%)*
Cc-cc			L	*Uncultured Faecalibacterium(~19%), Lachnospiraceae(~16%), Ruminococcaceae UCG-014(~14%), Generic Ruminococcaceae(~14%), Ruminococcaceae UCG-005(~3%), Lachnospiraceae sub. [Ruminococcus] torques group(~3%), Uncultured Negativibacillus(~2%), Ruminiclostridium 5(~2%), Ruminiclostridium 9(~2%), Ruminococcaceae sub. [Eubacterium] coprostanoligenes group(~2%)*
Cc-dd	36	H	H	*Lachnospiraceae(~15%), Ruminococcaceae UCG-014(~15%), Uncultured Faecalibacterium(~14%), Generic Ruminococcaceae(~10%), Lachnospiraceae sub. [Ruminococcus] torques group(~3%), Ruminiclostridium 9(~3%), Uncultured Negativibacillus(~3%), Mollicutes RF39(~3%), Lactobacillus(~3%), Butyricicoccus(~3%)*
Cc-ee			L	*Uncultured Faecalibacterium(~17%), Lachnospiraceae(~14%), Ruminococcaceae UCG-014(~7%), Lachnospiraceae sub. [Ruminococcus] torques group(~4%), Ruminococcaceae sub. [Eubacterium] coprostanoligenes group(~3%), Ruminococcaceae UCG-005(~2%), Ruminiclostridium 9(~2%), Negativibacillus(~2%), Ruminiclostridium 5(~2%), Tyzzerella(~2%)*
Cc-ff	F	L	H	*Uncultured Faecalibacterium(~18%), Lachnospiraceae(~14%), Ruminococcaceae UCG-014(~10%), Lachnospiraceae sub. [Ruminococcus] torques group(~4%), Ruminococcaceae sub. [Eubacterium] coprostanoligenes group(~3%), Ruminiclostridium 9(~3%), Uncultured Negativibacillus(~3%), Uncultured Clostridiales vadinBB60 group(~3%), Generic Ruminococcaceae(~2%), Ruminiclostridium 5(~2%)*
Cc-gg			L	*Lachnospiraceae(~20%), Ruminococcaceae UCG-014(~11%), Uncultured Faecalibacterium(~8%), Lachnospiraceae sub. [Ruminococcus] torques group(~7%), Generic Ruminococcaceae(~6%), Alistipes sp. CHKCI003(~4%), Lactobacillus(~4%), Mollicutes RF39(~3%), Ruminococcaceae UCG-005(~2%), Butyricicoccus(~2%)*

Table 3-3- Top Classifications through all days, Ceca. Lachnospiraceae, Uncultured Faecalibacterium, Generic Ruminococcaceae, and Ruminococcaceae UCG-014 were the common top classifications found in caca samples.

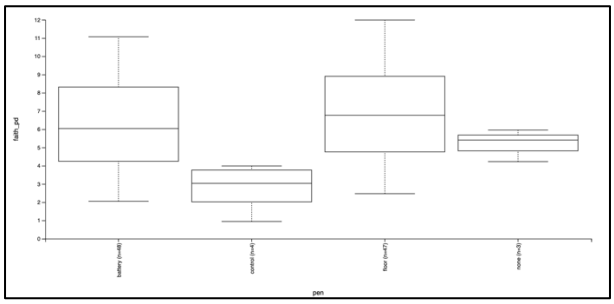
Supplementary Figures



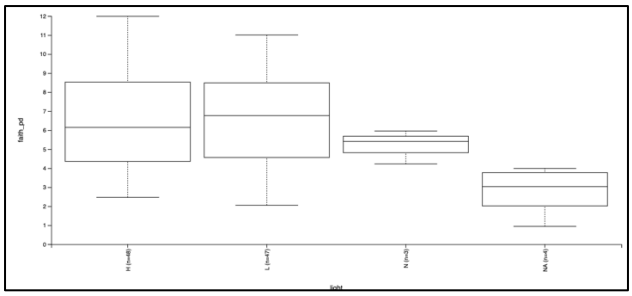
Supplement 1-Box plot of Faith's phylogenetic diversity over body site.



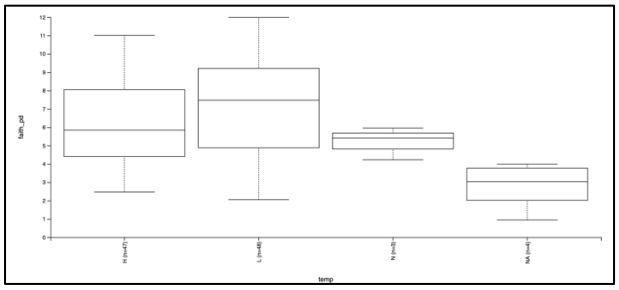
Supplement 2-Box plot of Faith's phylogenetic diversity over sample day.



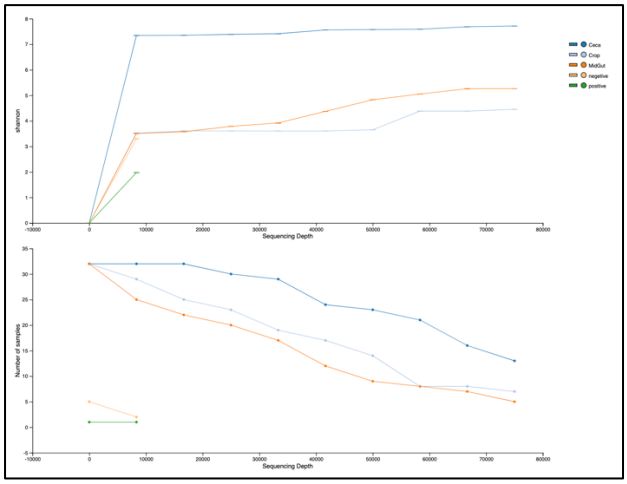
Supplement 3-Box plot of Faith's phylogenetic diversity over pen type.



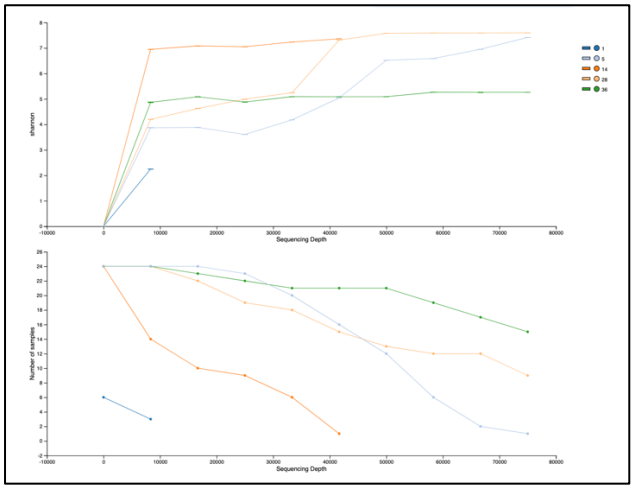
Supplement 4-Box plot of Faith's phylogenetic diversity over lighting level.



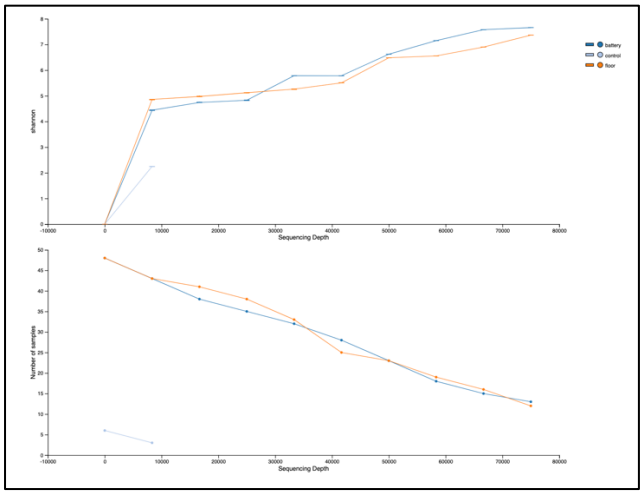
Supplement 5-Box plot of Faith's phylogenetic diversity over temperature.



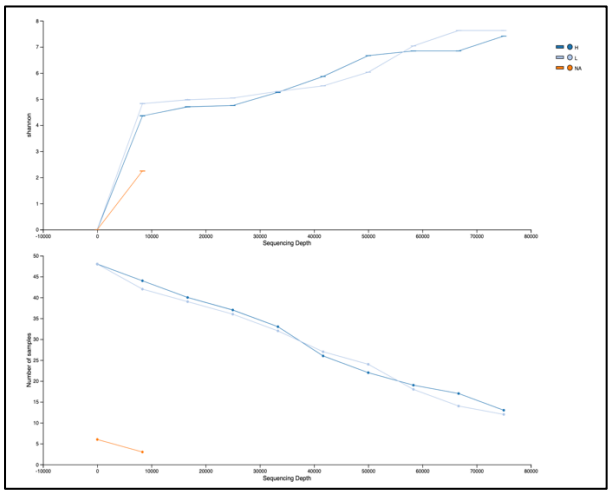
Supplement 6-Box plot of Faith's phylogenetic diversity over body site.



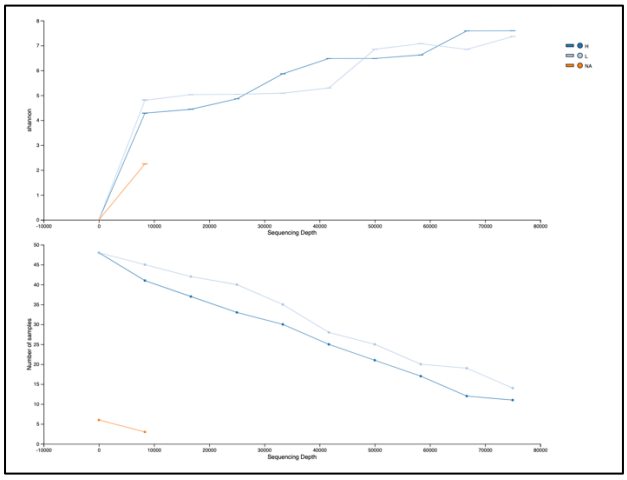
Supplement 7-Box plot of Faith's phylogenetic diversity over sample day.



Supplement 8-Box plot of Faith's phylogenetic diversity over pen type.



Supplement 9-Box plot of Faith's phylogenetic diversity over lighting level.



Supplement 10-Box plot of Faith's phylogenetic diversity over temperature level.

Definitions/abbreviations

Shannon index- (Shannon, 1948; Tuomisto, 2010)- Diversity index that predicts how diverse a community is. Originally proposed to compare differences between strings of text.

Faith's phylogenetic diversity-(Faith, 1992)-A phylogenetic generalization of species richness that measures average branch length of phylogenetic trees.

Jaccard coefficient-(Jaccard,1912)- The fraction of unique features in a sample set.

Pielou's evenness-(Pielou, 1966)- An index of diversity and species richness, on a scale of zero to one.

Operational taxonomic unit (OTU)-(Sokal & Sneath,1963)- an operational definition method used to classify groups of related entities by their similarity threshold.

References

[1] Food and Drug Administration. Veterinary feed directive. Federal Register. 2015;80(106):31708-35.

[2] Prescott JF, Sivendra R, Barnum DA. The use of bacitracin in the prevention and treatment of experimentally-induced necrotic enteritis in the chicken. The Canadian veterinary journal. 1978;19(7):181.

