Research in Veterinary Science 172 (2024) 105249

Contents lists available at ScienceDirect

Research in Veterinary Science

journal homepage: www.elsevier.com/locate/rvsc

The impact of different levels of functional oil supplementation in combination with salinomycin on growth performance and intestinal microbiota of broilers undergoing Eimeria challenge: An analysis of dynamics

Thaís Bastos Stefanello [a], Kátia Maria Cardinal [b], Catiane Orso [a], Carolina Haubert Franceschi [a], Jéssica Pereira Silva [a], Micheli Bertoni Mann [c], Jeverzon Frazzon [c], Priscila Oliveira Moraes [d], Andréa Machado Leal Ribeiro [a,*]

[a] Department of Animal Science, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil
[b] Department of Animal Science, Instituto Federal Farroupilha, Alegrete, RS, Brazil
[c] Institute of Food Science and Technology, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil
[d] Department of Animal Science and Rural Development, Universidade Federal de Santa Catarina, Florianópolis, Santa Catarina, Brazil

ARTICLE INFO

Keywords:
16S rRNA sequencing
Broiler
Coccidiosis
Eimeria
Microbiome

ABSTRACT

The effect of salinomycin sodium alone and in combination with functional oils on performance and microbiota of broiler infected *Eimeria* were evaluated. 512 broilers were randomly assigned to 4 treatments (8 replicates, 16 birds/pen): a Control group (any additives); Ionophore group: salinomycin supplementation at 66 ppm (SS66); Ionophore +0.075% Functional oil (FO) group (SS66 + FO supplementation at 750 ppm); and Ionophore +0.10% FO group (SS66 + FO supplementation at 1000 ppm). At 14 days of age, birds were gavaged with 1 mL of a saline solution containing sporulated oocysts of *E. tenella*, *E. acervulina* and *E. maxima*. Performance indices were measured weekly. At 28 days, intestinal content was collected for microbiota analysis. Broilers of Control group presented the worst performance indices. Broilers of Ionophore + FO (0.075% and 0.10%) groups exhibited a higher BW at 28 days of age. The supplementation of Ionophore +0.075% FO resulted in a higher relative proportion of *Firmicutes* and a lower proportion of *Actinobacteria* in the ileum-jejunum. *Lactobacillaceae* was the dominant family in the jejunal, and ileal microbiotas of broilers fed diets supplemented with Ionophore, Ionophore +0.075% FO and Ionophore +0.10% FO. The supplementation of ionophore yielded higher numbers of *Lactobacillaceae*, *Enterobactereaceae* and *Cloritridiaceae* in the cecal. Ionophore associated with FO controlled the *Lactobacillaceae*, *Enterobactereaceae* and *Cloritridiaceae* families present in the cecum. Therefore, the combination of salinomycin with functional oil showed synergistic effect on performance and modulation of intestinal microbiota of broilers challenged with *Eimeria*.

1. Introduction

Maintaining a healthy intestinal environment is a prerequisite for the efficient performance of broilers (Murugesan et al., 2015). However, the health and well-being of birds are constantly threatened by a series of pathogens and parasitic protozoa (Soutter et al., 2020), which represent an ongoing challenge to the world's poultry industry. Coccidiosis, an infection caused by protozoa of the genus *Eimeria*, is one of the most significant parasitic diseases in the chicken industry and a major problem worldwide, resulting in huge losses (Madlala et al., 2021). Studies have observed that chickens challenged with Eimeria spp. experience an increase in the number of pathogens, particularly *Escherichia coli* and *Enterococcus* spp., a reduction in *Lactobacillus*, a probiotic that benefits digestion and gut health; and imbalance in short-chain fatty acids, which can have a negative impact on nutrient absorption and gut health (Madlala et al., 2021; Cai et al., 2022).

For many years, the use of prophylactic doses of anticoccidials was the main choice for the treatment and prevention of this intestinal

* Corresponding author at: Department of Animal Science, Universidade Federal do Rio Grande do Sul Avenida Bento Gonçalves, 7712, Porto Alegre, RS 91540-000, Brazil.
E-mail address: aribeiro@ufrgs.br (A.M.L. Ribeiro).

https://doi.org/10.1016/j.rvsc.2024.105249
Received 25 January 2024; Received in revised form 24 March 2024; Accepted 25 March 2024
Available online 30 March 2024
0034-5288/© 2024 Elsevier Ltd. All rights reserved.

disease (Chapman et al., 2010; Ahmad et al., 2024). However, due to increasing changes in legislation and pressure on the poultry industry to reduce the use of antibiotic growth promoters (AGP) and anticoccidials, a demand for new alternative strategies to improve performance and disease resistance, including ways to establish a favorable gut microbiota (Yadav and Jha, 2019) has emerged.

Among the alternative nutritional strategies, the phytogenic feed additives comprise one of the scientific areas of great interest, since these products have beneficial bioactive compounds that can protect animals against bacteria and parasite infections (Sidiropoulou et al., 2020). Functional oils, including cashew nutshell liquid (CNSL) and castor oil, are substances that provide health benefits that extend beyond their nutritional value. The commercial of CNSL and Castor oil mixture contains bioactive substances such as cardanol, cardol, ricinoleic acid, terpenes, and phenols that, synergistically, exhibit potential antioxidant and antimicrobial activity and interact with the cell membrane of microorganisms limiting their growth (Andrade et al., 2011; Osmari et al., 2015; Pires et al., 2022).

Under a coccidiosis challenge, positive outcomes on performance and microbiota modulation were observed with the use of CNSL and Castor oil mixture, as its supplementation was able to compensate the negative effect caused by coccidiosis, showing a similar effect to the ionophore monensin on the performance during the rearing (up to 42 d) period (Moraes et al., 2019b). In addition, better energy utilization, higher survival rate of animals and lower intestinal damage caused by *Eimeria* were also observed with the same functional oil blend when it was compared to the control group without any additive (Murakami et al., 2014). Castor oil mixture also provided increased microbial diversity in broilers, higher richness and evenness of microbial taxa, especially Firmicutes and Bacteroidetes; and decreased *Clostridium perfringens* and *Escherichia coli* (Pires et al., 2022). Despite the positive results obtained with the use of CNSL and Castor oil mixture or other phytogenic feed additives, the process of removing AGP and anticoccidials from the poultry production chain is a gradual and often, both the resistance from producers and the high price of phytogenic additives products, makes their single use unfeasible. Additionally, in many situations, avian coccidiosis cannot be treated or controlled using just one compound but requires a combination of products and protocols to achieve the necessary efficiency (Pajić et al., 2023).

Therefore, the present study evaluated the single effect of salinomycin sodium and the impact of its combination with functional oils (CNSL - Castor oil blend) on the performance and microbiota of broiler chickens challenged with mixed *Eimeria* species.

2. Material and methods

2.1. Ethics statement

The work described here was conducted under protocol number 36475 approved by the Ethics Committee on Animal Use from the Universidade Federal do Rio Grande do Sul, Brazil, following the legislation for the protection of animals used for scientific purposes.

2.2. Treatments, bird husbandry and experimental design

A total of 512, one-day-old male broiler chicks (Cobb 500) were obtained from a commercial hatchery and housed in a controlled-temperature room, composed of 32 pens with 16 birds per pen. Each group was housed in a 1 m^2 pen equipped with two nipple drinkers and one tubular feeder. The nutritional program consisted of four phases (Table 1): pre-starter (1 to 7 d), starter (8 to 21 d), grower (22 to 35 d) and finisher (36 to 42 d), formulated to provide the nutritional requirements recommended by the Brazilian Tables of Poultry and Swine (Rostagno et al., 2017).

The experimental design was completely randomized with four treatments groups: Control group (basal diet without additives); Ionophore group (sodium salinomycin supplementation at 66 ppm - SS66); Ionophore +0.075% FO group (SS66 + FO supplementation at 750 ppm); Ionophore +0.10% FO group (SS66 + FO supplementation at 1000 ppm). All treatments groups were challenged with coccidiosis.

