The biological effects of microencapsulated organic acids and botanicals induces tissue-specific and dose-dependent changes to the microbiota

Kristina Feye
USDA-ARS Southern Plains Agricultural Research Center
Christina L. Swaggerty (christi.swaggerty@usda.gov)
Michael H. Kogut
USDA-ARS Southern Plains Agricultural Research Center
Steven C. Ricke
University of Arkansas Fayetteville
Andrea Piva
DIMEVET, University of Bologna
Ester Grilli
DIMEVET, University of Bologna

Research article

Keywords: Ileum, Jejunum, Microbiota, Microencapsulated, Natural botanicals, Organic acid

Posted Date: March 17th, 2020

DOI: https://doi.org/10.21203/rs.3.rs-17416/v1

Version of Record: A version of this preprint was published on November 2nd, 2020. See the published version at https://doi.org/10.1186/s12866-020-02001-4.

Abstract

Background: Microencapsulated organic acids and botanicals have the potential to develop into important tools for the poultry industry. A blend of vanillin, thymol, sorbic, and citric acids (AviPlus®P) has previously been shown to reduce *Salmonella* and other foodborne pathogens in chickens; however, changes to the microbiota of the jejunum and ileum have not been evaluated. Microbiota diversity is linked to, but not correlated with, the efficacy of natural products including organic acids and natural compounds; therefore, understanding the effects of these products on the microbiota is a necessary first step into evaluating the potential for wide-spread use within the poultry industry as an alternative to antibiotics.

Results: Day-of-hatch by-product breeder chicks (n=30) were placed into either the control (0 g/MT AviPlus®P; n=5) or one of two experimental groups (300 g/MT; n=5; 500 g/MT AviPlus®P; n=5). The experiment was conducted using two replicate pens therefore 10 chicks/treatment were used for all analysis. Chickens were sacrificed 15d post-hatch and total jejunum and ileum contents were individually collected and snap frozen until further processing and analysis. The DNA was extracted and sequenced using the Illumina 16S rDNA MiSeq V3 platform and the samples analyzed in QIIME2.2019.1. Samples were filtered for quality and chimeras using DADA2, with OTU frequencies less than three removed from the study. Alpha and beta diversity analytics indicated compartmentalization within the poultry gastrointestinal tract (GIT). Additionally, LEfSE and ANCOM analysis showed a reduction in *Enterobacteriaceae* with increased inclusion rate and an increase in *Clostridia*, both of which are associated with optimal GIT health.

Conclusion: The addition of a blend of organic acids and botanicals to the diet of chickens does not adversely impact the microbiota, and in fact, is associated with microbial changes within the ileum and jejunum that are largely thought of as beneficial. Promotion of a healthy GIT has the potential to limit colonization by *Salmonella* and other foodborne pathogens. The inclusion of the microencapsulated blend of organic acids and botanicals may be a viable antibiotic alternative for use in the poultry industry.

Background

The public concerns associated with the use of antibiotics in poultry production necessitates research into market perceived acceptable natural alternative compounds that promote feed efficiency and food animal health while reducing the burden of foodborne disease. Stepping back from the refined pharmacological fungal metabolites traditionally used in animal agriculture, plant secondary metabolites and essential oils are an attractive avenue of development for poultry production [1]. Research indicates bioactive natural compounds can decrease the microbial burden on the immune system and promote feed efficiency by improving digestibility and gastrointestinal (GIT) morphology in poultry [2–4]. Essential oils and other natural compounds have been documented to possess antibiotic, insecticidal, therapeutic, anti-inflammatory, and chemotherapeutic effects [1, 5].

Further refinements and extrusions of these compounds result in the development of pharmacological agents. Specifically, modifications to these isolated compounds result in known pharmacological agents and antibiotic potentiators [6]. For instance, isoflavones isolated from Lupinus argentus act as potentiators of the α-linolenic acid class of antibiotics, with documented increase in berberine and norfloxacin efficacy which is theorized to occur by the natural plant compound acting as a partial efflux pump antagonist [6]. The benefit of using crude essential oils and other natural compounds is that bioactive compounds are generally regarded as safe and can be multi-modal in their activation effects [1, 5, 7]. Previous research has documented the positive effects of essential oils on poultry production, gut health, and disease resistance [2, 8, 9].

The microbiota actively participates in homeostatic function, nutrient digestion, and biotransformation of compounds. The symbiotic relationship between host commensal microorganisms and the immune system facilitate immune tolerance and development and can have peripheral consequences to overall health and food animal feed efficiency [10–13]. Additionally, as the microbiota directly interact with feed matrices, any potential natural compounds must not negatively disrupt microbiota community structure and stability. Chemical disruption to the microbiota by antibiotic administration can result in a reduction of pharmacological activity of essential oils, enzymes, and other compounds [13–16]. This is important as the biotransformation of chemical compounds by the microbiota can result in the activation of antibiotics, potentiators, and may reduce the efficacy of essential oils and metabolites [14–16]. Sufficient evidentiary support must therefore demonstrate that the microbiota does not render the natural compounds inert nor that the biotransformation results in bactericidal effects that reduce diversity that corresponds with decreased absorption of nutrients and compounds [14–16].

Compartmentalization, or localization to a particular section the GIT, is important, though often overlooked in poultry feed amendment studies [17]. The activity of natural compounds should result in changes to the compartment of activity which would provide knowledge related to the changes within the microbial community structure and may ultimately provide insight into the biology driving these effects. Numerous microbiota studies have focused on the ceca, which may not be ideal as digestion also occurs in the foregut [18, 19]. Evidence suggests that the jejunum of feed-efficient animals, which is the site of nutrient absorption, has improved morphology, and increased enzymatic activity [20]. Likewise, the ileum, which absorbs any remaining vitamins and other nutrients not absorbed in the jejunum, is lighter and longer in more feed-efficient animals, with improved morphology [20]. While the ceca are heavily focused on in other studies with natural compounds and for the microbiota responses, it may not necessarily be the site biological activity occurs nor the site with the most biological ramifications if the microbial populations change [16, 17, 20]. Additionally, as microencapsulation technology continues to evolve, the targeted delivery of natural compounds through the harsh environment of the crop to their intended further location down the GIT may serve to improve biological activity [21].

