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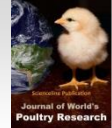

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Immune Profile of Broilers between Hatch and 9 Days of Age Fed Diets with Different Betaine and Fibre Concentrations

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ABSTRACT

An experiment was designed to determine the immune profile of broiler chickens between hatch and 9 days of age when fed diets with different fibre and betaine concentrations. A total of 240 day-old Cobb 500 male chickens were allocated to 16 cages with 15 chickens each. Treatments were arranged in a 2 x 4 factorial design, with 2 replicate cages per treatment. Treatments consisted of two feed formulations (low and high fibre diets) and four levels of betaine (0, 1, 3 or 5 kg/tonne). Before the start of trial (hatch), 10 broilers were weighed and blood samples were collected by cardiac puncture, then euthanised by cervical dislocation and jejunal samples collected for the determination of gene expression of claudin 1, claudin 5, occludin and interleukin 2 by PCR. Mononuclear cells populations in the blood samples were determined by flow cytometry. On days 4 and 9, five birds/cage (10 birds/treatment) were selected, euthanised and samples taken as per the start of hatch. Gene expression of claudin 1, claudin 5 and occludin was reduced between 4 and 9 days, independent of the group tested, while interleukin tended to increase between hatch and 4 days and decreased thereafter. Betaine inclusion reduced claudin 5 and occludin gene expression and increased CD8- CD28+ and CD8+ CD28+, suggesting it may aid in accelerating maturation of both the gastrointestinal tract and immune system for broilers in the early days post-hatch.

Key words: Betaine; Fibre; Gene expression; Maturation; Immune response

INTRODUCTION

The genetic selection imposed on broilers for improving weight gain and feed conversion ratio has reduced its immunocompetence and as a result, increased the disease susceptibility (Bridle et al., 2006). The generation of an unnecessary immune response represents a significant expense of energy and nutrients that could be targeted for other processes such as body weight gain. On other hand, failure to generate an immune response when required, is even more detrimental due to the morbidity and mortality it may cause. The immune system not only react to pathogenic challenges but also support the nervous system maintaining animal homeostasis (Tada, 1997).

At hatch, broilers do not have a fully developed immune system (Kogut, 2017). Both cellular and humoral immune systems develop during the first few days post hatch, including the development of the bursa of Fabricius and the thymus (Dibner et al., 1998) that increase proportionally more than other organs at early ages, receding as the animal gets older (Cazaban et al., 2015).

The gastrointestinal tract is not fully developed at hatch (Uni et al., 2003). The association in development of these two systems is linked because the development of the immune system depends on the availability of nutrients and energy absorbed via the gastrointestinal tract. Moreover, the gut functions as a sensory organ for the development of the immune system (Furness et al., 2013). Impaired nutrient absorption can influence the initial development of the immune system, compromising immunocompetence of the animal (Dibner et al., 1998). At the same time, maintenance of the immune status in the gastrointestinal tract has a high cost for the animal (Kogut et al., 2017).

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At hatch, gastrointestinal enterocytes are not tightly linked to each other, allowing the passage of large molecules via paracelullar routes directly into the interstitial spaces (Karcher and Applegate, 2008). Opening of paracellular passages is a route used by pathogenic bacteria and viruses while other bacteria actually increase rigidity of these junctions thereby reducing permeability (Sommer and Backhed, 2013). Gut-associated lymphoid tissues are agglomeration of immune cells which protect the body against the passage of microbiota from the gastrointestinal tract to other organs that are more concentrated in the hindgut and especially the ceca where the microbiota population is highest (Yegani and Korver, 2008).

The gut microbiota changes as soon as the animal starts to eat (Ballou et al., 2016). The composition of the feed influences the immune status of the animal and the species of bacteria that colonize the tract (Oakley and Kogut, 2016). The diet also influences the osmolarity of the intestines, with hyperosmolarity often stimulating inflammatory responses (Schwartz et al., 2009). Thus, the composition of the feed can influence the immune status of broilers. High fibre diets have been shown to increase the digesta transit time and stimulate bacteria growth due to a reduction in nutrient digestibility (Adeola et al., 2016) and also increase gut fermentation and possibly increase the inflammatory status of the digestive tract (Celi et al., 2017). On the other hand, fibre fermentation in the lower gut stimulates volatile fat acid such as butyrate, a trophic substance that stimulates villus growth (Rahmatnejad and Saki, 2016), increases gap junction protein expression, reduces inflammatory responses and endogenous losses during a coccidial challenge (Adedokun et al., 2012), lowers the presence of pathogenic bacteria in internal organs and improves animal performance (Rezaei et al., 2011).

