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Effect of a blend of essential oils, organic acids, tannins, vitamin E and zinc on the intestinal health of broiler chickens challenged with *Eimeria* spp., *Salmonella* Minnesota, *Escherichia* coli and *Clostridium perfringens*[1]

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This study aimed to evaluate the effects of (i) diets supplemented with a blend of organic acids, cinnamon essential oil, oregano essential oil, eugenol, thymol, curcumin, tannins, vitamin E, and zinc microencapsulated in vegetable fat and (ii) a challenge by *Eimeria* spp., *Salmonella* Minnesota, *Escherichia coli*, and *Clostridium perfringens*. Also, to evaluate the diet × challenge interaction effects on animal performance (1-21 and 22-42 days of age), weights of organs and primal cuts, and ileal morphometry in 42-day-old broiler chickens. The experiment was conducted according to a 2 × 2 factorial design (supplemented and unsupplemented diets × challenged and unchallenged broilers). Each treatment consisted of eight replications and eight birds per replicate. At 14 days of age, chickens in the challenge group (*n*=128) received orally 1mL of a suspension containing sporulated oocysts of *Eimeria* spp. (*E. acervulina*, *E. praecox*, *E. maxima*, *E. mitis*, *E. tenella*, and *E. necatrix*), and the other experimental group (*n*=128) received 1mL of saline solution orally. At 18 days of age, birds in the challenge group received 1mL of a suspension of *C. perfringens*, *E. coli*, and *S.* Minnesota, and unchallenged birds received 1mL of saline solution orally. From 1 to 21 days of age, microbial challenge reduced body weight, feed intake, weight gain and increased feed conversion. In the same period, supplemented birds had lower feed conversion. From 22 to 42 days of age, challenged birds had lower body weight, feed conversion, breast weight, thigh + drumstick weight, and heart weight. Supplemented birds had higher breast weight. Unchallenged birds fed the supplemented diet showed higher bursa weight, proventriculus weight, ileal villus height, and crypt depth. Unchallenged birds fed the unsupplemented diet had higher liver weight. Microbial challenges with *Eimeria* spp., *S.* Minnesota, *C. perfringens*, and *E. coli* impaired productive performance in the starter phase. They decreased the yield of primal cuts in 42-day-old broilers, partially explaining the recurring economic problems observed in the poultry sector. Overall, the studied blend was able to improve feed conversion in the starter phase, enhance digestive and absorption processes, and increase the yield of primal cuts.

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However, no effects were observed in challenged birds. The findings suggest that the studied effects are influenced by microbial conditions, blend composition, and inclusion level and may or may not result in beneficial outcomes.

INDEX TERMS: Coccidiosis, growth promoter, bacterial resistance, salmonellosis, intestinal health, broiler chickens, *Eimeria* spp., *Salmonella* Minnesota, *Escherichia coli*, *Clostridium perfringens*.

RESUMO.- [**Efeito da inclusão de *blend* de óleos essenciais, ácidos orgânicos, taninos, vitamina E e zinco sobre a saúde intestinal de frangos de corte submetidos ao desafio por *Eimeria* spp., *Salmonella* Minnesota, *Escherichia coli* e *Clostridium perfringens*.**] Este estudo teve como objetivo avaliar os efeitos de (i) dietas suplementadas com *blend* de ácidos orgânicos, óleos essenciais de canela, eugenol, timol e orégano, curcumina, taninos, vitamina E e zinco microencapsulados em gordura vegetal, e (ii) do desafio sanitário por *Eimeria* spp., *Salmonella* Minnesota, *Escherichia coli* e *Clostridium perfringens.* Além disso, avaliar a interação entre a dieta e o desafio sanitário sobre os seguintes parâmetros: desempenho animal (1-21 dias e 22-42 dias de idade), peso de órgãos e de cortes nobres e morfometria do íleo de frangos de corte com 42 dias de idade. O experimento foi conduzido em esquema fatorial 2 × 2 (dieta sem suplementação do *blend* e dieta suplementada com *blend* vs. animais sem desafio e animais desafiados por *Eimeria* spp., *S.* Minnesota, *E. coli* e *C. perfringens*). Cada tratamento foi composto por oito repetições e oito aves/repetição. Os frangos do grupo desafiado (n=128) aos 14 dias de idade receberam por via oral 1mL de solução contendo oocistos esporulados de *Eimeria* spp. (*E. acervulina*, *E. praecox*, *E. maxima*, *E. mitis*, *E. tenella* e *E. necatrix*); o outro grupo experimental (n=128) recebeu por via oral 1mL de solução salina. Aos 18 dias de idade os animais do grupo desafiado receberam por via oral 1mL de solução contendo cepas de *C. perfringens*, *E. coli* e *S.* Minnesota. Os animais do grupo não desafiado receberam novamente por via oral 1mL de solução salina. No período de 1-21 dias de idade o desafio sanitário reduziu o peso vivo, o consumo de ração, o ganho de peso e aumentou a conversão alimentar dos animais. Nesse mesmo período, os animais suplementados com o *blend* tiveram menor conversão alimentar. No período de 22-42 dias de idade, os animais desafiados apresentaram menor peso vivo e conversão alimentar. Os animais desafiados apresentaram menor peso de peito, coxa + sobrecoxa e coração, e os animais que consumiram a dieta suplementada com o *blend* apresentaram maior peso de peito. Os animais não desafiados e que consumiram a dieta suplementada com o *blend* apresentaram maior peso da bursa de Fabricius, do proventrículo, e maior altura de vilosidades e profundidade de cripta do íleo. Já os frangos não desafiados e que consumiram a dieta sem suplementação do *blend* apresentaram maior peso do fígado. O desafio sanitário por *Eimeria* spp., *S.* Minnesota, *C. perfringens* e *E. coli*, prejudicou o desempenho produtivo dos animais no período inicial, e o rendimento de cortes nobres de frangos de corte com 42 dias de idade, explicando em partes a causa dos problemas econômicos recorrentemente vistos no setor avícola. No geral o *blend* foi capaz de melhorar a conversão alimentar no período inicial, os processos digestivo e absortivo, bem como o rendimento de cortes nobres. Entretanto, não foi observado efeitos do *blend* em animais desafiados, o que sugere que os efeitos esperados são dependentes do tipo de desafio sanitário experimental, da composição dos componentes bioativos do *blend* e do nível de inclusão do *blend* que podem apresentar ou não resultados benéficos.

TERMOS DE INDEXAÇÃO: Coccidiose, melhorador de crescimento, resistência bacteriana, salmonelose, saúde intestinal, frangos de corte, *Eimeria* spp., *Salmonella* Minnesota, *Escherichia coli*, *Clostridium perfringens*.

INTRODUCTION

Broiler production is typically conducted at high stocking densities, which can facilitate the propagation of infectious diseases (Pant et al. 2018). The use of antimicrobials in livestock production is facing growing restrictions, driven by various factors such as the emergence of microbial resistance and changing consumer preferences (Fancher et al. 2020). As a result, the poultry industry has encountered great challenges in maintaining productivity, as birds become more susceptible to diseases without antibiotic growth promoters (Smith 2011, Fancher et al. 2020). Without subtherapeutic doses of antimicrobials, physiological stressors are more likely to induce harmful effects in birds. These effects include intestinal health problems, an elevated incidence of coccidiosis, and an increased occurrence of bacterial diseases such as colibacillosis, necrotic enteritis, and gangrenous dermatitis – the major bacterial diseases affecting broilers reared under antimicrobial-free conditions (Smith 2011, Fancher et al. 2020, Singer et al. 2023). These diseases are associated with high rates of comorbidity and mortality and constitute a significant cause of economic loss in poultry farming, as contaminated carcasses must be discarded for food safety reasons (Fancher et al. 2020).

Such issues have motivated the scientific community to search for alternative non-antibiotic strategies to improve bird performance and prevent colonization by zoonotic pathogens (Fancher et al. 2020). Efforts to reduce disease prevalence, minimize stress, and improve poultry gut and overall health are crucial to achieve better production performance. Some promising strategies to replace antimicrobials include genetic selection of more resistant birds and the use of phytogenic additives (e.g., essential oils and organic acids), functional nutrients (e.g., zinc and vitamin E), probiotics, and prebiotics (Casterlow et al. 2011, Neveling & Dicks 2021, Ayalew et al. 2022).

