Research Note: Orange corn altered the cecal microbiome in laying hens

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ABSTRACT Carotenoids, which are pigments known to have many health benefits, such as their antioxidant properties, are being researched for their potential as a feed additive for production animals. These pigments are found in varying quantities in different breeds of corn, and their impact on the chicken microbiome requires further investigation. This 35 d laying hen (Novagen White) feeding trial involved varying the levels and composition of carotenoids by changing the corn source: white (0.9 μg total carotinoids/g total diet), yellow (5.7 μg/g), and orange (24.9 μg/g). For each of the three corn diet treatments, 6 replicate cages were randomly assigned. The cecal microbial community composition of the hens was then studied by 16S rRNA gene amplicon sequencing. The composition of the cecal bacterial community, as determined by Bray-Curtis dissimilarity, was different ($P < 0.05$) in chickens fed the orange corn diet, compared to chickens on the white corn diet, but there was no statistical difference between animals fed yellow corn compared to the white or orange corn groups. There was no change in the alpha diversity between any of the groups. Within *Lactobacillus*, which is one of the most abundant genera, 2 amplicon sequence variants (**ASVs**) were decreased and one ASV was increased in the orange corn group compared to both the white and yellow corn groups. While previous studies showed that orange corn did not alter the community composition in broilers, it appears that orange corn based feed may alter the community composition of laying hens.

Key words: carotenoid, microbiome, hen, feed additive

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INTRODUCTION

The impact of the intestinal microbiota on its host has been a valuable area of study to improve animal health, disease resistance, feed efficiency, and animal welfare, while at the same time reducing the need to use antibiotics. An attempt to predictably and reproducibly alter the intestinal microbiome is still an active area of research (Cantu-Jungles et al., 2021). One approach to improve the composition of the intestinal microbial community in livestock is to utilize feed additives. For example, probiotics, prebiotics, and dietary acidifiers, among others, do not add nutrients to the diet, but they often have a major impact on the intestinal microbiome as well as the performance of the animal.

One feed additive that has been explored recently is the chemical family of carotenoids. Carotenoids are one of the most widespread groups of pigments that exist and are responsible for the colors red, orange, and yellow in the majority of fruits and vegetables. Unlike plants that synthesize these tetraterpene pigments naturally on their own, for animals the diet is the main source of carotenoids. The corn genotype used in this study contained higher levels of xanthophylls, specifically zeaxanthin, and lutein, which have various benefits. Some carotenoids can be converted by the body to various vitamins, which contributes to normal growth and development. Increased levels of dietary carotenoids have been associated with health benefits in humans (Bohn et al., 2021), including decreased incidences of several chronic diseases, and these benefits have been attributed to their antioxidant, antiapoptotic, and anti-inflammatory properties (Krinsky and Yeum, 2003). The supplementation of carotenoids in animal diets can help improve production performance, the quality of meat and eggs (Ortiz et al., 2021), as well as health in chickens (Abraham et al., 2021). Dietary carotenoids are deposited in the skin, feathers, and fat of chickens

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(Abraham et al., 2021). Therefore, the types of corn in animal feeds and their ability to provide animals with sufficient nutrients in order to sustain health and production are critical. With these numerous benefits, carotenoids present themselves as a potential feed additive in the agricultural industry.

In addition to direct health benefits, dietary carotenoids have also been shown to impact the intestinal microbiota. The carotenoids β-carotene, lycopene, capsaicin, and fucoxanthin, among others, have demonstrated interactions with the gut microbiota (Dingeo et al., 2020). However, the impact of the carotenoids in orange corn on microbial composition requires further research. The orange corn used in this study was bred to increase the dark orange color of the kernel to contain significantly higher levels of carotenoids (45−55 ug/g) than conventional yellow corn (∼15−20 ug/g). The eggs from birds utilized in this study demonstrated that orange corn based diets alter yolk color and zeaxanthin concentration in eggs (Ortiz et al., 2021). Given the benefits observed to the health of the bird when feeding orange corn, the objective of this study was to determine the impact of orange corn on the cecal microbiome. It was hypothesized that the antioxidative activity of carotenoids would alter the chemical profile of the digesta and the micro-organisms that grow in that environment. One previous study in broilers suggested that orange corn did not alter the microbiome (Abraham et al., 2021), but additional studies are needed to better understand how carotenoids affect the composition of the intestinal microbial community in laying hens.

