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Fermented soybean meal in broiler diets exposed to stress induced by corticosterone: Effect on growth performance, gut health and immune response

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Abstract

Aim of study: To investigate the effect of fermented soybean meal (FSBM) on responses of broiler chickens exposed to stress condition.

Material and methods: Two hundred and eighty-eight day-old male chickens were allocated to six treatments in a 2 × 3 completely randomized factorial design involving two factors: factor 1 was the subcutaneous injections of CORT or corn oil (as control) at 2 mg/kg body weight twice per day during 3 days; and factor 2 were 3 levels (0, 10 and 20%) of FSBM in replacement of the original soybean meal.

Main results: The replacement of soybean meal by FSBM increased FI and BWG without any significant effect on FCR (10 days), while FCR was increased significantly by CORT injection. Corticosterone injection caused a significant decrease in the ratio of villus height (VH) to crypt depth (CD) in the duodenum and jejunum. The height of villi in the duodenum increased significantly at 20% FSBM replacement. The antibody titers against Newcastle disease (28 and 2 day), coliform count (28 day) and activity of digestive enzymes (10 day) were not affected by either FSBM replacement or CORT injections. Corticosterone injection significantly increased tall-like receptor-4 (TLR4) and immunoglobulin A (IgA) expression, while decreased heat shock protein-70 (HSP70) expression. FSBM replacement down-regulated the expression of TLR4, HSP70, and IgA in small intestine compared to the control group. In stress condition induced by CORT injection, 10% FSBM replacement decreased HSP70 and IgA expression in the jejunum and ileum, while had no effects on TLR4..

Research highlights: Partial replacement of soybean meal by FSBM in diets had positive effects on performance, intestinal morphology and immune response in chicks exposed to stress.

Additional key words: probiotics; animal nutrition; *Bacillus subtilis*; Newcastle disease; gene expression.

Abbreviations used: BWG (body weight gain); CD (crypt depth); CORT (corticosterone); FCR (feed conversion ratio); FI (feed intake); FSBM (fermented soybean meal); HSP70 (heat shock protein-70); IgA (immunoglobulin A); TLR4 (tall-like receptor 4); VH (villi height).

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Introduction

Increasing the circulating corticosterone (CORT) level in response to the hypothalamic–pituitary–adrenal axis activation by stressors leads to a reduction in feed intake, body weight gain, relative weight of immune organs, and suppresses the innate immunity of broiler chickens (Hu et al., 2020). This neuroimmune dysfunction might influence the quality of the intestinal-immune barrier, thereby allowing pathogenic bacteria to migrate through the intestinal mucosa and generating an inflammatory infiltrate. This inflammation can change the intestinal nutrition absorption and consequently decrease growth performance (Ligeiro de Oliveira et al., 2008; Quinteiro-Filho et al., 2010). Harmful effects of stress on health and performance is well known in broiler chickens. To overcome the adverse effects, the use of feed additives is one of the recommendations (Ali et al., 2018). Antibiotics as a feed additive have been fed to poultry for a relatively long time in order to boost feed utilization and health status. However, biosafety concerns of poultry and human health, arising from resistance to the antibiotic and antibiotic residues contamination in poultry products, led to removal of growth promoter's antibiotics from poultry industry worldwide (Toghyani et al., 2010). In this regard, organic acids, probiotics, prebiotics, and fermented feeds are used to optimize gut health status and prevent gut disorders, and reduce the use of antibiotics (Kazi et al., 2022). Among the available approaches, solid-state fermentation of vegetal sources of protein is the most promising (Aljubori et al., 2017). In addition to the desired effect of enhancing gut health, fermented feeds have some nutritional benefits in animal feeding (Engberg et al., 2009). Fermentation is also reported as an effective strategy to abolish or reduce anti-nutritional agents and increase the nutritional quality of plant-based protein meals (Missotten et al., 2015; Ketnawa & Ogawa, 2019). Soybean meal (SBM) is the most valuable and commonly used plant protein source in poultry feeds (Chiang et al., 2010). However, due to some anti-nutritional factors such as phytic acid, oligosaccharides, trypsin inhibitor and allergenic proteins, which interfere with the accessibility to its nutrients, the use of SBM in poultry diets can be limited (Tousi-Mojarrad et al., 2014). The ability of fermentation in reducing trypsin inhibitor activity and other anti-nutritional factors in SBM have been reported previously (Sharawy et al., 2016). Besides of the mentioned advantages, the fermentation can produce a variety of nutrients such as vitamins, oligosaccharides and small-size peptides (Sharma et al., 2020). Among the different bacterial species, *Bacillus subtilis* is characterized as a safe strain for solid-state fermentation in both food and feed industry.

Therefore, the present study was conducted to investigate the effects of including soybean meal fermented with *Bacillus subtilis* (FSBM) in young broiler diets on growth performance, gut physiology and morphometric analysis, and the expression of some genes associated with the immune system in broiler chickens kept under stress conditions.

Material and methods

Preparation of FSBM

The strain of *B. subtilus* (Gallipro®200 with registration number DSM17299 in the European Union) with 4×10^9 spores per gram were obtained from Biochem Company. The following methods were tested to ferment SBM: 1) fermentation in a tray under aerobic condition with 30% of moisture for 24, 48 and 72 h (Tray), 2) fermentation in a bag under anaerobic conditions with 30% moisture for 24,

Table 1. Peptide content of fermented soybean meal (FSBM) prepared by tray[1], anaerobic[2] and float[3] methods using *Bacillus subtilis* spores based on standard glycine diagram.

Method and duration of fermentation	Absorption in 540 nm	Concentration (mg/mL)
Tray - 24 h	0.021	0.025
Tray - 48 h	0.531	1.270
Tray - 72 h	0.907	2.060
Anaerobic - 24 h	0.020	0.024
Anaerobic - 48 h	0.045	0.116
Anaerobic - 72 h	0.208	0.439
Float - 24 h	0.000	0.000
Float - 48 h	0.027	0.150
Float - 72 h	0.021	0.050

[1] Fermentation in tray under aerobic conditions with 30% of moisture for 24, 48 and 72 h. [2] Fermentation in bag under anaerobic conditions with 30% moisture for 24, 48 and 72 h. [3] Fermentation in bag under aerobic conditions with 70% moisture (as float form) for 24, 48 and 72 h.