Several non-antibiotic feed additives were investigated for their positive effects in broiler chickens. Phytogenic additives such as cinnamon essential oil, oregano essential oil, curcumin, eugenol, thymol, and tannins contain bioactive components, including polyphenols, terpenoids, phenolics, glycosides, and alkaloids, which were shown to exert beneficial effects on bird health (Barbieri et al. 2017, Abdelli et al. 2021). These bioactive components possess antimicrobial, antioxidant, and anti-inflammatory activities and improve

intestinal and hepatic functions (Abou-Elkhair et al. 2014, Abdelli et al. 2021, Kumar et al. 2022). Organic acids such as citric, fumaric, sorbic, and malic acids serve as alternatives to antibiotics and offer several benefits, contributing to the maintenance of gut morphology and microbial balance and increasing the rate of absorption of minerals and peptides (Khan & Iqbal 2016).

The antimicrobial properties of phytogenic additives are well described and partly explain their beneficial effects in birds. The antimicrobial action of plant extracts is attributed to their lipophilic nature, which allows these substances to penetrate bacterial cells and destabilize cell membranes (Yang et al. 2015). Thus, phytogenic additives directly affect bacteria, interfering with membrane integrity and function, leading to cell damage and eventual death (Li et al. 2021). These substances can also stimulate intestinal mucus secretion, which impairs pathogen adhesion and lends greater stability to intestinal microbiota (Chang & Yu 2022). Furthermore, numerous bioactive compounds in phytogenic additives are renowned for their antioxidant properties. These compounds neutralize oxidative substances, scavenge reactive oxygen species, and activate enzymes of the antioxidant defense system (Kruk et al. 2022, Piao et al. 2023).

Organic acids have an effective mechanism of action against bacteria. They act by lowering the intestinal pH, creating a slightly acidic environment in the intestinal lumen that is inhospitable for the growth and survival (bactericidal/bacteriostatic effect) of many pathogenic bacterial species (Tamblyn & Conner 1997, Khan & Iqbal 2016). Organic acids were shown to produce beneficial effects alone and in combination with essential oils, which can result in synergistic or additive effects on antibacterial activity (Zhou et al. 2007, Liu et al. 2017). According to Stefanello et al. (2020), the synergistic effects of organic acids and essential oils are mainly attributed to the modulation of the intestinal microbiota. The hydrophobic nature of essential oils increases the permeability of the bacterial membrane, facilitating, in turn, the influx of organic acids into the cytoplasm.

Vitamin E and zinc are two dietary components widely known to possess antioxidant properties. The mechanism of action of zinc has not been fully elucidated. It is believed to be related to the enhanced synthesis of metallothionein, a cystine-rich protein that acts as a scavenger/neutralizer of free radicals (Oteiza et al. 1996). The antioxidant effect of vitamin E is attributed to its ability to minimize the production of reactive oxygen species during fatty acid oxidation and inhibit the propagation of free radical reactions. In addition to having antioxidant effects, vitamin E possesses anti-inflammatory activity and contributes to general health by improving immune function (Rizvi et al. 2014).

Thus, the combined action of phytogenic additives, organic acids, and functional nutrients protects the intestinal epithelium, maintaining a favorable environment for intestinal health and reducing the risk of infections and intestinal disorders (Yang et al. 2018, Ribeiro et al. 2021). Another advantage provided by the combined use of these substances is that, unlike growth-promoting antibiotics, they do not cause antimicrobial resistance (Marshall & Levy 2011) and leave no traces in food products, representing a safe and sustainable approach to improving animal health and the quality of animal products (Stamilla et al. 2020).

The primary mechanism of action of antibiotic growth promoters involves modulation of the intestinal microbiota, thereby reducing opportunistic pathogens and subclinical infections (Plata et al. 2022). As the mode of action of feed additives is similar to that of antibiotics, they have been considered promising candidates to replace conventional growth promoters, prebiotics, and probiotics (Abou-Elkhair et al. 2018). Zhang et al. (2019a) demonstrated that dietary supplementation of broilers with essential oils and organic acids had a bacteriostatic effect on *Salmonella enteritidis*, concluding that the proposed treatment represented an effective replacement for antibiotics in the prevention and treatment of *Salmonella* infections. Pham et al. (2023) suggested that dietary supplementation of broilers with essential oils and organic acids could alleviate *Escherichia coli*-induced intestinal injury and inflammation. Pham et al. (2022) reported that treatment with essential oils and organic acids minimized intestinal damage, growth impairment, and alterations of the cecal microbiota in broilers challenged with *Eimeria* spp. and *Clostridium perfringens*. Furthermore, the cited authors proposed that these additives have great potential to replace antibiotic growth promoters. Bortoluzzi et al. (2019) observed that zinc supplementation of broiler chickens challenged with coccidiosis and necrotic enteritis minimized the severity of intestinal injury and reduced bird mortality. Moreover, the authors found that supplemented birds had lower expression of interleukin-8 and interferon-gamma genes, indicative of reduced inflammatory responses. Xu et al. (2023) showed that supplementing chicken diets with tannins can alleviate the negative effects of necrotic enteritis by improving antioxidant and anti-inflammatory capacity, regulating intestinal microbiota, and reducing intestinal permeability.

Given the foregoing, this study hypothesized that (i) the exposure of chickens to *Eimeria* spp., *Salmonella* Minnesota, *C.* perfringens, and *E. coli* negatively influences productive performance parameters, organ weights, primal cut weights, and ileal morphometry and (ii) dietary supplementation with a microencapsulated blend of organic acids, essential oils, curcumin, tannins, vitamin E, and zinc minimizes the harmful effects of these pathogens.

MATERIALS AND METHODS

Ethical approval. The experiment was conducted at the Iguatemi Experimental Farm, "Universidade Estadual de Maringá" (UEM), Paraná, Brazil, according to the guidelines of the local Animal Research Ethics Committee (CEUA Protocol No. 7533270721). Access to the experimental site was restricted to research team members for biosafety reasons.

Birds and experimental design. A total of 256 one-day-old male broilers (Cobb 500) were used in the experiment. Birds had an initial mean weight of 43g and were not vaccinated against coccidiosis. Individuals were housed in suspended cages ($1m^2$) equipped with an excreta collection tray in an air-conditioned environment under a 24-hour photoperiod (artificial lighting). The ambient temperature was initially set at 33°C and gradually reduced according to bird age, following the recommendations for the Cobb 500 line.

The experiment followed a completely randomized design with a 2 × 2 factorial arrangement, eight replicates per treatment, and eight birds per replicate. The first factor comprised a diet supplemented with a blend of organic acids, cinnamon essential oil, eugenol, thymol, oregano essential oil, curcumin, tannins, vitamin E, and

zinc microencapsulated in vegetable fat and an unsupplemented diet. The second factor comprised the birds challenged with *Eimeria* spp., *Clostridium perfringens*, *Escherichia coli*, *Salmonella* Minnesota, and the unchallenged birds.

At 14 days of age, birds in the microbial challenge group (n=128) received orally 1mL of a suspension containing sporulated oocysts of *Eimeria* spp. (2×10^4 *E. acervulina*, 2×10^4 *E. praecox*, 1.6×10^4 *E. maxima*, 4×10^4 *E. mitis*, 3×10^3 *E. tenella*, and 8×10^3 *E. necatrix*). The other experimental group (n=128) received 1mL of saline orally. Challenged and unchallenged broilers were housed separately to avoid cross-contamination but in the same facility to ensure that both groups were exposed to the same environmental conditions.

Before (days 7 and 14) and after (days 15 to 20) inoculation of *Eimeria* spp. oocysts and excreta were collected daily from challenged and unchallenged birds for qualitative analysis of oocysts. The results confirmed that challenged birds were contaminated and unchallenged birds were not. For a complementary diagnosis, the ileum was excised from two birds per treatment and replication and subjected to histological analysis to detect the presence of *Eimeria* spp. in the intestinal mucosa. For this, on the 4th (n=32 birds) and 11th (n=32 birds) days after inoculation, the selected birds were euthanized by cervical dislocation. Ileum specimens were collected, cut longitudinally, and thoroughly rinsed with ice-cold sterile saline. Then, samples were placed on a Styrofoam plate, fixed in Bouin's solution for 6 hours, and stored in flasks containing 70% ethanol. Subsequently, samples were dehydrated through a graded ethanol series, cleared with xylol, and embedded in paraffin. Longitudinal semi-serial sections (3µm thick) were prepared and stained with hematoxylin and eosin (HE). Histological images were captured using an Olympus BX 50 P1 optical microscope equipped with a 40× objective lens and coupled to an Olympus PMC 35 B digital camera.

