

***Laetiporus sulphureus*–fermented wheat bran enhanced the broiler growth performance by improving the intestinal microflora and inflammation status**

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ABSTRACT This study investigates the effects of *Laetiporus sulphureus*–fermented wheat bran (LS) as a feed supplementation on the immunomodulative properties in broiler chickens. Crude phenolic compounds, crude polysaccharides, crude triterpenoids, and ergosterol were determined in LS water extracts. In animal experiments, 400 male broilers (Ross 308) were randomly assigned into 5 groups fed with a corn–soybean–based diet (control) and a control diet replaced with 5% wheat bran (WB), 10% WB, 5% LS, and 10% LS, respectively. Each group had 4 replicates and 20 birds per pen (total of 80 birds/treatment). The results showed that the 5% LS-supplemented group had significantly higher BW in the finisher phase (22–35 D). Better feed conversion ratio ($P < 0.05$) of LS-supplemented groups was observed in both the finisher phase and the overall experimental period. The LS-supplemented groups had significantly lower coliform counts in the ileum than the other treatment and control groups ($P < 0.05$). The results of serum

immunoglobulin showed that LS supplementation significantly increased serum IgA concentration compared with the control and WB-supplemented groups ($P < 0.05$). Simultaneously, ileal IgA contents of the LS groups were significantly higher than in the WB and control groups ($P < 0.05$). Regarding proinflammatory cytokines, serum tumor necrosis factor alpha and IL-6 in the LS-supplemented groups were significantly lower than those in the 10% WB group ($P < 0.05$), whereas serum tumor necrosis factor alpha and IL-1 β in the 5% LS group were significantly lower than in both the control and WB-supplemented groups ($P < 0.05$). An investigation on the effects of LS on immune-related genes in broiler showed that chickens supplemented with 5% LS had lower levels of liver and jejunum *IL-1 β* and *NF- κ B* mRNA compared with the control group and WB groups ($P < 0.05$). In conclusion, LS supplementation can potentially enhance growth performance of broilers by improving intestinal microflora and inflammation status.

Key words: *Laetiporus sulphureus*, immunomodulation, broiler, solid state fermentation, wheat bran

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INTRODUCTION

Poultry production has been a major leading industry among animal husbandry industries in response to the growing demand for high-protein animal products. However, there are several issues, such as the rising cost of feedstuffs, infectious diseases, regulation of antimicrobials, and high-intensity production environments, that the poultry industry has had to confront (Lillehoj and Lee, 2012). Therefore, it is very important to

establish scientific solutions in taking sustainability, feed/food safety, animal welfare, and health into account, to create a future with good prospects for the poultry industry.

Developing alternative materials for major crop feedstuffs could be one solution for coping with the rising feed costs caused by the increasing demand for grains. Wheat bran (WB) is an agricultural byproduct produced by wheat processing. More than 6.5 million tons of wheat are produced globally per year, accompanied by the production of a large amount of wheat during processing for making flours. However, WB's high lignocellulosic content (44.0%) and low nutrition value (approximately 1,300 kcal/kg of ME) makes it unsuitable as supply to monogastric animals (Prückler et al., 2014). Furthermore, nonstarch polysaccharides (NSP) contained in WB tend to act as antinutritional

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compounds that could inhibit digestibility, causing pathogen proliferation in the gastrointestinal tract and inducing gut inflammation (Chen et al., 2015; Lai et al., 2015).

The solid-state fermentation (SSF) process uses low-cost agricultural and agroprocessing waste as substrates by applying NSP-degrading microorganisms to improve the utility of those by-products. Compared with the submerged fermentation, SSF is more efficient and cost effective in producing bioactive compounds (Hölker et al., 2004; Lee et al., 2020a). Filamentous fungi were reported to be the most suitable for SSF owing to their ability to endure low-moisture fermentation environments (Hernández et al., 2008; Lee et al., 2020a). Our previous studies had reported the application of SSF using filamentous fungi on agricultural byproducts which further exerted positive effects on monogastric animals. Lee et al. (2020a,b) used *Antrodia cinnamomea* on the SSF of WB and produced bioactive compounds such as phenolic compounds, polysaccharides, and triterpenoids, which exerted growth promoting, antioxidant, and anti-inflammation effects in broilers, which suggest the potential of SSF on the utilization of agroprocessing byproducts to produce bioactive compounds and functional feedstuffs.

In regular raising conditions, animals have a strong chance of encountering environmental viruses, bacteria, and fungi, which could cause oxidative damage and trigger lipid peroxidation, tissue damage, and inflammatory response (Cuzzocrea et al., 2001; Lee et al., 2017). To repair the damaged tissue and fight against infection, the inflammation process recruits immune cells to local sites by secreting inflammatory cytokines and chemokines; however, the prolonged inflammation may induce unnecessary energy consumption and retard the growth performance of animals (Lee et al., 2020a, 2017). Hence, diminishing the excessive inflammation, while maintaining the normal immune status in farm animals, will improve the quality and benefits in animal production.

Laetiporus sp. is one of the medicinal fungi traditionally used by Europeans to cure pyretic diseases, coughs, gastric cancer, and rheumatism (Ríos et al., 2012). In accordance with previous studies, submerged mycelial cultured *Laetiporus sulphureus* has been reported to produce functional polysaccharides (Jayasooriya et al., 2011; Lung et al., 2011) as well as mycophenolic acids (Fan et al., 2014) and being able to attenuate the excessive immune activation of the selected cells without causing cytotoxicity (Saba et al., 2015; Wang et al., 2017). Furthermore, our previous study showed that solid-state-fermented *L. sulphureus* can modulate lipopolysaccharide (LPS)-triggered inflammation process involving *TLR4* and *NF-κB* mediation in chicken peripheral blood monocytes (Lin et al., 2019). However, there are limited studies on the immunomodulatory effects of *L. sulphureus* in broiler chickens. Therefore, this study aims to inspect the bioactive component

in *L. sulphureus* solid-state-fermented WB (LS) and the effects of LS dietary supplementation on the growth performance and immunomodulation properties in broiler chickens.

