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Response of two broiler strains to dietary supplementation of fermented wheat bran with *Aspergillus niger* and *Aspergillus oryzae* under heat stress conditions

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Abstract Previous studies have documented the synthesis of prebiotics from agricultural wastes using *Aspergillus* fungi in solid-state fermentation. This study investigated the effects of dietary supplementation with fermented wheat bran (FWB), produced through solid-state fermentation with *Aspergillus niger* and *Aspergillus oryzae*, on broiler performance and intestinal morphology under cyclic heat stress conditions. The experiment followed a 4 × 2 factorial design, incorporating four FWB inclusion levels (0.00, 0.05, 0.10, and 0.15% of the diet, representing control and low, moderate, and high supplementation levels, respectively) and two broiler strains (Arian and Ross 308), with six replicates of six female chicks each. A total of 576 one-day-old chicks (288 Ross 308 and 288 Arian) was randomly assigned to treatment groups housed in wire mesh cages. From days 22 to 35, all birds were exposed to heat stress (HS). Dietary supplementation with 0.15% FWB significantly decreased feed intake ($P<0.05$) in both broiler strains during the period following HS exposure (35-42 d). Among Arian broilers, a high level of FWB supplementation resulted in poorer performance under HS conditions (22-35 days of age). In contrast, Ross broilers fed with 0.05% FWB demonstrated improved feed conversion ratio (FCR) throughout the rearing period ($P<0.05$). Additionally, all FWB supplementation levels were linked to a reduction in jejunal and ileal villus surface area in Arian chickens ($P<0.05$). These findings highlight the importance of exercising caution when recommending fermented wheat bran as a feed additive for broilers. Key factors such as the broiler strain, appropriate dosage, and the potential presence of mycotoxins—particularly ochratoxin A—must be carefully considered to ensure safety and effectiveness.

Keywords: *aspergillus*, intestinal morphology, plasma metabolites, solid-state fermentation

Introduction

To reduce the anti-nutritive compounds in dietary fiber sources and agricultural wastes such as wheat bran and to increase their nutritional value, approaches such as adding digestive enzymes and solid-state fermentation (SSF) have been suggested (Yang et al., 2009). The SSF method employs agricultural waste products as fermentation substrates with the enzymes realeased by microorganisms such as yeast, bacteria, and fungi. Filamentous fungi are considered the most suitable for SSF because they can thrive in fermentation media with low moisture content (Lee et al., 2020). *Aspergillus* is a genus of filamentous

fungi that, due to characteristics such as vast metabolic diversity, high secretory competence, excellent production yield and the capacity to perform modifications after translation, can produce a variety of secondary useful metabolites during the fermentation process, including digestive enzymes, citric acid and gluconic acid (Kurt et al., 2018). Thus, *A. niger* and *A. oryzae* have been widely used to enhance the nutritional value of agricultural wastes by interacting with substances like non-starch polysaccharides and proteins (Lateef et al., 2008).

The enzymes secreted by *A. oryzae* and *A. niger* could also modify anti-nutritive compounds such as saponins and hydrolyzable tannins in the feedstuffs (Dei et al., 2008). Aspergillus mycelium can be added to broiler diets as a probiotic, and Aspergillus fermentation products from solid-state fermentation (SSF) may exhibit prebiotic properties (Vosoogh Sharifi et al., 2022). The efficacy of different prebiotics depends on the defined fermentation profile developed by a particular microbial species used for fermentation and their content of non-fermentable oligosaccharides (Schoz-Ahrens et al., 2016). Therefore, prebiotics derived from wheat bran fermented by *Aspergillus* fungi may impact broiler performance and health differently than those produced by other bacteria and fungi. Research has shown that adding 1.50 g per kg diet of wheat bran cultured by *A. niger* strains in low-protein broiler diets (containing 95% of the recommended crude protein) improved weight gain and feed conversion ratio (FCR) during the initial 21 days of the rearing phase (Vosoogh Sharifi et al., 2022).

Heat stress (HS) negatively impacts on broiler performance (Sugiharo et al., 2017a) and carcass yield (Zeferino et al., 2016). Supplementation with 0.50% *Aspergillus awamori* did not enhance growth performance in broilers exposed to HS but mitigated lipid peroxidation levels in muscle caused by heat stress (El-deep et al., 2014). Supplementing antioxidant-rich prebiotics into broiler diets may offer protection against the adverse effects of HS. In this study, the antioxidant activities of *A. oryzae* and *A. niger* were demonstrated through their inhibitory effects on 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radicals and their Ferric Reducing Antioxidant Power (FRAP) assay outcomes. With fermented wheat bran known for its antioxidant properties, its potential role in alleviating HS in broilers merits further exploration. For instance, feeding broilers 1.0% *Chrysonilia crassa*-fermented bran between days 22 and 35 improved the feed conversion ratio (FCR) during days 21–25 and enhanced physiological tolerance to HS (Sugiharo et al., 2017b). However, another study reported no significant interaction between broiler diets containing fermented canola with *Lactobacillus salivarius* and rearing temperatures in terms of production performance or carcass yield (Aljubori et al., 2017).

This research investigated the impact of dietary fermented wheat bran (FWB), produced by fermenting wheat bran with *A. niger* and *A. oryzae* in a 1:1 ratio, on performance, blood metabolites, and intestinal morphology in two broiler strains reared in cages under heat stress (HS) conditions. The objective was to identify the optimal dietary inclusion level of FWB and assess its effectiveness in alleviating the adverse effects of HS.