Betaine is an osmoprotectant and is recognised as a chemical chaperone preventing protein denaturation and thus maintaining cell activity in hyperosmotic situations (Schwahn et al., 2003). Birds fed betaine have been shown to have reduced inflammatory responses when subsequently exposed to a coccidial challenge and maintain higher water flow and villus size (Kettunen et al., 2001), but similar effects were not observed when the betaine was offered at the same time as the coccidial challenge (Matthews and Southern, 2000).

The objective of the present study was thus to evaluate the proportion of circulating immune cells, and the tight protein and interleukin 2 gene expression in broilers at hatch, 4 and 9 days of age when fed diets with different levels of fibre and inclusion rates of betaine.

MATERIALS AND METHODS

Ethical approval

The study design was reviewed and approved by the Animal Use Ethics Committee of the Agricultural Science Campus of the Universidade Federal do Paraná (Protocol number 002/2015).

Animals, diets and experimental design

Two hundred and forty day-old male Cobb 500 broilers were sourced specifically for the experiment and housed in 16 stainless steel cages with 15 birds/cage. A further 10 birds were included for sampling at hatch, for measurement of the parameters of interest at the outset of the experiment. Room temperature was controlled with electric heaters to meet the breeder recommendations for the age of the bird (Cobb-Vantress Inc., 2015). Cage dimensions were 0.90 m × 0.40 m, and 0.30 m height. Birds had *ad libitum* access to water and feed. Treatments were designed in a 2 × 4 factorial arrangement with two levels of fibre concentration (low and high) and four inclusion rates of betaine (0, 1, 3 and 5 kg betaine/tonne of feed). Experimental diets were based on corn, soybean and rice bran as previously described (Santos et al., 2019) with Betaine (Vistabet® 96, AB Vista – Marlborough, United Kingdom) included at the targeted rate for each treatment by replacing the relevant weight of washed sand (Table 1).

Sample collection

At hatch, 10 birds that were not assigned to any treatment were weighed and separated for initial sampling. Blood was collected by cardiac puncture. Birds were then euthanized by cervical dislocation. The intestine was collected from the cardiac junction to the cloaca. One jejunal sample, defined as the section between the duodenal loop and 1cm distal of the Meckel`s diverticulum (Teirlynck et al., 2009), was taken from each bird. At 4 and 9 days of age, 10 birds per treatment were separated and the same collection process was followed for blood and jejunum collection.

Flow cytometry analysis

Approximately 2 mL of blood was collected into a heparin treated vacutainer tube. Initially, the mononuclear cells in the blood were isolated using Histopaque-1077 (Sigma Aldrich, St. Louis, USA), as described by Fair et al. (2008). Briefly, blood samples were centrifuged at 400 x g for 30 min after dilution with Phosphate-Buffered Saline (PBS) and Histopaque-1077, and white cells then collected as the layer above the erythrocytes. The white cells were then washed with PBS and centrifuged at 400 x g for 7 min. Samples were fixed with 1% paraformaldehyde and counted using a Neubauer counting chamber.

Once the cell concentration in the blood was determined, flow cytometry was performed as reported by Stabel et al. (2000). Briefly, 50µl of cell suspension (equivalent to 10^6 mononuclear cells) was incubated for 30 min at 37 °C with specific primary antibodies and fixed in paraformaldehyde for 30 min at 4 °C. This process was repeated for the secondary antibody. Monoclonal antibodies to the specific subset of cells were supplied by Southern Biotechnology. "CD4" specificity for T helper lymphocytes; "CD8β" specificity for T cytotoxic lymphocytes; "CD28" specificity for memory T lymphocytes; "Mo" specificity for monocytes; "MHCII" specificity for antigen-presenting cells and "TCR αβ Vβ1" specificity for T lymphocytes were prepared.

