Italian Journal of Animal Science

ISSN: (Print) (Online) Journal homepage: https://www.tandfonline.com/loi/tjas20

Effect of mannan-rich fractions and a mixture of sodium butyrate and zinc proteinate on performance, intestinal morphometry, and gene expression in broilers

M. A. S. Silva, V. Ribeiro Júnior, C. M. Silva, L. F. C. Silva, A. P. Del Vesco, G. M. Oliveira Junior, A. A. Calderano, F. C. Tavernari, A. Corassa & C. O. Brito

To cite this article: M. A. S. Silva, V. Ribeiro Júnior, C. M. Silva, L. F. C. Silva, A. P. Del Vesco, G. M. Oliveira Junior, A. A. Calderano, F. C. Tavernari, A. Corassa & C. O. Brito (2023) Effect of mannan-rich fractions and a mixture of sodium butyrate and zinc proteinate on performance, intestinal morphometry, and gene expression in broilers, Italian Journal of Animal Science, 22:1, 728-738, DOI: 10.1080/1828051X.2023.2240850

To link to this article: https://doi.org/10.1080/1828051X.2023.2240850

Published online: 09 Aug 2023.

ITALIAN JOURNAL OF ANIMAL SCIENCE
2023, VOL. 22, NO. 1, 728–738
https://doi.org/10.1080/1828051X.2023.2240850

Taylor & Francis
Taylor & Francis Group

RESEARCH ARTICLE

OPEN ACCESS Check for updates

Effect of mannan-rich fractions and a mixture of sodium butyrate and zinc proteinate on performance, intestinal morphometry, and gene expression in broilers

M. A. S. Silva[a], V. Ribeiro Júnior[b], C. M. Silva[a], L. F. C. Silva[c], A. P. Del Vesco[a], G. M. Oliveira Junior[a], A. A. Calderano[d], F. C. Tavernari[e], A. Corassa[f] and C. O. Brito[a]

[a]Department of Animal Science, Federal University of Sergipe, São Cristóvão, SE, Brazil; [b]Nucleus of Animal Science, Federal University of Sergipe, Nossa Senhora da Glória, SE, Brazil; [c]Alltech of Brazil, Maringá, PR, Brazil; [d]Department of Animal Science, Federal University of Viçosa, Viçosa-MG, SE, Brazil; [e]Brazilian Agricultural Research Corporation – EMBRAPA Swine and Poultry, Concórdia, SC, Brazil; [f]Department of Animal Science, Universidade Federal de Mato Grosso, Sinop-MT, Brazil

ABSTRACT

This study evaluated the effects of isolated or combined prebiotics blended with sodium butyrate and zinc proteinate on performance, intestinal morphometry, and expression of genes associated with nutrient transport and immune response in broilers. 1,400 birds were randomly assigned to 5 treatments (Negative control (NC) without antibiotic; Positive control (PC) with antibiotic; Prebiotic (Prebio) composed of mannan fractions; Blend, composed of sodium butyrate, hydrolysed yeast cell wall and zinc proteinate; and the combination Prebio + Blend), with eight replications and 35 birds per experimental unit. The PC led to higher weight gain (WG) and lowered feed conversion (FCR) from 1 to 7 days of life than the other treatments. The use of prebio and blend resulted in high WG from 22 to 42 days (1773; 1768.9 g, respectively). However, the combination of prebio + blend was similar to PC and NC treatments. The additives resulted in longer duodenal villi when administered alone and in ileal villi ($p = 0.001$) combined, with no effect on jejunal villi. Crypt depth and duodenal villus: crypt ratio was higher for both additives types. A significant difference among treatments in the expression of the sodium-dependent glucose transporter gene (*SGLT1*) was noted, with greater expression in birds that received NC. There was higher expression of the neutral and cationic amino acid transporter gene (*y + LAT-2*) with the prebio and blend, either alone or in combination. The prebio blend with sodium butyrate and zinc proteinate additives used separately in broiler diets supported beneficial changes in intestinal morphometry, nutrient absorption, and performance, indicating their value in antibiotic-free broiler production systems.

HIGHLIGHTS

- The additives in the diets of broilers from 1 to 42 days resulted in beneficial performance.
- The additives influenced the health of the birds, with a positive result in intestinal morphometry.
- The prebiotic and blend additives demonstrated increased gene expression of absorption of neutral amino acids.

ARTICLE HISTORY
Received 10 April 2023
Revised 26 June 2023
Accepted 20 July 2023

KEYWORDS
Blend; prebiotic; growth promoters; genes; immune response

Introduction

The use of antibiotics as growth promoters in animal diets has been reduced, as their use is associated with bacterial resistance to drugs, harming human health (Costa et al. 2018; Sweeney et al. 2018). Therefore, the development and use of additives to improve or maintain productive performance in antibiotic-free diets are necessary (Gibson et al. 2017; Corrigan et al. 2023). Much research has focused on the benefits provided by a variety of compounds with prebiotic action, among them, the cell wall of *Saccharomyces cerevisiae* yeasts, which is mainly made up of β-glucan (35–55%), mannan-protein (30-50%), and chitin (2–6%) (Munoz et al. 2023).

Research has shown that supplementation with these components increased weight gain, improved intestinal morphometry, and reduced pathogens such as *Clostridium perfringens* (Kim et al. 2011;

CONTACT C. O. Brito claudson@ufs.br

Hajiaghapour and Rezaeipour 2018). Another benefit observed with the yeast cell wall is the ability to modulate the immune system because of having in its structure pathogen-associated molecular patterns (PAMPs) that stimulate through pattern recognition receptors (PRRs) in intestinal epithelial cells of birds, the production of inflammatory-cytokines enhancing the immune response (Paul et al. 2013; Broom and Kogut 2018).

A new and alternative nutritional technology is additives produced from the metabolites of live bacteria (probiotics) or metabolites released after bacterial lysis (Danladi et al. 2022). These postbiotics provide the host with additional bioactivity, through sodium butyrate, with an effect similar to that of probiotics, but without using live cells (Goh et al. 2022).

Sodium butyrate, a natural product of microbial metabolism, represents one of the main sources for developing epithelial cells that line the intestine, thus stimulating the development of intestinal villi (Ahsan et al. 2016). Wu et al. (2018) reported that adding butyric acid to the birds' diet improved intestinal health, directly reflected in the performance of the animals. The zinc proteinate plays an important role in controlling the damage caused by pathogens such as *Clostridium perfringens*, helping to maintain intestinal integrity and the general health of birds (Bortoluzzi et al. 2019).

Several studies have reported positive effects of the combined use of prebiotics with live cell metabolites in poultry diets (Thanh et al. 2009; Loh et al. 2010). Kareem et al. (2017) observed a significant improvement in weight gain, feed conversion, and microbial ecology of birds supplemented with the combined use of inulin + RG14 metabolites. However, despite the benefits of these additives in poultry nutrition, are little information in the literature regarding the synergism of the prebiotic with the additives derived from cellular metabolites, sodium butyrate, and zinc proteinate on the intestinal morphology and gene expression of broiler chickens. Therefore, this study aimed to evaluate prebiotics and blend with sodium butyrate and zinc proteinate supplementation, alone and in combination, on performance, intestinal morphometry, and expression of genes associated with nutrient transport and immune response in broilers.

