Effect of live *Eimeria* vaccination or salinomycin on growth and immune status in broiler chickens receiving in-feed inclusion of gelatin and vitamin E

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ABSTRACT This experiment determined if 2% of gelatin, to improve the levels of proline and glycine in the diet, and 70 mg/kg of vitamin E supplementation would relieve the impaired performance of male Cobb broilers vaccinated for coccidiosis. Half of the chicks were vaccinated via water (live oocysts), while the other half received medication (salinomycin) in the feed until 35 d of age. The effects of coccidiosis vaccine on performance and mRNA levels of genes involved in mucin synthesis, cytokines, trefoil family factor-2 (**TFF2**), and metabolic processes (CD36) in the jejunum of broilers were measured. Vaccination negatively affected performance in the first 21 d; however, the inclusion of gelatin and vitamin E reduced this negative response. Additionally, supplementation with these nutrients led to an improvement in broilers receiving the coccidiostat ($P < 0.05$). From 21 to 35 d, birds treated with gelatin and coccidiosis vaccine experienced better body weight gain than birds without gelatin and vitamin E ($P < 0.05$). Vaccinated chickens had decreased body weight and decreased anti-inflammatory cytokine expression. Furthermore, they had increased inflammatory cytokine expression, mucin 2 expression, and TFF2 compared to salinomycin-fed broilers ($P < 0.05$). Transcripts for IL-1B, IFN-y, MUC2, TFF2 were decreased while mRNAs for IL-4 and IL-10 increased in salinomycin-fed broilers compared to vaccinated broilers ($P < 0.05$). In conclusion, broilers vaccinated against coccidiosis increase their pro-inflammatory immune status and mucin expression compared to broilers receiving salinomycin. These events may contribute to lower performance in vaccinated broiler chicks. Moreover, vitamin E and gelatin can minimize the vaccine's negative immune effects and promote better performance.

Key words: coccidiosis, nonessential, amino acids, vaccine, vitamin

INTRODUCTION

A healthy gut is fundamental for optimum performance, better feed efficiency, and overall health of poultry. The healthy gut can be compromised by coccidiosis, a parasitic disease that results in intestinal infection. *Eimeria* infection of broiler chickens can be controlled by vaccination (Williams, 2005; Shirley and Lillehoj, 2012). Mass administration of coccidiostat drugs has long been employed as a highly effective method of control, however drug resistance is widespread, and there is pressure demanding reduced drug usage in livestock production (Antonissen et al., 2016; Karavolias et al., 2018).

Vaccination for coccidiosis may be an alternative to anticoccidials. However, the vaccine has been shown to adversely affect the performance of broilers compared to those given a dietary anticoccidials (Lehman et al., 2009). The reduced performance due to the vaccines is associated with low infection rates (Lee et al., 2011) necessary to induce immunity, resulting in a decrease of absorbent intestinal surface area (Lehman et al., 2009). In response to vaccination, the intestinal mucosa increases production of mucin (Miller and Narva, 1979). Mucin is rich in amino acids such as glycine, serine, proline, and mainly threonine (Moran, 2008). Furthermore, pro-inflammatory cytokines can stimulate mucin production. The pyrogenic effect of the vaccine (fever) increases the basal metabolic rate by 10 to 15% for every 1°C rise in body temperature.

The objective of this study was to determine if the negative effects of the vaccination on broiler performance could be improved by providing additional

nutrients, such as gelatin and vitamin E for bird recovery. Gelatin is an ingredient that has a substantial content of nonessential amino acids (Boomgaardt and Baker, 1972) and can be an alternative to supply those nutrients required in greater amounts to produce mucin and recovery of the intestinal epithelium. To control inflammatory effects, nonsteroidal anti-inflammatory additives, such as vitamin E, could be used (Silva and Penna, 2012). Vitamin E supplementation preserves immune responses in individuals exposed to free radicals (Fialkow et al., 2007) and minimizes pro-inflammatory effects (Jiang, 2014). To investigate the questions defined above, an experiment was conducted to evaluate the use of gelatin and vitamin E in the feed to reduce the impact of the coccidiosis vaccine on the intestinal epithelium and growth performance of broiler chickens.

MATERIALS AND METHODS

All procedures were approved by the Ethics Committee on Animal Use of the Universidade Federal do Rio Grande do Sul, Brazil (number 35670).

Broiler Husbandry

A total of 560 one-day-old, male chicks (Cobb 500) were vaccinated according to the vaccine schedule at the hatchery (Languiru Group, Brazil). Chicks weighed 48 g and were randomly placed into 56 floor pens (10 birds per pen), respecting a variation between the replicates of $\pm$ 2.5% of total body weight and were raised until 42 d of age. Pens were covered with previously used wood shavings and were equipped with a tube feeder and nipple drinkers. Environmental temperature management to maintain the chickens in thermoneutral conditions during all growth stages were performed using air conditioning, fans, and exhaust fans. Birds had ad libitum access to water and mash feed.

Experimental Design, Diets, and Experimental Procedures

Broilers were distributed into eight treatments with 7 replicates (10 birds/each) in a completely randomized design. Half of the birds were vaccinated via water according the, against coccidiosis with a live attenuated vaccine (LIVACOX, 30−50 thousand oocysts (per liter of water) of each attenuated strain of *E. tenella, E. acervulina*, and *E. maxima*) on the first day of life. The other half (4 treatments) received a diet containing 60 mg/kg of salinomycin (Coxistac 12%) from the first day until 35 d of age. Two treatments in each group of coccidiostat or vaccine, received the addition of 70 mg per kg/feed of c, but 2% of gelatin, or both combined.

The control group received 35 mg of vitamin E and did not receive gelatin supplementation. The lower level of dietary vitamin E (35 mg/kg feed) used in this study was chosen to approximate concentrations found in commercial starter rations. Treatments are described in

Table 1. Experimental treatments.

