Establishment of Base Interactions of Select Genus Between Multiple Chick Sources in Differential Housing

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Research Article

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

Posted Date: September 22nd, 2021

DOI: https://doi.org/10.21203/rs.3.rs-907851/v1

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.

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.

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 biproducts 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 (Table 1). 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.

Alpha and Beta Diversity

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. 6 (2.6–2.12)) 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.

Differential OTUs Between Time Points and Treatments

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 (Table 2).

Alpha and Beta Diversity

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. 6 (2.6–2.12)) 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* [21, 24]; 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 (Fig. 6 (2.6–2.12)) Similarly, midgut samples diversified quickly after day zero (Fig. 6 (2.6–2.12)) spreading out before contracting back to a composition that was similar to day 5 and 14. Cecal samples possessed the more diverse communities overall.

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 associated with many health disorders including obesity in mice [22]. Furthermore once viewed at the family level Bacteroidales primarily split into two main major families: *Bacteroides* associated with meat and fat heavy diets, and *Prevotella* which is associated with fiber and plant matter heavy 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). Furthermore *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 [23], 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 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 mixed population. 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. Birds 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 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 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. 2.13). 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) facility for 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 [19]. Fastq files in Casava 1.8 paired end demultiplex format were read into QIIME2 to be joined and denoised using DADA2 [20]. 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. 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. Taxonomic classification plug-in classify-sklearn was run using the Silva 132 99% full length classifier sequences set to identify

Operational Taxonomic Units (OTUs). Taxonomic bar-plots were generated using the “taxa barplot” command.

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.

Declarations

Ethical approval and Consent to participate – This project was approved by Auburn University’s IACUC committee, protocol number 2018-3282

Consent for publication

Not applicable

Availabilty 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.

Competing interests

The authors declare that there are no competing interests.

Funding

USDA NIFA Hatch

Authors contribution

KSC major contributor to the design of the experiment and writing of the manuscript. LB, LM, JK all helped in husbandry of the birds asd wel as assited in sample collection. MB helped in the writing and reviewing of the manuscript. KM helped in all aspects of the experiment and is KSC major professor.

Acknowledgments

Thank you to the Macklin Lab team.

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Tables

Due to technical limitations, table 1 and 2 is only available as a download in the Supplemental Files section.