Methods and materials

Preparation of experimental FWB

Local isolates of *A. niger* (PFCC 92844) and *A. oryzae* (S-O3) were prepared from a fungi collection at the Pasteur Institute (Tehran, Iran), and individually grown in wheat bran using SSF as previously described (Subhosh Chandra et al., 2013). Briefly, each isolate was cultured on Sabouraud 4% dextrose agar (Difco; BD, Franklin Lakes, USA) for seven days at 28˚C. Spores were scraped from the agar surface and harvested by adding 10.0 mL sterile buffered saline supplemented with 0.05% Tween 20 (Da Mao Biotechnology Co., Ltd, Tianjin, China). The concentrations of the spore suspensions were adjusted to 1.0×10^6/mL, and then the suspension was utilized to inoculate wheat bran. For wheat bran SSF, 100 g of wheat bran and 50.0 mL double-distilled water were transferred to 1.0 L Erlenmeyer flasks and allowed to soak for 2 hours at room temperature while manually shaken every 30 minutes. The flask lids were tightly closed with thick cotton/ aluminum foil before autoclaving for 20 min at 121˚C. Spore suspension (10 mL) was used to inoculate the cooled sterile substrate. The cultures were transferred to an incubator for 15 days at 28˚C and shaken by hand twice daily. The fermented material was dried in an oven at 60˚C and then ground to a smooth powder that could pass through a 1.00 mm screen. The FWB was kept dry at room temperature until usage.

Evaluation of phytic acid content and antioxidant properties of substrates before and after SSF After extracting from the raw or FWBs by maceration technique (Heidari-Sureshjani et al., 2015), phenolic compounds were measured using the Folin-Ciocalteu reagent (Merck KGaA, Darmstadt-Germany) (McDonald et al., 2001). The 2,2-diphenyl-1-picrylhydrazyl (Merck KGaA, Darmstadt, Germany) free radical scavenging test was conducted following the method outlined by Brand-Williams et al. (1995). To assay ferric-reducing antioxidant power (FRAP), FRAP solution was added to the extract samples inside each of the specified wells of a 24-well plate. The absorbance of the plates at a wavelength of 593 nm was measured using an enzyme-linked immunosorbent assay reader (Synergy HTX Multi-Mode Reader, Biotech Instruments, Winooski, VT, USA). Based on these measurements, the calibration curve for $FeSO_4 \cdot 7H_2O$ (Merck KGaA, Darmstadt, Germany) was generated. Then, by putting the absorbance of each sample in the curve equation, its antioxidant capacity was calculated as FRAP value according to micromolar $7H_2O$, FeSO4 (Katalinic et al., 2006).

To analyze phytic acid content, phytate was extracted in about 3.0 g of the sample with 50.0 mL of 3%

trichloroacetic acid (Merck, Darmstadt, Germany) while being continuously shaken at 200 rpm for 1 hr. The supernatant was then aspirated after centrifugation, and the phytic acid content was determined following the method described by Nair et al. (1991).

Ethics of experiment

The Tarbiat Modares University Local Ethics Committee for Animal Experiments approved the experimental procedure (Approval ID: IR.MODARES.REC.1399.150).

Experimental design

The fermentation products were mixed in equal proportions to create the experimental dietary treatment known as FWB. A total of 576 1-day-old female chicks from the Arian and Ross 308 broiler strains were randomly allocated into 96 cages, with each cage containing an equal number of chickens across eight treatment groups. During the first 3 days of life, all chicks were given a commercial starter diet that did not contain wheat bran. Subsequently, all chicks were fed the experimental dietary treatments. From days 4 to 42, the control group received a diet containing 0.15% autoclaved, unfermented wheat bran (Table 1). Treatment groups received diets with 0.05%, 0.10%, or 0.15% fermented wheat bran (FWB), replacing the unfermented wheat bran in the control diet at corresponding levels. All chickens were allowed *ad libitum* access to feed.

Table 1. Ingredients and composition of the basal diet

	1-14 days of age	15-24 days of age	25-35 days of age	35-42 days of age
Ingredient (% as fed)				
Maize	54.58	62.72	66.60	68.55
Soybean meal (44% crude protein)	38.80	31.69	27.80	22.16
Autoclaved wheat bran	0.15	0.15	0.15	0.15
Corn gluten	1.00	0.00	0.00	0.00
Soybean oil	1.00	1.00	1.30	1.00
Limestone	1.16	1.07	1.00	1.01
Calcium phosphate	1.88	1.71	1.44	1.49
Vitamin premix	0.25	0.25	0.25	0.25
Mineral premix	0.25	0.25	0.25	0.25
DL-methionine	0.30	0.31	0.27	0.26
L-lysine	0.19	0.26	0.26	0.23
L-threonine	0.07	0.13	0.13	0.10
NaCl	0.33	0.21	0.21	0.21
Sodium bicarbonate	0.04	0.25	0.34	0.34
Sum	**100**	**100**	**100**	**100**
Calculated composition				
Metabolizable energy (kcal/kg)	2871	2950	3025	3025
Crude protein (%)	22.50	19.50	18.06	17.44
Lysin (%)	1.33	1.20	1.10	1.04
Methionine (%)	0.67	0.63	0.57	0.55
Methionine+cysteine (%)	1.00	0.92	0.85	0.82
Threonine (%)	0.89	0.82	0.76	0.72
Calcium (%)	0.96	0.87	0.78	0.78
Available phosphorus (%)	0.48	0.44	0.39	0.39
Na (%)	0.17	0.18	0.20	0.20
Anion-cation balance (mEq/kg)	240	230	225	220

Broilers were reared under heat stress from day 22 to day 35 of the experimental period, during which all Ross 308 and Arian chickens were exposed to a temperature of 33.0±1.0˚C from 10:00 AM to 5:00 PM for seven hours each day. The temperature reached 31˚C within approximately 45 minutes, marking the onset of the heat stress treatment. The average relative humidity fluctuated between 61.00% and 87.00% throughout the experiment. The study employed a factorial design consisting of 4×2 treatments, which included four different levels of FWB: 0.00%, 0.05%, 0.10%, and 0.15% in the diet, fed to two strains of broiler chickens, Arian and Ross 308. According to research by Jahanian Najafabadi et al. (2018), modifying the diet based on the Arian catalog, which entails extended use of starter and grower diets, improves the performance of Arian and Ross broiler chickens. In this study, the basic diet for both strains was developed following the recommendations of the Arian breeder (Table 1, Manual: Arian Broiler Chicken Management Guide Breeding, 2008).