Treatment	Dietary treatments	Protection
T1	Salinomycin	Coccidiostat
T2	Salinomycin + Vitamin E2	Coccidiostat
T3	Salinomycin + Gelatin1	Coccidiostat
T4	Salinomycin + Vitamin E + Gelatin[1]	Coccidiostat
T5	Vaccine	Vaccination
T6	Vaccine + Vitamin E[2]	Vaccination
T7	Vaccine + Gelatin1	Vaccination
T8	Vaccine + Vitamin E + Gelatin	Vaccination

[1]2% inclusion of gelatin is expressed on a total mixed diet basis
[2]70mg/kg of Vitamin E is expressed on kg of feed

Table 1. Four diets were used in the feeding program: pre-starter (1−7 d); starter (7−21 d); grower (21−35 d); and finisher (35−42 d). The diets, formulated according to Rostagno (2017), consisted of corn and soybean meal as main ingredients and were isonutritive (Table 2). The analysis of gelatin showed only the presence of 14 amino acids, with a peculiar characteristic of a high content of proline, hydroxyproline, and glycine.

Body weight (**BW**) averaged by pen was recorded weekly. Body weight gain (**BWG**), feed intake (**FI**), and feed conversion ratio (**FCR**) corrected for mortality bird weight were determined by feeding phase. Mortality was recorded daily. At 28 d of age, one chicken per replicate was euthanized for the collection of 5 cm of intestinal tissue located in the jejunum/ileal junction. Immediately after collection, samples were frozen in liquid nitrogen and stored at −80°C.

Gene Expression

Based on differential expression levels of genes, we tried to characterize the changes in the intestinal immune status and the choice of a panel of genes selected for further investigation is shown in Table 3. One ml per 100 mg tissue of Trizol reagent (Invitrogen, Carlsbad, CA) was used for RNA extraction according to the manufacturer's recommendations. Tissue was triturated using TissueLyser (Qiagen Retsch MM300 TissueLyser, MD) (tissue + Trizol) to complete dissociation. Chloroform (200 μL) was added and homogenized by hand for 1 min. The upper aqueous phase was obtained by centrifuging the homogenate at 12,000 g (320R Refrigerated bench centrifuge; Hettich, Tuttlingen, Germany) for 15 min at 4°C. The liquid phase was collected and transferred to a clean tube with 500 μL isopropanol. The solution was incubated at room temperature for 10 min and was then centrifuged at 12,000 g for 10 min at 4°C. The supernatant was discarded and the precipitate washed with 950 uL 75% ethanol. Pellets were dried for 15 min and then resuspended in RNase-free ultrapure water. The quality of RNA was determined by NanoDrop-1000 (Thermo Fisher Scientific, Waltham, MA) and the OD260/280 values of all samples were limited to a range of 1.9 to 2.0. No sample was excluded based on this characteristic. The integrity of total RNA (**mRNA**) was determined using ultraviolet

Table 2. Experimental diets with and without vitamin E and gelatin.

	Pre-starter (1−7 d)		Initial (8−21 d)		Grower (22−35 d)		Finisher (36−42 d)	
	control	w/ gelatin	control	w/ gelatin	control	w/ gelatin	control	w/ gelatin
Corn	56.64	56.71	58.91	60.70	61,.1	62.99	65.66	67.42
SBM	37.76	35.54	34.84	30.54	31.85	27.55	27.77	23.51
Soybean oil	1.38	1.42	2.67	2.67	3.45	3.80	3.42	3.77
L-Lysine HCl	0.30	0.30	0.38	0.38	0.24	0.29	0.27	0.32
DL-Methionine	0.36	0.36	0.35	0.35	0.28	0.33	0.27	0.31
L-Threonine	0.11	0.11	0.10	0.10	0.06	0.09	0.06	0.09
NaCl	0.52	0.52	0.50	0.50	0.47	0.47	0.46	0.46
Bicalcium Phosphate	1.89	1.90	1.59	1.59	1.32	1.36	1.11	1.15
Limestone	0.91	0.91	0.94	0.94	0.88	0.88	0.80	0.79
Choline	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05
Gelatin[1]	0.00	2.00	0.00	2.00	0.00	2.00	0.00	2.00
Constant[2]	. . . . .to 100%.						*calculated composition*	
ME, kcal/kg	2.960		3.050		3.150		3.200	
CP, %	22.4		21.21		19.9		18.4	
	Essential amino acids							
Dig Lys, %	1.324	1.324	1.275	1.275	1.131	1.131	1.060	1.060
Dig Met, %	0.651	0.655	0.588	0.627	0.552	0.592	0.525	0.525
Dig Met+Cis, %	0.953	0.953	0.876	0.876	0.826	0.826	0.774	0.774
Dig Thr, %	0.861	0.861	0.791	0.791	0.735	0.735	0.689	0.689
Dig Trp, %	0.257	0.254	0.241	0.217	0.225	0.200	0.211	0.211
Dig Arg, %	1.393	1.394	1.308	1.317	1.221	1.230	1.145	1.148
Dig Val, %	0.927	0.923	0.876	0.837	0.824	0.785	0.763	0.754
Dig Iso, %	0.865	0.860	0.814	0.763	0.762	0.711	0.711	0.645
Dig Leu, %	1.723	1.716	1.651	1.584	1.576	1.509	1.424	1.357
Dig His, %	0.536	0.532	0.509	0.466	0.482	0.439	0.442	0.399
	Nonessential amino acids							
Dig Gli, %	0.763	1.137	0.720	1.094	0.676	1.050	0.628	1.002
Dig Pro, %	1.226	1.396	1.183	1.360	1.137	1.316	1.010	1.180
Dig Ser, %	1.031	1.020	0.972	0.943	0.913	0.883	0.848	0.830

[1]Pure beef gelatin, Gelnex Company, Road 283, km 36. Ita, SC −Brazil 89760-000.

