Poultry Science®

Investigating antibiotic free feed additives for growth promotion in poultry: effects on performance and microbiota

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ABSTRACT The poultry industry is evolving towards antibiotic-free production to meet market demands and decelerate the increasing spread of the antimicrobial resistance. The growing need for antibiotic free products has challenged producers to decrease or completely stop using antimicrobials as feed supplements in broiler diet to improve feed efficiency, growth rate, and intestinal health. Natural feed additives (e.g., probiotics and phytobiotics) are promising alternatives to substitute antimicrobial growth promoters. The goal of our study was to characterize the effects of a Probiotic and an Essential Oils blend on broilers' performance and perform a time-series analysis to describe their excreta microbiome. A total of 320 Cobb 500 (1-day-old) chicks were raised for 21 d in 32 randomly allocated cages. Treatments consisted of 4 experimental diets: a basal diet, and a basal diet mixed with an Antibiotic (bacitracin methylene disalicylate), an essential oils blend (oregano oil, rosemary, and red pepper), or a Probiotic (*Bacillus subtilis*). Body weight (on 1, 10, and 21d), and feed intake (10d and 21d) were recorded and feed conversion ratio was calculated. Droppings were collected daily (1−21d) to characterize broilers' excreta microbiota by targeted sequencing of the bacterial 16S rRNA gene. The Probiotic significantly improved feed conversion ratio for starter phase 1 to 10d ($P = 0.03$), grower phase 10 to 21d ($P = 0.05$), and total period 1 to 21d ($P = 0.01$) compared to the Antibiotic. Feed supplements did not affect alpha diversity but did impact microbial beta diversity ($P < 0.01$). Age also impacted microbiome turnover as differences in alpha and beta diversity were detected. Furthermore, when compared to the basal diet, the probiotic and antibiotic significantly impacted relative abundance of *Bifidobacterium* (log2 fold change −1.44, $P = 0.03$), *Intestinimonas* (log2 fold change 0.560, $P < 0.01$) and *Ligilactobacillus* (log2 fold change −1.600, $P < 0.01$). Overall, Probiotic supplementation but not essential oils supplementation positively impacted broilers' growth performance by directly causing directional shifts in broilers' excreta microbiota structure.

Key words: broiler, probiotic, essential oil, microbiome, performance

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INTRODUCTION

Global chicken meat production is expected to reach over 102 million tons by 2024 and is estimated to continue increasing as promoted by globalization (OECD-FAO, 2023). The United States is the largest global poultry producer country, producing approximately 21 million tons of chicken in 2023, followed by the Brazil and China (USDA, 2024). The high demand for poultry meat led the industry toward more intensive production systems that, requiring rapid growth from broiler chickens, was aided using in-feed antimicrobial growth promoters (**AGP**) (Yadav and Jha, 2019).

Antimicrobial growth promoters are subtherapeutic doses of antibiotics and were widely used in animal production to improve feed efficiency, growth performance, and intestinal health (Mehdi et al., 2018). However, the

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increasing concern for antimicrobial resistance and consumer preference for antibiotic-free poultry products has led many countries to ban or restrict the use of AGP. Currently, in the United States, medically important antibiotics used in feed and water for food-producing animals are only allowed with the stipulations of the Veterinary Feed Directive (Stokka, 2016; Food and Drug Administration, 2021). The cessation or restriction of AGP use is associated with increases in enteritis (Buiatte et al., 2022), a decrease in growth performance, and negative economic impacts (Costa et al., 2017). Therefore, antibiotic-free alternative approaches to enhance intestinal health and improve growth performance are of great interest to the poultry industry (Ayalew et al., 2022). This includes probiotics and essential oils which have been increasingly investigated for use in poultry production worldwide (Vanbelle et al., 1990; Pourabedin and Zhao, 2015; Mehdi et al., 2018; Yadav and Jha, 2019; Suez et al., 2020; Tellez et al., 2020; Tellez-Isaias et al., 2021).

Probiotics are defined as "*live microorganisms which when administered in adequate amounts confer a health benefit on the host*" (Hill et al., 2014). They are hypothesized to modulate the intestinal microbiome to enhance microbial energy metabolism (Zhou et al., 2010; Bai et al., 2013; Khan et al., 2020). Probiotic supplementation may benefit poultry by lowering intestinal Enterobacteriales colonization through competitive exclusion, improving intestinal barrier function, and enhancing immune system maturation, thus promoting body weight gain and reduce mortality rate of growing broilers and layer hens (Walker, 2008; Molina, 2019).

Feed additives improve performance via several mechanisms including changes in intestinal morphology, and modulation of bacterial populations. In-feed supplementation with a *Bacillus spp.* based probiotic enhanced broilers' intestinal health by improving villi length to crypt depth ratio by approximately 49% in birds infected with *Clostridium perfringens* (Jayaraman et al., 2013). Moreover, *Bacillus subtilis* spores may create an anaerobic environment in the intestine, stimulating proliferation and competitive colonization of *Lactobacillus, Blautia, Fecalibacterium,* and *Bifidobacterium.* These genera then produce short chain fatty acids thus, decreasing or inhibiting the adhesion of pathogens such as *Escherichia coli*, and *Salmonella* (Wu et al., 2011; Yang et al., 2012; Jeong and Kim, 2014; Lee et al., 2015; Tellez et al., 2020; Rivera-Pérez et al., 2021). In addition to *Bacillus spp.* probiotics, other common and commercially available probiotic products in the poultry industry include *Lactobacillus, Enterococcus, Bifidobacterium,* and the yeast *Saccharomyces* (Suez et al., 2020).

Essential oils are plant-derived extracts that have drawn increasing attention in recent years as they possess various antioxidant, immune-modulatory, antimicrobial properties, and the ability to change the microbiome (Leyva-López et al., 2017). Research has demonstrated that incorporating oregano and thyme in poultry feed can bolster intestinal health, growth performance, microbiome diversity, and carcass quality in broilers chickens (Corduk et al., 2013; Zhang et al., 2021; Ayalew et al., 2022). These benefits may be attributed to carvacrol and thymol, 2 phenolic compounds known for antimicrobial activity against pathogenic bacteria and their positive impacts on metabolic activities, such as enzyme stimulation and lipid metabolism (Alagawany et al., 2018; Oniga et al., 2018). Specifically, supplementation with essential oils has been linked to a 5% increase in weight gain and a 1.5% improvement in feed efficiency (Bozkurt et al., 2009). Furthermore, these compounds have been shown to boost intestinal antioxidative capacity and immunity by enhancing the production of immunoglobulins, the activity of glutathione peroxidase enzymes, and the intestinal barrier function (Popović et al., 2016; Ruan et al., 2021). Additionally, the active components in essential oils can modulate intestinal microbiota composition, by increasing the abundance of Firmicutes and *Lactobacillus* (Corduk et al., 2013; Kim et al., 2023). While essential oils present a promising alternative to AGP, their effectiveness can be influenced by various factors, including their chemical composition, functional groups, and potential synergistic interactions with antibiotics, probiotics, and organic acids (Corduk et al., 2013; Leyva-López et al., 2017).

Before hatching, the chicken gut is initially colonized by a primitive assortment of microorganisms during the embryonic stage (Ding et al., 2017; Proszkowiec-Weglarz et al., 2022). These unstable microbial populations are acquired in the pre-hatching phase either from the hens' oviduct or the hatchery environment (Awad et al., 2016). As part of the dramatic changes in the microbiome structure during early chicken life, bacterial succession, and increased diversity over the first week of life are observed (Ranjitkar et al., 2016). The microbiome develops rapidly in the first 3 d post-hatch, indicating that age is an important factor of gut microbiome development, independent of dietary interventions or medications (Proszkowiec-Weglarz et al., 2022). Experiments have demonstrated the initial dominance Proteobacteria, while Firmicutes increases once the microbiome becomes more stable and then persists through life (Ballou et al., 2016).