At 18 days of age, after the first day of detection of *Eimeria* oocysts in the excreta of challenged birds (4 days post-inoculation), birds in the challenge group received orally 1mL of a suspension containing 6.3×10^6 colony-forming units (CFU) of *C. perfringens*, 10^6 CFU of *E. coli*, and 10^6 CFU of *S.* Minnesota. Unchallenged broilers received orally 1mL of saline.

Birds had *ad libitum* access to water and feed throughout the experimental period. Diets were based on corn and soybean meal and formulated to meet the nutritional requirements of birds, according to Rostagno et al. (2017) (Table 1). Supplementation was performed from 1 to 42 days of age (end of the experimental period). No anticoccidial drugs or antibiotics were added to feed or water.

Coccidiosis diagnosis. At 7 and 14 days of age (up to six days after *Eimeria* inoculation), a pooled sample of fresh excreta was collected daily from the excreta collection tray of challenged and unchallenged birds. This procedure was performed to confirm the infection in challenged broilers and the absence of infection in unchallenged broilers, as well as to identify the first day of oocyst shedding in infected birds.

The analysis was performed according to Gordon & Whitlock (1939), with some modifications. Approximately 2g of excreta was dissolved in 15mL of distilled water and centrifuged at 2500rpm for 2 min. Then, the supernatant was discarded, and the pellet was resuspended in 10mL of sucrose solution (1.18g mL^{-1}). The mixture was centrifuged at 2500rpm for 2 min, and an aliquot of the supernatant was placed on a histological slide for detection of oocysts. The slides were examined under an Olympus BX 50 P1 optical microscope equipped with a 40× objective lens and coupled to an Olympus PMC 35 B digital camera.

Broiler performance: Final live weight, feed intake, weight gain, and feed conversion. For performance analysis, each cage containing six birds was treated as an experimental unit (n=8). Performance parameters were assessed separately in the starter (1 to 21 days) and grower/finisher (22 to 42 days) phases.

Feed intake was calculated from the difference between the weight of feed provided on days 1 and 22 and the daily mean weight of leftovers at the end of each experimental period (21 and 42 days), as shown by the equation [Feed intake = (Feed provided − Leftovers)]. For calculation of weight gain, birds were weighed at 1, 21, and 42 days of age. Weight gain was calculated as [Weight gain = (Final weight − Initial weight)]. Feed conversion was calculated as the feed intake to weight gain ratio for the two experimental periods.

Weight of organs and primal cuts. At 42 days of age, six birds per treatment group were selected based on the mean weight of each replicate. Birds were weighed and subsequently slaughtered by cervical dislocation. The breast (with bones, without skin), thigh + drumstick (with bones, without skin), liver, spleen, heart, bursa of Fabricius, small and large intestines, cecum, pancreas, proventriculus, and gizzard were collected and weighed.

Morphometric analysis of the ileum. The ileum is the intestinal segment with the highest correlation with intestinal bacterial populations. Given that the supplement blend used in this study was aimed at controlling bacterial populations, morphometric analyses were focused on the ileum. At 42 days of age, six birds per treatment group were selected based on the mean weight of each replicate and slaughtered by cervical dislocation. Immediately after slaughter, ileum fragments measuring approximately 3cm were excised, cut longitudinally, and washed thoroughly with cold sterile saline. Specimens were placed on a Styrofoam plate and fixed in Bouin's solution for 6 hours. After fixation, ileum fragments were stored in 70% ethanol until processing. Subsequently, ileum samples were dehydrated through a graded ethanol series, cleared with xylol, and embedded in paraffin. Longitudinal semi-serial sections (3µm thick) were cut and stained with HE. Histological images were captured using an Olympus BX 50 P1 optical microscope equipped with a 40× objective lens and coupled to an Olympus PMC 35 B digital camera.

Morphometric measurements (villus height and crypt depth) were performed on 20 integral villi per bird using Image Pro Plus version 4.0 (Media Cybernetics). The villus/crypt ratio was calculated by dividing villus height by crypt depth. The results are expressed in millimeters.

Quantification of bacteria and detection of *Salmonella* spp. in the cecum. For quantification of enterobacteria, *C. perfringens*, sulfite-reducing clostridia, and *E. coli*, six 42-day-old chickens per treatment were selected based on the mean weight of the replicate. Birds were slaughtered by cervical dislocation, and the cecum and cecal tonsils were excised. Immediately after collection, samples were placed in sterile Nasco bags and stored at 8°C until analysis.

Microbial analyses were carried out by Integralab Laboratory following ISO 6579-1:2017 (detection of *Salmonella* spp.) (ISO 2017a), ISO 7937:2004 (quantification of *C. perfringens*) (ISO 2004), ISO 16649-2:2001 (quantification of *E. coli*) (ISO 2001), and ISO 21528-2:2017 (quantification of enterobacteria) (ISO 2017b).

Statistical analysis. The normality of the distribution of bird performance, organ weight, primal cut weight, and ileal morphometric data was evaluated using the UNIVARIATE procedure of SAS. The data were subjected to a two-way analysis of variance (ANOVA). The model included the effects of diet and microbial challenges as well as their interaction. Means were compared using Tukey's and Student's t-test (p<0.05). Analyses were performed using SAS 2002 version 9.00 (SAS Inst. Inc., Cary/NC). Bacterial counts were analyzed descriptively.

RESULTS

No oocysts were detected in excreta collected from challenged or unchallenged chickens at 7 or 14 days of age, confirming that birds did not have coccidiosis before inoculation (Fig.1-4). In challenged birds, oocyst shedding was detected at 18 days of age; that is, shedding started four days after inoculation of sporulated *Eimeria* oocysts, confirming the establishment of coccidiosis in challenged chickens (Fig.5). The lack of oocysts in excreta from unchallenged birds at 18 days of age validated their use as uninfected controls (Fig.6). Histological examination revealed the presence of *Eimeria* spp. at different phases of the intracellular cycle in the ileal villi of challenged birds at 11 days after inoculation (Fig.7 and 8).

Animal performance: Final live weight, feed intake, weight gain, and feed conversion ratio

In the starter phase (1-21 days), there were no significant interaction effects on final live weight (p=0.8459), feed intake

Table 1. Ingredient and nutrient composition of experimental diets fed to broiler chickens in the starter (1-21 days) and grower/finisher (22-42 days) phases

Ingredient composition (g kg−1)	Starter diets 1-21 days		Grower/finisher diets 22-42 days	
	UD	SD	UD	SD
Corn (78% crude protein)	575.823	575.823	644.338	640.122
Soybean oil	24.810	24.810	35.637	36.426
Soybean meal (46% crude protein)	352.215	352.215	277.431	281.153
Common salt	5.518	5.518	5.018	5.018
Calcitic limestone (38% Ca)	13.826	13.826	13.003	12.982
Dicalcium phosphate (20% Ca)	15.850	15.850	14.367	14.348
dl-Methionine (99%)	3.825	3.825	3.024	2.993
l-Threonine (98%)	1.152	1.152	0.792	0.743
l-Valine	0.525	0.525	0.353	0.289
l-Lysine (78%)	3.456	3.456	3.037	2.926
Vitamin–mineral premix*	2.000	2.000	2.000	2.000
Sannimix**	-	1.000	-	1.000
Kaolin	0.999		1.000	
Total	1000.000	1000.000	1000.000	1000.000
Nutrient composition (calculated)				
Crude protein (%)	20.999	20.999	17.985	17.985
Ether extract (%)	5.041	5.041	6.260	6.260
Calcium (%)	0.966	0.966	0.882	0.882
Total phosphorus (%)	0.701	0.701	0.639	0.639
Digestible phosphorus (%)	0.460	0.460	0.420	0.420
AME (kcal kg−1)	3000.000	3000.000	3150.000	3150.000
Total methionine + cystine (%)	1.027	1.027	0.873	0.873
Digestible methionine + cystine (%)	0.947	0.947	0.802	0.802
Total lysine (%)	1.402	1.402	1.173	1.173
Digestible lysine (%)	1.280	1.280	1.070	1.070
Total tryptophane (%)	0.260	0.260	0.216	0.216
Digestible tryptophane (%)	0.233	0.233	0.180	0.180
Total threonine (%)	0.935	0.935	0.786	0.786
Digestible threonine (%)	0.832	0.832	0.695	0.695
Total arginine (%)	1.404	1.404	1.176	1.176
Digestible arginine (%)	1.344	1.344	1.134	1.134
Sodium (%)	0.240	0.240	0.220	0.220
Potassium (%)	0.933	0.933	0.791	0.791