MATERIALS AND METHODS

Study Design

Experimental procedures were approved by the Purdue Animal Care and Use Committee (Protocol #1709001622). This study was completed at the Purdue Animal Sciences Research and Education Center (**ASREC**) in West Lafayette, IN. A total of 360 Novogen White laying hens at 32 wk of age were placed in cages measuring 1,440 in^2, with 20 birds in each cage. The experimental process was the same as described in Ortiz et al. (2021), but the current study focused on the cecal microbial community composition as determined by 16S rRNA gene amplicon sequencing. As described in Ortiz et al. (2021), the birds were fed either the white, yellow, or orange corn (56.5% of the whole diet) based on the assigned treatment, with 120 birds in each treatment. There were 6 replicate cages randomly assigned for each of the three diet treatments, with an empty cage between each of the treatments. Water was supplied ad libitum. The results described in Ortiz et al. (2021) are from the same birds that were used in this study.

Microbiome Library Preparation and Analysis

After 35 d on the dietary treatments, 72 layers (4 birds per pen) were euthanized, and their cecal contents were collected and stored immediately at 4°C. Samples were transported to the lab, homogenized, and a representative sample was placed in the DNA extraction plate and then all samples were frozen at −20°C until DNA extraction. Then 16S rRNA gene library preparation was completed as previously described (Abraham et al., 2021). Briefly, the cecal metagenomic DNA was isolated by utilizing the Qiagen (Valencia, CA) MagAttract PowerMicrobiome DNA/RNA kit. For the bead beating step, the Qiagen TissueLyzer was utilized. A 16S rRNA gene library was created from the extracted DNA according to the procedure described in Kozich et al. (2013). PCR amplification of the V4 region of the 16S rRNA gene was used to make the Illumina indexed amplicons, and gel electrophoresis was used to confirm amplification. The negative control, which used water as the DNA template, did not have observable PCR amplicons. The SequalPrep Normalization Plate kit (Invitrogen, Carlsbad, CA) was used to normalize the amplified DNA, which was then pooled into a single library for each 96-well plate. The KAPA Library Quantification Kit (Roche, Branford, CT) was used to determine the library concentration of four 96-well pools, and the Bioanalyzer (Agilent, Santa Clara, CA) with a high-sensitivity kit was used to determine the library average fragment length. The pooled samples together with the mock community and water samples were sequenced (Illumina, San Diego, CA; MiSeq v2 kit, 500 cycles). Using the Illumina software, the sequences were demultiplexed according to the oligonucleotide bar code sequence. Sequences were submitted in the National Center for Biotechnology Information Sequence Read Archive database under Bioproject PRJNA737418, with the BioSample numbers SAMN19693842 − SAMN19693915. QIIME2, version 2019.1 was used to analyze the 16S amplicon sequences. DADA2 was used to remove the low-quality sequences, as well as remove the first 13 bases of the forward and reverse reads due to low quality. Samples were rarefied and subsampled at 5,035 sequences per sample.