Chickens' gastrointestinal tract (**GIT**) consists of 3 parts which are colonized by microbes throughout: the upper segment, small intestine, and large intestine (Maki et al., 2019). The GIT harbors a variety of microorganisms that comprise an ecosystem with high relevance for bird and human public health (Carrasco et al., 2019). Although there is significant diversity between each GIT section, they are interconnected and thus influence each other's community composition (Maki et al., 2019; Yan et al., 2019). Therefore, we hypothesize that 1) supplementing broiler chickens' diet with either a Probiotic or an Essential Oil blend will improve growth performance, indicating that these natural alternatives can effectively replace AGP in poultry farming; and 2) these dietary supplements will influence the structure of broilers' excreta microbiota enabling the

identification of specific taxa potentially responsible for the phenotypic differences between the treatment groups. Here, we compare the effects of a commercially available Probiotic and an Essential Oils-based feed additives using a non-invasive sampling technique to understand 1) how these products impact production outcomes and 2) to describe and compare excreta microbiome diversity and composition to a basal diet and traditional AGP approaches.

MATERIALS AND METHODS

Ethical Approval

All experimental protocols were subjected to ethical approval following the Pennsylvania State University's Institutional Animal and Care Use Committee (**IACUC**) guidelines, in line with protocol no. PROTO202101779.

Experimental Design and Facility

The experiment was conducted at the Pennsylvania State University in the Poultry Education and Research Center (**PERC**), University Park, PA in March 2021. The study was conducted on 1-d broiler chickens (Cobb 500), using a complete randomized block design. A total of 320 male birds were individually weighed upon arrival and randomly allocated in 32 cages with 8 replicates per treatment and 10 broilers per cage. These animals were maintained in the study system from the day of hatch through d 21.

Dietary Treatments

Animals were fed a corn and soybean meal-based diet formulated in a mash form according to published nutrient recommendations for growing broiler chickens (Cobb-Vantress, 2018) (Table 1), which is termed basal diet. Two feed formulations were offered according to birds' age and growth stage: (starter 1 −10d) and grower (11−21d) and produced in a local feed mill in Pennsylvania. The feed additives were added to each standard diet to obtain a homogeneous distribution.

The experiment consisted of 4 experimental diets: a basal diet (negative control), a basal diet supplemented with a *Bacillus subtilis* strain Probiotic to contain 500,000 cfu/g (227 g/ton) (Calsporin, Calpis America, Inc., Peachtree City, GA), a basal diet supplemented with an essential oils blend product containing oregano oil, rosemary, and red pepper (100 g/ton of feed), (Activo, Ew Nutrition, Adel, IA), and a basal diet supplemented with bacitracin methylene disalicylate (50 g/ton of feed), (BMD; Zoetis, Pasipanny, NJ), as a comparison to a commonly used AGP in poultry diets (Table 2). Birds were fed ad libitum on the treatment diets and had free access to water during the 21d experimental period.

Table 1. Composition of the basal diet before supplementation with feed additives.

Ingredient	Starter (d1−10) (%)	Grower (d11−21) (%)
Corn	62.198	65.920
Soybean meal, solvent extracted	32.936	28.735
Limestone	1.345	1.196
Soybean meal, extruded	1.000	1.846
Monocalcium PH	0.947	0.840
Lysine Sulfate	0.358	0.333
DL-Methionine	0.336	0.306
Salt	0.233	0.234
Sodium bicarbonate	0.184	0.184
L-Threonine	0.140	0.089
Choline chloride	0.131	0.126
Vitamin premix[1]	0.050	0.050
Trace mineral premix[1]	0.050	0.050
Vitamin D	0.050	0.050
Tri-basic copper chloride	0.032	0.032
Phytase[2]	0.008	0.008
Calculated nutrient value		
ME (kcal/Kg)	2973	3024
Crude protein	21.16	19.68
Calcium	0.92	0.84
Available phosphorus	0.45	0.42
Sodium	0.16	0.16
Potassium	0.88	0.82
Dig. Lysine	1.22	1.12
Dig. TSAA	0.91	0.85
Dig. Threonine	0.83	0.73

[1]A proprietary commercial premix was used across all treatments. The premix was included at a level that meets or exceeds the vitamin and mineral requirements of the breed.
[2]Provides 500 FTU/kg.

Performance Measurements

All broiler chickens were individually weighed on d 1 and then at the end of each dietary phase on d 10 and 21. The feed consumed per pen was monitored at the end of each growth phase. Live body weight (**BW**), average daily gain (**ADG**), feed intake (**FI**), and feed conversion ratio (**FCR**; feed consumed/weight gain) were calculated at 3 periods: 1 to 10d (starter phase), 11 to 21d (grower phase), and 1 to 21d (total period). Body weight uniformity was defined as the percentage of individuals within ± 10% of the mean body weight, where a higher percentage indicates better uniformity.

Excreta Sampling

Excreta samples were collected daily during the entire experimental period (1−21d). The excreta collection was standardized by placing a sterile collection paper under the cage tray of each cage every day at the same time for 90 minutes. Collection papers were then folded, placed in a sterile plastic bag (Whirl-pak - Avantor, Philadelphia, PA), and stored at −80°C until DNA extraction.

DNA Extraction and PCR Amplification

Before DNA extraction, paper samples were randomized, thawed, and subjected to a preprocessing step. Briefly, 50 mL of molecular-grade water (Intermountain Life Science, West Jordan, UT) was added to the whirl-

Table 2. Experimental design of 21-d broiler chicken experiment.

Treatment	Description	Dose	No. of replicates	Total no. of birds
Basal Diet	Corn and soybean-base diet - Negative Control		8	80
Probiotic	*Bacillus subtilis* – (500,000 CFU/g of feed)	226.8 g/ton	8	80
Essential Oils	Blend of oregano, rosemary, and red pepper	100 g/ton	8	80
Antibiotic	Bacitracin methylene disalicylate - Positive Control	50 g/ton	8	80

pak disposable bag containing the collection papers and homogenized using a Stomacher 400 Circulator for 2 min. Then, 4 mL of the homogenized solution was collected and stored in microcentrifuge tubes. Genomic DNA was extracted using the Kingfisher instrument with the MagMAX microbiome Ultra kit (Thermo Fisher Scientific, Austin, TX), according to the manufacturer's instructions and 200 μL of homogenate as a starting sample. The extracted genomic DNA quality and quantity were assessed with a spectrophotometer (Nanodrop, Thermo Fisher Scientific Inc., Waltham, MA). For each extraction batch, a positive control comprised of ZymoBIOMICS Microbial Community DNA Standard (Zymo Research Corporation, Irvine, CA) and a negative control (reagents only) were included and carried through sequencing. As additional controls, one environmental (i.e., a paper placed inside of the cages without birds), and one autoclaved (a sample of the autoclaved paper batches) paper control were extracted and sequenced. The initial amplification of the hypervariable V4 region of the bacterial 16S rRNA was performed on genomic DNA following the Earth Microbiome Project protocol (Thompson et al., 2017). The amplification was carried out for 30 cycles using the primer set comprised of 515F (5′-GTG**Y**-CAGCMGCCGCGGTAA- 3′) and 806R (5′-GGAC-TAC**N**VGGGTWTCTAAT-3′) (Apprill et al., 2015; Parada et al., 2016). The thermal cycling conditions used were 98°C for 2 min, 98°C for 10 s, 56.5°C for 20 s, 72°C for 15 s (repeating steps 2-4, 30 times), and a final step of 72°C for 5 min. The amplification was checked on an agarose gel. The PCR products were sent to the Genomics Core Facility at the Pennsylvania State University (University Park, PA), subjected to library preparation, and sequenced on a 250 bp paired-end Illumina MiSeq platform (Illumina Inc., San Diego, CA).