Figures

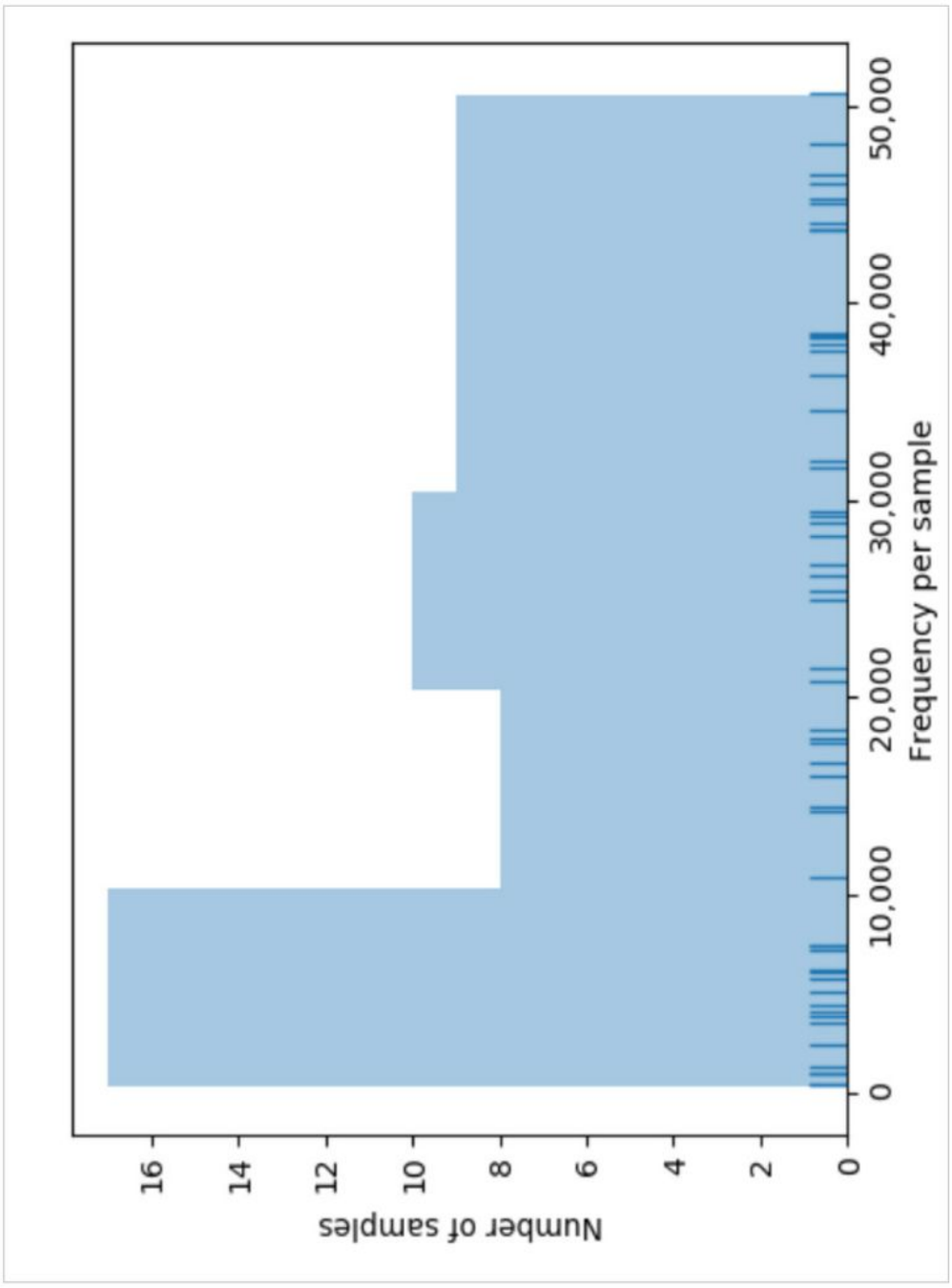

Figure 1

To that point universal target primers CS1/515F (5’-GTGYCAGCMGCCGCGGTAA-3’) and CS2/926R (5’-CCGYCAATTYMTTTRAGTTT-3’) were used to further expand potential targets.