Broiler weight and feed intake were measured weekly throughout the study. At 32 and 42 days of age, six broiler chickens from each treatment group (one bird from each cage) were selected and euthanized by decapitation (Bader et al., 2014). Ileal microbial counts and intestinal morphology were assessed in the birds slaughtered on day 32. At 42 days of age, before the final slaughter, blood samples were collected from the brachial vein and transferred into tubes containing heparin. The blood samples were centrifuged at 3000 × g for 15 minutes to separate the plasma, which was then stored at –20˚C until testing. The following blood plasma parameters were analyzed using commercial kits (Pars

Azmoon Co., Tehran, Iran): albumin, globulin, total protein, cholesterol (Ch), and triglycerides (TGs) (Ashayerizadeh et al., 2018).

Ileal microbial enumeration

Ileal contents were collected in sterile containers and then homogenized to determine microbial counts. Before conducting the culture, 1.0 g of the homogenized ileal contents was mixed with buffered peptone water at a 1:10 ratio. Subsequently, tenfold dilutions of each sample were prepared using 9.0 mL of a 0.10% peptone solution. The total number of aerobic mesophilic bacteria and Lactobacilli was quantified by counting colonies on plate count agar from Oxoid Ltd., Basingstoke, UK, and MRS agar from BBL® Microbiological Systems (Cockeysville, MD, USA), respectively. Coliforms and non-lactose-fermenting enterobacteria were assessed following cultivation on MacConkey's agar medium (BBL® Microbiological Systems, Cockeysville, MD, USA). The plate count agar plates and other culture media were incubated at 30°C for 2 to 3 days and 37°C for 1 day. The colony-forming units from two cultures per bird were counted and averaged, with the results expressed as log colony-forming units per gram of ileal content (Abdel-Raheem et al., 2012).

Histological analysis of the small intestine

Segments, approximately 5 cm long, were excised from the duodenum, jejunum, and ileum. The segments were fixed in 10% neutral buffered formalin for 1 day, dehydrated in alcohol solutions, cleared with methyl benzoate, and embedded in paraffin wax for standard preparation (Sohail et al., 2012). Sections (4 µm-thick) were prepared transversely, stained with Hematoxylin and Eosin, and then observed using a light microscope for morphometric analysis. For each sample, 10 complete and properly aligned crypt-villus units were chosen. Villus height (VH) was determined using light microscopy (Carl Zeiss, Jena, Germany) by measuring from the top of the villus to the point where it meets the crypt, and crypt depth (CD) was defined as the distance between two neighboring villi invaginations (Figure 1). The average VH and CD of 6 birds were calculated to determine the mean villus height for each treatment group. The surface area of the villi was determined by multiplying 2π by the average width and length of the villi. Furthermore, the goblet cells containing acidic mucin (Alcian Blue-reactive, blue-stained) were counted along the villi after staining four additional sections with Alcian Blue (Bahadoran et al., 2016). Goblet cells were counted in at least six villi and reported as the average number of cells per 100 mm of villus epithelium (Uni et al., 2003).

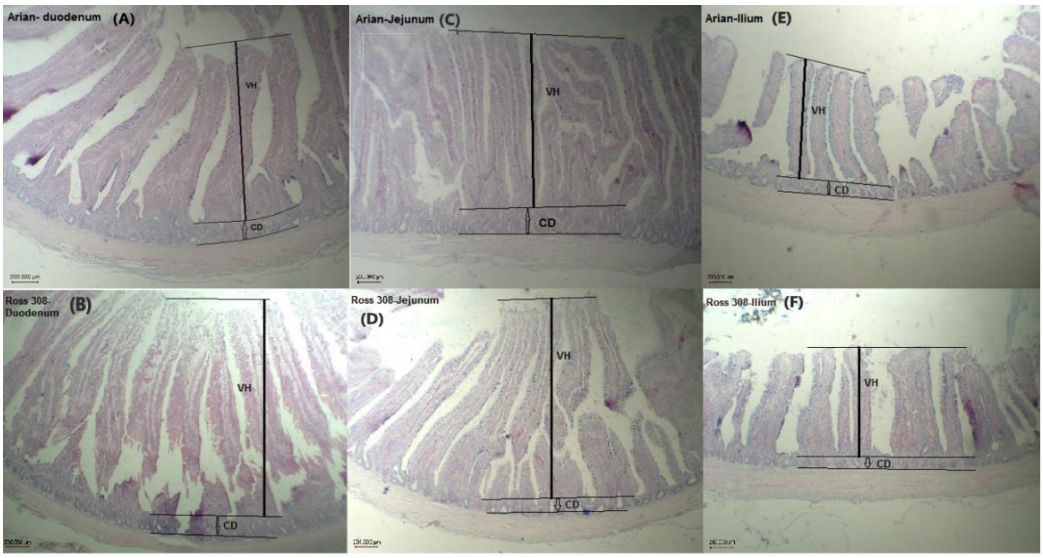

Fig. 1. Formalin-fixed, paraffin-embedded tissue sections of the duodenum [in Arian: top-left (**A**) and in Ross 308: down-left (**B**)], jejunum [in Arian: top-middle (**C**) and in Ross 308: down-middle (**D**)] and ileum [in Arian:top-right (**E**) and in Ross 308: down-right (**F**)] were stained by Hematoxylin and Eosin. Images were taken at × 40.00 magnification. The scale bar represents 0.20 mm.
*VH: Villus height; CD: Crypt depth.

Statistical analysis

Data were analyzed using the General Linear Model (GLM) procedure in SAS software (version 9.1.3; SAS Institute, Cary, NC, USA) in the following model:

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

where, Y_{ijk} is the observed characteristic, µ denotes the overall mean, A_i represents the main effect of FWB level, B_j indicates the main effect of the broiler chicken strains, AB_{ij} stands for the interaction effect between FWB level and strain type, and e_{ij} is the random error term associated with observation ijk. When the interaction term AB_{ij} was significant, the main effects were excluded from the interpretation. Comparisons between treatment means were performed using the Duncan's multiple range test at $P<0.05$.

Results

Total phenols, phytic acid, and antioxidant activities in the FWBs

According to the results presented in Table 2, the phenolic compounds extracted from *A. niger*-fermented wheat bran (FWB) were greater than those extracted from *A. oryzae*.