Statistical Analysis and Bioinformatics

This study used repeated measures randomized complete block design, with each cage containing 10 birds being considered an independent experimental unit. Effect sizes were calculated by comparing values for basal diet against probiotic and essential oils blend and probiotic against the antibiotic. Differences in FCR and BW were analyzed through preplanned contrasts using the R package emmeans (Lenth, 2023). The contrasts used are described as follows: 1) basal diet vs. all treatments combined (probiotic, essential oils, and antibiotic) (BD vs. ATC), 2) probiotic vs. antibiotic (PB vs. ATB), and 3) essential oils vs. antibiotic (EO vs. ATB). To account for multiple hypothesis testing, p-values were adjusted using the Benjamini-Hochberg (**BH**) procedure (Benjamini and Hochberg, 1995).

The bacterial 16S rRNA amplicon sequence reads were processed using quality profiling, adapter trimming, read filtering, and base correction with the Fastp tool (v. 0.23.1); To exclude unreliable reads, the minimum read length to be retained after trimming was 150 base pairs. To remove low bases, sliding window trimming was used with 4-base window that was trimmed if the average quality Phred score fell below 30. Tailing bases were trimmed if the Phred score fell below 20. Additionally, a strict criterion applied to reads containing "N" bases, indicating undetermined nucleotides, by setting the maximum number of "N" bases allowed in a read to 20. (Chen et al., 2018). After quality filtering, reads were dereplicated using the Dada2 (v. 1.18) R package (filtering parameters: maxN = 0, truncQ = 2, rm.phix = TRUE and maxEE = 2) (Callahan et al., 2016). Paired-end reads were merged, and chimeras were removed with the removeBimeraDenovo function. Amplicon sequence variants (**ASV**) were constructed, and taxonomy was assigned at the genus level with the database SILVA v138.1 (Quast et al., 2013). Contaminant ASVs were filtered with the decontam R package based on ASV frequencies in negative controls (Davis et al., 2018). Non-bacterial ASVs, ASVs unassigned at the phylum level, and ASVs with total relative abundance $< 1e^{-5}$ were removed from the final dataset.

To assess temporal turnover of the probiotic and essential oils on the broiler chicken excreta microbiota, statistical comparisons were performed for alpha diversity metrics (within-sample evenness or richness), beta diversity (between-sample diversity or community structure), and differential relative abundance of bacterial genera across treatments. Alpha diversity was calculated as the Shannon (considers evenness and richness) and Observed ASVs indexes. Kruskal–Wallis test was used to compare alpha diversity across treatment groups taking into account the temporal dynamics and interactions between time and treatment as independent variables. Dunn's test was performed for multiple comparisons and P-values were adjusted by using the BH method. A subset of 3 time points including d 1 (the start of the experiment), d 10 (when the diet was changed from starter to grower), and d 21 (the end of the experiment) was used to evaluate the impact of age on alpha diversity and beta diversity. Bacterial community data were transformed to center log-ratio (**CLR**) and visualized in a principal coordinates analysis (**PCA**) at the genus level with the microViz package (Barnett et al., 2021). Beta diversity differences were analyzed with permutational multivariate

ANOVA (**PERMANOVA**), with time, treatment, and their interaction using the Adonis test with 999 permutations on Aitchison distances. Pairwise comparisons were calculated using the vegan package (Oksanen et al., 2022). Differential relative abundance (determines if the fractional abundance of a taxa differs between a pairwise comparison) was performed between each treatment compared to the basal diet using linear models on log2-transformed total sum scaled data in the microViz R package. Differences at $P \leq 0.05$ were deemed to be significant. Data visualizations were performed with the microViz, ggplot2 R packages, and annotated using Adobe Illustrator (Wickham, 2009; Barnett et al., 2021).

RESULTS

Growth Performance

The descriptive growth performance results are depicted in Table 3. Uniformity in BW was numerically lower in the Probiotic group in the starter period compared to the other treatments, with values ranging from 76.25% (Probiotic), to 86.25% (Essential Oils). At 21d, BW uniformity was numerically higher in the Probiotic group although no statistically significant differences were observed. As shown in Table 4, birds fed Probiotic had a trending increase in BW compared to those fed Antibiotic in the starter phase ($P = 0.09$) and total period ($P = 0.07$). Across all experimental period, broilers fed the Probiotic improved FCR relative to those fed the Antibiotic (starter: $P = 0.03$; grower: $P = 0.05$; total period: $P = 0.01$). No differences were observed between essential oils and antibiotic or between basal diet and probiotic, essential oils and antibiotic.

Sequencing Results

Before sequence read preprocessing, the 723 sequenced samples resulted in 61,979,694 reads (average of 85,725.7 reads per sample). After read preprocessing and amplicon analysis through the Dada2 pipeline, 14,760,377 reads (average of 20,415.5 reads per sample) remained, encompassing a total of 5,367 taxa and 79 genera in the final dataset.

Taxonomic Classification

The most abundant phyla observed among all treatments and across all times were Firmicutes (88.69 ± 0.19%) and Proteobacteria (9.50 ± 0.20%) followed by Actinobacteriota (1.73 ± 0.034%) and Bacteroidetes (0.063 ± 0.0028%) (Figure 1). Birds fed Essential Oils had the largest abundance relative to Firmicutes (89.03 ± 0.16%) followed by Probiotic (88.69 ± 0.19%), Antibiotic (87.48 ± 0.19%) and basal diet (86.32 ± 0.18%). Moreover, the Antibiotic group showed the most relatively abundant Proteobacteria (10.25 ± 0.20%), followed by Probiotic (9.51 ± 0.20%), basal diet (9.24 ± 0.19%) and essential oil blend (7.98 ± 0.16%). We observed a time-dependent diet-independent development of the broiler microbiome (Figure 2): on 1d and 2d, the most relatively abundant phyla were Proteobacteria representing 65.78 ± 0.14% and 53.78 ± 0.15%, respectively. Proteobacteria decreased over time (d 5 [5.63 ± 0.044%], 10 [4.21 ± 0.097%] and 21 [0.86 ± 0.016%]), and the Firmicutes were the most dominant on 5d (94.03 ± 0.04%), 10d (93.04 ± 0.10%) and 21d (90.64 ± 0.11%). Supplementary data are available at https://github.com/gandalab/PoultryMicrobiome.

Across the entire dataset, Lactobacillaceae (31.10 ± 0.16%), Lachnospiraceae, (21.43 ± 0.16%), Enterococcaceae (17.19 ± 0.20%) and Ruminococcaceae (10.01 ± 0.12%) were the most dominant families. By treatment, probiotic, essential oils and antibiotic fed birds had similar relative abundances across families, however, Enterobacteriaceae (9.53 ± 0.18) was slightly more abundant relative to Ruminococcaceae (9.14 ± 0.11) in the excreta microbiota of chickens fed the Basal Diet (Figure 1).

On average, 19.39 ± 0.10% of all reads were not classified at genus level. The most relatively abundant genera independent of diet on d 1 were *Escherichia-Shigella* (58.54 ± 0.19%), followed by *Enterococcus* (16.04 ± 0.13%) and *Streptococcus* (11.31 ± 0.10%). Whereas on

Table 3. Descriptive statistics of broilers performance with different feed additives.