Reading was performed within 2 h of staining on a FACSCalibur flow cytometer (Becton Dickinson, United Kingdom), as reported by Beirão et al. (2012). Green fluorescence was detected on the FL1 channel (530/30 nm), and orange fluorescence was detected on the FL2 channel (585/42 nm). Cells were analysed at up to 10,000 events in the lymphocyte gate and data were analysed with FlowJo software (TreeStar Inc., United States).

Gene expression

Jejunal samples were immediately put in dry ice after sampling and stored at -80 ^{o}C. A 5 mm^2 section of each sample was collected and processed as described by Adedokun et al. (2012). RNA extraction was proceed using RNAzol® RT kit as per the manufacturer's protocol (Molecular Research Center Inc., Cincinnati, OH, USA). The RNA pellet was dissolved in nuclease-free water (Omnipure, EMD Chemicals Inc., Gibbstown, NJ) and its concentration determined using a 260/280 nm nanodrop spectrophotometer (Fisher Scientific, St. Louis, MO) and extracted RNA stored at -80°C (Beirão et al., 2012).

Primers were designed for real-time PCR with the Oligo Analyzer 3.1 tool from Integrated DNA Technologies for the (complementary DNA) cDNA sequence obtained from the NCBI database. Genes evaluated in this study were glyceraldehyde-3-phosphate dehydrogenase (GAPDH) which served as the housekeeping gene, claudin 1 and 5, occludin and interleukin 2. A list of the primers used in the present study is reported in table 2.

PCR analysis was performed as described by Bustin et al. (2009). Complementary DNA was synthesized from 1µg of total RNA using iScript Select cDNA Synthesis kit (BioRad) with Oligo $(dT)_{20}$ primer mix and GSP enhancer solution. The synthesis reaction was follows: 25 ^{o}C for 5 min, 42 ^{o}C for 1 h, and 70 ^{o}C for 15 min. The reaction for PCR were carried out on a RotorGene Real-Time PCR System (Quiagen Inc., Thousand Oaks, CA) in an EvaGreen fluorescent system. The following PCR conditions were used: 95 ^{o}C for 10 min, followed by 40 cycles of 60 ^{o}C for 10 sec and 95 ^{o}C for 10 sec. All samples were processed in triplicate and expressed relative to the house-keeping gene. For the analysis of gene expression at different ages, the expression was calculated based on the average expression of the target gene and housekeeping gene as reported by Pfaffi (2001) using the results from the low fibre diet without betaine inclusion as the reference.

Statistical analysis

Data was log-transformed prior to analysis as described by Beirão et al. (2012). Data were subjected to ANOVA using the GLM model for completely randomised design procedure of JMP Pro (SAS Institute Inc., Cary, NC, USA). Treatment means at hatch, 4 and 9 days of age were separated by Tukey`s Significant Difference test. Results at 4 and 9 days of age were also analysed using a two-way interaction considering fibre and betaine inclusion levels for the gene expression data and inclusion of a third factor, age, to generate the three-way interaction for the leukocyte population. A three-way interaction for gene expression was not considered as the samples were not collected from the same animals. Each animal served as the experimental unit. Statistical significance was accepted at $p < 0.05$.

Table 1. Ingredients and chemical composition of experimental diets (g/kg, as-fed basis).[1]

Ingredients	Low fibre diet	High fibre diet
Corn (8% CP[2])	533.60	463.80
Rice bran (12.5% CP[2])	-	70.00
Soybean oil	35.00	43.50
Soybean meal (46% CP[2])	385.00	376.50
Limestone	10.60	10.90
Dicalcium phosphate	18.00	17.50
Salt	4.60	4.60
Vitamin/Mineral premix[3]	2.00	2.00
Lysine HCl	2.05	2.05
DL Methionine	3.25	3.25
L- Threonine	0.65	0.70
Washed sand	5.00	5.00
Choline Cloride	0.25	0.20
Chemical composition		
Crude protein	220	220
Metabolizable energy, kcal/kg	3000	3000
Crude fibre	29.50	43.50
Sol. Arabinoxylane	0.53	0.60
Insol. Arabinoxylanse	19.21	21.81
Sol NSP[4]	19.88	19.30
Insol NSP[4]	89.48	93.74
Ether extract	60.00	77.00
Ash	34.00	39.00
Calcium	9.50	9.50
Phosphorous	6.80	7.60
Available Phosphorous	4.50	4.50
Sodium	2.00	2.00
Digestible Lysine	12.50	12.50
Digestible Methionine+Cysteine	9.10	9.10
Digestible Threonine	8.10	8.10