Table 2. Analysis of pH, urease activity index, and trypsin inhibitor in the original soybean meal (SBM) and fermented soybean meal (FSBM).

Item	SBM	FSBM
pH	7.00	6.87
Urease activity index[1]	0.07	0.06
Trypsin inhibitor units (per mg sample)[2]	13.40	3.40

[1] Calculated as: $pH_{blank} - pH_{sample}$. [2] One unit of trypsin activity is equivalent to an increase of 0.01 units of absorption at 410 nm in the reaction mixture under standard conditions.

48 and 72 h (Anaerobic) and 3) fermentation in a bag under aerobic condition with 70% moisture (as float form) for 24 and 48 h (Float). Based on glycinin and beta-conglycinins (Fig. 1) and peptide (Table 1) contents of FSBM, the method of fermentation in a tray under aerobic conditions with 30% moisture for 72 h was selected as the best one (greatest glycinin, beta-conglycinins and peptide contents). Briefly, this method consisted in the fermentation of SBM by soaking the SBM with water (30 water: 70 SBM) and inoculating 2×10^9 CFU *B. subtilus* spores per kg of SBM. The mixture was then cultured, mixed and fermented in a tray container (30 cm × 20 cm × 10 cm) for 72 h at 30°C. The pH, urease activity index and trypsin inhibitor concentration were measured in the original and fermented SBM (Table 2). Determination of urease was performed using AOAC methods (AOAC, 2005). Briefly, two sets of 0.2 g of grounded SBM (or FSBM) were weighed and added to a blank tube and test tube. Then, 10 mL of 0.05 M phosphate buffer solution (pH 7.0) and 10 mL of 0.5 M urea (made fresh in PBS prior to the assay) were added to blank and test tube respectively, mixed gently with SBM (or FSBM) samples and incubated in a water bath at 30°C, mixing/swirling every 5 min during incubation. At the end of 30 min incubation, the tubes were removed from water bath after mixing the contents one last time and kept on ice for 5 min to stop reaction. The pH of supernatant was measured using pH meter at 5 min after removal from the water bath. A 5-min interval between the preparation of the test and blank samples was needed. Urease activity (UA) was measured as the subtraction of the pH of blank tube from that of test tube (UA = pH test – pH blank). Trypsin inhibitor activity index was determined according to Liu (2019).

Animals and experimental diets

All animal procedures were in agreement with the Institutional Animal Care and Use Committee of the Guilan University, Rasht, Iran. Two hundred eighty-eight one-day-old male broiler chicks (Ross 308) were distributed into six experimental treatments in a 2 × 3 completely randomized factorial design with two levels of CORT injections (oil

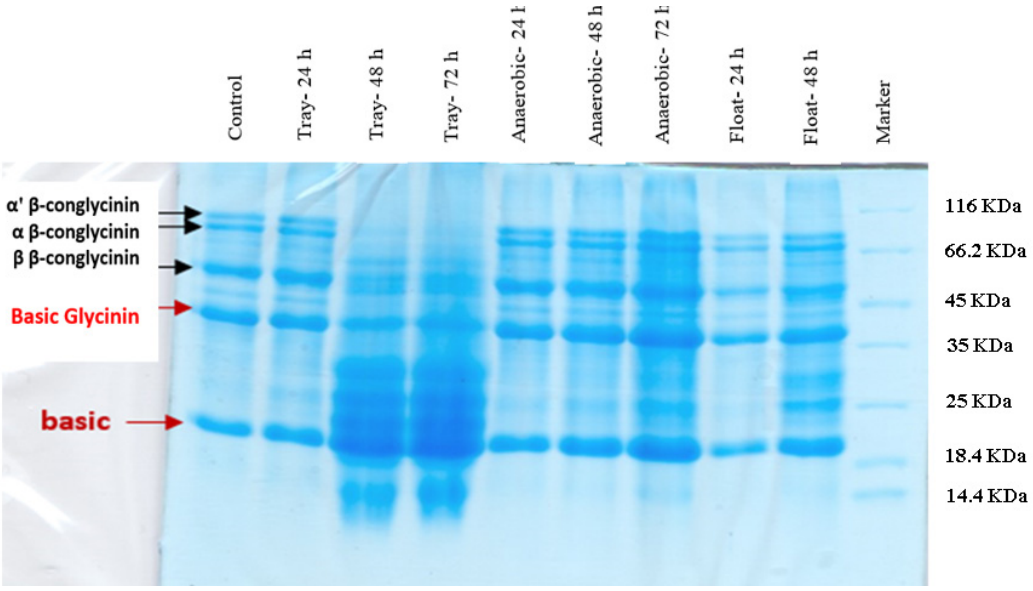

Figure 1. Glycinin and beta-conglycinins content of soybean meal fermented by tray, anaerobic and float methods using *Bacillus subtilis* spores at 0, 24, 48 and 72 h incubation.