Material and methods

The Ethics Committee Research in Animals Production (CEPAP) of the Federal University of Sergipe, Brazil, approved all procedures involving animals under protocol No. 21/2019.

Animals, experimental design, and diets

The experiment was carried out in the facilities of a poultry company in São Cristóvão, Sergipe, Brazil. All breeding techniques adopted followed the breeding manual of the used strain. At 1 day old, one thousand four hundred male and female Cobb broilers were housed in pens with concrete floors and bedded. The poultry litter was reused four times before the present study. Each pen, 4 m^2, represented an experimental unit. The lighting program adopted followed the lineage recommendations.

The birds were randomly distributed into five treatments, with eight replicates of 35 birds (20 females and 15 males) per experimental unit. The treatments adopted were Negative Control (NC), diets without antibiotics; Positive control (PC) with antibiotics; NC + Prebiotic (Prebio); NC + blend; and the combination Prebio + blend.

The inclusion rates for the additives were 0.4 kg/ton of prebiotic Actigen® and 1.0 kg/ton of Blend Viligen™, according to the manufacturer's recommendation. Viligen™ is composed of sodium butyrate (180 g/kg), hydrolyzed yeast, and zinc proteinate (10 g/kg), while Actigen® has active fractions of mannans derived from the *Saccharomyces cerevisiae* (150 g/kg) cell wall.

The antibiotics used in the PC diets were Methylene Disalicylate Bacitracin (55 ppm) from 1 to 21 days and Virginiamycin (15 ppm) from 22 to 42 days. The use of antibiotics followed the antibiotic use protocol of the poultry company, where the experiment was carried out.

The experimental diets were formulated based on corn and soybean meal to meet the nutritional requirements of the birds, as recommended by Rostagno et al. (2017), with four diet stages from ages 1 to 7; 8 to 21; 22 to 35, and 36 to 42 days (Table 1). Both antibiotics and natural additives were added to basal diets to replace corn starch partially.

Performance parameter

For performance analysis, the birds were weighed weekly, as well as the experimental rations provided and leftovers to determine body weight (g/bird), weight gain (g/day/bird), feed intake (g/day/bird) and feed conversion rate (g/g).

During all experimental periods, temperature and relative humidity were recorded every 15 min using Thermo-hygrometers (model ITR 157; Instrutherm Company, São Paulo, Brazil), placed at three different points in the shed (front, middle, and bottom). The ambient temperature for the birds started at

Table 1. Negative control diets for experimental ages, as-fed basis.

	Age, days			
Ingredients %	1 to 7	8 to 21	22 to 35	36 to 42
Corn	57.28	59.53	67.21	69.98
Soybean meal 45.5%	37.83	31.11	25.11	25.84
Extruded full-fat soybean	—	5.77	4.71	—
Meat and bone meal	1.55	0.91	0.28	—
Poultry fat	0.41	—	—	1.69
common salt	0.57	0.54	0.52	0.47
Limestone	1.18	1.17	1.15	1.14
Sodium bicarbonate	0.24	0.18	0.18	0.18
Biolys 55	0.19	0.14	0.25	0.15
DL-methionine	0.32	0.27	0.24	0.20
Threonine	0.04	0.01	0.01	0.01
Allzyme SSF	0.05	0.05	0.05	0.05
Anticoccidial[†]	0.05	0.05	0.03	0.03
Vitamin premix[‡]	0.05	0.05	0.05	0.05
Mineral premix[§]	0.10	0.08	0.07	0.06
Starch[¶]	0.14	0.14	0.14	0.14
Chemical composition				
Metabolizable energy (kcal/kg)	3,000	3,080	3,150	3,220
Crude protein[††]	23.67	23.17	19.88	19.19
Crude Fibre[††]	2.40	2.47	2.50	2.62
Calcium	0.970	0.880	0.770	0.719
Total phosphorus[††]	0.541	0.488	0.405	0.369
Phosphorus, available	0.435	0.393	0.344	0.321
Potassium	0.757	0.622	0.502	0.517
Sodium	0.230	0.220	0.210	0.190
Digestible arginine	1.53	1.39	1.77	1.08
Digestible isoleucine	0.89	0.86	0.72	0.69
Digestible lysine	1.26	1.17	1.05	0.93
Digestible methionine	0.64	0.58	0.51	0.47
Digestible Met + Cys	0.94	0.87	0.78	0.72
Digestible threonine	0.78	0.82	0.71	0.68
Digestible tryptophan	0.25	0.24	0.20	0.19
Digestible leucine	1.69	1.65	1.46	1.40
Digestible valine	0.98	1.37	0.81	0.77

[†]Anticoccidial: Semduramycin + Nicarbazine at ages 1 to 21 days and Diclazuril 0.5% from 22 to 42 days. [‡]Vitamin Premix: Folic Acid 800 mg, Pantothenic Acid 12000 mg, Sodium Selenite 250 mg, Vitamin A 9000 IU, Vitamin B1 1500 mg, Vitamin B12mg, Vitamin B2 6000 mg, Vitamin B6 3000 mg, Vitamin D3 2500 IU, Vitamin E 20000 IU, nicotinic acid 25,000mg, vitamin K3 2500 mg, biotin 60 mg, inert material q.s.p. 1000 *g*. [§]Mineral premix: Iron sulphate 100 mg, copper sulphate 20 mg, zinc oxide 100 mg, manganese oxide 160 mg, cobalt 2 mg, calcium iodate 2 mg, inert material q.s.p. 1000 *g*. [¶]In the medicated treatments, 55 ppm of Methylene Disalicylate Bacitracin was used at the age of 1 to 21 days and 15 ppm of Virginiamycin from 22 to 42 days. In the other treatments, the prebiotic (Actigen) and the Blend (Viligen). Both additives were used in partial substitution of starch. [††]Analyzed values were obtained by the infra-red-reflectance-spectroscopy method (NIRS-005), by the EvonikBrazil laboratory.

33 ± 0.5 °C and was reduced by 2 ± 0.5 °C each week, and remained at 27 ± 0.5 °C after day 21 of age until the end of the experiment. The relative humidity ranged from 68% to 81.0%.

Clostridium perfringens *colony counts and intestinal lesion score*

Poultry litter samples from all treatments were collected at 7, 21, and 42 days of age, stored at −18 °C, and sent to the lab to quantify (Colony forming unit – CFU) the amount of *C. perfringens* (CFU, g/litter). The counts were performed according to ISO (International Organisation for Standardization)–ISO 6887-1 (ISO 2017).

Necrotic enteritis lesion scoring and sample collection were performed, as described below. Eight birds per treatment (four males and four females), randomly selected, were slaughtered at 42 days of age, and a longitudinal incision was made, through which the digestive tract was carefully removed for the evaluation of intestinal lesions. Necrotic enteritis lesions were scored from 0 to 4 following the procedure of Lanckriet et al. (2010). Lesions were classified: 0, absence of macroscopic lesions; 1, thin or friable intestinal wall; 2, presence of necrosis or focal ulcerations; 3, major necrosis lesions; and 4, severe and extensive necrosis, typical of clinical conditions.

