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Intestinal health improvement with protected organic acids and essential oils for pullets raised under field conditions

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We investigated the dietary supplementation of protected organic acids and essential Oils P(OA+EO) in pullets raised under commercial conditions. A total of 208,000 pullets Lohmann at 2-weeks-old were assigned to receive 1 of 2 treatments: T1, control diet used in the farm; T2, control diet and P(OA+EO) at 300 g/t (fumaric, sorbic, citric and malic acids + thymol, eugenol and vanillin microencapsulated in hydrogenated vegetable fat - Jefo Nutrition Inc. Canada). P(OA+EO) was supplemented from 2 to 18 weeks and the trial lasted 21 weeks. At weeks 6, 12 and 21, 12 pullets/treatment were used for blood sampling and necropsy for *ISI – I See Inside*. A completely randomized design consisting of 2 treatments, each with 12 replicates of 1 hen/replicate, was used. To evaluate intestinal integrity, birds were inoculated with fluorescein-isothiocyanate labelled dextran (FITC-d) and blood samples were collected after 1.5 h. The macroscopic *ISI* score of alterations were classified to be presented as: overall health *ISI* (sum of the scores assigned for intestine, liver, proventriculus, annex glands, locomotor, and respiratory systems) and macro-intestinal *ISI* (sum of the scores assigned for duodenum, jejunum, ileum, and cecum). The histologic intestinal *ISI* alterations were evaluated in the ileum. A low *ISI* index represents better health status. Pullets on P(OA+EO) had lower ($P< 0.001$) levels of FITC-d recovered in the blood, which is related to reduced leaky gut. They also presented lower overall health *ISI* score at weeks 6 ($P = 0.002$) and 12 ($P = 0.003$), lower macro intestinal *ISI* score at weeks 6 ($P = 0.0001$) and 21 ($P = 0.004$) and, lower histologic intestinal *ISI* score of alterations at weeks 6 ($P = 0.09$), 12 ($P = 0.0006$), and 21 ($P< 0.0001$), which is associated to better overall health. In addition, at week 21, pullets on P(OA+EO) did not present *Eimeria* oocysts while the control treatment did ($P< 0.0001$). In conclusion, the

blend of protected organic acids and essential oils evaluated can be used to improve intestinal and overall health status in commercial pullets.

KEYWORDS

essential oil, intestinal health, intestinal permeability, organic acid, pullets

Introduction

The optimal productive performance in laying hens is closely correlated with gastrointestinal functionality and health. A more persistent laying period depends on proper intestinal development and maintenance of microbiota homeostasis. However, the early stages of a pullet's life are considered the most critical, a period characterized by a series of morphological and physiological changes, followed by hormonal changes and immunosuppression. In addition, under commercial rearing conditions, the intestinal environment is constantly exposed to various stimuli inherent to the environment, pathogens, and certain dietary components that result in an unbalanced intestinal ecosystem (a.k.a. dysbiosis) and may lead to a subsequent drop in layer performance (Cardoso Dal Pont et al., 2020). Over several decades, antibiotic growth promoters (AGP) have been used in commercial poultry production to alleviate the negative effects of these stimuli, maintaining the health and boosting production, mainly through modulating the microbiota and/or reducing the level of inflammation and immune stress (Niewold, 2007). However, the current reduction and/or withdrawal of these compounds from the poultry diets has triggered a search for natural strategies capable of maintaining the intestinal health of birds.

Among the most studied natural feed additives are organic acids (OA) and essential oils (EO), which represent a good alternative tool to be used in animal production. Organic acids have long been used as to improve growth performance of poultry. There are many ways that short chain organic acids can work to improve performance and health in the host. According to a review of D'Aquila et al. (2020) OA act as signaling molecules that influence diverse regulatory functions on host physiology, metabolism regulation, inflammation, and immunity. They also have antimicrobial benefits against some microorganism such as *E. coli*, *Salmonella* and *Campylobacter* (Khan and Iqbal, 2016; Polycarpo et al., 2017). Similar to OA, EO have gained interest due to their broad spectrum of antimicrobial, immunomodulatory and antioxidant properties (Gopi et al., 2014). The combination of the lipophilic nature of OA with the hydrophobic nature of EO can result in a synergistic effect, being more efficacious in improving intestinal health and modulating the microbiota (Stefanello et al., 2020; Adewole et al., 2021). Organic acids and EO in free form may be lost during feed processing, lose their activity, or be absorbed early in the gastrointestinal tract (GIT), such as the stomach and the proximal small intestine (Michiels et al., 2008; Choi et al., 2020). The microencapsulation of OA and EO using a lipid matrix offers the potential to not only protect them, but also to control their subsequent release as they pass through the GIT, maximizing their effectiveness (Hassan et al., 2018).