Table 2. Antioxidant indices and phytic acid content of fermented wheat bran

Fungi strain	Total phenolic compounds (mg/mL of dry substance extract)	Phytic acid (mg/g dry weight of sample)	DPPH assay (%)	FRAP assay (µmol reduced Fe (III) to Fe (II)/mL)
Uncultured wheat bran (Control)	1.40^{c}	3.30^{a}	48.31^{b}	0.50^{c}
A. niger (PFCC 92844)-FWB	4.59^{a}	1.76^{c}	54.58^{a}	0.59^{a}
A. Oryzae (S-O3)-FWB	2.43^{b}	2.17^{b}	51.60^{ab}	0.56^{b}
SEM	0.03	0.05	1.24	0.005
P-value	<0.01	<0.01	<0.01	<0.01

^{a,b}: within columns, mean values with common letter(s) are not different (P>0.05).

Additionally, in the extracts derived from wheat bran fermented with *A. niger* and *A. oryzae*, the levels of phytic acid reached 53.30% and 65.75% of the initial values in the non-FWB extract (3.30 mg per gram), respectively. Therefore, it can be inferred that the amount of phytases secreted by *A. niger* was higher than that produced by *A. oryzae*.

The antioxidant properties of the FWB extractions were superior to those of the unfermented sample. However, based on the FRAP test, wheat bran fermented with *A. niger* exhibited greater antioxidant activity than that fermented with *A. oryzae*.

Performance

The effects of feeding 0.15% FWB during the pre-HS period led to inconsistent results across the two broiler strains on feed conversion ratio (Table 3). During the HS period, the diet containing 0.15% FWB adversely affected the body weight gain (BWG) and worsened the FCR in Arian chickens compared to their respective control (P<0.05). During the post-HS period, dietary treatments did not significantly affect the FCR. However, across this phase as well as the entire experimental duration, the high-level dietary treatment reduced the feed intake (P<0.05). None of the levels of FWB significantly affected the total BWG of birds; however, low level of FWB (0.05%) improved the total FCR in Ross 308 chickens compared to the corresponding control (P<0.05).

Plasma metabolites

The concentrations of plasma metabolites are presented in Table 4. The effects of chicken strain, diet, and the interaction between broiler strain and diet on plasma albumin concentrations and the albumin-globulin ratio were not statistically significant. Broiler chickens receiving the diet with 0.10% fermented wheat bran (FWB) exhibited significantly decreased plasma cholesterol levels (P<0.05) and increased blood glucose concentrations (P<0.05) compared to the control groups. Ross broilers fed the diet containing 0.15% FWB showed a significant reduction in mean plasma total protein concentration compared to those on the control diet (P<0.05). The mean plasma globulin concentration was significantly lower in the 0.15% FWB-Ross group than in the respective control group. Additionally, plasma uric acid levels in Ross chickens fed 0.15% FWB were lower than those in the control broilers (P<0.05).

Intestinal microflora

Ross chickens demonstrated a greater abundance of ileal lactobacilli and fewer coliforms than Arian chickens, indicating enhanced digestive health (Table 5). Additionally, supplementation with fermented wheat bran (FWB) at all dietary levels significantly reduced the counts of the coliform bacteria in the ileal contents (P<0.05).

In both Arian and Ross strains, dietary treatment increased the population of ileal lactobacilli at a 0.10% dietary level, compared to the corresponding control groups.

Intestinal morphology

Tables 6 and 7 show the intestinal histological changes. Ross broilers fed 0.10% FWB showed significantly longer jejunal villi than those in the control-Ross group (P<0.05); however, this enhancement was not evident in the villi surface area. Feeding 0.05% FWB resulted in reduced crypt depth in the duodenum of both strains compared with the controls (P<0.05). However, the 0.15% FWB-Ross group showed a significant increase in the ileal crypt depth compared with the control-Ross group (P<0.05). Our findings indicated that chickens fed diets containing 0.15% and 0.10% FWB had lower levels of acidic goblet cells (aGCs) in the duodenum compared with those fed the basal diet (P<0.05). The number of jejunal and ileal aGCs was lower in the 0.15% FWB-Ross group than in the control-Ross group (P<0.05). The villus height to crypt depth (VH: CD) ratio in the duodenum and jejunum of broilers fed diets with 0.10% FWB significantly increased compared with the control groups (P<0.05).

Table 3. Effects of broiler strain, dietary treatments, and their interactions on the performance of broiler chickens during different periods of the experiment