[1]Betaine included at the expense of inert. [2]Crude Protein. [3]Supplied per kilogram diet: iron (Ferrous Sulphate), 60 mg; manganese (Manganese Sulphate and Manganese Oxide), 120 mg; zinc (Zinc Oxide), 100 mg; iodine (Calcium Iodate), 1 mg; copper (Copper Sulphate), 8mg; selenium (Sodium Selenite), 0.3mg, vitamin A, 9,600 IU; vitamin D_3 3,600 IU; vitamin E, 18mg; vitamin B_{12}, 15 mcg; riboflavin, 10 mg; niacin, 48 mg; d-pantothenic acid, 18 mg; vitamin K, 2 mg; folic acid, 1.2 mg; vitamin B_6, 4 mg; thiamine, 3 mg; d-biotin, 72 mcg. [4]Non-starch polysaccharides.

RESULTS

Average body weights were 47, 121 and 260 g at hatch, 4 and 9 days of age, respectively, which is close to the expected weight of the genetic line (Cobb-Vantress Inc., 2015) and no differences were observed between treatments. A detailed description of results has previously been published (Santos et al., 2019).

Claudin, occludin and interleukin 2 gene expression

Gene expression for claudin 1 and claudin 5, occludin and interleukin 2 was reduced ($p < 0.001$) at 9 days compared to hatch and 4 days of age (Table 3). At 4 days of age, inclusion of 1 and 3 kg/tonne of betaine reduced ($p < 0.05$) gene expression of claudin 5 and occludin while inclusion of 5 kg/tonne had intermediate results (Table 4). At day 9, reduction was dependent on the fibre content in the diet as betaine inclusion reduced ($p < 0.0001$) claudin 1 and 5, occludin and interleukin 2 gene expression in the low fibre diets but no effect was observed in high fibre diets. When betaine was not included in the diet, broilers fed the high fibre diets also presented lower ($p < 0.0001$) gene expression for claudin 1 and 5, occludin and interleukin 2 (Table 4).

Peripheral blood mononuclear cells

Peripheral blood mononuclear cells population varied depending on the age and inclusion of betaine, while fibre concentration had no effect. The population of CD8-CD28+ and CD8+CD28+ cells increased ($p < 0.0001$) from hatch to 4 days of age and fell thereafter, while CD8+CD28- cells further increased ($p < 0.0001$) at day 9 (Table 5). Betaine inclusion increased ($p < 0.0001$) the proportion of both CD8-CD28+ and CD8+CD28+ cells independent of the dose and age, but had no effect on the population of CD8+CD28- cells (Table 5). On the other hand, the proportion of CD4+TCRvβ1+ and CD4+TCRvβ1- cells increased ($p < 0.0001$) from hatch to 4 days of age and fell afterwards, whilst CD4-TCRvβ1+ increased ($p < 0.0001$) from hatch to 4 days and 4 to 9 days of age (Table 5). The impact of betaine on the proportion of CD4+TCRvβ1+; CD4+TCRvβ1- and CD4-TCRvβ1+ populations depended upon age (Table 6). At 4 days of age, inclusion of 3 kg/tonne of betaine increased ($p < 0.0001$) the proportion of CD4-TCRvβ1+; and inclusion of 5 kg/tonne increased ($p < 0.0001$) the proportion of both CD4+TCRvβ1+ and CD4+TCRvβ1- cells, but no effect of betaine inclusion was observed at 9 days of age. The proportion of both Mo+MHCII+ and Mo-MHCII+ cells compared with total cells increased ($p < 0.0001$) in birds at 4 days of age while that of Mo+MHCII- fell ($p < 0.0001$) between 4 and 9 days of age (Table 5). Age affected the CD4:CD8 ratio with the CD4+:CD8+ ratio falling ($p < 0.001$) between days 4 and 9, but no difference was observed between these ages and birds at hatch.