Recent studies that investigated the beneficial effects of protected OA and EO blends in broiler diets have reported their efficacy to improve growth performance and intestinal morphology (Liu et al., 2017; Yang et al., 2018), to improve intestinal health and barrier function (Stefanello et al., 2020; Bortoluzzi et al., 2021), to decrease *ISI* score for macro- and histologic alterations (Stefanello et al., 2020) and to increase enzyme activity (Yang et al., 2018; Yang et al., 2019). However, to our knowledge, only few studies have investigated the effect of the association of protected OA and EO in the pullet phase. Therefore, the objective of this study was to investigate the dietary supplementation of a protected blend of organic acids and essential oils P(OA+EO) on the intestinal health of pullets raised under commercial conditions until the beginning of production.

Materials and methods

Birds, housing, and treatments

The study was conducted in a commercial farm with a natural climate environment located in Primavera do Leste, Mato Grosso, Brazil. A total of 208,000 pullets Lohmann (2 weeks old) were vaccinated against coccidiosis at the hatchery and distributed in two barns in metal cages (13 birds per cage – 370 cm^2 per bird) with wire floor and linear feeders and nipple drinkers. The photoperiod during the trial was natural lightening. Each barn was randomly assigned to receive 1 of 2 treatments (T), as follows: T1, Control diet used in the farm; T2, control diet supplemented with a blend of protected organic acids and essential oils [P(OA+EO)] at 300 g/t. The P(OA+EO) consists of fumaric, citric, malic, and sorbic acids plus thymol, vanillin, and eugenol microencapsulated in a matrix of

triglycerides from hydrogenated vegetable oil (Jefo Nutrition Inc., Saint-Hyacinthe, QC, Canada) and the dosage used was according to the manufacture´s recommendation. The supplementation with P(OA+EO) was done from 2 to 18 weeks of age. Both treatments were supplemented with enramycin (10 and 5 ppm for pre-starter and starter phases, respectively). No additional antibiotic was used during the entire trial (21 weeks). At week 17 of age, all birds were transferred to production cages (wire floor, with linear feeder and nipple drinker). A four-phase feeding program was used with (starter, grower, developer, pre-laying) diets formulated according to genetic line recommendations. Water and feed in a mash form was provided *ad libitum*.

Samples collected

At weeks 6, 12 and 21 of age, 12 birds per treatment received fluorescein isothiocyanate-dextran (FITC-d) by oral gavage and after 1.5h, blood samples were collected to determine the serum concentration of FITC-d. After this time, they were euthanized by cervical dislocation, necropsied for macroscopic analysis, and to collect a section of ileum for histologic analysis.

Intestinal integrity – FITC-d analysis

Birds were orally gavaged with 0.2 mL/bird of FITC-d (3–5 kDa; 4,000 mol weight; Sigma-Aldrich, Brazil) dissolved in reverse osmosis water. Blood samples were collected 1.5 h after oral gavage. To detect FITC-d levels in the serum, the blood samples were kept at room temperature for 3 h to allow clotting, and centrifuged (500 × g for 15 min) to separate the serum. Fluorescence levels of diluted serum (1:1 in phosphate buffered saline, PBS) were measured at an excitation wavelength of 485 nm and emission wavelength of 528 nm (SpeedScan, Analytik Jena AG). The FITC-d concentration (µg/mL) in the serum was calculated based on a standard curve (Vicuña et al., 2015). FITC-d is a molecule with high molecular weight that is only detected in the blood when the intestinal mucosa is damaged, presenting a higher permeability.

Overall and intestinal health – macroscopic and histopathologic *I See Inside* analysis

The *I See Inside* (*ISI*) methodology was applied using the *ISISys* app (www.isiinstitute.com). Briefly, each organ alteration is scored from 0–3. The scores are based on the intensity of the observed alteration: score 0 (absence of alteration), score 1 (alteration of up to 25% of the area), score 2 (alteration of 25–50% of the area), and score 3 (alteration of more than 50% of the area). For each tissue alteration an impact factor (IF) is defined in the macroscopic (macro) and histologic analysis, according to the reduction of the organ functionality. The IF ranges from 1 to 3, with three being the worst impacting organ function. To obtain the final value of the *ISI* index, the IF of each alteration is multiplied by the respective score number, according to the formula $ISI = \Sigma(IF \times S)$, where IF = impact factor and S = Score. A low *ISI* score represents better health (Kraieski et al., 2017; Belote et al., 2019). The overall health *ISI* score considers the following organs/systems and their respective alterations: skin (foot pad lesions and oral mucosa lesions), locomotor (muscular hemorrhage, femoral head necrosis and tibial dyschondroplasia), respiratory (tracheitis, hydropericardium and airsacculitis), liver (color and size), kidney and pancreas (size), proventriculitis, gizzard erosion, yolk persistence, intestine (morphologic alteration on serosa or mucosa layer, cell debris and red thick mucus on mucosa, necrosis, presence of gas, presence of non-digested feed and *Eimeria* lesions). The macroscopic *ISI* score of alterations were classified to be presented as: macro-intestinal *ISI* (sum of the scores assigned for the 4 sections of the intestine) and overall health *ISI* (sum of the scores assigned for intestine plus other organs and system). For the histological analysis a section of ileum was collected from the distal two-thirds of the ileum (portion of the small intestine from Meckel's diverticulum to approximately 1 cm anterior to the ileo-cecal junction) by flushing with distilled water into plastic containers and immersed into 10% formalin for fixation. The samples were then embedded in paraffin following common histological analysis and stained with hematoxylin and eosin. In the ileum, 20 intact villi/birds were evaluated in 10× magnification (using 20× and 40× magnification to confirm alterations) under optical microscope (Nikon Eclipse E200, Sao Paulo, Brazil), considering the following alterations: lamina propria thickness, epithelial thickness, enterocytes proliferation, inflammatory cell infiltration in the epithelium, inflammatory cell infiltration in the lamina propria, goblet cells proliferation, congestion, and presence of *Eimeria* oocysts.