MATERIALS AND METHODS

Microorganisms and Inoculum Preparation

The *L. sulphureus* BCRC 35305 used for fermentation was purchased from the Bioresource Collection and Research Center, Food Industry Research and Development Institute (Hsinchu, Taiwan). Inoculum was prepared by shake flask culture with malt extract broth (MEB). Briefly, flasks filled with 100 mL of MEB were covered with tin foil and autoclaved at $121 \pm 1^\circ\text{C}$ for 30 min, followed by transferring *L. sulphureus* mycelium to the medium by punching out agar pieces (about 1-cm diameter) from precultured MEA plates, 100 mL of liquid media contained 5 pieces of agar. Flasks with agar pieces in MEB were incubated at 25°C on a rotary shaker incubator at 120 rpm for 5 D until used for fermentation.

Preparation of Solid-State-Fermented WB by L. sulphureus

Solid-state fermentation of *L. sulphureus* was performed in accordance to our previous study (Lin et al., 2019). Samples were collected and dried at 40°C for 2 D before being ground in a mill and stored at -20°C .

Extraction and Determination of Bioactive Compounds

Air-dried LS was weighed and added to distilled water. The mixture was then left to stand at 95°C for 1 h in a water bath. The solution was filtered (Advantec No. 1, Tokyo, Japan) to obtain the extracts of each sample, which were subsequently stored at -20°C for further analysis. The total phenolic contents were determined using the Folin-Ciocalteu reagent as per the method described by Kujala et al. (2000). The equation obtained from the standard gallic acid (GA) standard graph was used to determine the phenolic compounds of each sample (mg of GA equivalent per g DW of sample). Crude polysaccharide content was determined by phenol-sulfuric acid assay (Dubois et al., 1956) using the standard glucose calibration curve and expressed as mg of the glucose equivalent/g DM. The crude triterpenoid content of LS was determined as per the methods of Lu et al. (2011), and the content was calculated using the standard ursolic acid (U6753, Sigma) calibration curve (mg of the ursolic equivalent per g DW of sample). Ergosterol content was determined using HPLC. *L. sulphureus*-fermented WB extraction was filtered through a $0.22\text{-}\mu\text{m}$ membrane filter and subsequently analyzed using an HPLC instrument (Hitachi, Kyoto, Japan) equipped with a pump (L-2130), UV detector

(L-2490), column (Transgenomic CARBOSep CH0682 Pb, 300 mm × 7.8 mm), and computer system with HPLC D-2000 Elite. The sample injection volume was 20 µL. Chromatographic peaks in the samples were identified by comparing their retention times and UV spectra with the reference standard (45480, Sigma).

Enzyme Extraction and Assays

Three grams of samples from each fermentation group were weighted and diluted 10 times using the buffer and stirred on ice for 30 min and filtered through a filter paper (Advantec No. 1, Tokyo, Japan) to obtain extracellular enzyme solution. Laccase activity (EC 1.10.3.2) was determined by the oxidation of 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (Bourbonnais et al., 1995). One unit (U) of laccase activity was defined as 1 µmol of 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) oxidized per min. Xylanase and cellulase were determined by dinitrosalicylic acid method (Miller, 1959). One unit of xylanase and cellulase activity was defined as the quantity of enzyme required to liberate 1 µmol of reducing sugar (xylose/glucose) of crude filtrate per minute under standard assay conditions.

Experimental Birds and Housing

The animal trial was approved by the Animal Care and Use Committee of National Chung Hsing University, Taiwan (IACUC No. 105-140). A total of 400 1-day-old commercial Ross 308 male broiler chicks were randomly allocated to 1 of 5 treatments: control (corn-soybean meal), 5% corn replaced by 5% WB (5% WB), 10% corn replaced by 10% WB (10% WB), 5% corn replaced by 5% LS (5% LS), or 10% corn replaced by 10% LS (10% LS), with 4 replicates and 20 birds per pen (total of 80 birds/treatment). Ambient temperature was maintained at $34 \pm 1^\circ\text{C}$ for the first 7 D and then gradually decreased to $26 \pm 1^\circ\text{C}$ until the birds reached 21 D of age. A temperature of 26°C was maintained until the end of the 35 D. The experimental period included 2 phases: starter phase (1–21 D) and grower phase (22–35 D). At the beginning of the feeding trial, the average BW of the birds was even for all pens (approximately 43.4 ± 0.17 g/bird). All chicks were raised in a temperature-controlled house. The birds were kept in floor pens (2.5 × 4.0 m) with wire floors and rice bran litter material. In the starter phase, 2 fountain drinkers and 1 feed tray were included per pen. In the grower phase, nipple drinkers and a feeder were used as replacement. Vaccines for Marek's disease, Newcastle disease, and infectious bronchitis were provided to chicks immediately after birth. Water and feed were supplied ad libitum to the broilers. A feed formula (Table 1) was designed to meet the nutrient requirements of broilers informed by the NRC (1994). The proximate composition including DM, CP, and crude fat were analyzed as per the AOAC (1980). Starter and grower diets were offered to the birds from 1 to 21 D and from 22 to 35 D of age, respectively. Neither anticoccidial nor

antibacterial supplements were added to the feed mixtures. On day 21 and 35, the performance of the broilers was assessed by recording feed intake, BW of birds, BW gains, and feed conversion ratio (feed/gain, FCR).

Serum and Intestinal Content and Organ Collection

At 35 D, 4 birds were randomly selected from each pen for sampling. Five milliliter of blood samples were collected from the brachial vein. Blood samples were centrifuged at $2,000 \times g$ for 15 min, and the serum was stored at -20°C until analysis. After the blood samples were collected, the chickens were euthanized by exsanguination, and their abdominal cavities were opened. For each treatment group, the contents of the ilea and ceca of 3 birds were collected for the study. Furthermore, the liver, jejunum and ileum were harvested and submerged in RNA shield (Zymo, USA) for mRNA isolation.

Serum Characteristics

Serum samples were used for the determination of serum biochemical parameters, including white blood cell count, red blood cell count, hematocrit, mean corpuscular volume, mean cell hemoglobin, glucose, urea nitrogen, uric acid, triglyceride, cholesterol, high-density lipoprotein, low-density lipoprotein, glutamic oxalocetic transaminase, serum glutamic-pyruvate transaminase, mean corpuscular hemoglobin concentration, total protein, albumin, globulin, and hemoglobin. Analyses were performed using an Automatic Biochemical Analyzer (7150 autoanalyzer; Hitachi, Tokyo, Japan).