[3] Elwinger K, Berndtson E, Engström B, Fossum O, Waldenstedt L. Effect of antibiotic growth promoters and anticoccidials on growth of Clostridium perfringens in the caeca and on performance of broiler chickens. Acta Veterinaria Scandinavica. 1998;39(4):433-41.

[4] Van Immerseel F, Rood JI, Moore RJ, Titball RW. Rethinking our understanding of the pathogenesis of necrotic enteritis in chickens. Trends in microbiology. 2009;17(1):32-6.

[5] Tian J, Du J, Lu Z, Han J, Wang Z, Li D, Guan X, Wang Z. Distribution of microbiota across different intestinal tract segments of a stranded dwarf minke whale, Balaenoptera acutorostrata. MicrobiologyOpen. 2020;9(10):e1108.
[6] Wang W, Zheng S, Li L, Yang Y, Liu Y, Wang A, Sharshov K, Li Y. Comparative metagenomics of the gut microbiota in wild greylag geese (Anser anser) and ruddy shelducks (Tadorna ferruginea). Microbiologyopen. 2019;8(5):e00725.
[7] Li H, Li T, Beasley DE, Heděnec P, Xiao Z, Zhang S, Li J, Lin Q, Li X. Diet diversity is associated with beta but not alpha diversity of pika gut microbiota. Frontiers in microbiology. 2016;7:1169.
[8] Ferguson RM, Merrifield DL, Harper GM, Rawling MD, Mustafa S, Picchietti S, Balcàzar JL, Davies SJ. The effect of Pediococcus acidilactici on the gut microbiota and immune status of on-growing red tilapia (Oreochromis niloticus). Journal of applied microbiology. 2010;109(3):851-62.
[9] Little TJ, Hultmark D, Read AF. Invertebrate immunity and the limits of mechanistic immunology. Nature immunology. 2005;6(7):651-4.
[10] Brugman S, Ikeda-Ohtsubo W, Braber S, Folkerts G, Pieterse CM, Bakker PA. A comparative review on microbiota manipulation: lessons from fish, plants, livestock, and human research. Frontiers in nutrition. 2018;5:80.
[11] Segata N, Waldron L, Ballarini A, Narasimhan V, Jousson O, Huttenhower C. Metagenomic microbial community profiling using unique clade-specific marker genes. Nature methods. 2012;9(8):811-4.
[12] Human Microbiome Project Consortium. Structure, function and diversity of the healthy human microbiome. nature. 2012;486(7402):207.
[13] Langille MG, Zaneveld J, Caporaso JG, McDonald D, Knights D, Reyes JA, Clemente JC, Burkepile DE, Thurber RL, Knight R, Beiko RG. Predictive functional profiling of microbial communities using 16S rRNA marker gene sequences. Nature biotechnology. 2013;31(9):814-21.
[14] Nagpal R, Wang S, Solberg Woods LC, Seshie O, Chung ST, Shively CA, Register TC, Craft S, McClain DA, Yadav H. Comparative microbiome signatures and short-chain fatty acids in mouse, rat, non-human primate, and human feces. Frontiers in microbiology. 2018;9:2897.

[15] Kostic AD, Howitt MR, Garrett WS. Exploring host–microbiota interactions in animal models and humans. Genes & development. 2013;27(7):701-18.
[16] Gootenberg DB, Turnbaugh PJ. Companion animals symposium: humanized animal models of the microbiome. Journal of animal science. 2011 May 1;89(5):1531-7.
[17] Alzubaidy H, Essack M, Malas TB, Bokhari A, Motwalli O, Kamanu FK, Jamhor SA, Mokhtar NA, Antunes A, Simões MF, Alam I. Rhizosphere microbiome metagenomics of gray mangroves (Avicennia marina) in the Red Sea. Gene. 2016;576(2):626-36.
[18] Vandenkoornhuyse P, Quaiser A, Duhamel M, Le Van A, Dufresne A. The importance of the microbiome of the plant holobiont. New Phytologist. 2015;206(4):1196-206.
[19] Chasteen K S, Bailey MA, Munoz L R, Krehling JT, Brooks L, Macklin K S. Establishment of Base Interactions of Select Genus Between Multiple Chicks Sources in Differential Housing. Manuscript submitted for publication.
[20] Bolyen E, Rideout JR, Dillon MR, Bokulich NA, Abnet CC, Al-Ghalith GA, Alexander H, Alm EJ, Arumugam M, Asnicar F, Bai Y. Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. Nature biotechnology. 2019;37(8):852-7.
[21] Callahan BJ, McMurdie PJ, Rosen MJ, Han AW, Johnson AJ, Holmes SP. DADA2: high-resolution sample inference from Illumina amplicon data. Nature methods. 2016;13(7):581-3.
[22] Singh B: The Effects of Feeding Reduced-oil DDGS to Broilers when Challenged with C. perfringens and Eimeria spp. on Necrotic Enteritis and Intestinal Microbiome. Master's thesis. Auburn University, Poultry Science Department ; 2017.

Chapter 4 -The Effect of Lighting as a Stressor on the Broiler Microbiome in Immune Challenged Floor Raised vs Cage Raised Birds

Abstract

Background: Microbial communities are a product of both the history of their host and the environment in which the host resides. Sudden introduction of non-beneficial microbes or the over representation of a normally benign microbe can throw the gut into disarray. This study was aimed at observing these effects. Day old broilers were placed in both floor pens and battery cages in four different rooms. Birds in rooms 3 & 4 were challenged with 1 ml coccidiosis vaccine containing Eimeria spp. at 10x the recommended dose, then inoculated at days 18,19, and 20 with 10^7 CFU/ml C. *perfringens*. Room 3- normal starting lighting (3 fc), inoculated at day 18. Room 4- half normal starting lighting (1.5 fc), inoculated at day 18. Room 1- normal starting lighting (3 foot candles [fc]), not inoculated at day 18. Room 2- half normal starting lighting (1.5 fc), not inoculated at day 18. Lighting was adjusted down at day 7 (1 fc normal, 0.5 fc low) and day 12 (0.3 fc normal, 0.15 fc low). Samples of the crop, midgut (defined as the area between the duodenal loop and Meckel's diverticulum) and the ceca were taken at day 0 and 21. Samples were DNA extracted, amplified via PCR, and sequenced using 16S rRNA Illumina MiSeq protocol. Raw reads were then passed through the QIIME bioinformatics pipeline for analysis.

Results: Overall crop samples were uniform, retaining *Lactobacillus* in all samples and treatments. Inoculated (**IN**) crop samples displayed a slightly lower average proportion of *Lactobacillus* (avg. ~80.17%) compared to non-inoculated (**NI)** samples (avg. ~91.06%). Midgut were more variable in content with lighting implying a larger impact on composition by encouraging a higher proportional *Lactobacillus* presence. **IN** low light (**LL**) pens had higher ratios of *Lactobacillus* compared to normal light (**HL**) pens. Battery midgut seemed to trend towards being more similar to one another while floor midgut was highly variable in community

content. **NI** birds were more variable in community composition when compared with inoculated birds, with **IN** midgut being dominated by *Lactobacillus* and having higher ratios of uncultured *Faecalibacterium.* For ceca **LL** pens, both **IN** and **NI** samples closely resembled each other regardless of inoculation status with battery samples displaying almost the same ratios in both treatments. Similarly floor treatments also closely resembled one another though not to the same degree as battery samples. For ceca samples there was some differentiation between pen types with battery samples being more variable in top classifications than floor samples. Ceca samples lighting had the inverse effect than that of midgut, with normal light treatments being relatively uniform with one main top classification compared to low light treatments which displayed four separate top classifications.

Conclusions: Invasive introduction of non-beneficial bacteria had some effect on community composition and diversity. However due to contamination of samples with the positive control during DNA extraction it is unclear to what extent this effect impacts the host microbial communities. Further investigation is required in order to clarify trends and conclusions.

Keywords: Broiler Chickens, Microbiota, Influence, Beneficial, Diversity

Background

An increase in prevalence of gastrointestinal diseases such as necrotic enteritis in broilers due to the 2015 prohibition all subtherapeutic antibiotics[1] in production animals has generated an increased interest in the manipulation and management of the gut, its functions, and the microbial communities therein [2, 3, 4]. The gut is already well known to be a large factor in the overall health and survival of production animals, however the microbial communities contained within it are less well documented. With the rise in use of molecular techniques to trace these communities the pool of information has started to expand, studies designed to cover little-studied microbiomes have become more widespread [5, 6, 7, 8, 9] and previously inaccessible data streams have emerged [6, 7]. Plant and animal species that are of economic importance to humans receive a large portion of academic attention [10, 11, 12, 13, 14, 15, 16],

while less impactful species often have little available information outside of initial investigations[5,6,7,17,18].

This trial was conducted to further explore the effect of environments and organism populations on the communities within the broiler gut. Previous studies have noted inoculation with C. *perfringens* has a disruptive effect on the gut microbiome [19,20] and may even spur an uptick in volatile fatty acid (VFA) production in VFA producing bacteria [19, 20, 21, 22, 23, 24,25]. To this purpose day old broiler chicks were acquired from a commercial hatchery and placed in traditional floor pens or battery cages at different lighting levels. Birds were dosed with ADVENT® (Huvepharma) coccidiosis vaccine (containing E. acervulina, E. maxima, and E. tenella) at day 15 then subsequently inoculated with a C. *perfringens* strain and necropsied at day 26.

Results

Due to concerns over contamination present in the negative controls for this sample set, the classifications of *Proteus* and *Clostridium sensu stricto* 1 were filtered from the results during processing and before generation of alpha and beta diversity analyses as to form a clearer picture without potential outside influence. Because of the inoculation of birds with *Clostridium* sp. it was unclear how much of the above mentioned classifications in the samples was due to the contamination. The contamination could have been from the positive control which contains a strain of *Clostridium* regularly used in the investigating lab, however the presence could also be explained by accidental contamination from samples of inoculated birds. Future investigations will be conducted with a completely unrelated positive control to avoid the possibility of a similar incident in the future.

Succession in the Crop Microbiota

For the Kruskal-Wallis (pairwise) significance of Faith's phylogenetic diversity (PD) test between body site sample types crop samples averaged a branch length of 6.91 at the depth of 11,111 (8 samples); being not significantly different with a p-value of 0.757 when compared to midgut samples and being significantly shorter (p= 0.012) when compared to ceca samples at the depth of 11,111.

Because of the shallow sequencing depth presented in the crop samples lower accuracy reads may be present, with 8 out of 9 samples reaching the relatively shallow sampling depth of 11,111. Crop samples presented an extremely low average number of observed OTUs with the average crop sample OTU count being ~62, similarly the average midgut sample OTU count was ~57. In contrast the average ceca sample OTU count was ~235 at the sequencing depth of 11,111. Crop samples displayed similar evenness (Pielou's Evenness index) compared to midgut samples and much lower and less variable evenness when compared to cecal samples; crop samples displayed an average of 0.660 (high of 0.730, low 0.541), midgut samples at an average of 0.590 (high of 0. 690, low 0.534), and cecal samples displayed an average of 0.851 (high of 0. 925, low 0.570). When run with a Kruskal-Wallis (pairwise) significance test results were statistically significant when compared to midgut ($p = 0.0243$) and ceca ($p = 4.11x10^{-3}$).

Top classification across all days were represented by *Lactobacillus*.

When mapped as the Jaccard coefficient PCOA (Fig.4-2, 4-3) samples showed a tendency for unchallenged crop samples to be grouped closer to day 0 than challenged samples.

Shannon diversity (SD) score was average over all for all samples at ~3.83.