Statistical analysis

As a first step, data normality was verified using the Shapiro-Wilk normality test. Data were subjected to ANOVA to analyze parametric data (FITC-d data) and Kruskal Wallis to analyze non-parametric data (*ISI* data) using the XLSTAT software. A $P \leq 0.05$ was used to indicate statistical significance and a $P < 0.10$ was used to indicate tendency. The difference between the blood levels of FITC-d was analyzed by the unpaired t test. The statistical model used for variance analysis was:

$$Y_{ij} = \mu + T_i + e_{ij},$$

In which Y_{ij} = observed result, μ = overall mean of the experiment, T_i = treatment effect, and e_{ij} = random error associated with each observation.

Results

Overall health – *ISI* analysis

The overall health *ISI* score represents the sum of the macro intestinal scores and the scores assigned to the other organs and systems. Compared to the control group, pullets on P(OA+EO) presented the lowest overall health *ISI* score at weeks 6 (P = 0.002) and 12 (P = 0.003) (Figure 1). At week 6, this result was due to the lowest score for (P = 0.035) muscle hemorrhage. However, pullets on P(OA+EO) group presented greater score for (P = 0.003) femoral head necrosis (Table 1). At week 12, the lowest total *ISI* score for pullets on P(OA+EO) group was influenced by the lowest scores for muscle hemorrhage (P = 0.07), femoral head necrosis (P = 0.003), yellowish liver (P = 0.07), and airsacculitis (P = 0.001; Table 2).

Macroscopic intestinal health – *ISI* analysis

Pullets on P(OA+EO) had the lowest macro-intestinal *ISI* score in comparison to the control group at weeks 6 (P = 0.0001) and 21 (P = 0.004) (Figure 2), which indicates a better intestinal health status. This result may be attributed to the lowest alteration on serosa or mucosa layers in the duodenum (P = 0.062) and lowest cell debris and reddish on mucosa in the duodenum (P = 0.028), jejunum (P = 0.019) and ileum (P = 0.027) at week 6 (Table 1). Furthermore, the lower morphologic alteration on serosa or mucosa layers in the jejunum (P = 0.030) and lower cell debris and reddish on mucosa in the jejunum (P = 0.002) at week 21 may also explain the lowest macro-intestinal *ISI* score observed (Table 3). At week 12, pullets on P(OA+EO) group had the lowest (P = 0.010) morphologic alteration on the serosa or mucosa layers in the duodenum when compared to the control group (Table 2).

Histologic intestinal health – *ISI* analysis

Compared to the control group, the lowest histologic intestinal health *ISI* score (P = 0.09) of pullets on P(OA+EO) at week 6 was due to the lowest scores observed in the lamina propria thickness (P = 0.03), epithelial thickness (P = 0.08) and epithelial plasma cell infiltration (P = 0.06). At week 12, pullets on P(OA+EO) group presented the lowest histologic intestinal *ISI* score (P = 0.0006) due to the lowest scores observed for lamina propria thickness (P = 0.0002), lamina propria inflammatory infiltration (P = 0.003) and congestion (P< 0.0001). At week 21, pullets on P(OA+EO) had the lowest scores for all the parameters evaluated in the histologic *ISI*, such as, lamina propria thickness (P< 0.0001), epithelial thickness (P = 0.08), enterocytes proliferation (P = 0.05), epithelial plasma cell infiltration (P< 0.0001), lamina propria inflammatory infiltration (P< 0.0001), goblet cells (P = 0.02), congestion (P = 0.06), presence of *Eimeria* oocysts (P< 0.0001), and total intestinal *ISI* (P< 0.0001; Figure 3; Table 4). Alterations

