Characterization and evaluation of Colombian propolis on the intestinal integrity of broilers

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ABSTRACT Nutritional additives such as propolis seek to improve intestinal health as an alternative to the global ban on in-feed antibiotics used as growth promoters (**AGP**). The objective of this study was to evaluate the effect of propolis supplementation in diet of broilers. Four hundred and fifty straight-run Ross 308 AP broilers were fed with a basal diet (**BD**) throughout the whole experimental period. Birds were randomly distributed into 5 groups at d 14: negative control without antibiotics nor propolis (AGP-), positive control 500 ppm of Zinc Bacitracin as growth promoter (AGP +), and 3 groups supplemented with 150, 300, and 450 ppm of propolis. Every group included 6 replicates of 15 birds each. Propolis concentration was increased from d 22 to 42, in experimental groups to 300, 600, and 900 ppm of propolis, and 10% of raw soybean was included as a challenge in all groups during the same period. Analysis of productive parameters, intestinal morphometry, and relative quantification of genes associated with epithelial integrity by qPCR were performed at 21 and 42 d. The groups with the greatest weights were those that consumed diets including 150 (21 d) and 900 ppm (42 d) of propolis compared with all treatments. The lowest score of ISI was found at 300 (21 d) and 600 ppm (42 d). A lower degree of injury in digestive system was seen with the inclusion of 300 ppm (21 d) and 900 ppm (42 d). Up-regulation of *zonula occludens-1 (**ZO-1**)* was observed in jejunum of broilers supplemented with 150 and 300 ppm at 21 d. Up-regulation of *ZO-1* and *TGF-β* was also evidenced in ileum at all propolis inclusion levels at 42-day-old compared to AGP+ and AGP-. The beneficial effects were evidenced at inclusion levels of 150 ppm in the starter and 900 ppm in the finisher. According to the results, the Colombian propolis inclusion can improve productive performance, physiological parameters, and gene expression associated with intestinal integrity.

Key words: bee glue, intestinal health, performance, poultry, tight junction

2022 Poultry Science 101:102159
https://doi.org/10.1016/j.psj.2022.102159

INTRODUCTION

There are various interactions between an individual and its environment that impact its health and well-being. Those that take place in the intestine stand out since there are nutritional, microbiological, physiological, and immunological factors for the maintenance of intestinal health (Celi et al., 2017; Ducatelle et al., 2018). Any alteration of the homeostatic state of the intestine will generate different levels of stress, causing excessive energy expenditure that compromises the development of the chickens (Oakley and Kogut, 2016; Adedokun and Olojede, 2019). Therefore, the inclusion of additives in the diet that have anti-inflammatory, antibacterial, and trophic effects on the intestinal epithelium may improve the health, welfare, and performance of the birds. However, with the global limitations and impact of feeding on production costs, it is a priority to develop alternatives aimed at improving the efficiency in the use of nutrients without compromising intestinal integrity.

The intestinal barrier is formed by a layer of epithelial cells with intercellular junction complexes. These include tight junction (***TJ***) proteins such as claudins (***CLDN***), occludins (***OCL***), and zonula occludens (***ZO***) which separate the host tissue of luminal components to maintain intestinal homeostasis. TJs are a multiprotein complex that seals the space between adjacent epithelial cells, regulates fluid and solute permeability at the barrier (Chen et al., 2015; Awad et al., 2017), and mediates cellular interactions between enterocytes with

Received June 13, 2022.
Accepted August 24, 2022.

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other intestinal cells including intraepithelial lymphocytes, M cells, goblet cells, and dendritic cells which play specific roles in intestinal mucosal functions (Paradis et al., 2021). Inflammatory factors, commensal bacteria, and dietary components can cause alterations of the epithelial barrier and TJ proteins that allow contact or penetration of harmful luminal contents such as pathogenic bacteria, toxins, and antinutritional compounds (Hiippala et al., 2018).

The commercial animal industry has used antibiotics as growth promoters to improve nutrient utilization and productive performance in broilers; however, its regulation and subsequent prohibition in different countries have led to the search for other natural alternatives to maintain intestinal integrity (Yegani and Korver, 2008; Tarradas et al., 2020). According to data from the World Health Organization (**WHO**), an increase in the incidence of bacterial resistance to antimicrobials has been observed in agents considered potential pathogens with an impact on animal and human health. Among these pathogens, the following stand out: *Enterococcus* spp., *Escherichia coli, Campylobacter* spp., *Salmonella* spp., *Streptococcus pneumoniae, Staphylococcus aureus*, and *Clostridium* spp. (Marshall and Levy, 2011; Tarradas et al., 2020). However, with the regulations on the use of antibiotics as growth promoters, the resurgence of multifactorial diseases caused by *Clostridium perfringens* and *Eimeria* spp. has also been observed (Timbermont et al., 2011). As part of the natural alternatives that promote intestinal health, propolis has been proposed due to its anti-inflammatory, antibacterial, and antioxidant properties that contribute to the maintenance of intestinal integrity (Simone-Finstrom et al., 2017). It is possible to generate structural and functional changes associated with the modulation of gene expression in enterocytes with the addition of propolis to the diet of broilers, and thus contributes to maintaining intestinal health.

Propolis is a mixture of plant resin and beeswax which is part of the hives' protection mechanisms. This product allows bees to reduce the incidence of diseases and/or parasitism due to its antimicrobial and antiseptic properties (Simone-Finstrom et al., 2017; Saelao et al., 2020). The analyzes of propolis samples from various parts of the world have identified more than 300 chemical compounds, among which are polyphenols, resins, flavonoids, essential oils, and fatty acids. However, its components may vary depending on its origins (geographical region), surrounding trees, and/or flowers as well as the genetics of the hive. The therapeutic properties of propolis are associated with the presence and diversity of these secondary metabolites (Wang et al., 2013; Silva-Carvalho et al., 2015; Zabaiou et al., 2017; Saelao et al., 2020). Despite all the known effects of propolis, there is still limited information on its use as an additive in diets for broilers in terms of its inclusion levels and impact on indicators of intestinal integrity. Consequently, the objective of this study was to evaluate different levels of propolis inclusion in the diet of broilers and its impact on the morphometry of the intestinal epithelium, the cardiac index, and the expression of the *ZO-1, CLDN-3, OCL*, and *TGF-β* genes related to intestinal integrity in different parts of the intestine and their association with the body weight of broilers.