Determination of Cytokines and Immunoglobulins

IL-1 beta (IL-1β), IL-6, tumor necrosis factor alpha (TNF-α), IgA, and IgG in serum and secretory IgA (sIgA) in ileal and cecal contents were determined by using kits purchased from Cusabio Biotech Co., Ltd. The procedures were executed in accordance with the manual.

Microbial Parameters of Intestinal Contents

One gram of digesta from the ileum or cecum of 35-day-old chicken were collected and serially diluted in 9 mL PBS solution. *Escherichia coli* was cultured with chromogenic medium agar (CHROMagar ECC) under aerobic conditions at 37°C for 24 h, and lactic acid bacteria was cultured with Difco Lactobacilli MRS Agar under anaerobic conditions at 37°C for 48 h. The microflora numbers were calculated by plate count method, and the bacterial populations were expressed as log₁₀ CFU per gram of intestinal contents.

Table 1. Ingredients and chemical composition of the experimental diets for broilers.

Ingredients	Starter diet (1–21 D)					Finisher diet (22–35 D)				
	Control	5% WB	5% LS	10% WB	10% LS	Control	5% WB	5% LS	10% WB	10% LS
	g/kg									
Corn	524.9	458.7	458.4	392.5	391.9	549.5	482.9	482.9	416.3	416.3
Wheat bran	0	50.0	0	100.0	0	0	50.0	0	100.0	0
LS	0	0	50.0	0	100.0	0	0	50.0	0	100.0
Soybean meal, CP 44%	320.0	167.3	167.3	14.8	14.7	16.6	185.6	185.6	354.4	354.4
Fish meal, CP 60%	50.0	50.0	50.0	50.0	50.0	320.6	167.8	167.8	15.1	15.1
Full-fat soybean meal	41.4	209.8	210.1	377.9	378.7	30.0	30.0	30.0	30.0	30.0
Soybean oil	30.0	30.0	30.0	30.0	30.0	10.6	10.6	10.6	10.6	10.6
Limestone	11.6	11.6	11.6	11.5	11.5	12.2	12.2	12.2	12.2	12.2
Monocalcium phosphate	11.2	11.2	11.2	11.2	11.2	3.4	3.3	3.3	3.2	3.2
DL-Methionine	3.4	3.7	3.7	4.1	4.1	50.0	50.0	50.0	50.0	50.0
Salt	2.9	2.8	2.8	2.8	2.8	1.3	1.5	1.5	1.8	1.8
L-Lysine HCl	1.8	2.1	2.1	2.4	2.3	3.0	3.3	3.3	3.6	3.6
Choline-Cl (60%)	0.8	0.8	0.8	0.8	0.8	0.8	0.8	0.8	0.8	0.8
Vitamin premix ¹	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Mineral premix ²	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Total	1,000.0	1,000.0	1,000.0	1,000.0	1,000.0	1,000.0	1,000.0	1,000.0	1,000.0	1,000.0
Calculated nutrient value										
ME, kcal/kg	3,050.0	3,050.0	3,050.0	3,050.0	3,050.0	3,175.0	3,175.0	3,175.0	3,175.0	3,175.0
DM, %	88.28	88.85	89.11	89.42	89.95	88.31	88.88	89.15	89.45	89.98
CP, %	23.0	23.0	23.0	23.0	23.0	21.0	21.0	21.0	21.0	21.0
Crude fat, %	6.04	8.86	8.64	11.68	11.25	7.56	10.39	10.17	13.22	12.78
Calcium, %	1.05	1.05	1.05	1.05	1.05	0.90	0.90	0.90	0.90	0.90
Total phosphorus, %	0.73	0.73	0.73	0.72	0.72	0.68	0.67	0.67	0.67	0.67
Available phosphorus, %	0.50	0.50	0.50	0.50	0.50	0.45	0.45	0.45	0.45	0.45
Lysine, %	1.43	1.43	1.43	1.43	1.43	1.25	1.25	1.25	1.25	1.25
Methionine, %	0.73	0.74	0.74	0.76	0.76	0.65	0.66	0.66	0.67	0.67
Cysteine, %	0.34	0.32	0.32	0.31	0.31	0.31	0.30	0.30	0.29	0.29
Analyzed nutrient value										
DM, %	88.22	88.67	89.11	89.05	89.95	88.71	88.79	89.04	89.20	89.48
CP, % DM	23.54	23.48	23.45	23.52	23.40	21.83	21.77	21.67	21.25	21.24
Crude fat, % DM	6.32	8.89	8.63	11.44	11.23	7.23	10.33	10.73	12.49	12.74
Chemical analyzed value										
			WB					LS		
DM, %			88.9 ± 0.13					89.2 ± 0.15		
CP, % DM			19.0 ± 0.05					19.1 ± 0.07		
Ether extract, % DM			4.4 ± 0.41					3.7 ± 0.13		
ADF, % DM			11.7 ± 0.55					9.3 ± 0.53		
NDF, % DM			38.5 ± 0.77					35.6 ± 0.35		

Abbreviations: ADF, acid detergent fiber; LS, *Laetiporus sulphureus*-fermented wheat bran; NDF, neutral detergent fiber; WB, wheat bran.

¹Supplied per kg of diet: vit. A, 15,000 U; vit. D₃, 3,000 U; vit. E, 30 mg; vit. K₃, 4 mg; riboflavin, 8 mg; pyridoxine, 5 mg; vit. B₁₂, 25 µg; Ca-pantothenate, 19 mg; niacin, 50 mg; folic acid, 1.5 mg; biotin 60 µg.

²Supplied per kg of diet: Co (CoCO₃), 0.255 mg; Cu (CuSO₄·5H₂O), 10.8 mg; Fe (FeSO₄·H₂O), 90 mg; Zn (ZnO), 68.4 mg; Mn (MnSO₄·H₂O), 90 mg; Se (Na₂SeO₃), 0.18 mg.