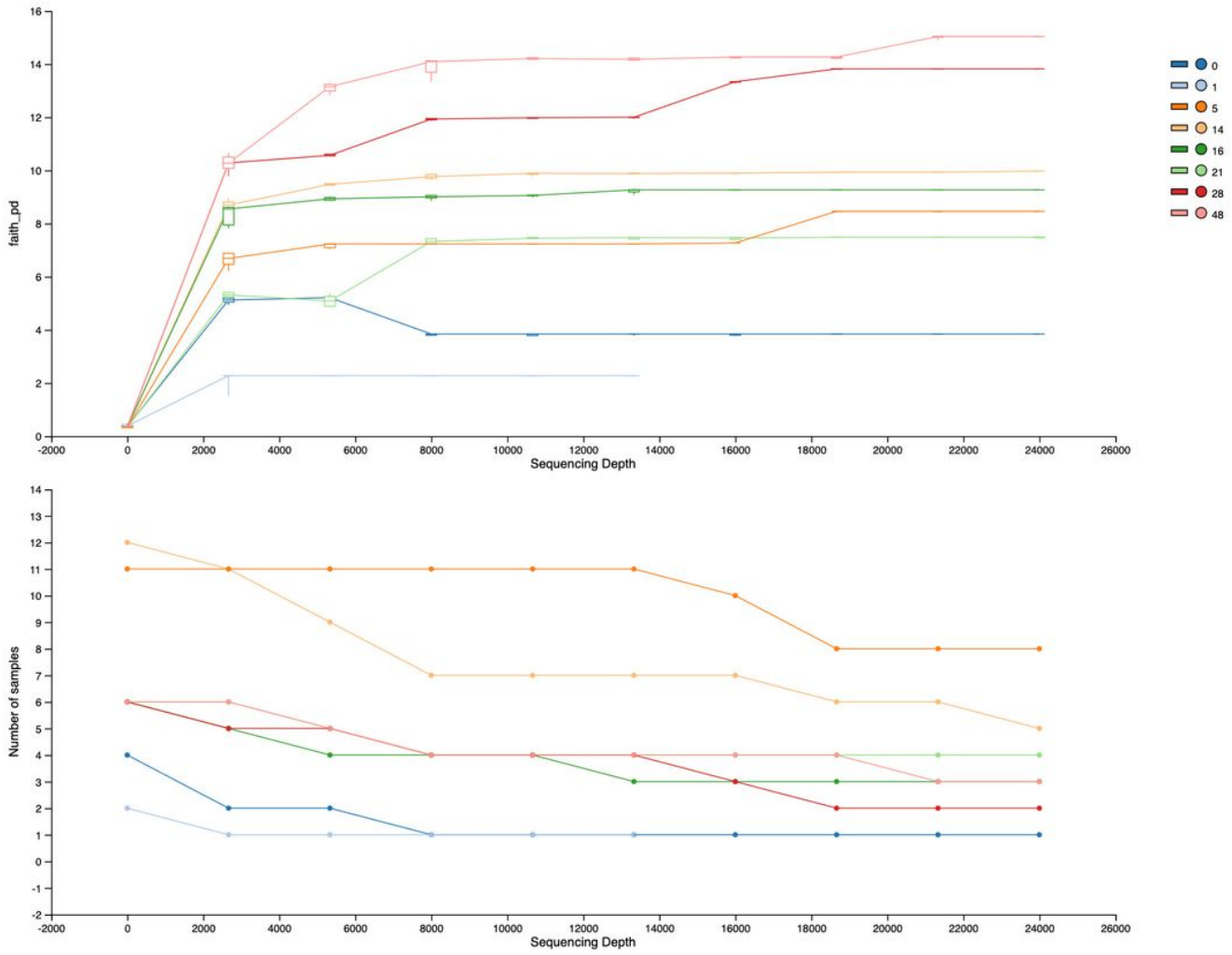

Figure 2

Sequencing depth

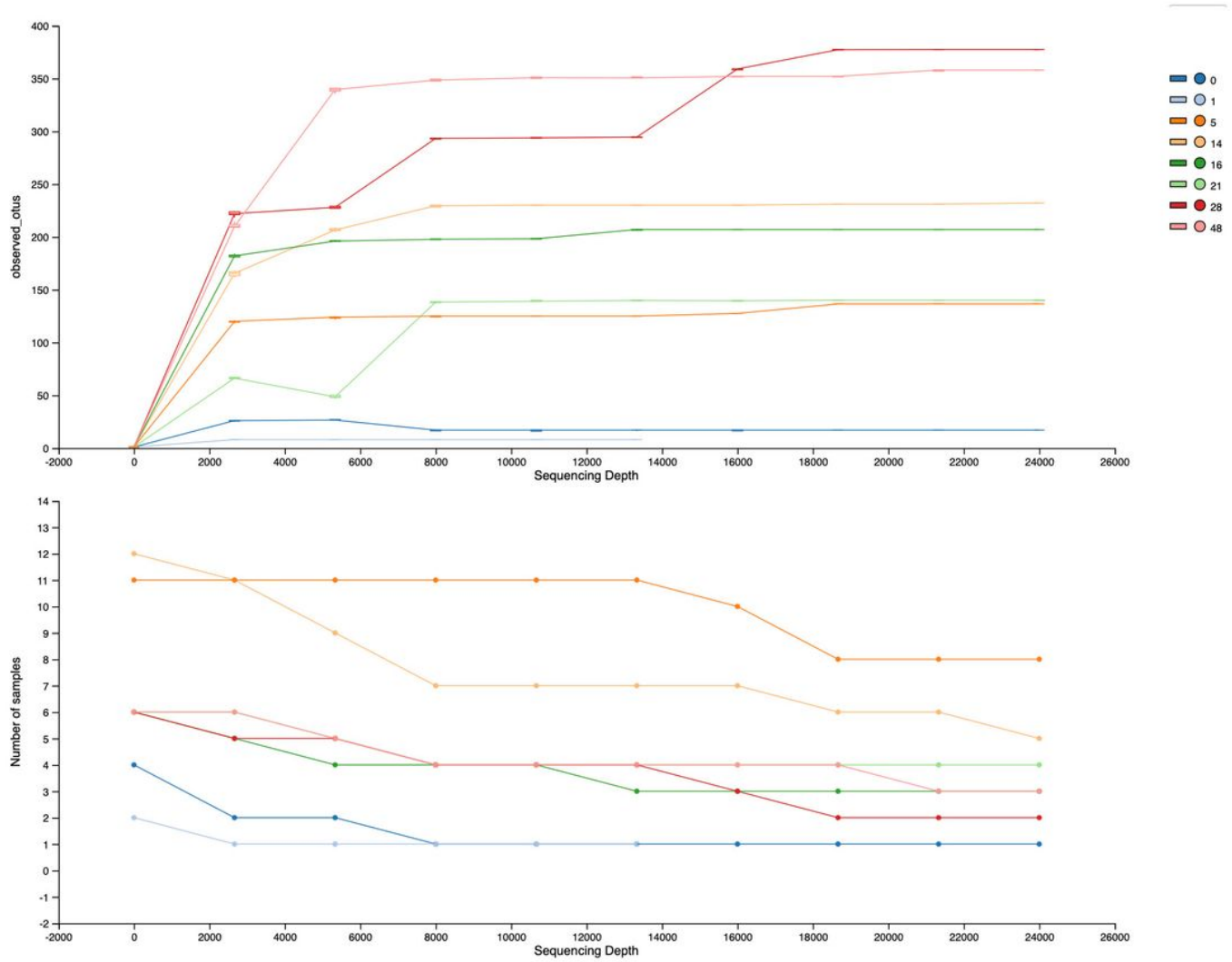