Table 3. Ingredients and calculated chemical composition of the diets.[1]

Ingredients (%)	Starter			Grower			Finisher		
	Control	10% FSBM	20% FSBM	Control	10% FSBM	20% FSBM	Control	10% FSBM	20% FSBM
Corn	52.64	52.84	52.84	58.84	58.84	58.84	62.50	58.84	62.50
Soybean meal	39.50	35.55	31.60	33.50	30.15	26.80	29.00	26.80	23.20
FSBM	0.00	3.95	7.90	0.00	3.35	6.70	0.00	6.70	5.80
Soybean oil	3.00	3.00	3.00	3.30	3.30	3.30	4.40	3.30	4.40
Dicalcium phosphate	1.95	1.95	1.95	1.75	1.75	1.75	1.65	1.75	1.65
Calcium carbonate	1.06	1.06	1.06	0.98	0.98	0.98	0.90	0.98	0.90
Sodium chloride	0.19	0.19	0.19	0.19	0.19	0.19	0.19	0.19	0.19
Sodium bicarbonate	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25
DL-Methionine	0.34	0.34	0.34	0.31	0.31	0.31	0.26	0.31	0.26
L-Lysine HCl	0.22	0.22	0.22	0.23	0.23	0.23	0.22	0.23	0.22
L-Threonine	0.10	0.10	0.10	0.09	0.09	0.09	0.08	0.08	0.08
Choline chloride	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05
Vitamin premix [2]	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25
Mineral premix [3]	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25
Chemical composition									
Metabolizable energy (kcal/kg)	2910	2910	2910	3000	3000	3000	3110	3110	3110
Crude protein (%)	22.00	22.00	22.00	19.70	19.70	19.70	18.00	18.00	18.00
Ether extract (%)	5.20	5.20	5.20	5.7	5.7	5.7	6.92	6.92	6.92
Crude fiber (%)	3.91	3.91	3.91	3.6	3.6	3.6	3.4	3.4	3.4
Apparent digestible lysine (%)	1.24	1.24	1.24	1.11	1.11	1.11	1.00	1.00	1.00
Apparent digestible valine (%)	0.93	0.93	0.93	0.84	0.84	0.84	0.76	0.76	0.76
Apparent digestible arginine (%)	1.37	1.37	1.37	1.21	1.21	1.21	1.09	1.09	1.09
Apparent digestible methionine + cystine (%)	0.92	0.92	0.92	0.84	0.84	0.84	0.75	0.75	0.75
Apparent digestible threonine (%)	0.84	0.84	0.84	0.75	0.75	0.75	0.63	0.63	0.63
Chlorine (%)	0.20	0.20	0.20	0.20	0.20	0.20	0.20	0.20	0.20
Sodium (%)	0.18	0.18	0.18	0.18	0.18	0.18	0.18	0.18	0.18
Calcium (%)	0.94	0.94	0.94	0.84	0.84	0.84	0.78	0.78	0.78
Available phosphorus (%)	0.47	0.47	0.47	0.42	0.42	0.42	0.40	0.40	0.40

[1] The rate of fermented soybean meal (FSBM) inclusion is based on the control group, for example in starter phase for the control group with 39.5% dietary soybean meal, 10% and 20% of soybean meal replacement are 3.95 and 31.6% FSBM, respectively. [2] The vitamin premix provided in kg of diet: vitamin A, 11000 IU; vitamin E, 65 mg; vitamin D, 4500 IU; vitamin K_3, 2.5 mg; vitamin B_{12}, 0.017 mg; Niacin, 60.0 mg; D panthotenic acid, 17 mg; Riboflavin, 6.5 mg; Pyridoxine, 4 mg; Thiamine, 3 mg; Folic acid, 1.5 mg; Biotin, 0.18 mg. [3] The mineral premix provided in kg of diet: Mn, 100 mg; Zn, 100 mg; Fe, 40 mg; Cu, 16 mg; I, 1 mg; Se, 0.3 mg.

injection vs. CORT injection) and three levels (0, 10 and 20%) of SBM replacement by FSBM. Each treatment was replicated four times with 12 birds per replicate (48 birds for each treatment). The breeding room temperature and relative humidity were maintained at 33°C and 65% for the first 3 d, gradually decreased to 21°C and 55% until 28 d of age and maintained at such conditions until the end of the experiment. From 7 to 9 days of age (for 3 days), the chicks received one of the subcutaneous injections (corn oil as control or CORT) at 2 mg/kg BW twice per day (Yang et al., 2015). All diets were formulated based on nutrient requirement recommendation of Ross 308 strain catalogue. The ingredients and chemical compositions (based on NRC, 1994) of the experimental diets are reported in Table 3. The birds had *ad libitum* access to water and feed throughout the experiment.

Growth performance

Chicks were weighed at the beginning of the experiment and at 10 days old. At 10 days of age, average feed intake (FI) and body weight gain (BWG) were measured and feed conversion ratio (FCR) was calculated by the

division of FI by BWG. Mortality was recorded through this period and was used to adjust FCR by the following formulas:

$$\text{Hen/day number} = (\text{Days of period} \times \text{Chicks in the end of period}) + (\text{Number of dead chicks} \times \text{Alive days})$$

$$\text{FI} = \frac{\text{Feed at the beginning of period - Feed at the end of period}}{\text{Hen} \cdot \text{day number}}$$

$$\text{BWG} = (\text{Chicks weight at the beginning of period - Chicks weight at the end of period + Dead chicks weight}) / \text{Hen} \cdot \text{day number}$$

Digestive enzymes, gut morphology and gene expression

At 10 days of age, 2 birds per replicate from each treatment (n = 8) were randomly selected and slaughtered to determine the digestive enzymes activity, morphological characteristics of the small intestine and gene expression.

For the digestive enzymes' activity assay, the digesta of the small intestine was collected into sterile tubes, then diluted to a ratio of 1 to 10 with Tris-HCl buffer and homogenized by a homogenizer. Subsequently, it was centrifuged with a refrigerated (4°C) centrifuge at 14000 rpm for 20 min. Supernatants were divided into 5 microtubes and kept at a -80°C until analyses (Feng et al., 2007). Digestive enzymes' activity was determined as reported by Routman et al. (2003). In brief, alpha-amylase activity was measured by hydrolysis of starch followed by determination of the amount of maltose produced. One unit of enzyme activity was defined and expressed as the amount of enzyme that produced one µmol of maltose/min, at 37°C. Pancreas lipase activity was assessed by titration, using as substrate the olive oil emulsion (Sigma) and the colipase excess extracted from poultry pancreas. One unit of enzymatic activity was defined and expressed as the quantity of enzyme that released 1 µmol of fatty acid per minute. The protease activity unit was defined as milligrams of azocasein degraded during 2 h of incubation at 38°C per mg of intestinal digesta protein or pancreas.