MATERIALS AND METHODS

Obtaining and Characterizing Propolis Extracts

Propolis was obtained from *Apis mellifera* beehives found in the central area of Colombia (Tequendama-Cundinamarca Region, Colombia, South America) at 1,300 meters above sea level (**MASL**). This region has an average temperature of 24°C and is characterized by an abundance of fruit crops (Figure 1). The propolis collected by beekeepers in the areas was processed to obtain ethanolic extracts (Bruschi et al., 2006). Each propolis sample was shaken daily twice a day for 25 d and kept in the dark to avoid the alteration of some light-sensitive metabolites. After 25 d, ultrasonic agitation was performed for 24 h and each of the samples was filtered. Once the ethanolic extract was obtained, it was kept in amber glass bottles until further processing. Producers were also surveyed to find out the botanical origin of the propolis used in the study.

Within the characterization analyzes of the product, the content of phenols was evaluated by the *Folin-Ciocalteu* method described by Singlenton and Rossi (1965). Likewise, the analysis of flavonoids was performed using the spectrophotometric method described by Tiveron et al. (2016). Antioxidant activity was also measured spectrophotometrically by quantifying the 2,2-diphenyl-1-picryl hydrazyl (**DPPH**) free radical inhibitory activity (Tiveron et al., 2016), while compound profiling was determined using the Agilent Technologies 6890 gas chromatograph (Agilent Co., Santa Clara, CA) coupled to a selective detector (AT 5973N MSD). The column used in the analysis was a DB-5MS (Agilent J & W Scientific, Folsom, CA) 5%-Ph-PDMS, 60 m × 0.25 mm × 0.25 mm.

Birds and Experimental Diets

The procedures performed on the birds were reviewed and endorsed by the Bioethics Committee of the Faculty of Veterinary Medicine and Zootechnics of the Universidad Nacional de Colombia through code number CB-FMVZ-UN-036-2021. A total of 450 straight-run Ross 308 AP broilers were raised in an experimental farm property of Universidad Nacional de Colombia, located at 2,554 MASL with an average temperature of 15°C. Standard management procedures for commercial operations were maintained according to the genetic recommendations. Temperature was 32°C at the start of the trial and gradually a range between 18 and 21°C was allowed after the third week of the growing period. Lighting period consisted of 12 h of light and 12 h of

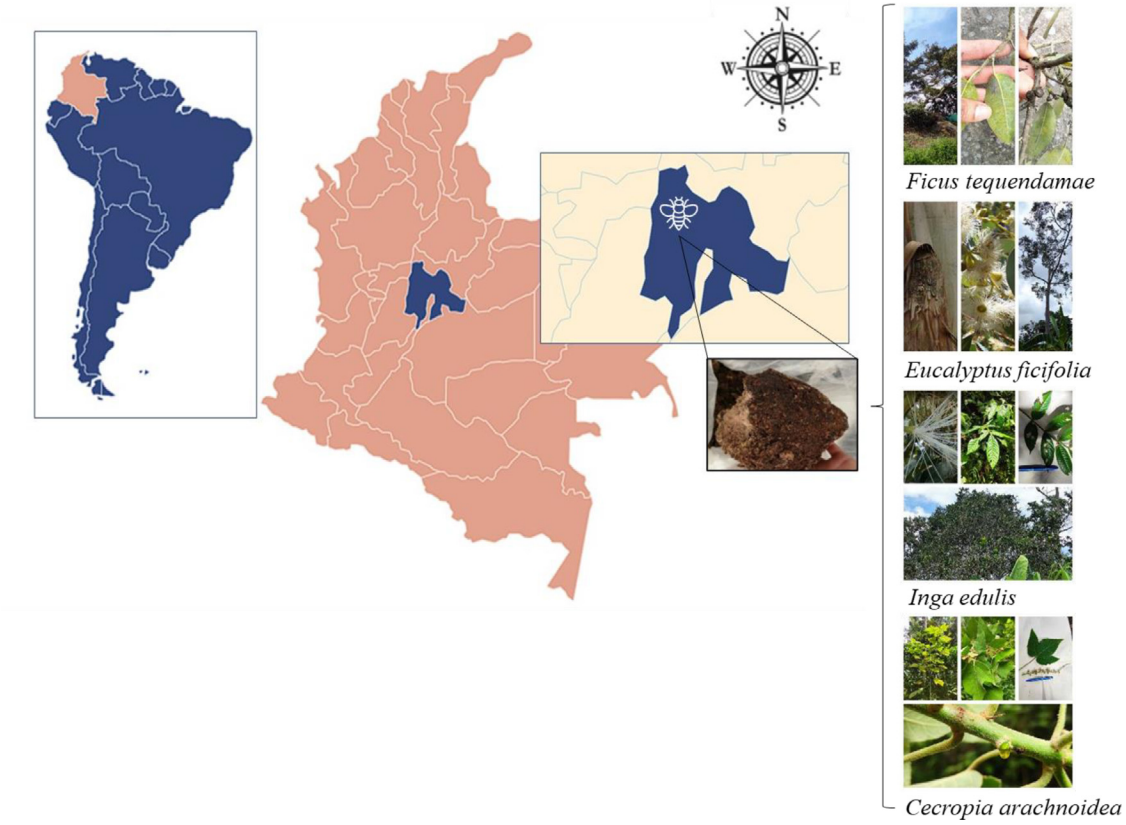

Figure 1. Geographical area in which both the propolis was obtained and the bioassay in the broilers was carried out. The figure shows the color of the propolis that was included in the diet and the plant resources identified in the ethnobotanical characterization.

darkness. Feed and water consumption were provided ad libitum.

Broilers were fed with a mixture of broiler starter during d 1 to13. A basal diet was formulated according to the requirements of the strain management guide (Table 1). At d 14, birds were randomly distributed into 5 experimental groups with 6 replicates of 15 birds each. The groups had a similar proportion between males and females.

Group 1 (negative control): basal diet without antibiotics as growth promoters (AGP-) nor inclusion of propolis.

Table 1. Ingredients and calculated composition of basal experimental diets using in feeding the experimental groups of broilers.

