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Physiological condition, gut morphology and immune responses of broilers supplemented with *Moringa oleifera* leaf extract, whey protein or their combination

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ABSTRACT

To improve growth performance and health, the broiler industry has recently incorporated plant sources and dairy milk byproducts as feed additives. The aim of this study was to investigate the effect of dietary supplementation of *Moringa oleifera* leaf extract (MOLE) and whey protein concentrate (WPC) or their combination on production performance, physiological status, gut integrity and immune responses of broilers. A total of 364 day old broiler chicks were used and assigned according to a completely randomized design with four dietary treatments, including CONT (basal feed as control), MOLE (basal feed + 1% MOLE), WPC (basal feed + 1% WPC), and MOLE-WPC (basal feed + 0.5% MOLE + 0.5% WPC). Daily weight gain and daily feed intake of broilers were higher in WPC and CONT groups ($P<0.05$) compared to MOLE group. The low-density lipoprotein (LDL) were lower in CONT and WPC ($P<0.05$) than in MOLE and MOLE-WPC groups. The serum glutamic oxaloacetic transaminase (SGOT) were lower in MOLE group ($P<0.05$) than in MOLE-WPC. In comparison to the CONT and WPC groups, the villi height and ratio of villi to crypt depth in the duodenum were more favorable in the MOLE and MOLE-WPC groups. The lactose negative enterobacteria have a lower number ($P<0.05$) in MOLE group. Histologically, the jejunum, ileum, and spleen were in better condition in the MOLE and MOLE-WPC groups ($P<0.05$) than in the other groups. In conclusion, supplementing MOLE, WPC or their combination improved the gut integrity and immune organ, but had no appreciable impact on production performance and physiological status in broilers.

Keywords: Blood parameter, Broiler, Immune response, Moringa oleifera, Whey protein

INTRODUCTION

The use of antibiotic growth promoter (AGP) in poultry feed has been prohibited as a result of the widespread concern about the harmful effects of antibiotic residues on consumers. Indeed, research demonstrates that removing AGP from poultry feed has a detrimental effect on the wellbeing and growth of chickens (Sugiharto, 2016). As a result, poultry nutritionists have been putting a lot of effort into coming up with a suggested AGP substitute for the poul-

try sector. The use of phytobiotics in poultry as an alternative to AGP has been recommended (Sugiharto, 2016), and one such phytobiotic is the leaf of the *Moringa oleifera* plant. The *M. oleifera* leaf contains a variety of bioactive chemicals such as vitamins and phenolic compounds that are beneficial for the health and growth performance of poultry (Khan et al., 2021).

M. oleifera has long been employed as an AGP substitute in broiler production. The populations of *Salmonella*, *Staphylococcus* spp., and *Eschericia coli* in ileum was decreased when 1% and 5% *M. oleifera* leaf extract (MOLE) was used in feed (Abu Hafsa et al., 2019). Total serum protein was also increased, and the blood profile was improved. Additionally, broiler chicks receiving MOLE had a favorable impact on growth index, according to Alabi (Alabi et al., 2017). Additionally, MOLE administration to broiler rations was found to reduce feed intake and feed conversion ratio (Ochi et al., 2015; Sugiharto and Toana, 2020). According to other studies, adding *M. oleifera* to broiler diet can boost antibody titre against Newcastle Disease and Infectious Bronchitis virus, lower MDA and ALT levels, and protect the liver from injury (Balami et al., 2018; Khan et al., 2021; Akram et al., 2020).

Whey is a by-product of cheese making which still has high nutritional value due to its essential amino acid content. Whey contains branched chain amino acids, especially leucine, which plays an important role as a modulator in protein metabolism. Whey protein concentrate also contains 65% lactose which helps increase the proliferation of lactic acid bacteria in the digestive tract (Rama et al., 2019). Therefore, whey protein is suggested to have prebiotic potentials so that it can provide healthful effects for the host (Zarei et al., 2020).