Figure 3

Sequencing depth

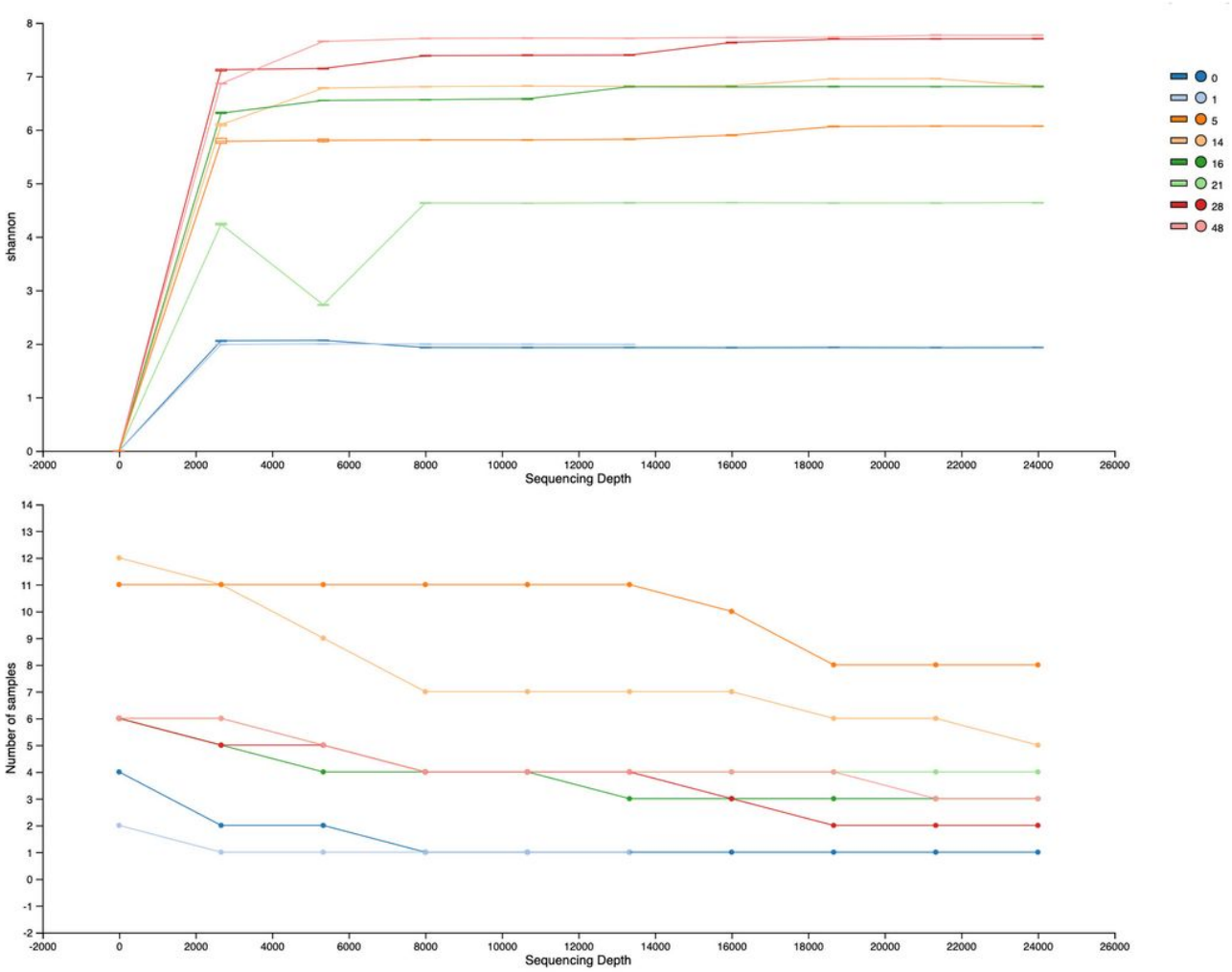

Figure 4

Sequencing depth

