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Blood and Intestine Profile of Broilers Fed *Averrhoa bilimbi* Fruit, Wheat Bran, and Yeast Blends

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ABSTRACT

A. bilimbi fruit filtrate, wheat bran, and _S. cerevisiae_ contain bioactive components favorable to broiler health. The use of these compounds in combination was expected to exert synergistic effects on broilers. The study investigated the effect of a combination of _A. bilimbi_ fruit filtrate, wheat bran, and _S. cerevisiae_ on haematological indices and intestinal selected bacteria and morphology of broilers. A total of 280 broiler chicks were randomly divided into 4 groups with 7 replications, including CONT (chicks offered diet without additive), TBLEND1, BLEND2, and BLEND3 (chicks offered diet with 0.25%, 0.5%, and 1% of the additive combination of _A. bilimbi_ fruit filtrate, wheat bran, and _S. cerevisiae_]), respectively. For data collection, the chicks were blood sampled at day 21 and 35, and slaughtered at day 35. The data were statistically treated with analysis of variance according to a completely randomized design. On day 21, the erythrocytes and haemoglobin levels were lower in BLEND2 and BLEND3 than those in CONT and BLEND1 ($p<0.05$). The leukocytes and lymphocytes values were lower in BLEND2 and BLEND3 than those in CONT ($p<0.05$). On day 35, erythrocytes were lower ($p<0.05$) in BLEND3 than that in CONT and BLEND1. The increased additive levels linearly decreased ($p<0.05$) erythrocytes, haemoglobin, and haematocrits values. At day 21, total triglyceride was lower ($p<0.05$) in BLEND3 than that in BLEND1 and BLEND2. The LDL level was lower ($p<0.05$) in BLEND3, whereas the HDL level was higher ($p<0.05$) in CONT than that in other groups. Creatinine was higher ($p<0.05$) in BLEND3 than in other groups. The ileal lactose negative _Enterobacteriaceae_ counts were lower in BLEND1, BLEND2, and BLEND3 than in CONT ($p<0.05$). The duodenal villi height to crypt depth ratio (VH/CD ratio) was higher ($p<0.05$) in BLEND1 than that in CONT and BLEND2. In the ileum, the VH/CD ratio linearly increased ($p<0.05$) with the elevated additive levels. In conclusion, the combination of _A. bilimbi_ fruit filtrate, wheat bran, and _S. cerevisiae_ was beneficial in reducing intestinal pathogen load and improving intestinal morphology of broilers.

Keywords: broiler chicken; feed additive; intestinal health; physiological conditions

INTRODUCTION

The broiler industry is a sector that plays an important role in meeting the needs of animal protein while also helping to boost the economy. However, the sustainability of the broiler industry, especially in Indonesia, is constrained by the prohibition on the use of in-feed antibiotics (antibiotic growth promoters/AGP), where the continuous provision of AGP can cause pathogenic bacteria to become resistant. Furthermore, AGP leaves an antibiotic residue on chicken meat (Abudabos *et al*., 2017) that may harm human health. Indeed, the prohibition of using AGP can implicate negative effects on broiler production and health (Sugiharto, 2016; Sugiharto & Ranjitkar, 2019). Various attempts have been conducted to find alternatives to AGP (Cimrin *et al*., 2020; Tufarelli *et al*., 2021). In this regard, herbal plants are being used as feed additives as part of the initiative to replace AGP for broiler chickens. While some studies on herbal plants have a positive effect, some have not observed the desired positive effect. However, this subject has been among the issues that need to be studied.

Averrhoa bilimbi is an herbal plant containing various compounds that may act as an antimicrobial, immunomodulator, antioxidant, and acidifier. Aside from that, it is widely available all year round and has a low economic value (Sugiharto, 2020). Although it is a plant rich in nutrient content, it appears as a neglected fruit crop. *A. bilimbi* fruit, besides being rich in vitamin C, has a high antioxidant effect and contains low calories. The previous report has shown that organic acids in *A. bilimbi* fruit, such as citric acid, improved broiler chickens' digestive tract (Pratama *et al*., 2021). Mareta *et al*. (2020) and Sugiharto *et al*. (2021) reported that fresh *A. bilimbi* fruit also contains lactic acid bacteria (LAB; 5.30 to 6.69 log cfu/mL), which are a good source of probiotics for broiler chickens. Due to its high LAB content, *A. bilimbi* L. fruit filtrate can also be used to ferment turmeric and black pepper powder in the study of Sugiharto *et al*. (2021).

Wheat bran is a cereal by-product that contains prebiotics and may have antioxidant properties (Gunenc *et al*., 2017). Wheat bran is a feed raw material used in both ruminant and poultry feeding. Wheat bran meets the daily digestible cellulose needs of ruminant animals. On the other hand, it appears as a feed that increases the nutritional value of feeds in all animals. Wheat bran fibers like arabinoxylan-oligosaccharides (AXOS) may act as prebiotics and thus have the ability to stimulate the growth and activity of probiotic microorganisms (Muller *et al*., 2020). In broilers, the administration of wheat bran in diets increased the LAB population in intestines compared to that in control (Feng *et al*., 2020**;** Lin & Lee, 2020). Concerning antioxidant properties, wheat bran contains a substantial amount of phenolic acids serving as antioxidants (Zhou *et al*., 2004). Besides its benefits, wheat bran contains high phytate (Salami et al., 2018), considered anti-nutrient in broilers. In most situations, the content of phytate in plant-based materials can be lowered by LAB fermentation (Sugiharto & Ranjitkar, 2019).

Saccharomyces cerevisiae has been widely applied as a probiotic for broiler chickens. A previous study has reported that *S. cerevisiae* improved digestion and absorption, as well as the immune status of broiler chickens (Elghandour *et al*., 2019). In a recent study on this subject, growth performance, immune status, and serum biochemical properties of live yeast (*S. cerevisiae*) in broilers were investigated (He *et al*., 2021). Researchers have stated that live yeast can be used as an alternative to antibiotics in broilers. Referring to Sugiharto *et al*. (2021), the *A. bilimbi* fruit filtrate was used to ferment wheat bran in the present study, considering its high LAB content (Mareta *et al*., 2020). Such fermentation was actually expected to lower the phytate level of wheat bran, while the presence of AXOS in wheat bran may support the growth of LAB originating from *A. bilimbi* fruit. With the A. bilimbi fruit filtrate-fermented wheat bran, *S. cerevisiae* was then combined with corroborating the probiotic benefits of broiler chickens. Overall, the synergistic and complementary effects of organic acids, LAB, and antioxidants originating from *A. bilimbi* fruit filtrate, prebiotics from wheat bran, and probiotic properties of *S. cerevisiae* were expected to favour the physiological and health of broilers. To the best of our knowledge, this is the first study investigating the combined use of *A. bilimbi* fruit filtrate, wheat bran, and *S. cerevisiae* on physiological conditions and intestinal health of broilers.

The study aimed to investigate the effect of a combination of *A. bilimbi* fruit filtrate, wheat bran, and *S. cerevisiae* on haematological indices and intestinal selected bacteria and morphology of broiler chickens. We hypothesized that a combination of *A. bilimbi* fruit filtrate, wheat bran, and *S. cerevisiae* would improve broiler chickens' physiological responses and intestinal health and functions.

MATERIALS AND METHODS

Preparation of Feed Additive

The ripe *A. bilimbi* fruit was harvested from surrounding the campus of Universitas Diponegoro, Tembalang, Semarang Regency. The fruit was thoroughly rinsed under the running water before being blended. An electric blender was used (with a medium speed) to crush the fruits. To obtain the *A. bilimbi* fruit filtrate, the juice was filtered through a cheesecloth. The pH values of *A. bilimbi* fruit filtrate ranged from 1.93 to 2.03.

Wheat bran was mixed with distilled water (3:1, g:mL) and stirred until homogeneous. The mixture was autoclaved at 121 °C for 15 minutes and then allowed to cool (Utama *et al*., 2019). The autoclaved wheat bran was subsequently added with the *A. bilimbi* fruit filtrate (1:4, g:mL) and incubated anaerobically using an anaerobic jar at 38 °C for 2 days. The fermented wheat bran was sun-dried, ground, and then added with *S. cerevisiae* concentrate (containing 9.82×10^{11} cfu/g; Angle Yeast Co. Ltd - Hubei, China) (2:1, g:g). The product was stored at room temperature until use. According to the viable count method with de Man Rogosa and Sharpe agar (incubated at 38 °C for 48 hours), the produced feed additive contained LAB of 5.47×10^{11} cfu/g, and the pH value was 4.65 (measured using portable pH meter, OHAUS ST300).