[2]Constant included 0.034% and 0.100% each vitamin & mineral premixes (provided the following per kg of premix: Vitamin premix = 34.520.000 IU vitamin A, 7.200. 000 IU vitamin D3, 8.600 mg vitamin K, 6.700 mg vitamin B1, 20.000 mg vitamin B2, 9.000 mg vitamin B6, 72.000 mcg vitamin B12, 34.000 mg pantothenic acid, 140.000 mg niacin, 2.800 mg folic acid, and 240 mg biotin. Mineral premix = cobalt 200 mg, 88.000 mg manganese, 95.535 mg zinc, 64.715 mg iron, 15.000 mg copper, 1795 mg iodine, 200 mg selenium). 70 mg/kg Vitamin E was add *on top* according to treatments. Coccidiostat Group feed with 0.05% Salinomycin (Bio-Cox 60 Granular, Alpharma Inc). Kaolin was add according to treatments and considered to have zero nutrient contribution. Vaccinated group feed without coccidiostat - birds vaccinated.

spectrophotometry and agarose gel electrophoresis. All samples had clear 28S and 18S ribosomal RNA bands, and no sample was excluded. The RNA samples were stored at −80°C until required for cDNA synthesis. The total concentration of RNA was determined by Qubit (Qubit 3.0 Fluorometer, MA) and next, 1 μg of RNA was treated with Deoxyribonuclease I (Fermentas Life Sciences, TX). The treated RNA was used for a reverse transcription (cDNA) reaction using the high GoScript Reverse Transcription Mix, Oligo(dT) (ThermoFisher), following the manufacturer's protocol. The cDNA was diluted at 1:100 in nuclease-free water and stored at −20°C for subsequent use for gene expression profiling.

Primers (**qRT-PCR**) were designed using Primer-BLAST on the NCBI website and GAPDH was used as reference genes on all PCR plates and genes tested. qRT-PCR was performed using an StepOnePlus Real-Time PCR System (Applied Biosystems, Foster City, CA) with a PowerUP SYBR Green PCR Master Mix (Thermo Fisher Scientific). The PCR mixture contained 5 μL PowerUP SYBR Green Supermix, 0.5 μL (10 mmol; SigmaAldric, MA) of each primer, and 1 μL of cDNA, along with H_2O for a total volume of 10 μL. The procedure was as follows: UDG activation − 50°C for 2 min; Dual-Lock DNA polymerase − 95°C for 2 min; followed by 40 cycles of denaturation at 95°C for 15 s; and annealing/extension for 1 min at 60°C. The individual measurements were performed in triplicate, and the relative gene expression was calculated using the 2−ΔΔCt method.

Statistical Analysis

All statistical analyses for gene expression were performed on transformed GAPDH-normalized data. SAS 9.3 software was used to perform the contrast analyses of the performance data. Contrasts were chosen to establish clearer comparisons, due to the great number of treatments. The partitioning of growth rate reduction for birds receiving the vaccine was calculated as described by Pastorelli et al. (2012). In brief, the relationship between variation in feed intake (**ΔFI**) and variation in weight gain (**ΔWG**) of vaccinated birds relative to medicated birds (expressed as a percentage) was analyzed using linear regression: ΔBWG = α + ΔFI. The analysis was carried out using the REG procedure in SAS. The intercept (α) represents ΔBWG related to

Table 3. Oligonucleotide primers used for quantitative reverse transcription-PCR of chicken cytokines.

Type	Gene	Primer Sequence[1]	Genbank accession no.[3]
Reference	GADPH[*,2]	F:GGTGGTGCTAAGCGTGTTAT	KO1458
		R:ACCTCTGTCATCTCTCCAC	
Pro-inflammatory	IL-1B	F:TGGGCATCAAGGGCTACA	AF111631
		R:TCGGGTTGGTTGGTGATG	
	IL-6	F:CAAGGTGACGGAGGAGGAC	AJ309540
		R:TGGCGAGGAGGGATTTCT	
	IL-17	F:CTCCGATCCCTTATTCTCCTC	AJ493595
		R:AAGCGGTTGTGGTCCTCAT	
	TNFSF15	F:CCTGAGTATTCCAGCAACGCA	AB194710
		R: ATCCACCAGCTTGATGTCACTAAC	
Th1	IFN-γ	F:AGCTGACGGTGGACCTATTATT	Y07922
		R:GGCTTTGCGCTGGATTC	
	IL-2	F:TCTGGGACCACTGTATGCTCT	AF000631
		R:ACACCAGTGGGAAACAGTATCA	
	IL-12	F:AGACTCCAATGGGCAAATGA	NM_213571
		R:CTCTTCGGCAAATGGACAGT	
Th2	IL-4	F:AACATGCGTCAGCTCCTGAAT	FJ907790.1
		R:TCTGCTAGGAACTTCTCCATTGAA	
	IL10	F:CGGGAGCTGAGGGTGAA	AJ621614
		R:GTGAAGAAGCGGTGACAGC	
	IL13	F:CCAGGGCATCCAGAAGC	AJ321735
		R:CAGTGCCGGCAAGAAGTT	
Chemokine	IL8	F:GGCTTGCTAGGGGAAATGA	AJ009800
		R: AGCTGACTCTGACTAGGAAACTGT	
Mucins	MUC2	F:ATGCGATGTTAACACAGGACTC	JX284122.1
		R:GTGGAGCACAGCAGACTTTG	
	Muc5ac	F:TGTGGTTGCTATGAGAATGGA	XM_025151100.1
		R:TTGCCATGGTTTGTGCAT	
	Muc13	F:GCATTCCTCAAGCAGAGGTG	XM_025152568.1
		R:CTCAGGCTGCCGTGATATTT	
Trefoil factor 2	TFF2	F:GCTGTAGCCCTCATCAGCTC	XM_010724393.2
		R:CTGGCAGCTATTTTGCACTG	
CD36 molecule	CD36	F:GAATTGCTGTGGAAGTGCTG	XM_025147445.1
		R:TGGTCCCAACAGACTCACTG	
Inducible nitric oxide synthase	iNOS	F:TGGGTGGAAGCCGAAATA	XM_034069034.1
		R:GTACCAGCCGTTGAAAGGAC	

[1]F: forward; R reverse.
[2]Glyceraldehyde-3-phosphate.dehydrogenase.
[3]ID: GenBank access number.
[*]Housekeeping gene.

changes in the animal's maintenance, not associated with ΔFI. The coefficients represent the extent of ΔBWG associated with ΔFI.