Table 1
Dietary compositions and nutrient levels of broilers (as-fed basis).

	Pre-starter (1–7 days)	Starter (8–21 days)	Grower (22–35 days)	Finisher (36–42 days)
Ingredient (%)				
Maize	44.53	46.37	48.94	58.80
Soybean meal	45.85	43.29	40.07	31.87
Soybean oil	5.022	6.122	7.117	6.045
L-Lysine	0.127	0.133	0.141	0.172
DL-Methionine	0.368	0.349	0.325	0.264
L-Threonine	0.072	0.067	0.062	0.047
NaCl	0.526	0.511	0.486	0.460
Limestone	0.915	0.825	0.780	0.653
Phosphate	2.188	1.927	1.678	1.290
Bicholine	0.050	0.050	0.050	0.050
Vit Premix[1]	0.034	0.034	0.034	0.034
Min Premix[2]	0.100	0.100	0.100	0.100
Inert/Ionophore/ Functional oil	0.205	0.205	0.205	0.205
Calculated nutrition levels, %				
EM, kcal/kg	2975	3050	3150	3200
CP	24.71	23.68	22.41	19.44
Ca	1.011	0.90	0.82	0.66
Available P	0.48	0.43	0.38	0.30
Dig Lys	1.364	1.30	1.23	1.06
Dig Met	0.680	0.65	0.61	0.52
Dig Met+Cis	1.009	0.96	0.91	0.79
Dig Thr	0.900	0.86	0.81	0.70
Dig Trp	0.287	0.27	0.25	0.21
(Na + K)-Cl, mEq/ kg[3]	228.39	216.91	202.51	166.96

[1] Vitamin premix containing the following per kilogram of diet: vitamin A, 10,000 IU; vitamin D3 (cholecalciferol), 3500 IU; vitamin E (DL-α-tocopheryl acetate), 60 mg; vitamin K (menadione), 3 mg; thiamine, 3 mg; riboflavin, 6 mg; pyridoxine, 5 mg; vitamin B12 (cyanocobalamin), 0.01 mg; niacin, 45 mg; pantothenic acid (D-calcium pantothenate), 11 mg; folic acid, 1 mg; biotin, 0.15 mg; choline chloride, 500 mg; ethoxyquin (antioxidant), 150 mg.
[2] Mineral premix containing the following per kilogram of diet: Fe, 60 mg; Mn, 100 mg; Zn, 60 mg; Cu, 10 mg; I, 1 mg; Co,0.2 mg; Se, 0.15 mg.[3]Dietary electrolyte balance.

The commercial blend of functional oils (Essential, Oligo Basics Agroind. Ltda, Cascavel, Brazil) contained a mixture of cashew nut shell liquid (*Anacardium occidentale*) and castor oil (*Ricinus communis* L.), containing as active components 4% of cardol, 20% of cardanol and 9% of ricinoleic acid. Both feed additives, the oil blend and sodium salinomycin (Coxistac 12% – Phibro Animal Health Corporation, Brazil), were included by replacing an inert ingredient (kaolin) in the basal diet for all phases varying the dosage of each treatment group.

2.3. Intestinal challenge

At 14 days of age, all chickens were inoculated by oral gavage with 1 mL of a saline solution containing sporulated oocysts of *E. tenella* (1×10^4 oocysts), *E. acervulina* (20×10^4 oocysts), and *E. maxima* (8×10^4 oocysts). The oocyst inoculum was acquired from the Laboratório Biovet (Laboratório BIO-VET LTDA, São Paulo, SP, Brazil) and consisted of field strains, but had been previously multiplied in the laboratory with the intention to simulate a coccidiosis infection similar to the one occuring in commercial breeding. The *Eimeria* inoculum dosage was chosen based on previous studies (Moraes et al., 2019a; Moraes et al., 2019b; Vieira et al., 2020), and was successful in causing a drop in performance without causing high mortality.

2.4. Performance

Feed intake and BW gain for each replicate were measured weekly.

2.5. Oocyst counts in litter

At 28 days of age, representative litter samples (a mixed sample of five handfuls taken in five sections per pen) were collectedand pooled. The mean number of oocysts per gram (OPG) of litter was determined according to Long and Rowell (1975).

2.6. Sample collection, DNA extraction, PCR amplification, and sequencing

At 28 days of age, three broilers, with an average weight close to the average of the replicate, were selected from four pens for each treatment (totaling 12 samples per treatment) and euthanized for individual collection of intestinal contents. A 10 cm portion of each segment: jejunum (descending duodenal loop to the Meckel's diverticulum), ileum (diverticulum to ileocecal insertion), and caecum were removed with the intestinal contents inside and immediately stored at −20 °C until further analysis.

Total microbial genomic DNA samples were extracted using the E.Z.N.A. Stool DNA Kit (Omega Bio-Tek, Norcross, Georgia, USA) according to the manufacturer's instructions. The genomic DNA was quantified using a Qubit® 3.0 Fluorometer (Life Technologies, Carlsbad, CA), and stored at −20 °C. After DNA extraction, the samples were sent to Imunova Análises Biológicas (Curitiba-PR, Brazil) for PCR amplification.

The V4 region of bacterial 16S rRNA gene was amplified using the universal primers 515F and 806R (Caporaso et al., 2010). Amplification was carried out according to the following program: initial denaturation at 94 °C for 3 min, followed by 18 cycles of 45 s at 94 °C, 30 s at 50 °C and 60 s at 68 °C and a final cycle at 72 °C for 10 min. Sequencing was performed by Illumina MiSeq (Illumina, San Diego, CA, USA). which generates paired end reads of 460 bp.

Raw sequencing reads of this study were deposited in the National Biotechnology Information Center (NCBI) under the accession number PRJNA854667.

2.7. Bioinformatic and statistical analysis

Sequencing reads were analyzed using the QIIME (Quantitative Insights Into Microbial Ecology) platform. Sequences were classified into bacterial genera through the recognition of operational taxonomic units (OTU) based on the homology of the sequences when compared to the SILVA 128 ribosomal sequence database (Yilmaz et al., 2014).

For diversity analysis, after the samples were rarefied, alpha diversity metrics (Shannon entropy, Simpson index, ACE, Chao1, Fisher and total number of observed OTUs), beta diversity metrics (weighted and unweighted UniFrac (Lozupone et al., 2011) and Bray-curtis dissimilarity (Clarke et al., 2006) were used. The statistical comparison between the groups in the alpha diversity analysis was performed using the nonparametric Wilcoxon test, accepting as statistically significant results values lower than 0.05 ($P < 0.05$). Statistical analyzes for beta diversity were performed using perMANOVA of the Adonis function, present in the vegan library, using 10,000 permutations (Anderson and Walsh, 2013). For beta diversity comparison between two groups, the values obtained by the per MANOVA analysis were corrected by Bonferroni. All figures and statistical analyzes were performed in R version 3.6 (https://www.R-project.org/). The calculation of phylogenetic diversity was performed by the spicy library, while the alpha and beta diversity were calculated using the phyloseq, vegan and microbiome libraries. Rarefaction curves, phylogenetic tree and correlation analyzes are performed using the Microbiome Analyst tool (Dhariwal et al., 2017).

All performance data were subjected to one-way analysis of variance (ANOVA) using XLSTAT statistical software (Addinsoft, Paris, France). When significant differences were present, a Tukey's test was performed to separate means and significance accepted at $P \leq 0.05$. The oocyst count data were performed using the nonparametric Kruskal-Wallis test.

3. Results

3.1. Performance

No performance differences were seen before the challenge (d 1 to 14). However, in the week following the challenge (d 15 to 21) the Control group, which was challenged with coccidiosis and fed a non-supplemented diet, had the worst performance in terms of BW, BW gain and FI, although these variables were not different from the Ionophore group. Also, the challenged broilers fed diets supplemented with Ionophore +0.075% FO exhibited a higher BW, BW gain and FI ($P < 0.05$) than other groups (Table 2).