	Before Heat Stress (4-21 d)			During Heat Stress (22-35 d)			After Heat Stress (36-42 d)			Total period (4-42 d)		
	FI (g/bird/period)	BWG (g/bird/ period)	FCR	FI (g/bird/ period)	BWG (g/bird/ period)	FCR	FI (g/bird/ period)	BWG (g/bird/ period)	FCR	Total FI g/bird/ period	Total BWG g/bird/ period	Total FCR
Strain												
Arian	958.49^{a}	663.67^{a}	1.44^{a}	2176.60	994.77^{b}	2.20a	1043.17	537.11	2.06	4178.26	2195.55^{b}	1.91^{a}
Ross	901.98^{b}	639.22^{b}	1.41^{b}	2151.68	1120.71^{a}	1.92b	1125.05	561.81	2.01	4178.71	2321.74^{a}	1.80^{b}
SEM	5.71	4.74	0.004	8.81	8.49	0.05	29.04	24.33	0.07	24.49	23.55	001
P-value	<0.01	0.01	<0.01	0.16	<0.01	<0.01	0.054	0.47	0.74	0.99	0.01	<0.01
Dietary treatment												
Control*	934.53^{ab}	660.75	1.41	21.48.06^{b}	104457^{b}	2.06	1169.17^{a}	539.93	2.17	4251.77^{a}	2245.25	1.89
0.05%FWB	921.80^{b}	642.23	1.43	216955^{ab}	1068.33^{ab}	2.04	1100.29^{ab}	563.30	2.03	4191.64^{a}	2273.85	1.85
0.01%FWB	956.86^{a}	665.15	1.44	2212.38^{a}	1099.94^{a}	2.02	1152.11^{a}	566.29	2.12	4321.35^{a}	2331.38	1.86
0.15%FWB	907.75^{b}	637.65	1.42	212657^{b}	1018.11^{b}	2.12	914.87^{b}	528.34	1.81	3949.18^{b}	2184.10	1.82
SEM	8.07	6.70	0.005	12.46	12.00	0.07	41.08	34.41	0.10	37.47	33.31	0.02
P-Value	<0.01	0.12	0.07	<0.01	0..01	0.23	<0.01	0.84	0.10	<0.01	0.18	0.41
Strain× diet												
Arian-Control	972.40^{a}	669.87	1.45^{a}	2162.90^{abc}	1007.48^{c}	2.15^{b}	1097.19	516.96	2.14	4232.49	2194.31	1.93^{a}
Arian0.05%FWB	944.39^{ab}	646.66	1.46^{a}	2232.56^{a}	1002.33^{c}	2.23^{ab}	1126.37	529.91	2.13	4303.32	2178.89	1.98^{a}
Arian0.01%FWB	977.84^{a}	695.70	1.40^{b}	218979^{ab}	1057.04^{bc}	207^{bc}	1091.84	547.65	2.07	4259.48	2300.39	1.86^{abc}
Arian0.15%FWB	939.30^{ab}	626.46	1.46^{a}	2121.17^{bc}	912.23^{d}	2.35^{a}	857.28	553.93	1.72	3917.75	2108.62	1.88^{ab}
Ross control	896.66^{cb}	651.64	1.38^{b}	2133.22^{bc}	1081.66^{ab}	1.98^{dc}	1241.16	562.89	2.20	4271.04	2296.19	1.86^{ab}
Ross 0.05%FWB	899.21^{cb}	637.80	1.41^{b}	210654^{c}	1134.33^{a}	1.86^{d}	1074.20	596.69	1.93	4079.96	2368.81	1.73^{c}
Ross 0.01%FWB	935.87^{ab}	634.61	1.48^{a}	2234.98^{a}	1142.84^{a}	1.96^{dc}	1212.37	584.93	2.12	4383.21	2362.37	1.86^{abc}
Ross 0.15%FWB	876.19^{c}	632.83	1.38^{b}	2131.96^{bc}	1124.00^{ab}	1.90^{d}	972.46	502.76	1.95	3980.62	2259.59	1.76^{bc}
SEM	16.14	13.39	0.01	24.92	24.00	0.15	58.09	48.66	0.14	74.94	66.61	0.04
P-Value	0.03	0.18	<0.01	<0.01	0.03	<0.01	0.31	0.63	0.48	0.11	0.78	0.04

*The control diet contained 0.15% autoclaved, unfermented wheat bran. Treatment groups received diets where fermented wheat bran replaced the unfermented wheat bran.
^{a,b,}: within columns, mean values with common letter(s) are not different (P>0.05).

Table 4. Effect of broiler strain, dietary treatments, and their interactions on plasma metabolites in broiler chickens at 42 d of age

	Albumin (g/dL)	Cholesterol (mg/dL)	Glucose (mg/dL)	Acid uric (mg/dL)	Protein (g/dL)	Triglycerides (mg/dL)	Globulin (g/dL)	Albumin: Globulin
Strain								
Arian	2.26	105.31	312.15^{a}	6.26^{a}	3.58	127.15^{a}	1.31	1.83
Ross	2.19	103.05	278.98^{b}	5.48^{b}	3.44	106.98^{b}	1.25	1.99
SEM	0.04	1.97	7.67	0.11	0.053	2.12	0.05	0.17
P-value	0.15	0.42	<0.01	<0.01	0.07	<0.01	0.39	0.49
Dietary treatment								
Control	2.26	111.80^{a}	259.87^{b}	6.18^{a}	3.66	120.97^{ab}	1.39	1.71
0.05%FWB	2.22	101.40^{ab}	304.73^{ab}	6.10^{ab}	3.48	123.68^{a}	1.26	1.89
0.01%FWB	2.26	100.42^{b}	322.07^{a}	5.85^{ab}	3.55	109.77^{b}	1.29	1.81
0.15%FWB	2.17	103.09^{ab}	295.60^{ab}	5.37^{b}	3.36	113.84^{ab}	1.18	2.23
SEM	0.05	2.79	10.85	0.16	0.07	2.99	0.08	0.24
P-Value	0.59	0.02	<0.01	<0.01	0.053	<0.01	0.29	0.45
Strain× diet								
Arian-Control	2.67	114.57	271.33	5.67^{abc}	3.65^{a}	131.63^{ab}	1.38^{a}	1.80
Arian0.05%FWB	2.14	100.67	318.00	6.41^{a}	3.33a^{b}	127.98^{abc}	1.20^{ab}	1.95
Arian0.01%FWB	2.33	99.27	323.67	6.74^{a}	3.53^{a}	110.77^{bcd}	1.20^{ab}	1.98
Arian0.15%FWB	2.32	105.73	324.62	6.23^{a}	3.80^{a}	138.22^{a}	1.48^{a}	1.58
Ross control	2.26	108.04	248.50	6.69^{a}	3.66^{a}	110.31^{bcd}	1.41^{a}	1.62
Ross 0.05%FWB	2.30	102.14	291.46	5.79^{ab}	3.62^{a}	119.38^{abc}	1.33^{ab}	1.83
Ross 0.01%FWB	2.18	101.57	310.47	4.95^{bc}	3.56^{a}	108.76^{dc}	1.38^{a}	1.65
Ross 0.15%FWB	2.03	100.45	265.60	4.51^{c}	2.92^{b}	89.46^{d}	0.88^{b}	2.87
SEM	0.07	5.85	25.34	0.23	0.11	4.24	0.11	0.33
P-Value	0.02	0.52	0.56	<0.01	<0.01	<0.01	<0.01	0.07

^{a,b,}: within columns, mean values with common letter(s) are not different (P>0.05).