RESULTS

Performance

Average mortality was 2.86% and there was no difference between groups. During the first 3 wk, temporary infection from vaccination created more stress than anticoccidial medication, evidenced by the reduced BWG and lower BW from 1 to 21 d ($P < 0.05$; Table 4). At 35 d of age, lowe BW and poorer FCR of vaccinated birds were observed ($P < 0.05$), however, no differences were observed for BWG among vaccine or coccidiostat birds in the period from 22 to 42 d ($P > 0.05$). No statistical differences were observed in any period between vaccine or coccidiostat groups for FI ($P > 0.05$).

Although no effect on performance was observed when gelatin or vitamin E was used alone, their concomitant use proved beneficial in the coccidiostat group ($P < 0.05$; Table 5).

To minimize the impact of the vaccine on performance, gelatin or vitamin E used in isolation were not enough in the period from 1 to 21 d, as well as from 1 to 42 d ($P > 0.05$) for most responses. However, vitamin E plus gelatin improved 42 d BW compared to the vaccine group without nutritional adjustment ($P < 0.05$). This higher final BW may be a reflex of a better FCR tendency from 1 to 21 d ($P < 0.07$). Gelatin inclusion alone improved performance of vaccinated birds in the period from 22 to 42 d; birds showed higher BWG and better FCR in this phase ($P < 0.05$).

Intestinal Gene mRNA Levels

The levels of inflammatory intestinal gene transcripts for IL-1β and IFN-γ (T helper 1 family) were increased in the vaccine chickens compared with coccidiostat-fed birds at d 28 ($P < 0.05$). On the contrary, those cytokines decreased in "vaccinated" chickens receiving gelatin and vitamin E ($P < 0.05$), while TNFSF15, IL-17F, IL-2, and IL-12 transcript levels did not show any differences (Figures 2 and 3). Transcript levels for IL-4 and IL-10 anti-inflammatory cytokines decreased in vaccine broilers compared to coccidiostat chickens ($P < 0.05$),

Table 4. Performance of broilers, vaccinated or not for coccidiosis receiving supplementation with vitamin E and gelatine, from 1 to 21 and 1 to 42 days of age.

Treatments	BW 21	BWG 1−21	FI 1−21	FCR 1−21	BW 42	BWG 1−42	FI 1−42	FCR 1−42
Coccidiostat	971	923	1,265	1.37	3,213	3,165	5,010	1.58
Coccidiostat + Vitamin E	1,048	1,000	1,317	1.32	3,240	3,163	5,028	1.60
Coccidiostat + Gelatin	1,102	1,053	1,308	1.24	3,293	3,250	5,125	1.57
Coccidiostat + Vitamin E + Gelatin	1,096	1,048	1,322	1.26	3,357	3,318	5,108	1.53
Vaccine	914	866	1,206	1.39	3,090	3,042	4,995	1.64
Vaccine + Vitamin E	891	842	1,216	1.44	3,060	3,011	4,960	1.64
Vaccine + Gelatin	891	843	1,169	1.39	3,162	3,104	4,959	1.59
Vaccine + Vitamin E + Gelatin	959	910	1,208	1.32	3,204	3,152	5,011	1.59
Contrasts				P value[1]				
Coccidiostat vs. Vaccine	0.046	0.032	ns	ns	0.031	ns	ns	ns
Coccidiostat vs. Coccidiostat + Vitamin E	0.008	0.011	ns	ns	ns	ns	ns	ns
Coccidiostat vs. Coccidiostat + Gelatin	<0.0001	<0.0001	ns	<0.001	ns	ns	ns	ns
Coccidiostat vs. Coccidiostat + Vitamin E + Gelatin	<0.0001	<0.0001	ns	0.003	0.012	0.012	ns	0.001
Vaccine vs. Vaccine + Vitamin E	ns	ns	ns	ns	ns	ns	ns	ns
Vaccine vs. Vaccine + Gelatin	ns	ns	ns	ns	ns	ns	ns	ns
Vaccine vs. Vaccine + Vitamin E + Gelatin	ns	ns	ns	0.070	0.046	ns	ns	ns
SEM[2]								
	15.5	15.6	16.0	0.01	26.3	32.9	52.6	0.01

[1]Probabilities lower than 0.05 are considered.
[2]Standard Error. ns = no significance.

Table 5. Performance of broilers, vaccinated or not for coccidiosis receiving supplementation with vitamin E and gelatine, from 22 to 42 d of age.