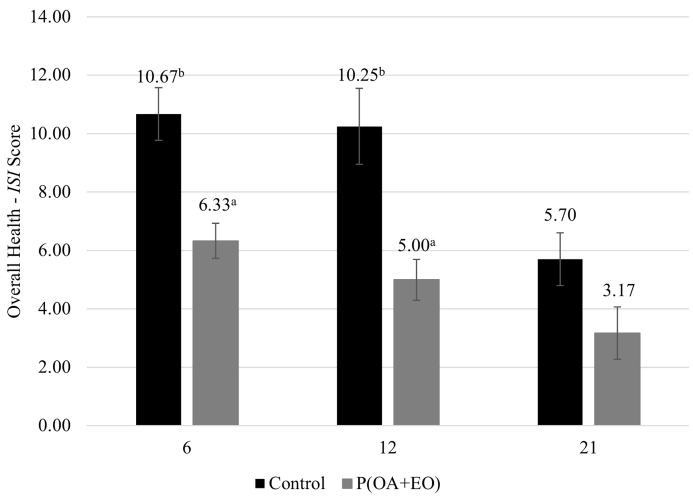

FIGURE 1
Overall health analyzed by the *ISI* methodology (sum of the scores given for intestine, liver, proventriculus, annex glands, locomotor, and respiratory system). Data are the mean value and standard error of each treatment at different ages (weeks). $^{ab}P<0.05$.

TABLE 1 Macroscopic intestinal and overall health – *ISI* score at 6 weeks of age.

Item	Control	P(OA+EO)	SEM	P-value
Duodenum				
Morphological alteration on serosa or mucosa layers	0.92^{B}	0.42^{A}	0.13	0.062
Cell debris and red thick mucus on mucosa	0.50^{b}	0.17^{a}	0.16	0.028
Jejunum				
Morphological alteration on serosa or mucosa layers	0.08	0.00	0.04	0.317
Cell debris and red thick mucus on mucosa	3.17^{b}	1.33^{a}	0.39	0.019
Ileum				
Morphological alteration on serosa or mucosa layers	0.08	0.00	0.04	0.317
Cell debris and red thick mucus on mucosa	1.83^{b}	0.33^{a}	0.34	0.027
Non-digested feed	0.00	0.33	0.12	0.148
Cecum				
Presence of gas	0.08	0.08	0.06	1.000
Total Intestinal health *ISI*	6.67^{b}	2.67^{a}	0.57	0.0001
Other organs and systems				
Muscle hemorrhage	0.67^{b}	0.17^{a}	0.12	0.035
Femoral head necrosis	1.00^{b}	1.92^{a}	0.16	0.003
Red liver	0.17	0.00	0.08	0.317
Tracheitis	0.83	0.75	0.10	0.610
Airsacculitis	0.25	0.25	0.17	1.000
Hydropericardium	0.33	0.50	0.17	0.623
Yolk persistence	0.08	0.00	0.04	0.317
Hypertrophic kidney	0.17	0.08	0.07	0.546
Hypertrophic pancreas	0.33	0.00	0.12	0.148
Gizzard erosion	0.17	0.00	0.06	0.148
Overall Health *ISI*	10.67^{b}	6.33^{a}	0.70	0.002

Only parameters that had alterations are presented.
P(OA+EO), Protected Organic Acids + Essential Oils at 300 g/t.
SEM, Standard error of the mean.
$^{ab}P< 0.05$; $^{AB}P< 0.10$.

in the ileum histology are illustrated as photomicrographs in Figure 4.

Intestinal integrity – FITC-d analysis

Pullets on P(OA+EO) had the lowest ($P< 0.001$) level of FITC-d recovered in the blood in all ages evaluated (6, 12 and 21 weeks of age) compared to the control group (Figure 5), indicating better intestinal integrity and decreased intestinal permeability.

Discussion

Due to the growing restriction to AGP, scientific studies have been conducted to evaluate the effects of alternative feed additives on the intestinal health of pullets during the production phase (Lee et al., 2015; Wang et al., 2019; Feng et al., 2021). However, few studies have explored the effects of these feed additives on the rearing phase. A more persistent laying period depends on the adequate development of the pullets' intestine and the maintenance of the microbiota homeostasis. Furthermore, according to Kogut et al. (2018), dietary components and poor-quality feed ingredients can also trigger a low-grade chronic inflammatory process known as sterile inflammation. The animal's inability to control this low-grade inflammation can also result in enteritis and dysbiosis. In this context, OA and EO represent an alternative as they act as signaling molecules that influence the host physiology, metabolism regulation, inflammation, and immunity. OA are essential for the maintenance of mucosal immunity by stimulating the expression of mucin (MUC2) synthesis and its secretion by goblet-like colon cells. They also stimulate antimicrobial peptides, so that they exert an innate defense against pathogens and modulate tight junction formation and their permeability potential through activation of adenosine monophosphate-activated protein kinase (D'Aquila et al., 2020). Similar to OA, EO have gained interest due to their broad spectrum of antimicrobial, immunomodulatory and

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TABLE 2 Macroscopic intestinal and overall health – *ISI* score at 12 weeks of age.