In Vivo Study

The *in vivo* experiment was approved by the Animal Ethics Committee of the Faculty of Animal, and Agricultural Sciences, Universitas Diponegoro (No. 57-02/A3/KEP/FPP) and was carried out under the basic animal husbandry and health guidelines mentioned in Legislation of the Republic of Indonesia No. 18, 2009.

Two hundred and eighty unsex broiler chicks (BW 48.16 ± 0.26 g; mean ± SD) (Lohmann) were randomly divided into 4 treatments with 7 replications. Each experimental unit contained 10 chicks. The treatments were CONT (chicks offered control diet, with no additive), BLEND1 (chicks offered diet administrated with 0.25% additive [a combination of *A. bilimbi* fruit filtrate, wheat bran, and *S. cerevisiae*]), BLEND2 (diet administrated with 0.50% additive), and BLEND3 (diet administrated with 1% additive). The additive was added and mixed at the end of the mixing process.

The birds were raised in an open-broiler house with a 1 m ×1 m pen for 10 birds for 35 days. A continuous light application was used for the duration of the experiment. The broiler house's temperature and relative humidity were regulated with light bulbs and plastic curtains. Using a manual feeder in each pen, the chicks were fed starter (days 1-21) and finisher (days 22-35) feeds, which were formulated according to Indonesian National Standards for Broiler Feed (SNI, 2015; Table 1). Throughout the experiment, the feed and drinking water (using a manual drinker) were given *ad libitum*. On days 4 and 18, the chicks were given Newcastle disease virus (NDV) vaccine *via* eye drops and drinking water, respectively. The Gumboro (infectious bursal disease)

Table 1. Ingredients and nutritional compositions of feeds provided to broilers treated with blends of *Averrhoa bilimbi* fruit filtrate, wheat bran, and *Saccharomyces cerevisiae*

Items (%, unless otherwise noticed)	Starter	Finisher
Yellow maize	53.50	61.00
Palm oil	2.32	2.95
Soybean meal (crude protein of 44.15%)	40.13	32.00
DL-methionine, 990 g	0.19	0.19
Bentonite	0.75	0.75
Limestone	1.00	1.00
Monocalcium phosphate	1.30	1.30
Premix[1]	0.34	0.34
Chlorine chloride	0.07	0.07
Salt	0.40	0.40
Calculated chemical ingredients		
ME, (kcal/kg)[2]	2,900	3,023
Crude protein	22.00	19.00
Crude fiber	5.47	5.53
Ca	1.14	1.11
P (available P)	0.57	0.58
Analyzed chemical ingredients		
Dry matter	90.00	89.40
Crude protein	19.00	18.75
Crude fat	3.17	5.270
Crude fiber	5.92	6.800
Ash	10.40	9.080

Note: [1]Provided per kg of feed: 1,100 mg Zn, 1,000 mg Mn, 75 mg Cu, 850 mg Fe, 4 mg Se, 19 mg I, 6 mg Co, 1,225 mg K, 1,225 mg Mg, 1,250,000 IU vit A, 250,000 IU vit D3, 1,350 g pantothenic acid, 1,875 g vit E, 250 g vit K3, 250 g vit B1, 750 g vit B2, 500 g vit B6, 2,500 mg vit B12, 5,000 g niacin, 125 g folic acid and 2,500 mg biotin. [2]ME (metabolizable energy) was predicted based on formula (Bolton, 1967): 40.81 {0.87 [crude protein + 2.25 crude fat + nitrogen-free extract] + 2.5}.

vaccine was also provided to the chicks by drinking water on day 12.

Sample Collection and Analysis

To avoid bias owing to gender differences, blood was taken from one of the male chicks reflecting the average body weight in each experimental unit via the brachial veins of the wings on days 21 and 35. The blood was separated into two tubes, one with ethylenediaminetetraacetic acid (EDTA) to measure the full blood profile, and the other with non-EDTA to make blood serum. The Prima Fully-Auto Hematology Analyzer (PT. Prima Alkesindo Nusantara, Jakarta, Indonesia) was used to assess the complete blood profile according to the manufacturer's protocols. The blood was allowed to sit at room temperature for 2 hours before being centrifuged at 5,000 rpm for 10 minutes to make the serum. The blood serum was stored in the freezer (at -10 °C) until the analysis.

One chick from each replicate was slaughtered, and their feathers were plucked on the 35th day. The visceral organs were separated after the abdomen was ripped open. The digesta samples were taken from the ileum and cecum and placed in a sterile tube for bacterial counts in the intestine to be determined. In addition, for measuring pH values (using Portable pH Meter OHAUS ST300), the digesta was also collected from the duodenum, jejunum, ileum, and cecum of broilers. For the intestinal histological examination, the duodenum, jejunum, and ileum segments were taken (about 2 cm). After that, these intestinal segments were immersed in a 10% neutral formalin buffer before being analyzed.

The hemagglutination inhibition (HI) procedure, developed by Villegas (1987), was used to measure the antibodies against NDV. In brief, the NDV antigen was incorporated with two-fold successive dilutions of the test samples. The dilutions were evaluated for full hemagglutination inhibition after adding chicken red blood cells. The geometric mean (Log_2) was used to quantify the antibody titers.

The total serum triglyceride was determined using glycerol-3-phosphate oxidase (GPO) and an enzymatic colorimetric method. Cholesterol oxidase/p-amino phenazone (CHOD-PAP) enzymatic colorimetric instrument was used to measure total cholesterol, high-density lipoprotein (HDL), and low-density lipoprotein (LDL) cholesterol. Total cholesterol was measured after enzymatic hydrolysis and oxidation, while triglycerides were measured after enzymatic separation with lipoprotein lipase. Both triglycerides and cholesterol employed a quinoneimine indicator made from 4-aminoantipyrine and 4-chlorophenol, which was created using hydrogen peroxide and peroxidase as a catalyst (DiaSys Diagnostic System GmbH, Holzheim, Germany). To precipitate the LDL, heparin was employed. The HDL remained in the supernatant after centrifugation was processed enzymatically using the CHOD-PAP method. The LDL concentration was determined based on the difference between total cholesterol and cholesterol in the supernatant.

The enzymes alanine aminotransferase (ALT) and aspartate aminotransferase (AST) was spectrophotometrically measured using the Reflotron system (Roche Diagnostics Corporation, Indianapolis, IN, USA). At room temperature, serum and ALT or AST reagents were combined. After 60 seconds of incubation at 37°C, the absorbance was measured at 340 nm. Total serum protein was determined using a photometric examination based on a biuret kit technique (total serum protein kit, DiaSys Diagnostic System GmbH, Holzheim, Germany), as directed by the manufacturer. Copper ions formed blue-violet complexes with proteins in an alkaline solution. The protein concentration was directly proportional to the color absorbance (measured at 545 nm). A photometric test was used to measure serum albumin using bromocresol green (DiaSys Diagnostic Device GmbH, Holzheim, Germany). In the presence of bromocresol green, serum albumin changed color from yellow-green to green-blue at a slightly acidic pH. Total protein minus albumin equals globulin concentration.

Uric acid levels were determined using an enzymatic colorimetric method that involved combining 4-aminoantipyrine with 2-hydroxy-2,4,6-tribromobenzoic acid (TBHBA; DiaSys Diagnostic Device GmbH, Holzheim, Germany) and hydrogen peroxide in the presence of peroxide to produce a chromogen that

measured at 520 nm. The absorption of the resulting red dye at 545 nm was proportionate to the serum creatinine level, as determined by the enzymatic colorimetric assay (DiaSys Diagnostic Device GmbH, Holzheim, Germany).

The measurement of the bacterial population in the gut was done according to Sugiharto *et al*. (2018). After aerobic incubation at 38 °C for 24 hours, the number of coliform and lactose negative *Enterobacteriaceae* was determined as a red and colorless colony on MacConkey agar (Merck KGaA, Darmstadt, Germany). *Enterobacteriaceae* were the sum of coliform and lactose negative *Enterobacteriaceae*. These bacteria are generally considered pathogens in broiler chickens (Shakouri *et al*., 2009; Morales-López *et al*., 2019). After anaerobic incubation at 38°C for 48 hours, the number of lactic acid bacteria on De Man, Rogosa, and Sharpe (MRS) agar (Merck KGaA) was estimated. According to Tunç *et al*. (2019), the histological measurement of the small intestinal segment of broilers was conducted. Hematoxylin and eosin were used to stain 5 μm intestinal slices. The villi heights and the depth of the crypt were measured using an optical microscope attached to a digital camera. Five measurements were taken for each bird to determine the mean villi height and crypt depth.