Intestinal morphometry

Six birds (three males and three females), randomly selected, aged 42 days per treatment, were slaughtered, and samples from the duodenum, jejunum, and ileum were collected, washed, and fixed in 10% buffered formalin for 24 h. Twelve sections of each sample were cut to a five μm, adhered to glass slides, and stained with Hematoxylin-Eosin (HE).

From each section, ten villi were randomly selected to measure the relationship between the villi's height and the crypt's depth. The samples were examined using the Optical microscope P1 Olympus BX 50 coupled to an Olympus PMC 35 B camera, using the 4× objective lens. The morphometric measurements of the duodenum, jejunum, and ileum were determined using the Image-Pro Plus computerised image analysis system, version 4.0 (Média Cibertecnics). This calculated the relationship between the villi height and the crypt depth.

Gene expression

Total RNAs from jejunal samples (2 cm from the mid-jejunum) were extracted from the tissues and homogenised with TrizolTM reagent (Invitrogen TM, Carlsbad CA, USA), according to the manufacturer's protocol. Ultraviolet spectrophotometry measured RNA concentration and purification (model Nanodrop2000 Q3 spectrophotometer; Thermo Scientific).

The RNA samples were treated with DNase I (Invitrogen, Carlsbad, CA, USA) to avoid contamination of the samples with DNA. For synthesising complementary DNA (cDNA), the GoScript Reverse Transcription System kit (Promega, Madison, Wi, USA)

was used, using 4 μL of DNase-treated RNA, according to the manufacturer's instructions.

For real-time polymerase chain reactions (qPCR), 5 μL of cDNA diluted to 40 ng, 0.5 μL of each primer at ten μM (final concentration: 200 nM), 12.5 μL of Sybr Green PCR Master Mix and enough water to complete the final volume of each reaction of 25 μL. The qPCR reactions were performed in a thermocycler (CFX96 Touch Real-Time PCR Detection System). The thermal cycling parameters for all genes were: 95 °C for 10 min, followed by 40 cycles of denaturation at 95 °C for 15 s and annealing/extension at 60 °C for 1 min. Dissociation curves were performed to ensure the specificity of the analyses.

The primers used in the reactions for the amplification of the toll-like receptor 2 (TLR2), Interleukin 1 beta genes (*IL-1β*) and Interleukin 2 (*IL-2*), glucose transporter 2 (*GLUT 2*), glucose transporter 1 Na^+/dependent (*SGLT1*), neutral and cationic amino acid transporter (*y + LAT2*) were designed based on gene sequences deposited at the National Centre for Biotechnology Information (NCBI) -www.ncbi.nlm.nih.gov (Table 2). The *β-actin* gene was used as an endogenous control, according to the protocol of Del Vesco et al. (2020) and Santana et al. (2021).

Statistical analysis

Data were submitted for analysis of variance using the PROC GLM of the SAS statistical package, version 8.2 (SAS, 2001).

The following model was used for performance, intestinal morphometry

$$Y_{ij} = \mu + G_i + \varepsilon_{ij}$$

Where, Y_{ij} = response variable of treatments *i*, replicate *j*, μ = overall mean effect, G_i= fixed effect of treatments, ε_{ij} = residual error. The results were expressed as mean ± standard error of the mean (SEM) and means were compared using Tukey's test at the 5% probability level. Descriptive statistics methods were used to present *C. perfringens* results.

All data on the relative expression of *TLR2, IL1β, IL2, GLUT2, SGLT1*, and *y-LAT2* genes were estimated by the 2-ΔΔCt method (Livak and Schmittgen 2001). The results were expressed as mean ± standard error of the mean (SEM), and the difference between the means was estimated using the Dunnett test, considering the differences in a significant level ($p < 0.05$).

Results

Performance parameter

Viability means, during the trial period, for negative control (NC), Positive control (PC), Prebiotic (Prebio), Blend and Prebio + Blend were 96.8 ± 3.5, 96.8 ± 4.1, 95.7 ± 3.2, 96.4 ± 3.6, and 96.7 ± 3.2%, respectively. For the early growth period, when examined as either day 1 to 7 or 1 to 21, birds that received the PC had greater weight gain than all other treatments. Feed intake was not different among groups for d1-7, but for d1-21, PC had the highest intake, with prebiotics having the lowest, while all other groups were intermediate. This influenced the feed conversion such that PC was most efficient from 1 to 7 days, but no differences were observed when calculated from d1-21 (Table 3). The individual prebio and blend groups had greater weight gain from 22 to 42 days (1773; 1768.9 g, respectively), while NC had the lowest weight gain during the same period. Intake and feed conversion were not significantly different among groups from 22-42d. However, when evaluated over the full experiment (d1-42), while weight gain and intake results mirror d22-42, the feed conversion was lower for the prebio and blend groups (1.70; 1.72,

Table 2. Sequence of primers used in the qRT-PCR reaction.

Genes†	No. Acess	Gene sequence (5′→ 3′)	Reference
TLR2	NM_204278.1	F: ACTGCCTGCAACGGTCAT R: CATCAGCTTCATTGTTGGTTTCTGT	Quinteiro-Filho et al. (2017)
IL1β	NM_204524.1	F: GTCAACATCGCCACCTACAA R: CGGTACATACGAGATGGAAACC	Del Vesco et al. (2020)
IL-2	GU119890.1	F: CCTCAAGAGTCTTACGGGTCTA R: AGTTGGTCAGTTCATGGAGAAA	El-Deep et al. (2019)
GLUT2	BI392505	F: CACACTATGGGCGCATGCT R: ATTGTCCCTGGAGGTGTTGGTG	Gilbert et al. (2007)
SGLT1	NM_207178	F: GCCATGGCCAGGGCTTA R: CAATAACCTGATCTGTGCACCAGTA	Gilbert et al. (2007)
y + LAT-2	XM_418326.6	F: CAGAAAACCTCAGAGCTCCCTTT R: TGAGTACAGAGCCAGCGCAAT	Gilbert et al. (2007)
β-Actina	L08165.1	F: GCCAACAGAGAGAAGATGAC R: CACCAGAGTCCATCACAATAC	Del Vesco et al. (2020)

†*TLR2* = Toll-like receiver 2; *IL-1β* = Interleukin 1 beta; *IL-2* = Interleukin 2; *GLUT2* = Glucose transporter 2; *SGLT1* = Glucose transporter 1 Na+/dependent; *y + LAT-2* = Neutral and cationic amino acid transporter.

Table 3. Performance of broilers fed experimental diets.