	Treatment[2]							
	Basal diet		Probiotic		Essential oils		Antibiotic	
Item[1]	Mean	SE	Mean	SE	Mean	SE	Mean	SE
BW (g)								
Starter	132.66	4.67	142.56	2.21	138.12	3.09	134.4	2.71
Grower	607.61	14.45	641.23	11.13	637.41	7.72	634.49	12.44
Total	740.27	18.72	783.8	11.51	775.54	10.18	768.89	14.18
FCR (g/g)								
Starter	1.41	0.05	1.29	0.02	1.31	0.01	1.31	0.02
Grower	1.42	0.02	1.38	0.007	1.40	0.02	1.41	0.01
Total	1.41	0.01	1.36	0.007	1.38	0.01	1.38	0.01
Uniformity (%)								
Starter	81.25		76.25		86.25		83.75	
Grower	55.12		67.5		53.16		53.84	
Total	54.54		68.75		65.82		67.94	

[1]BW: live weight; FCR: feed conversion ratio; Starter: starter phase: 1 to 10d; Grower: grower phase: 11 to 21d; Total: total period: 1 to 21d.
[2]SE: standard error.

Table 4. Effect of dietary supplementation on broiler performance.

	Contrasts[2]								
	BD vs. ATC			PB vs. ATB			EO vs. ATB		
Item[1]	Estimate	SE	*P*	Estimate	SE	*P*	Estimate	SE	*P*
BW(g)									
Starter	−10.14	11.44	0.38	−9.89	4.67	0.09	−4.44	4.67	0.38
Grower	17.2	40.53	0.82	−33.62	16.55	0.1	−3.82	16.55	0.82
Total	7.06	48.62	0.89	−43.52	19.85	0.07	−8.25	19.85	0.89
FCR(g/g)									
Starter	−0.09	0.1	0.55	0.11	0.04	0.03[3]	0.02	0.04	0.61
Grower	0.004	0.05	0.94	0.04	0.02	0.05[3]	0.02	0.02	0.33
Total	−0.002	0.03	0.95	0.04	0.01	0.01[3]	0.02	0.01	0.22

[1]BW: live weight; FCR: feed conversion ratio; Starter: starter phase: 1 to 10d; Grower = grower phase: 11d - 21d; Total = total period: 1 to 21d.
[2]Contrasts: BD vs. ATC = basal diet vs. all treatments combined; PB vs. ATB = probiotic vs. antibiotic; EO vs. ATB = essential oils vs. antibiotic; SE = standard error; *P* = *p*-values. The *p*-values were adjusted using the Benjamini-Hochberg (**BH**) procedure.
[3]Asterisk symbol represents statistical significance ($p \leq 0.05$).

d 10, *Escherichia-Shigella* (4.04 ± 0.09%), and *Streptococcus* (0.89 % ± 0.02) decreased, with *Enterococcus* (27.54 ± 0.19%) being the most relative abundant, followed by *Lactobacillus* (15.48 ± 0.08%). On d 21, *Lactobacillus* (17.81 ± 0.1%), *and Faecalibacterium* (14.68 ± 0.09) were the dominant genera (data available in the supplementary data and at https://github.com/gandalab/PoultryMicrobiome).

Alpha Diversity Analysis

No differences were seen in alpha diversity for Shannon index ($P = 0.78$) or Observed ASVs ($P = 0.85$) as shown in Figures 3A−3B. Across all treatments, alpha Shannon diversity ($P < 0.001$) and Observed ASVs ($P < 0.001$) showed a significant increase over the first 4 d ($P < 0.001$) (Figures 3C− 3D), followed by fluctuations and an overall decrease over the experimental period as shown in Figure 3E. Of the included fixed effects of treatment, age on Shannon and Observed ASVs ($P < 0.001$), and the interaction had a significant impact ($P < 0.001$) on the diversity of broilers' microbiomes.

A subset analysis was performed to compare the 3 time points representing the start of the experiment (1d), change of diet (10d), and end of the experiment (21d) − Figures 3F−3G. The interaction between age and treatment diet showed statistical differences for Shannon diversity ($P < 0.01$) and Observed ASVs ($P < 0.01$). Moreover, age was a significant factor for alpha diversity (Shannon diversity and Observed ASVs) ($P < 0.01$). Specifically, diversity increased from 1d to 10d ($P < 0.01$) and decreased from 10d to 21d ($P < 0.01$).

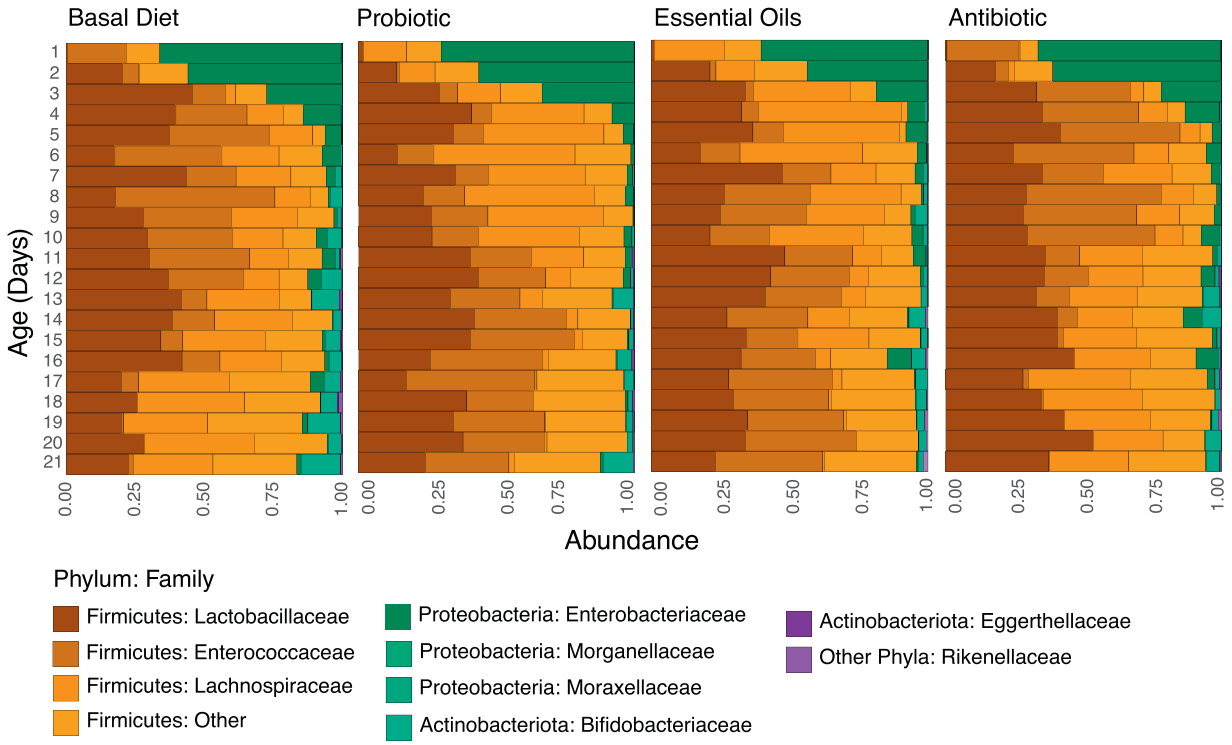

Figure 1. Relative abundance of the most abundant taxa among treatment groups (basal diet, probiotic, essential oils, and antibiotic) over the experimental period at the phylum: family level. Each color represents one phylum: family group, numbers in the y axis represent day of study and x axis the relative abundance.