UD = unsupplemented diet, SD = diet supplemented with a microencapsulated blend of organic acids, cinnamon essential oil, oregano essential oil, eugenol, thymol, curcumin, tannins, vitamin E, and zinc (Sannimix), AME = apparent metabolizable energy; * Vitamin and mineral levels provided by starter diets (per kg) = 2270.00 IU vitamin A, 8330 IU vitamin E, 591mg vitamin B1, 1490mg vitamin B2, 858mg vitamin B6, 3500mcg vitamin B12, 450mg vitamin K3, 2976mg calcium pantothenate, 8820mg niacin, 200mg folic acid, 20mg biotin, 86mg choline, 19mg zinc, 14mg iron, 20mg manganese, 3040mg copper, 290mg iodine, 50mg cobalt, 88mg selenium, 25mg ethoxyquin, and 20mg BHA. Vitamin and mineral levels provided by grower/finisher diets (per kg) = 2250.00 IU vitamin A, 500,000 IU vitamin D3, 7000 IU vitamin E, 450mg vitamin B1, 1000mg vitamin B2, 450mg vitamin B6, 3500µg vitamin B12, 420mg vitamin K3, 2500mg calcium pantothenate, 7000mg niacin, 180mg folic acid, 15mg biotin, 55mg choline, 12mg zinc, 12mg iron, 15mg manganese, 3000mg copper, 250mg iodine, 50mg cobalt, 72mg selenium, 40mg ethoxyquin, and 40mg BHA; ** Provided by kilogram of Sannimix = 50.00g citric acid, 135.00g fumaric acid, 35.00g sorbic acid, 35.00g malic acid, 15.00mg zinc, and 12.00mg vitamin E.

(p=0.9494), weight gain (p=0.8536), or feed conversion ratio (p=0.7332) (Table 2). However, there was a significant effect of microbial challenge on final live weight (p=0.0002), feed intake (p=0.0426), weight gain (p=0.0007), and feed conversion ratio (p=0.0002) (Table 2). Challenged birds showed lower live weight (about 20%), feed intake (about 6%), weight gain (about 21%) and higher (worse) feed conversion ratio (about 20%) than unchallenged birds. Also, it was observed that the diet influenced the feed conversion ratio (p=0.0067) since animals that consumed the diet supplemented with the blend had lower (better) feed conversion. Birds fed the supplemented diet had a 9% lower (better) feed conversion ratio (Table 2) than unsupplemented birds. There were no significant effects of diet on final live weight (p=0.3123), feed intake (p=0.5723), or weight gain (p=0.2286) (Table 2).

In the grower/finisher phase (22-42 days), there were no significant interaction effects on final live weight (p=0.9775), feed intake (p=0.5360), weight gain (p=0.4709), or feed conversion ratio (p=0.9580) (Table 3). Microbial challenge did influence final live weight (p=0.0101) and feed conversion ratio (p=0.0059) (Table 3). Challenged broilers had lower final live weight (about 11%) and lower feed conversion ratio (about 7%) than unchallenged broilers. No significant effects of microbial challenge were observed on feed intake (p=0.2850) or weight gain (p=0.1580). There was no significant effect of diet on final live weight (p=0.6070), feed intake (p=0.7975), weight gain (p=0.8381), or feed conversion ratio (p=0.8896) (Table 3).

Weight of organs and primal cuts of broilers at 42 days of age

There was an interaction effect between diet and microbial challenge on the weight of the bursa of Fabricius (p=0.0231) (Table 4). Unchallenged broilers fed the supplemented diet showed higher organ weight. There was no significant interaction effect on spleen weight (p=0.5166) or heart weight (p=0.0788) (Table 4). Microbial challenge influenced heart weight (p=0.0350) but not spleen weight (p=0.8695). Challenged broilers had lower heart weights than unchallenged broilers (Table 4). Diet did not exert significant effects on organ weights (p>0.05) (Table 4).

The weights of the liver, small and large intestine, cecum, pancreas, proventriculus, and gizzard of 42-day-old chickens are presented in Table 5. Diet and microbial challenge had an interaction effect on liver weight (p=0.0430) and proventriculus

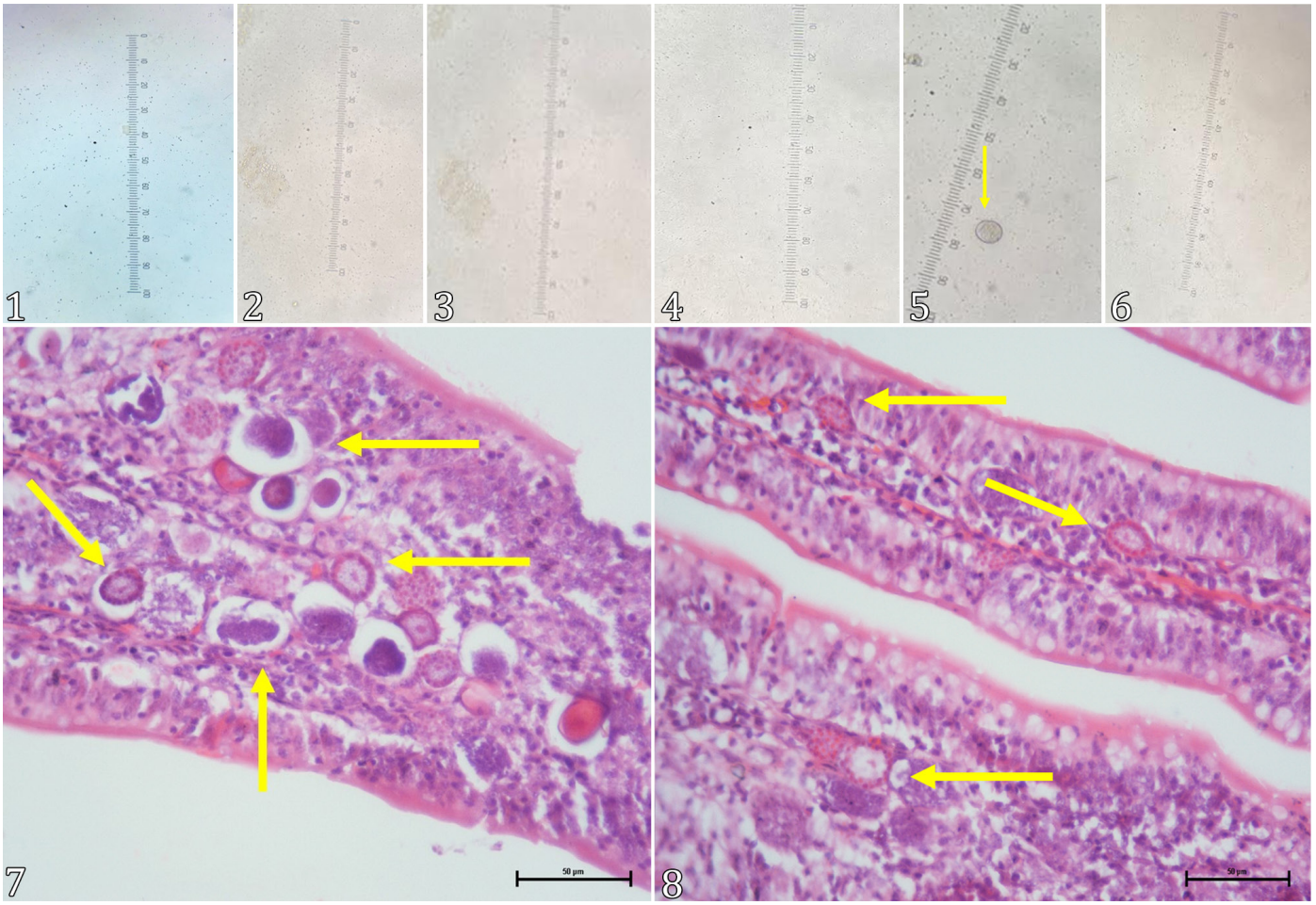

Fig.1-8. Qualitative coprological analysis of (**1**) unchallenged and (**2**) challenged broilers seven days prior to inoculation of sporulated oocysts of *Eimeria* spp. Qualitative coprological analysis of (**3**) unchallenged and (**4**) challenged broilers at 14 days of age (day of inoculation). Qualitative coprological analysis of (**5**) challenged and (**6**) unchallenged broilers at 18 days of age (four days post-inoculation). The yellow arrow indicates the presence of oocysts. (**7** and **8**) Histological images of ileal villi in broilers at 11 days post-inoculation, 40× magnification. Note the different structures of *Eimeria* (yellow arrows) in the ileal mucosa of challenged chickens. HE, bar = 50µm.

weight (p=0.0463). The highest liver weight was observed in unchallenged birds fed the unsupplemented diet. Unchallenged broilers fed the supplemented diet had higher proventriculus weight (10.32%) than challenged broilers fed the same diet (7.32%). There were no significant interaction effects on the weights of the small and large intestine (p=0.2102), cecum (p=0.4303), pancreas (p=0.4598), or gizzard (p=0.2626). Furthermore, there was no effect of microbial challenge on the weights of the liver (p=0.4625), small and large intestine (p=0.7781), cecum (p=0.9455), pancreas (p=0.1220), or gizzard (p=0.0616). Diet did not significantly affect the weights of the evaluated organs (p>0.05).