		Age			
Parameters	Treatments[†]	1–7	1–21	22–42	1–42
Weight gain (g)	NC	143.5[b]	888.3[b]	1642.8[b]	2535.2[b]
	PC	160.6[a]	942.1[a]	1673.4[ab]	2617.2[ab]
	Prebio	142.2[b]	892.3[b]	1773.0[a]	2669.3[a]
	Blend	146.5[b]	896.1[b]	1768.9[a]	2666.7[a]
	Prebio + blend	142.2[b]	894.9[b]	1741.1[ab]	2636.1[ab]
SEM		1.756	7.699	27.323	27.462
P-value		<0.001	<0.001	0.007	0.017
Feed intake (g)	NC	173.7	1335.0[ab]	3192.9	4536.3
	PC	178.8	1373.1[a]	3212.5	4592.2
	Prebio	174.3	1285.2[b]	3258.0	4542.1
	Blend	178.2	1326.9[ab]	3252.7	4581.0
	Prebio + blend	177.2	1316.0[ab]	3245.1	4561.1
SEM		1.445	15.266	39.333	48.339
P-value		0.063	0.006	0.748	0.914
Feed conversion (g/g)	NC	1.21[a]	1.50	1.95	1.79[a]
	PC	1.12[b]	1.46	1.92	1.76[ab]
	Prebio	1.23[a]	1.44	1.84	1.70[b]
	Blend	1.22[a]	1.48	1.84	1.72[b]
	Prebio + blend	1.25[a]	1.47	1.87	1.73[ab]
SEM		0.016	7.699	0.028	0.017
P-value		<0.001	0.204	0.060	0.013

[†]NC: diets without the use of antibiotics; PC: antibiotic diets; Prebio: diets enriched with Actigen Prebiotic; Blend = diets enriched with Viligen; Prebio + Blend: diets combined with additives. a,b Means in the same column with different superscript letters are significantly different by Tukey's Test ($p < 0.05$). SEM: Standard error of the mean.

Table 4. *C. perfringens* count (CFU/g) present in the aviary litter.

Treatments[†]	7 days	21 days	42 days
NC	820	55	160,000
PC	180	<10	250
Prebio	82	<10	<10
Blend	200,000	<10	<10
Prebio + blend	55,000	<10	<10

[†]NC: diets without the use of antibiotics; PC: antibiotic diets; Prebio: diets enriched with Actigen Prebiotic; Blend: diets enriched with Viligen; Prebio + Blend: diets combined with additives.

respectively) than birds without the use of additives, while the birds in the PC and combination groups were intermediate. Similar results have occurred from the 1 to 42-day phase, and the ongoing use of prebio or blend led to positive outcomes.

In the phases studied, the combined use of prebiotic and blend did not provide additive results that could differentiate them from other treatments ($p > 0.05$), but provided a weight gain of 100.9 g compared to birds that received the control diet and 18.9 g to birds with antibiotics.

C. perfringens *count and intestinal lesion score*

According to the data collected at seven days, the lowest concentration of *Clostridium perfringens* oocysts in the poultry litter was observed in the prebio group (82 CFU/g), followed using the antibiotic in the PC group (180 CFU/g) (Table 4). In contrast, the highest concentrations were in treatments containing blend (200,000 CFU/g) and in the blend plus prebio combination (55,000 CFU/g).

The negative control collections from the litter at 21 days showed a decrease in the concentration of CFUs with values lower than 10 CFU/g. Likewise, at 42 days, the use of the post-biotic and the prebiotic, alone or in combination, resulted in concentrations of *C. perfringens* below 10 CFU/g, lower than using antibiotics (250 CFU/g). The presence of intestinal lesions caused by necrotic enteritis, observed between treatments (Figure 1), did not show significant differences among treatments ($p = 0.099$).

Intestinal morphometry

Using prebiotics and blending alone or in combination resulted in a greater height of the duodenal villus ($p < 0.0001$) when compared to the PC (Table 5). However, birds in the PC group had a smaller crypt depth (165.7 μm), although it was not different from birds that received the blend (186.3 μm). The negative control birds had the greatest crypt depth. The duodenal villus: crypt ratio showed no difference among the prebiotic (8.6), blend (9.1), or PC (9.2) groups, and all, including the Prebio + Blend combination, provide a higher villus: crypt ratio compared to birds without any additive (6.9).

The treatment with prebiotic birds had the highest villus: crypt ratios ($p < 0.001$) because of the smaller crypt depths in the jejunum and ileum. Birds fed the

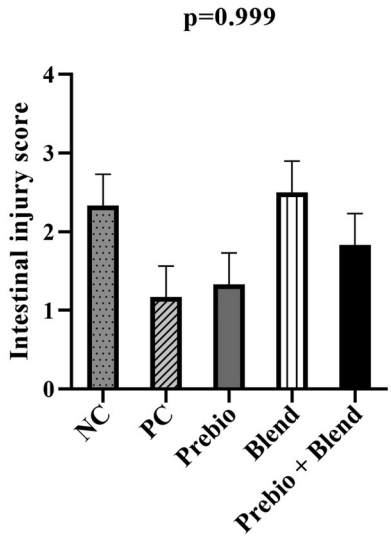

Figure 1. Effects of experimental treatments on the intestinal lesion (duodenum to ileum) score of broilers at 42 days ($n = 40$). NC: Negative control diets without antibiotics; CP: Positive control diets with antibiotics; Prebio - diets with actigen prebiotic; blend – diets with Viligen; Prebio + blend: diets combined with additives.

combined Prebio + blend had an additive effect on the villus height in the ileum. Also, it is possible to observe that NC had the deepest crypts, contributing to the lowest Villus: Crypt ratio in both jejunum and ileum.

Gene expression

The relative expression of the immune response-associated genes *TRL2, IL1-β,* and *IL2* (Figure 2) and the nutrient absorption genes, such as the glucose transporter *GLUT2* (Figure 3), were not significantly different among treatments. However, the expression of sodium-dependent glucose transporter (*SGLT1*) had a lower expression in birds that received antibiotics (PC) as compared to all other treatments and was highest in the negative control birds (NC). However, NC was not different from the Prebio, Blend, and combination treatments. For the expression of the gene *y + LAT2*, a transporter of neutral and cationic amino acids (*y-LAT2*), we observed that the use of prebio and blend, alone or in combination, was twice as high ($p < 0.001$) as in both NC and PC birds.

Discussion

The diets with antibiotics (PC) resulted in improvements in the performance of the birds in the initial phase (1 to 21 days) when compared with the evaluated additives, including the treatment without any additive (NC). These results corroborate the literature findings, in which antibiotics' impact on weight gain and feed conversion of birds in the initial period (1 to 21 days) was observed (Chen et al. 2009; Mokhtari et al. 2015; Kazemi et al. 2019).

Rehman et al. (2020) verified that supplementation with prebiotics (MOS) and protein (multistrain probiotic) increased feed consumption of the birds since, with the intestinal microbiota balanced, the initial development of the intestine provided greater feed consumption in broilers during the initial phase (Hamasalim 2016).

The Prebio or Blend additives provided effectiveness when used alone in the growth phases (22 to 42 days), with about 8% greater weight gain of the birds, compared to the diet without additives (NC) and 5% in the treatment PC, which consequently influenced feed conversion in the complete production cycle (1 to 42 days).