Table 2. Primers used for real-time PCR

Genes	Forward primer sequence (5'-3')	Reverse primer sequence (5'-3')	Fragment size (base pair)
GAPDH[1]	TCTGGAGAAACCAGCCAAGT	GAGACAACCTGGTCCTCTGTG	104
CLDN1[2]	CCAATGAAGAGGGCTGAT	GTGCATGGAGGATGACCA	185
IL2[3]	TCCCAGGTAACACTGCAGAGTTT	TTGGAAAATATCAAGAACAAGATTCATC	92
CLDN5[4]	ATCTGTGCGCCTTTGAGACT	GCGACCTGCAATGAGTTCG	149
OCLN[5]	GTGGGTTCCTCATCGTCATC	GTTCTTCACCCACTCCTCC	156

[1]Glyceraldehyde 3-phosphate dehydrogenase; [2]Claudin 1; [3]Interleucine 2; [4]Claudin 5; [5]Occludin

Table 3. Claudin 1, Claudin 5, Occludin and Interleukin 2 gene expression (log of the proportion of GADPH expression) on jejunal samples of broilers at hatch, 4 and 9 days of age.[1]

Age (days)	Claudin 1	Claudin 5	Occludin	Interleukin 2
hatch	-0.055[a]	-0.588[a]	-0.176[a]	-0.798[a]
4	-0.374[a]	-0.687[a]	-0.469[a]	-0.108[a]
9	-2.828[b]	-2.521[b]	-2.143[b]	-2.184[b]
SEM	0.109	0.211	0.137	0.202
p-value	<0.001	<0.001	<0.001	<0.001

[1]Means and standard error of the mean (SEM) represent 10 birds at hatch, 4 and 9 days of age. [a,b]Mean within columns with different superscripts are statistically different ($p < 0.05$).

Table 4. Claudin 1, Claudin 5, Occludin and Interleukin 2 gene expression (log of the proportion of GADPH expression) on jejunal samples of broilers at 4 and 9 days of age and fed diets with different fibre concentration and betaine inclusion.[1]

Treatment		Claudin 1		Claudin 5		Occludin		Interleukin 2	
		Age (days)							
		4	9	4	9	4	9	4	9
Fibre									
Low[2]		-0.145	-1.820	-1.008	-2.081	-0.918	-1.591	-0.482	-3.087
High[3]		-0.136	-2.526	-0.734	-2.768	-0.605	-2.143	-0.472	-3.769
SEM		0.095	0.125	0.147	0.145	0.143	0.117	0.098	0.142
Betaine (kg/tonne)									
0		-0.001	-1.277	-0.359[a]	-1.680	-0.237[a]	-1.354	-0.356	-2.255
1		-0.228	-2.279	-1.161[b]	-2.780	-1.095[b]	-2.021	-0.500	-3.672
3		-0.182	-2.678	-1.183[b]	-2.632	-1.050[b]	-1.918	-0.504	-4.005
5		-0.153	-2.432	-0.774[ab]	-2.660	-0.660[ab]	-2.071	-0.549	-3.781
SEM		0.135	0.178	0.208	0.204	0.203	0.166	0.139	0.203
Fibre × Betaine(kg/tonne)									
Low	0		-0.134[a]		-0.426[a]		-0.304[a]		-0.874[a]
	1		-1.886[b]		-2.455[b]		-1.723[bc]		-3.218[b]
	3		-2.854[b]		-2.618[b]		-1.990[bc]		-4.160[b]
	5		-2.611[b]		-2.932[b]		-2.426[bc]		-4.097[b]
High	0		-2.548[b]		-2.934[b]		-2.666[c]		-3.635[b]
	1		-2.934[b]		-3.105[b]		-2.518[bc]		-4.126[b]
	3		-2.502[b]		-2.646[b]		-1.846[bc]		-3.850[b]
	5		-2.176[b]		-2.388[b]		-1.564[bc]		-3.466[b]
SEM			0.229		0.293		0.238		0.291
p-value									
Fibre		0.928	0.001	0.167	0.002	0.111	0.002	0.940	0.001
Betaine		0.658	<0.001	0.021	0.001	0.013	0.046	0.776	<0.001
Fibre × Betaine		0.388	<0.001	0.945	<0.001	0.866	<0.001	0.604	<0.001

[1]Means and standard error of the mean represent 10 birds/treatment at 4 and 9 days of age. [2]Corn-soybean meal diet. [3]Corn-rice bran-soybean meal diet. [a-c]Mean within columns with different superscripts are different ($p < 0.05$).