Based on a completely randomized design (CRD), analysis of variance (ANOVA, SPSS 16.0 version) was employed to examine the data of the experiment statistically. Duncan's multi-range test was used after the treatment's notable impact ($p<0.05$) was discovered. The effect of increasing concentrations of additives in feeds on the observed parameters was also investigated using linear regression.

RESULTS

Complete blood counts of broilers

Data on complete blood counts of broilers are presented in Table 2. Looking at the blood parameters on the 21st day, it was seen that feed additive affects most of the parameters statistically. However, the application did not affect haematocrits, MCV, RDW-SD, RDW-CV, MPV, heterophils, and thrombocytes values. On day 21, the numbers of erythrocytes and concentration of haemoglobin were lower in BLEND2 and BLEND3 than those in CONT and BLEND1 chicks ($p<0.05$). Regression analysis further showed that the increase in the levels of dietary additives linearly decreased the levels of erythrocytes and haemoglobin in the blood ($p<0.05$).

The values of MCH and MCHC were lower and higher, respectively, in BLEND3 than those in other treatment groups. The increase in dietary additive levels linearly decreased ($p<0.05$) and increased ($p<0.05$) the MCH and MCHC values, respectively. Leukocytes and lymphocytes values were lower in BLEND2 and BLEND3 than in CONT ($p<0.05$), but were not different from the BLEND1 group. Further, increasing the dietary additive levels was accompanied by the decreased ($p<0.05$) leukocytes and lymphocytes values at day 21. At the measurement of day 35, the numbers of erythrocytes were lower ($p<0.05$) in BLEND3 than that in CONT and BLEND1, but did not vary from that in BLEND2 chicks. Regression analysis showed that the increased levels of dietary additive were associated with the linear decrease ($p<0.05$) in erythrocytes, haemoglobin, and haematocrits values.

Antibody Titers Against NDV and Serum Biochemical Variables of Broilers

Antibody titers toward NDV vaccine in broiler chickens are presented in Table 3. There was no substantial effect of dietary treatments on the antibody titers against NDV vaccine determined both at day 21 and day 35.

Table 4 shows the serum biochemical variables of broiler chickens. When looking at serum biochemical parameters in Table 4 at day 21, it was seen that the effect of new additives on total triglyceride, LDL, HDL, and creatinine values were statistically significant. On day 21, the total triglyceride level was lower ($p<0.05$) in BLEND3 than in BLEND1 and BLEND2, but did not differ from CONT. The LDL level was lower ($p<0.05$) in BLEND3, whereas HDL level was higher ($p<0.05$) in CONT as compared to that in other groups. The LDL and HDL levels linearly decreased ($p<0.05$) with the increased levels of dietary additive content. Creatinine was higher ($p<0.05$) in BLEND3 than in other treatment groups. Its levels in serum also linearly increased ($p<0.05$) with the increased additive levels in feeds. On day 35, there was no influence of the dietary additive on the measured serum biochemical indices.

pH Values of Intestinal Segments of Broilers

pH values of the intestine of the broiler are provided in Table 5. Table 5 showed that the effects of new additives on ileum are statistically significant. The ileum of BLEND1 birds had higher pH values than that in the ileum of other treatment groups ($p<0.05$). The pH values of duodenum, jejunum, and cecum were not different among the groups of broiler chickens.

Bacterial Populations in Intestine of Broilers

The data on selected bacterial populations in the intestine of broilers are presented in Table 6. The numbers of negative lactose *Enterobacteriaceae* were lower in the ileum of BLEND1, BLEND2, and BLEND3 than that in CONT group ($p<0.05$). In the cecum, LAB numbers were lower in BLEND1 than in other treatment groups ($p<0.05$).

Intestinal Morphology of Broilers

The data on villi height (VH), crypt depth (CD) as well as villi height to crypt depth ratio (VH/CD ratio) were provided in Table 7. The VH/CD ratio was higher ($p<0.05$) in the duodenum of BLEND1 than in CONT and BLEND2, but did not differ from BLEND3 chicks. In the ileum, the VH/CD ratio linearly increased ($p<0.05$) with the elevated levels of dietary additives. Other parameters of intestinal morphology were not different across the treatment groups of broilers.

Table 2. Complete blood counts of broilers treated with blends of *Averrhoa bilimbi* fruit filtrate, wheat bran, and *Saccharomyces cerevisiae*

Items	Treatments				SEM	p value	
	CONT	BLEND1	BLEND2	BLEND3		A	L
Day 21							
Erythrocytes (10^{12}/L)	2.94[a]	2.72[a]	2.32[b]	2.28[b]	0.08	<0.01	<0.01
Haemoglobin (g/dL)	10.6[a]	9.93[ab]	8.86[bc]	7.93[c]	0.30	<0.01	<0.01
Haematocrits (%)	33.2	34.8	39.9	29	1.23	0.31	0.11
MCV (fl)	126	129	130	129	0.66	0.08	0.08
MCH (pg)	35.9[a]	36.5[a]	36.8[a]	27.3[b]	0.86	<0.01	<0.01
MCHC (g/dL)	28.8[b]	28.5[b]	30.8[b]	34.7[a]	0.62	<0.01	<0.01
RDW-SD (10^{-15} L)	60.6	61.9	63.5	59.2	1.14	0.62	0.82
RDW-CV (%)	12.7	12.7	12.9	12.2	0.21	0.71	0.49
MPV (10^{-15} L)	7.8	9.06	8.60	8.47	0.20	0.17	0.4
Leukocytes (10^9/L)	81.4[a]	70.1[ab]	62.1[b]	60.1[b]	2.81	0.02	<0.01
Heterophils (10^9/L)	4.78	5.07	3.64	3.57	0.36	0.53	0.12
Lymphocytes (10^9/L)	76.6[a]	65.0[ab]	58.6[b]	56.0[b]	2.6	0.02	<0.01
Thrombocytes (10^9/L)	33.7	10.7	11.0	14.1	4.91	0.3	0.19
Day 35							
Erythrocytes (10^{12}/L)	3.39[a]	3.34[a]	3.11[ab]	2.67[b]	0.10	0.04	<0.01
Haemoglobin (g/dL)	12.1	11.7	11.3	9.64	0.37	0.10	0.02
Haematocrits (%)	41.1	39.9	38.6	33.1	1.23	0.09	0.02
MCV (fl)	122	124	125	107	0.66	0.47	0.28
MCH (pg)	35.5	36.1	36.3	35.7	0.29	0.81	0.76
MCHC (g/dL)	29.3	29.3	29.3	29.9	0.24	0.93	0.59
RDW-SD (10^{-15} L)	49.4	48.6	44.2	50.2	1.79	0.66	0.91
RDW-CV (%)	10.7	10.4	10.9	10.6	0.20	0.84	0.89
MPV (10^{-15} L)	9.14	9.07	9.27	9.03	0.11	0.88	0.89
Leukocytes (10^9/L)	102	88.6	80.0	95.6	4.69	0.39	0.51
Heterophils (10^9/L)	5.14	7.07	5.64	4.07	0.52	0.23	0.33
Lymphocytes (10^9/L)	96.9	81.6	74.4	91.3	4.54	0.31	0.56
Thrombocytes (10^9/L)	14.3	13.4	15.7	13.6	0.74	0.70	0.99

Note: [a,b,c]Means in the same row with different superscripts differ significantly ($p<0.05$). CONT= chicks offered control diet, with no additive; BLEND1= chicks offered diet administrated with 0.25% additive; BLEND2= diet administrated with 0.50% additive; BLEND3= diet administrated with 1% additive; MCV= mean corpuscular volume; MCH= mean corpuscular haemoglobin; MCHC= mean corpuscular haemoglobin concentration; RDW-SD= red blood cell distribution width-standard deviation; RDW-CV= red blood cell distribution width-coefficient variation; A= analysis of variance; L= linear regression; SEM= standard error of the means.