There are numerous studies in the literature that evaluate the role of essential oils and other natural products. For example, thymol has been evaluated for its anti-inflammatory and microbiota modulating effects in poultry with promising results [22] and vanillin has been shown to exhibit antibiotic-like effects [23, 24]. Organic acids have also shown promise as a feed amendment in poultry [25]. Sorbic acid has anti-fungal properties and combined with prebiotics can improve feed efficiency [26, 27] while citric acid can be used as an antimicrobial in food processing and has minimal effects in vivo when included at high concentrations [28]. In another study, citric and acetic acid combinations improved body weight gain, feed conversion, and feed efficiency at a lower inclusion rate [29].

The objectives of this study were to evaluate the effects of feeding broilers a diet supplemented with a microencapsulated blend of organic acids (25% citric and 16.7% sorbic), and botanicals (1.7% thymol and 1% vanillin; AviPlus®P) on the microbiota populations in the jejunum and ileum. Broilers were used because the real-life effects of these compounds in production animals are an important research question to ask in order to determine the mechanism of action. The commercial broiler by-product chickens used in this study are representative of the birds used in poultry production. These two tissues were selected because they are two important organ systems associated with feed efficiency and production in broilers. Only organ specific effects can truly be delineated using in vivo models. By evaluating community structure and composition, it will be possible to determine if there are any effects on the microbiota due to bioactivity of organic acids and botanicals.

Results

Diversity analysis

For each study, a total of 5 samples per animal were collected. In total, this was a total of 10 animals included in the bioinformatics analyses per treatment for all analytics. Evenness and richness are two essential components to alpha diversity. Alpha diversity speaks to the community structure and evenness of the microbial ecosystem without taking into account differences in speciation [13]. Shannon's diversity index is classically associated with numerous microbial studies and is used to calculate Pielou's evenness [13]. Therefore, taken together, both metrics are competently able to assess changes in alpha diversity due to location or treatment. In the present study, the effect of treatment was not significant for either the ileum or the jejunum. However, the effect of location was significant ($P < 0.05$; Fig. 1A and Fig. 1B) for Shannon's diversity index and Pielou's evenness comparing the ileum and jejunum. There was a significant ($P < 0.05$) decrease in Shannon's diversity index and Pielou's evenness metric for the ileum. Meaning, species richness and the even distribution of that richness across the ileum is less than that of the jejunum.

Specific to beta-diversity, the qualitative metrics Bray-Curtis Dissimilarity Index and Weighted Unifrac Distance Matrix were statistically significant for the interaction of treatment and location ($P < 0.05$; Fig. 2A and Fig. 2B). Beta diversity indicates there may be compositional differences that are arising, with Bray-Curtis being a function of total assessment and the Weighted Unifrac Distance Matrix considering phylogenetic branch length and are considered qualitative as the total reads and counts leading to those diversity differences are not considered [13]. There is a clear difference in beta diversity for both matrices between the ileum and jejunum ($Q < 0.05$). Additionally, there are significant changes to diversity between the tissues within treatment, which is to be expected (Table 1). Specific to the comparison between the 300 and 500 g/MT treatments, the 500 g/MT treatment was statistically significant between the ileum and jejunum ($Q = 0.024$). The 300 g/MT treatment also exhibited this difference. Likely, the effects of the local microbiota drive these differences, as indicated by the alpha diversity analysis.

Analysis of communities of the microbiota (ANCOM)

Because of the qualitative differences in beta diversity, and how there may be tissue-specific effects driving these differences, it became necessary to sort the data and use ANCOM (Analysis of Communities of the Microbiota) to delineate the potential changes to compositional diversity. In the ileum, there was no difference in treatment by organ. Therefore, the differences in treatment observed in the beta diversity index are likely due to tissue-specific effects, not the localized effect of treatments. However, there were dose-dependent responses observed in the jejunum at the Phylum level (Fig. 3). Lactobacilliaceae predominated for all three treatment groups, with a stepwise increase in this population from the control (NTC) to the 300 g/MT and finally to the 500 g/MT group (Fig. 3A, B, C, respectively). This corresponds with a stepwise increase in Staphylococcaceae from 0% (rounded number) of the total operational taxonomic units (OTU) associated with treatment at 500 g/MT to 3% in the NTC group. Additionally, the Enterobacteriaceae populations, commonly associated with the pathobiont E. coli and the foodborne pathogen Salmonella, decrease in the 500 g/MT treatment group. In both the 300 g/MT and 500 g/MT treatment group, the OTU identified as Aerococcaceae did not fluctuate by treatment. In the 300 g/MT treatment group, Ruminococcaceae were the most abundant. Therefore, there were significant changes in the microbial consortia statistically associated with the treatment groups in the jejunum. These effects did not occur in the ileum, which suggests that the substantial difference in microbial consortia between tissues likely drives the beta diversity effects observed for the ileum.

Linear discriminant analysis effect size (LEfSE) analysis

Least discriminant analysis effect size (LEfSE) accounts for the underlying grouping by population whereas ANCOM considers the changes to the entirety of the microbial consortia. Therefore, LEfSE can indicate different changes more directly corresponding to treatment effects. As the 300 g/MT treatment was the intermediary treatment, the NTC and 500 g/MT score were compared (Fig. 4). As supported by the ANCOM analysis, the NTC exhibited an increase in Aeromonas, Gammaproteobacteriaceae and Enterobacteriaceae populations. Meanwhile, relative to 300 g/MT, the 500 g/MT treatment group had less Clostridiaceae and Micrococcoaceae. When parsing out important veterinary pathogens, Enterobacteriaceae demonstrated a stepwise decrease (from NTC onward) (Fig. 5). However, for Clostridiaceae, there was a large increase in that population for 500 g/MT as compared to the NTC and 300 g/MT treatments (Fig. 6).

Discussion

Research into the biology of aging in the microbiota and dysbiosis provides some clues to the metrics of microbiota stabilization to maintain a homeostatic balance. Some evidence suggests that the stability of the microbiota is defined over time via volatility metrics and the microbiota-by-age score (MAZ) score; however similar changes observed in stable systems by compartment can indirectly support what is or is not viewed as a stable microbiota that may contribute to a loss in homeostasis [30, 31]. Dysbiosis associated with aging and inflammation typically results in the de-compartmentalization of the gastrointestinal microbiota [16, 32]. As seen with the aforementioned studies, the results shown herein found a strong compartmentalization of the microbial ecology by tissue irrespective of treatment. Additionally, while differences exist from the NTC, there is not a collapse and shrinkage in diversity or bloom of

populations associated with significant pathology by treatment. Enterobacteriaceae, which contains numerous foodborne and zoonotic microorganisms, were reduced as the inclusion of the microencapsulated blend increased from 0 g/MT to 500 g/MT. Ultimately, data presented herein demonstrated the microencapsulated blend or organic acids and botanicals results in changes to the microbiota that may be beneficial to the host. These data are in agreement with an earlier study showing thymol altered the microbiota in poultry with promising results [22]. While we cannot speak to potential changes in MAZ score or stability over time, data presented does indicate the microbiota is biologically functional at 15 days post-hatch in chicks on a diet supplemented with a microencapsulated blend of organic acids and botanicals.