In order to perform the gut morphology analyses, the mid-part of the duodenum, jejunum, and ileum sections from each animal were aseptically removed, flushed with 0.9% physiological saline solution, fixed with 4% formaldehyde-phosphate buffer and kept at 4°C until microscopic assessment of mucosal morphology. Each sample was prepared after staining with hematoxylin and eosin using standard paraffin embedding procedures. Villus height (VH) and crypt depth (CD) were measured by ImageJ software package (Awad et al., 2009; Young & Morrison, 2018).

Regarding gene expression, the expression of tall-like receptor-4 (TLR4), heat shock protein-70 (HSP70), and IgA, as candidate proteins whose expression is induced by heat stress, were investigated and analyzed by qRT-PCR in the different sections of the small intestine at 10 days of age. Total RNA was extracted from the three parts (duodenum, jejunum, and ileum) of intestine tissue in chicken according to the manufacturer's instructions (AccuZol, Bioneer kit). The concentration and quality of the RNA was determined by measuring the absorbance at 230, 260, and 280 nm using a Nanodrop spectrophotometer (Thermo Scientific, 2000). The 260/280 and 260/230 ratios of absorbance values were used to assess the purity of RNA. A 260/280 ratio of ~ 2.0 and 260/230 ratio in the range of 2.0-2.2 was accepted as the best quality of RNA. Lower ratios may indicate the presence of phenol, protein, carbohydrates, or other contaminants that absorb at or near 260, 230 or 280 nm. One microgram of total RNA was reverse transcribed into cDNA, using Thermo scientific kit, and the following reagents were added into a sterile, nuclease-free tube on ice according to the manufacturer's instructions (Bioneer). The qRT-PCR analysis was performed using the SYBR Green® Supermix (Bio-Rad) on a CFX96 real-time PCR Detection System (Bio-Rad). The qRT-PCR results were analyzed using the ΔCt value (Ct gene of interest – Ct GAPDH for each sample). The Ct is the number of cycles required for the fluorescent signal to cross the threshold. The relative gene expression was obtained using the ΔΔCt method (ΔCt sample – ΔCt calibrator), with the control group used as a calibrator to compare treatment sample gene expression, where the relative gene

Table 4. The primer sequences for RT-PCR amplification.

Gene	NCBI number	Primers	Sequence	Production size (bp)
TLR4	417241	Forward	5′-TAAGGAGTGGCAACAGCTCG-3′	138
		Reverse	5′-GAACAGCCCGTTCATCCTCA-3′	
HSP70	423504	Forward	5′-CCCCACCAACACCATCTTTG-3′	129
		Reverse	5′-TTGTACTCCACCTGCACCTT-3′	
IgA	416928	Forward	5′-AAGGTCTCCGTGGAGGATTG-3′	124
		Reverse	5′-TGACGTGAGAGGCTTTACCG-3′	
GAPDH	347193	Forward	5′-CAGAACATCATCCCAGCGTC-3′	132
		Reverse	5′-GAAGAGGCCACCACACGACAG-3′	

Table 5. Effects of corticosterone (CORT) injection and replacement of soybean meal (SBM) by fermented soybean meal (FSBM)[1] on broiler chickens performance parameters from 1 to 10 days of age.

Effects[2]	Daily weight gain (g/bird. d)	Daily feed intake (g/bird. d)	Feed conversion ratio
CORT			
CORT 0 mg (oil)	23.40	30.98	1.32 [b]
CORT 4 mg/day	23.19	32.00	1.38 [a]
SEM	0.375	0.375	0.017
p-value	0.710	0.670	0.041
FSBM replacement			
0% of FSBM repl.	21.20 [b]	28.80 [b]	1.36
10% of FSBM repl.	24.30 [a]	32.90 [a]	1.35
20% of FSBM repl.	24.40 [a]	32.70 [a]	1.34
SEM	0.460	0.459	0.020
p-value	0.001	0.010	0.860
CORT × FSBM replacement			
CORT 0 × 0% of FSBM repl.	21.1	28.2	1.34
CORT 0 × 10% of FSBM repl.	24.4	32.5	1.33
CORT 0 × 20% of FSBM repl.	24.6	32.5	1.34
CORT 4 mg/day × 0% of FSBM repl.	21.3	29.6	1.37
CORT 4 mg/day × 10% of FSBM repl.	24.1	33.4	1.38
CORT 4 mg/day × 20% of FSBM repl.	24.1	33.1	1.37
SEM	0.650	0.649	0.029
p-value	0.880	0.760	0.980

[1] The rate of FSBM inclusion is based on the control group, for example in starter phase for the control group with 39.5% dietary soybean meal, 10% and 20% of soybean meal replacement are 3.95 and 31.6% FSBM, respectively. [2] Each treatment replicated four times with 12 birds per replicate. [a–b] Means followed by different letters in the same column is significantly different ($p < 0.05$).

expression was expressed as fold change = $2^{-\Delta\Delta Ct}$ (Livak & Schmittgen, 2001). By using of $2^{-\Delta\Delta CT}$ method, the data are presented as the fold change in gene expression normalized to an endogenous reference gene (we used GAPDH in the current study) and relative to the untreated control. For the untreated control sample, ΔΔCT equals zero and 2^0 equals one, so that the fold change in gene expression relative to the untreated control equals one. The primer sequences are shown in Table 4.

Antibody titer against Newcastle disease and microbial count

After vaccination at 6 and 18 days of age, 24 birds (1 chick/replicate) at 28 and 42 days of rearing period were randomly selected, and blood samples were taken from the wing vein. Serum was separated and processed for HI (hemagglutination inhibition) test for Newcastle disease virus (NDV) (Thayer & Beard, 1998).