Ingredients, g/kg	Pre start 1−12 d	Starter 13−21 d	Growth 22−42 d
Corn	539.1	566.5	621.8
Soybean meal 49% (CP)	277.1	254.0	203.2
Extruded soybean	100.0	100.0	100.0
Palm oil	17.9	21.7	18.8
Fishmeal	20.0	15.0	15.0
Dicalcium phosphate	14.3	13.6	12.0
Calcium carbonate	12.2	11.7	10.9
Vitamin-mineral trace	2.0	2.0	2.0
Sodium chloride	3.5	3.5	3.5
Sodium bicarbonate	3.0	3.0	3.0
L-Lysine HCl	1.9	1.2	1.8
DL-Metihionine	2.1	1.5	1.5
L-Tryptophan	1.1	0.3	0.6
Choline chloride 60%	1.0	1.0	1.0
	Calculated composition %		
Crude protein (CP)	22.61	21.14	19.40
ME, kcal/g	3,000	3,050	3,100
Fat	6.67	6.95	6.50
Calcium	0.96	0.90	0.82
Total phosphorus	0.80	0.76	0.64
Available phosphorus	0.48	0.45	0.41
DEB, mEq/kg	257	247	224
Digest lysine	1.28	1.15	1.07
Digest methionine	0.53	0.45	0.44
Total SAA	0.81	0.72	0.70
Digest threonine	0.83	0.74	0.70

Abbreviations: ME, metabolizable energy; DEB, dietary electrolyte balance; SAA, sulfut.

Group 2 (positive control): basal diet + 500 ppm of Zinc Bacitracin (AGP+)

Group 3: basal diet + 150 ppm of propolis

Group 4: basal diet + 300 ppm of propolis

Group 5: basal diet + 450 ppm of propolis

Propolis was solubilized in a 15% propylene glycol solution and added with oil to the premix. Then, it was incorporated into the complete feed mix. From d 22 to 42, the inclusion of propolis was increased in groups 3, 4, and 5 to 300, 600, and 900 ppm of propolis, respectively and 10% of raw soybean was included in the diet as a challenge (pro-inflammatory) in all experimental groups during the same period.

Heart, gizzard, proventriculus, small intestine, lymphoid organs, and liver samples were randomly collected from 6 birds of each group at 21 and 42 d for the analyses described below.

Cardiac Index, Relative Organ Weight, and Productive Parameters of Broilers

The cardiac index (**CI**) was calculated from the heart samples obtained during the necropsy of birds. This was done to determine the presence of pulmonary arterial hypertension (**PAH**) due to hypobaric hypoxia from the high-altitude conditions in which the experiment was performed. For this calculation, the weight of the right ventricle was taken and divided by the total ventricular weight. Chickens with CI above 30 were designated as hypertensive and below 26 as healthy (Moreno de Sandino and Hernández, 2006). In the necropsy, the weight of the organs of the digestive system (gizzard, proventriculus, intestine, and liver with glad bladder) was also obtained. The birds were not fed 12 h before the sampling. Body weight was recorded as the basis for the calculation of relative organ weight (%). Throughout the experiment, the productive parameters: weight, feed intake, average daily gain, feed conversion rate (**FCR**), and mortality were registered weekly until d 42.

Morphometry and Intestinal Integrity Analysis

From the samples of duodenum, jejunum, ileum, cecum, liver, and lymphoid organs, sections were made and preserved in 10% neutralized formalin. Histological slides were stained with hematoxylin-eosin (**H&E**). Histological sections were examined under a light microscope (Olympus, Tokyo, Japan). ImageView version x64 software was used to measure the villus height, villus width, and crypt depth at 21 and 42 d of ten well-formed villi in the duodenum, jejunum, and ileum per group. To estimate the ratio of the villus to crypt (**H:C**), the villus height was divided by the crypt depth. Intestinal integrity analyzes were also performed using the modified "I See Inside" (**ISI**) method according to the following parameters and impact factors (**IF**:1-3):

i. Intestine: Lamina propria thickness (IF:2), Epithelial thickness (IF:1), Enterocytes proliferation (IF:1), Inflammatory cell infiltration in the epithelium (IF:1), Inflammatory cell infiltration in the lamina propria (IF:3), Goblet cells proliferation (IF:2), Congestion (IF:2), Presence of oocysts (IF:3) and bacteria (IF:3), crypt dilation (IF:2), and necrosis (IF:3) (Maximum Score = 69).

ii. Liver: Congestion (IF:1), Cell vacuolization (IF:2), Bile-duct proliferation (IF:2), Immune cells infiltration (IF:1), Necrosis (IF:3), Lymphocytic aggregate (IF:2) and Pericholangitis (IF:3) (Maximum Score = 42).

The scores assigned for each parameter ranging from 1 to 3 and the maximum ISI score were calculated according to the formula reported in previous studies (Brudnicki et al., 2017; Kraieski et al., 2017; Belote et al., 2019).

Measurement of Gut Integrity Gene Expression

The total amount of RNA was extracted from 20 mg of intestinal mucosa from the jejunum and ileum using the commercial Invitrap Spin Universal RNA mini kit (Invitek Molecular GmbH, Berlin, Germany) following the manufacturer's instructions. The concentration of each sample was quantified across wavelengths at 260 nm and 280 nm (A260/280) using a UV spectrophotometer (NanoDrop, Thermo Scientific, Waltham, MA). Cleanup of contaminating DNA was performed using DNase I (New England Biolabs, Ipswich, MA). For cDNA synthesis, the High-Capacity cDNA Reverse Transcription kit (Applied Biosystems, Grand Island, NY) was used in a final volume of 10 μL which included 1.6 μL of H_2O, 1 μL RT-Buffer (10X), 1 μL RT-Random Primers (100 mM), 0.4 μL dNTP's mix (25X), 0.5 μl RNAse inhibitor (20U/μL), and 0.5 μL MultiScribe Reverse Transcriptase (50 U/μL). The reactions were incubated at 25°C for 10 min and 37°C for 120 min and finished at 85°C for 5 min. For real-time PCR, the Light Cycler Thermocycler (Roche, Mannheim, Germany) and the SYBR green PCR (Roche) detection methodology were used. Relative expression of genes encoding *ZO-1, CLDN-3, OCL*, and transforming growth factor-β (***TGF-β***) proteins was measured in the jejunum and ileum from the cDNA obtained by RT and subsequently diluted with ultrapure water (1:10). Specific primers for β-Actin were used as housekeeping genes (5′- TCT GGC ACC ACA CTT TCT ACA-3′and 5′- CAC AGG ACT CCA TAC CCA AGA-3′) and for the genes of interest (ZO-1: 5′- CAA CTG GTG TGG GTT TCT GAA - 3′ and 5′- TCA CTA CCA GGA GCT GAG AGG TAA- 3, CLDN-3: 5′- CCA GGT GAA GAA GAT GCG GA-3′ and 5′- GGT GTG AAA GGG TCA TAG AAG GC, OCL: 5′- CAG CAC CTA CCT CAA CCA GTA CAT-3′ and 5′ AGG CAG AGC AGG ATG