Likely, as the compartmental activation of the microbiota has also been demonstrated in other studies, it is resulting in the physiological effects as different compartments convey different biological processes [20, 33]. While feed efficiency, nutrient absorption, enzymatic activity and other GIT health parameters were not measured in this study, future studies should determine if there are changes to the nutritional uptake of feed. Since 15 d-of-age could be considered a semi-developed state for the gut and microbiota [34], future studies will also be needed to evaluate the microbiota populations later in the grow-out period. Evaluating the volatility and maturation of the GIT, as well as the terminal production age GIT of the birds would address bird health issues and highlight any potential food safety concerns.

In numerous pharmacological studies, the biotransformation of drugs by the microbiota resulting in their absorption by the jejunum is linked to increased diversity and biological activity of that microbial population [20, 35]. It would be expected based on human studies that the jejunum would likely be the most bioactive site for absorption, making it the most logical site to observe treatment effects [10]. Likely, these effects diffuse outward or the metabolites are further transformed by downstream local microbial populations. For Salmonella, profound changes to the microbial ecology are required to shift the oxidation-reduction potential of the GIT environment [36, 37]. An earlier study showed the microencapsulated blend used in the current study reduced Salmonella load in the ceca (Ester Grilli, personal communication); however, advanced microbiome analyses were not conducted for that study. Therefore, it would be interesting to evaluate whether or not the stepwise changes observed in the current study in the jejunum and ileum are also observed during a Salmonella infection trial. From a management perspective, understanding the adjustment of the inclusion rate would provide useful information to producers to most effectively manage problematic barns versus those with a lower risk of contamination. Therefore, repeating this dose-and-compartment-response study under challenge conditions would provide insightful information.

Importantly, as with the maintenance of the compartmentalization of the GIT environment, data presented herein indicates that both dose and location effects are unique and significant. This could be valuable as tools evaluating the effects of the products may be developed off of microbiota data and missing significant changes could be a missed opportunity. Previous studies also indicate microbial changes may be occurring in the ileum [38]. However, the reductionist, classical microbiological approach undertaken only evaluated a few culture-dependent microorganisms and did not take into account compositional nor diversity changes. Therefore, this culture-independent study is unique as it provides novel insight into the microbial shifts in two very bioactive components of the GIT. Further studies will be required to evaluate the changes in the immune status, GIT barrier function, feed efficiency and nutrient utilization. The European Union Commission and European Food Safety Authority (EFSA) have recognized the microencapsulated blend of organic acids and botanicals used in the current study (AviPlus® P; identification number 4d3) for its ability to improve growth rate and feed efficiency in healthy poultry, and the findings presented herein begin to offer mechanistic insight into those benefits.

As with any laboratory experiment, there are limitations that prohibit the inclusion of all variables encountered in the field and/or farm. One specific limitation to the current study is that the microbiome results are qualitative which did not allow us to directly consider cell counts and 16S rDNA copy number. Future studies should focus on using a greater statistical power for compositional analyses [39, 40]. Different populations can contribute varying copy numbers of 16S rDNA to the analysis; therefore, using a quantitative method may be more important in the future [41]. As microbiome studies become more common, advances such as using long read technology will become necessary to truly understand the microbial shifts due to treatment, and not sequencing based on a small region (V3 or V4 is most common). Despite these limitations, components of our study such as the observed changes in beta diversity are likely to remain consistent and are indicative of potentially optimal microbiota changes. Further, the data showed that shifts in dispersion and mean, as analyzed by ANISOM, occurred by treatment. This type of metric will likely stand the test of time and will be essential in delineating the biological role of the microbiota and how it is affected by treatment.

Conclusion

The addition of a blend of organic acids and botanicals to the diet of chickens does not adversely impact the microbiota, and in fact, is associated with microbial changes within the ileum and jejunum that are largely thought of as beneficial. Promotion of a healthy GIT has the potential to limit colonization by Salmonella and other foodborne pathogens. The inclusion of the microencapsulated blend of organic acids and botanicals may be a viable antibiotic alternative for use in the poultry industry and contributes, in part, to improved growth rate and feed efficiency in healthy chickens.

Methods

Experimental design, animal husbandry, and tissue collection

The experiments were conducted in accordance with the recommended code of practice for the care and handling of poultry and followed the ethical principles according to the Guide for the Care and Use of Laboratory Animals [42]. All bird studies were under the approved experimental procedures outlined in protocol #2017008 and were approved by the USDA/ARS Institutional Animal Care and Use Committee and overseen by Dr. Roger B. Harvey (attending veterinarian). Day-of-hatch by-product male breeder chicks were obtained from a commercial hatchery (Timpson, TX, USA) and were vaccinated with a killed autogenous Salmonella vaccine per standard commercial practices by the commercial hatchery in accordance with their standard practices.

The chicks were transported in standard chick boxes and placed in a BL2 building in floor pens (3 m x 3 m) containing wood shavings and provided supplemental heat and ad libitum access to food supplied in hanging feeders and fresh water through nipple drinkers. Chickens were provided 24 hr of continual light at placement to ensure sufficient water and food intake, then transitioned to 18 hr of light and 6 hr of darkness for the remainder of the study. The temperature of the pens was maintained at 35 °C for day 1 to 3, 32 to 34 °C for day 4 to 7, and 29 to 31 °C for day 8 to 15. Chickens were monitored each morning (08:00) for mortality, behavioral changes, litter quality, and feed and waterers were checked to ensure they were in proper working order. No mortality, behavioral changes, or other animal welfare concerns were observed during the study. The chicks were not treated with any medications or other therapeutic interventions during the study.