There were no significant interaction effects of diet and microbial challenge on breast weight (p=0.0797) or thigh + drumstick weight (p=0.9964) in chickens aged 42 days (Table 6). However, microbial challenge significantly affected breast weight (p=0.0001) and thigh + drumstick weight (p=0.0004). Challenged broilers had a 15% lower breast weight and 14% lower thigh + drumstick weight than unchallenged broilers (Table 6). It was also found that diet influenced breast weight (p=0.0003). Birds fed the blend-supplemented diet had a higher

Table 2. Main and interaction effects of dietary supplementation and microbial challenge with *Eimeria* spp., *Salmonella* Minnesota, *Escherichia coli*, and *Clostridium perfringens* on the performance of broiler chickens in the starter phase (1-21 days of age)

Challenge group	Diet group	Final live weight (kg)	Feed intake (kg)	Weight gain (kg)	Feed conversion
UB	UD	0.87 ± 0.02	1.16 ± 0.07	0.81 ± 0.06	1.45 ± 0.04
	SD	0.90 ± 0.04	1.14 ± 0.02	0.85 ± 0.04	1.32 ± 0.02
CB	UD	0.68 ± 0.05	1.09 ± 0.02	0.64 ± 0.05	1.74 ± 0.12
	SD	0.72 ± 0.07	1.07 ± 0.07	0.68 ± 0.07	1.58 ± 0.06
Main effects					
Microbial challenge	UB	0.88^{a} ± 0.03	1.15^{a} ± 0.05	0.83^{a} ± 0.05	1.39^{b} ± 0.08
	CB	0.70^{b} ± 0.06	1.08^{b} ± 0.04	0.66^{b} ± 0.06	1.66^{a} ± 0.12
Diet	UD	0.78 ± 0.11	1.12 ± 0.06	0.72 ± 0.10	1.59^{a} ± 0.18
	SD	0.81 ± 0.11	1.11 ± 0.06	0.77 ± 0.11	1.45^{b} ± 0.15
p-value					
Microbial challenge		0.0002	0.0426	0.0007	0.0002
Diet		0.3123	0.5723	0.2286	0.0067
Microbial challenge × Diet		0.8459	0.9494	0.8536	0.7332

UB = unchallenged broilers, CB = challenged broilers, UD = unsupplemented diet, SD = diet supplemented with a microencapsulated blend of organic acids, cinnamon essential oil, oregano essential oil, eugenol, thymol, curcumin, tannins, vitamin E, and zinc (Sannimix); ^{a,b} Means within columns followed by the same lowercase letter are not significantly different by Tukey's test or Student's t-test (p<0.05). Results are presented as mean and standard deviation. Each cage of six birds was treated as an experimental unit (n=8 cages per treatment).

Table 3. Main and interaction effects of dietary supplementation and microbial challenge with *Eimeria* spp., *Salmonella* Minnesota, *Escherichia coli*, and *Clostridium perfringens* on the performance of broiler chickens in the grower/finisher phase (22-42 days of age)

Challenge group	Diet group	Final live weight (kg)	Feed intake (kg)	Weight gain (kg)	Feed conversion
UB	UD	2.92 ± 0.16	3.35 ± 0.22	1.84 ± 0.06	1.82 ± 0.07
	SD	2.61 ± 0.17	3.26 ± 0.11	1.80 ± 0.04	1.82 ± 0.04
CB	UD	2.57 ± 0.10	3.17 ± 0.08	1.88 ± 0.11	1.69 ± 0.08
	SD	2.87 ± 0.19	3.21 ± 0.21	1.90 ± 0.09	1.69 ± 0.03
Main effects					
Microbial challenge	UB	2.90^{a} ± 0.16	3.30 ± 0.16	1.82 ± 0.05	1.82^{a} ± 0.05
	CB	2.59^{b} ± 0.13	3.19 ± 0.15	1.89 ± 0.09	1.69^{b} ± 0.06
Diet	UD	2.72 ± 0.22	3.26 ± 0.18	1.86 ± 0.08	1.76 ± 0.10
	SD	2.77 ± 0.23	3.24 ± 0.15	1.85 ± 0.09	1.75 ± 0.08
p-value					
Microbial challenge		0.0101	0.2850	0.1580	0.0059
Diet		0.6070	0.7975	0.8381	0.8896
Microbial challenge × Diet		0.9775	0.5360	0.4709	0.9580

UB = unchallenged broilers, CB = challenged broilers, UD = unsupplemented diet, SD = diet supplemented with a microencapsulated blend of organic acids, cinnamon essential oil, oregano essential oil, eugenol, thymol, curcumin, tannins, vitamin E, and zinc (Sannimix); ^{a,b} Means within columns followed by the same lowercase letter are not significantly different by Tukey's test or Student's t-test (p<0.05). Results are presented as mean and standard deviation. Each cage of six birds was treated as an experimental unit (n=6 cages per treatment).

breast weight (0.74kg) than birds fed the unsupplemented diet (0.65kg) (Table 6). Thus, the blend afforded a 15% increase in breast yield. There was no significant effect of diet on thigh + drumstick weight (p=0.9099) (Table 6).

Ileal morphometry of 42-day-old broilers

Diet and microbial challenge exerted significant interaction effects on ileal villus height (p<0.0001) and crypt depth (p<0.0001) in 42-day-old broilers (Table 7). Unchallenged birds fed the supplemented diet, and challenged birds fed the unsupplemented diet had higher villus height and crypt depth (Table 7). There was no significant interaction effect on villus/crypt ratio (p=0.4717), and the main effects of diet (p=0.1288) and microbial challenge (p=0.2893) were not significant (Table 7).

Table 4. Main and interaction effects of dietary supplementation and microbial challenge with *Eimeria* spp., *Salmonella* Minnesota, *Escherichia coli*, and *Clostridium perfringens* on the spleen, bursa of Fabricius, and heart weights of broiler chickens at 42 days of age

Challenge group	Diet group	Spleen (g)	Bursa of Fabricius (g)	Heart (g)
UB	UD	2.51 ± 0.40	2.61^{b} ± 0.73	13.19 ± 1.40
	SD	2.50 ± 0.65	4.07^{a} ± 1.41	15.10 ± 2.14
CB	UD	2.71 ± 0.88	3.79^{b} ± 0.78	12.90 ± 2.10
	SD	2.40 ± 0.24	3.19^{b} ± 1.05	12.20 ± 1.10
Main effects				
Microbial challenge	UB	2.52 ± 0.52	3.34 ± 1.32	14.14^{a} ± 1.99
	CB	2.56 ± 0.64	3.49 ± 0.93	12.55^{b} ± 1.61
Diet	UD	2.61 ± 0.66	3.20 ± 0.95	13.05 ± 1.69
	SD	2.46 ± 0.47	3.63 ± 1.27	13.65 ± 2.21
p-value				
Microbial challenge		0.8695	0.7337	0.0350
Diet		0.5380	0.3150	0.4044
Microbial challenge × Diet		0.5166	0.0231	0.0788

UB = unchallenged broilers, CB = challenged broilers, UD = unsupplemented diet, SD = diet supplemented with a microencapsulated blend of organic acids, cinnamon essential oil, oregano essential oil, eugenol, thymol, curcumin, tannins, vitamin E, and zinc (Sannimix); ^{a,b} Means within columns followed by the same lowercase letter are not significantly different by Tukey's test or Student's *t*-test (p<0.05). Results are presented as mean and standard deviation. Each cage of six birds was treated as an experimental unit (n=6 cages per treatment).