The study by Zadegan et al. (2022) reported that the use of whey in broiler feed increased the activity of antioxidant enzymes and reduced lipid peroxidation. Other studies suggest that dietary supplementation using whey protein has emerging health benefits including reducing malondialdehyde (MDA) activity (reduced lipid peroxidation), serum triglycerides and cholesterol, increasing catalase (CAT), superoxide dismutase (SOD) and glutathione peroxidase (GPx) enzyme activity, as well as facilitating microbiota proliferation throughout the small intestine (Ashour et al., 2019; Greenhalgh et al., 2022). Although it did not show a significant effect on performance, PCV, hemoglobin, lymphocytes and polymorphonuclear leukocytes (PMN), the use of whey protein in feed had positive effects on platelets and leukocytes in broiler chickens (Szczurek et al., 2013; Kanza et al., 2013).

Combining plant extract with other healthy ingredients to have a synergistic effect on the host is quite frequent (Sugiharto, 2021). In broiler chicken production, the combination use of MOLE and whey protein has, however, never been tried before. It was hypothesized that using MOLE and whey protein together will produce greater impact than using just one of them. Determining the impact of MOLE, whey protein, or their combination on broiler growth performance, physiological state, gut morphology, and immunological responses was thereof the goal of the current investigation.

MATERIALS AND METHODS

The current experiment was approved by the Committee of Animal Ethic of the Faculty of Animal and Agricultural Sciences, Universitas Diponegoro (58-04d/A-6/KEP-FPP).

Animals and Experimental Diets

A total of 364 day-old Cobb broiler chicks, purchased from a local hatchery, were employed in this present study. The initial weight of chicks averaged 45.75 ± 0.96 g. All chicks were raised in broiler open-sided house following the standard broiler rearing management practice. Rice husk was used as a litter. Continuous lighting was provided to the chicks throughout the study period. For the entire duration of the study, the chicks were given unlimited access to food and

Table 1. Feed formulation on starter and finisher period

Items	Compositions (%)	Items (%)	Compositions (%)
Starter period		**Finisher period**	
Yellow maize	57.9	Yellow maize	61.0
Palm oil	2.55	Palm oil	2.95
Soybean meal	34.8	Soybean meal	32.0
DL-methionine	0.19	DL-methionine	0.19
Bentonite	1.00	Bentonite	0.75
Limestone	1.34	Limestone	1.00
Monocalcium phosphate	1.51	Monocalcium phosphate	1.30
Premix[2]	0.27	Premix[2]	0.34
Chlorine chloride	0.07	Chlorine chloride	0.07
Salt	0.40	Salt	0.40
Chemical compositions:		Chemical compositions:	
ME (kcal/kg)[1]	3385	ME (kcal/kg)[1]	3594
Crude protein	15.94	Crude protein	15.61
Crude fibre	3.44	Crude fibre	3.96
Crude fat	2.01	Crude fat	2.74
Ash	6.00	Ash	5.07

[1]Metabolizable energy was calculated according to Bolton formula: 40.81 {0.87 [crude protein + 2.25 crude fat + nitrogen-free extract] + 2.5}

[2]Premix contained (per kg of diet) of Vitamin A 7750 IU, Vitamin D3 1550 IU, Vitamin E 1.88 mg, Vitamin B1 1.25 mg, Vitamin B2 3.13 mg, Vitamin B6 1.88 mg, Vitamin B12 0.01 mg, Vitamin C 25 mg, folic acid 1.50 mg, Ca-D-pantothenate 7.5 mg, niacin 1.88 mg, biotin 0.13 mg, Co 0.20 mg, Cu 4.35 mg, Fe 54 mg, I 0.45 mg, Mn 130 mg, Zn 86.5 mg, Se 0.25 mg, L-lysine 80 mg, choline chloride 500 mg, DL-methionine 900 mg, CaCO3 641.5 mg, dicalcium phosphate 1500 mg

water. The chicks were divided at random into 4 treatment groups, each of which had 7 replications/pens, and each pen had 12 chicks in it. The chicks were raised on commercial pre-starter feed from the time of arrival to day seven, which contained 23% crude protein, 5% crude fat, 5% crude fiber, and 7% ash (according to the feed label). From day 8 onward, the chicks were given formulated starter and finisher feeds as shown in Table 1. From day 0 to 42 days old, the treatments using MOLE and whey protein concentrate (WPC) as a feed addition were used. The the treatment groups included CONT (basal feed as control), MOLE (basal feed + 1% MOLE), WPC (basal feed + 1% WPC), and MOLE-WPC (basal feed + 0.5% MOLE + 0.5% WPC).