Table 3. Antibody titers toward Newcastle disease virus of broilers treated with blends of *Averrhoa bilimbi* fruit filtrate, wheat bran and *Saccharomyces cerevisiae*

Items (Log_2 GMT)	Treatments				SEM	p value	
	CONT	BLEND1	BLEND2	BLEND3		A	L
Day 21	2.28	1.57	1.85	1.85	0.19	0.63	0.56
Day 35	3.57	3.29	4.71	2.14	0.35	0.07	0.38

Note: CONT= chicks offered control diet, with no additive; BLEND1= chicks offered diet administrated with 0.25% additive; BLEND2= diet administrated with 0.50% additive; BLEND3= diet administrated with 1% additive; GMT= geometric mean titer; A= analysis of variance; L= linear regression, SEM= standard error of the means.

DISCUSSION

Performance of Broilers

The combined use of *A. bilimbi* fruit filtrate, wheat bran, and *S. cerevisiae* linearly increased the final body weight of broilers in this current study. Indeed, the effect of such an additive was more pronounced when it was given at a concentration of 1% of the diet. At day 35, broilers in the CONT group weighed 1689.48 ± 56.38 g (mean ± SD), BLEND1 1709.86 ± 94.41 g, BLEND2 1673.40 ± 112.16 g, and BLEND3 1825.46 ± 84.88 g. Concerning feed intake, the CONT chicks consumed 2534.20 ± 129.33 g, BLEND1 2464.73 ± 99.35 g, BLEND2 2361.11 ± 154.79, and BLEND3 2550.02 ± 111.57 g during the experiment. In this case, the BLEND2 group consumed less feed than the CONT and BLEND3 groups. Interestingly, the feed conversion ratio (FCR) of broilers was found, at which broilers were administrated with a combination of *A. bilimbi* fruit filtrate, wheat bran, and *S. cerevisiae* had better FCR than the control broilers. The FCR values of CONT was 1.54 ± 0.05, BLEND1 1.49 ±

Table 4. Serum biochemical variables of broilers treated with blends of *Averrhoa bilimbi* fruit filtrate, wheat bran, and *Saccharomyces cerevisiae*

Items	Treatments				SEM	p value	
	CONT	BLEND1	BLEND2	BLEND3		A	L
Day 21							
Total cholesterol (g/dL)	138	145	141	122	4.02	0.20	0.16
Total triglyceride (g/dL)	83.4^{bc}	104^{ab}	110^{a}	80.6^{c}	4.19	0.02	0.97
LDL (g/dL)	25.4^{a}	41.4^{a}	35.9^{a}	11.25^{b}	4.09	0.01	0.04
HDL (g/dL)	125^{a}	96.4^{b}	95.4^{b}	98.9^{b}	4.32	0.04	0.04
Total protein (g/dL)	2.74	3.14	3.28	2.79	0.1	0.15	0.75
Albumin (g/dL)	1.24	1.36	1.39	1.27	0.03	0.17	0.63
Globulin (g/dL)	1.50	1.77	1.89	1.52	0.08	0.19	0.82
A/G ratio	0.83	0.77	0.78	0.84	0.02	0.48	0.77
Uric acid (mg/dL)	7.54	9.63	8.13	9.4	0.52	0.44	0.39
Creatinine (mg/dL)	0.06^{b}	0.07^{b}	0.08^{b}	0.13^{a}	0.01	<0.01	<0.01
AST (U/L)	258	250	259	225	7.6	0.38	0.20
ALT (U/L)	2.15	3.4	2.98	2	0.25	0.14	0.70
Day 35							
Total cholesterol (g/dL)	113	119	119	114	1.64	0.43	0.70
Total triglyceride (g/dL)	60.5	71.5	67.6	68.6	3.03	0.64	0.46
LDL (g/dL)	14.5	19.7	19.3	13.3	3.43	0.72	0.76
HDL (g/dL)	96.1	93.0	86.1	106	4.78	0.53	0.58
Total protein (g/dL)	3.16	3.10	2.94	3.02	0.07	0.73	0.37
Albumin (g/dL)	1.36	1.37	1.28	1.34	0.03	0.78	0.62
Globulin (g/dL)	1.81	1.73	1.68	1.66	0.05	0.73	0.31
A/G ratio	0.76	0.79	0.77	0.81	0.02	0.73	0.37
Uric acid (mg/dL)	4.70	5.34	5.39	5.30	0.17	0.48	0.24
Creatinine (mg/dL)	0.05	0.05	0.05	0.06	<0.01	0.52	0.17
AST (U/L)	301	286	299	291	9.84	0.93	0.85
ALT (U/L)	4.13	4.06	3.47	4.56	0.29	0.63	0.79

Note: ^{a,b,c}Means in the same row with different superscripts differ significantly (p<0.05). CONT= chicks offered control diet, with no additive; BLEND1= chicks offered diet administrated with 0.25% additive; BLEND2= diet administrated with 0.50% additive; BLEND3= diet administrated with 1% additive; LDL= low-density lipoprotein; HDL= high-density lipoprotein; A/G ratio= albumin to globulin ratio; AST= aspartate aminotransferase; ALT= alanine aminotransferase; A= analysis of variance; L= linear regression; SEM= standard error of the means.

Table 5. pH values of the intestinal segments of broilers treated with blends of *Averrhoa bilimbi* fruit filtrate, wheat bran, and *Saccharomyces cerevisiae*

Items	Treatments				SEM	p value	
	CONT	BLEND1	BLEND2	BLEND3		A	L
Duodenum	6.63	6.79	6.93	6.71	0.06	0.4	0.48
Jejunum	6.16	6.52	6.15	5.98	0.12	0.43	0.39
Ileum	5.48^{b}	6.80^{a}	5.98^{b}	5.45^{b}	0.16	<0.01	0.49
Cecum	7.14	7.05	7.35	7.3	0.09	0.65	0.36

Note: ^{a,b}Means in the same row with different superscripts differ significantly (p<0.05). CONT= chicks offered control diet, with no additive; BLEND1= chicks offered diet administrated with 0.25% additive; BLEND2= diet administrated with 0.50% additive; BLEND3= diet administrated with 1% additive; A= analysis of variance; L= linear regression, SEM= standard error of the means

0.04, BLEND2 1.45 ± 0.03 and BLEND3 1.43 ± 0.04. This finding suggested that a combination of *A. bilimbi* fruit filtrate, wheat bran, and *S. cerevisiae* could increase the digestibility and improve the nutrient utilization by broilers.

Mareta *et al*. (2020) and Pratama *et al*. (2021) previously revealed that *A. bilimbi* fruit filtrate improved physiological circumstances, gastrointestinal health, and broiler growth rate. Li *et al*. (2018) also found that wheat bran positively impacted the gut bacterial environment, morphology, and final body weight of broilers. Likewise, the LAB and *S. cerevisiae* have long promoted broiler growth (Sugiharto, 2016). In the present study, the synergistic effects of acid from *A. bilimbi* fruit filtrate, prebiotic from wheat bran, LAB from *A. bilimbi* fruit, and probiotic *S. cerevisiae* on broiler chicks most likely improved health, nutrient utilization, and therefore growth promotion. In support of this, Sugiharto (2016) and Sapsuha *et al*. (2021) suggested that the combined use of some bioactive compounds, such as probiotics, prebiotics, organic acids, and plant-derived products, resulted in a better effect on growth compared to that of

Table 6. Bacterial populations in the intestine of broilers treated with blends of *Averrhoa bilimbi* fruit filtrate, wheat bran, and *Saccharomyces cerevisiae*

Items (log cfu/g)	Treatments				SEM	p value	
	CONT	BLEND1	BLEND2	BLEND3		A	L
Ileum							
LAB	11.40	9.20	10.20	10.40	0.29	0.06	0.50
Coliform	8.42	6.87	7.03	6.85	0.34	0.29	0.13
Lactose negative *Enterobacteriaceae*	8.60[a]	5.67[b]	6.48[b]	6.45[b]	0.33	0.01	0.09
Enterobacteriaceae	8.64	7.53	7.16	6.96	0.35	0.35	0.09
Cecum							
LAB	11.5[a]	11.1[b]	11.5[a]	11.5[a]	0.07	0.04	0.49
Coliform	8.41	7.08	7.80	7.33	0.22	0.17	0.21
Lactose negative *Enterobacteriaceae*	7.93	7.50	8.32	6.67	0.29	0.23	0.26
Enterobacteriaceae	8.68	7.94	8.95	7.87	0.21	0.16	0.45

Note: [a,b]Means in the same row with different superscripts differ significantly ($p<0.05$). CONT= chicks offered control diet, with no additive; BLEND1= chicks offered diet administrated with 0.25% additive; BLEND2= diet administrated with 0.50% additive; BLEND3= diet administrated with 1% additive; LAB= lactic acid bacteria; A= analysis of variance; L= linear regression; SEM= standard error of the means.