Table 5. Main and interaction effects of dietary supplementation and microbial challenge with *Eimeria* spp., *Salmonella* Minnesota, *Escherichia coli*, and *Clostridium perfringens* on the liver, intestine, cecum, pancreas, proventriculus, and gizzard weights of broiler chickens at 42 days of age

Challenge group	Diet group	Liver (g)	Small and large intestine (g)	Cecum (g)	Pancreas (g)	Proventriculus (g)	Gizzard (g)
UB	UD	47.50^{a} ± 7.10	66.57 ± 4.15	7.23 ± 1.02	4.38 ± 0.45	8.29^{ab} ± 1.43	30.79 ± 5.09
	SD	41.93^{b} ± 3.54	70.37 ± 5.31	7.92 ± 1.17	5.00 ± 0.77	10.32^{a} ± 2.52	32.71 ± 2.98
CB	UD	41.69^{b} ± 3.86	69.20 ± 8.94	7.66 ± 1.92	4.17 ± 0.55	8.01^{ab} ± 0.90	29.55 ± 3.79
	SD	44.75^{ab} ± 4.21	66.25 ± 6.14	7.41 ± 1.45	4.41 ± 0.58	7.32^{b} ± 0.81	28.02 ± 2.20
Main effects							
Microbial challenge	UB	44.71 ± 6.09	68.47 ± 4.96	7.57 ± 1.11	4.69 ± 0.69	9.31 ± 2.22	31.75 ± 4.10
	CB	43.22 ± 4.17	67.72 ± 7.45	7.53 ± 1.63	4.29 ± 0.56	7.67 ± 0.89	28.78 ± 3.06
Diet	UD	44.60 ± 6.24	67.88 ± 6.78	7.44 ± 1.48	4.28 ± 0.50	8.15 ± 1.15	30.17 ± 4.33
	SD	43.34 ± 3.99	68.31 ± 5.88	7.66 ± 1.29	4.70 ± 0.72	8.82 ± 2.37	30.36 ± 3.50
p-value							
Microbial challenge		0.4625	0.7781	0.9455	0.1220	0.0186	0.0616
Diet		0.5359	0.8714	0.7085	0.0959	0.3097	0.8961
Microbial challenge × Diet		0.0430	0.2102	0.4303	0.4598	0.0463	0.2626

UB = unchallenged broilers, CB = challenged broilers, UD = unsupplemented diet, SD = diet supplemented with a microencapsulated blend of organic acids, cinnamon essential oil, oregano essential oil, eugenol, thymol, curcumin, tannins, vitamin E, and zinc (Sannimix); ^{a,b} Means within columns followed by the same lowercase letter are not significantly different by Tukey's test or Student's *t*-test (p<0.05). Results are presented as mean and standard deviation. Each cage of six birds was treated as an experimental unit (n=6 cages per treatment).

Quantification of bacteria and identification of *Salmonella* spp. in the cecum

As shown in Figure 9, both challenged and unchallenged birds (42 days of age) fed the blend-supplemented diet showed a reduction in *Escherichia coli*, enterobacteria, and sulfite-reducing clostridia counts in the cecum. *Clostridium perfringens* count was lower in challenged broilers fed the supplemented diet than in the unsupplemented diet. Within the unchallenged group, *C. perfringens* count was lowest in unsupplemented broilers. The cecum of challenged supplemented birds was found to contain *Salmonella* Yoruba, and that of challenged unsupplemented birds was found to contain *Salmonella* Minnesota. In unchallenged broilers, regardless of the diet, *Salmonella* Corvallis was identified in the cecum. As expected, challenged broilers had higher counts of *E. coli*, enterobacteria, *C. perfringens*, and sulfite-reducing clostridia than unchallenged broilers (Fig.10).

Table 6. Main and interaction effects of dietary supplementation and microbial challenge with *Eimeria* spp., *Salmonella* Minnesota, *Escherichia coli*, and *Clostridium perfringens* on the breast and thigh + drumstick weights of broiler chickens at 42 days of age

Challenge group	Diet group	Breast (g)	Thigh + drumstick (g)
UB	UD	0.68 ± 0.06	0.57 ± 0.04
	SD	0.81 ± 0.08	0.58 ± 0.05
CB	UD	0.62 ± 0.03	0.50 ± 0.05
	SD	0.66 ± 0.04	0.50 ± 0.04
Main effects			
Microbial challenge	UB	0.75[a] ± 0.10	0.58[a] ± 0.04
	CB	0.64[b] ± 0.04	0.50[b] ± 0.03
Diet	UD	0.65[b] ± 0.06	0.54 ± 0.06
	SD	0.74[a] ± 0.10	0.54 ± 0.06
p-value			
Microbial challenge		0.0001	0.0004
Diet		0.0003	0.9099
Microbial challenge × Diet		0.0797	0.9964

UB = unchallenged broilers, CB = challenged broilers, UD = unsupplemented diet, SD = diet supplemented with a microencapsulated blend of organic acids, cinnamon essential oil, oregano essential oil, eugenol, thymol, curcumin, tannins, vitamin E, and zinc (Sannimix); [a,b] Means within columns followed by the same lowercase letter are not significantly different by Tukey's test or Student's *t*-test ($p<0.05$). Results are presented as mean and standard deviation. Each cage of six birds was treated as an experimental unit ($n=6$ cages per treatment).

Table 7. Main and interaction effects of dietary supplementation and microbial challenge with *Eimeria* spp., *Salmonella* Minnesota, *Escherichia coli*, and *Clostridium perfringens* on the ileal morphometry of broiler chickens at 42 days of age

Challenge group	Diet group	Villus height (mm)	Crypt depth (mm)	Villus/crypt ratio
UB	UD	0.76[b] ± 0.15	0.09[c] ± 0.03	9.14 ± 3.48
	SD	0.99[a] ± 0.16	0.12[a] ± 0.03	8.32 ± 2.32
CB	UD	1.02[a] ± 0.25	0.12[a] ± 0.03	8.49 ± 2.22
	SD	0.80[b] ± 0.11	0.10[b] ± 0.02	8.20 ± 2.10
Main effects				
Microbial challenge	UB	0.87 ± 0.19	0.10 ± 0.03	8.34 ± 2.16
	CB	0.91 ± 0.22	0.11 ± 0.02	8.73 ± 2.97
Diet	UD	0.89 ± 0.24	0.11 ± 0.03	8.82 ± 2.92
	SD	0.89 ± 0.16	0.11 ± 0.03	8.26 ± 2.20
p-value				
Microbial challenge		0.1211	0.0613	0.2893
Diet		0.9321	0.1287	0.1288
Microbial challenge × Diet		<0.0001	<0.0001	0.4717

UB = unchallenged broilers, CB = challenged broilers, UD = unsupplemented diet, SD = diet supplemented with a microencapsulated blend of organic acids, cinnamon essential oil, oregano essential oil, eugenol, thymol, curcumin, tannins, vitamin E, and zinc (Sannimix); [a,b] Means within columns followed by the same lowercase letter are not significantly different by Tukey's test or Student's *t*-test ($p<0.05$). Results are presented as mean and standard deviation. Each cage of six birds was treated as an experimental unit ($n=6$ cages per treatment).

DISCUSSION

The growing concern about antibiotic-resistant bacteria and the transfer of antibiotic waste into food intended for human consumption led to the banning of antibiotic growth promoters in animal production; as a result, there has been an increase in enteric diseases (Bade et al. 2021). Alternative strategies, such as the use of probiotics, prebiotics, functional nutrients (e.g., vitamin E, zinc), phytogenic additives (e.g., essential oils, curcumin, tannins), and organic acids, have been extensively investigated (Yang et al. 2018, Bortoluzzi et al. 2020, Akter et al. 2022). In this study, we evaluated the dietary effects of a blend of organic acids, essential oils (cinnamon and oregano), eugenol, thymol, curcumin, tannins, vitamin E, and zinc, microencapsulated in vegetable fat on the performance parameters of broiler chickens challenged with *Eimeria* spp., *Salmonella* Minnesota, *Clostridium perfringens*, and *Escherichia coli* at 1-21 and 22-42 days of age.

There were no significant effects of diet on live weight, feed intake, or weight gain in either growth stage (1-21 and 22-42 days). However, diet influenced the feed conversion ratio in the starter phase (1-21 days of age). Broilers fed the supplemented diet had a lower feed conversion ratio, which is considered a positive effect. Therefore, the feed efficiency of supplemented birds was about 9% higher than that of unsupplemented birds. This result may be attributed to the numerous functions of blend constituents, such as digestion stimulation, increased release of endogenous digestive enzymes, maintenance of intestinal morphology and integrity, and modulation of intestinal microbiota (Khan & Iqbal 2016, Yang et al. 2018, Iqbal et al. 2021, Ma et al. 2021).