ACG AT-3′, and TGF-β: 5′- CGG CCG ACG ATG AGT GGC TC-3′and 5′- CGG GGC CCA TCT CAC AGG GA-3′) previously reported (Barshira and Friedman, 2006; Lucke et al., 2018; Metzler-Zebeli et al., 2019). The final reaction volume was 10 μL and the amplification conditions were as follows: 95°C for 10 min, 45 cycles of 95°C for 15 s, 60°C for 10 s, and 72°C for 15 s. The specificity of the products was confirmed by the dissociation curves (Tm) obtained with the software. The standardized formulas were used to calculate the Ct value and the standard error between replicates reported by Willems et al. (2008). The difference in relative gene expression level was analyzed using the 2-DDCt method (Schmittgen and Livak, 2008)

Experimental Design and Statistical Analysis

The study was conducted under a completely randomized design. For the analysis PFREQ, MEANS, and GLM packages of the statistical software SAS (Statistical Analysis System v 9.4) for Windows (SAS Institute Inc, Cary, NC) and the bioconductor package of the statistical software R (R Foundation for Statistical Computing, Vienna, Austria, URL https://www.R-project.org/) were used. ANOVA and Tukey test were performed to identify whether statistical differences existed between groups in the relative organs weight, weight, and morphometry of the villi. The statistical model was as follows: $y_{ij} = \mu + \tau_i + \varepsilon$ were Yij was the response variable, μ is the average, τ_j is effect of the i-th treatment, and εij is the experimental error. For FCR and CI variables, a non-parametric model was conducted to verify differences in scale based on the Wilcoxon-rank. $P < 0.05$ were considered statistically significant.

RESULTS

Characterization of Propolis

The propolis used in the diet supplementation of the broilers was characterized by having brown tones (Figure 1). Regarding plant origin, 14 plants (7 native) were identified as the primary sources of resins, among which the following stand out: eucalyptus (*Eucalyptus ficifolia*), pines (*Pinus* spp), rubber trees (*Ficus tequendamae*), acacias (*Acacia mangium*), mangoes (*Magnifera indica*), arrayanes (*Myrcia popayanensis*), guamos (*Inga edulis*), yarumo (*Cecropia arachnoidea*), and citrus trees. The contents of phenols and flavonoids were: 97.51 mg GAE/g EEP and 38.73 mg GAE/g EEP, respectively. Essential oils such as epizonarene and α-calacorene, alkaloids such as caffeine, and different terpenoids were also detected. The complete analysis of the components of the propolis in the selected collection area is shown in Table 2. The antioxidant activity was determined by quantifying the inhibitory activity of the DPPH free radical which was 21.66.

Table 2. Profile of propolis components from the Tequendama region of central Colombia (South America) by gas chromatography-mass spectroscopy.

Compound	tR (min)	Relative quantity (%)
	Aromatic acids	
Benzoic acid	26.4	2.7
Acetophenone	10.9	0.7
	Terpenoides	
α-Pinene	8.1	0.2
Cadin-3, 5-diene (sesquiterpene)	18.1	0.1
Ar-curcumene (sesquiterpene)	18.6	0.1
α-candiol (sesquiterpene)	21.3	0.2
Lanosterol (triterpenoid)	37.7	2.5
α-Amirone	38.9	2.5
Lupenone (triterpene)	39.2	2.6
Lupeol	39.8	7.2
	Essential oils	
Epizonarene	18.9	0.1
α-Calacorene	19.7	0.1
	Alkaloid	
Caffeine	23.6	0.1
	Fatty acids	
Palmitic acid	49.4	0.7
Ethyl palmitate	25.1	1.4
Ethyl linoleate	26.7	0.9
Ethyl oleate	53.5	2.9
	Flavonoids	
Ferulic acid	3.8	2.6
Quercetin	4.5	1.8
Caffeic acid	3.3	7.8
Ursolic acid	8.8	<1

tR (min): Retention time (minutes).

CI, Relative Organ Weight, and Productive Parameters of Broilers

In CI, relative weight of organs, and body weight of the necropsied broilers at the 2 ages evaluated did not show statistically significant differences between the groups. However, it was observed that at 21-day-old, the CI was lower in group 3 to 150 ppm (17.8%), while the group AGP- had the highest CI (24.8%). It was also observed that the group with the highest relative weight of the gizzard was group 4 to 300 ppm (3.2%). Likewise, these inclusions groups (150 and 300 ppm) had the highest relative weight of the intestine (5.5 and 4.5%) and liver (3.1 and 3.3%), respectively (Table 3).

At 42-day-old, the lowest CI was found in the group 5 to 900 ppm (18.8%) and the highest was in the group 4 to 600 ppm (22.8%). Group 4 to 600 ppm had the lowest relative weight of the gizzard (5.9%) and groups 3 to 300 ppm and 5 to 900 ppm had the highest relative weights in the intestine (10.1%) and liver (6.9 and 7.7%), respectively.

According to the parameters established for CI, all the necropsied birds included in the study were classified as chickens without PAH. However, depression, hydropericardium, ascites, and/or cyanotic crest, chin, and nails were observed in individuals who died during the experiment.

At 21-day-old, the group with the highest body weight of the necropsied broilers was group 4 to 300 ppm (923 g). At 42-day-old, group 5 to 900 ppm had the highest body weight of necropsied broilers (2,685 g). It was

Table 3. Relative organ weight, cardiac index − CI, and weight of broilers supplemented with different levels of propolis during their productive cycle (21 and 42-day-old).