It is important to highlight that the combination of Prebio + blend resulted in similar feed conversion to birds that received diets with antibiotics (PC). This result shows that despite their different modes of action, their beneficial effects were similar, making these additives beneficial when antibiotic-free broiler production is required (Ahiwe et al. 2021). Kareem et al. (2016) observed that birds supplemented by a combination of prebiotic + blend (postbiotic - metabolite products from probiotic) had better results for weight gain (WG = 2284.31 g) compared to the group that received an antibiotic (WG= 2198.34 g).

The present study demonstrated that Prebio or Blend use leads to low feed conversion values, indicating that the isolated supplementation of those additives may be an important strategy to improve bird performance.

Among the additives evaluated, the Blend (Viligen) is composed of sodium butyrate (180 g/kg) and zinc proteinate (10 g/kg), and the Prebio (Actigen) includes mannan-rich fractions (150 g/kg). Butyrate is a short-chain fatty acid readily absorbed in the intestine of birds, being metabolised even faster than the glucose molecule, providing energy for epithelial cells (Wu et al. 2018). Mannan is a well-known immunomodulator promoting beneficial intestinal microflora's predominance (Mathis et al. 2012).

Biswas et al. (2019) infer that providing substrates that stimulate the growth of beneficial bacteria to the host results in greater efficiency in feed digestibility and utilisation, providing greater growth. Zhang et al. (2011) report that using sodium butyrate for broilers benefits the growth performance of broilers in terms

of increased feed intake, body weight gain, and significant improvement in feed conversion ratio.

Yeast mannan oligosaccharides also improved the performance of broilers supplemented for 52 days (Mathis et al. 2012), and possibly positively modulating bacteria in the gut. (Hamasalim 2016). Bozkurt et al. (2008) observed that mannans derived from the cell wall of yeasts included at 1.0 g/kg in diets for broilers could replace antibiotics in the feed, improving the performance and carcase yield of broilers.

Table 5. Intestinal morphometry of broilers fed experimental diets at 42 days of age.

Treatments[†]	Villus height (μm)	Crypt depth (μm)	Villus: crypt
		Duodenum	
NC	1523.6^{ab}	228.8^{a}	7.0^{c}
PC	1453.0^{b}	165.8^{d}	9.2^{a}
Prebio	1627.7^{a}	190.0^{bc}	8.6^{ab}
Blend	1632.9^{a}	186.3^{cd}	9.1^{a}
Prebio + blend	1598.7^{a}	210.7^{ab}	7.8^{bc}
SEM	30.721	5.350	0.275
P-value	<0.0001	<0.0001	<0.0001
		Jejunum	
NC	1269.5	216.1^{a}	6.1^{c}
PC	1302.6	187.3^{b}	7.9^{b}
Prebio	1246.5	144.2^{d}	8.9^{a}
Blend	1279.8	165.2^{c}	7.8^{b}
Prebio + blend	1267.1	161.1^{c}	8.0^{b}
SEM	21.259	4.288	0.212
P-value	0.496	<0.0001	<0.0001
		Ileum	
NC	869.8^{b}	196.9^{a}	4.7^{d}
PC	887.6^{b}	171.8^{b}	5.5^{c}
Prebio	906.1^{b}	118.3^{c}	8.0^{a}
Blend	781.6^{c}	158.1^{b}	5.3^{cd}
Prebio + blend	1079.5^{a}	164.1^{b}	6.6^{b}
SEM	22.420	5.232	0.192
P-value	<0.0001	<0.0001	<0.0001

[†]NC: diets without the use of antibiotics; PC: antibiotic diets; Prebio: diets enriched with Actigen Prebiotic; Blend: diets enriched with Viligen; Prebio + Blend: diets combined with additives. a,b,c,d Means in the same column with different superscripted letters are significantly different by Tukey's Test ($p < 0.05$). SEM: Standard error of the mean.

The combination of additives did not result in a synergistic effect on broiler performance, mainly for weight gain. Few studies evaluated the synergism of prebiotics, a blend of sodium butyrate and zinc proteinate for broilers, as an alternative to antibiotics. However, Kareem et al. (2016) reported a synergistic effect of the prebiotic combination inulin + blend (postbiotic - metabolite products from probiotic) for broilers, with better results for feed conversion (1.77 vs. 1.89 g/g) and weight gain (2190 vs. 2020.46 g) for diets with antibiotics. These results were attributed to organic acids, bacteriocins, hydrogen peroxide and vitamins (postbiotics), and inulin which helps to modulate the intestinal health of birds.

Using Prebio and Blend alone or in combination resulted in lower CFU counts of *Clostridium perfringens* in the aviary litter as the age of the birds increased. Although no effect of treatments was observed for the intestinal lesion score, it is possible to observe that both the use of isolated prebiotics and in combination with the blend resulted in lower values than those found in the negative control treatment. Similarly, Mathis et al. (2012) observed a reduction in the *C. perfringens* count in the faeces of chickens supplemented with a biotic rich in mannan fractions derived from the yeast cell wall (Actigen) with *C. perfringens* from 21 to 23 days.

Associated with these positive results on the health of the birds, the use of the Prebio and blend with sodium butyrate and zinc proteinate affected the intestinal morphology, with positive changes in the height of the villi, the depth of the crypts, and the villus: crypt ratio in the three segments of the small intestine.

However, the effect was not synergistic, except for ileum villus height. When Kareem et al. (2016)

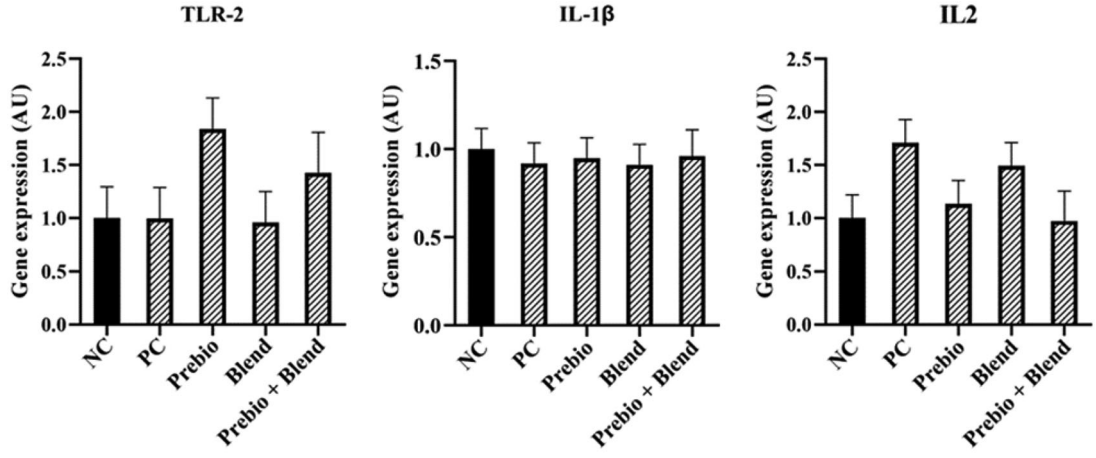

Figure 2. Relative expression of the immune system genes, Toll-like receptor 2 (TLR2), Interleukin 1β (*IL-1β*) and Interleukin 2 (*IL-2*) from the jejunum of broilers at 42 days of age ($n = 25$). NC: diets without the use of antibiotics; PC: antibiotic diets; Prebio: diets enriched with actigen prebiotic; blend: diets enriched with Viligen; Prebio + blend: diets combined with additives.