Two independent trials were conducted using chicks from a different hatch-out. At placement, chicks weighed 44.85 g ± 0.60 and 44.78 g ± 0.62 for trials one and two, respectively. Chickens from the two hatches were maintained separately to ensure proper biological replication of the experiment. The two replicates of the experiment were handled as follows: chickens (n = 15) were randomly selected and placed into one of three groups: the control (0 g/MT AviPlus®P; n = 5 chickens) or one of the experimental groups (300 g/MT; n = 5 chickens; 500 g/MT AviPlus®P; n = 5 chickens). The experiment was conducted using two replicate pens therefore 10 chickens/treatment were used for all analyses. Chickens assigned to the control pen were allowed ad libitum access to a balanced, un-medicated corn and soybean meal-based starter diet that met or exceeded the established nutrient requirements [43]. Chickens assigned to the supplement-fed pen were given free access to the same starter diet mixed with 300 or 500 g/metric ton (MT) of a microencapsulated blend of citric (25%) and sorbic (16.7%) acids, thymol (1.7%), and vanillin (1.0%) (AviPlus®P, Vetagro S.p.A., Reggio Emilia, Italy). The feed was mixed in small batches for 15 min (34 g AviPlus®P/113 kg feed and 56.7 g AviPlus®P/113 kg feed for the 300 and 500 g/MT, respectively) using a Wenger AB batch mixer (Sebetha, KS). All chickens assigned to the control pens were evaluated first followed by those in the 300 and 500 g/MT groups. For both experimental replicates, chickens on the control, 300, and 500 g/MT diet (n = 5 per group/experiment; n = 10 total) were euthanized by cervical dislocation and necropsied at 15-days-of-age. Total content from the jejunum and ileum were collected and immediately flash frozen in liquid nitrogen to preserve activity followed by transfer to − 80 °C until further processing and analysis. Samples were collected at day 15 based on previous studies and proprietary knowledge of the product tested (Ester Grilli, personal communication).

DNA extraction and microbiome analysis

The DNA was extracted and sequenced as per standard laboratory guidelines [40]. Briefly, the tissue (ileum or jejunum) contents were thawed, homogenized, and 0.3 g removed followed by extraction using the Qiagen Stool Kit (Qiagen, Hilden, Germany). The DNA was eluted and stored at -20 °C until the library preparations commenced. Using the amplicon sequence variance index primers and protocol, the library was prepared as previously described [44]. Normalization and library clean-up were also performed prior to sequencing [40, 44]. The Illumina MiSeq 16S rDNA Microbiome Library (v2; Illumina, San Diego, CA, USA) was constructed and sequenced as per standard company guidelines. The sequences were exported from Illumina BaseSpace [45], de-multiplexed, and prepared for import into QIIME2.2019.1 [46].

The sequences were filtered for quality and chimera using DADA2, with Q30 being the cut off range for sequence quality [47]. Additionally, in order to remove any potential chimeras that escaped detection, OTUs with a frequency of less than 3 were removed from the analyses. Alpha and beta analyses were performed using the standard QIIME2.2019.1 pipeline, with ANOSIM (analysis of similarities) selected as it considers dispersion and the mean difference in beta diversity per group. In order to refine the analyses, the tissue data was then sorted into either a “ileum” or “jejunum” dataset for compositional analysis. Differential abundance was evaluated using the plugin ANCOM [48], which considers the compositional changes associated with treatment. Finally, LEfSE analysis was performed per standard practices [49] to determine which populations were enriched by treatment using least discriminate analysis, which is inversely related to ANCOM data [50].

Statistical analyses

Compositional microbiota studies are necessarily heterogeneous and represent the changes of a microbial consortia and structure by treatment. Necessarily, the use of statistically sound plugins to evaluate this compositional data are important as standard statistical practices are irrelevant if they do not take into account the compositional nature of the data. Alpha and beta diversity parameters were considered significant if the main effect was $P < 0.05$. Pairwise differences between the main effect of treatment were considered significant if $Q < 0.05$, which takes into account the false discovery rate associated with this class of data. Finally, for ANCOM, proc GLM was used in the background, with the central $\log_2$ ratio of the effect (W) significant of $Q < 0.05$. LEfSE was considered significant if $LDA > 2$ and $Q < 0.05$.

Abbreviations

ANCOM: analysis of communities of the microbiota; ANOSIM: analysis of similarities; GIT: gastrointestinal tract; LEfSe: linear discriminant analysis effect size; NTC: control; OTU: operational taxonomic unit.

Declarations

Ethics approval and consent to participate

All bird studies were under experimental protocol #2017008 and were approved by the USDA/ARS Institutional Animal Care and Use Committee (IACUC) and overseen by Dr. Roger B. Harvey, attending veterinarian. The IACUC operates under the Animal and Plant Health Inspection Service (APHIS) establishment number 334299.

Consent for publication

Not applicable.

Availability of data and materials

Data is freely available at www.github.com/RickeLab/BMCMicroSubmission26Feb2020. Please contact SCR if you request additional information.

Competing interests

AP and EG are Vetagro employees but did not participate in the analysis of this study. All other authors declare no conflict of interest.

Funding

This research was supported, in part, by Vetagro S.p.A (Agreement number 58-3091-8-005) and the USDA/ARS (3091-32000-035-00D). CLS, AP, EG conceived the study. KMF performed the microbiome analytics. KMF and CLS wrote the manuscript and prepared the paper for publication. MHK, AP and EG edited the manuscript.

Author's contributions

CLS, AP, EG conceived the study. KMF performed the microbiome analytics. KMF and CLS wrote the manuscript and prepared the paper for publication. MHK, SCR, AP and EG edited the manuscript. All authors read and approved the final manuscript.

Acknowledgements

The authors thank M. Reiley Street, Jr. (U.S. Department of Agriculture, Agricultural Research Service [USDA/ARS], College Station, TX) for outstanding technical support and assistance with daily animal care. The USDA is an equal opportunity provider and employer. Mention of trade names or commercial products in this publication is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the USDA.