In the second week post-challenge (d 21 to 28), broilers supplemented with Ionophore +0.075% FO and Ionophore +0.10% FO yielded significantly higher BW, BW gain and FI than the Control group. However, FI was not different between the Control and the Ionophore group. Similarly to the previous week, the Control broilers performed worse than all the other treatments, not recovering from the intestinal challenge with coccidiosis.

From d 29 to 35 and d 35–42, broilers fed diets supplemented with Ionophore +0.075% FO and Ionophore +0.10% FO were heavier ($P < 0.05$) than broilers from the other treatments. The Control group could not compensate the loss of performance caused by the challenge, which resulted in a lower final BW. Also, broilers in the Ionophore group were lighter than broilers fed with Ionophore + FO.

During the 42 d period, a significant difference in BW gain was observed among treatment groups. The treaments fed with Ionophore +0.075% FO and Ionophore +0.10% FO exhibited a higher BW gain compared to the Control and Ionophore groups. The group treated with ionophore alone displayed a reduction of 6% and 5% in BW gain in comparison to the treatments supplemented with Ionophore +0.075% FO and Ionophore +0.10% FO, respectively.

3.2. Oocyst litter count

The oocyst counts in the litter of broilers that received diets supplemented with Ionophore alone, Ionophore +0.075% FO and Ionophore +0.10% FO were found to be lower than those of the broilers in the Control group ($P < 0.1$; Fig. 1).

3.3. Operational taxonomic

A total of 412,688 frequencies were obtained from 35 samples (3 birds per treatment and 3 individual intestinal segments) with an average of 11,791 per sample. Sample 15,609 (Control - jejunum) showed low sequencing depths and was disregarded from subsequent analyses.

3.4. Alpha diversity analysis

The alpha diversity index indicates the richness and uniformity of a sample. Statistically significant microbiota modulations ($P < 0.05$) were observed between the Control and the Ionophore groups using the Simpson Index (Fig. 2). Furthermore, a trend ($P = 0.093$) was observed between the Ionophore and Ionophore +0.075% FO groups. No significant differences were found for the other applied alpha diversity tests considering the pool of intestinal segments. Conversely, independently of the statistical significance, Shannon's, ACE, Chao1 and Fisher's graphics showed a larger distribution and diversity in Ionophore +0.075% FO group, while showing a lower, but more uniform α-diversity species for the microbiota of the Ionophore +0.10% FO group.

Table 2
Growth performance of broilers chickens fed four different treatments*.

	Control	Ionophore	Ionophore + 0.075% FO	Ionophore + 0.10% FO	SEM	*P*-value
1-7d						
BW1d	43	43	43	43	0.09	0.901
BW	132	134	139	137	1.96	0.651
FI	100	96	105	100	1.55	0.301
BWG	89	91	96	94	1.95	0.641
FCR	1.132	1.069	1.097	1.068	0.02	0.438
7-14d						
BW	354	362	373	353	5.31	0.551
FI	289	285	295	284	3.98	0.790
BWG	221	228	234	217	3.84	0.434
FCR	1.312	1.252	1.264	1.314	0.01	0.258
14-21d						
BW	620[b]	650[b]	695[a]	647[b]	9.76	**0.047**
FI	469[b]	473[b]	531[a]	497[b]	7.58	**0.007**
BWG	267c	287[bc]	322[a]	293[b]	6.27	**0.011**
FCR	1.776	1.661	1.653	1.705	0.03	0.474
22-28d						
BW	1095[c]	1196[b]	1273[a]	1270[a]	17.41	**<0.0001**
FI	754b	835a	861a	880a	17.36	**0.045**
BWG	475c	546b	578ab	624a	13.94	**0.000**
FCR	1.610	1.530	1.509	1.429	0.04	0.505
29-35d						
BW	1895[c]	1993[b]	2092[a]	2106[a]	22.27	**0.000**
FI	1222	1124	1186	1238	30.12	0.569
BWG	791	787	840	836	15.23	0.475
FCR	1.608	1.429	1.414	1.490	0.05	0.599
35-42d						
BW	2794[b]	2825[b]	2978[a]	2984[a]	28.27	**0.017**
FI	1447	1373	1446	1447	14.95	0.204
BWG	899	846	886	878	14.88	0.654
FCR	1.614	1.632	1.641	1.659	0.02	0.905
1-42d						
FI	4282	4186	4424	4446	49.642	0.206
BWG	2742[b]	2786[b]	2955[a]	2941[a]	29.255	**0.010**
FCR	1.546	1.489	1.478	1.498	0.013	0.261

Each value represents the mean of eight replicates. *Control group (no additives in coccidiosis-challenged birds); Ionophore group (sodium salinomycin -SS66- in coccidiosis-challenged birds); Ionophore +0.075% FO group (SS66 + FO at 750 ppm in coccidiosis-challenged birds); Ionophore +0.10% FO group (SS66 + FO at 1000 ppm in coccidiosis-challenged birds).
a,b Different letters in same row indicate significant differences between the respective means ($P < 0.05$; Tukey test).

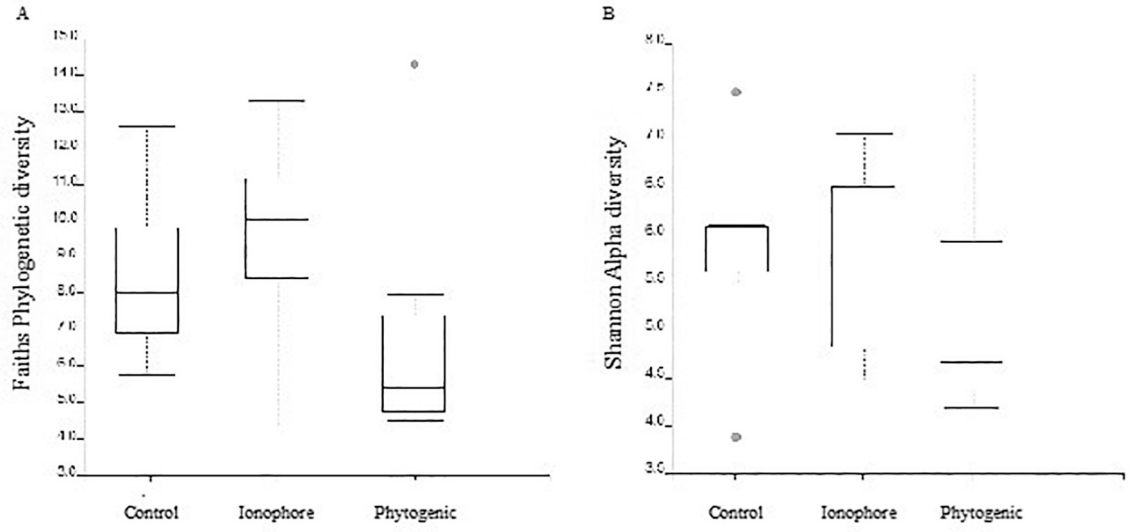

Fig. 1. Number of *Eimeria* spp. OPG in litter at d 28. Each value represents the mean x 10^3 / g per gram of litter of eight replicates. Control group (no additives in coccidiosis-challenged birds); Ionophore group (sodium salinomycin -SS66- in coccidiosis-challenged birds); Ionophore +0.075% FO group (SS66 + FO at 750 ppm in coccidiosis-challenged birds); Ionophore +0.10% FO group (SS66 + FO at 1000 ppm in coccidiosis-challenged birds). [a,b] Different letters indicate significant differences between the respective means ($P < 0.1$; Kruskal-Wallis test).