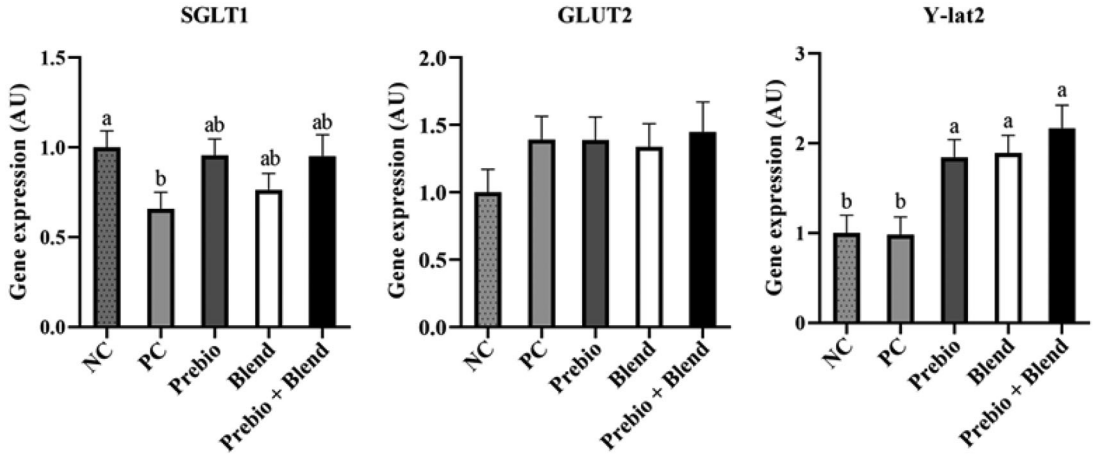

Figure 3. Relative expression of nutrient uptake genes, glucose transporter 2 (*GLUT2*), sodium-dependent glucose transporter (*SGLT1*), and neutral and cationic amino acid transporter (*y-LAT2*) from the jejunum of broilers aged 42 days ($n = 25$). Means with different letters a, b differ significantly ($p < 0.05$). NC: diets without the use of antibiotics; PC: antibiotic diets; Prebio: diets enriched with actigen prebiotic; blend = diets enriched with Viligen; Prebio + blend: diets combined with additives.

supplemented broilers with different levels of prebiotic + blend (postbiotic - metabolite products from probiotic) combined, the authors reported that in all dietary groups, there was a synergistic effect with an increase in the duodenal and ileal villi of the birds, compared to the control treatment.

Butyrate, one constituent of the postbiotic (product of microbial metabolism) used in this trial, represents an important energy source for the cells lining the intestine, stimulating differentiation and apoptosis (Elgeddawy et al. 2020), resulting in improvements in intestinal function. The zinc proteinate is also associated with better bowel function in broilers (Bortoluzzi et al. 2019).

Thus, using these blends, with two components together may potentiate such effects, as observed in the duodenum of birds supplemented Postbio in this trial, where the villus height was 12% higher compared to birds treated with antibiotics. In the jejunum and ileum segments, the effect of the Prebio was more pronounced. These results agree with previous research; Wu et al. (2018) showed longer villi in birds supplemented with sodium butyrate compared to the group that received diets with antibiotics.

It is possible to infer that the changes generated in the intestinal morphology of the birds provided an increase in the absorptive surface area in the intestine, which possibly affected the nutrient absorption, potentiating the positive results for growth and feed conversion of birds supplemented with prebiotic and blend (Table 3).

As observed in the study of Kareem et al. (2017) found that blend RG14 (postbiotic - metabolite products from probiotic) addition to broiler diets improved intestinal villi, reduced Enterobacteriaceae strains, and reduced faecal pH, resulting in increased performance parameters, and better immune response and intestinal health of birds.

Contrary to previous positive results of prebiotics and postbiotics (product of microbial metabolism) on the intestinal health of chickens (Rosyidah et al. 2014; Kareem et al. 2016), an immunomodulatory effect was not observed with the additives used in the present study, as they did not affect the expression of *Toll-Like 2, IL1-β* and *IL2*.

In the present study, the additives, combined or not, inhibited the development of pathogenic *C. perfringens* strains, and the health challenge was mitigated using Prebio and Blend, justifying the absence of effects of genes *Toll-Like 2, IL1-β*, and *IL2*. In addition, the absence of severe ulcerations, observed in the enteric necrosis score, did not lead to an immune response.

Intestinal nutrient transporters showed *SGLT1* was more highly expressed when the birds did not receive any additive in the diets (NC), followed by treatments with Prebio, Blend, and the combination Prebio + Blend.

Pacific et al. (2017) demonstrated that administering prebiotics to chickens, regardless of concentration, increased the gene expression of nutrient transporters, such as *SGLT1*. The sodium-dependent glucose transporter (*SGLT1*) is the main glucose transporter in the intestinal epithelium (Kaminski and Wong 2018). It can catch glucose from the diet to take it to the bloodstream (Zhang et al. 2011). Jahromi et al. (2016) reported that the absorption of glucose in the gut of broilers could be improved with probiotics, which

promotes high stimulation of D-glucose absorption in the brush border membrane of enterocytes, with an associated increase in the *SGLT1* co-transporter.

However, the low activity of SGLT1 indicates lower absorption of nutrients through the small intestine, a fact observed in PC, which could explain the reduced performance for birds observed in this treatment.

The use of additives alone or in combination allowed an increase in the expression of the *y + lat2* gene. According to Fotiadis et al. (2013), this gene is associated with the increase in the absorption of neutral amino acids. Corresponding to this response, the blend and the Prebio alter the mucosal architecture in terms of longer villi, which was reflected in the positive performance of birds at 42 days of age.

Thus, the results observed in this trial for the growth of the birds, gut morphology, and gene expression can likely be attributed to beneficial changes in the intestinal function of the chickens fed with the additives, which may be an indication of improvement in the absorption of some nutrients from the diet (Kazemi et al. 2019). Healthy animals use and convert the nutrients present in their diet more efficiently. The positive effect of the use of prebiotics and blend with sodium butyrate and zinc proteinate alone or in combination on the intestinal microbiota and, consequently, the animal's weight gain and feed conversion were observed in the present study.

Conclusion

In this trial, using a prebiotic or blend with sodium butyrate and zinc proteinate in the diets of broilers from 1 to 42 days resulted in beneficial changes to intestinal morphometry and performance. This indicates that these materials may be beneficial in antibiotic-free broiler production systems.

Ethical statement

The Ethics Committee Research in Animals Production (CEPAP) of the Federal University of Sergipe, Brazil, approved all procedures involving animals under protocol No. 21/2019.