RNA Isolation and Quantitative Reverse Transcription PCR

The collected liver, jejunum, and ileum were processed to extract RNA. The procedure for RNA isolation and purification was performed by following the manual of the kit purchased from AllBio Science, Inc., Taiwan. RNA concentration was determined spectrophotometrically and diluted to 50 ng/µL. Total RNA concentration and purity, cDNA synthesis, and qPCR analysis (StepOnePlus Real-Time PCR System; Roche Diagnostics Ltd.) were determined according to Lin et al. (2014). Gene-specific primers were designed based on the genes of *Gallus gallus* (Supplementary Table 1). Gene expression data from the same treatment groups were normalized to housekeeping gene b-actin and calculated for the means and SD.

Statistical Analysis

Data were subjected to ANOVA as a completely randomized design using the GLM procedure of the SAS software (version 9.2; Statistical Analysis System).

The mathematic model used was

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

where Y_{ij} = observed response of the birds in each pen; μ = overall mean; T_i = fixed effect of LS or WB supplementation; and ε_{ij} = residual error when a pen was regarded as an experimental unit, $\varepsilon_{ij} \sim N(0, \sigma_e^2)$.

The mean values were compared between each groups using the least square means with the significant level at $P < 0.05$. We further proceeded to develop the regression of the variable of interest based on the level of LS or WB supplementation.

Table 2. Bioactive contents and enzyme activities in aqueous extracts of products solid-state-fermented wheat bran by *Laetiporus sulphureus* (LS).¹

Item	Total phenolics	Crude polysaccharides	Crude triterpenoids	Ergosterol	Laccase	Cellulase	Xylanase
	(mg of GAE ² /g DW ³)	(mg of GCE ⁴ /g DW)	(mg of UAE ⁵ /g DW)	(mg/g DW)	(U/g DW)	(U/g DW)	(U/g DW)
LS	7.8 ± 0.37	13.4 ± 0.68	2.9 ± 0.05	97.3 ± 0.54	504.2 ± 8.07	2.1 ± 0.12	12.7 ± 0.22

¹Values are expressed as the mean ± SD of 4 independent experiment (n = 4).

²GAE = gallic acid equivalent.

³DW = dry weight.

⁴GCE = glucose equivalent.

⁵UAE = ursolic acid equivalent.

RESULTS

Bioactive Contents and Enzyme Activities in Solid-State-Fermented WB by *L. sulphureus*

The major functional compounds and enzyme activities of LS are presented in Table 2. The total phenolic compounds, crude polysaccharides, crude triterpenoids, and ergosterol were 7.8 ± 0.37 mg of GA equivalent/g DW, 13.4 ± 0.68 mg of glucose equivalent/DW, 2.9 ± 0.05 mg of UA equivalent/g DW, and 97.3 ± 0.54 mg/g DW, respectively. Laccase, cellulase, and xylanase activities of LS were 504.2 ± 8.07 U/g DW, 2.1 ± 0.12 U/g DW, and 12.7 ± 0.22 U/g DW, respectively.

Growth Performances

The effects of LS supplementation on growth performances of broilers are displayed in Table 3. The results of the 5% LS and 10% LS group in starter phase (1–21 D) tend to have higher BW, weight gain, and better FCR over other groups; however, there was no significance among the groups ($P > 0.05$). In the finisher phase (22–35 D), the 5% LS group had significantly

higher BW than the control group and 10% WB group ($P < 0.05$). The 5% LS and 10% LS groups tended to have better, but not significant, FCR compared with the other groups ($P > 0.05$). The data of the overall experimental period (1–35 D) implied that 5 and 10% LS supplementation provided significantly better FCR compared with other groups ($P < 0.05$). Regressions were calculated for those variables that responded significantly to varying levels of LS or WB supplementation. Feed consumption is defined as Y_2 (g), then $Y_2 = 1,358-64.165X$ ($P = 0.0619$) under LS supplementation of during 1–21 D (Supplementary Table 2).

Selected Intestinal Microbial Count

The effects of LS supplementation on ileal and cecal lactic acid bacteria and coliform counts in 35-day-old broiler chickens are presented in Table 4. The results showed that ileal coliform counts were significantly lower in the LS supplemented groups (5 and 10%) than in the other groups ($P < 0.05$). The 5 and 10% LS-supplemented groups tended to have higher ileal and cecal lactic acid bacteria counts over all other treatment groups and control group ($P > 0.05$).

Table 3. Effects of *Laetiporus sulphureus*-fermented wheat bran on growth performance of broilers.

Items	Experimental diets					P Value	SEM
	Control	5% WB	10% WB	5% LS	10% LS		
1–21 D							
BW (g/bird) ¹	1,005	1,009	1,007	1,025	1,039	0.291	7.10
Weight gain (g/bird)	958	962	960	986	978	0.690	6.75
Feed consumption (g/bird) ²	1,321	1,346	1,303	1,326	1,294	0.281	7.73
FCR	1.31	1.34	1.34	1.27	1.25	0.058	0.01
22–35 D							
BW (g/bird)	2,394 ^b	2,408 ^{a,b}	2,354 ^b	2,499 ^a	2,452 ^{a,b}	0.030	12.51
Weight gain (g/bird)	1,421	1,463	1,348	1,433	1,414	0.464	13.93
Feed consumption (g/bird)	2,137	2,156	2,091	2,142	2,092	0.806	20.41
FCR	1.51	1.48	1.54	1.47	1.46	0.225	0.01
1–35 D							
Weight gain (g/bird)	2,379	2,425	2,308	2,438	2,405	0.048	12.61
Feed consumption (g/bird)	3,457	3,502	3,393	3,468	3,395	0.547	23.00
FCR	1.45 ^a	1.44 ^a	1.46 ^a	1.41 ^b	1.41 ^b	0.011	0.01

^{a, b}Means within the same rows without the same superscript letter are significantly different ($P < 0.05$).

Abbreviations: FCR, feed conversion rate; LS, *Laetiporus sulphureus*-fermented wheat bran; WB, wheat bran.

¹Results are provided as the means of 80 birds in each control and treatment group (n = 80).

²Results are provided as the means of 4 replicates (20 birds/replicate) in each control and treatment group (n = 4).