The MOLE was bought from the local pharmaceutical industry (PT. Boroburur, Semarang, Central Java). The MOLE contains total flavonoid of 14.295% and antioxidant capacity 65.615%. The WPC was purchased from Davisco (80% crude protein; Davisco Foods International, Le Sueur, Minnesota, USA). The all chicks were vaccinated against Newcastle Disease (ND), Infectious Bronchitis (IB), Avian Influenza (AI) and Infectious Bursal Disease (IBD) to protect the birds from the field virus infection during the trial. At day 4th, the chicks were vaccinated with Medivac ND-IB by eye drop and Medivac ND-AI by 0.15 ml subcutan injection. At day 11st, Medivac Gumboro A were applied by drinking water.

Data Collection and Laboratory Analysis

Productivity Parameters. The daily body weight gain, daily feed intake, and feed efficiency of the chicks were all measured. Data on daily weight gain were calculated by weighing the chickens at their final weight (42 days of age), deducting by their initial weight, and then dividing by the treatment period (42 days). Daily feed intake was determined by dividing the total feed consumption during treatment by the number of treatment days. Feed efficiency was determined

by dividing the body weight during treatment by the total feed consumed and then multiplied with 100%.

The Blood Profile. At the end of experiment, 1 chicken from each experimental unit was randomly selected for blood collection through the brachial vein using steril syringe. One mililitre (ml) of blood was put into the EDTA tube for routine blood testing and 3 ml blood was placed at non-EDTA tube for serum formation. The routine blood profile testing of chicks was determined automatically with a hematology analyzer (Prima Fully-auto Hematology Analyzer, PT. Prima Alkesindo Nusantara, Jakarta, Indonesia).

The anticoagulant-free blood was sentrifuged at 3,000 rpm (10 minutes) for serum separation. Serum samples were stored at freezer for later analysis of total cholesterol, triglyceride, high-density lipoprotein (HDL) and low-density lipoprotein (LDL), uric acid, and creatinine. Triglyceride levels were determined by the enzymatic colorimetric assay method of Werner et al. (1981). Low density lipoprotein (LDL) were precipitated by adding magnesium ions and phospotungstic through the sample. For this purpose one part of the sample and three parts of precipitant were used. By using centrifugation, HDL will be left in the supernatant. The cholesterol level was determined based on the procedure described by Lopes-Virella et al. (1997). The total protein and albumin levels were measured with spectrophotometric assays (Sigma-Aldrich, St. Loius, USA). The data of globulin were calculated from the serum total protein minus serum albumin.

Specific reagent were used for determination of serum SGOT, SGPT, uric acid and creatinine. SGPT and SGOT enzyme activity was determined using kinetic enzymatic reaction. The principle of SGPT kinetic examination is L-alanine aminotransferase catalyzes the transamination of L-alanine and α-ketoglutarate to form l-glutaate and pyruvate. The piruvate then reduced to lactate by lactate dehudrogenase (LDH) enzyme and nicotinamide adenine dinucleotide (NADH) was oxidized to NAD. The amount of oxidized NADH resulting from the decreased of absorption were directly proportional to ALT activity and measured photometrically with 340 nm wavelength. The SGOT determination was according to aminotransferase (AST) catalization from L-aspartate and α-ketoglutarate to L-glutamate and oxaloasetate. Oxaloacetate was reduced to malate dehydrogenase enzyme (MDH), then nicotinamide adenine dinucleotide (NADH) was oxidized to NAD. The amount of oxidized NADH is directly proportional to AST activity and measured photometrically with 340 nm wavelength. The creatinine was determined using enzymatic spectrophotometry method. A 10 µl of serum sample was diluted with 740 µl distilled water then being fortified with 500 µg/ml creatinine-D3 solution. Ethanol was then added to the above of the mixture to precipitate outs the proteins. After vortexing for 1 minute and centrifugation at 12,000 rpm for 10 minutes, the supernatant was collected. The creatinine level was observed and presented in mg/dl. Enzymatic determination of uric acid results from the oxidation of uric acid by uricase, which converts its substrate to allantoin. The sample were observed by colorimetric method according to Badoeidalfard et al. (2019).