Table 7. Morphology of small intestine of broilers treated with blends of *Averrhoa bilimbi* fruit filtrate, wheat bran, and *Saccharomyces cerevisiae*

Items	Treatments				SEM	p value	
	CONT	BLEND1	BLEND2	BLEND3		A	L
Duodenum							
Villi height (μm)	952	1094	1047	1044	34.3	0.55	0.46
Crypt depth (μm)	123	113	130	114	4.82	0.58	0.82
VH/CD ratio	7.97[b]	9.85[a]	8.20[b]	9.23[ab]	0.27	0.04	0.39
Jejunum							
Villi height (μm)	968	842	876	1032	39.9	0.33	0.54
Crypt depth (μm)	111	104	124	119	3.62	0.22	0.18
VH/CD ratio	8.89	8.51	7.12	8.75	0.53	0.46	0.65
Ileum							
Villi height (μm)	607	636	719	734	32.2	0.93	0.11
Crypt depth (μm)	109	87.9	98.3	99.9	3.00	0.08	0.51
VH/CD ratio	5.57	7.23	7.29	7.43	0.32	0.11	0.04

Note: [a,b]Means in the same row with different superscripts differ significantly ($p<0.05$). T0= chicks offered control diet; with no additive; T1= chicks offered diet administrated with 0.25% additive; T2= diet administrated with 0.50% additive; T3= diet administrated with 1% additive; VH= villi height; CD= crypt depth; A= analysis of variance; L= linear regression; SEM= standard error of the means.

the single compound alone. In this study, no mortality was found throughout the experimental period.

Complete Blood Counts of Broilers

In general, the data on erythrocyte indices were within the normal values as described by Scarth (2006). Both at days 21 and 35, the numbers of erythrocytes and haemoglobin concentration decreased with the increased levels of additive in feeds. In general, erythrocytes and haemoglobin have been associated with oxygen transport functions and support cellular metabolism for energy production (Stier *et al*., 2013). About leukocyte indices, in this study, the numbers of leukocytes and lymphocytes of broiler were higher, whereas heterophils were within the normal ranges (Scarth, 2006). Owing to the pathogens-host interaction, the high numbers of leukocytes and lymphocytes seemed to be the response of broilers against the high pathogenic loads, especially virus in broiler house during the experiment (Asheg *et al*., 2002). The interesting result was found at day 21, at which leukocytes and lymphocytes values decreased with the increased levels of additive in feeds.

In most situations, the increased levels of leukocytes and lymphocytes were attributed to the infections in birds (Akhtar *et al*., 2015; Hidanah *et al*., 2018). Indeed, infections have long been thought to be attributed to a rise in metabolic rate in animals due to a greater need for energy for maintenance and recovery (Kostadinović *et al*., 2011). Taking all the above facts together, the decreased levels of erythrocytes and haemoglobin in additive fed-broilers seemed to be the response of the birds toward the decreased potential of infections. This finding was actually in agreement with Sugiharto *et al*. (2020), reporting the decrease in erythrocytes and haemoglobin levels, concomitant with the decrease in leukocytes and lymphocytes, in broiler chickens when provided with antibiotics, zinc bacitracin, and fermented feed.

Our current finding further showed that the increased levels of dietary additive linearly decreased MCH and increased MCHC levels in the blood of broilers, particularly at day 21. In line with this, Pop *et al*. (2017) documented that dietary supplementation with artemisinin (serving as an antimicrobial agent) decreased the MCH and increased MCHC values of broiler chickens. This may suggest that the reduced pathogenic load may decrease MCH and increase MCHC values of broilers (Nasr El-Deen *et al*., 2018). In our study, MCHC values varied between 28.5% and 34.7%. A study stated that the feed additive did not affect the MCHC values of broiler chickens statistically, while the MCHC values varied between 23.33%-26.33% (Aka-Tanimo *et al*., 2020).

It is generally known that MCH denotes the average quantity of oxygen-carrying hemoglobin within an erythrocyte. In this regard, the lower MCH in the additive-supplemented chicks could be attributed to the lower metabolic rate (energy need) for the protection from infection. Concerning MCHC, Richardson *et al*. (2020) noticed that the increase in MCHC was associated with the decreased oxygen transport, and hence reduced metabolic rate of animals. Owing to the potential of additive in reducing the pathogenic load of broilers, our inference was actually supported by the fact in this study that the birds administrated with feed additive showed substantially lower content of lactose negative *Enterobacteriaceae* in their ileum.

Antibody Titers Against NDV and Serum Biochemical Variables of Broilers

At both measurement times, the titers of antibodies against NDV vaccine did not differ across the experimental groups. However, there was a strong tendency that the chicks received 0.5% additive in feeds exhibited higher antibody titers against NDV at day 35 than the other birds. Indeed, the respective chicks had antibody titers against NDV of 4.71 $\log_2$ GMT, which was classified as protective against the virus (considered as protective when the titers were at least 4 $\log_2$ GMT; van Boven *et al*., 2008). The immunomodulatory effect of *A. bilimbi* fruit (Sugiharto, 2020), wheat bran (Shang *et al*., 2020a), and *S. cerevisiae* (Elghandour *et al*., 2019) seemed to be responsible for the enhanced antibody titers against NDV in broilers. Yet, this inference should be interpreted with caution as the chicks that received 0.25 and 1% additive in feeds were not protected from NDV.

In general, the data on biochemical parameters of broilers presented in this study were in accordance with the results revealed by Pratama *et al*. (2021) and Sugiharto *et al*. (2021) at both times of determination. On day 21, the LDL cholesterol level was lower in BLEND3 than that in other treatment groups. This suggested that supplementation of additives, particularly at 1% in feed, reduced bad cholesterol in birds. Considering that LDL carries cholesterol and triglycerides from the liver to the peripheral tissues, the reduced LDL level could therefore be expected to reduce the total cholesterol and triglycerides in the circulation and their contents in broiler meats. A previous study by Taherpour *et al*. (2009) reported that dietary supplementation of probiotics, prebiotics, and organic acids lowered the LDL-cholesterol of broilers. Another study conducted on this subject reported that its probiotic contribution caused a significant decrease in LDL-cholesterol levels (Alaqil *et al*., 2020). In this regard, the synergistic effect of organic acid derived from A. bilimbi fruit filtrate, prebiotic from wheat bran, and probiotic *S. cerevisiae* increased the LDL clearance in the liver and thus lowered the liver the LDL level in serum.

The HDL-cholesterol has generally been considered as good cholesterol for broilers. In this study, the level of HDL was substantially lower in the additive-supplemented birds compared to control. This was not expected as HDL serves a crucial function in transporting the surplus fatty acids and cholesterol from peripheral tissues and blood to the liver, which is then excreted through feces. Yet, at day 21 the total triglycerides and cholesterol levels did not vary between control and the treated birds. Because blood cholesterol (total cholesterol, LDL-cholesterol, and HDL-cholesterol) belong to the "fast turnover cholesterol pool" (Konjufca *et al*., 1997), the frequent changes and fluctuating levels of such cholesterol fractions in blood could be understood in the current study.

Our data showed that creatinine linearly increased with the enhanced level of additives in feed. In general, creatinine levels in the blood are linked to kidney function and the amount of protein broken down (Del Vesco *et al*., 2015), suggesting that the elevated creatinine level in broiler blood appeared to be caused by kidney damage. It seemed, however, not the case in our study as the level of uric acid (an indicator of kidney damage; Abdel-Sattar *et al*., 2019) did not differ among the treatment groups. Studies in humans revealed that creatinine is one of the major non-enzymatic antioxidants in human plasma (Fumagalli *et al*., 2011). In this regard, the increased creatinine levels in the serum of broilers, with the increased dietary additive levels, seemed to be attributed to the increased capacity of chickens in scavenging free radicals. The content of phenolic acids in *A. bilimbi* fruit filtrate (Sugiharto, 2020) and wheat bran (Zhou *et al*., 2004; Shang *et al*., 2020b) as well as a-D-mannans in *S. cerevisiae* (Elghandour *et al*., 2019) possibly contributed to the improved antioxidative activity of broiler chickens in the present study.

pH Values of Intestinal Segments of Broilers

Our current data showed that the ileal pH value was higher in broilers supplemented with 0.25% feed additive when compared with birds in other groups. The reason for the increased pH in the respective birds was unknown, but the excessive buffering response of the ileum to acids (derived from the additive) appeared to raise the pH value of the ileum. Increased intestinal (jejunum) pH values were also observed in the study of Pratama *et al*. (2021) when broiler chickens were offered with the fermented *A. bilimbi* fruit filtrate.