Succession in the Midgut Microbiota

Midgut samples averaged a PD score of 7.15 (high 9.63, low 2.68) at the sampling depth of 11,111 (8 of 9 samples). Midgut samples were significantly less diverse than cecal samples with the Kruskal-Wallis (pairwise) significance of PD (p= 0. 0152). Midgut samples presented a much lower average number of observed OTUs with the average midgut sample OTU count being ~57 compared to the average cecal sample OTU count being ~235 at the sequencing depth of 11,111. Midgut samples exhibited much lower and more variable evenness compared to ceca samples, with midgut samples displaying the lowest average evenness score of 0.590 (high of 0.690, low 0.534) vs ceca (0.851) and crop (0.660) . A Kruskal-Wallis (pairwise) significance test was statistically significant with $p = 2.32x10^{-3}$ when compared to ceca samples and p= 0.0243 when compared to crop. For midgut samples the top classifications varied slightly with the constant top genus being *Lactobacillus* for 6 of 9 samples, *Clostridioides* for 2 samples, and the remaining sample topped by *Lachnospiraceae*. Similarly to crop samples, challenged midgut samples plotted on a Jaccard coefficient PCOA (Fig.4-2, 4-3) trended towards differentiation.

Shannon diversity score was moderate over samples at sampling depth of 11,111, the average being 3.34 (high 4.67, low 2.18).

Succession in the Cecal Microbiota

Cecal samples were the most diverse of samples taken with an average branch length of 10.96 with Faith's phylogenetic diversity (PD) test, being significant (p=0. 0152) when compared to midgut samples and (p=0. 0119) crop samples using a Kruskal-Wallis (pairwise) significance. Ceca samples presented a much higher average number of observed OTUs a with the average cecal sample OTU count being ~235. Ceca samples exhibited much higher and less variable evenness (Pielou's Evenness index) compared to midgut or crop samples, with cecal samples displaying an average of 0.850 (high of 0.925, low 0.568) vs midgut (0.590) and crop (0.660) samples. Results were significant when run with a Kruskal-Wallis (pairwise) significance test when compared to crop ($p = 4.11x10^{-3}$) and midgut ($p = 2.32x10^{-3}$). In cecal communities the top classifications were *Lachnospiraceae*, *Ruminococcaceae* UCG-014, *Ruminococcaceae*, and *Faecalibacterium* sub. bacterium ic1379. When plotted in a Jaccard coefficient PCOA (Fig. 4-3) ceca samples showed less differentiation compared to crop or midgut samples, with ceca samples falling closer to day 0 than other sample types regardless of pen type or inoculation status. Shannon diversity score for ceca samples was relatively high compared with crop (3.830) and midgut (3.34) samples, the average being 6.91.

Trends in Pen Types

Floor birds were placed on fresh pine shavings to minimize influence of previous flocks. Battery samples averaged a PD score of 8.51 (high 12.15, low 5.69) at the sequencing depth of 11,111. Floor samples averaged a PD score of 8.33 (high 12.90, low 2.68). Samples were not significantly diverse from one another when analyzed using Kruskal-Wallis (pairwise) significance test($p>0.05$). Battery samples presented an average 114 of observed OTUs (high 300, low 35) at the sequencing depth of 11,111. Floor samples averaged 124 observed OTUs (high 336, low 17). For battery samples evenness was relatively high with an average of 0.714, floor samples displayed a slightly lower average evenness of 0.686. Samples did not display significantly different evenness when compared with Kruskal-Wallis (pairwise) significance test ($p>0.05$). For battery crop samples, top classifications were consistent between treatments with

Lactobacillus being top for all samples. For battery midgut samples, top classifications were mostly consistent between treatments with top genus being *Lactobacillus* for all sample points save for normal-lighting non-inoculated battery (HNB) birds having *Clostridioides* as the top classification. For battery ceca samples, top classifications were evenly split between *Lachnospiraceae* for normal-lighting non-inoculated battery (HNB) and normal-lighting inoculated battery (HIB),and *Ruminococcaceae* UCG-014 for low-lighting non-inoculated battery (LNB) and low-lighting inoculated battery (LIB).

For floor crop samples, top classifications were consistent between treatments with *Lactobacillus* being top for all samples. For floor midgut samples, top classifications were mostly consistent between treatments with top genus being *Lactobacillus* for all sample points save for normal-lighting non-inoculated floor (HNF) samples having uncultured *Faecalibacterium* as the top classification. Because of low levels of quality samples normal-lighting inoculated floor (HIF) ceca samples were not included in the results. For floor ceca samples, top classifications were highly varied with *Lachnospiraceae* being top or sharing top classification for normal-lighting non-inoculated floor (HNF), low-lighting inoculated floor (LIF),and low-lighting non-inoculated floor (LNF). *Ruminococcaceae* UCG-014 shared top classification for LIF. Finally for LNF the top classification was also shared by *Faecalibacterium* sub. bacterium ic1379 and generic *Ruminococcaceae*. Shannon diversity score for battery samples (4.66) was similar to floor samples (4.77).

Trends in Lighting levels

Normal lighting (HL) samples averaged a PD score of ~8.33 (high 12.15, low 6.67) at the sequencing depth of 11,111. Low Light (LL) samples averaged a PD score of ~8.51(high 12.90, low 2.68). Samples were not significantly different from one another when analyzed using Kruskal-Wallis. HL samples presented an average ~115 of observed OTUs (high 300, low 33) at the sequencing depth of 11,111. LL averaged ~123 observed OTUs (high 336, low 17). For evenness samples did not display significantly different evenness when compared with Kruskal-Wallis (pairwise) significance test ($p>0.05$) with HL displaying an average evenness of 0.700 and LL pens an average of 0.696. HL crop samples showed *Lactobacillus* as the sole top classification for all treatments. HL midgut samples displayed either *Lactobacillus* (HIF,HIB), *Clostridioides* (

HNB), or *Lachnospiraceae* (HNF) as top classifications. HL ceca sample top classifications were represented by *Lachnospiraceae* (HNF, HNB, HIB).

LL crop samples showed *Lactobacillus* as the top classification for all sample days. LL midgut samples were dominated by *Lactobacillus* (LNB, LIB, LNF, LIF). LL ceca top classifications were more variable than HL ceca samples with *Lachnospiraceae* representing or sharing top representation for LNF and LIF, *Ruminococcaceae* UCG-014 representing top classification for LNB, LIB, and LIF, and generic *Ruminococcaceae* sharing top classification for LNF.

HL Shannon diversity (SD) score was moderate, the average being 4.62 (high 7.49, low 2.95). LL Shannon diversity (SD) score was also moderate, the average being 4.83(high 7.42, low 2.18).

Trends in Inoculation Status

Overall non-inoculated samples reached a notably deeper sequencing depth (~36,000) compared to inoculated samples (~16,000).

Non-inoculated (**NI**) samples averaged a PD score of ~9.25 (high 12.90, low 6.23) at the sequencing depth of 11,111. Inoculated (**IN**) samples averaged a PD score of ~7.36(high 11.77, low 4.61). **NI** samples were significantly different from **IN** when analyzed using Kruskal-Wallis ($p=0.0153$). **NI** samples presented an average ~142 of observed OTUs (high 336, low 49) at the sequencing depth of 11,111. **IN** samples averaged ~90 observed OTUs (high 210, low 30). Samples did not display significantly different evenness when compared with Kruskal-Wallis (pairwise) significance test with **NI** displaying an average evenness of 0.731 and **IN** pens an average of 0.668.

NI crop samples showed *Lactobacillus* as the sole top classification for all treatments. **NI** midgut samples displayed either *Lactobacillus* (LNF,LNB), *Clostridioides* (HNB), or *Lachnospiraceae* (HNF) as top classifications. **NI** ceca sample top classifications were represented by or shared top representation with *Lachnospiraceae* (HNF, HNB, LNF). LNF shared top spots with generic *Ruminococcaceae* and *Faecalibacterium* sub. bacterium ic1379. LNB was characterized by *Ruminococcaceae* UCG-014.

IN crop samples showed *Lactobacillus* as the top classification for all sample days. **IN** midgut samples were dominated by *Lactobacillus* (HIB, HIF, LIB, LIF). **IN** ceca top classifications were as variable as **NI** ceca samples with *Lachnospiraceae* representing or sharing top representation

for HIB and LIF, and *Ruminococcaceae* UCG-014 representing top classification for LIB and LIF. **NI** Shannon diversity (SD) score was moderate, the average being 5.05 (high 7.49, low 3.16). **IN** Shannon diversity (SD) score was also moderate, the average being 4.38(high 7.09, low 3.02).

Discussion

Overall crop samples were uniform, retaining *Lactobacillus* in all samples and treatments. **IN** crop samples displayed a slightly lower average proportion of *Lactobacillus* (avg. ~80.17%) compared to **NI** samples (avg. ~91.06%). **IN** samples were also similar to day 0 compared to **NI** samples when plotted as a Jaccard PCOA (Fig. 4-3, 4-4) with biplot analysis indicating heavy influence by *Clostridiaceae* 1 and *Clostridioides*. Lighting level and pen type seemed to have little effect on the microbial community present, however since results were heavily filtered to remove suspected contamination this may not have originally been the case.

Similarly to crop samples, **IN** midgut samples did not differentiate as much from day 0 starting point as **NI** samples when plotted on the Jaccard PCOA(Fig. 4-3, 4-4) with biplot analysis again indicating heavy influence by *Clostridiaceae* 1 and *Clostridioides* especially in the **IN** samples. **NI** midgut were more variable in content with lighting implying a larger impact on composition by encouraging *Lactobacillus* growth. **IN** samples though more uniform also seemed to be slightly affected by lighting level, with low light pens having slightly higher ratios of *Lactobacillus* compared to normal light pens. Battery pens seemed to trend towards being more similar while floor pens were highly variable in community content. The affinity of *Lactobacillus* for lower lighting seems to be a repeating trend when compared to the previous trial that examined lighting levels [26] where lactobacillus seemed to favor lower lighting and higher temperature pens; the bacterium also appeared frequently in low temperature pens with low lighting but was notably less prevalent in low temperature/high light pens.

Similarly to the previous trial [26] cecal samples both lighting and pen type seemed to have some effect on population proportions, with low light pens being more variable than normal light pens. Furthermore in low light pens, both **IN** and **NI** samples closely resembled each other regardless of inoculation status with battery (LIB, LNB) displaying almost the same ratios in both treatments. Floor treatments (LIF, LNF) also closely resembled one another though not to the same degree as battery samples. Additionally ,ceca samples continued to maintain the

same evenness between treatment types with little differentiation (Fig. 4-3, 4-4) as seen in the previous chapters.

Pen type seemed to have little effect on community content in crop or midgut samples, though battery midgut samples seemed less variable than floor midgut samples. For ceca samples there was some differentiation between pen types with battery samples being more variable in top classifications than floor samples.

Lighting had little apparent effect on crop samples. For midgut samples low light treatments (4 of 4) were dominated by *Lactobacillus* in contrast with normal light treatments (2 of 4). Moreover normal light treatments that possessed *Lactobacillus* as the highest proportioned community member did so at a lower ratio than low light pens. For ceca samples lighting had the inverse effect, with normal light treatments being relatively uniform with one main top classification compared to low light treatments which displayed four separate top classifications and ratios. Overall there seems to be a trend of *Lactobacillus* being notably more prevalent in low light environments, regardless of ambient temperature[26]. Whether or not this is due to stress of the bird in higher lighting environments or stress of the bacteria is unclear. Though very few papers were found on the subject when investigated further, there is some slight indication that higher light levels have some unquantified effect on select microbes [27] and *Lactobacillus* in particular; a pilot study from 2017 measured metabolites produced by three *Lactobacillus* strains grown in both light and dark conditions and though overall growth was not affected the metabolites produced were different between environments [28]. Due to the lack of information on this particular phenomenon an additional study would be required to investigate further.