Discussion

In alignment with our findings, Sousa et al. (2021) discovered that after solid-state fermentation (SSF), aqueous extracts of oilseed cakes fermented by *Aspergillus oryzae* exhibited lower antioxidant potential values compared to those fermented by *A. niger*. Table 2 shows that the amount of phenolic compounds released by the two fungal species differed, potentially affecting the antioxidant potential after solid-state fermentation (Sousa et al., 2021).

Our growth performance results differed from those of Yamamoto et al. (2007), who observed an increased growth rate in broilers fed *A. awamori*-fermented distillation by-products. Moreover, Kang et al. (2015) reported that the body weight gains of broiler chickens treated with dietary fermented rice bran were significantly greater than those receiving the basal diet.

The inclusion of antioxidant-rich FWB in the diet failed to enhance broiler chicken performance during or following heat stress. The underlying mechanisms behind the ineffectiveness of dietary supplementation in promoting growth under heat-stress conditions remain uncertain. According to Salami et al. (2015), the efficacy of dietary supplementation in heat-stressed broilers is influenced by various factors, including dosage, duration, diet composition, bird age, and the severity of the stress. The reduced feed intake (FI) in broilers fed high levels of FWB (Table 3) may be due to increased concentration of compounds such as ochratoxin-A, nucleic acids, and some metabolites in the diet (Chiou et al., 2001).

Table 5. Effect of broiler strain, dietary treatments, and their interactions on microbial counts in the ilium

	Total aerobic mesophilic bacteria	Coliform and lactose-negative enterobacteria	Lactobacilli
Strain			
Arian	7.04[a]	6.40[a]	7.16[a]
Ross	6.63[b]	6.07[b]	7.04[b]
SEM	0.03	0.03	0.02
P-value	<0.01	<.0001	0.001
Dietary treatment			
Control	6.87[b]	7.14[a]	6.55[c]
0.05%FWB	6.40[c]	5.51[c]	7.11[b]
0.01%FWB	7.35[a]	6.08[b]	7.52[a]
0.15%FWB	6.74[b]	6.22[b]	7.22[b]
SEM	0.05	0.04	0.03
P-Value	<0. 01	<0.01	<0.01
Strain× diet			
Arian-Control	7.14	7.02[b]	6.98[d]
Arian0.05%FWB	6.57	5.56[d]	6.96[d]
Arian0.01%FWB	7.48	6.86[b]	7.61[a]
Arian0.15%FWB	6.99	6.15[c]	7.08[dc]
Ross control	6.58	7.25[a]	6.13[e]
Ross 0.05%FWB	6.22	5.46[d]	7.26[bc]
Ross 0.01%FWB	7.22	5.30[e]	7.43[ab]
Ross 0.15%FWB	6.49	6.28[c]	7.35[ab]
SEM	0.07	0.05	0.05
P-Value	0.11	<0.01	<0.01

[a,b]: within columns, mean values with common letter(s) are not different (P>0.05).

The hypocholesterolemic effects of FWB supplementation at a moderate level (Table 4) support the findings of Lin et al. (2020), who proposed that the addition of *A. niger* to broiler diets impacts blood lipid levels by reducing triglycerides (TGs) and cholesterol (Ch) concentrations in plasma. A similar reduction in lipid levels was observed upon substituting soybean meal with fermented rapeseed meal (Ashayerizadeh et al., 2018) or fermented soybean meal in broiler diets (Sembratowicz et al., 2020). The cholesterol-lowering effects of Aspergillus spp. have been attributed to the deconjugation of bile acids, which facilitates cholesterol removal by creating new bile salts and thus promoting biliary secretion, resulting in reduced levels of cholesterol in the blood plasma (Liong et al., 2005). Furthermore, lactobacilli in the microbiome can lower total cholesterol and triglyceride values in blood plasma by promoting the excretion of bile acids in feces and inhibiting the activity of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) (Liong et al., 2005). The decrease in plasma TG levels may also be linked to the increased presence of lactic acid bacteria in the broiler gut (Kannan et al., 2005). The hyperglycemic effects of FWB at a moderate substitution level (0.10%, Table 3) - may be attributed to enhanced intestinal glucose absorption (Chichlowski et al., 2008). Increased glucose uptake following prebiotic consumption is associated with improved passive absorption and transport through the gastrointestinal lining (Kannan et al., 2005).

A high level of fermented wheat bran (FWB) negatively affected the blood protein and uric acid concentrations in Ross broiler chickens, as shown in Table 4. Fermented rice bran dietary supplementation did not affect albumin levels, according to Kang et al. (2015). In contrast to our current findings, An et al. (2022) reported that feeding broiler chickens a diet containing 7.00% fermented wheat bran (FWB) increased serum total protein and globulin levels compared with a control diet. The contradictory results observed may be attributed to heat stress during the chicken breeding process in our experiment or the specific fungal strains used for fermentation. Additionally, there is a possibility of ochratoxin-A being present in the wheat bran fermented with *A. niger*. In a similar study conducted at our research center, the presence of this toxin was confirmed when oak kernel was used as a substrate for fermentation by the same fungal strain during solid-state fermentation (unpublished data). Therefore, under HS exposure, elevated toxin levels in the diet beyond a certain threshold may negate the benefits of prebiotic supplements.

Reduced plasma uric acid concentrations in broilers have been attributed to decreased protein consumption (Darsi et al., 2012). The decrease in feed intake by broilers receiving 0.15% FWB over the entire experimental period (Table 3) could reduce plasma uric acid levels by restricting dietary protein consumption.