The antibody titers against ND and AI were determined using hemagglutination inhibition (HI) assay according to OIE (2021a) and OIE (2021b). The ND and AI antibody titers test was performed in U-based microtiter plates using 4 HA units of LaSota antigen and AI H5N1 antigen. The two folds serial dilution of the test samples were mixed with an equal volume of ND and AI antigen. Chicken red blood cells (CRBC) were added and subsequently the number of dilutions were examined for the presence of complete inhibition of the hemagglutination. The thiobarbituric acid method (Placer et al., 1966) was used to determine the serum MDA level. The MDA values was measured as a lipid peroxidation index. The serum samples was homogenized in 4.0 mL potassium chloride (KCl) and 0.1 mM butylated hydroxytoluene. Then, 200 µL of the homogenized sample were extracted and mixed with 2 ml of thiobarbituric acid solution. The

Table 2. Performance of broilers (days 0-42)

Items	CONT	MOLE	WPC	MOLE-WPC	SEM	P value
DWG (g)	45.34[a]	41.43[b]	45.69[a]	43.22[ab]	0.53	0.008
DFI (g)	76.49[ab]	74.74[bc]	77.05[a]	73.74[c]	0.40	0.006
FE (%)	58.09	54.57	57.31	56.46	0.53	0.106

[a,b,c]Means marked with superscript letters in the same row are significantly different (P<0.05). CONT: control (basal feed), MOLE: basal feed + 1.0% M. oleifera, WPC: basal feed + 1% whey protein concentrate, MOLE-WPC: basal feed + 0.5% M. oleifera + 0.5% whey protein concentrate, SEM : standard error mean, DWG: daily weight gain, DFI: daily feed intake, FE: feed efficiency

mixed samples were placed in a waterbath for 60 minutes at 95 °C until the developing of the pale-red pigment. The samples were placed at cooler room under running water. Then, 3 mL of n-butanol was added, and the sample was vortexed for 60 seconds. The mixture was then centrifuged at 4000 rpm for 15 minutes, and the supernatant was extracted. MDA values were determined using colorimeter spectrophotometer against pure n-butanol.

Digestive Tract's pH and Gut Microbiota. Measurement of intestine pH was carried out on the duodenum, ileum and cecum segment using digital pH-meter (ST300 Portable pH-Meter, OHAUS). The pen tip of pH-meter was attached to the duodenum, ileum dan cecum mucosa until the intestinal pH values appeared on the pH-meter screen. The lactose negative enterobacteria and total coliform were counted on MacConkey agar (Merck KGaA, Darmstadt, Germany) as colorless and red colonies after incubation for 24 hours at 38°C. The lactic acid bacteria colony was enumerated using de Man, Rogosa and Sharpe agar (MRS) from Merck KGaA and followed by anaerobic incubation at 38°C for 48 hours.

Lymphoid Organ Weight and Histology Parameters. The lymphoid organ relative weight (bursa of Fabricius, spleen and thymus) was determined by calculated lymphoid organ weight divided by the live body weight and multiplied by 100%. The histologic analysis was conducted to the spleen and small intestinal segment (duodenum, jejunum and ileum). Histologic organ were prepared by 5 µm transverse cutting and Hematoxilin-Eosin (HE) stained. The measurement of villus height and crypt depth of each intestinal segment were performed using an optical microscope equipped with a digital camera (Leica Mycrosystems GmBH, Wetzlar, Germany). The villus-crypt ratio (VH/CD) was determined by calculating the villus height divided by crypt depth. The lesion of spleen and small intestine were graded according to the severity of tissue injury. For injury distribution, scores were according to the injury type, focal or multifocal injury, loss of cilia, epithelial cell hypertrophy/ hyperplasia, inflammatory infiltration and necrosis. A severity score was assigned where 0 (no lesion, 0% cells damage); 1 (mild, 5-25% of cells damage); 2 (moderate, 26-50% cells of damage), 3 (severe, >50% of cells damage) (Agusetyaningsih et al., 2022; Alabi et al., 2020). The villi height and crypt depth were measured by calculating the average of 3 observations site per small intestine sample.