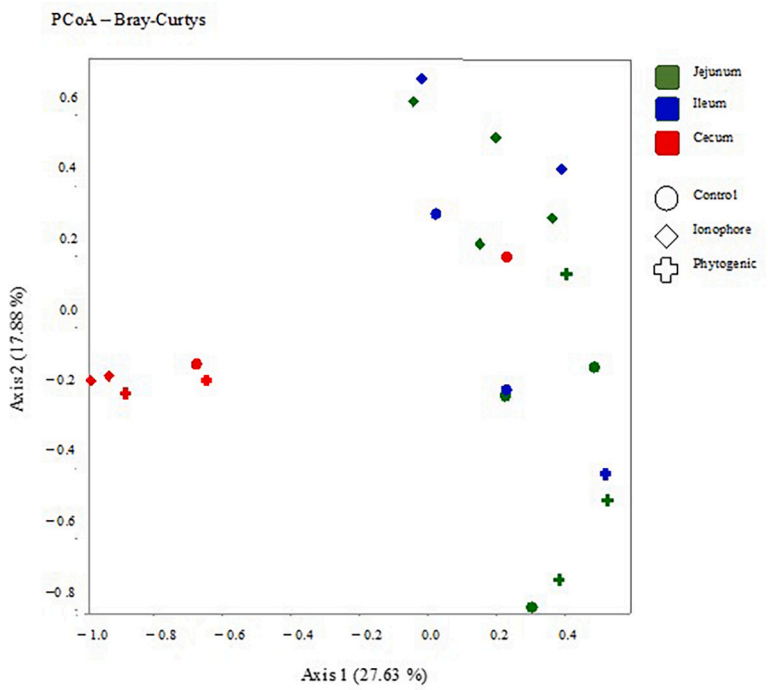

Fig. 2. Alpha diversity, estimated by the parameters Shannon entropy (A), Simpson index (B), ACE (C), Chao1 (D), Fisher (E) and total number of observed OTUs (F). Statistical tests were performed using the Wilcoxon test. Differences that presented a *p* value lower than 0.05 were considered statistically significant. Control group (no additives in coccidiosis-challenged birds); Ionophore group (sodium salinomycin -SS66- in coccidiosis-challenged birds); Ionophore +0.075% FO group (SS66 + FO at 750 ppm in coccidiosis-challenged birds); Ionophore +0.10% FO group (SS66 + FO at 1000 ppm in coccidiosis-challenged birds).

In addition, the graphic of the total number of observed OTUs showed a higher distribution of the number of different species in the Ionophore +0.075% FO group, which can be translated into a greater richness of an ecosystem.

3.5. Beta diversity analysis

Analysis of similarity (PERMANOVA) were performed on the weighted and unweighted Unifrac distances and Bray-Curtis metric obtained from the beta diversity workflow in QIIME2 (Figs. 3, 4). The PERMANOVA test based on treatment groups resulted in a *p*-value of 0.791 and a test statistic of 0.621 for weighted, a p-value of 0.561 and test statistic of 0.966 for unweighted Unifrac analysis and of p-value 0.194 and a test statistic of 1.248 for Bray-Curtis. These results indicate that no significant differences in dissimilarity were found based on treatment groups, and that the treatments had a similar microbial composition, also indicating that grouping based on treatment was weak (i.e., the differences could be explained by randomness). Despite the lack of homogeneity in the dispersion, the PcoA graphics showed that the microbial populations of the broilers challenged with coccidiosis

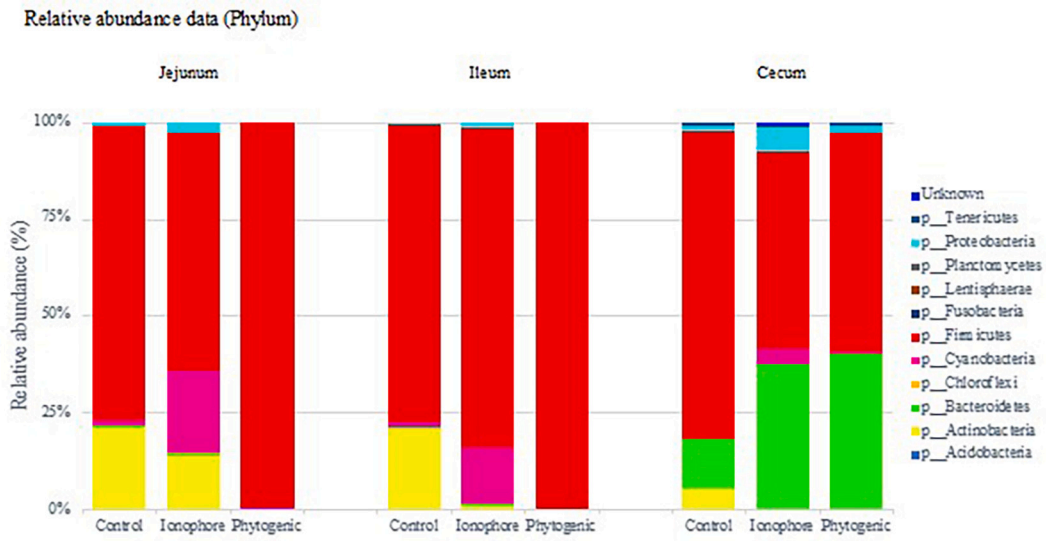

Fig. 3. Beta diversity, estimated by Weighted Unifrac dissimilarity (A) (*p*-value = 0.791) and the Unweighted Unifrac dissimilarity (B) (p-value = 0.561). Colored ellipses were automatically added using the Rggforce library. Control group (no additives in coccidiosis-challenged birds); Ionophore group (sodium salinomycin -SS66- in coccidiosis-challenged birds); Ionophore +0.075% FO group (SS66 + FO at 750 ppm in coccidiosis-challenged birds); Ionophore +0.10% FO group (SS66 + FO at 1000 ppm in coccidiosis-challenged birds).

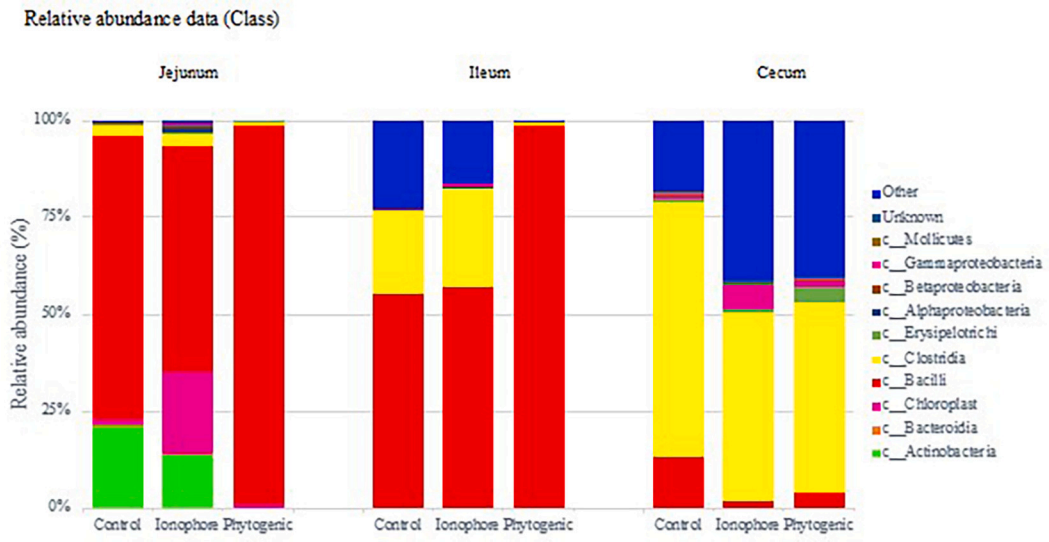

Fig. 4. Beta diversity, estimated by Bray-Curtis dissimilarity (p-value = 0.194). Colored ellipses were automatically added using the Rggforce library. Control group (no additives in coccidiosis-challenged birds); Ionophore group (sodium salinomycin -SS66- in coccidiosis-challenged birds); Ionophore +0.075% FO group (SS66 + FO at 750 ppm in coccidiosis-challenged birds); Ionophore +0.10% FO group (SS66 + FO at 1000 ppm in coccidiosis-challenged birds).

that received the additives exhibited a closer microbial composition.