Table 6. Effect of broiler strain and dietary FWB levels on duodenum morphology

	VH (µm)	VTd (µm)	CD (µm)	GN	VH:CD	SA (mm^2)
Strain						
Arian	1702.1	204.2	177.5	8.9	10.3	1.1
Ross	1583.3	185.4	180.8	9.2	9.8	0.9
SEM	47.6	10.6	6.3	0.2	0.6	0.06
P-value	0.09	0.22	0.71	0.29	0.55	0.05
Diet						
Control	1454.2^{b}	187.5^{ab}	250.0^{a}	10.1^{a}	6.0^{b}	0.88^{b}
0.05%FWB	1533.3^{ab}	195.8^{ab}	138.3^{b}	9.7^{ab}	11.7^{a}	0.94^{b}
0.01%FWB	1795.8^{a}	233.3^{a}	153.3^{b}	8.4^{bc}	11.9^{a}	1.32^{a}
0.15%FWB	1787.5^{a}	162.5^{b}	175.0^{b}	8.1^{c}	10.5^{a}	0.89^{b}
SEM	67.34	15.02	8.90	0.3	0.80	0.09
P-Value	<0.01	0.02	<0.01	<0.01	<0.01	<0.01
Strain× diet						
Arian-Con	1525.0	183.3	250.0^{a}	10.53	6.35	0.89
Arian-0.05%FWB	1741.7	200.0	163.3^{bc}	9.73	10.71	1.10
Arian-0.01%FWB	1800.0	258.3	153.3^{bc}	7.73	11.80	1.46
Arian-0.15%FWB	1741.7	175.0	143.3^{bc}	7.60	12.17	0.96
Ross-Control	1383.3	191.7	250.0^{a}	9.60	5.58	0.86
Ross-0.05%FWB	1325.0	191.7	113.3^{c}	9.73	12.71	0.79
Ross-0.01%FWB	1791.7	208.3	153.3^{bc}	9.07	11.94	1.17
Ross-0.15%FWB	1833.3	150.0	206.7^{ab}	8.53	8.89	0.83
SEM	95.2	21.2	12.6	0.4	1.1	0.1
P-Value	0.07	0.57	<0.01	0.07	0.15	0.67

^{a,b}: within columns, mean values with common letter(s) are not different (P>0.05).
VH: Villus height; VT: Villus thickness; CD: Crypt depth; GN: Acidic goblet cell numbers in 100 µm of villus height; SA: Villus surface area (The average width of the villi × the average length of the villi × π).

Research has shown that adding fermented wheat bran to broilers' diet, particularly at 0.05% and 0.10%, can have beneficial effects on the intestinal microflora (Table 5). Studies have found that dietary supplementation using 5% *Leitiporus sulphureus*-fermented wheat bran significantly boosted the Lactobacillaceae population compared to a control group (Lin and Lee, 2020). The enzymes produced by Aspergillus fungi break down complex carbohydrates, and the subsequent fermentation of the resulting simple sugars into volatile fatty acids creates acidic conditions within the gut contents (Zia et al., 2013). This environment is optimal for the growth of Lactobacillus bacteria, which can inhibit the proliferation of harmful bacteria by producing lactic acid and further lowering the pH of the intestinal contents (Sakata et al., 2003). Additionally, the antimicrobial properties of glucose oxidase derived from *A. niger* may help reduce the total coliform bacteria in the ileal content (Wang et al., 2022). The increased bioavailability of antioxidant compounds released after solid-state fermentation (SSF) may also play a role in modulating the intestinal flora (Bertsch et al., 2020).

The shape and morphology of the intestine, particularly the villi and crypts of Lieberkühn, serve as indicators of the digestive tract's functionality and overall health (Muir et al., 2000). Enhanced intestinal morphometric features reported in earlier studies on fermented products were attributed to the beneficial effects of small peptides formed during the fermentation of large proteins (Hu et al., 2016), which helped mitigate the adverse effects of anti-nutritional compounds on intestinal structure (Özdoğan et al., 2012). Aligning with the findings of Lin et al. (2020), chickens fed a diet supplemented with fermented wheat bran (FWB) exhibited increased villi height in the jejunum compared to the control group. However, despite the observed rise in villi height in the jejunum and ileum (Table 7), this benefitial effect was offset by a reduction in the villi thickness in these segments. Consequently, while the jejunal absorption surface area remained unchanged in the Ross strain (as shown in Table 7), it was significantly reduced in the Arian strain following FWB supplementation. The combination of diminished villus thickness (VT) and increased crypt depth (CD) in the jejunum and ileum of chickens fed moderate to high levels of FWB raises concerns about possible harmful substances in the dietary treatments.

Low to moderate dietary FWB supplementation reduced duodenal crypt depth in both broiler strains (Table 6). In contrast, a high level of FWB increased ileal crypt depth in the Ross strain, while moderate levels elevated jejunal crypt depth in both strains (Table 7). Deeper crypts are typically linked to accelerated cellular shedding and epithelial cell turnover, which are induced by bacterial toxins in the intestine (Pelicano et al., 2003). In a healthy gut, cell turnover occurs at a slower rate, reflected by relatively shallow crypts (Pelicano et al., 2003).

Goblet cells synthesize and secrete a mucus layer that hinders bacterial translocation and protects the lamina from harmful substances such as toxins andpathogens (Deplancke and Gaskins, 2001). High dietary doses of fermented wheat bran (0.15%) negatively affected the population of intestinal aGCs in the jejunum and ileum of Ross broilers (Table 7). Mair et al. (2010) discovered that the number of aGCs in the jejunal villi significantly decreased when prebiotics were added to the diet of piglets. Reduced total number of aGCs could contribute to a higher risk of intestinal bacterial translocation in broiler chickens, potentially resulting in inflammatory reactions in birds (Deplancke and Gaskins, 2001). Given the potential presence and toxicity of ochratoxin-A in the experimental fermented wheat bran, dietary supplementation at a 0.15% level is strongly discouraged. Tong et al. (2020) found that administering ochratoxin A at 50 µg/kg body weight per day decreased the intestinal goblet cells in broiler chickens.