Groups	Gizzard (%)	Proventriculus (%)	Gut (%)	Liver (%)	CI (%)	Weight (g)[1]
			Day 21			
AGP-	3.0 ± 0.2	0.5 ± 0.02	4.8 ± 0.2	2.6 ± 0.1	24.8 ± 0.2	883 ± 41
AGP+	3.0 ± 0.2	0.5 ± 0.03	4.9 ± 0.2	3.0 ± 0.1	23.6 ± 0.1	857 ± 18
150 ppm	3.1 ± 0.1	0.5 ± 0.03	5.5 ± 0.1	3.1 ± 0.2	17.8 ± 0.1	915 ± 18
300 ppm	3.2 ± 0.2	0.6 ± 0.04	5.4 ± 0.3	3.3 ± 0.2	21.3 ± 0.2	923 ± 17
450 ppm	3.1 ± 0.2	0.5 ± 0.07	4.5 ± 0.2	2.9 ± 0.2	19.6 ± 0.1	853 ± 19
			Day 42[2]			
AGP-	6.0 ± 0.4	1.1 ± 0.04	9.7 ± 0.3	6.8 ± 0.5	20.2 ± 0.1	2,567 ± 82
AGP+	6.4 ± 0.3	1.0 ± 0.1	10.2 ± 0.6	6.2 ± 0.3	21.8 ± 0.1	2,590 ± 66
300 ppm	6.1 ± 0.4	1.1 ± 0.1	10.1 ± 1.5	6.9 ± 0.3	20.6 ± 0.2	2,541 ± 137
600 ppm	5.9 ± 0.3	1.0 ± 0.03	10.0 ± 0.4	6.7 ± 0.2	22.8 ± 0.2	2,596 ± 64
900 ppm	6.5 ± 0.2	1.1 ± 0.05	10.1 ± 0.3	7.7 ± 0.4	18.8 ± 0.1	2,685 ± 136

Abbreviations: AGP-, negative control basal diet without antibiotics as growth promoters nor inclusion of propolis; AGP+, positive control basal diet with Zinc Bacitracin (500 ppm) as growth promoters.

No statistically significant differences were found between the groups ($P > 0.05$).

Data are shown as mean ± SEM (Standard error of mean).

[1]Body weight of necropsied broilers.

[2]From day 21 to 42, 10 % raw soybean was included as a challenge.

noted that, this group (900 ppm) also had the highest accumulated body weight (2,717 g), FCR (1.40), and feed intake (4,684 g) respect to control groups AGP- and AGP+ (Table 4).

Intestinal Morphometry and Intestinal Integrity Index

At 21 d, the chickens treated with the inclusion of propolis were found to have an increase in some of the morphometric parameters observed. These included the height of the duodenal, jejunal, and ileum villi in the group with the highest level of propolis inclusion (450 ppm) compared to the control AGP- ($P < 0.05$). The highest H:D ratio in the duodenum and jejunum was found at 450 ppm, with statistically significant differences in the jejunum compared to the other treatments ($P < 0.05$).

After 42 d, the duodenum had no statistically significant differences between the groups. In the jejunum and ileum, AGP+ and group 4 to 300 ppm had the highest villi compared to the other groups ($P < 0.05$). The average of the measurements and the statistically significant differences between the groups are shown in Table 5. In ileum, the highest H:D ratio was found in group 5 to 900 ppm, with statistically significant differences compared to AGP- ($P < 0.05$). Despite not finding significant differences between the groups in duodenum and jejunum, the H:D ratio was higher in the 5 to 900 ppm group vs. what was observed in the AGP-.

In the ISI score, all experimental groups had a low injury score in the 2 ages. The group 4 to 300 ppm at 21 d and to 600 ppm at 42 d had the lowest injury score in the intestinal sections respect to the control groups AGP+ and AGP-. This was represented by a decrease in the hypertrophy and proliferation score of goblet cells in the ileum and cecum. AGP+ obtained the highest score given the presence of bacteria and necrosis in the cecum. The measurements and significant differences between groups in the intestinal sections and liver are shown in Table 6.

Table 4. Productive parameters of broilers supplemented with different levels of propolis during their productive cycle (21- and 42-day-old).

Groups	Weight (g)	Feed intake (g)[1]	FCR	Average daily gain (g)	M (%)
		Day 21			
AGP-	843.8 ± 8.7	1,056 ± 8.1	1.34 ± 0.1	37.93 ± 0.4	-
AGP+	857.9 ± 6.1	1,092 ± 23.1	1.35 ± 0.1	38.01 ± 0.3	-
150 ppm	860.4 ± 7.8	1,028 ± 3.36	1.30 ± 0.0	37.97 ± 0.4	-
300 ppm	854.0 ± 11.2	1,040 ± 27.7	1.32 ± 0.1	38.43 ± 0.5	-
450 ppm	851.3 ± 11.9	1,068 ± 16.7	1.34 ± 0.1	38.29 ± 0.5	-
		Day 42[2]			
AGP-	2,706 ± 44.7	4,530 ± 72.0	1.56 ± 0.1	88.72 ± 2.2	20
AGP+	2,692 ± 22.0	4,601 ± 70.1	1.39 ± 0.1	89.08 ± 1.1	14
300 ppm	2,712 ± 34.9	4,505 ± 23.5	1.38 ± 0.0	89.25 ± 1.4	18
600 ppm	2,683 ± 40.4	4,587 ± 80.2	1.36 ± 0.1	87.10 ± 1.5	19
900 ppm	2,717 ± 38.0	4,684 ± 72	1.40 ± 0.1	88.85 ± 1.5	17

Abbreviations: AGP-, negative control basal diet without antibiotics as growth promoters nor inclusion of propolis; AGP+, positive control basal diet with Zinc Bacitracin (500 ppm) as growth promoters; FCR, feed conversion rate; M (%), accumulated mortality percentage.

No statistically significant differences were found between the groups ($P > 0.05$).

Data are shown as mean ± SEM (Standard error of mean).

[1]Feed intake was adjusted at 21 d with 800 g and 42 d with 2900 g.

[2]From day 21 to 42, 10% raw soybean was included as a challenge.

Table 5. Morphometry of the villi of the small intestine of broilers supplemented with different levels of propolis at two times during the productive cycle (21- and 42-day-old).