Disclosure statement

No potential conflict of interest was reported by the author(s).

Funding

The authors would like to thank the Coordination for the Improvement of Higher Education Personnel (CAPES) for the financial support provided (Code 001). Coordenação de Aperfeiçoamento de Pessoal de Nível Superior.

Data availability statement

The data that support this study cannot be publicly shared due to ethical or privacy reasons and may be shared upon reasonable request to the corresponding author if appropriate.

References

Ahiwe EU, Dos Santos TT, Graham H, Iji PA. 2021. Can probiotic or prebiotic yeast (*Saccharomyces cerevisiae*) serve as alternatives to in-feed antibiotics for healthy or disease-challenged broiler chickens? A review. J Appl Poult Res. 30(3):100164. doi: 10.1016/j.japr.2021.100164.

Ahsan U, Cengiz Ö, Raza I, Kuter E, Chacher MFA, Iqbal Z, Umar S, Çakir S. 2016. Sodium butyrate in chicken nutrition: the dynamics of performance, gut microbiota, gut morphology, and immunity. Worlds Poult Sci. 72(2):265–275. doi: 10.1017/S0043933916000210.

Biswas A, Mohan N, Raza M, Mir NA, Mandal A. 2019. Production performance, immune response and blood biochemical parameters in broiler chickens fed diet incorporated with prebiotics. J Anim Physiol Anim Nutr. 103(2): 493–500.

Bortoluzzi C, Lumpkins B, Mathis GF, França M, King WD, Graugnard DE, Dawson KA, Applegate TJ. 2019. Zinc source modulates intestinal inflammation and intestinal integrity of broiler chickens challenged with coccidia and *Clostridium perfringens*. Poult Sci. 98(5):2211–2219. doi: 10.3382/ps/pey587.

Bozkurt M, Kucukyilma K, Catli AU, Cinar M. 2008. Growth performance and slaughter characteristics of broiler chickens fed with antibiotic, mannan oligosaccharide and dextran oligosaccharide supplemented diets. Int J Poult Sci. 7(10):969–977. doi: 10.3923/ijps.2008.969.977.

Broom LJ, Kogut MH. 2018. The role of the gut microbiome in shaping the immune system of chickens. Vet Immunol Immunopathol. 204:44–51. doi: 10.1016/j.vetimm.2018.10.002.

Chen KL, Kho WL, You SH, Yeh HS, Tang SW, Hsieh CW. 2009. Effects of *Bacillus subtilis* var. natto and *Saccharomyces cerevisiae* mixed fermented feed on the enhanced growth performance of broilers. Poult Sci. 88(2): 309–315. doi: 10.3382/ps.2008-00224.

Corrigan A, Leigh RJ, Walsh F, Murphy R. 2023. Microbial community diversity and structure in the cecum of laying hens with and without mannan-rich fraction supplementation. J Appl Poult Res. 32(2):100342. doi: 10.1016/j.japr.2023.100342.

Costa T, Linhares I, Ferreira R, Neves J, Almeida A. 2018. Frequency and antibiotic resistance of bacteria implicated in community urinary tract infections in North Aveiro between 2011 and 2014. Microb Drug Resist. 24(4):493–504. doi: 10.1089/mdr.2016.0318.

Danladi Y, Loh TC, Foo HL, Akit H, Md Tamrin NA, Naeem Azizi M. 2022. Effects of postbiotics and paraprobiotics as replacements for antibiotics on growth performance, carcass characteristics, small intestine histomorphology,

immune status and hepatic growth gene expression in broiler chickens. Anim. 12(7):917. doi: 10.3390/ani12070917.

Del Vesco AP, Khatlab AdS, Santana TP, Pozza PC, Menck Soares MA, Brito CO, Barbosa LT, Gasparino E. 2020. Heat stress effect on the intestinal epithelial function of broilers fed methionine supplementation. Livest Sci. 240:104152. doi: 10.1016/j.livsci.2020.104152.

Elgeddawy SA, Shaheen HM, El-Sayed YS, Elaziz MA, Darwish A, Samak D, Batiha GE, Mady RA, Bin-Jumah M, Allam AA, Alagawany M, et al. 2020. Effects of the dietary inclusion of a probiotic or prebiotic on florfenicol pharmacokinetic profile in broiler chicken. J Anim Physiol Anim Nutr. 104:549–557. doi: 10.1111/jpn.13317

El-Deep MH, Dawood MAO, Assar MH, Ijiri D, Ohtsuka A. 2019. Dietary Moringa oleifera improves growth performance, oxidative status, and immune related gene expression in broilers under normal and high temperature conditions. J Therm Biol. 82:157–163. doi: 10.1016/j.jtherbio.2019.0.

Fotiadis D, Kanai Y, Palacín M. 2013. The SLC3 and SLC7 families of amino acid transporters. Mol Aspects Med. 34(2-3): 139–158. doi: 10.1016/j.mam.2012.10.007.

Gibson GR, Hutkins R, Sanders ME, Prescott SL, Reimer RA, Salminen SJ, Scott K, Stanton C, Swanson KS, Cani PD, et al. 2017. Expert consensus document: the International Scientific Association for Probiotics and Prebiotics (ISAPP) consensus statement on the definition and scope of prebiotics. Nat Rev Gastroenterol Hepatol. 14(8):491–502. doi: 10.1038/nrgastro.2017.75.

Gilbert ER, Li H, Emmerson DA, Webb KE, Jr, Wong EA. 2007. Developmental regulation of nutrient transporter and enzyme mRNA abundance in the small intestine of broilers. Poult Sci. 86(8):1739–1753. doi: 10.1093/ps/86.8.1739.

Goh JXH, Tan LTH, Law JWF, Ser HL, Khaw KY, Letchumanan V, Lee LH, Goh BH. 2022. Harnessing the potentialities of probiotics, prebiotics, synbiotics, paraprobiotics, and postbiotics for shrimp farming. Rev. Aquac. 14(3):1478–1557. doi: 10.1111/raq.12659.

Hajiaghapour M, Rezaeipour V. 2018. Comparison of two herbal essential oils, probiotic, and mannan-oligosaccharides on egg production, hatchability, serum metabolites, intestinal morphology, and microbiota activity of quail breeders. Livest Sci. 210:93–98. doi: 10.1016/j.livsci.2018.02.007.

Hamasalim HJ. 2016. Synbiotic as feed additives relating to animal health and performance. AiM. 06(04):288–302. doi: 10.4236/aim.2016.64028.

International Organization for Standardization [ISO]. 2017. Microbiology of the food chain - Preparation of test samples, initial suspension and decimal dilutions for microbiological examination. Part 1: general Rules for the Preparation of the Initial Suspension and Decimal Dilution. ISO Norm :6887–6881.

Jahromi MF, Altaher YW, Shokryazdan P, Ebrahimi R, Ebrahimi M, Idrus Z, Tufarelli V, Liang JB. 2016. Dietary supplementation of a mixture of *Lactobacillus* strains enhances performance of broiler chickens raised under heat stress conditions. Int J Biometeorol. 60(7):1099–1110. doi: 10.1007/s00484-015-1103-x.