Statistical Analysis

The pen was considered the experimental unit for microbiome analysis. Therefore, the pen replicates were joined into a combined pen sample, with equal number of sequences from all animals from the same pen. This was accomplished with the feature-table group function in QIIME2, using the rarified ASV table as input. Then, alpha and beta diversity were calculated. Alpha diversity metrics were compared using the Kruskal-Wallis test as implemented in QIIME2. The 99% clustered Silva database (version 132), which was trained with the primers from this experiment, was used to assign taxonomy to the amplicon sequence variants (**ASV**). The

differentially abundant ASVs according to the diet on a pairwise basis were determined using DESeq2 v1.26 (Love et al., 2014) (function DESeq in R). QIIME2 output files were imported into R with the qiime2R package v0.99.20, and R was utilized to generate figures. QIIME2 commands and R scripts are available at www.github.com/john2929/OrangeCorn for reproducibility.

RESULTS AND DISCUSSION

The corn used in the diet altered the cecal bacterial community composition, as measured by Bray-Curtis ($P = 0.003$, Figure 1A) and Unweighted UniFrac ($P = 0.028$, Figure 1C). Specifically, the community composition (Bray-Curtis) of chickens fed orange corn was different than chickens fed white corn (q value = 0.033) while the animals fed yellow corn were not statistically different from the white or orange corn groups. Community composition as measured by Weighted UniFrac found that microbial communities from chickens raised on the different corn diets were not significantly different ($P > 0.05$, Figure 1B). While differences in beta diversity were observed in this study, a previous study fed similar corn diets to broiler chickens (using the same corn sources), but no significant difference in beta diversity was observed (Abraham et al., 2021).

The major phyla found in the hen cecal community included Bacteroidetes and Firmicutes (Figure 1D). Bacteroidetes numerically increased, while Firmicutes decreased in average relative abundance in hens raised on the yellow and orange corn diets compared to the white corn diet. In a previous study, Bacteroidetes was reported to be lower in the jejunal microbial communities of chicks given a dietary supplement (including β-carotene and other supplements), compared to chicks without such supplements (Gong et al., 2020). While our study did not observe this effect, our study sampled a different site of the digestive tract and used a different feed supplement. In the current study, the major families found in the ceca included *Bacteroidaceae, Lactobacillaceae*, and *Ruminococcaceae, Rikenellaceae*, and *Prevotellaceae* (Figure 1E) and the major genera were Bacteroides, Lactobacillus, and *Rikenellaceae* RC9 gut group (Figure 1F). Numerically, the average relative abundance of *Lactobacillus* decreased as a genus in the hens raised on the yellow and orange corn diets compared to the white corn diet. The relative abundances of *Rikenellaceae* RC9 gut group and *Tannerellaceae* were highest in the orange corn diet and lowest in the white corn diet.

Ruminococcaceae UCG-014 was lowest in the white corn diet groups compared with yellow and orange corn diet groups (Figure 2). Two *Lactobacillus* ASVs decreased in relative abundance and one *Lactobacillus* ASV increased in relative abundance in the orange compared to both the yellow and white corn diet groups. *Ruminococcus torques* group had one enriched ASV in the white corn group compared to both the yellow and orange corn diet groups. In the white corn diet group compared with the orange corn diet group, there was an increase in one ASV assigned to *Lachnospiraceae*, while there was a decrease in one ASV assigned to each of *Tannerellaceae, Butyricimonas, Bacteroides*, and *Ruminococcaceae* UCG-014. This is a contrast to a previous study involving broilers, in which one ASV