Item	Control	P(OA+EO)	SEM	P-value
Duodenum				
Morphological alteration on serosa or mucosa layers	1.75^{b}	1.00^{a}	0.16	0.010
Cell debris and red thick mucus on mucosa	0.00^{A}	0.50^{B}	0.14	0.070
Jejunum				
Morphological alteration on serosa or mucosa layers	0.08	0.08	0.06	1.000
Cell debris and red thick mucus on mucosa	1.50	1.00	0.26	0.441
Ileum				
Morphological alteration on serosa or mucosa layers	0.17	0.08	0.07	0.546
Cell debris and red thick mucus on mucosa	0.17	0.33	0.14	0.546
Cecum				
Presence of gas	0.08	0.17	0.07	0.546
Total Intestinal health *ISI*	3.75	3.17	0.36	0.410
Other organs and systems				
Muscle hemorrhage	0.25^{B}	0.00^{A}	0.07	0.070
Femoral head necrosis	1.83^{b}	0.50^{a}	0.22	0.003
Yellow liver	0.50^{B}	0.00^{A}	0.14	0.070
Tracheitis	0.67	0.42	0.10	0.229
Airsacculitis	3.25^{b}	0.50^{a}	0.47	0.001
Hydropericardium	0.00	0.17	0.08	0.317
Yolk persistence	0.00	0.08	0.04	0.317
Hypotrophic pancreas	0.00	0.17	0.08	0.317
Overall Health *ISI*	10.25^{b}	$5{,}00^{a}$	0.89	0.003

Only parameters that had alterations are presented.
P(OA+EO), Protected Organic Acids + Essential Oils at 300 g/t.
SEM, Standard error of the mean.
$^{ab}P< 0.05$; $^{AB}P< 0.10$.

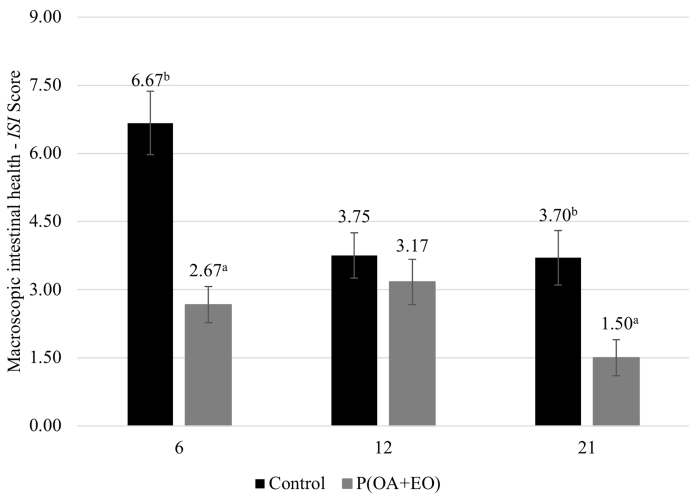

FIGURE 2
Intestinal health analyzed by the macroscopic *ISI* methodology (sum of the scores given for duodenum, jejunum, ileum, and cecum). Data are the mean value of each treatment at different ages (weeks). $^{ab}P<0.05$.

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TABLE 3 Macroscopic intestinal and overall health – *ISI* score at 21 weeks of age.

Item	Control	P(OA+EO)	SEM	P-value
Duodenum				
Morphological alteration on serosa or mucosa layers	0.50	0.25	0.12	0.327
Cell debris and red thick mucus on mucosa	0.80	0.83	0.25	0.949
Jejunum				
Morphological alteration on serosa or mucosa layers	0.60[b]	0.08[a]	0.12	0.030
Cell debris and red thick mucus on mucosa	1.80[b]	0.17[a]	0.29	0.002
Ileum				
Cell debris and red thick mucus on mucosa	0.00	0.17	0.09	0.374
Total Intestinal health *ISI*	3.70[b]	1.50[a]	0.41	0.004
Other organs and systems				
Yellow liver	1.00	0.67	0.31	0.608
Tracheitis	0.10	0.25	0.11	0.498
Airsacculitis	0.60	0.50	0.25	0.849
Hypertrophic kidney	0.10	0.00	0.05	0.284
Oral mucosa lesions	0.20	0.08	0.07	0.451
Gizzard erosion	0.00	0.17	0.06	0.193
Overall Health *ISI*	5.70	3.17	0.68	0.710

Only parameters that had alterations are presented.
P(OA+EO), Protected Organic Acids + Essential Oils at 300 g/t.
SEM, Standard error of the mean.
[ab]P< 0.05.

antioxidant properties (Gopi et al., 2014). The mode of action in which OA and EO modulate the intestinal microbiota and immunity seems complementary (Yang et al., 2015; Dittoe et al., 2018). Thus, the beneficial effects observed with the supplementation of a blend of OA and EO in poultry diets are due to the synergistic effects they can produce together (Wang et al., 2019; Stefanello et al., 2020; Bortoluzzi et al., 2021). In addition, the technology of protection plays a key role in allowing OA and EO to be slowly released in the intestine and reach the final portion of the digestive tract, where potentially pathogenic microbiota is found (Choi et al., 2020).