	Duodenum (μm)				Jejunum (μm)				Ileum (μm)			
Groups	Height	Width	Depth[1]	H:D[2]	Height	Width	Depth[1]	H:D[2]	Height	Width	Depth[1]	H:D[2]
						Day 21						
AGP-	1,068 ± 5.7^{a}	148 ± 1.2^{b}	162 ± 1.0^{ab}	6.88 ± 0.5^{ab}	645 ± 4.0^{a}	91 ± 0.9^{a}	138 ± 1.1^{b}	4.98 ± 0.2^{a}	444 ± 2.5^{a}	122 ± 1.1^{b}	129 ± 1.0^{b}	3.63 ± 0.2^{a}
AGP+	1,039 ± 6.7^{a}	125 ± 1.4^{a}	141 ± 1.0^{ab}	7.97 ± 0.5^{ab}	699 ± 3.0^{b}	96 ± 0.9^{ab}	131 ± 1.1^{ab}	5.83 ± 0.3^{a}	511 ± 2.1^{b}	70 ± 0.6^{a}	127 ± 0.7^{ab}	4.12 ± 0.1^{a}
150 ppm	1,111 ± 7.0^{a}	123 ± 1.2^{a}	187 ± 1.4^{b}	6.06 ± 0.4^{a}	558 ± 3.5^{a}	98 ± 0.5^{ab}	134 ± 0.8^{b}	4.40 ± 0.4^{a}	744 ± 3.8^{c}	102 ± 1.1^{b}	124 ± 0.7^{ab}	6.33 ± 0.3^{b}
300 ppm	1,159 ± 3.3^{ab}	107 ± 1.0^{ab}	149 ± 0.8^{ab}	8.0 ± 0.5^{ab}	531 ± 3.1^{a}	93 ± 0.8^{b}	119 ± 1.2^{a}	4.92 ± 0.4^{a}	507 ± 2.6^{ab}	79 ± 0.6^{b}	120 ± 1.2^{a}	4.50 ± 0.3^{a}
450 ppm	1,184 ± 6.2^{b}	115 ± 0.9^{a}	137 ± 0.7^{a}	8.9 ± 0.7^{b}	713 ± 2.4^{b}	88 ± 0.5^{ab}	95 ± 0.6^{a}	7.88 ± 0.5^{b}	487 ± 2.3^{b}	83 ± 0.7^{b}	122 ± 0.7^{a}	4.23 ± 0.3^{a}
						Day 42						
AGP-	1,125 ± 6.6	134 ± 1.0	148 ± 1.0	7.93 ± 0.5	726 ± 203^{a}	87 ± 0.6	115 ± 1.4	7.68 ± 1.0	669 ± 71^{b}	117 ± 0.9	182 ± 1.4^{b}	4.05 ± 0.3^{a}
AGP+	1,104 ± 6.41	111 ± 1.0	138 ± 1.8	8.44 ± 0.5	982 ± 146^{c}	89 ± 0.7	112 ± 0.8	9.17 ± 0.4	777 ± 136^{a}	92 ± 0.8	138 ± 0.8^{a}	5.84 ± 0.2^{b}
300 ppm	1,167 ± 5.0	120 ± 1.1	126 ± 0.5	8.25 ± 0.5	896 ± 117^{ab}	76 ± 0.8	124 ± 0.8	7.63 ± 0.4	521 ± 138^{b}	87 ± 0.8	100 ± 1.2^{a}	5.90 ± 0.6^{b}
600 ppm	896 ± 5.2	133 ± 1.4	129 ± 0.7	7.34 ± 0.5	906 ± 169^{bc}	87 ± 0.6	115 ± 0.9	8.45 ± 0.5	780 ± 130^{a}	84 ± 0.9	147 ± 1.3^{a}	5.60 ± 0.2^{b}
900 ppm	1,133 ± 6.8	123 ± 1.0	125 ± 0.7	9.43 ± 0.6	777 ± 162^{a}	84 ± 0.6	94 ± 0.9	8.88 ± 0.6	716 ± 148^{a}	104 ± 0.8	129 ± 1.2^{a}	6.48 ± 0.7^{b}

Abbreviations: AGP-, Negative control basal diet without antibiotics as growth promoters nor inclusion of propolis; AGP+, positive control basal diet with Zinc Bacitracin (500 ppm) as growth promoters.
^{ab}Different letters indicate significant differences ($P < 0.05$).
Data are shown as mean ± SEM (Standard error of mean).
[1]Crypt depth.
[2]Villus height-to-crypt depth ratio.

Relative RNA Expression of the ZO-1, CLDN-3, OCL, and TGF-β Genes

In the jejunum of 21 d chickens, *ZO-1* expression was 4 times higher in groups 4 to 300 ppm and 5 to 450 ppm in comparison to the control groups (AGP- and AGP+). Group 5 to 450 ppm had an upregulation of *CLND-3* expression when compared to the other groups. The expression of TGF-β was not stimulated by the treatments provided (Figure 2).

At 21 d in the ileum, positive expression of *TGF-β* was observed in the groups with inclusion of propolis compared AGP-. At 42 d in the ileum, a positive expression of *ZO-1* was evidenced in the groups including propolis, highlighting the 16-fold higher expression in group 4 to 600 ppm compared to the control groups AGP- and AGP+ (Figure 2).

DISCUSSION

Propolis is a natural compound which is characterized by having antibacterial, antifungal, antioxidant, immunomodulatory, and antitumor properties (Lotfy, 2006). The present study shows that the propolis inclusion in the broiler diet improves the body weight both at the starter and the finisher compared to the negative control group (AGP -) and the group with AGP (AGP+). This was observed with lower inclusion (150 ppm) at 21 d and with higher inclusion (900 ppm) at 42 d when compared to controls. Thus, it is possible that lower doses of propolis are needed to improve the body weight of the broilers when there is no challenge. With the raw soybean inclusion, which has antinutritional factors such as lecithins and trypsin inhibitors with proinflammatory effects (Leeson and Summers, 2001; Fasina et al., 2006), higher doses of propolis are needed to improve the body weight and feed conversion. Broilers fed with propolis have a higher resistance to stressors as heat stress (Seven and Seven, 2008), infections with *Eimeria* spp. (Biavatti et al., 2003), and lead toxicity (Seven et al., 2012). Some propolis components such as polyphenols may have anti-inflammatory and antioxidant properties, modulating arachidonic acid pathways to produce eicosanoids and inhibit the gene expression of lipoxygenases (**LOX**) and cyclooxygenases (**COX**; Jung et al., 2008; Doiron et al., 2017). The caffeic phenylethyl species (**CAPE**) could promote the synthesis of anti-inflammatory cytokines (IL-10 and IL-4) and inhibit the production of inflammatory cytokines (IL-8 and TNF-a; Moura et al., 2011). The antioxidative effects of propolis have also been related to behavioral changes such as reduction of feather pecking (Abdel-Rahaman and Mosaad, 2013; Mahmoud et al., 2015), improving health, welfare, and performance.

The increase in body weight evidenced in the present study is a function that has been attributed to propolis, both in broilers (Shalmany and Shivazad, 2006) as well as in other poultry species (Bonomi et al., 2002; Denli et al., 2005; Abdel-Rahaman and Mosaad, 2013). Feed intake was also improved in broilers fed propolis at 250

Table 6. Score using the "I See Inside" methodology - ISI of histological alterations of the intestine and liver of broilers supplemented with different levels of propolis at 21- and 42-day-old.