Treatments	BW 42	BWG 22−42	FI 22−42	FCR 22−42
Coccidiostat	3,213	2,240	3,406	1.52
Coccidiostat + Vitamin E	3,240	2,192	3,381	1.54
Coccidiostat + Gelatin	3,293	2,191	3,392	1.54
Coccidiostat + Vitamin E + Gelatin	3,357	2,270	3,431	1.51
Vaccine	3,090	2,224	3,377	1.52
Vaccine + Vitamin E	3,060	2,168	3,280	1.51
Vaccine + Gelatin	3,162	2,260	3,329	1.47
Vaccine + Vitamin E + Gelatin	3,204	2,243	3,347	1.49
Contrasts		P value[1]		
Coccidiostat vs. Vaccine	0.031	ns	ns	ns
Coccidiostat vs. Coccidiostat + Vitamin E	ns	0.077	ns	ns
Coccidiostat vs. Coccidiostat + Gelatin	ns	ns	ns	ns
Coccidiostat vs. Coccidiostat + Vitamin E + Gelatin	0.012	ns	ns	ns
Vaccine vs. Vaccine + Vitamin E	ns	ns	ns	ns
Vaccine vs. Vaccine + Gelatin	ns	0.019	ns	0.010
Vaccine vs. Vaccine + Vitamin E + Gelatin	0.046	0.083	ns	ns
SEM[2]				
	18.3	10.5	15.7	0.007

[1]Probabilities lower than 0.05 are considered.
[2]Standard Error. ns = no significance.

but IL-4 transcript levels in the vaccine group were greater when vitamin E and gelatin were given ($P < 0.05$; Figure 4).

Intestinal MUC2 gene transcription was increased in the vaccine without supplementation vs. coccidiostat broilers ($P < 0.05$), but vitamin E and vitamin E + gelatin lowered MUC2 expression ($P < 0.05$) in this group. No differences between treatments were observed for MUC13 and MUC5ac ($P > 0.05$; Figure 5). Similar to that observed for MUC2, TFF2 transcription was higher in the vaccine chicks ($P < 0.05$) in relation to the coccidiostat group and vitamin E + gelatin decreased its expression. iNOS and CD36 transcription were not significantly affected by either diet supplementation or vaccination ($P > 0.05$; Figure 6).

DISCUSSION

Vaccination for coccidiosis negatively influenced broilers performance compared to birds receiving coccidiostat. This better performance at d 21 and 42 in medicated versus vaccinated chickens was also demonstrated by Lehman et al. (2009), who reported that broilers immunized with Paracox vaccine had a poor performance during the first 3 wk post-hatching compared with chickens receiving salinomycin. Niewold (2007) hypothesized that antibiotics may stimulate growth by inhibiting the production and circulation of cytokines. Cytokine release causes an acute phase response leading to a loss of appetite and muscle tissue catabolism. Certainly, inflammation leads to performance decreases,

but equally may act by shifting the microbiota composition toward one that is less capable of evoking an inflammatory response (Humphrey and Klasing, 2003). Antibiotics could also simply lower the total microbial load, leading to less inflammation and a lower energetic cost for the animal.

Coccidiosis vaccines can induce a mild transient form of coccidiosis, usually occurring between 14 and 28 d post-hatching, which can impair broiler performance (Lehman et al., 2009). This transient form of coccidiosis usually occurs around 21 d (third cycle of oocysts) and reflects in low performance. Silva et al. (2009) similarly reported a vaccine-induced reduction in BWG at 21 d of age but observed no differences in BWG between vaccinated and unvaccinated birds at the final phase (36 d of age). One of the negative responses often associated with a coccidiosis vaccination is a decline in BWG, and in the current study, we observed this response clearly at 21 d. Lower body weight was observed at 42 d in the vaccinated birds, however, similar BWG was observed between the groups from 22 to 42 d. As the poultry cycle is short, despite a possible compensatory gain at this phase, the gain was not enough to achieve the same BW as broilers receiving anticoccidials.

Treatments with salinomycin plus amino acid and vitamin E supplementation improved broiler performance at 21 d. This result suggests that despite the good support of salinomycin, essential amino acids can be conditionally important factors in animals' performance even in the absence of a defined challenge (as the vaccine). Additionally, vitamin E can protect cells against free radical oxidative processes (Tappel, 1972) and act as an immunomodulator (Boa-Amponsem et al., 2000). The negative effects of the vaccine were more pronounced until 21 d of age and supplementation with gelatins and vitamin E reflected better BWG and FCR in the period from 22 to 42 d. The amino acid supply and the antioxidant and immunomodulatory effect of vitamin E seem to allow birds to recover better from vaccine effects in the compensatory period.

The intercepts from vaccine groups were negative, suggesting that between vaccinated and unvaccinated groups, the fraction due to changes in maintenance was negative and independent of FI. Therefore, under vaccination effect, broilers experienced an increase in maintenance requirements, explaining the lower BWG. This change in maintenance for the vaccine group was -1.23% of the total BWG variation (Figure 1B).