In the weighted Unifrac PcoA analysis, the treatment groups were well separated with 38.9% and 26.1% variation by the principal components PcoA1 and PcoA2, respectively, whereas for the unweighted Unifrac were well separated with 13.8% and 5.9% variation by the principal components PcoA1 and PcoA2, respectively. In PcoA based on Bray–Curtis diversity metric, the treatment groups were well separated with 32.3% and 15.1% variation by the principal components PcoA1 and PcoA2, respectively.

3.6. Relative abundance

To elucidate the effect of feed additives associated with *Eimeria* infection on the composition of the intestinal microbiota (jejunum, ileum, and cecum), the bacteria at the phylum and family levels to characterize the dynamics of microbial taxonomic distribution were analyzed.

All sequences were classified into nine phyla, although four phyla were more common (>1%): *Firmicutes*, *Actinobacteria*, *Bacteroidetes*, and *Proteobacteria* (Fig. 5).

Jejunum And Ileum: Broilers fed diets supplemented with Ionophore +0.075% FO showed a higher proportion of *Firmicutes* and lower abundance of *Actinobacteria*, compared to the other groups. *Actinobacteria* was more abundant in the jejunum and ileum of the Control group. *Proteobacteria* was more abundant in the jejunum of the Control group.

Lactobacillaceae was the dominant family in the jejunal, and ileal microbiotas of the broilers fed diets supplemented with Ionophore, Ionophore +0.075% FO and Ionophore +0.10% FO (Fig. 6). Control group birds showed a lower proportion of *Lactobacillaceae* and a higher abundance of *Corynebacteriaceae*.

Cecum: Broilers fed diets supplemented with Ionophore showed a

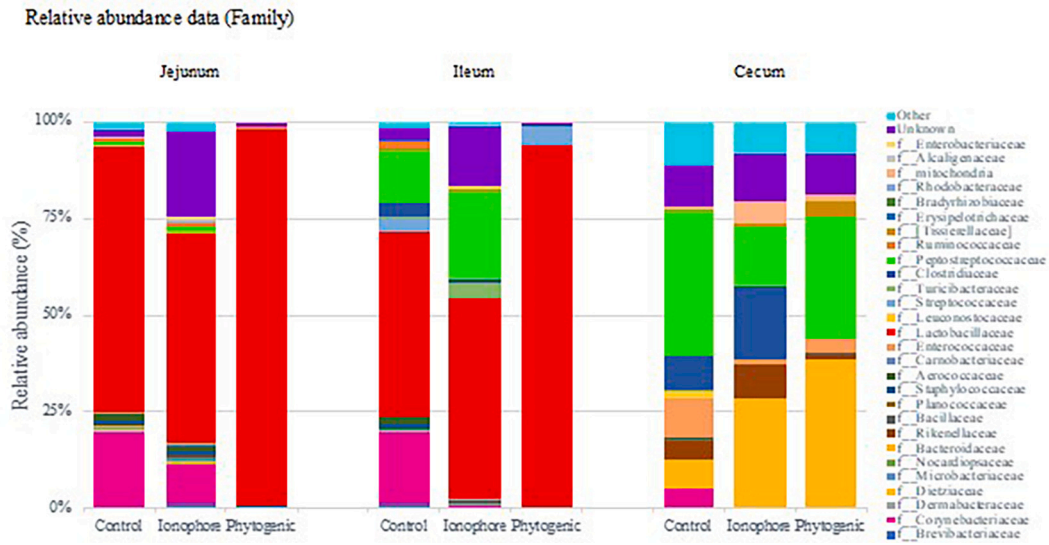

Fig. 5. Bar graph of the relative abundances of the gut microbiota at the phylum level. The relative abundances of the gut bacteria presented here were calculated by averaging the data obtained from the replicates within each group. Minor bacterial genera and unassigned values were included as "others". Control group (no additives in coccidiosis-challenged birds); Ionophore group (sodium salinomycin -SS66- in coccidiosis-challenged birds); Ionophore +0.075% FO group (SS66 + FO at 750 ppm in coccidiosis-challenged birds); Ionophore +0.10% FO group (SS66 + FO at 1000 ppm in coccidiosis-challenged birds).

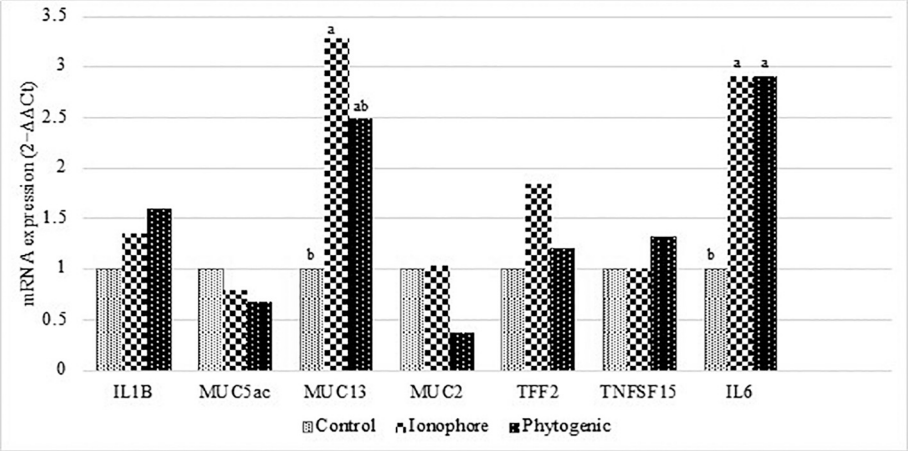

Fig. 6. Bar graph of the relative abundances of the gut microbiota at the family level. The relative abundances of the gut bacteria presented here were calculated by averaging the data obtained from the replicates within each group. Minor bacterial genera and unassigned values were included as "others". Control group (no additives in coccidiosis-challenged birds); Ionophore group (sodium salinomycin -SS66- in coccidiosis-challenged birds); Ionophore +0.075% FO group (SS66 + FO at 750 ppm in coccidiosis-challenged birds); Ionophore +0.10% FO group (SS66 + FO at 1000 ppm in coccidiosis-challenged birds).

higher relative abundance of Firmicutes, while the Bacteroidetes were more abundant for the Ionophore +0.075% FO group. In addition, broilers challenged and supplemented with additives showed a cecal microbiota with a higher percentage of *Proteobacteria* when compared to the Control group, except the ionophore +0.075% FO group.

Ruminococcaceae was the dominant family in the cecal microbiotas of all groups, followed by *Lachnospiraceae*. Ionophore group birds exhibited a greater abundance of *Lactobacillaceae* compared to the other groups (14% vs. 2%, 6% and 1% for Control, Ionophore +0.075% FO, and Ionophore +0.10% FO groups, respectively).

The main results regarding family, genus and species can be observed in the table on supplementary material (S1). The main observations within the families were an increase in *Lactobacillaceae* and *Ruminococcaceae* in all intestinal portions with Ionophore and Ionophore +0.075% FO treatments, and reduction with Ionophore +0.10% FO. Reduction of *Enterobacteriaceae* and *Erysipelotrichaceae* in all intestinal portions with Ionophore, Ionophore +0.075% FO and Ionophore +0.10% FO treatments. The Ionophore +0.075% FO treatment showed the best results in terms of *Lactobacillaceae* and *Ruminococcaceae* abundance and lower abundance of *Enterobacteriaceae* and *Erysipelotrichaceae*. Observin the genus, the Ionophore +0.075% FO treatment showed the greatest increase in *Lactobacillus* in all parts of the intestine. The Ionophore +0.10% FO treatment showed an increase in gut health-related genera in the cecum, such as *Ruminococcus*, *Bacteroides*, *Blautia*, and *Faccalibacterium*. The Control treatment had the highest abundance of *Corynebacterium* in all parts of the intestine.