Inoculation status had no apparent outstanding effect on crop community proportions. For midgut samples non-inoculated birds were more variable in community composition when compared with inoculated birds, with **IN** being dominated by *Lactobacillus* and having higher ratios of uncultured *Faecalibacterium.* In ceca samples both **IN** and **NI** samples shared similar top classifications, **NI** samples displayed slightly lower ratios of the classifications present.

Conclusion

Invasive introduction of non-beneficial bacteria had some effect on community composition and diversity. However due to contamination of samples with the positive control it is unclear to what extent this effect impacts the host microbial communities. Similarly to the previous chapter ("*The Effect of Lighting and Temperature as Stressors on the Broiler Microbiome in Floor Raised vs Cage Raised Birds*"), the lighting level present did impact the percentage of *Lachnospiraceae*, *Ruminococcaceae* UCG-014, and generic *Ruminococcaceae* present in ceca samples regardless of pen type. Lighting also seemed to have an impact on percentage of *Lactobacillus* present in midgut samples, low light pens having a higher ratio than normal lighting. Because of the misstep of contamination this trial should be rerun to confirm the conclusions made in this paper and in order to make broader conclusions, however there does seem to be a strong link between lighting level and *Lactobacillus* presence in particular that warrants additional investigation.

Methods

Bird and Farm Management

Unsexed and unvaccinated day-old broiler chicks were obtained from a commercial hatchery and transported to the Auburn University Poultry Research Farm. Birds were split into eight groups:

A-Floor, normal starting lighting (3 foot candles [fc]), no inoculation

B-Battery, normal starting lighting (3 fc), no inoculation

C- Floor, half normal starting lighting (1.5 fc), no inoculation

D- Battery, half normal starting lighting (1.5 fc), no inoculation

E- Floor, normal starting lighting (3 fc), inoculation

F- Battery, normal starting lighting (3 fc), inoculation

G- Floor, half normal starting lighting (1.5 fc), inoculation

H- Battery, half normal starting lighting (1.5 fc), inoculation

These groups were randomly placed in two pen types in four rooms: traditional floor(1 pen per room), and battery cages (3 per room). Floor pens received 30 birds each. Batteries received 10

per cage. Rooms **1** and **3** lighting was started at a normal lighting of 3 foot candles as per industry standard, reduced to 1.5 fc at day 7 and again reduced to 0.3 at day 12. Rooms **2** and **4** lighting was started at a reduced 1.5 fc, being further reduced to 0.3 fc at day 7 and again to 0.15 fc at day 12. At day 15, birds in treatments **E**, **F**, **G**, and **H** were challenged using a 1 mL oral gavage of ADVENT® (Huvepharma) coccidiosis vaccine (containing E. acervulina, E. maxima, and E. tenella) diluted with sterile water and administered at a 10 x recommended dose per bird. On days 18, 19, and 20 previously challenged birds were inoculated with a NetB toxin producing C. *perfringens* strain diluted to 10^7 CFU/mL. The C. *perfringens* strain was grown from a frozen bacterial isolate streaked on to 5% sheep blood tryptic soy agar (TSA) incubated anaerobically at 37°C for approximately 24 hours. From the TSA plate a typical C. *perfringens* colony was removed and placed in brain-heart infusion broth to be incubated anaerobically for another 24 hours at 37°C. Inoculum was assumed to have grown to 10^9 CFU and was verified by serially diluting and spread plating inoculum after grow up.

Birds were fed a standard starter diet from day 0 to day 14, then grower from day 15 for the remainder of the trial. Necropsies to acquire samples of the crop, midgut (defined as the section between the duodenal loop and Meckel's diverticulum) and entire ceca were conducted at day 0 and 26. Five birds per group were euthanized via CO_2 asphyxiation and sampled using aseptic technique. Samples were individual taken by treatment and type then placed on ice after acquisition. After transport to the lab samples were placed in -80^{o}C freezer until DNA extraction.

DNA Extraction

After removal from the -80^{o}C freezer, ceca and midgut samples were thawed and extracted using the Omega Bio-tek E.Z.N.A. Stool DNA Extraction kit according to manufacturer's instructions, with one modification in that for step 4 DNA was incubated at 54^{o}C overnight followed by 10 minutes at 70^{o}C to insure cell breakdown as per recommendations from Omega Bio-tek trouble-shooting staff. Extracted DNA was tested for concentration and purity with a NanoDrop® ND-1000 Spectrophotometer, the desired concentration being a 260nm/280nm ratio between 1.8 - 2.0. Afterwards, DNA was place in a 2^{o}C refrigerator to await further processing.

Polymerase Chain Reaction (PCR) Amplification

DNA amplification via PCR was performed using a BioRad iQ5 thermocycler by touchdown protocol in order to maximize the amount of DNA amplified (Fig. 4-1). To that point universal target primers CS1/515F (5'-GTGYCAGCMGCCGCGGTAA-3') and CS2/926R (5'-CCGYCAATTYMTTTRAGTTT-3') were used to further expand potential targets. Amplification was confirmed with agarose gel electrophoresis on a 2% gel made with Tris-acetate-EDTA (TAE) buffer using Lonza® 100 bp Extended Range DNA Ladder. Gels were run for 75v for ~1 hour or until satisfactory visual conformation. Following confirmation, DNA PCR product was stored in a 2^{o}C refrigerator.

Illumina MiSeq Sequencing

PCR product was subsequently pooled by type and treatment, labeled, and sent to University of Illinois Chicago DNA Services Facility (UIC DNAS) facility under temperature-controlled conditions for 16S rRNA Illumina gene sequencing under a Illumina MiSeq protocol.

Data Analysis and Statistics

Following sequencing, raw FASTQ files were uploaded to the Illumina BaseSpace cloud database. Raw FASTQ files were downloaded from BaseSpace and uploaded to the Alabama Supercomputer (ASC) for more in-depth memory heavy analysis with QIIME2 pipeline [29]. Fastq files in Casava 1.8 paired end demultiplex format were read into QIIME2 to be joined and denoised using DADA2 [30]. Denoising and dereplication proceeded based on demultiplexing stats with forward reads being truncated at 220 base pairs and a max error rate of 4. The resulting feature table and representative sequences table were further filtered to exclude eukaryotic sequences and then used to determine optimum sampling depth in order to retain the most features without excluding a large number of samples.

The highest feature frequency per sample being 59,051, the lowest being 3,936 and the mean frequency being 20,740. For diversity analysis a sampling depth of 8200 was chosen to retain as many samples as possible. Reads with a frequency less than 5 were removed from sampling. A phylogenetic diversity analyses tree was generated using the q2-phylogeny plug-in. Taxonomic classification plug-in classify-sklearn was run using the Silva 132 99% full length classifier sequences set to identify Operational Taxonomic Units (OTUs). Using the classified data,

filtering was preformed using the “filter table” function to remove sequences identified as belong to Eukaryotic organisms to eliminate host DNA. Filtering for Proteus and “Clostridium_sensu_stricto” identified sequences was also conducted due to high numbers present in the negative control. The QIIME2 diversity core-metrics-phylogenetic command was used to generate alpha and beta diversity analysis. QIIME2 “diversity alpha-rarefaction” command was used to generate an alpha rarefaction curve. A PCOA biplot was generated using the “diversity pcoa_biplot” command to establish a directional key for the prominent taxa responsible for some of the biggest shifts in the Jaccard graph. Taxonomic bar-plots were generated by loading taxonomic data into R Studio and generating barplots using the ggplots package.

Figures

Reaction conditions	1 x Rxn volume (µL)
Stage 1[1]	
Master Mix[2]	12.5
DdH_2O[3]	10.75
DNA Template[4]	1.0
Forward Primer[5]	0.5
Reverse Primer[6]	0.5
BSA[7]	0.25
Stage 2[8]	
Primers[9]	0.4
Stage 1 PCR Yield	1.0

[1]Performed at Auburn University Laboratory (Auburn, AL).

[2]Master Mix = Lucigen EconoTaq Plus 2x Master Mix (Middleton, WI): 0.1 units/µL of EconoTaq DNA Polymerase, Reaction Buffer (pH 9.0), 400 µM dATP, 400 µM dGTP, 400 µM dCTP, 400 µM dATP, 3 mM MgCl2, and a proprietary mix of PCR Enhancer/Stabilizer and blue and yellow tracking dyes.

[3]DdH_2O = double distilled H_2O.

[4]DNA Template = pooled jejunum tissue sample (5 birds/pen, each 2 cm in length, 15 cm proximal from Meckel's diverticulum) bacterial DNA extracted using Wizard Genomic Purification Kit (Madison, WI).

[5]Forward Primer = Eurofins Genomics CS1/515F Universal Target 16S rRNA Gene (Louisville, KY): 5'-GTGYCAGCMGCCGCGGTAA-3', expected amplicon size with CS1 456, annealing temperature 50°C.

[6]Reverse primer = Eurofins Genomics® CS2/926R Universal Target 16S rRNA Gene (Louisville, KY): 5'-CCGYCAATTYMTTTRAGTTT-3', expected amplicon size with CS2 456, annealing temperature 50°C.

[7]BSA (Bovine Serum Albumin) = Purified BSA 100x (Lawrenceville, GA).

[8]Performed at University of Illinois Chicago DNA Services Facility (Chicago, IL).

[9]Primers = Fluidigm's AccessArray Barcoded Primers (San Francisco, CA): Read 1: Standard CS1: A+CA+CTG+ACGACATGGTTCTACA; Index Read: CS2rc: A+GAC+CA+AGTCTCTGCTACCGTA; Read 2: Standard CS2: T+AC+GGT+AGCAGAGACTTGGTCT (A "+" preceding a base indicates a Locked Nucleic Acids(LNA)-base).

Cycle Step	Temperature	Time	Cycles
Stage 1[1]			
Initial Denaturation	94°C	5 min	1x
Variable Temperature Annealing			15x, reduce 1°C/cycle
Denaturation	94°C	30 sec	
Annealing	60°C	30 sec	
Extension	72°C	30 sec	
Constant Temperature Annealing	94°C		30x
Denaturation	94°C	30 sec	
Annealing	55°C	30 sec	
Extension	72°C	30 sec	
Final Extension	72°C	7 min	1x
Hold	4°C	∞	
Stage 2[2]			
Initial Denaturation	95°C	5 min	1x
Variable Temperature Annealing			28x
Denaturation	95°C	30 sec	
Annealing	60°C	30 sec	
Extension	72°C	30 sec	

[1]Performed at Auburn University Laboratory (Auburn, AL) using a Bio-Rad IQ5 thermocycler (Hercules, CA).

[2]Performed at University of Illinois Chicago DNA Services Facility (Chicago, IL) in preparation for Illumina MiSeq (San Diego, CA).

Figure 4-1- Reaction conditions and primers for amplification of bacterial populations by polymerase chain reaction (PCR) via touchdown protocol [21].