Exposure of broilers to *Eimeria* spp., *S.* Minnesota, *E. coli*, and *C. perfringens* caused a significant reduction in final live weight (21 days), feed intake, and weight gain in the starter phase. Furthermore, feed conversion ratio increased by 20% compared with unchallenged birds. A reduction in feed intake was expected in challenged chickens; under these conditions, the intake of essential nutrients is insufficient, limiting development (Miska & Fetterer 2018).

The reduced performance of challenged broilers was possibly related to structural and functional changes caused by protozoa and bacteria in the intestinal mucosa during infection (Paris & Wong 2013, Gottardo et al. 2016, Feng et al. 2022). As a result, digestion and nutrient absorption are impaired, leading to decreased performance. According to Gottardo et al. (2016), *Eimeria* infection is a conditional factor for necrotic enteritis because of the destruction of mucous mass. The structural and functional alterations caused by *Eimeria* infection compromise the natural barrier function of the intestinal environment, facilitating colonization by various other pathogens, such as *C. perfringens*, *E. coli*, and *Salmonella* (Qin et al. 1995, Gottardo et al. 2016, Feng et al. 2022), further impairing performance. Such conditions increase energy expenditure for the renewal of intestinal cells and activation of immune responses, worsening the feed conversion ratio.

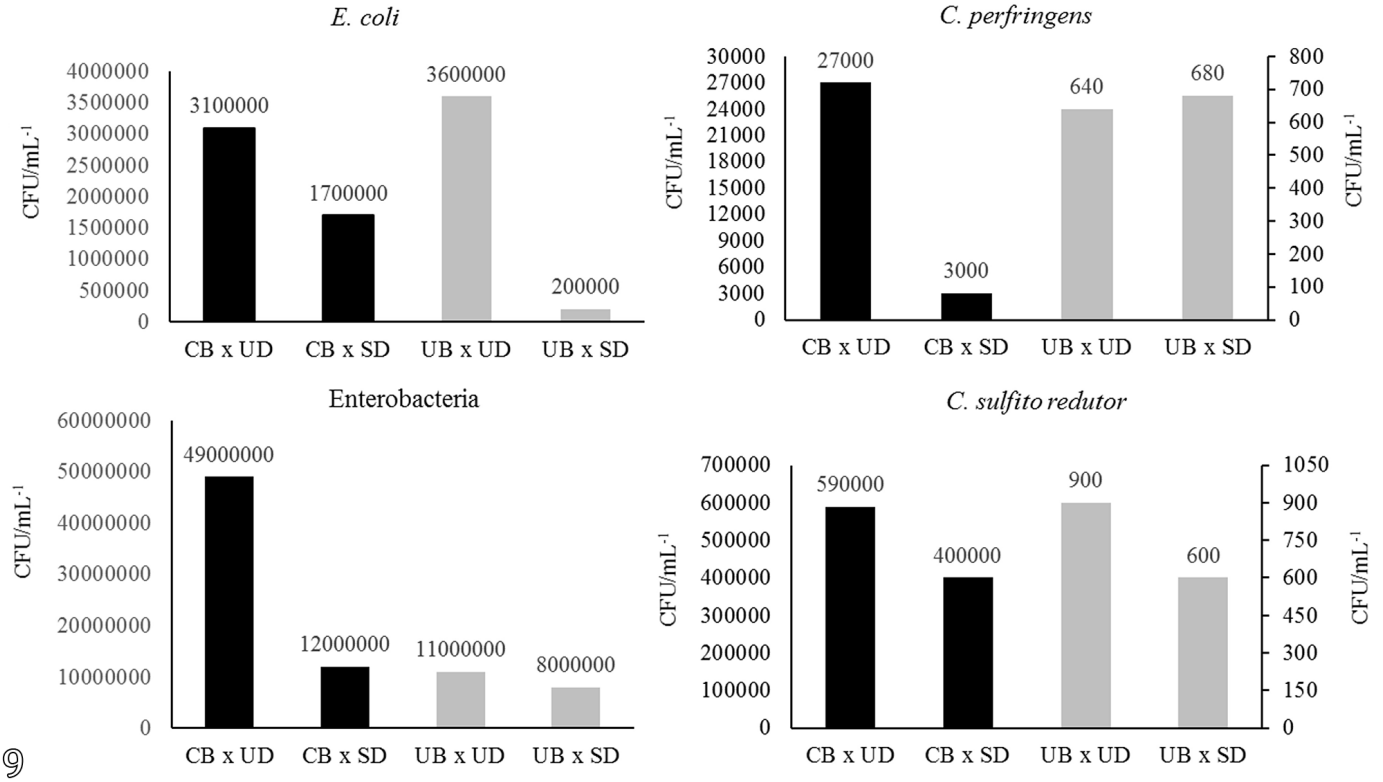

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Fig.9. Effects of microbial challenge and diet on the counts of *Escherichia coli*, enterobacteria, *Clostridium perfringens*, and sulfite-reducing clostridia in the cecum of broilers aged 42 days. The results are descriptive and expressed as colony-forming units (CFU) mL^{-1}. CB = broilers challenged with *Eimeria* spp., *C. perfringens*, *E. coli*, and *Salmonella* Minnesota; UB = unchallenged broilers; UD = unsupplemented diet; SD = diet supplemented with a blend (Sannimix) of organic acids, essential oils (cinnamon and oregano), eugenol, thymol, curcumin, tannins, vitamin E, and zinc, microencapsulated in vegetable fat.

Part of the nutrients and energy obtained through the diet is diverted to repairing the intestinal mucosa (Maiorka 2004).

Regarding the grower/finisher phase (22-42 days), challenged broilers had lower live weight, feed conversion ratio, breast weight, and thigh + drumstick weight. The feed conversion ratio expresses the efficiency with which animals convert consumed feed into live weight. Our results suggest that challenged broilers diverted the absorbed nutrients to other physiological and metabolic processes, not necessarily those related to muscle mass deposition, which became evident by the decrease in live weight and weight of primal cuts. Microbial analysis of the cecum of 42-day-old birds showed that even after 24 days post-inoculation, the bacterial load of challenged birds was higher than that of unchallenged birds. Given these results, it can be said that when birds are affected in the starter phase, they may not recover promptly, leading to increased costs in control measures and losses in protein yield in the finisher phase.

Adding the blend to broiler diets led to an increase in breast weight. Essential oils, organic acids, tannins, vitamin E, and zinc are important nutritional components with antioxidant activity (Song et al. 2017, Qui 2023, Xu et al. 2023). These nutrients can interact and neutralize oxidative substances and reduce oxygen concentration, preventing the production of oxidative species; furthermore, they activate enzymes involved in the antioxidant defense system (Oteiza et al. 1996, Rizvi et al. 2014, Kruk et al. 2022, Piao et al. 2023). It is widely known that increased levels of reactive oxygen species are one of the main causes of reduced meat yield and quality. Oxidative stress leads to a high protein degradation rate, consequently reducing meat's total protein content and essential amino acid content (Thanatsang et al. 2020). The blend not only improved the conversion ratio of feed into meat by influencing various metabolic mechanisms but also enhanced the antioxidant status of birds, controlling protein oxidation and consequently resulting in increased breast yield.

Orlowski et al. (2018) demonstrated that phytogenic additives improve breast muscle mass and reduce fat percentage. Flees et al. (2021) observed that chickens supplemented with phytogenic additives via water exhibited increased expression of the target of the rapamycin (*mTOR*) gene in muscle. *mTOR* activation by the additive led to an increase in protein synthesis, resulting in higher breast yield. Orlowski et al. (2018) and Flees et al. (2021) argued that phytogenic additives can increase muscle protein synthesis and reduce fat deposition via modulation of the peripheral intermediate metabolism, increasing adipose tissue lipolysis and favoring muscle protein synthesis over hepatic lipogenesis.