References

1. Hashemi SR, Davoodi H. Phytogenics as new class of feed additive in poultry industry. J Anim Vet Advances. 2010;9(17):2295-304.
2. Wallace RJ, Oleszek W, Franz C, Hahn I, Baser KH, Mathe A, et al. Dietary plant bioactives for poultry health and productivity. Br Poult Sci. 2010;51(4):461-87.
3. Madhupriya V, Shamsudeen P, Manohar GR, Senthikumar S, Soundarapandiyan V, Moorthy M. Phyto feed additives in poultry nutrition - A review. Inter J Sci Environ Tech. 2018;7(3):815-22.
4. Yang Y, Iji PA, Choct M. Dietary modulation of gut microflora in broiler chickens: a review of the role of six kinds of alternatives to in-feed antibiotics. World's Poult Sci J. 2009;65(1):97-114.
5. Kabera JN, Semana E, Mussa AR, He X. Plant secondary metabolites: biosynthesis, classification, function, and pharmacological properties. J Pharm Pharmacol. 2014;2:377-92.
6. Morel C, Stermitz FR, Tegos G, Lewis K. Isoflavones as potentiators of antibacterial activity. J Agric Food Chem. 2003;51(19):5677-9.
7. Dias DA, Urban S, Roessner U. A historical overview of natural products in drug discovery. Metabolites. 2012;2(2):303-36.
8. Windisch W, Schedle K, Plitzner C, Kroismayr A. Use of phytogenic products as feed additives for swine and poultry. J Anim Sci. 2008;86(14 Suppl):E140-8.
9. Steiner T. Managing Gut Health: Natural Growth Promoters as a Key to Animal Performance. Press NU, editor. Nottingham, UK. 2006;1-98.
10. Clemente JC, Ursell LK, Parfrey LW, Knight R. The impact of the gut microbiota on human health: an integrative view. Cell. 2012;148(6):1258-70.
11. Tremaroli V, Backhed F. Functional interactions between the gut microbiota and host metabolism. Nature. 2012;489(7415):242-9.
12. Le Roy CI, Woodward MJ, Ellis RJ, La Ragione RM, Claus SP. Antibiotic treatment triggers gut dysbiosis and modulates metabolism in a chicken model of gastro-intestinal infection. BMC Vet Res. 2019;15(1):37.
13. Feye KM, Baxter MFA, Tellez-Isaias G, Kogut MH, Ricke SC. Influential factors on the composition of the conventionally raised broiler gastrointestinal microbiomes. Poult Sci. 2020;99(2):653-9.
14. Choi MS, Kim JK, Kim DH, Yoo HH. Effects of Gut Microbiota on the Bioavailability of Bioactive Compounds from Ginkgo Leaf Extracts. Metabolites. 2019;9(7).
15. Bicas JL, Fontanille P, Pastore GM, Larroche C. Characterization of monoterpene biotransformation in two pseudomonads. J Appl Microbiol. 2008;105(6):1991-2001.

16. Marin L, Miguelez EM, Villar CJ, Lombo F. Bioavailability of dietary polyphenols and gut microbiota metabolism: antimicrobial properties. Biomed Res Int. 2015;2015:905215.
17. Ricke SC. Impact of prebiotics on poultry production and food safety. Yale J Biol Med. 2018;91:151-9.
18. Jin S-H, Corless A, Sell JL. Digestive system development in post-hatch poultry. World's Poult Sci J. 1998;54(4):335-45.
19. Sklan D. Development of the digestive tract of poultry. World's Poult Sci J. 2001;57(4):415-28.
20. Metzler-Zebeli BU, Magowan E, Hollmann M, Ball MEE, Molnar A, Witter K, et al. Differences in intestinal size, structure, and function contributing to feed efficiency in broiler chickens reared at geographically distant locations. Poult Sci. 2018;97(2):578-91.
21. Piva A, Pizzamiglio V, Morlacchini M, Tedeschi M, Piva G. Lipid microencapsulation allows slow release of organic acids and natural identical flavors along the swine intestine. J Anim Sci. 2007;85(2):486-93.
22. Abd El-Hack ME, Alagawany M. Performance, egg quality, blood profile, immune function, and antioxidant enzyme activities in laying hens fed diets with thyme powder. J Anim Feed Sci. 2015;24:127-33.
23. Govindasami T, Pandey AC, Palanivelu N, Pandey A. Synthesis, Characterization and Antibacterial Activity of Biologically Important Vanillin Related Hydrazone Derivatives. Int J Organic Chem. 2011;01(03):71-7.
24. Harini ST, Kumar HV, Rangaswamy J, Naik N. Synthesis, antioxidant and antimicrobial activity of novel vanillin derived piperidin-4-one oxime esters: preponderant role of the phenyl ester substituents on the piperidin-4-one oxime core. Bioorg Med Chem Lett. 2012;22(24):7588-92.
25. Dittoe DK, Ricke SC, Kiess AS. Organic Acids and Potential for Modifying the Avian Gastrointestinal Tract and Reducing Pathogens and Disease. Front Vet Sci. 2018;5:216.
26. Kubo I, Lee SH. Potentiation of Antifungal Activity of Sorbic Acid. J Agric Food Chem. 1998;46(10):4052-5.
27. Pirgozliev V, Murphy TC, Owens B, George J, McCann ME. Fumaric and sorbic acid as additives in broiler feed. Res Vet Sci. 2008;84(3):387-94.
28. Centeno C, Arija I, Viveros A, Brenes A. Effects of citric acid and microbial phytase on amino acid digestibility in broiler chickens. Br Poult Sci. 2007;48(4):469-79.
29. Islam MR, Khandaker ZH, Chowdhury SD, Islam KMS. Effect of citric acid and acetic acid on the performance of broilers. J Bangeldesh Agri Univ. 2008;6(2):315-20.
30. Faith JJ, Guruge JL, Charbonneau M, Subramanian S, Seedorf H, Goodman AL, et al. The long-term stability of the human gut microbiota. Science. 2013;341(6141):1237439.
31. Subramanian S, Blanton LV, Frese SA, Charbonneau M, Mills DA, Gordon JI. Cultivating healthy growth and nutrition through the gut microbiota. Cell. 2015;161(1):36-48.
32. Li H, Qi Y, Jasper H. Preventing Age-Related Decline of Gut Compartmentalization Limits Microbiota Dysbiosis and Extends Lifespan. Cell Host Microbe. 2016;19(2):240-53.
33. Rehman T. Role of the Gut Microbiota in Age-Related Chronic Inflammation. Endocrine Metabolic Immune Disorders - Drug Targets. 2012;12:361-7.
34. Pan D, Yu Z. Intestinal microbiome of poultry and its interaction with host and diet. Gut Microbes. 2014;5(1):108-19.
35. Yamauchi K, Tarachai P. Changes in intestinal villi, cell area and intracellular autophagic vacuoles related to intestinal function in chickens. Br Poult Sci. 2000;41(4):416-23.
36. Bratburd JR, Keller C, Vivas E, Gemperline E, Li L, Rey FE, et al. Gut Microbial and Metabolic Responses to Salmonella enterica Serovar Typhimurium and Candida albicans. mBio. 2018;9(6).
37. Rivera-Chavez F, Baumler AJ. The Pyromaniac Inside You: Salmonella Metabolism in the Host Gut. Annu Rev Microbiol. 2015;69:31-48.
38. Hasan A, Adem Y. Influence of dietary thymol and carvacrol preparation and/or an organic acid blend on growth performance, digestive organs and intestinal microbiota of broiler chickens. African J Microbiol Res. 2011;5(8):979-84.
39. La Rosa PS, Brooks JP, Deych E, Boone EL, Edwards DJ, Wang Q, et al. Hypothesis testing and power calculations for taxonomic-based human microbiome data. PLoS One. 2012;7(12):e52078.
40. Feye KM, Rubinelli PM, Chaney WE, Pavlidis HO, Kogut MH, Ricke SC. The Preliminary Development of an in vitro Poultry Cecal Culture Model to Evaluate the Effects of Original XPC(TM) for the Reduction of Campylobacter jejuni and Its Potential Effects on the Microbiota. Front Microbiol. 2019;10:3062.
41. Vandeputte D, Kathagen G, D'Hoe K, Vieira-Silva S, Valles-Colomer M, Sabino J, et al. Quantitative microbiome profiling links gut community variation to microbial load. Nature. 2017;551(7681):507-11.
42. National Research Council. Guide for the Care and Use of Laboratory Animals. 8th ed. Washington DC: National Academies Press;2011.
43. National Research Council. Nutrient requirements of poultry. 9th. Washington DC: National Academy Press. 1994;19-34.
44. 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. Appl Environ Microbiol. 2013;79(17):5112-20.
45. Illumina. https://login.illumina.com/platform-services-manager/?rURL=https://basespace.illumina.com&clientId=basespace&clientVars=aHR0cHM6Ly9iYXNlc3BhY2UuaWxsdW1pbmEuY29tL2Rhc2hib2FyZA&redirectMe Accessed 9 March 2020.
46. Bolyen E, Rideout JR, Dillon MR, Bokulich NA, Abnet CC, Al-Ghalith GA, et al. Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. Nat Biotechnol. 2019;37(8):852-7.
47. Callahan BJ, McMurdie PJ, Rosen MJ, Han AW, Johnson AJ, Holmes SP. DADA2: High-resolution sample inference from Illumina amplicon data. Nat Methods. 2016;13(7):581-3.