4. Discussion

Previous studies have demonstrated that the inclusion of CNSL and castor oil in poultry diets improves growth performance, metabolizable energy, and gut morphometry (Bess et al., 2012; Murakami et al., 2014). Additionally, under an intestinal challenge with coccidiosis, the functional oils have been shown to enhance energy utilization, increase survival rates, and reduce the intestinal lesions caused by Eimeria in supplemented broilers (Murakami et al., 2014) In our laboratory, we observed encouraging positive outcomes on performance and microbiota in broilers challenged with coccidiosis when fed a diet supplemented with a blend of CNSL and castor oil. When compared to the widely used ionophore anticoccidial, sodium monensin (100 ppm), the functional oil blend (1500 ppm) had a similar effect on rearing performance during the period up to 42 days of age and was able to offset the negative effects caused by coccidiosis (Moraes et al., 2019b). Furthermore, the CNSL and castor oil blend was found to be an effective option for modulating the intestinal microbiota, displaying antimicrobial action against gram-positive bacteria, particularly *Clostridium perfringens* and *Staphylococcus aureus*, in a coccidiosis challenge. The pursuit of alternative options to synthetic anticoccidial drugs is a crucial area of research for scientist, producers, and the poultry industry (Qaid et al., 2021), particularly in light of evolving legislation and growing pressure to reduce the use of AGP and anticoccidials. However, it should be acknowledged that the transition away from these compounds in the poultry industry is a gradual process and, in many cases, the most practical approach to facilitating these changes is through a transitional phase in which AGP and/or anticoccidials are combined with an alternative additive. Furthermore, in a previous study it was found that the addition of 1.0 kg/t of CNSL potentiated the beneficial effect of salinomycin on the performance of broiler during coccidiosis challenge yielding satisfactory results in substituting virginiamycin (Moraes et al., 2023).

Our laboratory has been focused on evaluating a phytogenic solution with anticoccidial properties that could potentially be used in poultry diets, specifically the blend of CNSL and Castor oil. Cashew nut shell liquid contains cardanol and cardol, which possess antioxidant properties and act as natural ionophores, disrupting the lipid layers of gram positive bacterial cell wall (Sosa et al., 2020; Paramashivappa et al., 2001). These compounds belong to the phenols group and possess high antibacterial activity due to their high solubility in biological membranes, making them notorious bactericides (Nazzaro et al., 2013). Conversely, castor oil, which is rich in ricinoleic acid, can interact with the microbial membrane and inhibit microbial growth by dissolving chitin, a component of cell membranes.

4.1. Performance and oocysts

In the present study, at the end of d 42, the broilers in the Control group did not have sufficient time to recover from the negative effects of coccidiosis, as evidenced by their low final BW. Additionally, the group of broilers supplemented with only an ionophore also exhibited a low final BW, statistically similar to the group without additive. However, when an ionophore was combined with functional oils at both dosages (0.075% and 0.10%), a significant increase in final weight at the end of 42 days of age was observed. These results suggest a synergistic effect between the ionophore sodium salinomycin and the functional oils on growth performance under the conditions of this study.

Although Moraes et al. (2019a) reported that the use of functional

oils decreased the excretion of oocysts, similar to the effect of the ionophore monensin, in this study, no synergistic effect was observed in terms of oocyst excretion. All treatments with ionophores resulted in lower oocyst excretion compared to the control group, but there was no significant difference between the treatments with ionophores. It is important to highlight that in this study we observed that CNSL acted as an immune system modulator, directing the host's immune response against the pathogen. Unlike monensin, which acted by causing parasite death.

Other studies have also investigated the synergistic effects of combining natural additives and anticoccidial drugs. For example, Malik et al. (2016) found that the combination of berberine and the synthetic anticoccidial amprolium resulted in a synergistic increase in effectiveness against coccidian oocysts as evidenced by a reduction in the number of oocysts shed in the feces and an improvement in weight gain and feed conversion. This synergistic effect may be explained by the different mechanisms of action of these two compounds. In this study, it was observed that prior to the challenge, the inclusion of an ionophore alone or in combination with functional oil did not have a significant impact on performance. However, following the intestinal challenge, it is hypothesized that the inclusion of these additives from the first day of age helped to prepare the broilers for the subsequent intestinal stress.

4.2. Microbioma

Firmicutes, *Actinobacteria*, *Bacteroidetes* and *Proteobacteria* were the most abundant phyla in all treatments. Some recent in vivo experiments have suggested that phytogenic products, such as functional oils, modify the composition of intestinal microbiota, increasing the relative abundance of *Firmicutes* in the gut (Salaheen et al., 2017; Li et al., 2018). In the jejunal and ileal environments, broilers of the Ionophore +0.075% FO group showed a higher proportion of *Firmicutes* and less abundance of *Actinobacteria*, compared with other treatments. The Firmicutes phylum is a diverse group of bacteria that possess a variety of metabolic activities, including the decomposition of polysaccharides and the production of butyrate (Ducatelle et al., 2018). In the current study, it was found that the treatment group had an increased relative abundance of *Lactobacillaceae* and a decreased relative abundance of *Corynebacteriaceae* in the segments of the small intestine. On the other hand, the challenged birds that did not receive additives had a reduced percentage of *Lactobacillaceae* in the jejunum and ileum when compared to the other groups. Strains of Lactobacillus influence the immune system by prompting immune cells to release inflammatory signaling molecules, like tumor necrosis factor-alpha, interferon-gamma, and interleukin-12, thereby activating an immune response-modulating effect when challenged by Eimeria species. The growth of these Lactobacillus strains contributes positively to weight gain, reduces tissue damage scores, and enhances mucosal barrier function (Walsh et al., 2021). The Lactobacillaceae family, which belongs to the Firmicutes phylum and the Bacilli class, is abundant in the small intestine of broilers, and is considered an indicator of intestinal health. These bacteria produce lactic acid as a product of carbohydrate metabolism (Bhogoju et al., 2018) and can create an inhibitory environment for the growth of many bacteria (pH-sensitive bacteria) by disrupting the bacterial outer membrane, thus potentiating the antimicrobial activity of host lysozyme (Alakomi et al., 2000).

In the present study, the cecal environment was found to be more complex and diverse compared to the small intestine. The small intestine is characterized by a higher concentration of Lactobacillaceae, while the cecum is known to contain a higher proportion of carbohydrate-fermenting bacteria such as *Lachnospiraceae* and *Ruminococcaceae*, which is in agreement with previous studies (Glendinning et al., 2019).

In the study conducted by Martynova-Van Kley et al. (2012), it was observed that the challenge of Eimeria spp. stimulated the proliferation of *Lactobacillus* spp. in the cecum. While *Lactobacillus* spp. is generally considered a beneficial group for chicken performance, its elevated abundance in the cecum may suggest an alteration in the normal microbiota of this region. The use of antimicrobials, such as salinomycin, can impede the maturation of the intestinal microbiota, and increase the population of opportunistic bacteria in the cecum (Gao et al., 2017). In this study, it was observed that in the cecal environment, in the group that received only the ionophore, there was an increase in the relative abundance of *Lactobacillaceae*, *Enterobactereaceae* and *Cloritridiaceae*, in addition to a reduction in *Ruminococcaceae* and *Lachnospiraceae*, when compared to the other treatments.

The relative abundance of *Ruminococcaceae* and *Lachnospiraceae* is associated with the production of short-chain fatty acids, which is reduced during intestinal challenges. Previous research has shown that the use of certain antibiotics, such as tylosin and enramycin, can increase the abundance of *Ruminococcaceae,* while salinomycin and monensin can reduce it. The present study found that the addition of a fiunctional oil in combination with an ionophore resulted in an increase in the *Lachnospiraceae* fand *Ruminocococeae* when compared to the the treatment with ionophore alone. Furthermore, a previous study (Pires et al., 2022) that used the same functional oil in combination with with ionophores (semduramicin and nicarbazin), found that after the removal of ionophores at 42 days, there was an increase in the abundance of *Ruminococcaceae, Lachnospiraceae* and *Rikenellaceae*.