The antigen titre for running the HI test was determined by standard haemagglutinin (HA) technique using NDV vaccine as antigen. An HI test is a serum samples examination for the presence of HI antibodies to NDV. Two-fold serial dilutions of the test samples were mixed with an equal volume of NDV antigen. Chicken red blood cells were added and subsequently the dilutions were examined for the presence of complete inhibition of the hemagglutination. The reciprocal of the highest dilution of the NDV antigen causing 100% agglutination of an equal volume of standardized red blood cells was taken as the HA titre of the antigen (Alexander & Gough, 2003).

Digesta samples for bacteriological analysis were taken from the ileum at 28 days of age and transported to the laboratory immediately on ice (4°C). Serial dilutions of the rinse diluent were prepared in sterile physiological saline solution. Total aerobic bacterial populations were enumerated on plate count MacConkey agar. MacConkey agar is a selective and differential culture medium for gram-negative and enteric bacteria. Coliforms are defined as aerobic or facultatively anaerobic, gram negative, non-sporeforming rods capable of fermenting lactose to produce gas and acid. Lactose fermenters turn red or pink on McConkey agar. For counting the CFUs, serial dilutions of the rinse

diluent (100 µL) were overlaid on the surface of the agar, and incubated at 37 ºC for 24 h (Dickens et al., 2000).

Statistical analyses

All analyses on growth performance records and data related to gut health, digestive enzymes activity, microbial count, small intestine morphology and relative expression of TLR4, HSP70 and IgA mRNA were carried out using GLM procedures of SAS (SAS Institute., 2001). Comparisons of means were analyzed by Tukey's tests at $p<0.05$. The statistical model was as follows:

$$Y_{ijk} = \mu + A_i + B_j + AB_{ij} + e_{ijk}$$

where: Y_{ijk} is the observed value for a particular trait, µ is the overall mean, A_i is the main effect of factor A (type of injection), B_j is the main effect of factor B (rate of SBM replacement by FSBM), AB_{ij} is the interaction effect of two factors, and e_{ijk} is random error associated with the ijkth measuring. Additionally, linear and quadratic contrasts were used to test linear and quadratic effects, respectively, of FSBM on BWG, FI, and FCR.

Since data coming from RNA-Seq had a skewed distribution, unequal variances for the individual genes and the presence of extreme values, the log transformation was used to convert genes expression data into normal distribution. As the normalized counts x_{ij} can be equal to zero, we shift them by one before log transforming them, i.e.:

$$x_{ij}^{\log} = \log(x_{ij} + 1)$$

Results

The effect of CORT injection and SBM replacement by FSBM on growth performance from 1 to 10 d of age of the chicks is shown in Table 5. Corticosterone injection did not affect FI and BWG but led to significant increase in FCR compared to the control group with oil injection ($p<0.05$). On the other hand, the replacement of part of SBM by FSBM increased BWG and FI ($p<0.05$) but did not

Table 6. Effects of corticosterone (CORT) injection and replacement of soybean meal (SBM) by fermented soybean meal (FSBM)[1] on broiler chickens histomorphology (villus height, VH and crypt depth, CD) of duodenum, jejunum and ileum at 10 days of age.

Effects[1]	Duodenum			Jejunum			Ileum		
	VH (µm)	CD (µm)	VH:CD	VH (µm)	CD (µm)	VH:CD	VH (µm)	CD (µm)	VH:CD
CORT									
CORT 0 mg (Oil)	1399	167	8.39^{a}	1084	131	8.29^{a}	623.0	105	5.97
CORT 4 mg/day	1344	174	7.85^{b}	1028	136	7.60^{b}	565.2	105	5.41
SEM	28.52	3.65	0.18	25.50	2.33	0.22	22.40	3.87	0.09
p-value	0.183	0.170	0.043	0.070	0.130	0.040	0.086	0.980	0.132
FSBM replacement									
0% of FSBM repl.	1308^{b}	165	7.94	1025	136	7.50	588	109	5.61
10% of FSBM repl.	1371^{ab}	174	7.91	1037	131	7.93	576	103	5.57
20% of FSBM repl.	1435^{a}	172	8.52	1006	133	8.35	618	106	5.89
SEM	34.90	4.40	0.22	1.25	2.85	0.27	27.50	4.75	0.27
p-value	0.050	0.370	0.110	0.080	0.480	0.130	0.544	0.930	0.660
CORT × FSBM replacement									
CORT 0 × 0% of FSBM repl.	1326	162	8.21	1080	130	7.84	605	106	5.77
CORT 0 × 10% of FSBM repl.	1366	167	8.18	1079	127	8.54	600	106	5.66
CORT 0 × 20% of FSBM repl.	1505	172	8.79	1152	136	8.48	665	103	6.48
CORT 4 mg/day × 0% of FSBM repl.	1290	169	7.70	1030	143	7.26	571	106	5.45
CORT 4 mg/day × 10% of FSBM repl.	1376	181	7.63	996	136	7.33	553	101	5.49
CORT 4 mg/day × 20% of FSBM repl.	1365	173	8.24	1059	129	8.21	572	108	5.30
SEM	49.40	6.32	0.31	35.60	4.04	0.38	36.90	0.71	0.358
p-value	0.360	0.640	0.980	0.290	0.084	0.450	0.730	0.750	0.382

[1] The rate of FSBM inclusion is based on the control group, for example in starter phase for the control group with 39.5% dietary soybean meal, 10% and 20% of soybean meal replacement are 3.95 and 31.6% FSBM, respectively. [2] Each treatment was replicated 4 times with 12 birds per replicate. $^{a–b}$ Means followed by different letters in the same column is significantly different ($p < 0.05$)

Table 7. Effects of corticosterone (CORT) injection and replacement of soybean meal (SBM) by fermented soybean meal (FSBM)[1] on broiler chickens immune response and digestive enzyme activity at different ages.