Groups	Duodenum	Jejunum	Ileum	Cecum	Intestinal ISI[1]	Liver	ISI total score[2]
			Day 21				
AGP-	16 ± 1.0	9 ± 1.0	13 ± 1.5[b]	13 ± 1.5	12.8	6 ± 0.6	18.8
AGP+	13 ± 1.5	10 ± 1.3	11 ± 1.3[ab]	15 ± 1.5	12.3	5 ± 0.2	17.3
150 ppm	15 ± 2.1	11 ± 1.5	9 ± 1.0[a]	14 ± 0.8	12.3	5 ± 0.7	17.3
300 ppm	12 ± 1.2	10 ± 1.3	9 ± 0.8[ab]	11 ± 0.9	10.5	6 ± 0.8	16.5
450 ppm	15 ± 1.9	12 ± 0.7	10 ± 0.7[ab]	15 ± 1.7	13.0	6 ± 1.2	20.0
			Day 42				
AGP-	12 ± 1.3	12 ± 0.8	11 ± 0.6	13 ± 2.5	12.0	9 ± 1.2	19.0
AGP+	11 ± 1.1	12 ± 1.0	8 ± 2.0	20 ± 2.7	13.0	11 ± 1.0	25.0
300 ppm	11 ± 1.0	9 ± 1.3	9 ± 0.9	19 ± 0.8	13.0	9 ± 1.2	22.0
600 ppm	13 ± 1.5	9 ± 0.4	5 ± 2.0	17 ± 1.2	11.0	9 ± 0.3	20.0
900 ppm	13 ± 1.7	11 ± 2.5	9 ± 1.7	17 ± 0.0	12.5	11 ± 3.5	23.5

Abbreviations: AGP-, negative control basal diet without antibiotics as growth promoters nor inclusion of propolis; AGP+, positive control basal diet with Zinc Bacitracin (500 ppm) as growth promoters.

No statistically significant differences were found between the groups ($P > 0.05$).

Data are shown as mean ± SEM (Standard error of mean).

[ab]Different letters indicate significant differences ($P > 0.05$).

[1]Intestinal ISI represents the sum of the average score of all alterations of duodenum, jejunum, ileum, and cecum. The maximum score of injury per section is 69.

[2]ISI total score represents the sum of the intestinal ISI + liver score. The maximum score of injury is 111 (69 + 42).

(Shalmany and Shivazad, 2006) and 400 mg/kg (Hassan and Abdulla, 2011). The increase of this parameter was also observed in this study at 900 ppm, which has been attributed to the improvement of food flavor by flavonoids and benzoic acids of propolis, which in turn increase the digestibility of proteins (Seven et al., 2012). These results suggest that propolis could represent a viable alternative to replace AGP in the diet of broilers, with a dose cost per ton of 4.5 USD of propolis vs. 3.1 USD of Zinc Bacitracin as AGP (unpublished data).

The intestinal epithelium adjusts rapidly to the lumen conditions. Therefore, the increase of height and H:D ratio in some sections of the intestine in the propolis-supplemented groups may correspond to a positive effect of propolis, enlarging absorptive surface area and thus enhancing nutrient digestibility in the intestine. This effect of propolis has been reported in a previous study (Prakatur et al., 2019), in which a higher villus height was observed in the groups with propolis similar to what we evidenced in the duodenum and ileum at the 2 ages

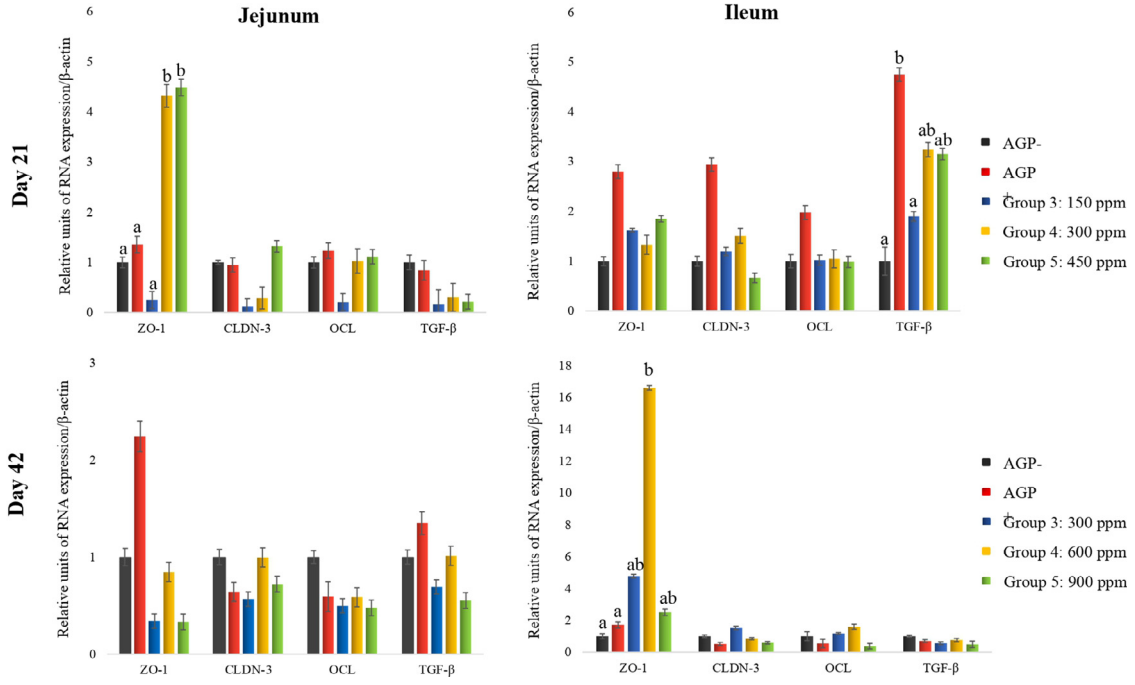

Figure 2. Relative expression levels of the zonula occludens (ZO-1), claudin 3 (CLDN-3), occludins (OCL), and transforming growth factor-β (TGF-β) genes in the jejunum (A and C) and ileum (B and D) of broilers supplemented with different levels of propolis. From d 21 to 42, 10% raw soybean was included as a challenge. To determine the difference in the expression of the genes of interest, the 2-DDCt method was used. The bars correspond to the SEM (Standard error of mean). Abbreviations: AGP-, negative control basal diet without antibiotics as growth promoters nor inclusion of propolis; AGP+, positive control basal diet with Zinc Bacitracin (500 ppm) as growth promoters.

evaluated. This fact could also be associated with the higher body weight of broilers supplemented with the higher level of propolis at d 42 in our research.