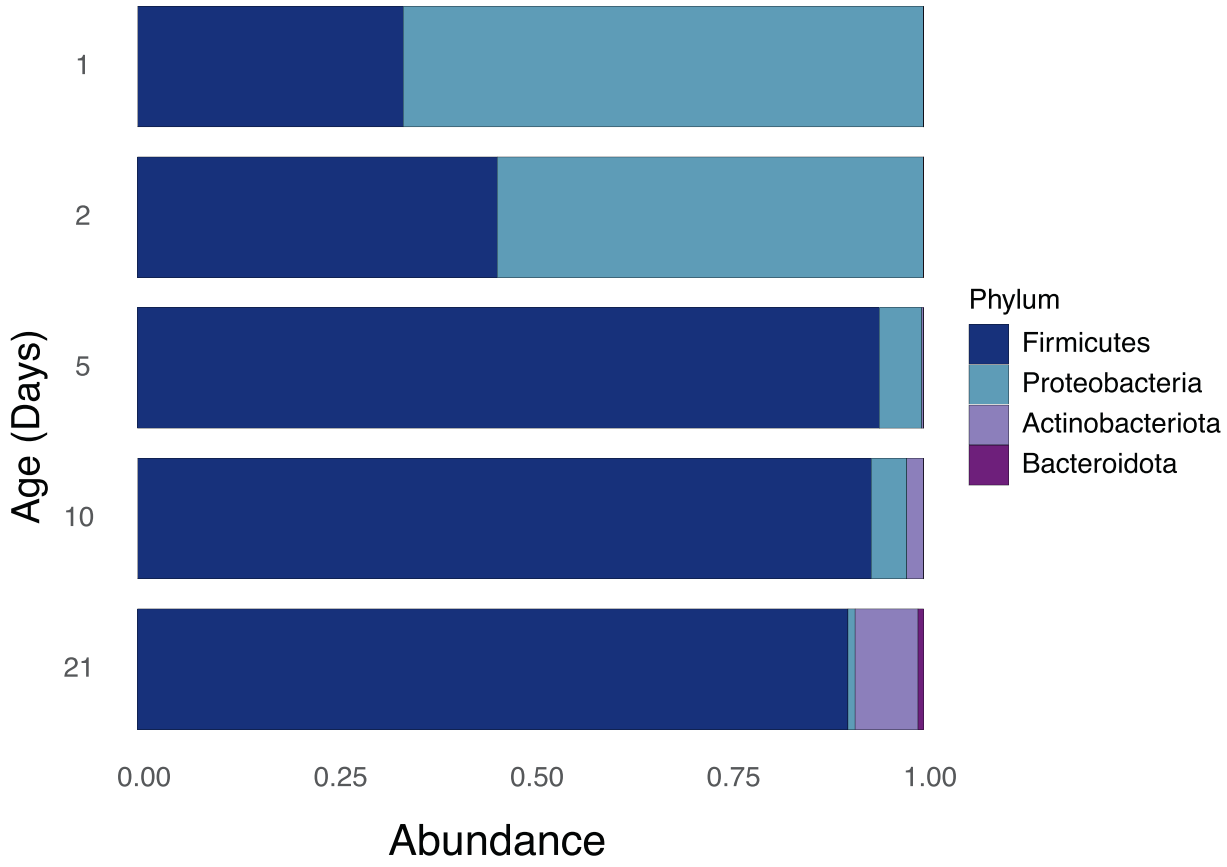

Figure 2. Relative abundance in 5 different ages (1, 2, 5, 10, and 21d) at the phylum level. Each color represents one phylum, numbers in the y axis represent day of study and x axis the relative abundance.

Likewise, when comparing d 1 to d 21, a significant fluctuation (increased from 1d to 10d and decreased from 10d to 21d) in Shannon diversity and Observed ASVs was observed ($P < 0.01$) - Figures 3F−3G).

Beta Diversity Analysis

Differences in bacterial community beta diversity were significant for the factors age ($P < 0.01$) and treatment ($P < 0.01$), but not for their interaction ($P = 0.61$). Pairwise comparison showed differences between the Basal Diet and Antibiotic ($P < 0.01$) and between the basal diet and the probiotic ($P = 0.03$).

When a subset analysis was performed to compare the 3-time points 1, 10, and 21, a clear clustering by age was observed with a statistically significant difference ($P < 0.01$) - Figure 4A which was independent of diet. Pairwise comparisons showed differences between 1d and 10 d ($P < 0.01$), 10d and 21d ($P < 0.01$) and, 1d and 21 ($P < 0.01$). The PCA (Figure 4B), displays the top 5 bacterial taxa loadings (taxa that contributes to each PCA axis) across the entire dataset (all time points included − 1−21d). The PCA suggested that *Escherichia-Shigella* were associated with the first days of the experimental period. As the birds progressed in the study, greater relative abundances of *Enterococcus* were associated with that period. Finally, the relative abundances of *Blautia* and *Fecalibacterium* progressively increased until the end of the experimental period.

Differential Relative Abundance

Across all time points, supplementing chicken diets with Probiotic or Antibiotic significantly changed the relative abundance of specific bacterial genera compared to the basal diet, while there were no taxa affected by essential oils compared to the basal diet (Figure 5). *Bifidobacterium* was relatively less abundant in Probiotic supplemented birds compared to the basal diet (log2 fold change −1.44, $P = 0.03$). *Intestinimonas* was significantly more relatively abundant in the antibiotic supplemented birds (log2 fold change 0.560, $P < 0.01$) than in the basal diet while *Bifidobacterium* (log2 fold change −1.580, $P < 0.01$) and *Bacilli Ligilactobacillus* (log2 fold change −1.600, $P < 0.01$) were significantly less relatively abundant in the antibiotic supplemented birds when compared to the basal diet.

DISCUSSION

Increasing concerns about intensive AGP use in the poultry industry has resulted in an urgent need for alternative products to promote animal growth performance and enhance intestinal health (Dibner and Richards, 2005). Probiotics and essential oil supplementation in broiler diets are popular options proposed to improve