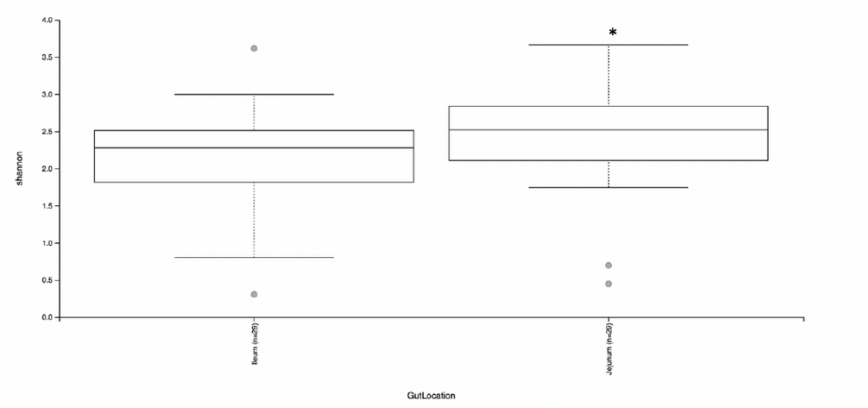
48. Mandal S, Van Treuren W, White RA, Eggesbo M, Knight R, Peddada SD. Analysis of composition of microbiomes: a novel method for studying microbial composition. Micro Ecol Health Dis. 2015;26:27663.
49. Galaxy Community Hub. https://galaxyproject.org. Accessed 9 March 2020.
50. Segata N, Izard J, Waldron L, Gevers D, Miropolsky L, Garrett WS, et al. Metagenomic biomarker discovery and explanation. Genome Biol. 2011;12(6):R60.

Tables

Due to technical limitations, the tables are only available as a download in the supplemental files section.

Figures

A



B

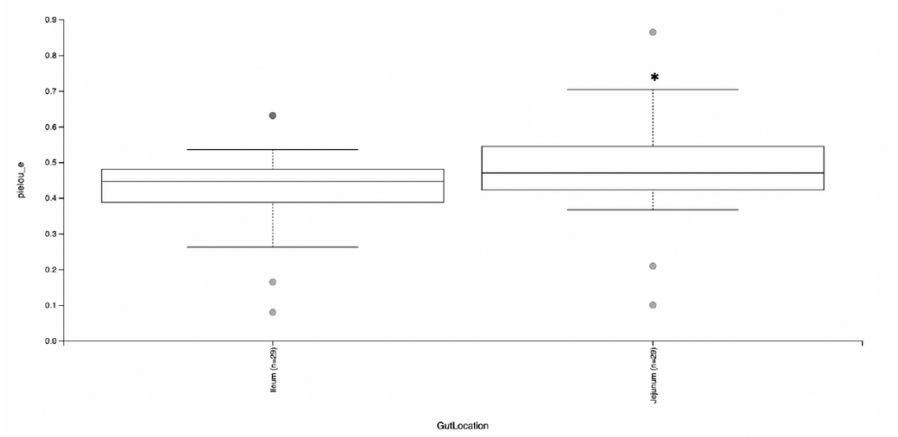
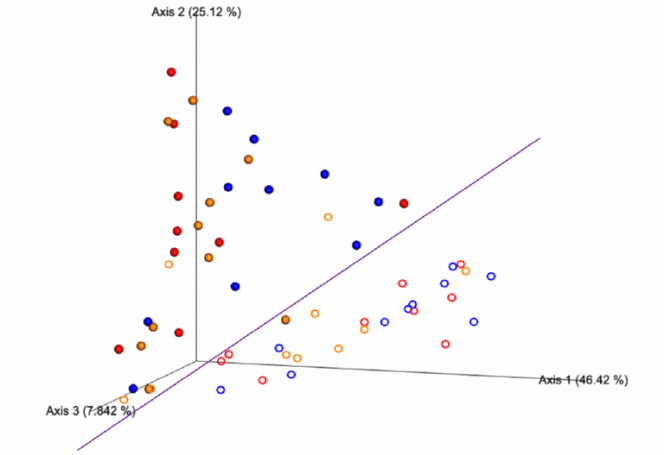


Figure 1

(A) Shannon Diveristy Index of Gut Location. (B) Pielou's Evenness. The astric represents a significant difference between the ileum and jejenum ($P < 0.05$). Evidence suggests compartmentalization was maintained.