Table 4. Effects of LS supplementation on intestinal microflora in 35-day-old broilers.¹

Items	Experimental diets					P Value	SEM
	Control	5% WB	10% WB	5% LS	10% LS		
Coliform	log cfu/g						
Ileum	7.23 ^a	7.11 ^a	7.39 ^a	6.55 ^b	6.94 ^b	0.02	0.09
Cecum	8.98	8.64	8.38	8.34	8.67	0.07	0.14
Lactic acid bacteria	log cfu/g						
Ileum	8.17	8.33	8.37	8.54	8.64	0.18	0.06
Cecum	8.28	8.43	8.49	8.80	8.74	0.39	0.08
L:C							
Ileum	1.17	1.23	1.18	1.31	1.27	0.43	0.02
Cecum	0.91	0.99	0.96	1.04	1.05	0.06	0.01

^{a, b} Means within the same rows without the same superscript letter are significantly different ($P < 0.05$).

Abbreviations: L:C, ratio of lactic acid bacteria to coliform; LS, *Laetiporus sulphureus*-fermented wheat bran; WB, wheat bran.

¹Results are provided as the means of 4 replicates (6 birds/replicate) in each control and treatment group ($n = 6$).

Serum Profiles

The effects of LS supplementation on serum profiles in 35-day-old broilers are shown in Table 5. All selected indices showed no significant differences among groups, in spite of the slight low-density lipoprotein and cholesterol-lowering effects of the WB and LS groups compared with the control group ($P > 0.05$). Regressions were calculated for those variables that responded significantly to varying levels of LS or WB supplementation. If LS or WB supplementation defined as X (g/kg) and triglycerides level is defined as Y_1 , then $Y_1 = 27 + 10.7X$ ($P = 0.0474$) under WB supplementation at 35 D ago (Table 5). White blood cell is defined as Y_3 (cells/ μ L), then $Y_3 = 0.17 + 0.00012X$ ($P = 0.0088$) under LS supplementation at 35 D ago (Supplementary Table 2).

Serum IgA and IgG Concentration

The effects of LS supplementation on serum concentration of IgA and IgG in 35-day-old broilers are illustrated in Figure 1. The 5% and 10% LS supplementation significantly increased the serum IgA concentration compared with the control and WB supplemented groups ($P < 0.05$), whereas no significant differences was observed on serum IgG among the groups ($P > 0.05$).

Secretory IgA Concentrations in the Ileum and Cecum

Effects of LS supplementation on the ileal and cecal sIgA concentration are shown in Figure 2. *L. sulphureus*-fermented WB supplementation significantly improved the

Table 5. Effects of LS supplementation on serum profiles in 35-day-old broilers.¹

Items	Experimental diets					P Value	SEM
	Control	5% WB	10% WB	5% LS	10% LS		
WBC (cells/ μ L)	196,467	196,283	195,725	197,100	193,700	0.263	413.02
RBC (10^6 cells/ μ L)	2.67	2.62	2.59	2.67	2.53	0.635	0.03
Ht (%)	35.24	33.85	33.30	34.50	32.52	0.337	0.39
MCV (fl)	131.64	129.38	128.69	129.89	129.58	0.578	0.58
MCH (pg)	35.12	34.42	34.09	36.05	34.42	0.217	0.25
	mg/dL						
GLU	209.67	202.80	207.83	203.00	206.2	0.959	3.33
BUN	1.80	2.17	2.50	2.00	2.20	0.933	0.22
UA	2.80	2.88	2.33	2.37	2.17	0.689	0.16
TG	34.00	33.00	38.50	37.00	35.80	0.185	0.64
CHOL	145.67	144.67	142.50	139.83	136.80	0.851	2.67
HDL	74.00	75.75	74.33	73.17	73.75	0.983	1.23
LDL	70.17	66.33	59.60	59.83	56.17	0.166	1.89
	U/L						
SGOT	415.33	411.50	411.50	395.25	400.67	0.999	21.30
SGPT	7.00	6.25	6.00	5.80	6.00	0.831	0.29
	g/dL						
MCHC	27.71	26.62	26.49	27.03	26.58	0.251	0.19
TP	3.67	3.35	3.18	3.42	3.70	0.250	0.08
ALB	1.58	1.52	1.48	1.53	1.70	0.530	0.04
GLO	1.85	1.80	1.78	1.80	1.86	0.964	0.04
Hb	9.20	9.00	9.43	9.57	9.30	0.436	0.09

Abbreviations: ALB: albumin; BUN: urea nitrogen; CHOL: cholesterol; GLO: globulin; GLU: glucose; Hb: hemoglobin; HDL: high-density lipoprotein; Ht: hematocrit; LDL: low-density lipoprotein; LS: *Laetiporus sulphureus*-fermented wheat bran; MCH: mean cell hemoglobin; MCHC: mean corpuscular hemoglobin concentration; MCV: mean corpuscular volume; RBC: red blood cell; SGOT: glutamic oxaloacetic transaminase; SGPT: serum glutamic-pyruvate transaminase; TG: triglyceride; TP: total protein; UA: uric acid; WBC: white blood cell.

¹The results are provided as the means of 6 replicates in each control and treatment groups ($n = 6$).

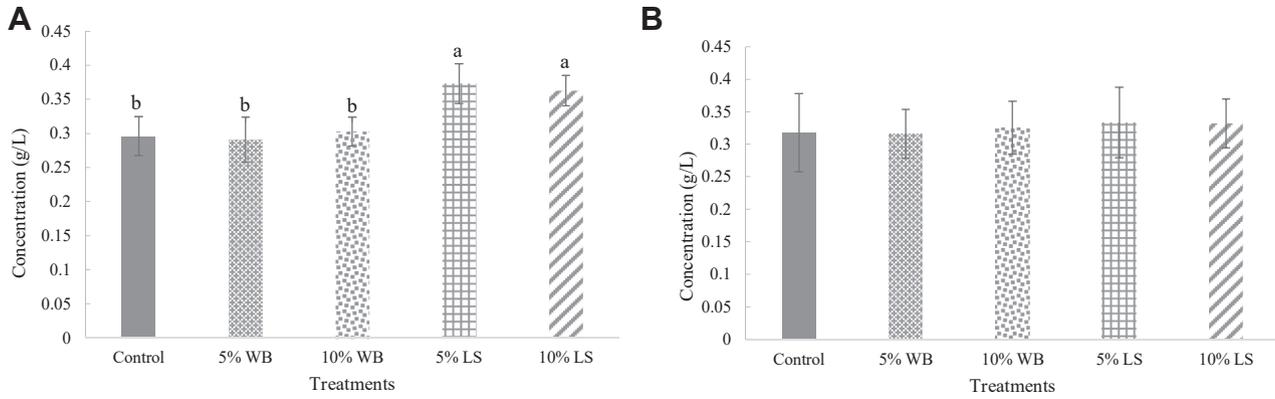


Figure 1. Effects of LS supplementation on serum (A) IgA and (B) IgG concentration in 35-day-old broilers. Values are expressed as the mean \pm SD of 6 samples ($n = 6$). ^{a, b}Means among groups without the same letter are significantly different ($P < 0.05$). Abbreviations: LS, *Laetiporus sulphureus*-fermented wheat bran; WB, wheat bran.

ileal concentration of sIgA over the control group and WB-supplemented groups ($P < 0.05$). The cecal sIgA concentration in the LS-supplemented groups tended to be higher than that in all other groups; however, it was not statistically significant ($P > 0.05$).