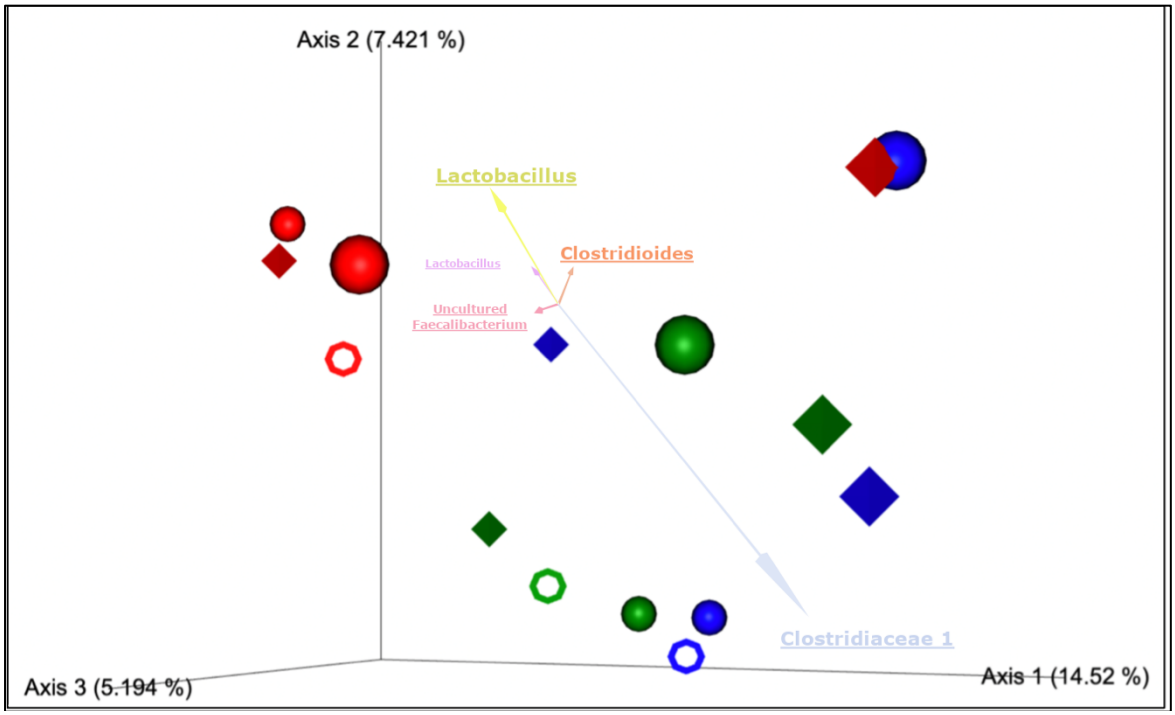


Figure 4-2-Jaccard PCOA-Floor (Diamond(♦)= normal light, , Sphere(o)= low light, Ring(◎)= day 0. red= ceca, blue = midgut, green= crop. Large icon= inoculated, small icon=non-inoculated. Midgut samples showed the most differentiation from day 0, whereas ceca samples did not show much differentiation from day 0. Crop samples differentiated moderately compared to midgut and ceca. When tied to a PCOA biplot using the "diversity pcoa_biplot"command in QIIME2 a directional key was generated using the prominent taxa responsible for some of the biggest shifts in the graph. These taxa were Clostridiaceae 1, Clostridioides, Lactobacillus, and Uncultured Faecalibacterium.

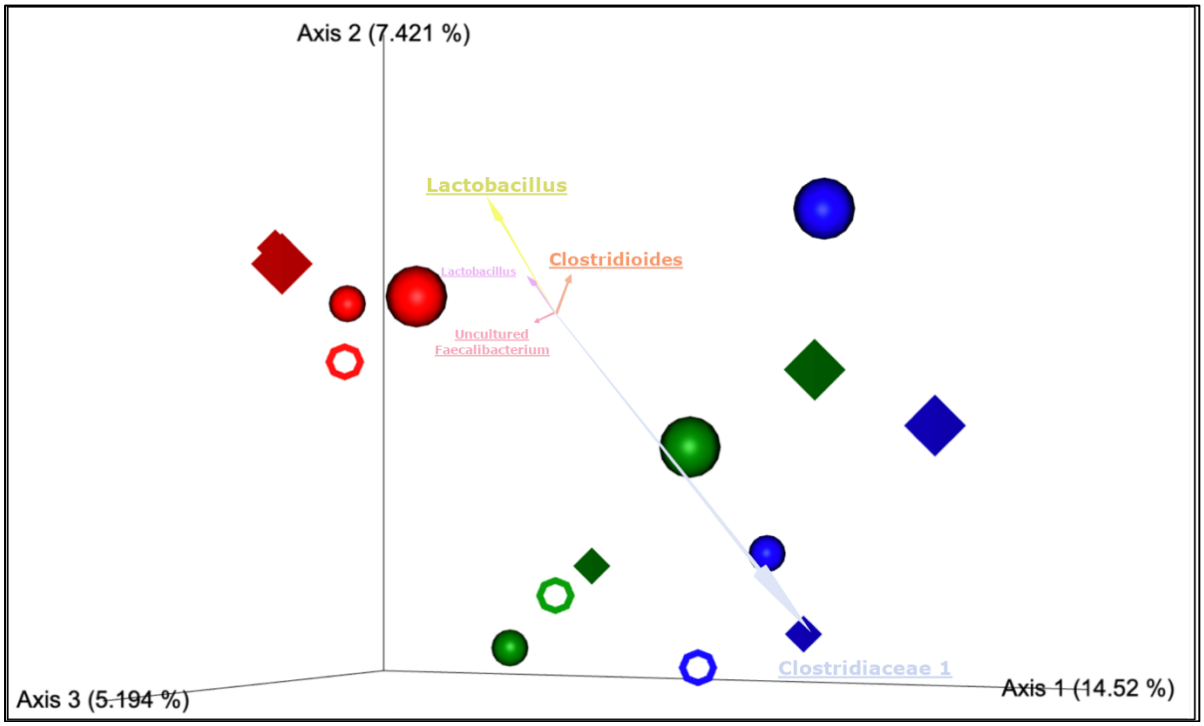


Figure 4-3-Jaccard PCOA-Battery(Diamond(♦)= normal light, , Sphere(o)= low light, Ring(◎)= day 0. red= ceca, blue = midgut, green= crop. Large icon=inoculated, small icon=non-inoculated). Similarly to floor samples, midgut battery samples showed the most differentiation from day 0, whereas ceca samples differentiated little from day 0. Crop samples differentiated

moderately compared to midgut and ceca. When tied to a PCOA biplot using the "diversity pcoa_biplot"command in QIIME2 a directional key was generated using the prominent taxa responsible for some of the biggest shifts in the graph. These taxa were Clostridiaceae 1, Clostridioides, Lactobacillus, and Uncultured Faecalibacterium.

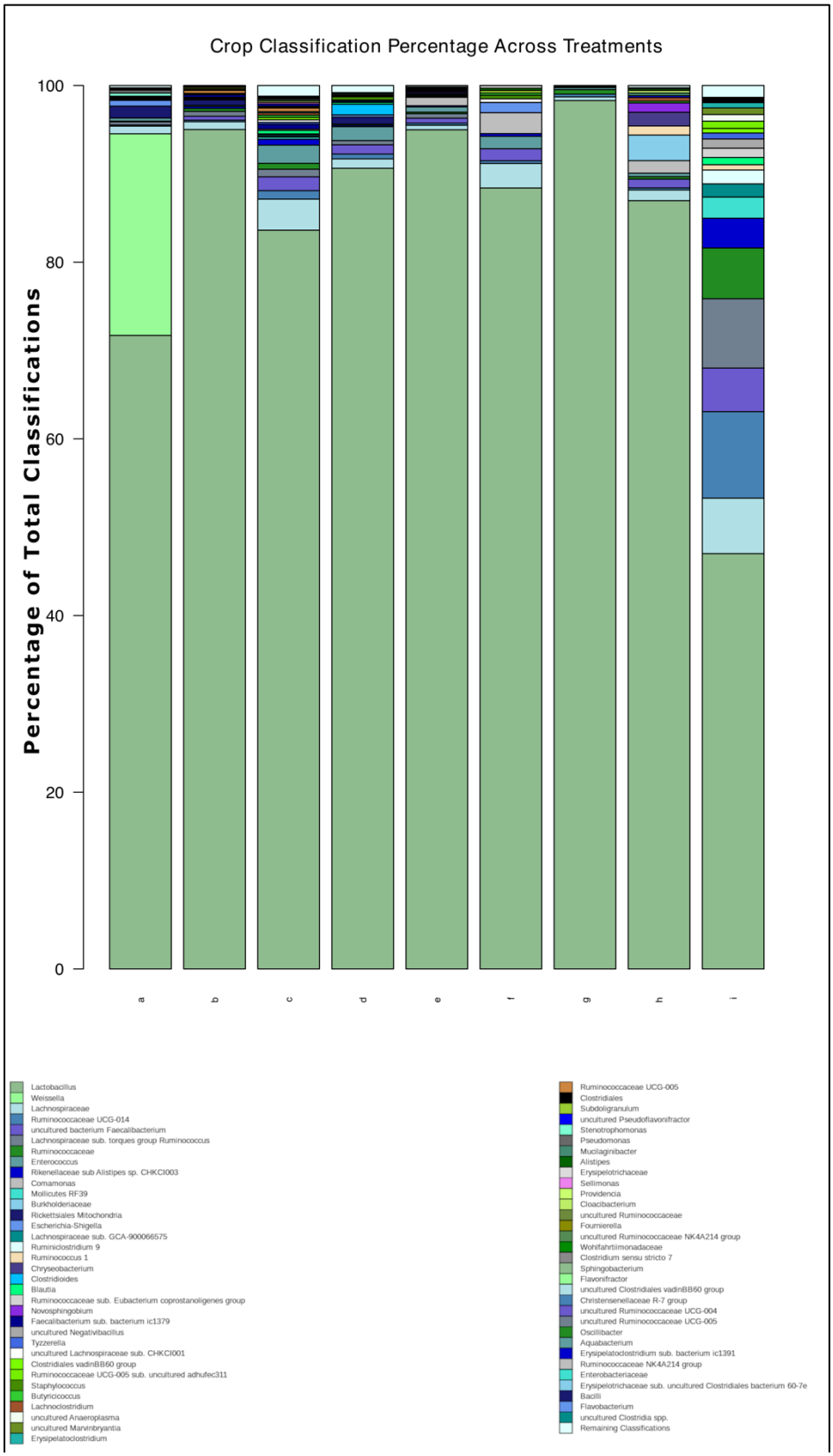


Figure 4-4-Top Classification Percentages, Crop. Letters reference corresponding columns in Table 4-1.