Table 5. Leukocyte population determined by flow cytometry (log of the proportion of the population) on blood samples of broilers fed diets with different fibre concentration and betaine inclusion at hatch, 4 and 9 days of age.[1]

Leukocytes	CD8- CD28+	CD8+ CD28+	CD8+ CD28-	Mo+ MHCII-	Mo+ MHCII+	Mo- MHCII+	CD4- TCRvβ1+	CD4+ TCRvβ1+	CD4+ TCRvβ1-	CD4:CD8 Ratio
Age (days)										
hatch	0.553[b]	0.012[c]	-1.425[c]	-0.496[a]	0.044[b]	-0.266[b]	-0.375[c]	0.360[c]	-0.128[c]	2.91[ab]
4	1.141[a]	0.603[a]	-0.399[b]	-0.457[a]	0.476[a]	0.028[a]	0.286[b]	0.959[a]	0.568[a]	3.15[a]
9	0.726[b]	0.276[b]	0.106[a]	-0.839[b]	0.430[a]	0.007[a]	0.382[a]	0.563[b]	0.307[b]	2.01[b]
SEM	0.028	0.029	0.031	0.053	0.032	0.034	0.028	0.028	0.031	0.15
Fibre										
Low[2]	0.930	0.445	-0.139	-0.647	0.469	0.045	0.366	0.774	0.415	2.60
High[3]	0.902	0.437	-0.111	-0.682	0.432	-0.015	0.308	0.756	0.443	2.49
SEM	0.026	0.027	0.031	0.052	0.031	0.033	0.026	0.027	0.029	0.14
Betaine (kg/tonne)										
0	0.824[b]	0.286[b]	-0.224	-0.801	0.431	-0.043	0.259	0.690	0.339	2.84
1	0.971[a]	0.495[a]	-0.164	-0.660	0.497	0.028	0.370	0.773	0.436	2.35
3	0.977[a]	0.466[a]	-0.079	-0.602	0.475	0.042	0.318	0.774	0.465	2.48
5	0.964[a]	0.516[a]	-0.112	-0.537	0.404	0.039	0.384	0.823	0.515	2.50
SEM	0.037	0.037	0.044	0.073	0.043	0.046	0.036	0.038	0.041	0.21
p-value										
Age	<0.001	<0.001	<0.001	<0.001	<0.001	<0.010	<0.001	<0.001	<0.001	<0.001
Fibre	0.719	0.822	0.727	0.560	0.356	0.172	0.120	0.629	0.457	0.489
Betaine	0.010	<0.001	0.107	0.082	0.435	0.536	0.074	0.107	0.027	0.423
Fibre × Betaine	0.949	0.497	0.561	0.162	0.058	0.224	0.318	0.852	0.985	0.385
Fibre × Age	0.402	0.849	0.428	0.188	0.519	0.903	0.632	0.413	0.478	0.437
Age × Betaine	0.118	0.377	0.545	0.393	0.125	0.191	0.011	0.050	0.036	0.216
Fibre×Age×Betaine	0.671	0.684	0.962	0.974	0.712	0.962	0.611	0.688	0.554	0.082

[1]Means and standard error of the mean represent 10 birds at hatch day of age and 10 birds/treatment at 4 and 9 days of age. [2]Corn-soybean meal diet. [3]Corn-rice bran-soybean meal diet. [a-c]Mean within columns with different superscripts are different ($p < 0.05$).

DISCUSSION

At hatch, the paracellular junctions between enterocytes in the intestine are not well developed, allowing the paracellular transport of large molecules from the yolk sac. This route of absorption reduces rapidly as these paracellular junctions tighten. The results from the present experiment support this hypothesis as the gene expression for claudin 1 and claudin 5 and occludin was greater between hatch and 4 days compared with birds at day 9. This suggests a greater rate of production of these proteins to increase the strength of these junctions during the first few days post hatch. The percentage of enterocyte membrane involved in tight junctions increases in the 3 days after hatch (Karcher and Applegate, 2008), supporting that during these early days post hatch, the tight junctions become more rigid.