Kaminski NA, Wong EA. 2018. Differential mRNA expression of nutrient transporters in male and female chickens. Poult Sci. 97(1):313–318. doi: 10.3382/ps/pex262.

Kareem KY, Loh TC, Foo HL, Akit H, Samsudin AA. 2016. Effects of dietary postbiotic and inulin on growth performance, IGF1 and GHR mRNA expression, faecal microbiota and volatile fatty acids in broilers. BMC Vet Res. 12(1):163. doi: 10.1186/s12917-016-0790-9.

Kareem KY, Loh TC, Foo HL, Asmara SA, Akit H. 2017. Influence of postbiotic RG14 and inulin combination on cecal microbiota, organic acid concentration, and cytokine expression in broiler chickens. Poult Sci. 96(4):966–975. doi: 10.3382/ps/pew362.

Kazemi SA, Ahmadi H, Karimi Torshizi MA. 2019. Evaluating two multistrain probiotics on growth performance, intestinal morphology, lipid oxidation and ileal microflora in chickens. J Anim Physiol Anim Nutr (Berl). 103(5):1399–1407. doi: 10.1111/jpn.13124.

Kim GB, Seo YM, Kim CH, Paik LK. 2011. Effect of dietary prebiotic supplementation on the performance, intestinal microflora, and immune response of broilers. Poult Sci. 90(1):75–82. doi: 10.3382/ps.2010-00732.

Lanckriet A, Timbermont L, De Gussem M, Marien M, Vancraeynest D, Haesebrouck F, Ducatelle F, Van Immerseel F. 2010. The effect of commonly used anticoccidials and antibiotics in a subclinical necrotic enteritis model. Avian Pathol. 39(1):63–68. doi: 10.1080/03079450903505771.

Livak KJ, Schmittgen TD. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. Methods. 25(4):402–408. doi: 10.1006/meth.2001.1262.

Loh TC, Thanh NT, Foo HL, Hair-Bejo M, Azhar BK. 2010. Feeding of different levels of metabolite combinations produced by *Lactobacillus plantarum* on growth performance, fecal microflora, volatile fatty acids and villi height in broilers. Anim Sci J. 81(2):205–214. doi: 10.1111/j.1740-0929.2009.00701.x.

Mathis GF, Lumpkins B, Pierce JL, Hooge DM. 2012. Effects of dietary antibiotics, actigen yeast cell wall derivative, or both on broiler chicken live performance in a fifty-two-day pen trial on built-up litter. J Poult Sci. 49(4):313–318. doi: 10.2141/jpsa.0110175.

Mokhtari R, Yazdani A, Kashfi H. 2015. The effects of different growth promoters on performance and carcass characteristics of broiler chickens. J. Anim. Vet. Adv. 7(8):271–277.

Munoz LR, Bailey MA, Krehling JT, Bourassa DV, Hauck R, Pacheco WJ, Chaves-Cordoba B, Chasteen KS, Talorico AA, Escobar C, et al. 2023. Effects of dietary yeast cell wall supplementation on growth performance, intestinal *Campylobacter jejuni* colonization, innate immune response, villus height, crypt depth, and slaughter characteristics of broiler chickens inoculated with *Campylobacter jejuni* at d 21. Poult Sci. 102(5):102609. doi: 10.1016/j.psj.2023.102609.

Pacific S, Song J, Zhang C, Wang Q, Glahn RP, Kolba N, Tako E. 2017. Intra amniotic administration of raffinose and stachyose affects the intestinal brush border functionality and alters gut microflora populations. Nutrients. 9(3):304–314. doi: 10.3390/nu9030304.

Paul MS, Brisbin JT, Abdul-Careem MF, Sharif S. 2013. Immunostimulatory properties of toll-like receptor ligands

in chickens. Vet Immunol Immunopathol. 152(3-4):191–199. doi: 10.1016/j.vetimm.2012.10.013.

Quinteiro-Filho WM, Calefi AS, Cruz DSG, Aloia TPA, Zager A, Astolfi-Ferreira CS, Piantino Ferreira JA, Sharif S, Palermo-Neto J. 2017. Heat stress decreases expression of the cytokines, avian β-defensins 4 and 6 and Toll-like receptor 2 in broiler chickens infected with *Salmonella enteritidis*. Vet Immunol Immunopathol. 186:19–28. doi: 10.1016/j.vetimm.2017.02.006.

Rehman A, Arif M, Sajjad N, Al-Ghadi MQ, Alagawany M, Abd El-Hack ME, Alhimaidi AR, Elnesr SS, Almutairi BO, Amran RA, et al. 2020. Dietary effect of probiotics and prebiotics on broiler performance, carcass, and immunity. Poult Sci. 99(12):6946–6953. doi: 10.1016/j.psj.2020.09.043.

Rostagno HS, Albino LFT, Hannas MI, Donzele JL, Sakomura NK, Perazzo FG, Saraiva A, Teixeira ML, Rodrigues PB, Oliveira RF. 2017. Brazilian tables for poultry and swine: composition of feedstuffs and nutritional requeriments. 4 ed. Viçosa (MG) Animal Science Departament UFV.

Rosyidah M, Loh T, Foo H, Cheng X, Bejo M. 2014. Effect of feeding metabolites and acidifier on growth performance, faecal characteristics and microflora in broiler chickens. J Anim Vet Adv. 10(21):2758–2764.

Santana TP, Gasparino E, Khatlab AS, Brito CO, Barbosa LT, Lamont LT, Del Vesco AP. 2021. Effect of prenatal ambient temperature on the performance physiological parameters, and oxidative metabolism of Japanese quail (*Coturnix coturnix japonica*) layers exposed to heat stress during growth. Sci Rep. 11(1):9809. doi: 10.1038/s41598-021-89306-0.

Statistical Analyses System. 2001. User's guide: statistics. [CD-ROM] Version 8.2. USA: SAS Institute.

Sweeney MT, Lubbers BV, Schwarz S, Watts JL. 2018. Applying definitions for multidrug resistance, extensive drug resistance and pandrug resistance to clinically significant livestock and companion animal bacterial pathogens. J Antimicrob Chemother. 73(6):1460–1463. doi: 10.1093/jac/dky043.

Thanh N, Loh TC, Foo HL, Hair-Bejo M, Azhar B. 2009. Effects of feeding metabolite combinations produced by *Lactobacillus plantarum* on growth performance, faecal microbial population, small intestine villus height and faecal volatile fatty acids in broilers. Br. Poult. Sci. 250(3): 298–306.

Wu W, Xiao Z, An W, Dong Y, Zhang B. 2018. Dietary sodium butyrate improves intestinal development and function by modulating the microbial community in broilers. PloSOne. 13(5):e0197762. doi: 10.1371/journal.pone.0197762.

Zhang WH, Jiang Y, Zhu QF, Gao F, Dai SF, Chen J, Zhou GH. 2011. Sodium butyrate maintains growth performance by regulating the immune response in broiler chickens. Br Poult Sci. 52(3):292–301. doi: 10.1080/00071668.2011.578121.