A)



B)

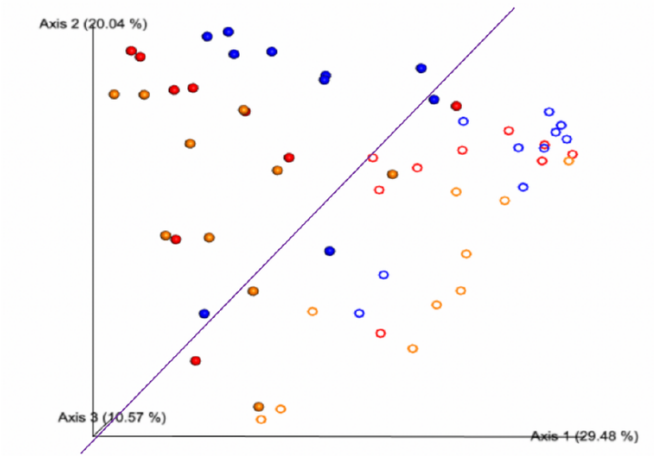


Figure 2

Beta Diversity Matrix. (A) Weighted Unifrac Distance Matrix. (B) Bray-Curtis Dissimilarity Index. Shape Coding: Sphear: Ileum; Ring; jejunum. Color: Red: 0 g/MT; Blue; 300 g/MT; Gold: 500 g/MT. Significant differences exist for compartmentalization. The effect of treatment was demonstrated throughout the study.

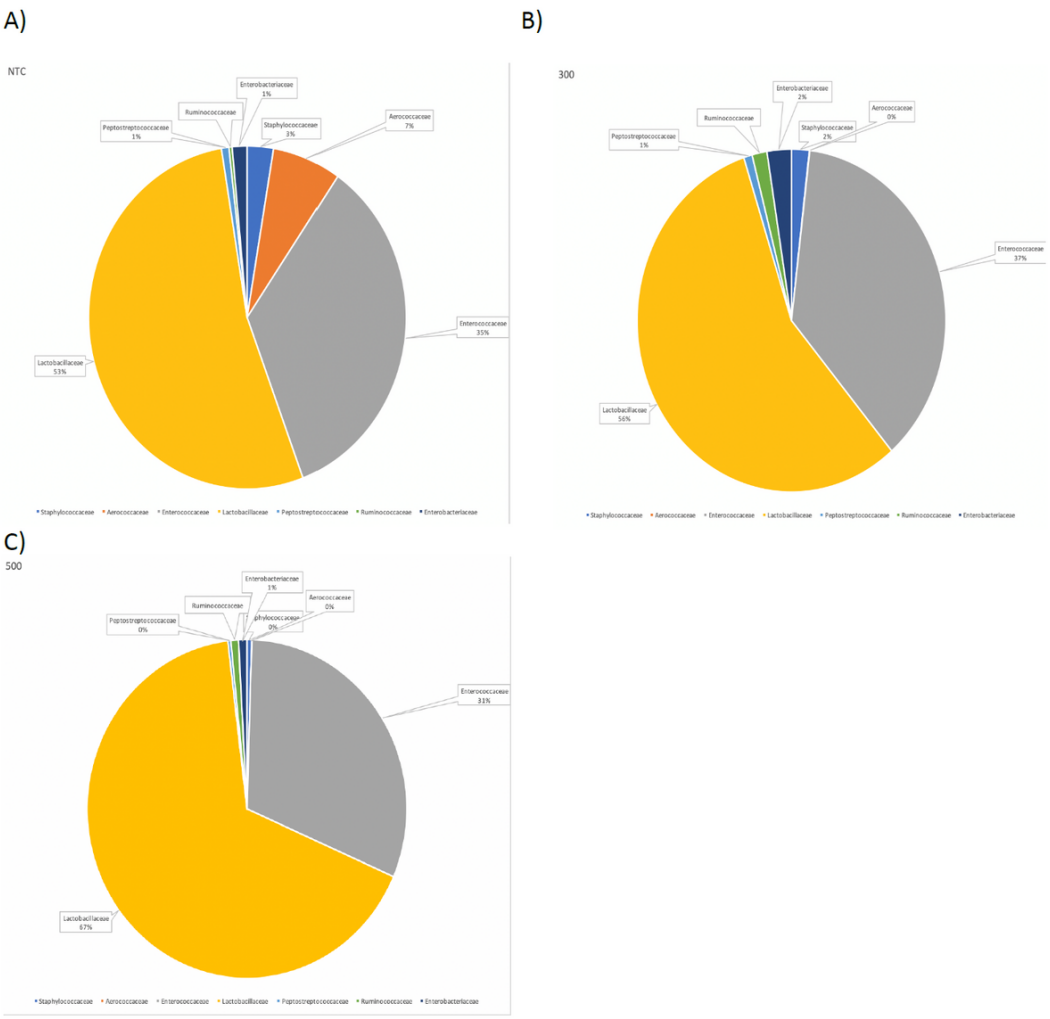


Figure 3

A) NTC; B) 300 g/MT AviPlus®P, C) 500 g/MT AviPlus®P. The legends for the specific OTUS associated with treatment as defined by ANCOM ($Q < 0.05$) is listed on the figure. Significant fluctuations occurred with increasing doses of AviPlus®P.