Regardless of the type of feed additive, there is still a lot of debate in the poultry industry about how to assess intestinal health in the field. In the present study, *I See Inside* (*ISI*) was the

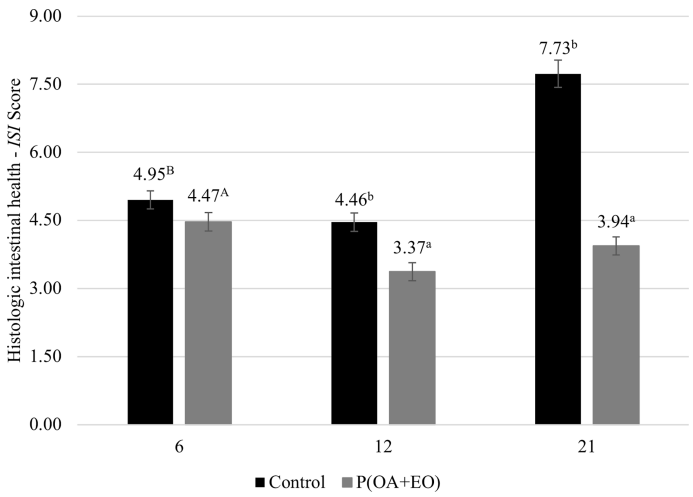

FIGURE 3
Intestinal health analyzed by the histologic *ISI* methodology (sum of the scores given for the alterations in the ileum). Data are the mean value and standard error of each treatment at different ages (weeks). [ab]P<0.05; [AB]P<0.10.

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TABLE 4 Histologic intestinal health – *ISI* score at 6, 12, and 21 weeks of age.

Item	Control	P (AO+EO)	SEM	P-value
Week 6				
Lamina propria thickness	1.94[b]	1.75[a]	0.04	0.03
Epithelial thickness	0.58[B]	0.50[A]	0.02	0.08
Enterocytes proliferation	0.56	0.49	0.02	0.12
Epithelial plasma cell infiltration	0.59[B]	0.51[A]	0.02	0.06
Lamina propria inflammatory infiltration	1.06	0.99	0.06	0.60
Goblet cells proliferation	0.06	0.12	0.02	0.13
Congestion	0.13	0.10	0.02	0.54
Total intestinal *ISI*	4.95[B]	4.47[A]	0.13	0.09
Week 12				
Lamina propria thickness	1.70[b]	1.29[a]	0.06	0.0002
Epithelial thickness	0.24	0.20	0.02	0.36
Enterocytes proliferation	0.19	0.02	0.02	0.81
Epithelial plasma cell infiltration	0.43	0.39	0.03	0.56
Lamina propria inflammatory infiltration	1.31[b]	0.95[a]	0.06	0.003
Goblet cells proliferation	0.23	0.25	0.03	0.67
Congestion	0.32[b]	0.06[a]	0.03	<0.0001
Total intestinal *ISI*	4.46[b]	3.37[a]	0.16	0.0006
Week 21				
Lamina propria thickness	2.19[b]	1.48[a]	0.06	<0.0001
Epithelial thickness	0.31[B]	0.23[A]	0.02	0.080
Enterocytes proliferation	0.32[b]	0.23[a]	0.02	0.050
Epithelial plasma cell infiltration	0.52[b]	0.30[a]	0.03	<0.0001
Lamina propria inflammatory infiltration	2.27[b]	1.27[a]	0.07	<0.0001
Goblet cells proliferation	0.22[b]	0.10[a]	0.03	0.02
Congestion	0.53[B]	0.33[A]	0.04	0.06
Presence of *Eimeria* oocysts	1.36[b]	0.00[a]	0.09	<0.0001
Total intestinal *ISI*	7.73[b]	3.94[a]	0.22	<0.0001

Only parameters that had alterations are presented.
P(OA+EO), Protected Organic Acids + Essential Oils at 300 g/t.
SEM, Standard error of the mean.
[ab]P< 0.05; [AB]P< 0.10.

methodology chosen to assess overall and intestinal health. In this regard, two points must be highlighted: 1) the macro alterations are the last stage of an inflammatory process, while the first signs are seen at a histological level. Thus, taking both analyses together, we can have a broader idea of the actual health status of the animals. 2) The *ISI* parameters are mainly related to proliferative enteritis process, such as the morphological alterations in the serosa or mucosa layers in the macro analysis and, the inflammatory cell infiltration and enterocytes proliferation in the histological analysis, respectively. These parameters have a great impact on the reduction of an organ's functionality, and unlike the measurement of villi and crypt length, the *ISI* goes deeper generating more information about the real status of the low-grade inflammation. Therefore, the *ISI* methodology is an important tool to assess intestinal health and has been used with different types of intestinal challenge models in poultry (Kraieski et al., 2017; Belote et al., 2019; Adewole et al., 2021; Belote et al., 2021).