Table 7. Effect of broiler strain and dietary FWB levels on jejunum and ileum morphology

	Jejunum						Ileum					
	VH (µm)	VT (µm)	CD (µm)	GN	VH:CD	SA (mm^2)	VH (µm)	VT (µm)	CD (µm)	GN	VH:CD	SA (mm^2)
Strain												
Arian	1195.8^{b}	268.7^{a}	164.2	12.7	7.5	0.99^{a}	1503.3	303.3^{a}	123.3^{b}	10.7^{b}	12.4^{a}	1.40^{a}
Ross	1259.9^{a}	200.0^{b}	167.1	13.0	7.7	0.79^{b}	1500.1	295.9^{b}	135.8^{a}	13.4^{a}	11.4^{b}	1.21^{b}
SEM	21.07	6.75	4.90	0.29	0.24	0.03	20.95	9.57	3.33	0.25	0.31	0.01
P-value	0.04	<0.01	0.68	0.39	0.62	<0.01	0.91	0.004	0.01	<0.01	0.03	<0.01
Diet												
Control	1128.1^{b}	341.7^{a}	143.3^{b}	13.4	6.02^{b}	1.20^{a}	1340.2^{b}	346.5^{a}	121.7^{b}	11.8	11.20	1.46^{a}
0.05%FWB	1237.5^{a}	170.8^{c}	161.7^{ab}	12.2	7.70^{b}	0.67^{b}	1326.7^{b}	253.3^{b}	121.7^{b}	12.3	11.32	1.06^{b}
0.01%FWB	1345.8^{a}	187.5^{c}	189.2^{a}	13.7	9.43^{a}	0.80^{b}	1740.0^{a}	266.7^{b}	136.7^{a}	12.8	12.88	1.43^{a}
0.15%FWB	1200.0^{ab}	237.5^{b}	168.3^{ab}	12.1	7.24^{b}	0.89^{b}	1600.0^{a}	260.0^{b}	138.3^{a}	11.4	12.08	1.26^{ab}
SEM	29.80	9.55	6.93	0.41	0.34	0.05	29.63	13.54	4.71	0.35	0.44	0.06
P-Value	<0.01	<0.01	<0.01	0.02	<0.01	<0.01	<0.01	<0.01	0.02	0.06	0.04	<0.01
Int												
Arian-Con	1100.0^{b}	433.3^{a}	133.3	12.3^{ab}	5.8^{c}	1.49^{a}	1377.3^{b}	400.0^{a}	133.3^{bcd}	10.4^{d}	10.3^{ab}	1.73^{a}
Arian-0.05%FWB	1316.7^{ab}	175.0^{cd}	170.0	11.9^{ab}	7.8^{abc}	0.72^{bc}	1333.3^{b}	280.0^{b}	100.0^{d}	10.3^{d}	13.4^{a}	1.17^{bc}
Arian-0.01%FWB	1166.7^{b}	183.3^{cd}	193.3	12.9^{ab}	8.8^{ab}	0.67^{c}	1960.0^{a}	226.7^{b}	160.0^{ab}	10.2^{d}	12.2^{ab}	1.39^{ab}
Arian-0.15%FWB	1200.0^{b}	283.3^{b}	160.0	13.6^{ab}	7.6^{abc}	1.07^{b}	1346.7^{b}	306.7^{ab}	100.0^{d}	12.0^{dc}	13.5^{a}	1.29^{abc}
Ross-Control	1156.2^{b}	250.0^{cb}	153.3	14.5^{a}	6.3^{bc}	0.90^{bc}	1307.0^{b}	293.0^{ab}	110.0^{dc}	13.2^{bc}	12.1^{ab}	1.19^{bc}
Ross-0.05%FWB	1158.3^{b}	166.7^{d}	153.3	12.5^{ab}	7.6^{abc}	0.61^{c}	1320.0^{b}	226.7^{b}	143.3^{abc}	14.4^{ab}	9.2^{b}	0.94^{c}
Ross-0.01%FWB	1525.0^{a}	191.7^{cd}	185.0	14.4^{a}	10.1^{a}	0.92^{bc}	1520.0^{b}	306.7^{ab}	113.3^{dc}	15.3^{a}	13.5^{a}	1.46^{ab}
Ross-0.15%FWB	1200.0^{b}	191.7^{cd}	176.7	10.7^{b}	6.8^{bc}	0.72^{bc}	1853.3^{a}	213.3^{b}	176.7^{a}	10.9^{d}	10.6^{ab}	1.24^{bc}
SEM	42.1	13.5	9.8	0.6	0.5	0.07	41.9	19.1	6.7	0.5	0.6	0.08
P-Value	<0.01	<0.01	0.19	<0.01	0.19	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01

^{a,b}: within columns, mean values with common letter(s) are not different (P>0.05).

VH: Villus height; VT: Villus thickness; CD: Crypt depth; GN: Acidic goblet cell numbers in 100 µm of villus height; SA: Villus surface area (The average width of the villi × the average length of the villi × π).

Conclusions

The dietary inclusion of 0.15% fermented wheat bran (FWB) during heat stress (HS) negatively impacted the performance parameters in Arian broilers, which also reduced the feed intake in both Arian and Ross chickens during the pre- and post-HS periods. However, only Ross chickens demonstrated improved feed conversion ratios (FCR) throughout the rearing period when fed 0.05% FWB. Importantly, all levels of FWB supplementation led to a reduction in jejunal surface area (SA) in Arian broilers. These findings suggested that fungal-produced toxins or other compounds present in FWB may have diminished its potential prebiotic benefits, especially at higher inclusion levels. The observed variation in responses between the broiler strains points to differences in toxin resistance; Ross chickens may have counteracted the effects of low-dose toxins (at 0.05% FWB supplementation) through a more robust immune system and distinct gut microbial composition, thereby preserving intestinal surface area. These findings highlight the need for appropriate and tailored nutritional interventions, especially based on the broiler strain, to maximize performance under heat stress conditions. Furthermore, the lack of benefits from FWB supplementation underscores the complexity of mitigating heat stress through dietary approaches. Future investigations should evaluate ochratoxin concentrations before incorporating *Aspergillus*-fermented products into broiler diets and explore alternative feed additives that might offer more consistent benefits in improving heat-stressed broiler performance. The results emphasized the importance of considering strain-specific responses when designing dietary strategies for broilers under heat stress, which could aid in optimizing the productivity and welfare in commercial poultry operations. The study also underscores the need to explore other dietary interventions, such as alternative prebiotic sources or feed additives, that could offer more consistent benefits in heat-stressed broilers. Lastly, studies focusing on the long-term effects of FWB supplementation on broiler health, immunity, and gut morphology under varying environmental conditions could provide valuable insights for optimizing poultry nutrition strategies.

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