Effects[2]	Antibody titers against Newcastle disease (log 2)		Coliform count (Log 10 CFU/g digesta)	Digestive enzyme activity (unit/mg digesta)		
				Amylolysis	Proteolysis	Lipolysis
	28 d	42 d	28 d	10 d		
CORT						
CORT 0 mg (oil)	3.66	2.50	6.13	106.91	9.49	49.77
CORT 4 mg/day	3.08	2.29	6.03	90.97	8.75	46.01
SEM	0.26	2.26	0.21	10.89	0.27	3.59
p-value	0.088	0.235	0.730	0.356	0.063	0.478
FSM replacement						
0% of FSM repl.	2.87	2.13	6.48	101.90	9.11	47.95
10% of FSM repl.	3.81	2.81	6.19	100.30	8.87	44.98
20% of FSM repl.	3.43	2.50	5.58	94.59	9.37	50.74
SEM	0.32	0.28	0.26	13.04	0.34	4.51
p-value	0.079	0.215	0.062	0.911	0.593	0.684
CORT × FSM replacement						
CORT 0×0% of FSM repl.	3.00	2.12	6.56	106.96	9.84	51.01
CORT 0×10% of FSM repl.	4.25	3.25	6.11	127.25	9.08	46.94
CORT 0×20% of FSM repl.	3.87	2.63	6.73	86.50	9.54	51.34
CORT 4 mg/day×0% of FSM repl.	2.87	2.12	6.41	96.87	8.39	44.89
CORT 4 mg/day×10% of FSM repl.	3.37	2.30	6.27	73.37	8.66	43.01
CORT 4 mg/day×20% of FSM repl.	3.00	2.50	5.41	102.68	9.20	50.14
SEM	0.452	0.429	0.365	19.700	0.507	6.820
p-value	0.588	0.505	0.802	0.173	0.440	0.928

[1] The rate of FSBM inclusion is based on the control group, for example in starter phase for the control group with 39.5% dietary soybean meal, 10% and 20% of soybean meal replacement are 3.95 and 31.6% FSBM, respectively. [2] Each treatment replicated four times with 12 birds per replicate.

modify FCR. Interaction effects between CORT injection and FSBM replacement on performance parameters were not significant. Figure 2 shows the test for the response of BWG, FI, and FCR to doses of FSBM, where we appreciate that the response for BWG and FI was quadratic, while for FCR was linear.

The effects of CORT and FSBM replacement on VH, CD, and ratio VH:CD are presented in Table 6. Corticosterone injection led to significant ($p<0.05$) decrease in the VH:CD ratio of the duodenum and jejunum at 10 days of age but did not affect VH and CD in any section of small intestine. Villus height was greater ($p<0.05$) for FSBM replacement at 20% than for FSBM at 0% in the duodenum, with FSBM at 10% showing intermediate values. The interaction effect of CORT injection and FSBM replacement on the VH, CD, and VH:CD ratio was not significant ($p>0.05$) for any part of the small intestine.

The effects of CORT and FSBM replacement on Newcastle titer and digestive enzymes activity (amylolysis, proteolysis, and lipolysis) coliform count of broiler chicks are presented in Table 7. Newcastle titer, digestive enzymes activity and coliform count were not affected by neither CORT injection nor FSBM ($p>0.05$). The interaction effect of CORT injection and FSBM replacement was also not significant on Newcastle titer, digestive enzymes activity and coliform count ($p>0.05$).

Table 8 shows the effect of CORT and FSBM replacement on TLR4, HSP70, and IgA gene expression in the different sections (duodenum, jejunum and ileum) of small intestine at 10 days of age, respectively. Corticosterone injection significantly increased and decreased TLR4 and IgA expression in duodenum, respectively, but did not affect HSP70 expressions compared to the control group (with oil injection) ($p>0.05$). HSP70 gene expression in the duodenum increased in response in the treatment with 10% SBM replacement by FSBM; however, TLR4 and IgA gene expression decreased in treatments with 10% and 20% SBM replacement by FSBM, respectively, compared with the control group.

Table 8. Effects of corticosterone (CORT) injection and replacement of soybean meal (SBM) by fermented soybean meal (FSBM)[1] on broiler chickens gene expression associated with immune response in different part of intestine in broiler chicken.

Effects	TLR4[2]			HSP70[3]			IgA[4]		
	Duodenum	Jejunum	Ileum	Duodenum	Jejunum	Ileum	Duodenum	Jejunum	Ileum
CORT									
CORT 0 (oil, control)	0.704^{b}	0.743^{b}	0.694	1.285	0.420^{a}	0.428^{a}	0.218^{a}	0.201^{b}	0.392^{b}
CORT 4 mg/day	1.497^{a}	1.168^{a}	0.682	1.468	-0.085^{b}	-0.353^{b}	0.048^{b}	0.623^{a}	0.838^{a}
SEM	0.027	0.061	0.036	0.061	0.050	0.069	0.021	0.034	0.028
p-value	0.0001	0.0001	0.815	0.056	0.0001	0.0001	0.0001	0.0001	0.0001
FSBM replacement									
0% of FSBM repl.	1.216^{a}	1.076^{a}	0.851^{a}	1.075^{b}	0.805^{a}	0.375^{a}	0.216^{a}	0.953^{a}	1.046^{a}
10% of FSBM repl.	1.026^{b}	0.685^{b}	0.378^{b}	1.846^{a}	-0.003^{b}	0.125^{a}	0.090^{b}	0.388^{b}	0.100^{c}
20% of FSBM repl.	1.060^{b}	1.106^{a}	0.835^{a}	1.210^{b}	-0.297^{b}	-0.386^{b}	0.095^{b}	-0.105^{b}	0.700^{b}
SEM	0.034	0.075	0.044	0.075	0.050	0.0847	0.025	0.041	0.035
p-value	0.004	0.003	0.0001	0.0001	0.0001	0.002	0.007	0.0001	0.000
CORT × FSBM replacement									
CORT 0×0% of FSBM repl.	1.000^{ab}	1.000^{a}	1.000^{ab}	1.000^{c}	1.000^{a}	1.000^{a}	1.000^{a}	1.000^{a}	1.000^{a}
CORT 0×10% of FSBM repl.	0.430^{c}	0.300^{b}	0.476^{cd}	1.720^{ab}	-0.063^{c}	0.590^{a}	-0.413^{d}	0.060^{c}	-0.350^{c}
CORT 0×20% of FSBM repl.	0.680^{b}	0.930^{a}	0.570^{cd}	1.140^{c}	0.440^{b}	-0.303^{b}	0.070^{c}	-0.450^{d}	0.530^{b}
CORT 4 mg/day×0% of FSBM repl.	1.430^{a}	1.150^{a}	0.703^{bc}	1.150^{c}	0.623^{b}	-0.250^{b}	-0.560^{d}	0.910^{ab}	1.090^{a}
CORT 4 mg/day×10% of FSBM repl.	1.620^{a}	1.530^{a}	0.280^{d}	1.973^{a}	-0.130^{c}	-0.340^{b}	0.593^{b}	0.716^{b}	0.553^{b}
CORT 4 mg/day×20% of FSBM repl.	1.440^{a}	1.280^{a}	1.100^{a}	1.283^{bc}	-0.923^{d}	-0.470^{b}	0.120^{c}	0.246^{c}	0.870^{a}
SEM	0.048	0.107	0.628	0.106	0.0756	0.119	0.036	0.0591	0.501
p-value	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001