At the macro-intestinal level (sum of the scores given to duodenum, jejunum, ileum, and cecum), the dietary supplementation of P(OA+EO) significantly reduced the *ISI* score of pullets at week 6 and 21, mainly due to the reduction in the morphologic alteration on serosa or mucosa layers, cell debris, and reddish on mucosa. At week 12, pullets on P(OA+EO) had significant lower morphologic alteration on serosa or mucosa layers. Similar results were found by Stefanello et al. (2020) who observed that broilers supplemented with P(OA+EO) and challenged with coccidiosis and *C. perfringens* presented lower macro-intestinal *ISI* score than broilers that were challenged but didn't receive P(OA+EO). Taken together, this suggests that P(OA+EO) can balance the intestinal ecosystem and reduce the dysbiosis.

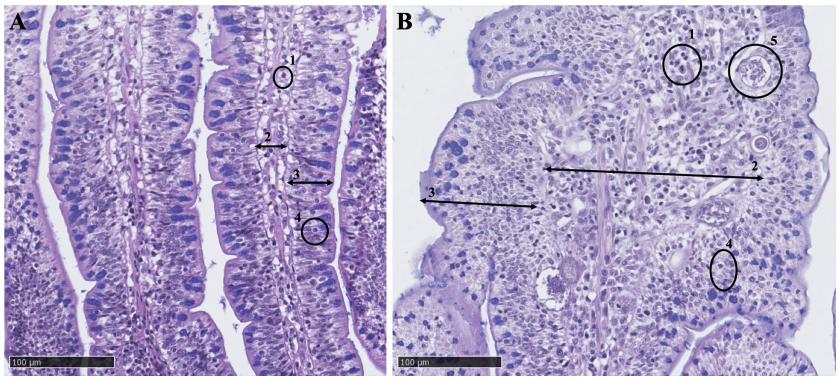

FIGURE 4
Photomicrography of ileum sections of pullets stained with hematoxylin and eosin. Alcian blue was used to stain goblet cells. **(A)** 1, Low *ISI* score of inflammatory cells infiltration in the lamina propria; 2, lamina propria thickness; 3, epithelial thickness; 4, inflammatory cells infiltration in the epithelium in pullets on P(OA+EO) group (200X). **(B)** 1, High *ISI* score of inflammatory cells infiltration in the lamina propria; 2, lamina propria thickness; 3, epithelial thickness; 4, inflammatory cells infiltration in the epithelium and 5, presence of oocysts in pullets in the control group (200X).

Macroscopic intestinal alterations are commonly observed in the last stage of an inflammatory response. The results observed in the histological *ISI* evaluation of the intestine allows for an earlier and deeper analysis of morphologic alterations and dysbiosis. For example, although there was no significant difference between treatments for macro-intestinal *ISI* score at week 12, the histologic intestinal *ISI* scores show significant differences at all ages evaluated. Regardless of age, pullets in the control group had the worst histologic *ISI* score, suggesting that these animals were continuously under dysbiosis. On the other hand, the signs of dysbiosis were attenuated in pullets supplemented with P(OA+EO). Reduced lamina propria thickness, epithelial thickness, epithelial plasma cell infiltration, lamina propria inflammatory infiltration and congestion were the parameters that mainly explained the lowest histologic *ISI* score of pullets supplemented with P(OA+EO). Belote et al. (2019) reported that lamina propria thickness and inflammatory cell infiltration are the most appropriate parameters to compare feed additives focused in improving intestinal health due to their great correlation with growth performance. In addition, at week 21, the lowest histologic *ISI* score of pullets on P(OA+EO) was also due to the reduced enterocytes proliferation, goblet cells proliferation and presence of *Eimeria*. It is well known that the *Eimeria* life cycle is complex counting with intra- and extracellular stages that induce inflammatory response, increase epithelial turnover (El-Shall et al., 2022) and reduce absorption capacity (Shirley and Lillehoj, 2012), which is a sign of dysbiosis. During an inflammatory response, an exacerbation

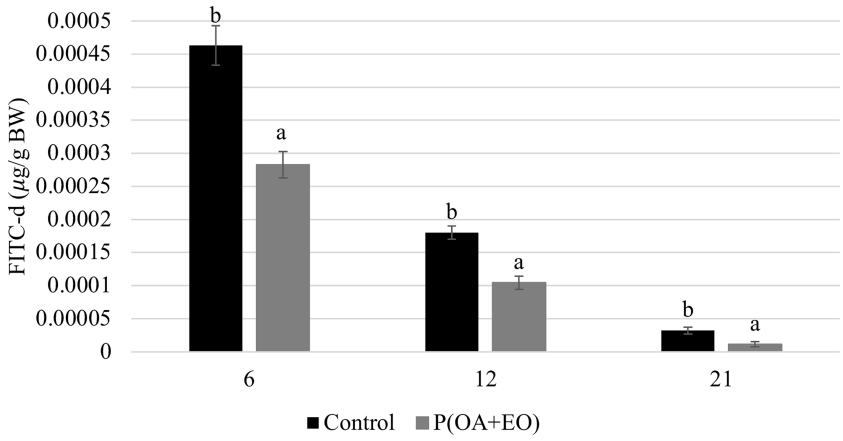

FIGURE 5
Blood levels of FITC-d by week. Data (mean value and standard error) standardized per g of body weight. $^{ab}P<0.05$.