The current study also evaluated the blend's effect and microbial challenge's effect on organ weight (liver, spleen, heart, bursa of Fabricius, small and large intestine, cecum, pancreas, proventriculus, and gizzard). Microbial challenge reduced heart weight in 42-day-old chickens, indicating a possible negative effect of these bacteria (*S.* Minnesota, *E.*

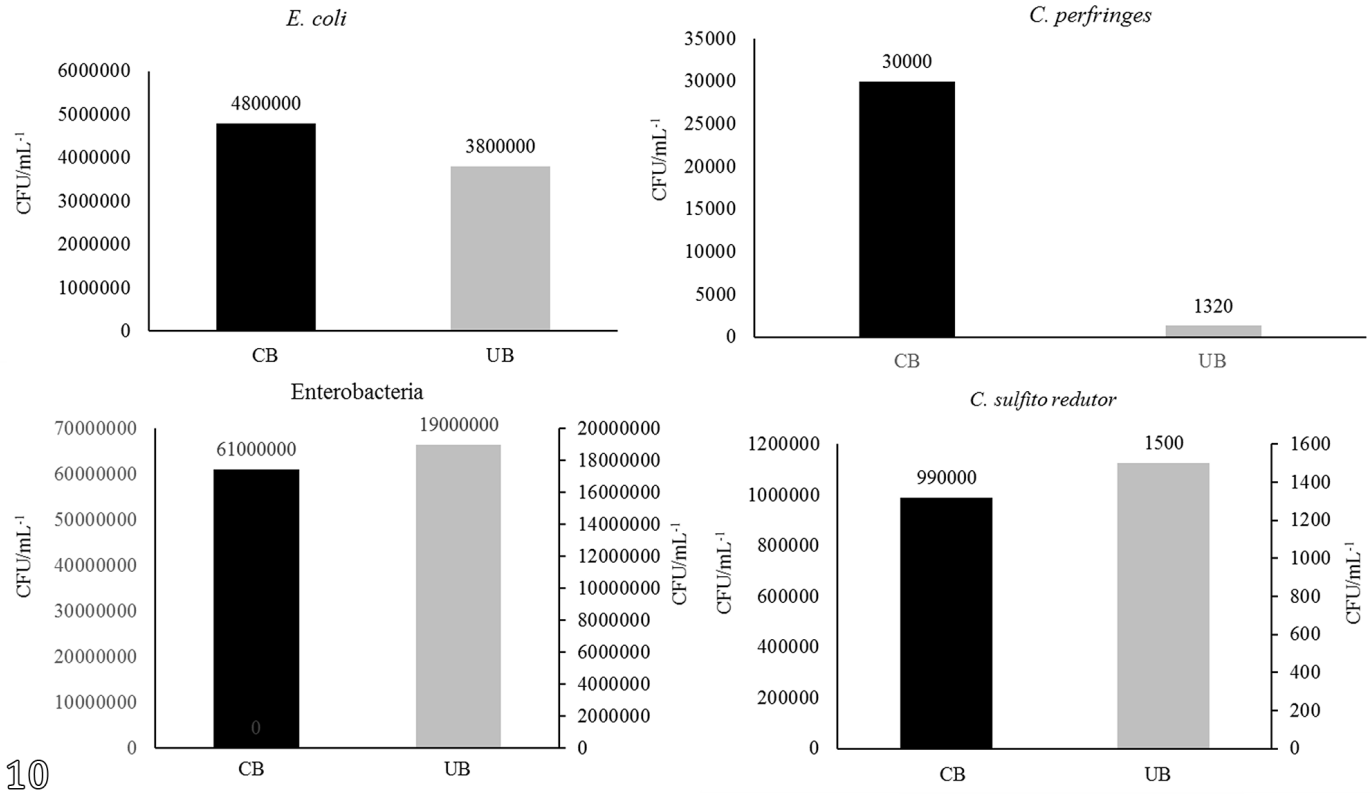

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Fig.10. Effects of microbial challenge on the counts of *Escherichia coli*, enterobacteria, *Clostridium perfringens*, and sulfite-reducing clostridia in the cecum of broilers aged 42 days. The results are descriptive and expressed as colony-forming units (CFU) mL^{-1}. CB = broilers challenged with *Eimeria* spp., *C. perfringens*, *E. coli*, and *Salmonella* Minnesota; UB = unchallenged broilers; UD = unsupplemented diet; SD = diet supplemented with a blend (Sannimix) of organic acids, essential oils (cinnamon and oregano), eugenol, thymol, curcumin, tannins, vitamin E, and zinc, microencapsulated in vegetable fat.

coli, and *C. perfringens*) on the cardiovascular health of birds. Furthermore, unchallenged broilers fed the unsupplemented diet had higher liver weight. The liver is one of the main organs responsible for metabolism and is the major organ for detoxification and immune defense (Zhang et al. 2019b). The liver's size depends on how much work it performs (Parsaie et al. 2007). Thus, the higher liver weight in these birds might be related to greater liver activity in the body's different metabolic processes stimulated by blended components.

The highest bursa of Fabricius weight was observed in unchallenged broilers fed the supplemented diet. The weight of lymphoid organs can be used as an indication of the body's ability to supply lymphoid cells during immune responses (Willis et al. 2013). According to Willis et al. (2013), in chickens, the weight of the bursa of Fabricius reflects the anatomical response to the alteration of the immune system during stress. A lower weight of this organ has been associated, among other causes, with organ atrophy caused by lymphocyte depletion or micronecrosis and cell migration (Nakamura et al. 1986). On the other hand, a higher organ weight indicates better health status and reduced immunosuppression (Willis et al. 2013). Therefore, it is suggested that the higher bursa of Fabricius weight observed in supplemented chickens indicates better immunological and health conditions attributed to the immunomodulatory effect (Huang et al. 2018, Hidayati et al. 2020, Shojadoost et al. 2021, Phillips et al. 2023) of the essential oils, organic acids, vitamin E, and zinc present in the blend.

Valdivieso-Ugarte et al. (2019) stated that the immunomodulatory activity of essential oils may be attributed to their ability to modify cytokine secretion, which probably occurs through the regulation of NF-κB and the MAPK signaling pathway or through their ability to influence the expression of inducible nitric oxide synthase (iNOS) and prostaglandin secretion. Yang et al. (2018) showed that supplementing broiler diets with essential oils and organic acids increased immunoglobulin A levels in duodenal and ileal mucosa. According to Lee & Han (2018), dietary vitamin E supplementation can increase lymphocyte proliferation, immunoglobulin levels, antibody responses, natural killer cell activity, and interleukin 2 production. Khatun et al. (2020) demonstrated that dietary supplementation with essential oils, arginine, and vitamin E altered the expression of cytokines that can positively influence immune function in broilers. Cui et al. (2004) suggested that zinc strongly influences broilers' immune system; zinc deficiency limits lymphoid organ development and the population of mature T lymphocytes in the blood. Yuan et al. (2023) found that tannin supplementation improved the immune function of broilers challenged with lipopolysaccharides, as evidenced by increased serum concentrations of immunoglobulins A and M.

We observed that unchallenged broilers fed the supplemented diet had higher proventriculus weight and higher ileal villus height and crypt depth. According to Assis et al. (2021), a higher percentage of organs responsible for digestion (e.g., proventriculus and gizzard) and nutrient metabolism (e.g., liver and ileum) is favorable for increased feed use efficiency; in other words, under these conditions, birds can perform better. Villus height and crypt depth are important measurements for assessing gut health and functionality. Higher villus height and crypt depth generally indicate a greater capacity for absorption and cell renewal, reflecting increased intestinal function (Bravo et al. 2014). The major function of the ileum is to absorb water and minerals (Richards-Rios et al. 2020). Although the ileum itself does not play a significant role in amino acid absorption, the ileal microbiota contributes greatly to this function (Richards-Rios et al. 2020). The intestinal microbiota also contributes to maintaining intestinal homeostasis, promoting epithelial renewal, and providing defense against opportunistic pathogens (Feitosa et al. 2020). Furthermore, the microbiota influences intestinal motility and nutrient absorption (Kogut 2019). According to Abdelli et al. (2021), the performance of production animals is related to intestinal health and functionality, which are continuously modulated by diet, intestinal integrity, intestinal microbiota, and the immune system. Phytogenic additives, organic acids, and several so-called functional nutrients contribute to maintaining the integrity of the intestinal environment and microbiota. Assessment of the cecal bacterial load of 42-day-old chickens further demonstrated the beneficial immunomodulatory and antibacterial effects of the blend, as fewer pathogens were detected in the cecum of challenged and unchallenged broilers fed the blend. Thus, our results suggest that blend supplementation can improve digestion, nutrient absorption, and immune response by modulating different organs and metabolic pathways in healthy, unchallenged birds.

CONCLUSION

The microbial challenge, caused by *Eimeria* spp., *Salmonella* Minnesota, *Clostridium perfringens*, and *Escherichia coli*, hindered broiler performance during growth (1-21 days) and decreased the yield of primal cuts (42 days). Dietary supplementation with a blend of essential oils, organic acids, and nutrients improved feed conversion ratio in the starter phase and primal cut yield in the finisher phase. However, the blend did not have significant effects on challenged birds, indicating that its effects may depend on the type of microbial challenge, the composition of bioactive compounds, and the level of dietary supplementation. Further research is needed to understand these effects better.

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