In this study, an increase in ileum pH was associated with a decrease in the number of LAB in the intestinal segment. This may be explained by the fact that an acidic environment is more conducive to the growth

of intestinal LAB (Sugiharto, 2016). In another study conducted, the effect of probiotic and enzyme addition on mixed feed was found to be significant on ileal pH. The highest ileal pH was found in the control group (Kırkpınar *et al*., 2018). The excessive buffering action of ileum was not observed in broilers, given either 0.5% or 1.0% of the additive in the current study. The increased LAB population in the ileum (due to the increased LAB derived from *A. bilimbi* fruit filtrate) most likely counteracted the buffering action of the ileum, resulting in a lower pH value of the ileum. Furthermore, increased levels of *S. cerevisiae* in conjunction with higher additive levels can lower ileum pH by secreting organic acids such as lactic and acetic acid (Elghandour *et al*., 2019).

Regardless of the feed additive effect, the pH values of broiler gut segments in this investigation were generally within normal values. The pH values of duodenum ranges from 5.30 to 6.53 (Nkukwana *et al*., 2015; Ndelekwute *et al*., 2018), jejunum from 5.70 to 6.30 (Nkukwana *et al*., 2015; Lan *et al*., 2020), ileum from 5.80 to 6.79 (Józefiak *et al*., 2008; Nkukwana *et al*., 2015; Ndelekwute *et al*., 2018) and cecum from 5.95 to 6.86 (Nkukwana *et al*., 2015; Ndelekwute *et al*., 2018; Pratama *et al*., 2021). Several factors are affecting the pH values of intestinal segments of broilers, including compositions of diets, feed forms, the retention time of digesta, buffering activity of diets, ages, dietary additive (for instance, organic acids), intestinal microbial populations, etc. (Józefiak *et al*., 2008; Nkukwana *et al*., 2015; Ndelekwute *et al*., 2018; Lan *et al*., 2020; Pratama *et al*., 2021).

Bacterial Populations in Intestine of Broilers

It was apparent that the numbers of negative lactose *Enterobacteriaceae* were substantially lower in the ileum of additive-supplemented broilers than that in control. There was also a trend that *Enterobacteriaceae* counts linearly decreased with the increased levels of a combination of *A. bilimbi* fruit filtrate, wheat bran, and *S. cerevisiae* in diets. These findings indicated that the feed additive could serve as an antibacterial agent in the intestine of broilers. The published studies have reported the antibacterial activities of *A. bilimbi* fruit filtrate (Mareta *et al*., 2020; Pratama *et al*., 2021) and *S. cerevisiae* (Elghandour *et al*., 2019) on broilers. The synergistic effects of the *A. bilimbi* fruit filtrate and *S. cerevisiae* were thought to multiply the antibacterial activity of the additive used in this study. The reduced lactose negative *Enterobacteriaceae* with dietary feeding additive was actually concomitant with the reduced numbers of *Enterobacteriaceae* in the ileum of broilers.

In the cecum, the number of LAB was lower in broilers supplemented with 0.25% additive as compared to other birds. The same tendency was also shown in the ileum, at which the numbers of LAB tended to be lower in broilers administrated with 0.25% additive compared to other chickens. Several factors are determining the numbers of LAB in the intestine, including acid condition (low pH values), availability of simple sugar in the intestine as the substrate for the growth of LAB (Sugiharto, 2016; Sugiharto & Ranjitkar, 2019), as well as the survival of LAB during the passage through the intestine (Verruck & Prudencio, 2019). The increased pH values in the ileum may not be conducive to LAB growth, resulting in a low number of LAB in the respective gut section. The LAB will naturally move through the intestinal segments with the food/digesta (Vesa *et al*., 2000; Verruck & Prudencio, 2019). Hence, the lower numbers of LAB in the ileum may be attributable to the lower LAB numbers in the cecum of broilers.

The diversity and numbers of bacteria in the intestine vary among broiler chickens. In this study, the ileal numbers of LAB and coliform were higher than those formerly reported by Józefiak *et al*. (2008) and Pratama *et al*. (2021). However, in the cecum, the LAB and coliform counts reported in this study agreed with Pratama *et al*. (2021). These facts further confirmed the variations in LAB and coliform counts among broiler chickens. Several factors influence bacteria populations in broiler intestines, including feed nutritional contents, broiler house hygienic conditions, gut pH, and so on (Józefiak *et al*., 2008; Sugiharto, 2016; Sugiharto & Ranjitkar, 2019).

Intestinal Morphology of Broilers

Literature suggests that a greater VH/CD ratio was associated with the broiler's increased capacity to absorb the nutrients deriving from the digestion process (Ruangpanit *et al*., 2020). In this study, the duodenum and ileum of additive-administrated broilers showed a higher VH ratio to CD than that of control, suggesting that the additive could improve the intestinal function in absorbing the nutrients. One study observed that probiotic and synbiotic additives affect the VH/CD ratio statistically; VH/CD ratio was higher in the probiotic group (Awad *et al*., 2009).

A previous study by Pratama *et al*. (2021) reported the efficacy of the fermented *A. bilimbi* fruit filtrate in improving the intestinal morphology (increased villi height of jejunum) of broilers. They also suggested that the presences of organic acids and LAB were responsible for the improved intestinal microbial ecology and morphology of broilers. Likewise, wheat bran has been reported to improve intestinal morphology (increased villi height, decreased crypt depth, and increased VH/CD ratio) of broiler chickens (Shang *et al*., 2020b). Moreover, Helal *et al*. (2021) noticed that feeding *S. cerevisiae* increased villi height and reduced crypt depth in the broiler. Taken the facts together, it could therefore be inferred that the complementary and synergistic effects of *A. bilimbi* fruit filtrate, wheat bran, and *S. cerevisiae* may improve the intestinal morphology of broiler chickens.

CONCLUSION

The combination of *A. bilimbi* fruit filtrate, wheat bran, and *S. cerevisiae* was potential in reducing the pathogen load in the intestine of broiler, which was reflected by the lower numbers of lactose negative *Enterobacteriaceae* and *Enterobacteriaceae* in the ileum. However, the additive did not increase good bacteria (LAB) populations in the intestine. The combination of *A. bilimbi* fruit filtrate, wheat bran, and *S. cerevisiae* also

improved blood cell indices and serum biochemistry parameters. Moreover, the additive, especially at the level of 0.25%, improved the intestinal morphology as indicated by the broiler's higher VH/CD ratio.

CONFLICT OF INTEREST

The authors state that they have no conflict interest.

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REFERENCES

Abdel-Sattar, W. M., K. M. Sadek, A. R. Elbestawy, & D. M. Mourad. 2019. The protective role of date palm (*Phoenix dactylifera* seeds) against aflatoxicosis in broiler chickens regarding carcass characteristics, hepatic and renal biochemical function tests and histopathology. World Vet. J. 9:59-69. https://doi.org/10.36380/scil.2019.wvj9

Abudabos, A. M., A. H. Alyemni, Y. M. Dafalla, & R. U. Khan. 2017. The effect of phytogenics on growth traits, blood biochemical and intestinal histology in broiler chickens exposed to *Clostridium perfringens* challenge. J. Appl. Anim. Res. 46:691-695. https://doi.org/10.1080/09712119.2017.1383258

Aka-Tanimo, H. A., D. O. Oshibanjo, V. O. Adelowo, M. A. Akwashiki, I. W. Azi, O. C. James, S. Haruna, & A. Y. Sunday. 2020. Growth performance, carcass characteristics and hematology indices of broiler chicken fed graded levels of *(Mangifera indica)* mango leaf meal. Asian J. Res. Anim. Vet. Sci. 6:20-27.