Proinflammatory Cytokine Concentrations in Serum

The effects of LS supplementation on serum concentration of proinflammatory cytokines in 35-day-old broilers are demonstrated in Figure 3. The 10% WB supplementation significantly elevated serum TNF- α and IL-6 concentration compared with the control group ($P < 0.05$), whereas both the LS-supplemented groups had lowered levels of these cytokines compared with the WB groups ($P < 0.05$). Serum TNF- α and IL-1 β were significantly decreased in the 5% LS group compared with the control group and WB-supplemented groups ($P < 0.05$).

Selected Immune-Related Gene Expression in the Liver, Jejunum, and Ileum

To understand the molecular mechanism of immunomodulation activities of LS in 35-day-old broilers, selected immune-related mRNA levels were examined in the liver, jejunum, and ileum of the broilers. Figure 4A shows that 10% WB supplementation significantly elevated the expression of all selected genes in the liver ($P < 0.05$), whereas downregulation of proinflammatory genes was observed in the LS supplementation groups compared with the WB groups ($P < 0.05$). The liver IL-1 β mRNA level of 5% LS group was also significantly reduced compared with the control group and WB groups ($P < 0.05$). In the jejunum (Figure 4B), mRNA levels of IL-1 β were increased significantly by the supplementation of 10% WB, and the LS supplementation groups had lower mRNA levels of these genes at the same time compared with the control and WB groups. Moreover, in comparison with the 10% WB group, IL-6 mRNA expression in both the LS groups were

significantly lower ($P < 0.05$), whereas the 5% LS group had lower TNF- α mRNA level ($P < 0.05$). Regarding the ileum (Figure 4C), the WB supplementation groups showed slight elevation on the selected genes ($P > 0.05$). Furthermore, the 5% LS group had significantly lower expressions of all selected genes than the 10% WB group ($P < 0.05$); in particular, IL-6 and IL-1 β were even lower than in both the WB groups ($P < 0.05$). Both the LS groups had slightly lower expression of selected mRNAs than the control group; however, there are no significant effects ($P > 0.05$).

DISCUSSION

L. sulphureus, a fungus species that had long been used for medical purposes, had been identified as having various compounds, including mycophenolic compounds, triterpenoid compounds, and polysaccharides (Hwang et al., 2008; Fan et al., 2014; He et al., 2015), that give it the therapeutic properties. In addition, medical fungus produces ergosterol, known as provitamin D2, which can potentially exert anti-inflammatory effects by inhibiting NF κ B expression (Ma et al., 2003). Therefore, we first identified the crude phenolic compounds, crude triterpenoid compounds, crude polysaccharide compounds, and ergosterol contents to verify the components in LS. Furthermore, our previous study proved that *L. sulphureus* SSF for 12 D by using WB as substrate had the most efficient production of bioactive compounds and had exerted in vitro immunomodulation properties on chicken peripheral blood monocytes (Lin et al., 2019). In this study, the results of the bioactive compounds in LS validated that we could proceed to the stage of the animal trial to test the hypothesis that LS could potentially modulate the immune response in broilers.

Phytochemical and fungal-derived phenolic compounds and polysaccharides were reported to exert growth-promoting effects. Starčević et al. (2015) reported that broiler chickens supplemented with 5 g/kg tannic acid (a kind of phenolic compound) had higher final BW than those of the control group. Phenolic compound

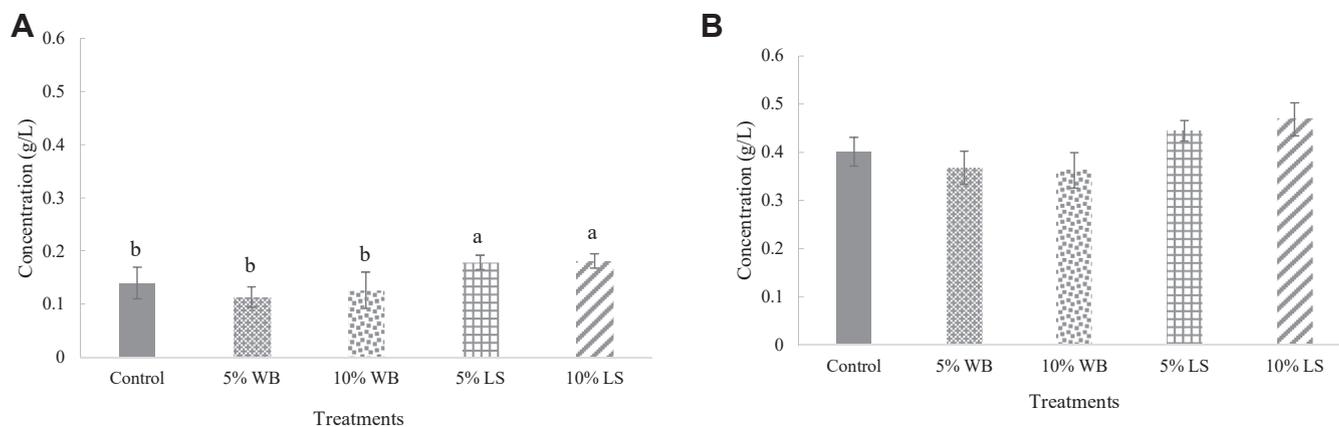


Figure 2. Effects of LS supplementation on (A) ileal and (B) cecal secretory IgA (sIgA) in 35-day-old broilers. Values are expressed as the mean \pm SD of 6 samples ($n = 6$). ^{a, b}Means among groups without the same letter are significantly different ($P < 0.05$). Abbreviations: LS, *Laetiporus sulphureus*-fermented wheat bran; WB, wheat bran.