In previous studies, it has been demonstrated that the blend of CNSL and castor oil has an impact on gram-positive bacteria and modulates the intestinal microbiota by reducing the abundance of pathogenic bacteria (Moraes et al., 2019a, 2019b; Vieira et al., 2020; Pires et al., 2022). The synergistic effect observed between the blend of functional oils and the ionophore in our study can be attributed to the mechanism of action of these additives. Specifically, the antimicrobial properties of salinomycin and functional oils are related to their ability to permeate through the bacterial cell membrane. As reported by Abbas et al. (2012), the liquid components of CNSL, cardol and anacardic acid, have a similar action to that of monovalent ionophores (such as salinomycin, monensin and others) by causing damage to the bacterial cell membrane. Additionally, ricinoleic acid, a component of castor oil, exhibits antimicrobial properties by denaturing and coagulating proteins in the bacterial cell wall.

This study demonstrated the efficacy of utilizing ionophores and functional oils in broilers challenged with coccidiosis. The combination of salinomycin sodium and CNSL – Castor oil blend exhibited a synergistic effect on modulation of the microbiota in broilers challenged with sporulated oocysts of *Eimeria*. The positive effect of this combination may be attributed to the complementary mechanism of action against intestinal challenges, leading to improved intestinal health and control of opportunistic bacterial growth. Therefore, due to the cost-effectiveness and observed results, it is recommended to include the CNSL and Castor oil blend at the level 0.075% in broiler chicken diets.

CRediT authorship contribution statement

Thaís Bastos Stefanello: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Supervision, Validation, Visualization, Writing – original draft. **Kátia Maria Cardinal:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Methodology, Data curation. **Catiane Orso:** Conceptualization, Data curation, Formal analysis, Methodology, Supervision, Validation, Visualization. **Carolina Haubert Franceschi:** Visualization, Validation, Supervision, Conceptualization, Data curation, Methodology. **Jéssica Pereira Silva:** Validation, Supervision, Methodology, Investigation, Formal analysis, Data curation, Conceptualization, Visualization. **Micheli Bertoni Mann:** Visualization, Validation, Supervision, Methodology, Formal analysis, Data curation. **Jeverzon Frazzon:** Data curation, Formal analysis, Methodology, Validation, Visualization. **Priscila Oliveira Moraes:** Visualization, Validation, Supervision, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Andréa Machado Leal Ribeiro:** Validation, Supervision,

Project administration, Methodology, Investigation, Formal analysis, Data curation, Conceptualization, Visualization, Writing – original draft, Writing – review & editing.

Declaration of competing interest

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.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.rvsc.2024.105249.

References

Abbas, R.Z., Iqbal, Z., Khan, A., Sindhu, Z.U.D., Khan, J.A., Khan, M.N., Raza, A., 2012. Options for integrated strategies for the control of avian coccidiosis. Int. J. Agric. Biol. 14, 1014–1020.

Ahmad, R., Yu, Y.H., Hua, K.F., Chen, W.J., Zaborski, D., Dybus, A., Cheng, Y.H., 2024. Management and control of coccidiosis in poultry—A review. Animal Bioscience 37 (1), 1. https://doi.org/10.5713/ab.23.0189.

Alakomi, H.L., Skyttä, E., Saarela, M., Mattila-Sandholm, T., Latva-Kala, K., Helander, I. M., 2000. Lactic acid permeabilizes gram-negative bacteria by disrupting the outer membrane. Appl. Environ. Microbiol. 66, 2001–2005. https://doi.org/10.1128/AEM.66.5.2001-2005.2000.

Anderson, M.J., Walsh, D.C.I., 2013. PERMANOVA, ANOSIM, and the mantel test in the face of heterogeneous dispersions: what null hypothesis are you testing? Ecological monographs 83, 557–564.

Andrade, T.D.J.A.D.S., Araújo, B.Q., Citó, A.M.D.G.L., Da Silva, J., Saffi, J., Richter, M.F., Ferraz, A.D.B.F., 2011. Antioxidant properties and chemical composition of technical cashew nutshell liquid (CNSL). Food Chem. 126, 1044–1048. https://doi.org/10.1016/j.foodchem.2010.11.122.

Bess, F., Favero, A., Vieira, S.L., Torrent, J., 2012. The effects of functional oils on broiler diets of varying energy levels. J. Appl. Poultry Res. 21, 567–578. https://doi.org/10.3382/japr.2011-00481.

Bhogoju, S., Nahashon, S., Wang, X., Darris, C., Kilonzo-Nthenge, A., 2018. A comparative analysis of microbial profile of Guinea fowl and chicken using metagenomic approach. PloS One 13, e0191029. https://doi.org/10.1371/journal.pone.0191029.

Cai, H., Liao, S., Qi, N., Sun, M., 2022. Single and combined effects of clostridium butyricum and coccidiosis vaccine on growth performance and the intestinal microbiome of broiler chickens. Front. Microbiol. 13, 811428 https://doi.org/10.3389/fmicb.2022.811428.

Caporaso, J.G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F.D., Costello, E.K., Fierer, N., Peña, A.G., Goodrich, J.K., Gordon, J.I., Huttley, G.A., Kelley, S.T., Knights, D., Koenig, J.E., Ley, R.E., Lozupone, C.A., McDonald, D., Muegge, B.D., Pirrung, M., Reeder, J., Sevinsky, J.R., Turnbaugh, P.J., Walters, W.A., Widmann, J., Yatsunenko, T., Zaneveld, J., Knight, R., 2010. QIIME allows analysis of high-throughput community sequencing data. Nat. Methods 7, 335–336. https://doi.org/10.1038/nmeth.f.303.

Chapman, H.D., Jeffers, T.K., Williams, R.B., 2010. Forty years of monensin for the control of coccidiosis in poultry. Poult. Sci. 89, 1788–1801. https://doi.org/10.3382/ps.2010-00931.

Clarke, K.R., Somerfield, P.J., Chapman, M.G., 2006. On resemblance measures for ecological studies, including taxonomic dissimilarities and a zero-adjusted Bray–Curtis coefficient for denuded assemblages. J. Exp. Mar. Biol. Ecol. 330, 55–80. https://doi.org/10.1016/j.jembe.2005.12.017.

Dhariwal, A., Chong, J., Habib, S., King, I.L., Agellon, L.B., Xia, J., 2017. MicrobiomeAnalyst: a web-based tool for comprehensive statistical, visual and meta-analysis of microbiome data. Nucleic Acids Res. 45, 180–188. https://doi.org/10.1093/nar/gkx295.

Ducatelle, R., Goossens, E., De Meyer, F., Eeckhaut, V., Antonissen, G., Haesebrouck, F., Van Immerseel, F., 2018. Biomarkers for monitoring intestinal health in poultry: present status and future perspectives. Vet. Res. 49, 43. https://doi.org/10.1186/s13567-018-0538-6.

Gao, P., Ma, C., Sun, Z., Wang, L., Huang, S., Su, X., Xu, J., Zhang, H., 2017. Feed-additive probiotics accelerate yet antibiotics delay intestinal microbiota maturation in broiler chicken. Microbiome 5 (1), 91. https://doi.org/10.1186/s40168-017-0315-1.

Glendinning, L., Watson, K.A., Watson, M., 2019. Development of the duodenal, ileal, jejunal and caecal microbiota in chickens. Anim Microbiome 1, 17. https://doi.org/10.1186/s42523-019-0017-z.

Li, Y., Fu, X., Ma, X., Geng, S., Jiang, X., Huang, Q., Hu, C., Han, X., 2018. Intestinal microbiome-metabolome responses to essential oils in piglets. Front. Microbiol. 9, 1988. https://doi.org/10.3389/fmicb.2018.01988.

Long, P.L., Rowell, J.G., 1975. Sampling broiler house litter for coccidial oocysts. Br. Poultry Sci. 16, 583–592. https://doi.org/10.1080/00071667508416233.