ID	Day	Status	Lighting	Pen	Top Classifications Crop
a	0	Non-inoculated	NA	NA	*Lactobacillus(~72%), Weissella(~23%), Rickettsiales Mitochondria(~1%), Lachnospiraceae(~1%), Escherichia-Shigella(~1%)*
b	21	Non-inoculated	N	F	*Lactobacillus(~95%), Lachnospiraceae (~1%), Rickettsiales Mitochondria(~1%), Lachnospiraceae sub. torques group Ruminococcus(~1%), uncultured Faecalibacterium(~1%)*
c	21	Non-inoculated	N	B	*Lactobacillus(~84%), Lachnospiraceae(~4%), Enterococcus(~2%), uncultured Faecalibacterium(~2%), Ruminococcaceae UCG-014(~1%), Lachnospiraceae sub. torques group Ruminococcus(~1%), Rikenellaceae sub Alistipes sp. CHKCI003(~1%), Ruminococcaceae(~1%), Faecalibacterium sub. bacterium ic1379(~1%)*
d	21	Non-inoculated	L	F	*Lactobacillus(~91%), Enterococcus(~2%), Clostridioides(~1%), Lachnospiraceae (~1%), uncultured Faecalibacterium(~1%), Rickettsiales Mitochondria(~1%), Ruminococcaceae UCG-014(~1%), Lachnospiraceae sub. torques group Ruminococcus (~1%)*
e	21	Non-inoculated	L	B	*Lactobacillus(~95%), Comamonas(~1%), Enterococcus(~1%), uncultured Faecalibacterium(~1%), Lachnospiraceae sub. torques group Ruminococcus(~1%), Lachnospiraceae(~1%)*
f	21	Inoculated	N	F	*Lactobacillus(~88%), Lachnospiraceae(~3%), Comamonas(~2%), Enterococcus(~1%), uncultured Faecalibacterium(~1%), Escherichia-Shigella(~1%)*
g	21	Inoculated	N	B	*Lactobacillus(~98%), Ruminococcaceae(~1%)*
h	21	Inoculated	L	F	Lactobacillus*(~87%),* Burkholderiaceae*(~3%),* Chryseobacterium*(~2%),* Comamonas*(~1%),* Lachnospiraceae*(~1%),* Novosphingobium*(~1%),* Ruminococcus 1*(~1%),* uncultured Faecalibacterium*(~1%)*
i	21	Inoculated	L	B	*Lactobacillus(~47%), Ruminococcaceae UCG-014(~10%), Lachnospiraceae sub. torques group Ruminococcus(~8%), Lachnospiraceae(~6%), Ruminococcaceae(~6%), uncultured Faecalibacterium(~5%), Rikenellaceae sub Alistipes sp. CHKCI003(~3%), Mollicutes RF39(~2%), Ruminiclostridium 9(~2%), Lachnospiraceae sub. GCA-900066575(~2%), Ruminococcaceae sub. Eubacterium coprostanoligenes group(~1%), uncultured Negativibacillus(~1%)*

Table 4-1-Top Classifications, Crop. Lactobacillus was dominant classification across all treatments sampled with little variation.

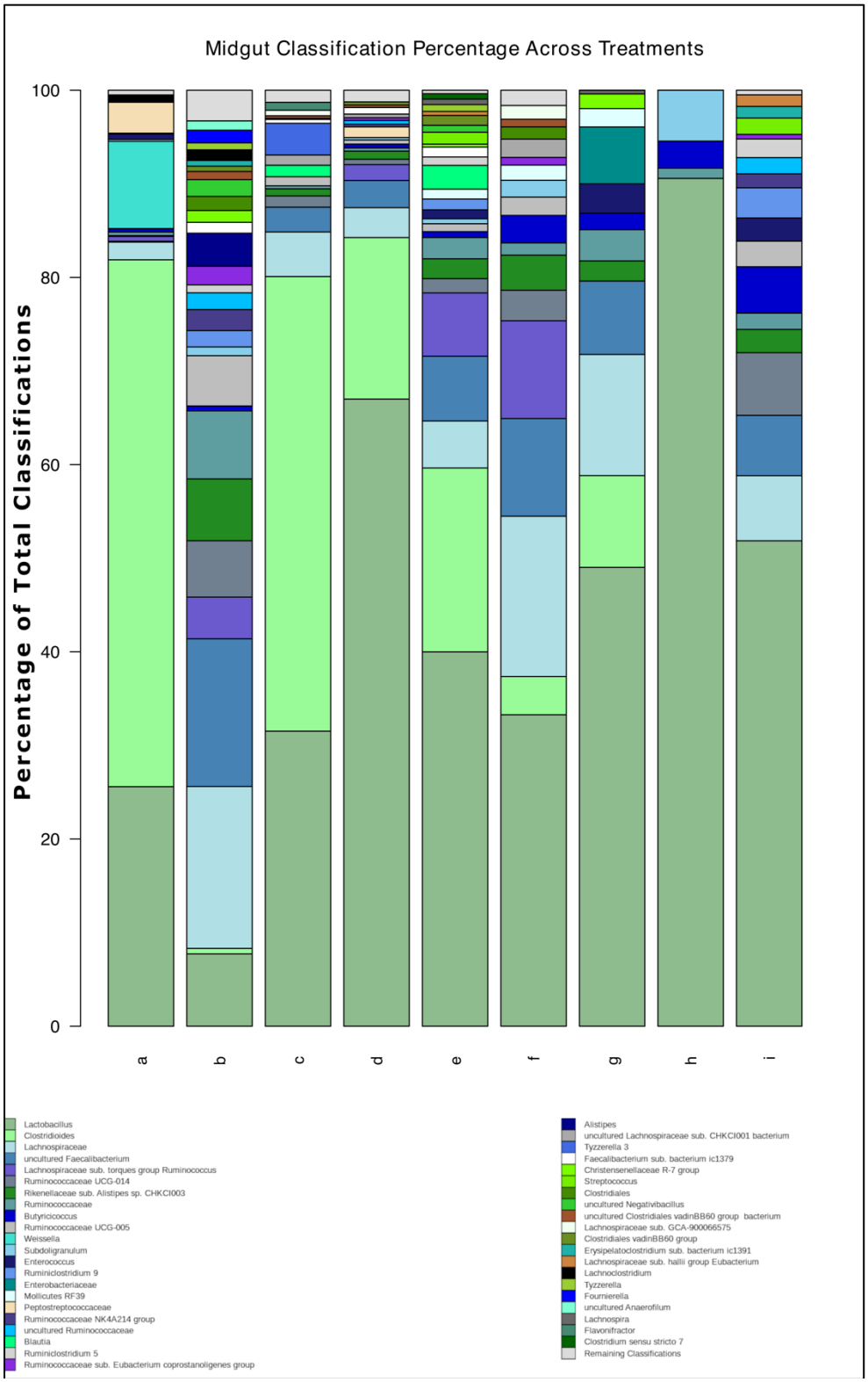


Figure 4-5-Top Classification Percentages, Midgut. Letters reference corresponding columns in Table 4-2.

ID	Day	Status	Lighting	Pen	Top Classifications Midgut
a	0		NA	NA	*Clostridioides(~56%), Lactobacillus(~26%), Weissella(~9%), Peptostreptococcaceae(~3%), Lachnospiraceae(~2%), Enterococcus(~1%), Lachnospiraceae sub. torques group Ruminococcus(~1%), Lachnoclostridium(~1%)*
b	21	Non-inoculated	N	F	*Lachnospiraceae(~17%), uncultured Faecalibacterium(~16%), Lactobacillus(~8%), Ruminococcaceae(~7%), Rikenellaceae sub. Alistipes sp. CHKCI003(~7%), Ruminococcaceae UCG-014(~6%), Ruminococcaceae UCG-005(~5%), Lachnospiraceae sub. torques group Ruminococcus(~5%), Alistipes(~4%), Ruminococcaceae NK4A214 group(~2%)*
c				B	*Clostridioides(~59%), Lactobacillus(~32%), Lachnospiraceae(~5%), Tyzzerella 3(~3%), uncultured Faecalibacterium(~3%), Blautia(~1%), Ruminococcaceae UCG-014(~1%), uncultured Lachnospiraceae sub. CHKCI001 bacterium(~1%), Ruminococcaceae UCG-005(~1%), Flavonifractor(~1%)*
d			L	F	*Lactobacillus(~67%), Clostridioides(~17%), Lachnospiraceae(~3%), uncultured Faecalibacterium(~3%), Lachnospiraceae sub. torques group Ruminococcus(~2%), Peptostreptococcaceae(~1%), Rikenellaceae sub. Alistipes sp. CHKCI003(~1%), Faecalibacterium sub. bacterium ic1379(~1%), Ruminococcaceae UCG-014(~1%)*
e				B	*Lactobacillus(~40%), Clostridioides(~20%), uncultured Faecalibacterium(~7%), Lachnospiraceae sub. torques group Ruminococcus(~7%), Lachnospiraceae(~5%), Blautia(~3%), Ruminococcaceae(~2%), Rikenellaceae sub. Alistipes sp. CHKCI003(~2%), Ruminococcaceae UCG-014(~2%), Streptococcus(~1%)*
f		Inoculated	N	F	*Lactobacillus(~33%), Lachnospiraceae(~17%), uncultured Faecalibacterium(~11%), Lachnospiraceae sub. torques group Ruminococcus(~11%), Clostridioides(~4%), Rikenellaceae sub. Alistipes sp. CHKCI003(~4%), Ruminococcaceae UCG-014(~3%), Butyricicoccus(~3%), Ruminococcaceae UCG-005(~2%), uncultured Lachnospiraceae sub. CHKCI001 bacterium(~2%)*
g				B	*Lactobacillus(~49%), Lachnospiraceae(~13%), Clostridioides(~10%), uncultured Faecalibacterium(~8%), Enterobacteriaceae(~6%), Ruminococcaceae(~3%), Enterococcus(~3%), Rikenellaceae sub. Alistipes sp. CHKCI003(~2%), Mollicutes RF39(~2%), Butyricicoccus(~2%), Christensenellaceae R-7 group(~2%)*
h			L	F	*Lactobacillus(~91%), Subdoligranulum(~5%), Butyricicoccus(~3%), Ruminococcaceae(~1%)*
i				B	*Lactobacillus(~52%), Lachnospiraceae(~7%), Ruminococcaceae UCG-014(~7%), uncultured Faecalibacterium(~5%), Butyricicoccus(~3%), Ruminiclostridium 9(~3%), Ruminococcaceae UCG-005(~3%), Enterococcus(~3%), Rikenellaceae sub. Alistipes sp. CHKCI003(~3%), Ruminiclostridium 5(~2%)*

Table 4-2-Top Classifications, Midgut. Lactobacillus, Clostridioides, and Lachnospiraceae were the dominant classifications present in midgut samples.