The compounds and the doses of propolis evaluated in this study do not represent a harmful to the health of the birds due to no signs of tissue damage were observed in the intestine or liver of the propolis inclusion groups neither before nor after the pro-inflammatory challenge. As mentioned above, the antioxidant properties of propolis promote a state of epithelial protection, possibly modulating the production of cytokines involved in tissue damage and triggered by inflammatory processes. The phenolic mixtures available in propolis can reduce free radicals in inflamed tissues and thus reduce oxidative stress (Paulino et al., 2015).

In this study, the groups with propolis inclusion had the lowest rate of intestinal injury compared to AGP+ after the proinflammatory challenge. It is noteworthy that the histological changes observed in the caecum of AGP+ group may be associated with dysbiosis and, therefore, with damaged intestinal integrity. The protective capacity of propolis has been reported previously in other animal models such as rats with induced gastric ulcers, where a lower degree of mucosal injury was seen in the individuals treated with propolis (Mendonça et al., 2020). It has also been shown that the inclusion of propolis can have a hepatoprotective effect associated with the decrease in lipid oxidation of the hepatocyte cell membrane particularly attributed to phenolic components (Nirala et al., 2008; Babinska et al., 2012). The low scores of liver injury in the treated groups in this study suggest this effect. It has also been shown that propolis supplementation of broilers may modulate liver metabolism by reducing the activity of some enzymes such as catalase and superoxide dismutase (Seven et al., 2009, 2010).

The immune response and intestinal epithelium integrity mediators can be regulated by supplementation of additives as propolis in the broiler diet. The present study showed a possible effect of propolis as a modulator of the expression of genes associated with TJ proteins in jejunum and ileum, reflected in up-regulation of *ZO-1* in the groups with an inclusion of 300 and 450 ppm of propolis at the starter compared to AGP-. The modulating effect of *ZO-1* was also seen in all groups with propolis inclusion after the addition of raw soybeans as a proinflammatory. *ZO-1* is a protein which participates in maintenance of claudin-based barrier function (Citi, 2020), and could be regulated by some compound from propolis. Previous studies have shown that flavonoids and polyphenols can maintain the structure of intestinal barrier by regulating the TJ protein expression (Suzuki and Hara, 2009, 2011) through the *AMPK-ERK1/2* pathway (Wang et al., 2016). However, the expression of other genes which codify TJ proteins measured in this research (*OCL* and *CLDN-3)* was similar in the groups with propolis inclusion and AGP- group in the 2 ages and intestinal sections. It is important to consider that TJs are a broad family of proteins responsible for intestinal integrity, so those that could mediate the actions of propolis should continue to be researched.

Likewise, the modulatory effect on anti-inflammatory factors such as *TGF-β* was also evident by the up-regulation in the ileum of the propolis-included groups before the pro-inflammatory challenge. After the challenge, the expression levels of this factor were similar to those of AGP-. *TGF-β* is a cytokine involved in the immune regulation and the maintenance of intestinal homeostasis which, along with other molecules, may be associated with sterile inflammation or chronic low-grade inflammation in absence of an infection. This response is commonly caused by antinutritional factors with a proinflammatory effect such as lectins, toxoalbumins, and trypsin inhibitors found in some sources of food which are not digested and that produce intestinal viscosity (Leeson and Summers, 2001; Fasina et al., 2006). However, there are multiple mediators in addition to TGF-B whose activation also depends on nutritional components that trigger a food-induced immune response (**FIIR**), affecting the physiological development of the bird and reducing its efficiency. Therefore, future studies are required to evaluate a broader transcriptomic profile of gene families that can mediate the mechanisms of action of propolis as well as the synthesis of proteins involved in the process.

The study was carried out in a high-altitude area, so the birds were exposed to hypobaric hypoxia. The high mortality rate was associated with the presentations of clinical signs compatible with PAH. All experimental groups showed signs of PAH such as depression, cyanosis, and ascites. In addition, no dietary restriction programs were performed in the study, because the objective was to evaluate different levels of propolis inclusion and the effects on the intestinal integrity and performance. The results showed that the propolis inclusion levels did not have an effect in reducing the prevalence of PAH. Further research are needed to evaluate the direct effect on cells due to the fact that the flavonoids from propolis modulate the expression of hypoxia-inducible factor-1 (**HIF-1**; Hattori et al., 2011), which has been reported as a mediator of molecular responses in PAH (Semenza, 2001). Other propolis compounds such as CAPE can modulate the expression of HIF-1 by regulating the *AKT/ERK* pathway (Cheng et al., 2019) and inhibiting the activation of the NF-κB-dependent inflammatory pathway (Natarajan et al., 1996; Wu et al., 2013).

Finally, the concentration of polyphenols has also been related to the brown color of propolis, which was characteristic in this study. One of the difficulties reported to compare the results of research of propolis as a feed additive in broilers, is that the studies do not report the analysis of the compounds (Mahmoud et al., 2016). In the present study, Colombian propolis compounds are described and native botanical sources such as acacias (*Acacia mangium*), mangoes (*Magnifera indica*), myrtle (*Myrcia popayanensis*), guamos (*Inga edulis*), and yarumo (*Cecropia arachnoidea*) from which bees feed, are also identified. The diversity and quantity of biomass in the tropical countries may allow bees to synthesize bioactive compounds from several botanical

sources, possibly increasing the concentration, availability, and diversity of compounds in propolis with potential beneficial effects on human and animal health. For this reason, it is important to report the compounds of propolis obtained in the different countries, particularly those with a large botanical variety such as countries in South America.

In conclusion, the results of this research demonstrate a beneficial effect of the propolis inclusion in broiler feed, improving some production parameters and cellular responses associated with intestinal integrity. This study also showed that propolis did not have a detrimental effect on any of the parameters evaluated, so it is postulated as a potential alternative to replace AGP in poultry. The results of this research and the comparison with other studies, should consider that the variability of the effects of propolis in birds could exist due to several factors. According to the results of this study, the inclusion of 900 ppm had the greatest positive effect in the finishing broilers. Future studies will be necessary to generate more information on the effects of propolis and the feasibility of its inclusion in human and animal nutrition in countries with the potential to produce this natural additive.

ACKNOWLEDGMENTS

The financial support for the current study was provided by the Ministerio de Ciencia, Tecnología e Innovación − Minciencias de Colombia and Gobernación de Cundinamarca, university-industry alliance (Universidad Nacional de Colombia and Campo Colombia) within the framework of the grant Colombia Bio − 828 de 2019.

DISCLOSURES

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

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