of enterocytes and goblet cells proliferation can be translated into a continuous need to repair the damaged tissue, resulting in immature enterocytes, and increased epithelial thickness. Sanches et al. (2020), working with broilers challenged with *Eimeria* spp. vaccine and *C. perfringens* and Belote et al. (2019), working with broilers challenged with *Eimeria* spp. vaccine, observed similar results, such as greater histologic intestinal *ISI* scores for parameters related to proliferative enteritis. Belote et al. (2019) concluded that the greater the *ISI* score, the worse the growth performance of broilers challenged with *Eimeria*. Although there are many studies reporting that EO have an effect on diminishing the oocyst output through inhibition or impairment of the invasion, replication, and development of *Eimeria* species in the intestinal tissue of chickens (Zhai et al., 2018; El-Shall et al., 2022), it is important to note that we don't expect P(OA+EO) to act directly in the parasite. We can hypothesize that the better intestinal health status of the pullets supplemented with P(OA+EO) was a key factor to modulate the dysbiosis resulting from *Eimeria* proliferation.

The overall health *ISI* score is the sum of the intestinal score and the scores assigned to other organs and systems. Pullets on P(OA+EO) group presented better overall health status, as they had significant lower scores at weeks 6 and 12, compared to the control group. This result was due to the lowest *ISI* scores for intestine, muscle hemorrhage, femoral head necrosis, yellowish liver, and airsacculitis. Intestinal health can influence the overall health of birds in many ways (Diaz Carrasco et al., 2019; Yadav and Jha, 2019). The intestine has constant communication to the external environment, which means that the intestinal microbiota is under the influence of the different factors (i.e., nutrition, biosecurity, weather, etc.) that the pullet experiences. On the other hand, the intestinal microbiota and its products can modulate the physiological and metabolic response of the animal, increasing the inflammatory response and the loss of intestinal barrier integrity (Yadav and Jha, 2019). Besides, the intestinal microbiota and its toxins can translocate to other organs causing a broader infection (Wideman, 2016).

The better intestinal and overall health observed in the present study in pullets supplemented with P(OA+EO) is in accordance with the lower blood recovery of FITC-d at all ages evaluated. In a state of intestinal homeostasis, FITC-d cannot cross the intestinal barrier and reach the bloodstream, as it must cross through the paracellular space between two enterocytes. In a healthy gut, the paracellular space is protected by tight junction proteins. When the intestinal integrity is lost because of a greater cell turnover and inflammatory process – as observed in the control group of the present study – the expression of the tight junction proteins is reduced, increasing the blood levels of FITC-d (Vicuña et al., 2015). The increase in intestinal permeability favors the translocation of bacteria and toxins from the intestinal lumen to the bloodstream and other organs. Similar to the results observed in the present study, the supplementation of P(OA+EO) in poultry diets has shown a significant improvement of the intestinal barrier integrity through reduced permeability, increased expression of tight junction proteins, mucin-2, and immunoglobulin A (Liu et al., 2017; Yang et al., 2018; Wang et al., 2019; Yang et al., 2019; Stefanello et al., 2020).

Conclusions

In the present study, pullets supplemented with P(OA+EO) had better intestinal and overall health during the rearing period (2-18 weeks). In addition, we observed a positive carry-over effect at the initial laying phase (up to 21 weeks) for pullets on P(OA+EO). Our results suggest that P(OA+EO) may be a strategic tool to support the development of the intestine of pullets raised under field conditions.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

Ethical review and approval were not required for the animal study because this was a commercial study where the animals were under the normal management of the farm.

Author contributions

MM: draft and revision of the protocol, interpretation of results, draft and revision of the manuscript, and final approval for paper publication. MV: draft and revision of the protocol, interpretation of results, draft and revision of the manuscript, and final approval for paper publication. TS: interpretation of results, draft and revision of the manuscript. FJ: draft and revision of the protocol, *in vivo* trial, trial conduction. GS: revision of the protocol, *in vivo* trial, trial conduction. JT: revision of the protocol, *in vivo* trial, trial conduction. CN: revision of the protocol, *in vivo* trial, trial conduction. LA: revision of the protocol, *in vivo* trial, trial conduction. ES: I See Inside methodology analysis and final approval for paper publication. All authors contributed to the article and approved the submitted version.

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Conflict of interest

MdSV, MLdM, TBS and ES were employed by Jefo Nutrition Inc. FBJ was employed by Safeeds Animal Nutrition. GMdMS, JMNT, CYN and LCRVA were employed by Grupo Mantiqueira.

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