Intestinal gene transcripts for IL-β and IFN-γ were increased, whereas IL-4 and IL-10 transcripts were decreased in chickens compared with coccidiostat birds at 28 d. It seems that, as Niewold (2007) proposed, salinomycin may activate anti-inflammatory pathways in the avian gut, as evidenced by increased transcription of the counter-regulatory cytokines IL-4 and IL-10 compared with cocci-vaccine. At the same time, the vaccine increased pro-inflammatory cytokines released by antigen-recognizing cells, including IL-1β. Intestinal inflammation can contribute to the reductions in the performance of broilers vaccinated. The relative effects of
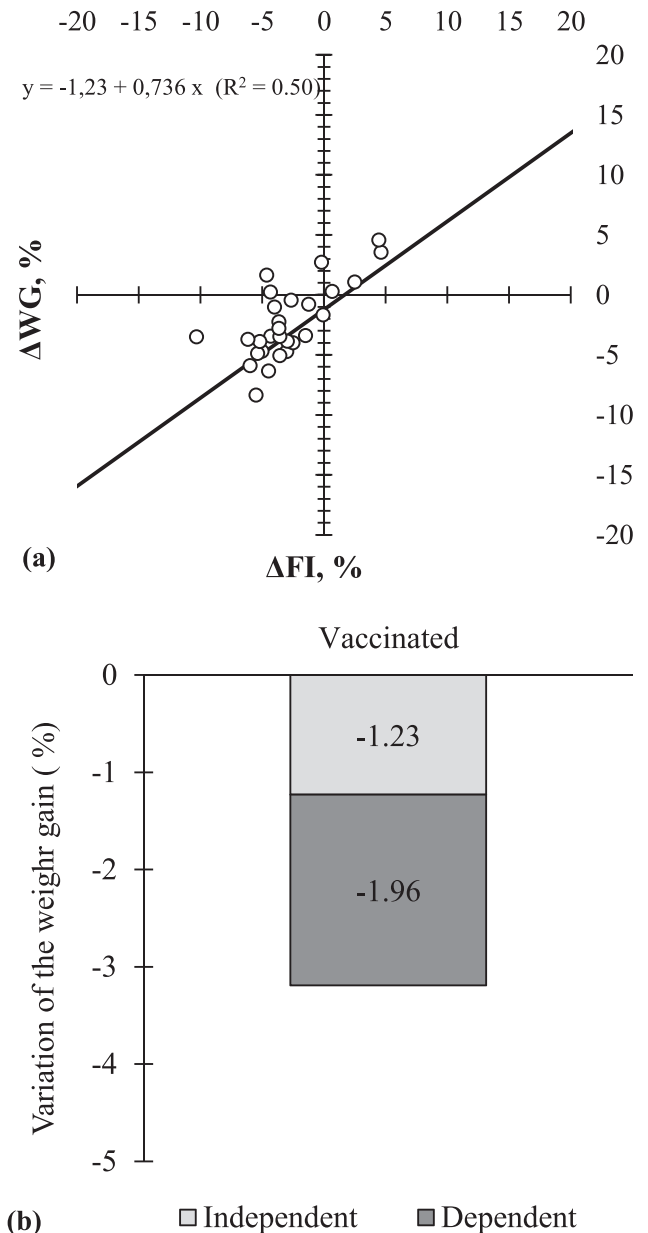

Figure 1. Relationship between the reduction in feed intake and growth rate (based on Pastorelli et al. 2012): (A) Relationship between the variation in growth (ΔWG, Weight Gain 1 to 42 d) and in feed intake (ΔFI, Feed Intake 1 to 42 d) of chickens vaccinated for coccidiosis. Responses are expressed as results protected by coccidiostats broilers relative to the vaccinated chickens. The lines represent the linear model adjustments. (B) Partitioning of the reduction in the average growth rate resulted from vaccination between the fraction due to changes in maintenance requirement (☐ not associated with a reduction in feed intake) or due to the change in feed efficiency (▨ associated with a reduction in feed intake).

coccidiosis vaccination on intestinal levels of pro-inflammatory cytokine transcript, may reflect the heightened inflammatory status induced by the live parasites.

The correlation between variation (Δ) in weight gain and feed intake caused by vaccination was $R^2 = 0.509$ ($P < 0.05$) (Figure 1A). The variation in weight gain (ΔWG) showed a linear relationship with variation in feed intake (ΔFI). The intercepts of the equations were different from zero and negative. The partition of the
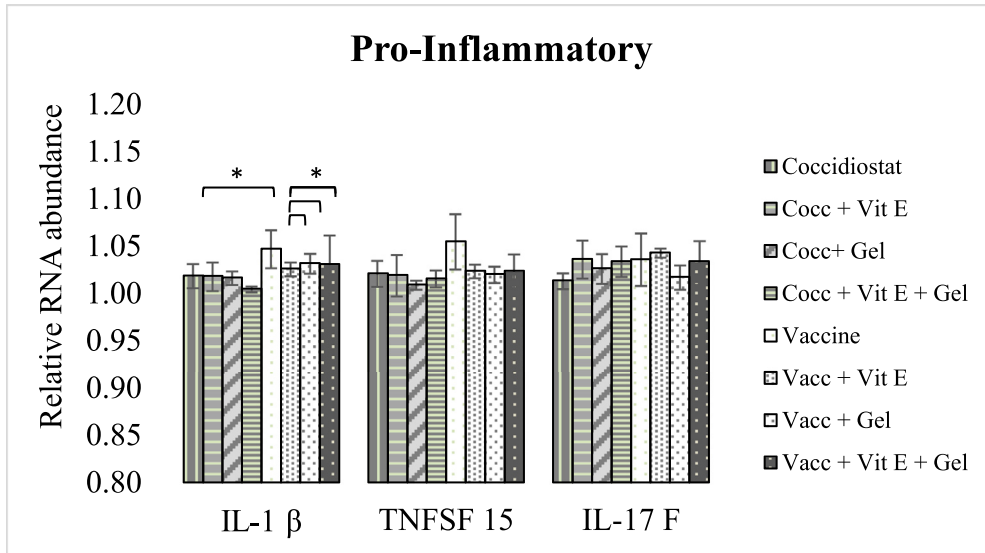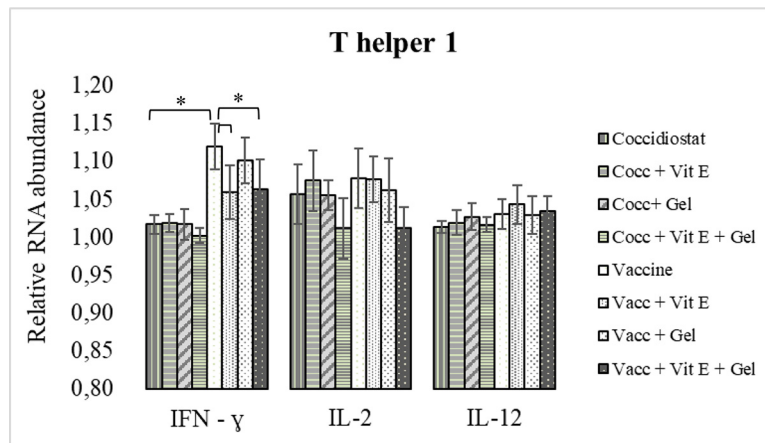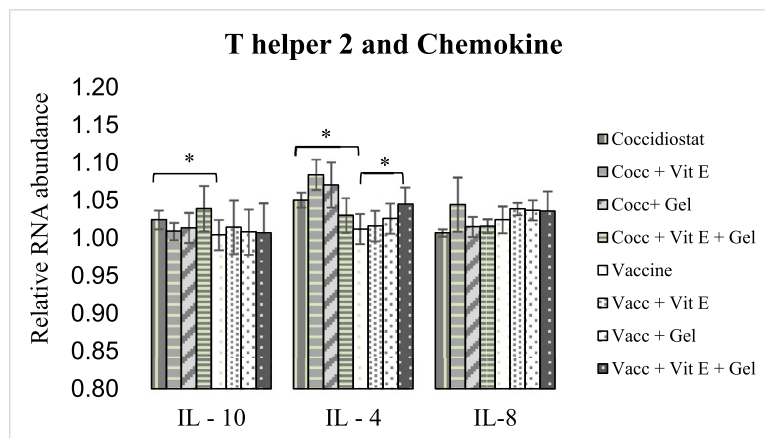
Pro-Inflammatory