Gene expression of interleukin 2 tended to increase gene expression between hatch and 4 days and reducing afterwards. Interleukin 2 is a proinflammatory interleukin involved in the proliferation of lymphocytes by stimulating the transition of activated T cells from the G_1 to the S phase (Han et al., 2010). An increase in interleukin gene expression between hatch and 4 days of age may be related to a transitory proinflammatory status as birds may be reacting to the presence of digesta in the gastrointestinal tract. Most of the materials in the gastrointestinal tract, although not pathogenic, are capable of stimulating an immune response (Huges, 2005).

At hatch the gastrointestinal tract of chickens is empty, with absorption of fat and readily available proteins in the yolk sac taking place majorly from the yolk sac membrane and the intestine directly into the blood (Noy and Sklan, 2001). Once birds hatch and start to consume feed with more complex nutrients (more complex proteins, carbohydrates, fibre, etc), the intestine may react immunologically due to the presence of these nutrients in the lumen. Given at hatch the digestive tract of chicken is not completely developed (Geyra et al., 2001), the low digestibility and absorption rates can result in a hyperosmotic solution that further contributes to a proinflammatory response (Hubert et al., 2004; Schwartz et al., 2009). It is estimated that 3% of the metabolised energy of the chicken could be directed towards the

maintenance of a feed-induced immune response (Kogut, 2017).

At 4 days of age, the inclusion of 1 and 3 kg/tonne of betaine in the diet reduced gene expression for claudin 5 and occludin, which suggests there has been a quicker development of the gastrointestinal tract and the demand for tight junction formation has reduced. Tight junction formation is affected by enterocyte differentiation and replication (Karcher and Applegate, 2008). Betaine inclusion increases villus height, modulates immune response and improves gastrointestinal tract recovery after a coccidial challenge (Kettunen et al., 2001; Klasing et al., 2002). As an osmoprotectant, betaine may help the gastrointestinal tract maintain its physiology when facing a transitory hyperosmotic period. At 9 days of age, gene expression of claudin 1, claudin 5, occludin and interleukin 2 were higher in broilers fed the low fibre diet and these were reduced by both betaine inclusion or fibre addition. Fibre interferes with the inflammatory responses in broilers but it is dependent upon the type of fibre used (Teirlynck et al., 2009). Inclusion of soluble fibre increases inflammatory responses while insoluble fibre reduce these inflammatory responses and stimulate villus growth (Rezaei et al., 2011; Adedokun et al., 2012). Fibre composition in rice bran is mainly represented by insoluble fibre.

Cytotoxic T cells are classified dependent on the presence of CD8 and CD28 in the superficia of their membrane. T cells that express both CD8 and CD28 are recognised as T cells in initial stages of activation or memory cells (CD8+CD28+) and T cells that express CD28 but not CD8 are recognised as non-activated T cells (CD8-CD28+) (Nabeshima et al., 2002). When T cells are activated for a long period of time, they lose the expression of CD28 while maintaining CD8 expression and are classified as activated cytotoxic T cells (CD8+CD28-). These cells are specific to target antigens and cannot replicate (Nabeshima et al., 2002; Pawelec et al., 2004).

The proportion of cytotoxic T cells in the leukocyte population increased between hatch and 4 days, and a reduction in this percentage was observed for CD8-CD28+ and CD8+CD28+ between 4 and 9 days, while CD8+CD28- increased further. The initial increase in the population of cytotoxic T cells may be related to the development of the immune system. Beirão et al. (2012) observed that under normal commercial conditions the levels of cytotoxic T cells increase in proportion to other T cells from day of age to 5 weeks This suggests that the changes noted in the present study is related to an early development of the immune system. Betaine inclusion increases the proportion of CD8+CD28+ and CD8-CD28+ suggesting it aids in the development of the immune status but no effect was observed in the population of CD8+CD28- as the effect of betaine is likely not related to specific antigens.