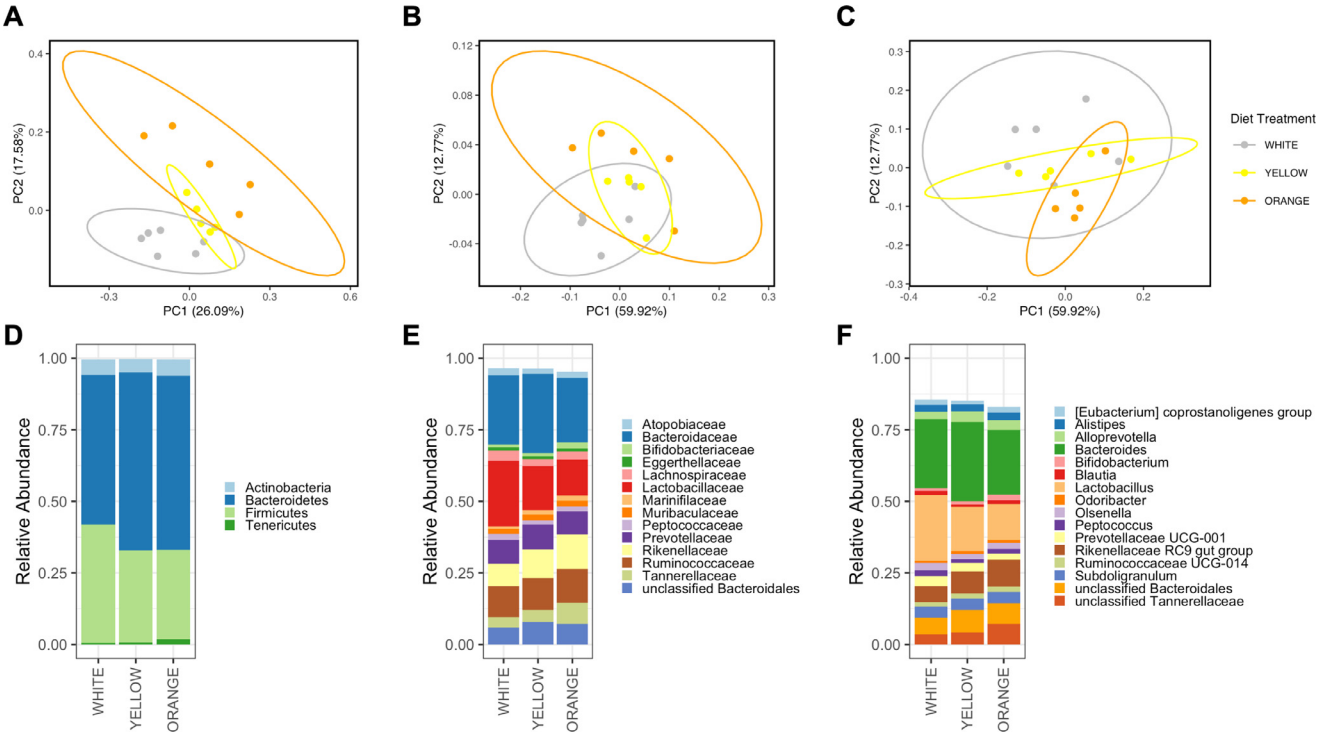

Figure 1. Principal coordinates analysis based on Bray-Curtis dissimilarity matrix (A), Weighted UniFrac (B), and Unweighted UniFrac (C). Samples are colored as per corn type in the diet. Ellipses indicate a 95% CI of the range of individual samples. Relative abundance according to diet treatment (white, yellow, orange) classified at the phylum (D), family (E), and genus (F) level.