Figure 2. Effect of coccidiosis vaccination or coccidiostat on pro-inflammatory intestinal cytokine transcript levels. At 28 d post-hatch, intestinal tissues were removed and the levels of transcript for IL1β, TNFSF15, IL-17F, and GAPDH were quantified by real time RT-PCR. The mRNA levels for each cytokine were normalized to the GAPDH internal control. Vertical bars represent mean ± SD normalized mRNA levels (n = 7 repetition/treatment). Contrast Bars are significantly different ($P < 0.05$).

T helper 1

Figure 3. Effect of coccidiosis vaccination or coccidiostat on T helper 1 intestinal cytokine transcript levels. At 28 d post-hatch, intestinal tissues were removed and the levels of transcript for IFN-γ, IL-2, IL-12, and GAPDH were quantified by real time RT-PCR. The mRNA levels for each cytokine were normalized to the GAPDH internal control. Vertical bars represent mean ± SD normalized mRNA levels (n = 54). Contrast Bars are significantly different ($P < 0.05$).

T helper 2 and Chemokine

Figure 4. Effect of coccidiosis vaccination or coccidiostat on T helper 2 intestinal cytokine transcript levels. At 28 d post-hatch, intestinal tissues were removed and the levels of transcript for IL-10, IL-4, IL-8, and GAPDH were quantified by real time RT-PCR. The mRNA levels for each cytokine were normalized to the GAPDH internal control. Vertical bars represent mean ± SD normalized mRNA levels (n = 7). Contrast Bars are significantly different ($P < 0.05$).
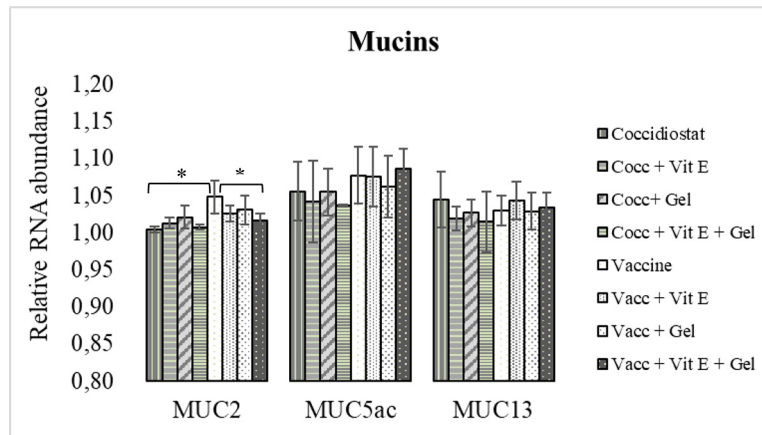
Figure 5. Effect of coccidiosis vaccination or coccidiostat on intestinal mucins transcript levels. At 28 d post-hatch, intestinal tissues were removed and the levels of transcript for MUC2, MUC5ac, MUC13, and GAPDH were quantified by real time RT-PCR. The mRNA levels for each prime were normalized to the GAPDH internal control. Vertical bars represent mean ± SD normalized mRNA levels (n = 7). Contrast Bars are significantly different ($P < 0.05$).

effects on ΔWG corrected for the average ΔFI is presented in Figure 2B.

A beneficial effect of nutritional supplementation in the reduction of inflammatory status was observed: broilers receiving gelatin and vitamin E had lower IL-1β and IFN-γ intestinal levels of cytokine transcripts. The parasites invade in the intestinal epithelium to replicate and produce oocysts which causes an inflammatory response in the intestine of the host. Vitamin E is absorbed in its unesterified form through the intestinal epithelium and is readily incorporated into cellular membranes where it acts as an anti-inflammatory immunomodulator in chickens (Boa-Amponsem et al., 2000). This effect can be seen in the lower expression of the IFN-γ cytokine in the group treated with vitamin E.

Coccidiosis vaccination skewed the immune balance toward a pro-inflammatory/Th1 state, while salinomycin favored an anti-inflammatory status. Infection with Eimeria protozoa upregulates the expression of pro-inflammatory cytokines while decreasing the expression of anti-inflammatory cytokines (Engberg et al., 2000; Lillehoj et al., 2007; Lillehoj and Li, 2004; Lee et al., 2011).

The exposure to vaccine was not enough for detecting cytokine expression of IL-6 and IL-13 (data not shown). Additionally, an increase in iNOS expression was not observed in any group at 28 d and this can be related to the time when the acute phase of infection occurs, that is between 4 and 7 d post vaccination (Allen and Fetterer, 2002). In the current experiment, coccidiosis vaccination did not increase the gene expression of nitric oxide in the intestinal mucosa, which is in agreement with the findings of Perez-Carbajal et al. (2010), suggesting that the transient coccidiosis elicited by vaccination is not as severe as the natural infection.