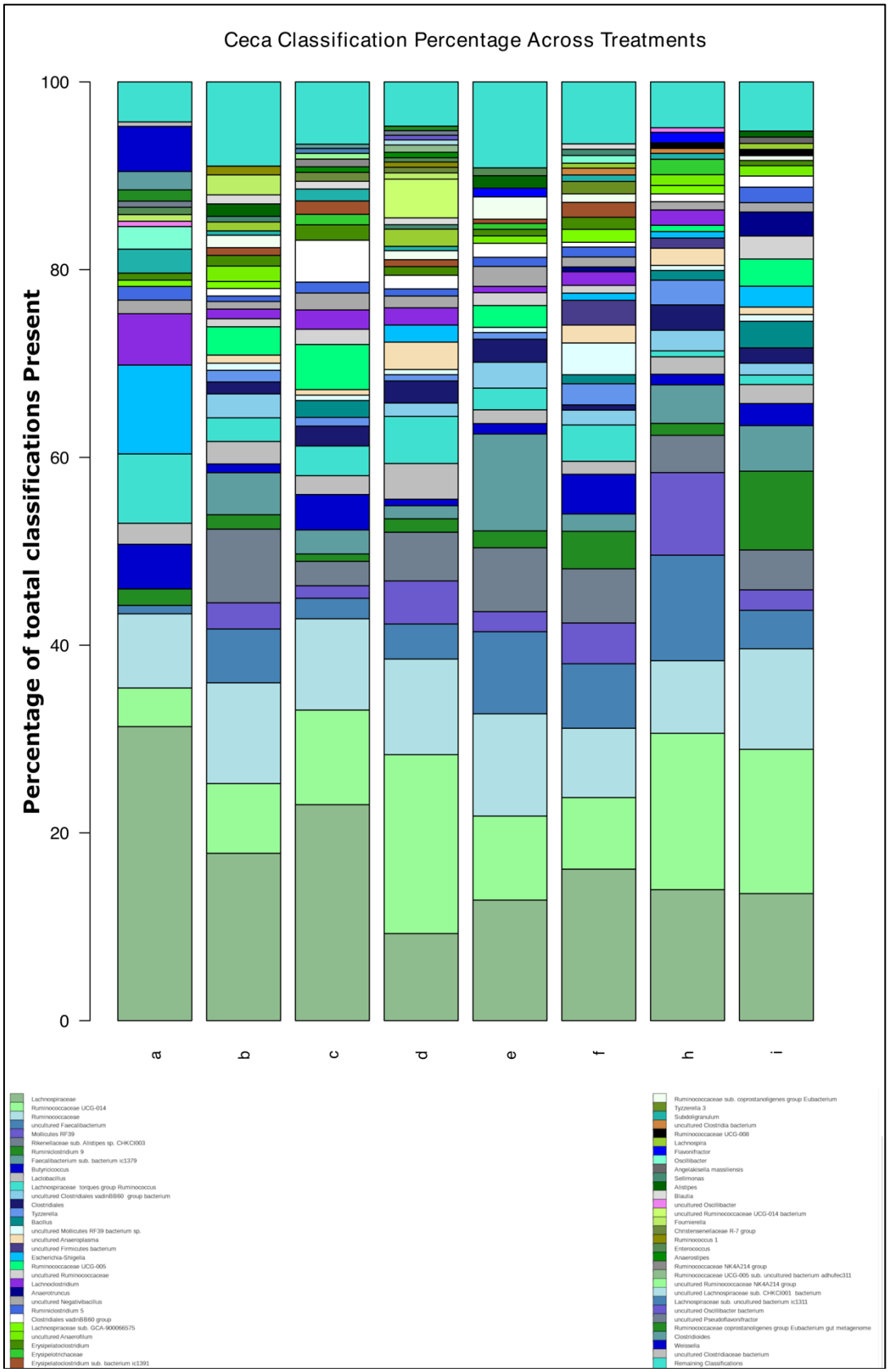
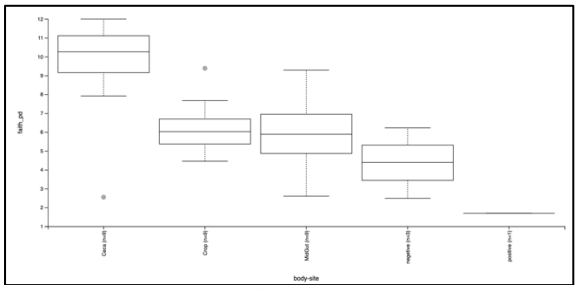


Figure 4-6-Top Classification Percentages, Ceca. Letters reference corresponding columns in Table 4-3.

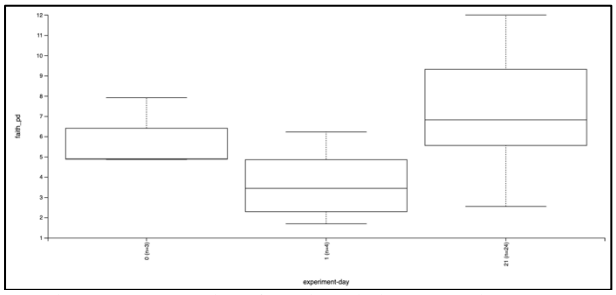
ID	Day	Status	Lightin	Pen	Top Classifications Ceca
a	0		NA	NA	*Lachnospiraceae(~31%), Escherichia-Shigella(~10%), Ruminococcaceae(~8%), Lachnospiraceae torques group Ruminococcus(~7%), Lachnoclostridium(~6%), Weissella(~5%), Butyricicoccus(~5%), Ruminococcaceae UCG-014(~4%), Subdoligranulum(~3%), Oscillibacter(~2%)*
b	21	Non-inoculated	N	F	*Lachnospiraceae(~17%), Ruminococcaceae(~11%), Rikenellaceae sub. Alistipes sp. CHKCI003(~8%), Ruminococcaceae UCG-014(~7%), uncultured Faecalibacterium(~6%), Faecalibacterium sub. bacterium ic1379(~5%), Ruminococcaceae UCG-005(~3%), Mollicutes RF39(~3%), uncultured Clostridiales vadinBB60 group bacterium(~3%), Lachnospiraceae sub. torques group Ruminococcus(~3%)*
c				B	*Lachnospiraceae(~23%), Ruminococcaceae UCG-014(~10%), Ruminococcaceae(~10%), Ruminococcaceae UCG-005(~5%), Clostridiales vadinBB60 group(~5%), Butyricicoccus(~4%), Lachnospiraceae torques group Ruminococcus(~3%), Rikenellaceae sub. Alistipes sp. CHKCI003(~3%), Faecalibacterium sub. bacterium ic1379(~3%), uncultured Faecalibacterium(~2%)*
d			L	F	*Ruminococcaceae UCG-014(~19%), Ruminococcaceae(~10%), Lachnospiraceae(~9%), Rikenellaceae sub. Alistipes sp. CHKCI003(~5%), Lachnospiraceae torques group Ruminococcus(~5%), Mollicutes RF39(~5%), uncultured Ruminococcaceae UCG-014 bacterium(~4%), Lactobacillus(~4%), uncultured Faecalibacterium(~4%), uncultured Anaeroplasma(~3%)*
e				B	*Lachnospiraceae(~13%), Ruminococcaceae(~11%), Faecalibacterium sub. bacterium ic1379(~10%), Ruminococcaceae UCG-014(~9%), uncultured Faecalibacterium(~9%), Rikenellaceae sub. Alistipes sp. CHKCI003(~7%), uncultured Clostridiales vadinBB60 group bacterium(~3%), Clostridiales(~5%), Ruminococcaceae sub. coprostanoligenes group Eubacterium(~2%), Ruminococcaceae UCG-005(~2%)*
f		Inoculated	N	F	*Lachnospiraceae(~16%), Ruminococcaceae UCG-014(~8%), Ruminococcaceae(~7%), uncultured Faecalibacterium(~7%), Rikenellaceae sub. Alistipes sp. CHKCI003(~6%), Mollicutes RF39(~4%), Butyricicoccus(~4%), Ruminiclostridium 9(~4%), Lachnospiraceae torques group Ruminococcus(~4%), uncultured Mollicutes RF39 bacterium sp. (~3%)*
g				B	NA
h			L	F	*Ruminococcaceae UCG-014(~17%), Lachnospiraceae(~14%), uncultured Faecalibacterium(~11%), Mollicutes RF39(~9%), Ruminococcaceae(~8%), Faecalibacterium sub. bacterium ic1379(~4%), Rikenellaceae sub. Alistipes sp. CHKCI003(~4%), Clostridiales(~3%), Tyzzerella(~3%), uncultured Clostridiales vadinBB60 group bacterium(~2%)*
i				B	*Ruminococcaceae UCG-014(~15%), Lachnospiraceae(~14%), Ruminococcaceae(~11%), Ruminiclostridium 9(~8%), Faecalibacterium sub. bacterium ic1379(~5%), Rikenellaceae sub. Alistipes sp. CHKCI003(~4%), uncultured Faecalibacterium(~4%), Ruminococcaceae UCG-005(~3%), Bacillus(~3%), Anaerotruncus(~3%)*

Table 4-3-Top Classifications, Ceca. Top classifications present were Lachnospiraceae, Ruminococcaceae UCG-014, Ruminococcaceae, and Faecalibacterium sub. bacterium ic1379.

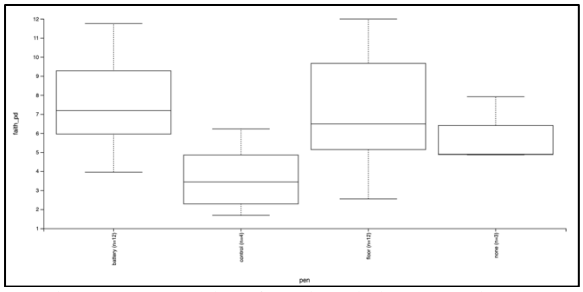
Supplementary Figures



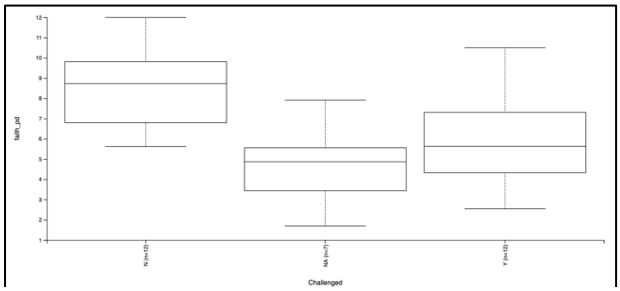
Supplement 11-Box plot of Faith's phylogenetic diversity over body site.



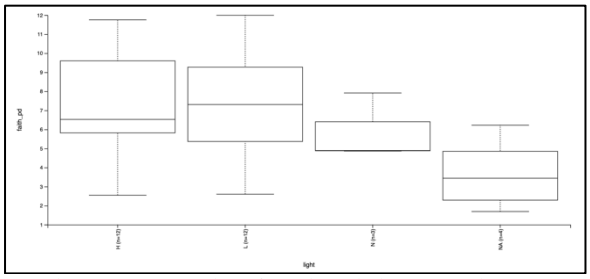
Supplement 12-Box plot of Faith's phylogenetic diversity over sample day.



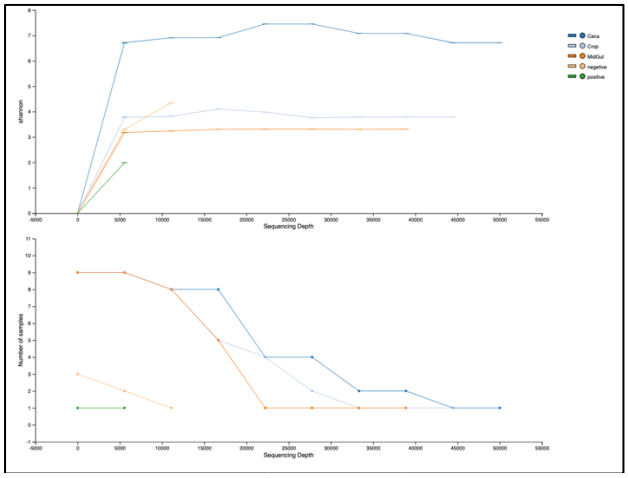
Supplement 13-Box plot of Faith's phylogenetic diversity over pen type.



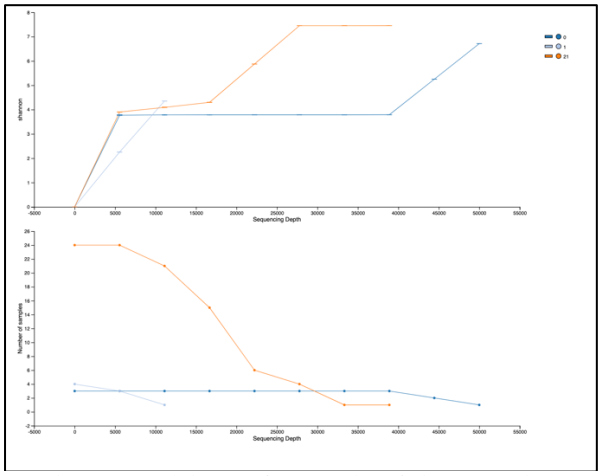
Supplement 14-Box plot of Faith's phylogenetic diversity over challenge status.