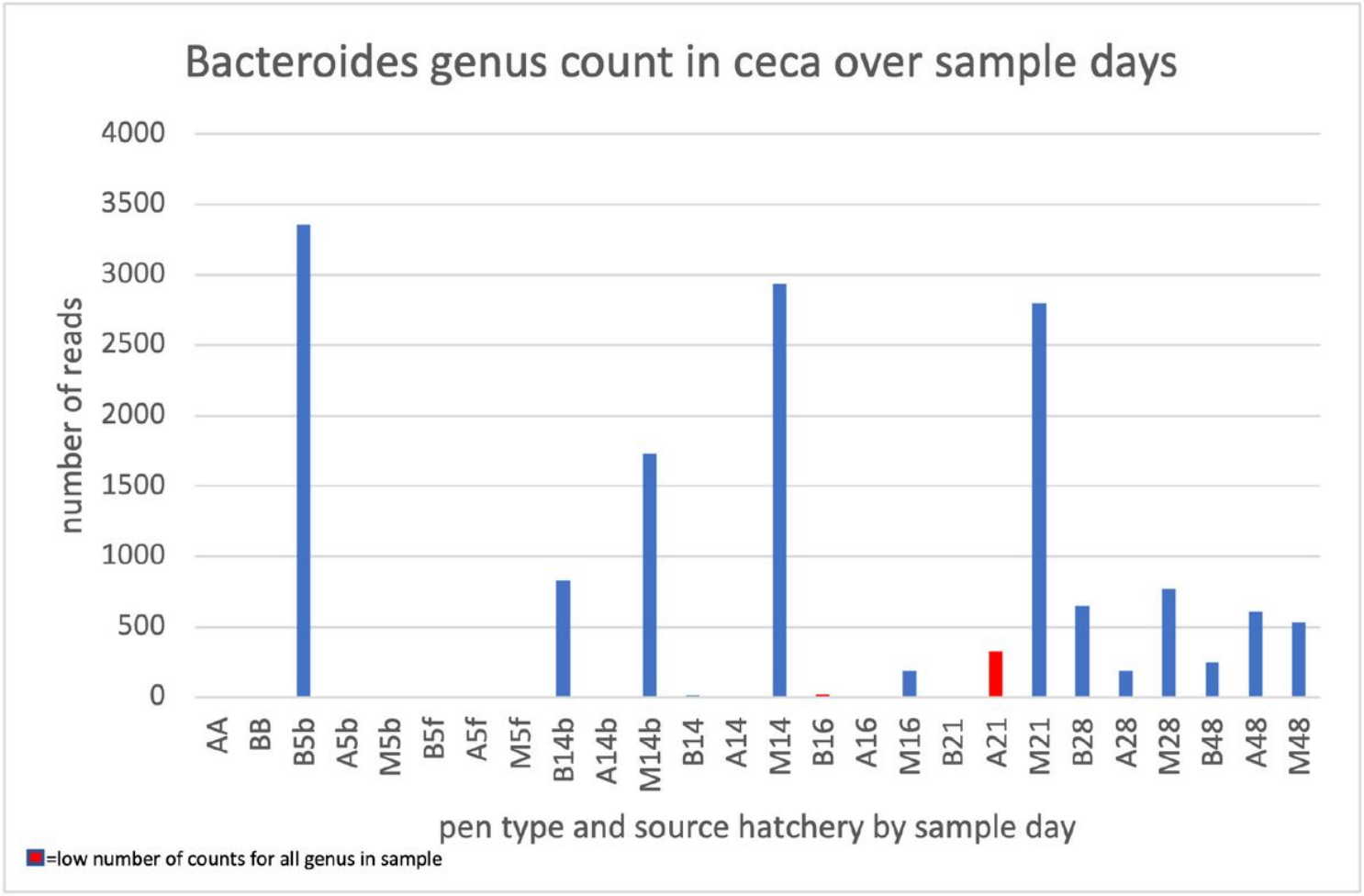

Figure 5

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.