Increased mucin production is a reflection of the intestinal inflammatory process caused by the vaccine. Supplementation with vitamin E and gelatin provided better recovery of the intestinal epithelium, explained by the lower production of mucin. The vaccine group
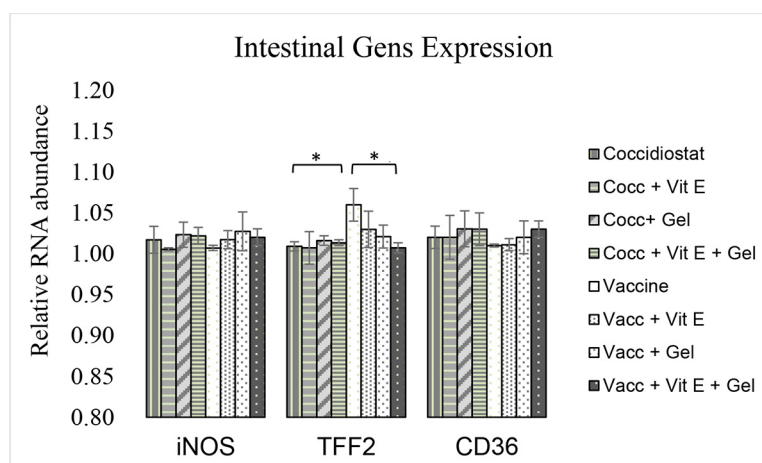

Figure 6. Effect of coccidiosis vaccination or coccidiostat on intestinal gens transcript levels. At 28 d post-hatch, intestinal tissues were removed and the levels of transcript for iNOS, TFF, CD36, and GAPDH were quantified by real time RT-PCR. The mRNA levels for each prime were normalized to the GAPDH internal control. Vertical bars represent mean ± SD normalized mRNA levels (n = 7). Contrast Bars are significantly different ($P < 0.05$).

also showed higher expressions of mucin genes than the coccidiostat group, as indicated by the higher MUC2 intestinal gene transcripts in the vaccine group. After a coccidiosis infection, broilers increase secretory mucin (MUC2) production and need to regenerate the surface of the damaged intestinal enterocytes. Coccidiosis infection has been shown to increase mucin production through inflammatory cytokine production leading to increased mucogenesis (Collier et al., 2008). Challenged gut demands more amino acids. Supplementation with gelatin and vitamin E allowed the intestinal mucosa to recover early and consequently produced less mucin.

Mucin is composed of high amounts of nonessential amino acids, and although broilers are able to synthesize them, this may not be fast enough to satisfy the higher demand after vaccination (Lehman et al., 2009). It was proven to be beneficial to supply these amino acids in the form of gelatin, facilitating chickens' recovery from coccidial vaccination.

Although it is not clear which amino acids provide the benefit during the vaccine challenge, proline and glycine are both extremely important to the improved performance. Glycine is readily synthesized by the chick, but this synthesis may not be rapid enough to satisfy the needs for fast tissue growth (Corzo et al., 2004; Jiang et al., 2005; Dean et al., 2006), especially after tissue damage caused by infection. Mucin is rich in amino acids such as glycine, serine, proline, and mainly threonine (Moran, 2008). Intestinal challenges can be demanding more quantities of these amino acids which can be found in gelatin. Despite the importance of these 2 conditionally nonessential amino acids, their requirements under varied conditions are still not fully known.

According to Forder et al. (2012), when mucosa deteriorates as a result of coccidiosis challenge, the expression of MUC2 becomes impeded, preventing replenishment of the mucus layer and increasing the chance of new infections and damage. However, the vaccine did not cause a sufficient degree of lesions to prevent replenishment of the mucus layer as this group showed greater expressions of MUC2 compared to the salinomycin group and also to the vaccinated group receiving gelatin and vitamin E supplementation. MUC2 has been shown to have NF-ƙB binding sites within the promoter region which are upregulated in the intestinal tract during inflammation (Kim and Ho, 2010). This architecture suggests that a low level of infection is enough to stimulate the physical gut barrier function through the upregulation of MUC2. Trefoil family factor genes (**TFF2**) interact with mucin to maintain intestinal barrier function (Suzuki et al., 2006) and play an important role in the repair of the damaged intestinal mucosa, and their expression increases after mucosal damage (Kurt-Jones et al., 2007). TFF2 mRNA transcripts were significantly increased by the vaccine. The vitamin E and gelatin helped establish the intestinal barrier function though increasing TFF gene expression.

The CD36 transcription gene was not affected by treatments. Levels of CD36 are significantly suppressed when the broilers are challenged by Eimeria. The main function of CD36 is to measure lipid transport and enterocyte metabolism. The challenge caused by the vaccine is not strong enough to affect metabolism in the intestinal epithelium.

The relative effects of coccidiosis vaccination on intestinal levels of cytokine transcripts may reflect the heightened inflammatory status. Coccidiosis vaccines can be a good alternative to prevent coccidiosis in broiler flocks, but can induce intestinal epithelium damage, impairing performance and also increasing the metabolic costs associated with activation of the immune system. As seen, the weight gain depression that occurred between days 1 and 21 post vaccination, can be relieved by using better nutritional support. In conclusion, results reported herein indicate that coccidiosis vaccination had a significant adverse impact on overall BW, BWG, and FCR of vaccinated birds, although overall FI was not impaired by vaccination. Given diets with additional gelatin, vaccinated broilers benefited from additional amino acids, especially proline and glycine, provided by gelatin. Broilers that received the inclusion of vitamin E and gelatin had a significant improvement in performance, even when not vaccinated.

DISCLOSURES

SUPPLEMENTARY MATERIALS

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.psj.2022.102206.

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