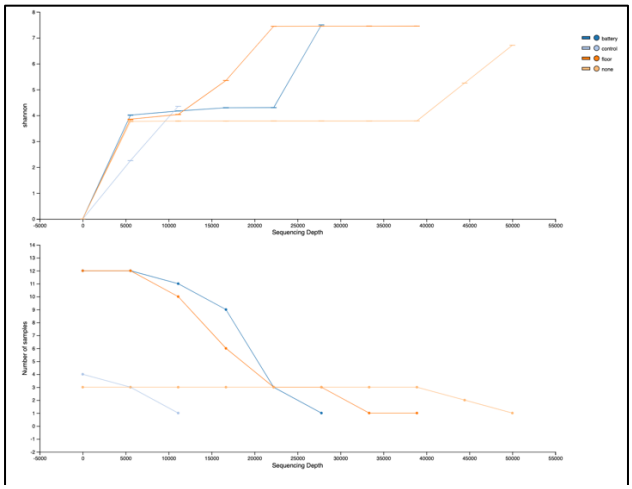
Supplement 15-Box plot of Faith's phylogenetic diversity over lighting level.



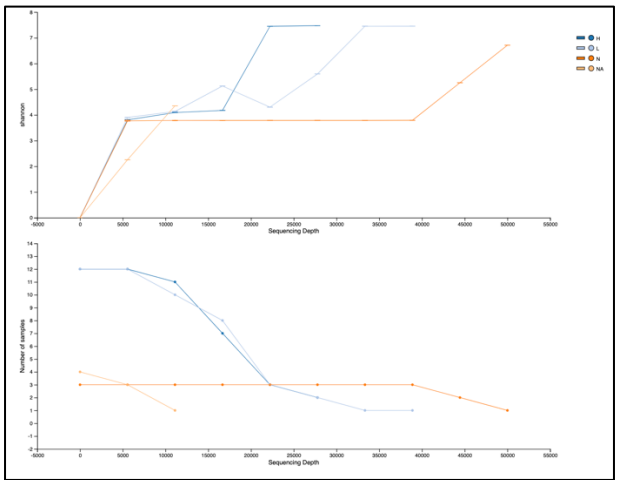
Supplement 16-Alpha rarefication plot of Shannon index over body site.



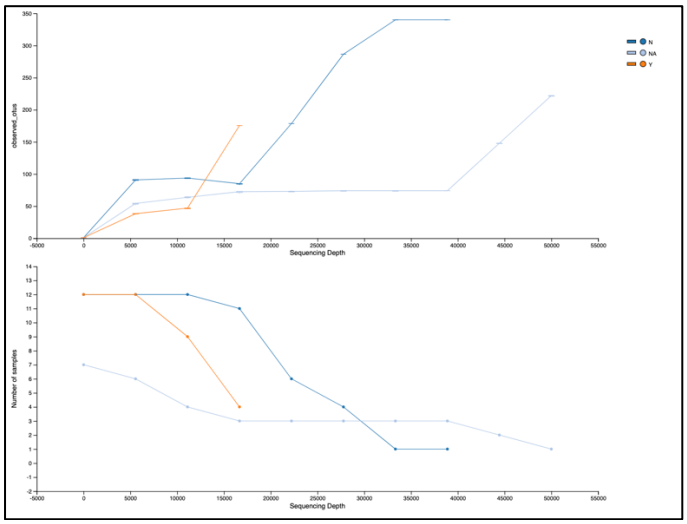
Supplement 17-Alpha rarefication plot of Shannon index over sample day.



Supplement 18-Alpha rarefication plot of Shannon index over pen type.



Supplement 19-Alpha rarefication plot of Shannon index over lighting level.



Supplement 20-Alpha rarefication plot of Shannon index over challenge status.

Definitions/abbreviations

Shannon index- (Shannon, 1948; Tuomisto, 2010)- Diversity index that predicts how diverse a community is. Originally proposed to compare differences between strings of text.

Faith's phylogenetic diversity-(Faith, 1992)-A phylogenetic generalization of species richness that measures average branch length of phylogenetic trees.

Jaccard coefficient-(Jaccard,1912)- The fraction of unique features in a sample set.

Pielou's evenness-(Pielou, 1966)- An index of diversity and species richness, on a scale of zero to one.

Operational taxonomic unit (OTU)-(Sokal & Sneath,1963)- an operational definition method used to classify groups of related entities by their similarity threshold.

References

[1] Food and Drug Administration. Veterinary feed directive. Federal Register. 2015;80(106):31708-35.

[2] Prescott JF, Sivendra R, Barnum DA. The use of bacitracin in the prevention and treatment of experimentally-induced necrotic enteritis in the chicken. The Canadian veterinary journal. 1978;19(7):181.

[3] Elwinger K, Berndtson E, Engström B, Fossum O, Waldenstedt L. Effect of antibiotic growth promoters and anticoccidials on growth of Clostridium perfringens in the caeca and on performance of broiler chickens. Acta Veterinaria Scandinavica. 1998;39(4):433-41.

[4] Van Immerseel F, Rood JI, Moore RJ, Titball RW. Rethinking our understanding of the pathogenesis of necrotic enteritis in chickens. Trends in microbiology. 2009;17(1):32-6.

[5] Tian J, Du J, Lu Z, Han J, Wang Z, Li D, Guan X, Wang Z. Distribution of microbiota across different intestinal tract segments of a stranded dwarf minke whale, Balaenoptera acutorostrata. MicrobiologyOpen. 2020;9(10):e1108.
[6] Wang W, Zheng S, Li L, Yang Y, Liu Y, Wang A, Sharshov K, Li Y. Comparative metagenomics of the gut microbiota in wild greylag geese (Anser anser) and ruddy shelducks (Tadorna ferruginea). Microbiologyopen. 2019;8(5):e00725.
[7] Li H, Li T, Beasley DE, Heděnec P, Xiao Z, Zhang S, Li J, Lin Q, Li X. Diet diversity is associated with beta but not alpha diversity of pika gut microbiota. Frontiers in microbiology. 2016;7:1169.
[8] Ferguson RM, Merrifield DL, Harper GM, Rawling MD, Mustafa S, Picchietti S, Balcàzar JL, Davies SJ. The effect of Pediococcus acidilactici on the gut microbiota and immune status of on-growing red tilapia (Oreochromis niloticus). Journal of applied microbiology. 2010;109(3):851-62.
[9] Little TJ, Hultmark D, Read AF. Invertebrate immunity and the limits of mechanistic immunology. Nature immunology. 2005;6(7):651-4.
[10] Brugman S, Ikeda-Ohtsubo W, Braber S, Folkerts G, Pieterse CM, Bakker PA. A comparative review on microbiota manipulation: lessons from fish, plants, livestock, and human research. Frontiers in nutrition. 2018;5:80.
[11] Segata N, Waldron L, Ballarini A, Narasimhan V, Jousson O, Huttenhower C. Metagenomic microbial community profiling using unique clade-specific marker genes. Nature methods. 2012;9(8):811-4.
[12] Human Microbiome Project Consortium. Structure, function and diversity of the healthy human microbiome. nature. 2012;486(7402):207.
[13] Langille MG, Zaneveld J, Caporaso JG, McDonald D, Knights D, Reyes JA, Clemente JC, Burkepile DE, Thurber RL, Knight R, Beiko RG. Predictive functional profiling of microbial communities using 16S rRNA marker gene sequences. Nature biotechnology. 2013;31(9):814-21.
[14] Nagpal R, Wang S, Solberg Woods LC, Seshie O, Chung ST, Shively CA, Register TC, Craft S, McClain DA, Yadav H. Comparative microbiome signatures and short-chain fatty acids in mouse, rat, non-human primate, and human feces. Frontiers in microbiology. 2018;9:2897.

[15] Kostic AD, Howitt MR, Garrett WS. Exploring host–microbiota interactions in animal models and humans. Genes & development. 2013;27(7):701-18.
[16] Gootenberg DB, Turnbaugh PJ. Companion animals symposium: humanized animal models of the microbiome. Journal of animal science. 2011 May 1;89(5):1531-7.
[17] Alzubaidy H, Essack M, Malas TB, Bokhari A, Motwalli O, Kamanu FK, Jamhor SA, Mokhtar NA, Antunes A, Simões MF, Alam I. Rhizosphere microbiome metagenomics of gray mangroves (Avicennia marina) in the Red Sea. Gene. 2016;576(2):626-36.
[18] Vandenkoornhuyse P, Quaiser A, Duhamel M, Le Van A, Dufresne A. The importance of the microbiome of the plant holobiont. New Phytologist. 2015;206(4):1196-206.
[19] Singh B. The Effects of Feeding Reduced-oil DDGS to Broilers when Challenged with C. perfringens and Eimeria spp. on Necrotic Enteritis and Intestinal Microbiome. Master's thesis. Auburn University, Poultry Science Department ; 2017.
[20] Antonissen G, Eeckhaut V, Van Driessche K, Onrust L, Haesebrouck F, Ducatelle R, Moore RJ, Van Immerseel F. Microbial shifts associated with necrotic enteritis. Avian Pathology. 2016;45(3):308-12.
[21] Wei S, Morrison M, Yu Z. Bacterial census of poultry intestinal microbiome. Poultry science. 2013;92(3):671-83.
[22] Whelan RA, Doranalli K, Rinttilä T, Vienola K, Jurgens G, Apajalahti J. The impact of Bacillus subtilis DSM 32315 on the pathology, performance, and intestinal microbiome of broiler chickens in a necrotic enteritis challenge. Poultry science. 2019;98(9):3450-63.
[23] Dec M, Puchalski A, Urban-Chmiel R, Wernicki A. Screening of Lactobacillus strains of domestic goose origin against bacterial poultry pathogens for use as probiotics. Poultry science. 2014;93(10):2464-72.
[24] Eeckhaut V, Van Immerseel F, Croubels S, De Baere S, Haesebrouck F, Ducatelle R, Louis P, Vandamme P. Butyrate production in phylogenetically diverse Firmicutes isolated from the chicken caecum. Microbial biotechnology. 2011;4(4):503-12.
[25] Sengupta R, Altermann E, Anderson RC, McNabb WC, Moughan PJ, Roy NC. The role of cell surface architecture of lactobacilli in host-microbe interactions in the gastrointestinal tract. Mediators of inflammation. 2013;2013.

[26] Chasteen K S. The Effect of Lighting and Temperature as Stressors on the Broiler Microbiome in Floor Raised vs Cage Raised Birds. Master's thesis. Auburn University, Poultry Science Department ; 2021.
[27] Robertson JB, Davis CR, Johnson CH. Visible light alters yeast metabolic rhythms by inhibiting respiration. Proceedings of the National Academy of Sciences. 2013;110(52):21130-5.
[28] Xu M, Zhong F, Zhu J. Evaluating metabolic response to light exposure in Lactobacillus species via targeted metabolic profiling. Journal of microbiological methods. 2017;133:14-9.
[29] Bolyen E, Rideout JR, Dillon MR, Bokulich NA, Abnet CC, Al-Ghalith GA, Alexander H, Alm EJ, Arumugam M, Asnicar F, Bai Y. Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. Nature biotechnology. 2019;37(8):852-7.
[30] Callahan BJ, McMurdie PJ, Rosen MJ, Han AW, Johnson AJ, Holmes SP. DADA2: high-resolution sample inference from Illumina amplicon data. Nature methods. 2016;13(7):581-3.