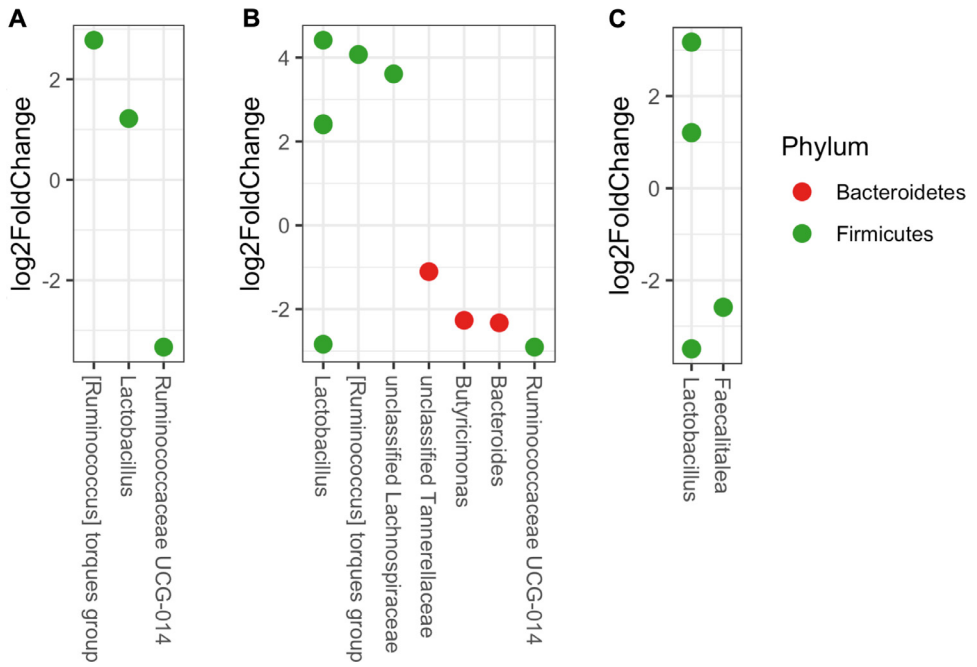

Figure 2. Pairwise $\log_2$ fold change in ASV relative abundance compared between the diet groups. Log_2 fold change values larger than zero show an increase in the (A) white compared with yellow, (B) white compared with orange and (C) yellow compared with orange corn groups. Each point represents one ASV. The points are colored according to their phylum assignment. Abbreviation: ASV, amplicon sequence variant.

assigned to *Bacteroides* increased in the white corn diet group compared with the orange corn diet group, one ASV assigned to the *Faecalitalea* was decreased in the yellow corn diet compared to the orange corn group, and no ASVs were enriched in the orange corn group (Abraham et al., 2021). It appears that the cecal communities in hens and broilers are quite different (which could be due to age, genetics, and diet composition) and the corn type in the diet in these 2 systems selected different bacteria potentially because carotenoids have low selective specificity toward the bacterial community, similar to what has been described as selection specificity for dietary fibers (Cantu-Jungles et al., 2021).

The alpha diversity was not statistically different ($P > 0.5$) when comparing the microbial communities from chickens raised on white, yellow, and orange corn diets. Similar to a previous study in broilers, the different corn diets did not significantly change the alpha diversity in the chicken cecal microbiome (Abraham et al., 2021). Previous swine studies have also reported community alpha diversity to be not affected by the various carotenoid levels in corn (González-Prendes et al., 2019; Li et al., 2019).

The data in this study both agree and disagree with previous findings. Alpha diversity has repeatedly not been altered by increased carotenoids in animal diets (González-Prendes et al., 2019; Li et al., 2019; Abraham et al., 2021). However, the community composition (beta diversity) in this study was statistically altered by orange corn compared to white corn based diets, while in a previous study, beta diversity was unaltered by the level and composition of carotenoids in the corn (Abraham et al., 2021). This disagreement may be due to the study design differences (age, breed, stocking density, and diet formulation (even though the corn source was the same) between the birds in the 2 studies. In both studies, the dietary treatment period was similar: 5 wk in the current study and 6 weeks in the previous study (Abraham et al., 2021). These study design differences were reflected in the fact that the cecal microbial composition of the hens in the current study had an entirely different composition than the broilers of the previous study – the major genera that were present in this study are *Bacteroides, Lactobacillus*, and Rikenellaceae RC9 gut group, while the major genera present in the broiler study are *Alistipes, Bacteroides, Faecalibacterium*, and Ruminococcaceae UCG-014. Given the study design differences between the studies previously completed, the relationship between dietary carotenoids and the chicken microbial community remains unclear. Additional research is needed to better understand how carotenoids impact the microbiome of both broiler and layer chickens.

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DISCLOSURES

E. Rocheford and T. Rocheford are shareholders and officers of NutraMaize LLC.

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