GA (75–100 mg/kg) was also shown to positively affect the feed conversion efficiency in broiler chicken in the overall experimental period (Samuel et al., 2017). Polysaccharides of *Lentinus edodes*, an edible mushroom, had been reported as a potential growth promoter in broilers (Guo et al., 2004). The growth-promoting effects of phenolic compounds and polysaccharides might be contributed by their antioxidant effect that could dampen the growth-retarding factors, such as lipid peroxidation and oxidative stress (Guo et al., 2004; Samuel et al., 2017). Furthermore, many reports had proved the ability of *L. sulphureus* to secrete lignocellulose-degrading enzymes, such as xylanase, β -1,3-1,4-glucanase and manganese peroxidase (Mtui and Masalu, 2008; Hong et al., 2009). These enzymes produced during the SSF process were able to break down the lignocellulosic cell walls in botanical resources and release the encapsulated starch and proteins, further improving the nutrient using efficiency of broilers as well as the FCR (Lin et al., 2017). According to our results, broiler growth performance, including BW in the finisher phase and FCR in the overall experimental period, was positively influenced by the supplementation of LS, which could be because of the effects of lignocellulose-degrading

enzymes, phenolic compounds, and polysaccharides contained in LS.

Coliform counts in 35-day-old birds supplemented with LS were significantly suppressed compared with other groups. The results could be because of the antimicrobial effects of *L. sulphureus* as stated in past studies. Ethanol extracts of *L. sulphureus* which contained phenolic and flavonoid compounds were found to inhibit *E. coli* ATCC 35218 (Turkoglu et al., 2007); similar pathogen-inhibiting properties were also reported by Petrović et al. (2013) who demonstrated the inhibition activity of *L. sulphureus* methanol extracts of LS against *Staphylococcus aureus*, *Bacillus cereus*, and *Salmonella enteritidis*. From the other angle, the inhibited ileal coliform count might be because of the increment of intestinal sIgA (Figure 2). Secretory IgA is one of many important factors that protect the mucosal surface of the intestine and defend against enteric pathogens and toxins from inflicting the intestinal epithelium. Studies have shown that sIgA exerted an anti-infective function by preventing S-fimbriated *E. coli* strains from adhering to human epithelial cells (Schroten et al., 1998). Furthermore, increased intestinal sIgA secretion was reported to result from the influence of consuming phenolic- and triterpenoid-rich herbs or medicinal fungus. Liu et al.

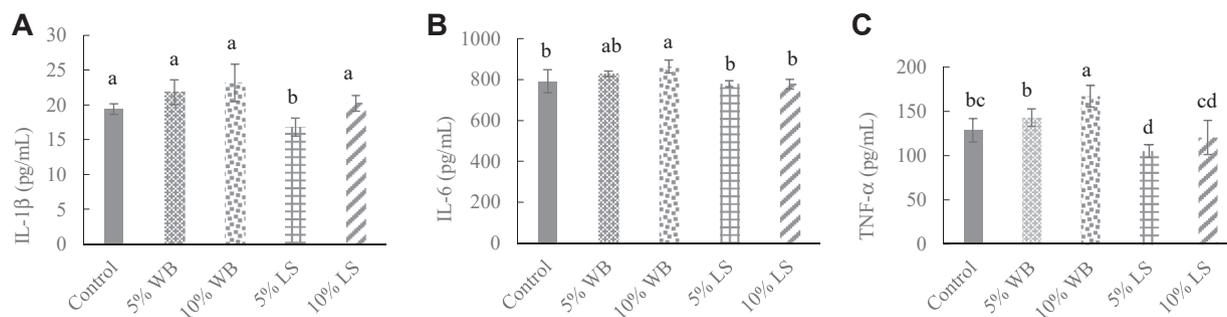


Figure 3. Effects of LS supplementation on serum (A) IL-1 β , (B) IL-6, and (C) TNF- α concentration in 35-day-old broilers. Values are expressed as the mean \pm SD of 6 samples ($n = 6$). ^{a-d}Means among groups without the same letter are significantly different ($P < 0.05$). Abbreviations: LS, *Laetiporus sulphureus*-fermented wheat bran; TNF- α , tumor necrosis factor- α ; WB, wheat bran.