Helper T cells are classified by the presence of CD4 and TCRvβ1 phenotypes. Helper T cells with CD4+ and TCRvβ1- (CD4+TCRvβ1-) reaction are recognised as classical helper T cells and are responsible for activation of lymphocytes and adaptative immune response (Chan et al., 1988). When TCRvβ1 is expressed (CD4+ TCRvβ1+) cells tends to migrate to the mucosa where they are responsible for IgA production (Cihak et al., 1991). Other helper T cell that express TCRvβ1, but not CD4, are classified as cytotoxic mucosal T cells (CD4- TCRvβ1+) and have an important role in cellular immunity (Lillehoj, 1994). Birds challenged by *Salmonella* spp. have reduced concentrations of CD4 and CD8 in the peripheral blood (Flores et al., 2012) which actually increases their susceptibility to *Salmonella* infection (Kamalavenkatesh et al., 2005). Inclusion of betaine increased the percentage of helper T cells at 4 days but not at 9 days of age. As the objective of the present study was not to measure the response to a specific pathogen, but to evaluate the immune system development in young birds, it could be concluded that betaine may play a role as an immune modulator during the transition period following hatch and improve immune competence.

Monocytes (Mo+ MHCII+) are precursors of macrophages in different tissues and express Mo marker in the membrane and represents a connection between the innate and adaptative immune responses. There are other cells that are not monocytes (Mo-) that may also be antigen-presenting cells when expressing the MHCII marker in its membrane (Mo-MHCII+). Monocytes that do not present the MHCII marker reduce lymphocyte activity, classified as suppressor monocytes (Mo+MHCII-) (Masternak and Reith, 2002). An increase in the proportion of both Mo+MHCII+ and MO-MHCII+ between hatch and 4 days of age and the reduction of suppressor monocytes (Mo+MHCII-) shows how the chick prepares for any immune response during these early stages. The betaine and fibre concentration in the diet did not affect the proportion of these populations.

The ratio between CD4 and CD8 cells may be used as an indicator of cell-mediated immune response (Cheng et al., 2001). Higher ratios of CD4:CD8 are usually an indication of immunocompetence (Beirão et al., 2012), whilst reduced values are usually associated with

challenging environmental factors, immunization or challenges such as bronchitis (Beirão et al., 2012). Genetic selection for higher growth and egg production is also associated with a reduction in CD4:CD8 ratio (Cheng et al., 2001). In the present evaluation, the CD4:CD8 ratio was reduced (p <0.001) at day 9 compared with day 4 but the results were still greater than 1.5 which is considered as a normal ratio (Cheng et al., 2001) when cellular immune mechanisms are not impaired and survivability is not compromised (Reid and Tervit, 1995; Levinson and Jawetz, 1996). A reduction of the CD4:CD8 ratio can be associated with a natural exposure to immune estimulators in the environment as the ratio is reduced in birds raised commercially compared with birds raised specifically to be pathogen-free (Flores et al., 2012). Some degree of immune challenge may be required to develop the immune system by increasing the relationship between helper and cytotoxic cells (Beirão et al., 2012). Neither fibre concentration nor betaine inclusion affected the CD4:CD8 ratio in the current study. A lack of response may be related to the development of the immune system being a result of exposure to a moderate bacterial challenge, but not a challenge by specific pathogens.

CONCLUSION

Betaine inclusion reduced expression of claudin 5 and occludin, and increased the proportion of cytotoxic T cells, non-activated helper T cells, and helper T cells. These results suggest that betaine can be used as a modulator of immune maturation during the early days post hatch, probably not by acting directly on these immune cells but helping the birds to support and adapt to this initial period and then reducing the inflammatory response associated with this adaptation process. Fibre content had no effect on the gene expression and leukocyte population in broilers until 9 days of age. As fibre will affect the microbiota population in the gut, it is possible that the effect of fibre on the immune status of broilers takes longer to develop. In both cases, the fluctuation of the local gene expression for tight junction proteins and leukocyte populations shows that gut development is closely linked with development of the systemic immune system due to the role of the gut as a sensory organ.

DECLARATIONS

Authors' contributions

Tiago Tedeschi dos Santos, Suelen Baal and Ana Vitória Fisher da Silve designed the trial and developed it. Celso Favero worked on Laboratory analysis. Mike Bedford, Sophie Lee and Tiago Tedeschi dos Santos analysed the statistical data and wrote the article. All authors confirmed the final version of the original article for publishing in this journal.

Competing interests

All authors declare no conflicts of interest.

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