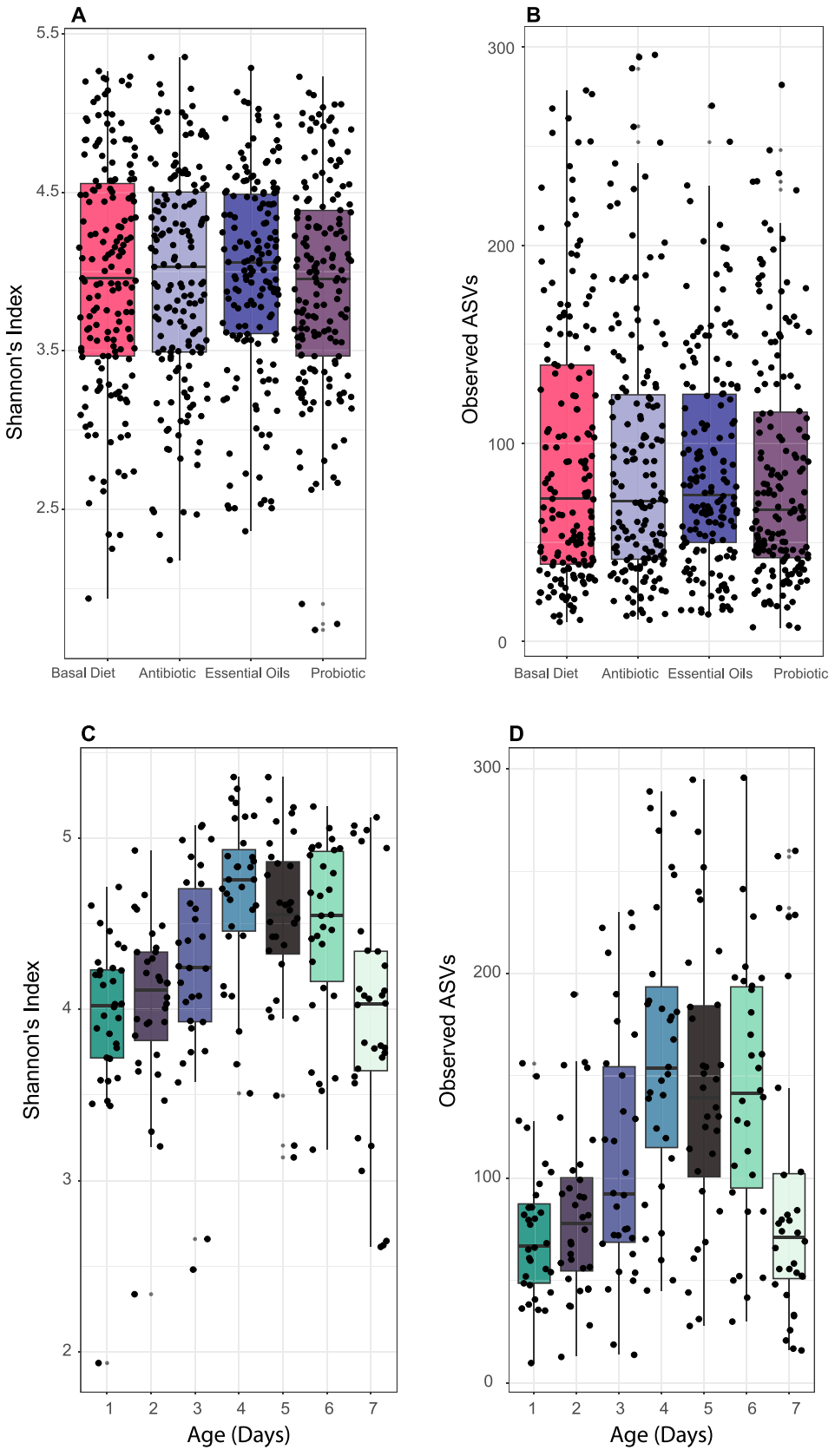

Figure 3. Alpha diversity comparisons. Alpha diversity calculated as the Shannon and Observed ASVs indexes and treatment groups were compared with Kruskal–Walli's test. A subset of 3 d including d 1 (the start of the experiment), d 10 (when the diet was changed from starter to grower), and d 21 (the end of the experiment) was done to evaluate the impact of the diet on alpha diversity. (A–B) Boxplot of alpha diversity (Shannon index – A and Observed ASVs – B) of the basal diet, Antibiotic, Essential Oils, and Probiotic treatments including all time points. Colors represent treatments. (C–D) Boxplots of alpha diversity (Shannon index – C and Observed ASVs – D) of the first week (1–7d) of broilers life. Colors represents the age in d. (E) Line plot of alpha diversity (Shannon index), mean and standard errors are shown, and lines represent the hypothetical variation of alpha diversity over the experimental period. Time was the only significant factor. Colors represent the treatments. (F–G) Boxplots of alpha diversity (Shannon index – F and Observed ASVs – G) of 3-time points including 1, 10, and 21d. Colors represents the age in days. Each boxplot indicates the interquartile range (**IQR**) with the median represented by the horizontal line within the box, while whiskers extend to display the full range of the data excluding outliers, which are depicted as individual points.

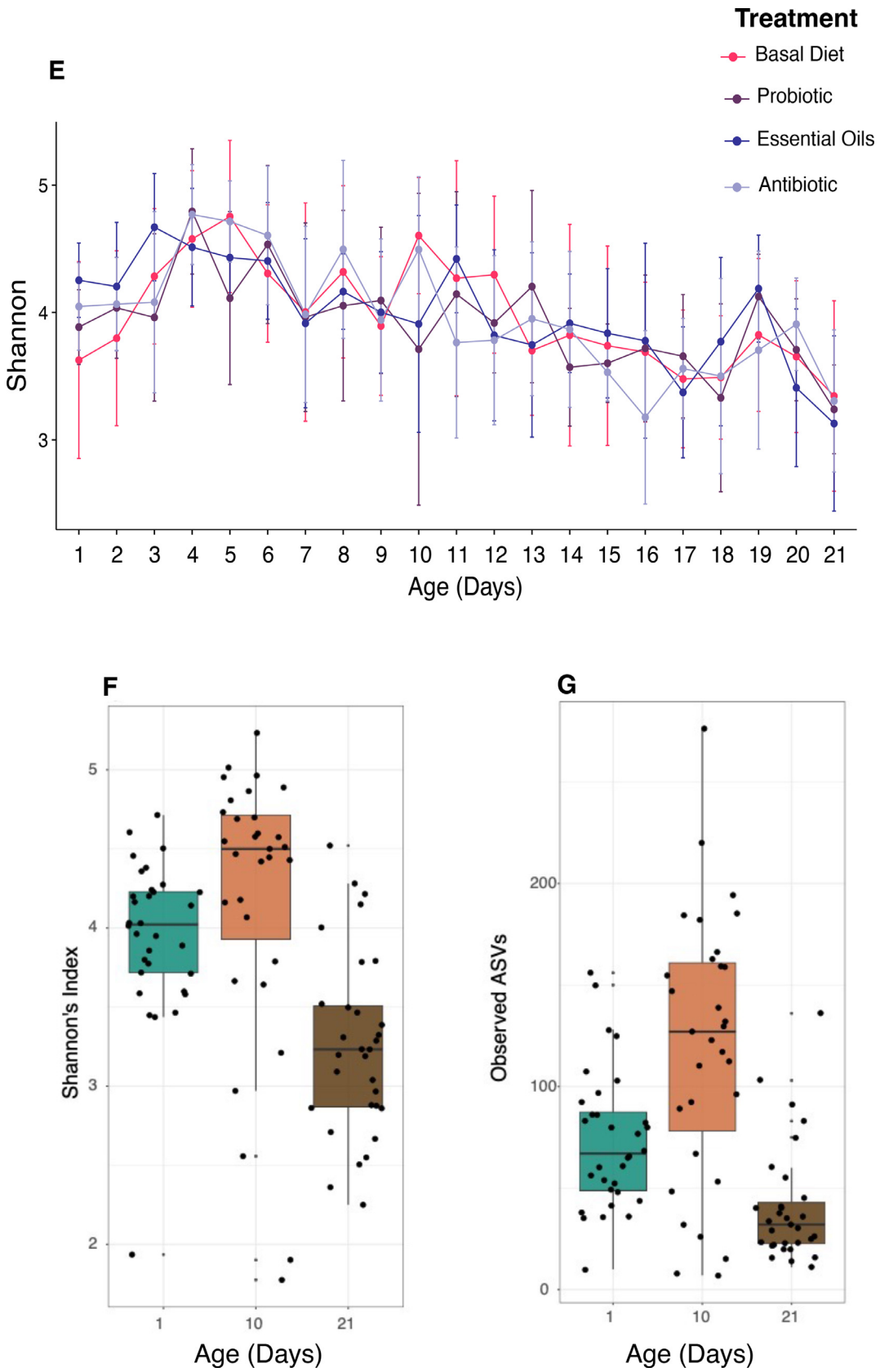

Figure 3 Continued.