[1] The rate of FSBM inclusion is based on the control group, for example in starter phase for the control group with 39.5% dietary soybean meal, 10% and 20% of soybean meal replacement are 3.95 and 31.6% FSBM, respectively. [2] TLR4: Tall-like receptor 4. [3] HSP70: Heat shock protein-70. [4] IgA: Immunoglobulin A. $^{a–b}$ Means followed by different letters in the same column is significantly different ($p < 0.05$).

In the jejunum, CORT injection significantly decreased ($p>0.05$) the expression of HSP70 and increased both TLR4 and IgA expressions compared to the control group (Table 8), and FSBM replacement significantly decreased HSP70 and IgA expression. TLR4 expression in the jejunum was increased by 0% and 20% FSBM replacement compared with 10% FSBM replacement group ($p>0.05$).

Regarding the effects of CORT injection in the ileum, it significantly increased IgA expression and decreased the expression of HSP70 compared to the control group ($p>0.05$). Corticosterone injection did not affect the expression of TLR4 in the ileum. The replacement of 20% of SBM by FSBM in broiler diets significantly decreased HSP70 and IgA expression in the ileum (Table 8).

Regarding the interaction effect of CORT injection and SBM replacement by FSBM in the three segments studied, HSP70 and IgA genes expression increased significantly in compared to CORT injected chicks in the duodenum ($p<0.05$). No significant differences were observed in TLR4 gene expression for the interaction effect between 10% and 20% FSBM and CORT injection treatments compared to CORT injected without FSBM group. The combined data from CORT injection and FSBM replacement showed that CORT injection and FSBM replacement significantly decreased HSP70 and IgA genes expression in the jejunum in comparison to CORT injected chicks ($p<0.05$). However, there was no significant difference in TLR4 expression between CORT-injected chicks fed diet with 10% FSBM replacement with CORT injected chicks fed SBM ($p<0.05$) in the jejunum. The results of the interaction effect of CORT injection and FSBM replacement on expression of TLR4, HSP70, and IgA were variable in the ileum (Table 8). A significant reduction in HSP70 expression was observed in the ileum of CORT-injected chicks fed diet with 10% FSBM replacement. However, 20% FSBM replacement plus CORT injection led to a significant increase in TLR4 and IgA expression in the ileum compared to non-CORT-injected chicks fed FSBM.