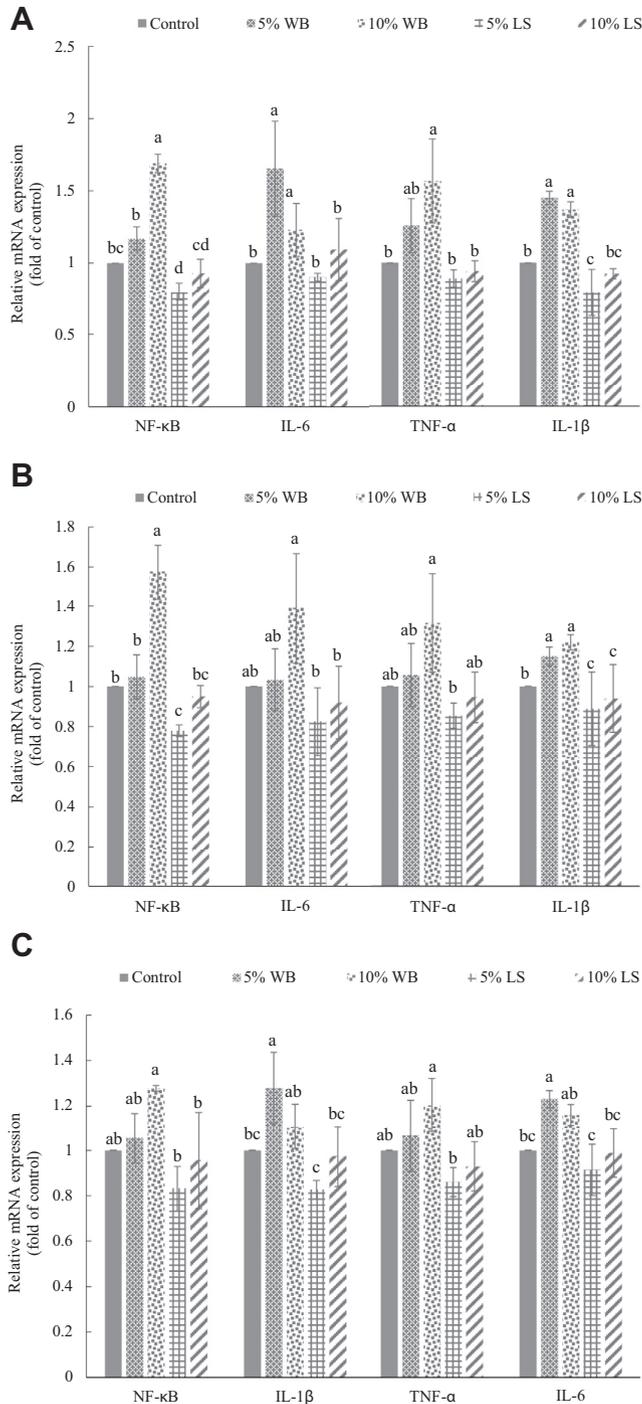


Figure 4. Effects of LS supplementation on mRNA expression levels of the selected immune-related genes in the (A) liver, (B) jejunum, and (C) ileum of 35-day-old broilers. Values are expressed as the mean \pm SD of eight samples ($n = 8$). ^{a-d}Means among groups without the same letter within the same sampling day are significantly different ($P < 0.05$). Abbreviations: LS, *Laetiporus sulphureus*-fermented wheat bran; NF- κ B, nuclear factor kappa-B; TNF- α , tumor necrosis factor- α ; WB, wheat bran.

(2018) showed that Chinese herbal formula Fei-Xi-Tiao-Zhi-Fang significantly upregulated intestinal sIgA concentration, while *Cordyceps militaris* also showed to increase the ileal sIgA level in *E. coli* (ATCC 25922)-infected mice (Zhang et al., 2018). These outcomes shared similar results with our study.

Besides sIgA, serum concentration of IgA in broilers was also elevated by the supplementation of LS. The results are in common with those reported by Liu et al. (2016), which showed that *Ganoderma lucidum* supplementation elevated serum IgA in aflatoxin B1-challenged chickens. To investigate the immunomodulation effects of LS in broiler chickens deeper, proinflammatory cytokines along with mRNA were tested. The results showed that serum TNF- α and IL-6 in the 5% LS group were significantly lower than both the control and WB-supplemented groups, accompanied with lower levels of proinflammatory mRNA expressed in the liver and intestinal tissues. The previously mentioned increment of serum IgA might be one of the contributors to this result because serum IgA was reported to dampen excessive immune response and downregulate the release of TNF- α and IL-6 in human monocytes (Wolf et al., 1994; Monterio, 2014).

The immunomodulation properties of medicinal fungus have been widely reported; they clearly state that these effects originate from their phenolic compounds, triterpenoid compounds, and polysaccharides. *G. lucidum* has been claimed to dampen the progression of inflammatory breast cancer by downregulating the expression of *Nf- κ B* (Martinez-Montemayor et al., 2008; Suarez-Arroyo et al., 2013). Lee et al. (2020b) also reported the effects of *A. cinnamomea*-fermented powder in downregulating the liver expression of genes involving inflammatory pathways such as *NF- κ B*, *iNOS*, *TNF- α* , *IL-1 β* , *NLRP3*, and *COX2* in broiler chickens receiving high-NSP diets. Regarding *L. sulphureus*, most of the studies focused on its in vitro anti-inflammation effects. *L. sulphureus*-fermented WB-derived triterpene eburicoic acid has been described by Wang et al. (2017) as being able to inhibit LPS-induced activation of *NF- κ B* pathways and downregulate the inflammatory response in RAW 264.7 cells. Similarly, Saba et al. (2015) reported that acetyl eburicoic acid from LS can reduce the proinflammatory cytokines secreted by LPS-induced RAW 264.7 cells. Our previous study on LS ethanol extracts containing phenolic compounds, triterpenoid compounds, and polysaccharides also mentioned that it was able to attenuate LPS-induced IL-1 β and *iNOS* secretion by suppressing *NF- κ B* expression (Lin et al., 2019). In this study, we focused on the in vivo immunomodulatory effects of LS by evaluating the proinflammatory cytokine and mRNA in the broilers.

Interestingly, proinflammatory genes and cytokines were triggered by WB supplementation, although the serum profiles indicate no adverse health effects (Table 5). As previously mentioned, the WB is mainly composed of NSP, which may induce gut inflammation in broiler chickens (Chen et al., 2015) and associate with *NF- κ B* activation (Vlantis et al., 2016). Applying SSF on WB enabled *L. sulphureus* to degrade the NSP and increase the nutrient value of WB, while secreting secondary metabolites such as polysaccharides and phenolic compounds to serve as functional compounds and exert immunomodulatory

effects. Ruiz et al. (2007) proved that polyphenolic flavonoid compound quercetin is able to inhibit TNF-induced NF- κ B transcription factor recruitment in murine epithelial cells. Polysaccharides such as *Astragalus mongholicus* polysaccharides had also been claimed to inhibit LPS-induced production of TNF- α and IL-8 mRNA in IEC-6 cells by potentially suppressing NF- κ B (Yuan and Sun, 2008). The results of these studies were similar to our data which showed that chickens supplemented with 5% LS had lower levels of jejunum IL-1 β and NF- κ B mRNA than those in the control group and WB groups ($P < 0.05$). However, as per our results, compared with the 5% LS group, the 10% LS group had less favorable effects on the results of growth performance, proinflammatory cytokines, and mRNA expressions, which might be the relatively high NSP content received by the birds in the 10% LS group.

In conclusion, LS supplementation can improve growth performance and exert immunomodulatory functions to dampen potential environment-induced inflammation, while 5% LS supplementation had a more ideal outcome. The aforementioned results indicate the potential of LS to be used as functional feedstuff in poultry immunomodulation.

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SUPPLEMENTARY DATA

Supplementary data associated with this article can be found in the online version at <http://doi.org/10.1016/j.psj.2020.04.011>.

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