bird health (Walker, 2008). Several reports demonstrate that supplementing feed with either probiotics containing *Bacillus* species, or mixtures of essential oils can improve body weight and feed efficiency, as well as alter the microbiota composition in broiler chickens (Walker, 2008; Mountzouris et al., 2010; Bai et al., 2013; Hernandez-Patlan et al., 2019; Zhang et al., 2021; Ruan et al., 2021; Kim et al., 2023). However, the lack of scientific data on excreta microbiome time-series studies on broilers fed diets containing different compounds available in the

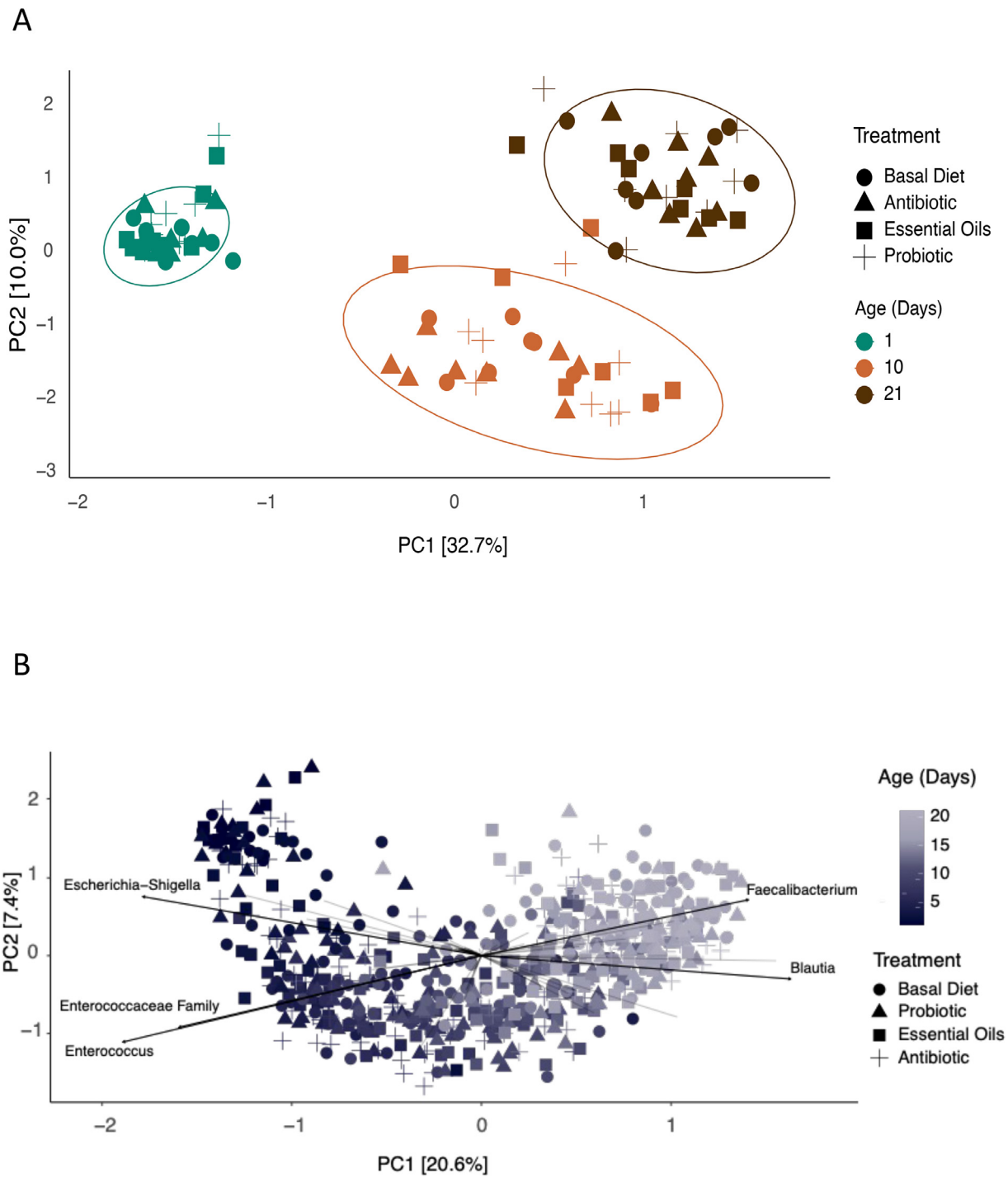

Figure 4. Beta diversity comparisons. Beta diversity differences were analyzed with permutational multivariate ANOVA (**PERMANOVA**) with time, treatment, and their interaction using the Adonis test with 999 permutations on Aitchison distances. (A) Principal component analysis (**PCA**) centered log-ratio-transformed microbiome data at genus level in a subset of samples representing 3 different time points of the experiment (starter, change of diet, and the end of the experiment) 1, 10, and 21 d shown by the colors and treatments are represented by shape. (B) Principal component analysis (PCA) centered log-ratio-transformed microbiome data at the genus level showing the 5 top bacterial loadings. Treatments are represented by shape and age is represented by the color.

poultry industry hinders the application of these alternatives in practical settings. In this study, we supplemented broilers' feed with an Antibiotic, a Probiotic, or an Essential Oil blend, profiled the dynamic changes in the excreta microbiota over 21 d, and evaluated the impact of feed additives on growth performance. The Probiotic significantly improved the FCR when compared to Antibiotic. We observed significant changes in the composition of the chickens' excreta microbial beta diversity between treatments and over time and found that age is a major driver of changes in broiler chickens' excreta microbiota.

Broiler Microbiota Varies Overtime Independent of Diet

Age is a major driver in structuring the microbiome diversity and composition (Ballou et al., 2016;

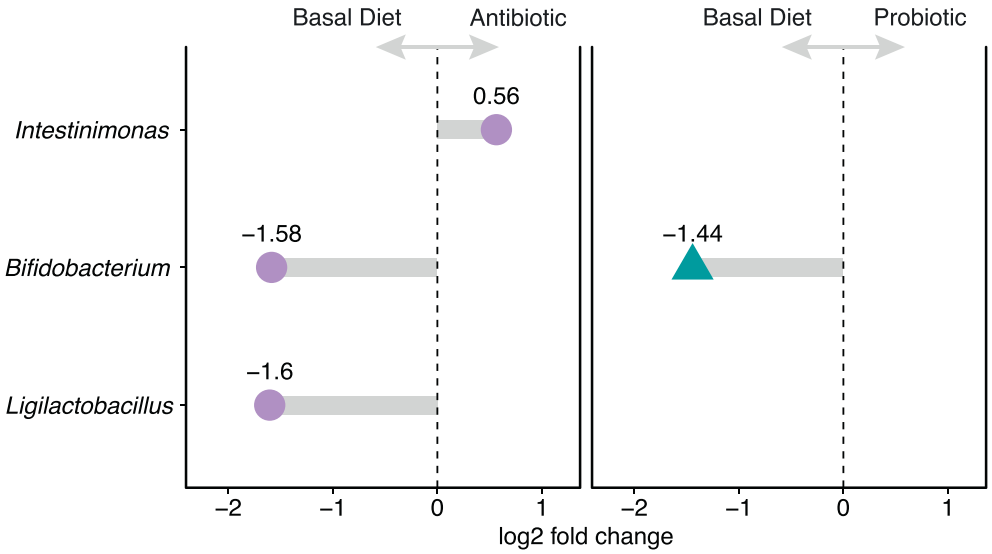

Figure 5. Differential relative abundance. Data were assessed with linear models on log2- transformed total sum scaled data in the microViz R package at the genus level. The effect size (log2 fold change) is shown for each bacterial genus with the family name. Significant differences are shown in purple circles or green triangles (positive log2 fold change is more relatively abundant in treatment groups and negative log2 fold change is less relatively abundant in treatment groups compared to basal diet).