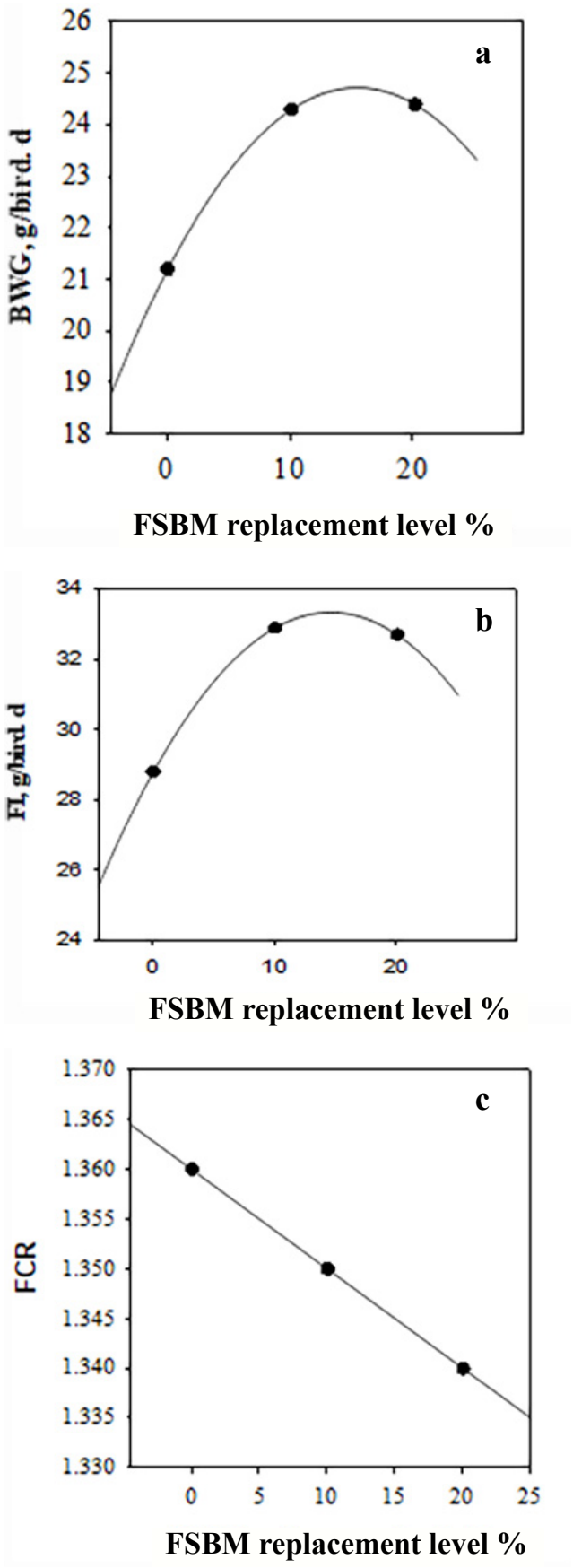

Figure 2. Test for the response of body weight gain (BWG, a), feed intake (FI, b), and feed conversion ratio (FCR, c) to doses of fermented soybean meal (FSBM).

Discussion

Previous studies (Ligeiro de Oliveira et al., 2008; Quinteiro-Filho et al., 2010) have reported that stressful conditions can change the nutrients absorption capacity in the gut and, consequently, can lead to a reduction in growth performance, which are in line with our results. In the present study, CORT injection led to a significant reduction in duodenal and jejunal VH:CD ratio, as well as a lower VH in jejunum ($p=0.07$) compared with non-CORT injection group. In terms of gene expression, CORT injection led to up-regulation of TLR4 gene expression in the duodenum and jejunum but not in the ileum. Although limited information is available on the effect of stress situations on TLRs in broiler chickens, it has been reported that the expression of TLR4 is elevated to protect the intestine from injury by heat stress (Huang, 2017). The expression of IgA also increased by CORT injection in the jejunum and ileum in the present study. Bos et al. (2001) reported that IgA is the primary protective immunoglobulin of intestinal secretions and is synthesized by plasma cells in lamina propria to defend and regulate the homeostasis in stress condition which is parallel to the results of the current study in which IgA expression in the jejunum and ileum increased by CORT injection. We also found that the HSP70 expression by different parts of intestine was suppressed by CORT injection. Hasan-Siddiqui et al. (2020) reported that the gene expression of HSP70 in different sections of the small intestine was different according to the duration of heat stress, and that after 24 h exposure to heat stress the HSP70 expression decreased, because the heat tolerance capacity of the chicks improved. Yang et al. (2009) also reported that decrease in HSP70 expression could be related to the stress duration.

Regarding the use of fermented ingredients, similar or even a better nutritive value of solid-state fermented protein products compared to their unfermented forms have been reported in the literature (Tang et al., 2012; Xu et al., 2012). As stated by several studies (Wang et al., 2010; Canibe & Jensen, 2012; Shahowna et al., 2013), fermentation can increase feedstuff palatability and the digestibility of organic matter, nitrogen, amino acids, fiber and calcium. In the present study, the replacement of 10% or 20% of SBM by FSBM in diets of broiler chicks led to an increase in FI and BWG, which is in agreement with the result of Chiang et al. (2010). Additionally, in the current study, broilers fed with diets in which 20% of SBM was replaced by FSBM showed significantly higher ($p<0.05$) VH in duodenum and a tendency to a greater VH also in jejunum ($p=0.08$), compared with those chicks fed diets with unfermented SBM. These findings are in line with the results of previous studies with SBM (Feng et al., 2007; Chiang et al., 2010; Tang et al., 2012; Xu et al., 2012). VH in ileum was not affected by FSBM. It has been reported that increased levels of small peptides (Tang et al., 2012) and reduced trypsin inhibitors (Feng et al., 2007) in FSBM compared with unfermented SBM, in line with our

results (Fig. 1 and Table 1), appeared to be responsible for improved small intestinal structure and function. Fermented feeds with lower levels of toxins and antigenic materials have also been suggested to benefit the intestinal morphology of broiler chickens (Chiang et al., 2010; Xu et al., 2012). Wang et al. (2003), in a study with pigs, also reported that dietary addition of small peptides improved intestinal morphology as determined by greater VHs and lower CDs in the duodenum, jejunum and ileum. Considering the digestive enzymes activity results, we did not find any significant differences between the SBM and FSBM treatments, probably due to the thermal treatment during processing of soybean, especially during the desolventizing/toasting process which reduces its heat-labile antinutritional factors (including trypsin inhibitor, glycinin, β-conglycinin), to a level with minimum adverse effect on the digestive enzymes activity. Regarding the antibody titer against NDV and coliform bacteria population, although no significant differences were detected among treatments, at 28 days tended to be higher and lower, respectively, in animals fed FSBM compared with control animals. These results agree with previous studies (Sugiharto & Ranjitkar, 2019; Zhu et al., 2020; Liu et al., 2022). In addition, regarding gene expression, our findings showed that FSBM inclusion in broiler diet decreased the expression of TLR4, HSP70, and IgA expression genes in different sections of small intestine.

Looking at the benefits of feeding fermented ingredients under stress conditions, Sugiharto & Ranjitkar, (2019) reported that fermented feed exhibits beneficial influences on gut ecosystems and morphology, immune functions as well as growth performance of bird in stress condition. In the present study, we did not observe these benefits of using FSBM in stressed animals compared with non-stressed animals in terms of performance, gut morphology and physiology, probably because of the relatively short duration of the stress condition in our study. However, FSBM replacement in stress condition induced by CORT injection decreased HSP70 gene expression in the jejunum and ileum, while increased IgA and TLR4 expression in all parts of the intestine, which is in line with the findings of Sugiharto et al. (2015) and can be related to the immune reactivity of FSBM.

In summary, findings of the current study regarding the ability of FSBM replacement in controlling the inflammatory response via down-regulating HSP70 and IgA expression in the jejunum and ileum in stress situation, suggest positive effects of replacement of FSBM on growth performance, intestine morphology and immune response in broiler chicks exposed to the stress induced by CORT injection for a longer period of time.

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