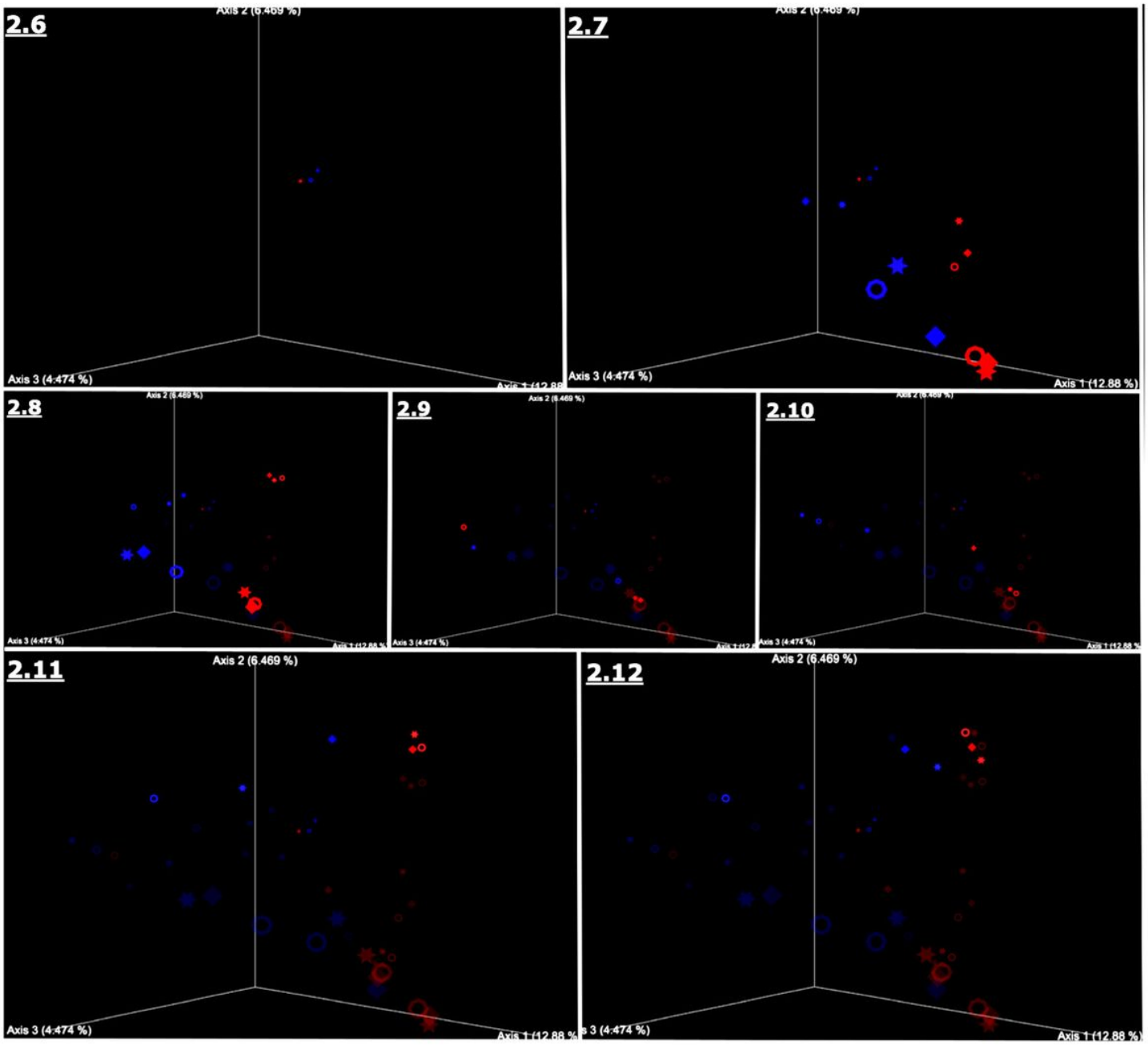

Figure 6

(2.6-2.12)- Jaccard coefficient plots left to right over time day 0 to day 48 ([Diamond(•)= hatchery A, Circle(⍰)=hatchery B, Star(⍰) = mixed], [red= ceca, blue = midgut], [Battery = large icons (Day 5-14), Floor = small icons])

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).

Figure 7

Reaction conditions

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Table1.pdf
- Table2.pdf