Proszkowiec-Weglarz et al., 2022). Here, we observed fluctuations in alpha diversity throughout the 21-d experiment. The detected increase in alpha diversity in the first 4 d aligns with findings from Ballou et al. (2016) and Ijaz et al. (2018), who reported rapid development of the microbiome in the first days independent of different feeding strategies. These changes may happen due to birds being exposed to environmental microorganisms immediately after hatching and as their gastrointestinal system rapidly develops during the initial days of life (Proszkowiec-Weglarz et al., 2022). The fluctuations could possibly be explained by age-related successional changes in gut microbiome composition through the establishment of more stable bacterial taxa by around 28 d of life (Shang et al., 2018; Li et al., 2022).

Ballou et al. (2016) reported that Enterobacteriaceae prevalence was pronounced in the first days of broilers' life, while Firmicutes increased after a week. Likewise, in our experiment, we found Proteobacteria to be predominant in the birds' first days, while Firmicutes increased around d 10. *Escherichia-Shigella, Enterococcus,* and *Streptococcus* were the most predominant genera when the birds were younger while Lachnospiraceae family and *Fecalibacterium* genus were the most predominant taxa by 21 d of age, as indicated in the PCA plot. Lachnospiraceae and *Fecalibacterium* belong to the Firmicutes phylum, which has been positively associated with host intestinal health (Zhang et al., 2012; Ballou et al., 2016; Bilal et al., 2021; Mohamed et al., 2022; Li et al., 2022). Several bacteria present in this group are involved in the production of short chain fatty acids and the degradation of sugar and polysaccharides, favoring host energy regulation and mucosal integrity thereby promoting improvement in animal growth performance (Rajput et al., 2013; Ma et al., 2018; Zhang et al., 2020).

Given the importance of antimicrobial resistance concerns in the poultry industry, essential oils and probiotics represent a potentially new generation of natural AGP alternatives. (Wu et al., 2011; Jeong and Kim, 2014; Weinroth et al., 2018; Ma et al., 2018; Tellez et al., 2020; Bilal et al., 2021). Here, we showed that the use of an Antibiotic significantly changed microbial composition (beta diversity) and impacted the relative abundance of *Intestinimonas*. This genus belongs to the phylum Firmicutes and is a butyrate-producing bacterium positively correlated with optimal gut health and animal performance (Bui et al., 2016; Li et al., 2022). We, however, did not find significant differences in growth performance for the Antibiotic group in this study. Moreover, we observed that *Bifidobacterium* was more relatively abundant in the basal diet than in the antibiotic or probiotic supplemented diets. A possible explanation is that the Antibiotic (bacitracin methylene disalicylate) used in this study is known to deplete Gram-positive bacteria, which may explain the impact on *Bifidobacterium* and *Ligilactobacillus* abundance in the excreta microbiota of broilers on this treatment since *Bifidobacterium* is a common colonizer of chickens' GIT (Rychlik, 2020; Li et al., 2022). On the contrary, Song et al. (2014) observed that *Bifidobacterium* was relatively more abundant in the ileum microbiome of broilers fed Probiotics. Perhaps, these differences are due to the type of sample or probiotic used in the study as we used *Bacillus subtilis* strains while the probiotic they used a mixture of bacteria including *Bacillus licheniformis, Bacillus subtilis*, and *Lactobacillus plantarum.*

Chicken microbiota studies often rely on different samples including cecal, ileal, and excreta content. Although excreta samples are fast and noninvasive, it presents unique challenges due to the distinctive digestive physiology and diversity of microbial communities and functionality (Oakley et al., 2014; Kers et al., 2019). The rapid time of digesta through the GIT, taking approximately 2 h through the and the short length of an adult chicken colon, and the constant excreta content being eliminated limits the time for microbial fermentation (Warriss et al.,

2004). This rapid process, combined with the necessity of collecting samples from the cage tray or floor without control over their exposure time to air, introduces variability in the microbial composition observed. In this present study, we assure that time was rigorously controlled over the sample collection (~90 min per cage) to assure accuracy and consistency across samples.

Antibiotic-Free Feed Additives Improve Broilers' Performance

The development of AGP alternatives able to maintain the production and disease management demands previously met by AGP represents a major research theme applicable to the poultry industry (Hafez and Attia, 2020). Current studies build on existing evidence of the beneficial effects of a *Bacillus subtilis*-based probiotic on FCR with effects mainly observed during the starter phase (Ma et al., 2018). Here, we observed an improvement in FCR in the Probiotic group compared to the Antibiotic in all feeding phases. Likewise, in the experiments of Li et al. (2016) and Jayaraman et al. (2017) improvement on FCR were observed in birds fed probiotics. A comparative experiment using ducks supplemented with BMD and *Bacillus subtilis* (Zhang et al., 2022) displayed a reduction of FCR, and an improvement of intestinal morphology on ducks fed probiotic (*Bacillus subtilis*). Contrarily, Rivera-Pérez et al. (2021) observed no differences in FCR between the antibiotic (**BMD**) and probiotic (*Bacillus subtilis* QST713) treated groups from d 1 to 21. One potential explanation for this change in FCR may be changes in microbial composition (beta diversity) as observed in the present study, or increases on enzyme secretion profile and other postbiotic substances produced by *Bacillus subtilis*, such as propionic and butyric acid, that result in improved nutrient digestibility (Ma et al., 2018; Qiu et al., 2021).

It has been indicated that feed supplemented with probiotics containing *Bacillus spp.* or essential oils may improve BW, ADG and FCR during the whole life of broiler chickens (Bai et al., 2013; Corduk et al., 2013; Su et al., 2021; Zhang et al., 2021). However, no significant improvement in BW between the Probiotic or Essential Oils compared to the other treatment groups was observed. Likewise, Corduk et al. (2013) and Liu et al. (2023) did not show significant differences in BW of broilers when fed essential oils and probiotic. It is valid to mention that these divergences in results may depend on several distinct aspects, including type of probiotics, the dose, food storage, method of production, host traits, the length of the experiment, or biosecurity aspects of management on the farm (Zhou et al., 2010; Corduk et al., 2013; Rajput et al., 2013; Lee et al., 2015; Skoufos et al., 2016).

CONCLUSIONS

In summary, we demonstrated an improvement in FCR across growing phases in Probiotic but not Essential Oils supplementation, in all the growing phases, and changes in the excreta microbiota of broiler chickens in response to dietary supplementation. Additionally, we observed a dynamic turnover in the excreta microbiota assembly through age, thus supporting age as a major factor structuring the successional dynamics of broilers' excreta microbiota. This study provides evidence for the benefits of feed additives supplementation as an alternative to AGP used in broiler production, which may aid in promoting sustainable production practices. However, the use of these feed additives in broiler production is still in its early stages, and further studies to evaluate the health outcomes, mechanisms, and consequences for antimicrobial resistance prevalence will be necessary to better understand the role of feeding AGP alternatives on the gastrointestinal tract of broilers.

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Availability of Data and Materials: Sequencing data are available at PRJNA928060. All tools used in the analysis are publicly available as described in Methods and References. Performance and microbiome analysis codes, and complementary tables are available at GitHub (https://github.com/gandalab/PoultryMicrobiome).

DISCLOSURES

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

SUPPLEMENTARY MATERIALS

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.psj.2024.103604.

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