Akhtar, M., M. M. Awais, M. I. Anwar, S. Ehtisham-ulHaque, A. Nasir, M. K. Saleemi, & K. Ashraf. 2015. The effect of infection with mixed Eimeria species on hematology and immune responses following Newcastle disease and infectious bursal disease booster vaccination in broilers. Vet. Q. 35:21-26. https://doi.org/10.1080/01652176.2014.991048

Alaqil, A. A., A. O. Abbas, H. S. El-Beltagi, H. El-Atty, K. Abd, G. M. Mehaisen, & E. S. Moustafa. 2020. Dietary supplementation of probiotic *Lactobacillus acidophilus* modulates cholesterol levels, immune response, and productive performance of laying hens. Animals 10:1588. https://doi.org/10.3390/ani10091588

Asheg, A., M. Levkut, V. Revajova, Z. Sevcikova, L. Kolodzieyski, & J. Pistl. 2002. T lymphocyte subpopulations and B lymphocyte cells in caecum and spleen of chicks infected with *Salmonella enteritidis*. Acta Histochem. 104:435-439. https://doi.org/10.1078/0065-1281-00666

Awad, W. A., K. Ghareeb, S. Abdel-Raheem, & J. Böhm, J. 2009. Effects of dietary inclusion of probiotic and synbiotic on growth performance, organ weights, and intestinal histomorphology of broiler chickens. Poult. Sci. 88:49-56. https://doi.org/10.3382/ps.2008-00244

Bolton, W. 1967. Poultry Nutrition. MAFF Bulletin No.174. HMSO, London.

Cimrin, T., R. I. Tunca, M. D. Avsaroglu, T. Ayasan, & S. Kücükersan. 2020. Effects of an antibiotic and two phytogenic substances (cinnamaldehyde and 1,8-cineole) on yolk fatty acid profile and storage period-associated egg lipid peroxidation level. R. Bras. Zootec. 49:e20190270. https://doi.org/10.37496/rbz4920190270

Del Vesco, A. P., E. Gasparino, D. O. Grieser, V. Zancanela, D. M. Voltolini, A. S. Khatlab, S. E. F. Guimarães, M. A. M. Soares, & A. R. O. Neto. 2015. Effects of methionine supplementation on the expression of protein deposition-related genes in acute heat stress-exposed broilers. PLoS ONE 10:e0115821. https://doi.org/10.1371/journal.pone.0115821.t002

Elghandour, M. M. Y., Z. L. Tan, S. H. Abu Hafsa, M. J. Adegbeye, R. Greiner, E. A. Ugbogu, J. C. Monroy, & A. Z. M. Salem. 2019. *Saccharomyces cerevisiae* as a probiotic feed additive to non and pseudo-ruminant feeding: A review. J Appl. Microbiol. 128:658-674. https://doi.org/10.1111/jam.14416

Feng, Y., L. Wang, A. Khan, R. Zhao, S. Wei, & X. Jing. 2020. Fermented wheat bran by xylanase-producing Bacillus cereus boosts the intestinal microflora of broiler chickens. Poult. Sci. 99:263-271. https://doi.org/10.3382/ps/pez482

Fumagalli, S., F. Fattirolli, L. Guarducci, T. Cellai, S. Baldasseroni, F. Tarantini, D. Di Bari, G. Masotti, & N. Marchionni. 2011. Coenzyme Q10 terclatrate and creatine in chronic heart failure: a randomized, placebo-controlled, double-blind study. Clin. Cardiol. 34:211-7. https://doi.org/10.1002/clc.20846

Gunenc, A., C. Alswiti, & F. Hosseinian. 2017. Wheat bran as a prebiotic source and antioxidant activities of fiber fractions. J. Food Res. 6:1-10. https://doi.org/10.5539/jfr.v6n2p1

He, T., S. Mahfuz, X. Piao, D. Wu, W. Wang, H. Yan, T. Ouyang, & Y. Liu. 2021. Effects of live yeast (*Saccharomyces cerevisiae*) as a substitute to antibiotic on growth performance, immune function, serum biochemical parameters and intestinal morphology of broilers. J. Appl. Anim. Res. 49:15-22. https://doi.org/10.1080/09712119.2021.1876705

Helal, F., A. El-Badawi, S. El-Naggar, M. Shourrap, O. Aboelazab, & S. A. Hafsa. 2021. Probiotics role of Saccharomyces cerevisiae and Bacillus subtilis in improving the health status of rabbits' gastrointestinal tract. Bull. Natl. Res. Cent. 45:1-9. https://doi.org/10.1186/s42269-021-00522-0

Hidanah, S., E. K. Sabdoningrum, R. S. Wahjuni, & S. Chusniati. 2018. Effects of meniran (*Phyllanthus niruri* L.) administration on leukocyte profile of broiler chickens infected with *Mycoplasma gallisepticum*. Vet. World. 11:834-839. https://doi.org/10.14202/vetworld.2018.834-839

Józefiak, D., S. Kaczmarek, & A. Rutkowski. 2008. A note on the effects of selected prebiotics on the performance and ileal microbiota of broiler chickens. J. Anim. Feed Sci. 17:392-397. https://doi.org/10.22358/jafs/66633/2008

Kırkpınar, F., Z. Açıkgöz, S. Mert, & O. Işık. 2018. Effects of dietary probiotic, prebiotic and enzyme mixture supplementation on performance, carcass, organ, ileal pH and viscosity of broilers. J. Anim. Prod. 59:1-9. https://doi.org/10.29185/hayuretim.469862

Konjufca, V. H., G. M. Pesti, & R. B. Bakalli. 1997. Modulation of cholesterol levels in broiler meat by dietary garlic and copper. Poult. Sci. 76:1264-1271. https://doi.org/10.1093/ps/76.9.1264

Kostadinović, L. M., S. T. Pavkov, J. D. Lević, T. A. GalonjaCoghill, G. K. Dozet, & N. C. Bojat. 2011. Effect of sulphachloropyrazine on antioxidative systems in blood and liver of broilers. Acta Vet. Brno. 80:165-170. https://doi.org/10.2754/avb201180020165

Lan, R. X., S. Q. Li, Z. Zhao, & L. L. An. 2020. Sodium butyrate as an effective feed additive to improve growth performance and gastrointestinal development in broilers. Vet. Med. Sci. 6:491-499. https://doi.org/10.1002/vms3.250

Li, B., J. Leblois, B. Taminiau, M. Schroyen, Y. Beckers, J. Bindelle, & N. Everaert. 2018. The effect of inulin and wheat bran on intestinal health and microbiota in the early life of broiler chickens. Poult. Sci. 97:3156-3165. https://doi.org/10.3382/ps/pey195

Lin, W. C. & T. T. Lee. 2020. *Laetiporus sulphureus*-fermented

wheat bran enhanced the broiler growth performance by improving the intestinal microflora and inflammation status. Poult. Sci. 99:3606-3616. https://doi.org/10.1016/j.psj.2020.04.011

Mareta, I., G. Nathaniel, T. Yudiarti, E. Widiastuti, H. I. Wahyuni, & S. Sugiharto. 2020. Effect of *Averrhoa bilimbi* fruit filtrate and shrimp paste mixture on performance, gut microbes and blood profile of broilers. Indonesian Journal of Animal and Veterinary Science 25:182-189. https://doi.org/10.14334/jitv.v25i4.2515

Morales-López, S., J. A. Yepes, J. C. Prada-Herrera, & A. Torres-Jiménez. 2019. Enterobacteria in the 21st century: a review focused on taxonomic changes. J. Infect. Dev. Ctries. 13:265-273. https://doi.org/10.3855/jidc.11216

Müller, M., G. D. A. Hermes, C. E. Emanuel, J. J. Holst, E. G. Zoetendal, H. Smidt, F. Troost, F. G. Schaap, S. O. Damink, J. W. E. Jocken, K. Lenaerts, A. A. M. Masclee, & E. E. Blaak. 2020. Effect of wheat bran derived prebiotic supplementation on gastrointestinal transit, gut microbiota, and metabolic health: a randomized controlled trial in healthy adults with a slow gut transit. Gut Microbes 12:1704141. https://doi.org/10.1080/19490976.2019.1704141

Nasr El-Deen, N. A. M., S. A. M. El-Mandrawy, & M. A. Mahmoud. 2018. Trial in using *Clostridium butyricum* as prophylactic for Salmonellosis in broiler chicken: Hematobiochemical, immunological and pathological alterations. Ann. Clin. Pathol. 6:1130.