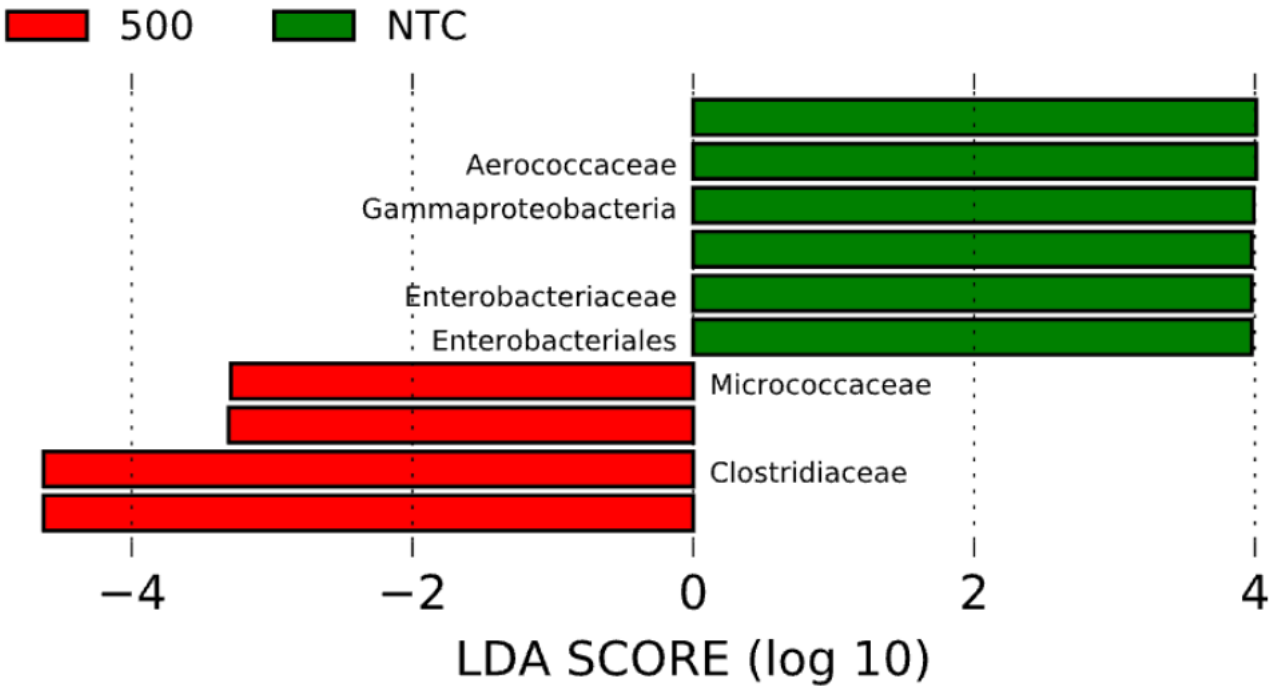


Figure 4

LEfSE Analysis. Missing OTUs are not defined within the Family taxonomical designation. The 500 and NTC group are relative to 300. An LDA > 2 with a Q < 0.05 is considered significant and is graphically represented.

Figure 5

Enterobacteriaceae LEfSE Relative Abundance Data. The dotted lines are the median and the solid lines are the class mean. The relative abundance significant by LEfSE of each animal is displayed. The NTC has on average a greater abundance of Enterobacteriaceae, with a stepwise decrease in this

population as AviPlus®P inclusion increases.

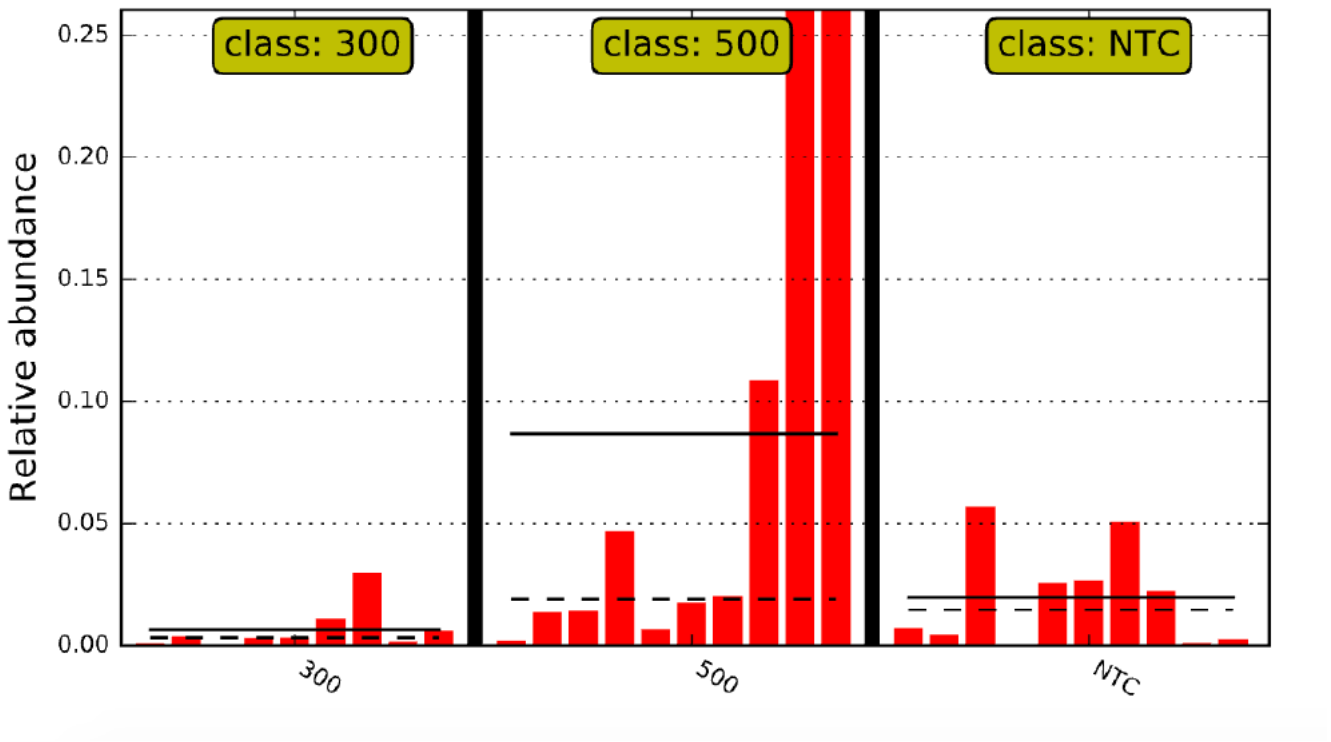


Figure 6

Clostridiaceae LEfSE Relative Abundance Data. The dotted lines are the median and the solid lines are the class mean. The relative abundance significant by LEfSE of each animal is displayed. The 300 g/MT treatment has on average a lower abundance of Clostridiaceae, with an increase in this population as AviPlus at the 500 g/MT level.

Supplementary Files

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

- Table2.pdf
- MCROD2000185Checklist.pdf
- Table1.pdf