Lozupone, C., Lladser, M.E., Knights, D., Stombaugh, J., Knight, R., 2011. UniFrac: an effective distance metric for microbial community comparison. ISME J. 5, 169–172. https://doi.org/10.1038/ismej.2010.133.

Madlala, T., Okpeku, M., Adeleke, M.A., 2021. Understanding the interactions between Eimeria infection and gut microbiota, towards the control of chicken coccidiosis: a review. Parasite 28, 48. https://doi.org/10.1051/parasite/2021047.

Malik, T.A., Kamili, A.N., Chishti, M.Z., Tanveer, S., Ahad, S., Johri, R.K., 2016. Synergistic approach for treatment of chicken coccidiosis using berberine—A plant natural product. Microb. Pathog. 93, 56–62. https://doi.org/10.1016/j.micpath.2016.01.012.

Martynova-Van Kley, M.A., Oviedo-Rondon, E.O., Dowd, S.E., Hume, M., Nalian, A., 2012. Effect of Eimeria infection on Cecal microbiome of broilers fed essential oils. Int. J. Poult. Sci. 11, 747–755. https://doi.org/10.3923/ijps.2012.747.755.

Moraes, P.O., Andretta, I., Cardinal, K.M., Ceron, M., Vilella, L., Borille, R., Frazzon, A.P., Frazzon, J., Santin, E., Ribeiro, A.M.L., 2019a. Effect of functional oils on the immune response of broilers challenged with Eimeria spp. Animal 13, 2190–2198. https://doi.org/10.1017/S1751731119000600.

Moraes, P.O., Cardinal, K.M., Gouvêa, F.L., Schroeder, B., Ceron, M.S., Lunedo, R., Frazzon, A.P.G., Frazzon, J., Ribeiro, A.M.L., 2019b. Comparison between a commercial blend of functional oils and monensin on the performance and microbiota of coccidiosis-challenged broilers. Poult. Sci. 98, 5456–5464. https://doi.org/10.3382/ps/pez345.

Moraes, P.O., Silva Pires, P.G., Benetti Filho, V., Lima, A.L.F., Kindlein, L., Taschetto, D., Wagner, G., 2023. Intestinal health of broilers challenged with Eimeria spp. using functional oil blends in two physical forms with or without anticoccidials. Sci. Rep. 13 (1), 14612. https://doi.org/10.1038/s41598-023-41743-9.

Murakami, A.E., Eyng, C., Torrent, J., 2014. Effects of functional oils on coccidiosis and apparent metabolizable energy in broiler chickens. Asian Australas. J. Anim. Sci. 27, 981–989. https://doi.org/10.5713/ajas.2013.13449.

Murugesan, G.R., Syed, B., Haldar, S., Pender, C., 2015. Phytogenic feed additives as an alternative to antibiotic growth promoters in broiler chickens. Front Vet Sci. 2, 21. https://doi.org/10.3389/fvets.2015.00021.

Nazzaro, F., Fratianni, F., De Martino, L., Coppola, R., De Feo, V., 2013. Effect of essential oils on pathogenic bacteria. Pharmaceuticals 6 (12), 1451–1474.

Osmari, M.P., De Matos, L.F., Salab, B.L., Díaz, T.G., 2015. Líquido da casca da castanha de caju: Características e aplicabilidades na produção animal Cashew nut shell liquid: Characteristics and applicability in animal production. Maringá 9, 143–149.

Pajić, M., Todorović, D., Knežević, S., Prunić, B., Velhner, M., Andrić, D.O., Stanimirovic, Z., 2023. Molecular investigation of Eimeria species in broiler farms in the province of Vojvodina, Serbia. Life 13 (4), 1039. https://doi.org/10.3390/life13041039.

Paramashivappa, R., Kumar, P.P., Vithayathil, P.J., Rao, A.S., 2001. Novel method for isolation of major phenolic constituents from cashew (Anacardium occidentale L.) nutshell liquid. J. Agric. Food Chem. 49, 2548–2551. https://doi.org/10.1021/jf001222j.

Pires, P.G.D.S., Torres, P., Teixeira Soratto, T.A., Filho, V.B., Hauptli, L., Wagner, G., Haese, D., Pozzatti, C.D., Moraes, P.O., 2022. Comparison of functional-oil blend and anticoccidial antibiotics effects on performance and microbiota of broiler chickens challenged by coccidiosis. PloS One 17, e0270350. https://doi.org/10.1371/journal.pone.0270350.

Qaid, M.M., Al-Mufarrej, S.I., Azzam, M.M., Al-Garadi, M.A., 2021. Anticoccidial effectivity of a traditional medicinal plant, Cinnamomum verum, in broiler chickens infected with Eimeria tenella. Poult. Sci. 100, 100902 https://doi.org/10.1016/j.psj.2020.11.071.

Rostagno, H.S., Albino, L.T., Hannas, M.I., Donzele, J.L., Sakomura, N.K., Perazzo, F.G., Brito, C.O., 2017. Tabelas brasileiras para aves e suínos: composição de alimentos e exigências nutricionais. In: 4a, edição.. MG: Departamento de Zootecnia, Universidade Federal de Viçosa, Viçosa, p. 488.

Salaheen, S., Kim, S.W., Haley, B.J., Van Kessel, J.A.S., Biswas, D., 2017. Alternative growth promoters modulate broiler gut microbiome and enhance body weight gain. Front. Microbiol. 8, 2088. https://doi.org/10.3389/fmicb.2017.02088.

Sidiropoulou, E., Skoufos, I., Marugan-Hernandez, V., Giannenas, I., Bonos, E., Aguiar-Martins, K., Lazari, D., Blake, D.P., Tzora, A., 2020. In vitro Anticoccidial study of oregano and garlic essential oils and effects on growth performance, fecal oocyst output, and intestinal microbiota in vivo. Front Vet Sci. 7, 420. https://doi.org/10.3389/fvets.2020.00420.

Sosa, B., Moro, S., Gomes, E.B., Cardoso, R.L.M., Cardoso, M.S., Boscolo, L.M., Bittencourt, F., 2020. Essential oils in diets for Nile tilapia juveniles: productive performance and plasmatic biochemistry. Aquacult. Res. 51, 2758–2765. https://doi.org/10.1111/are.14614.

Soutter, F., Werling, D., Tomley, F.M., Blake, D.P., 2020. Poultry coccidiosis: design and interpretation of vaccine studies. Front Vet Sci. 7, 101. https://doi.org/10.3389/fvets.2020.00101.

Vieira, A.M., Soratto, T.A.T., Cardinal, K.M., Wagner, G., Hauptli, L., Lima, A.L.F., Dahlke, F., Peres Netto, D., Moraes, P.O., Ribeiro, A.M.L., 2020. Modulation of the intestinal microbiota of broilers supplemented with monensin or functional oils in response to challenge by Eimeria spp. PloS One 15, e0237118. https://doi.org/10.1371/journal.pone.0237118.

Walsh, M.C., Jacquier, V., Schyns, G., Claypool, J., Tamburini, I., Blokker, B., Geremia, J. M., 2021. A novel microbiome metabolic modulator improves the growth performance of broiler chickens in multiple trials and modulates targeted energy and

amino acid metabolic pathways in the cecal metagenome. Poult. Sci. 100 (3), 100800 https://doi.org/10.1016/j.psj.2020.10.054.
Yadav, S., Jha, R., 2019. Strategies to modulate the intestinal microbiota and their effects on nutrient utilization, performance, and health of poultry. J Anim Sci Biotechnol. 10, 2. https://doi.org/10.1186/s40104-018-0310-9.
Yilmaz, P., Parfrey, L.W., Yarza, P., Gerken, J., Pruesse, E., Quast, C., Schweer, T., Peplies, J., Ludwig, W., Glöckner, F.O., 2014. The SILVA and "all-species living tree project (LTP)" taxonomic frameworks. Nucleic Acids Res. 42, 643–648. https://doi.org/10.1093/nar/gkt1209.