Ndelekwute, E. K., E. D. Assam, & E. M. Assam. 2018. Apparent nutrient digestibility, gut pH and digesta viscosity of broiler chickens fed acidified water. MOJ Anat. Physiol. 5:250-253. https://doi.org/10.15406/mojap.2018.05.00203

Nkukwana, T. T., V. Muchenje, P. J. Masika, & B. Mushonga. 2015. Intestinal morphology, digestive organ size and digesta pH of broiler chickens fed diets supplemented with or without *Moringa oleifera* leaf meal. S. Afr. J. Anim. Sci. 45:362-370. https://doi.org/10.4314/sajas.v45i4.2

Pop, L. M., L. C. Ştefănuţ, A. F. Tăbăran, A. L. Paştiu, Z. Kalmár, C. A. Magdaş, V. Mircean, & A. Györke. 2017. Influence of dietary artemisinin supplementation on productive performance and haematological parameters of broiler chickens. R. Bras. Zootec. 46:130-137. https://doi.org/10.1590/s1806-92902017000200008

Pratama, A. R., I. Mareta, T. Yudiarti, H. I. Wahyuni, E. Widiastuti, & S. Sugiharto. 2021. Administration of fermented *Averrhoa bilimbi* L. fruit filtrate through drinking water on growth performance, haematological indices, intestinal ecology and carcass characteristics of broiler chickens. Trop. Anim. Sci. J. 44:79-89. https://doi.org/10.5398/tasj.2021.44.1.79

Richardson, S. L., A. Hulikova, M. Proven, R. Hipkiss, M. Akannic, N. B. A. Roy, & P. Swietach. 2020. Single-cell o2 exchange imaging shows that cytoplasmic diffusion is a dominant barrier to efficient gas transport in red blood cells. Proc. Natl. Acad. Sci. 117:10067-10078. https://doi.org/10.1073/pnas.1916641117

Ruangpanit, Y., K. Matsushita, K. Mukai, & M. Kikusato. 2020. Effect of trehalose supplementation on growth performance and intestinal morphology in broiler chickens. Vet. Anim. Sci. 10:100142. https://doi.org/10.1016/j.vas.2020.100142

Salami, S. A., E. A. Agbonlahor, A. O. Salako, B. A. Sideeq, J. O. Agboola, & J. O. Atteh. 2018. Nutritive values of wheat bran-based broiler diet supplemented with different classes of enzymes. Trop. Agric. 95:246-256.

Sapsuha, Y., E. Suprijatna, S. Kismiati, & S. Sugiharto. 2021. Combination of probiotic and phythobiotic as an alternative for antibiotic growth promoter for broilers chickens-a review. Livest. Res. Rural Dev. 33:1-8.

Scarth, L. L. 2006. The Merck Veterinary Manual Online (8th edition). Reference Reviews 20:40-40. https://doi.org/10.1108/09504120610647492

Shakouri, M. D., P. A. Iji, L. L. Mikkelsen, & A. J. Cowieson. 2009. Intestinal function and gut microflora of broiler chickens as influenced by cereal grains and microbial enzyme supplementation. J. Anim. Physiol. Anim. Nutr. (Berl). 93:647-658. https://doi.org/10.1111/j.1439-0396.2008.00852.x

Shang, Q. H., S. J. Liu, T. F. He, H. S. Liu, S. Mahfuz, X. K. Ma, & X. S. Piao. 2020a. Effects of wheat bran in comparison to antibiotics on growth performance, intestinal immunity, barrier function, and microbial composition in broiler chickens. Poult. Sci. 99:4929-4938. https://doi.org/10.1016/j.psj.2020.06.031

Shang, Q., D. Wu, H. Liu, S. Mahfuz, & X. Piao. 2020b. The impact of wheat bran on the morphology and physiology of the gastrointestinal tract in broiler chickens. Animals 10:1831. https://doi.org/10.3390/ani10101831

SNI (Indonesian National Standard). 2015. Standard for broiler feed (SNI 8173.2:2015 and SNI 8173.3:2015). National Standardization Agency of Indonesia, Jakarta, Indonesia.

Stier, A., P. Bize, Q. Schull, J. Zoll, F. Singh, B. Geny, F. Gros, C. Royer, S. Massemin, & F. Criscuolo. 2013. Avian erythrocytes have functional mitochondria, opening novel perspectives for birds as animal models in the study of ageing. Front. Zool. 10:33. https://doi.org/10.1186/1742-9994-10-33

Sugiharto, S. 2016. Role of nutraceuticals in gut health and growth performance of poultry. J. Saudi Soc. Agric. Sci. 15:99-111. https://doi.org/10.1016/j.jssas.2014.06.001

Sugiharto, S. 2020. The potentials of two underutilized acidic fruits (*Averrhoa bilimbi* L. and *Phyllanthus acidus* L.) as phytobiotics for broiler chickens. J. Adv. Vet. Res. 10:179-185. https://advetresearch.com/index.php/AVR/article/view/464

Sugiharto, S. & S. Ranjitkar. 2019. Recent advances in fermented feed toward improved broiler chicken performance, gastrointestinal tract microecology and immune response: a review. Anim. Nut. 5:1-10. https://doi.org/10.1016/j.aninu.2018.11.001

Sugiharto, S., E. Widiastuti, I. Isroli, T. Yudiarti, T. A. Sartono, & H. I. Wahyuni. 2020. Effect of feeding fermented mixture of cassava pulp and *Moringa oleifera* leaf meal on immune responses, antioxidative status, biochemistry indices, and intestinal ecology of broilers. Vet. World. 13:392-399. https://doi.org/10.14202/vetworld.2020.392-399

Sugiharto, S., E. Widiastuti, A. R. Pratama, H. I. Wahyuni, T. Yudiarti, & T. A. Sartono. 2021. Hematological and intestinal responses of broilers to dietary supplementations of lactic fermented turmeric, black pepper or a mixture of both. Acta Univ. Agric. et Silvic. Mendelianae Brun. 69:101-110. https://doi.org/10.11118/actaun.2021.011

Sugiharto, S., T. Yudiarti, I. Isroli, & E. Widiastuti. 2018. Effect of feeding duration of *Spirulina platensis* on growth performance, haematological parameters, intestinal microbial population and carcass traits of broiler chicks. S. Afr. J. Anim. Sci. 48:98-108. https://doi.org/10.4314/sajas.v48i1.12

Taherpour, K., H. Moravej, M. Shivazad, M. Adibmoradi, & B. Yakhchali. 2009. Effects of dietary probiotic, prebiotic and butyric acid glycerides on performance and serum composition in broiler chickens. Afr. J. Biotechnol. 8:2329-2334. https://doi.org/10.5897/AJB09.178

Tufarelli, V., P. Baghban-Kanani, S. Azimi-Youvalari, B. Hosseintabar-Ghasemabad, M. Slozhenkina, I. Gorlov, A. Seidavi, T. Ayaşan, & V. Laudadio. 2021. Effects of horsetail (*Equisetum arvense*) and spirulina (*Spirulina platensis*) dietary supplementation on laying hens productivity and oxidative status. Animals. 11:335. https://doi.org/10.3390/ani11020335

Tunc, M. A., S. Yildirim, & M. A. Yorük. 2019. Effects of tarragon (*Artemisia dracunculus*) powder on broiler performance parameters and histopathology of internal organs. Austral. J. Vet. Sci. 51:113-118. https://doi.org/10.4067/S0719-81322019000300113

Utama, C. S., Z. Zuprizal, C. Hanim, & W. Wihandoyo. 2019. Effect of heating time on the chemical quality of wheat pollard as a prebiotic potential agent. Jurnal Aplikasi Teknologi Pangan 8:113-122. (full article in Indonesian language). https://doi.org/10.17728/jatp.5262

van Boven, M., A. Bouma, T. H. F. Fabri, E. Katsma, L. Hartog, G. Koch. 2008. Herd immunity to Newcastle disease virus in poultry by vaccination. Avian Pathol. 37:1-5. https://doi.org/10.1080/03079450701772391

Verruck, S. & E. S. Prudencio. 2019. Survival of *Bifidobacterium* ssp. during gastrointestinal passage and their mechanism of action for pathogenic bacteria inhibition in the gut: A concise review. Food Biol. 8:1-6. https://doi.org/10.25081/fb.2019.v8.3856

Vesa, T., P. Pochart, & P. Marteau. 2000. Pharmacokinetics of *Lactobacillus plantarum* NCIMB 8826, *Lactobacillus fermentum* KLD, and *Lactococcus lactis* MG 1363 in the human gastrointestinal tract. Aliment. Pharmacol. Ther. 14:823-828. https://doi.org/10.1046/j.1365-2036.2000.00763.x.

Villegas, P. 1987. Avian Virus Diseases Laboratory Manual. College of Veterinary Medicine, University of Georgia, Athens, Georgia, USA.

Zhou, K., L. Su, & L. L. Yu. 2004. Phytochemicals and antioxidant properties in wheat bran. J. Agric. Food Chem. 52:6108-6114. https://doi.org/10.1021/jf049214g