Statistical Analysis

The SPSS program version 16.0 was used to analyze the data. The analysis was carried out based on a one-way ANOVA with 5% significance level. The Duncan multiple range test was used to evaluate the variations among the treatment groups. The histopathological lesion scores on spleen and small intestinal segments were analysed non-parametrically using Kruskal-Wallis analysis method.

RESULTS

Performance of Broilers

Data on the growth performance of broilers

Table 3. Complete blood counts of broilers

Items	CONT	MOLE	WPC	MOLE-WPC	SEM	P value
Haemoglobin (g/dL)	10.78	10.07	10.07	10.00	0.23	0.63
Erythrocytes (10^6/µL)	2.66	2.93	2.77	2.53	0.92	0.49
Haematocrit (%)	32.28	35.64	33.35	30.71	1.09	0.46
MCV	122.48	122.18	121.32	122.27	0.46	0.84
MCH	43.97	34.41	36.54	39.35	1.76	0.25
MCHC	36.08	28.30	30.37	32.42	1.40	0.24
RDW-SD	43.97	34.41	36.54	39.35	1.42	0.75
RDW-CV	36.08	28.30	30.37	32.4	0.29	0.80
MPV	9.77	9.87	9.11	9.84	0.16	0.30
PDW	9.65	13.98	10.78	12.01	0.60	0.06
Leukocytes (10^3/µL)	114.57	98.14	89.25	91.28	4.66	0.21
Heterophils (10^3/µL)	13.07	13.14	13.07	6.92	1.36	0.29
Lymphocytes (10^3/µL)	101.50	85.00	76.21	84.35	3.79	0.11
Eosinofil (10^3/µL)	0.00	0.00	0.00	0.00	0.00	-
Thrombocytes (10^3/µL)	22.42	24.28	30.71	22.71	1.73	0.30

CONT: control (basal feed), MOLE: basal feed + 1.0% M. oleifera, WPC: basal feed + 1% whey protein concentrate, MOLE-WPC: basal feed + 0.5% M. oleifera + 0.5% whey protein concentrate, SEM : standard error mean, MCV: mean corpuscular volume, MCH: mean corpuscular hemoglobin, MCHC: mean corpuscular hemoglobin concentration, RDW-SD: red cell distribution width-standard deviation, RDW-CV: red cell distribution-coefficient variation, MPV: mean platelet volume, PDW: platelet distribution width

Table 4. Blood biochemical parameters

Items	CONT	MOLE	WPC	MOLE-WPC	SEM	P value
Total cholesterol (mg/dL)	97.71	103.10	100.47	99.51	2.39	0.89
HDL (mg/dL)	31.95	31.07	29.74	24.01	2.12	0.57
LDL (mg/dL)	53.57^{b}	63.14^{a}	57.28^{ab}	65.28^{a}	1.54	0.01
Triglyceride (mg/dL)	60.97	44.52	67.24	51.11	4.82	0.36
Total protein (g/dL)	3.17	2.86	3.22	3.01	0.10	0.64
Albumin (g/dL)	1.18	1.14	1.17	1.15	0.24	0.93
Globulin (g/dL)	1.99	1.99	2.05	1.85	0.09	0.61
Uric acid (mg/dL)	6.27	5.68	5.67	5.96	0.25	0.83
Creatinine (mg/dL)	0.05	0.05	0.06	0.05	0.01	0.09
SGOT (g/dL)	275.59^{ab}	225.97^{b}	282.31^{ab}	345.77^{a}	15.12	0.03
SGPT (g/dL)	5.19	6.20	4.81	2.72	0.49	0.08
MDA (nmol/ml)	2.19	1.99	2.22	2.37	0.07	0.30
ACR	21.68	21.84	17.75	21.70	0.67	0.07

^{a,b}Means marked with superscript letters in the same row are significantly different (P<0.05). CONT: control (basal feed), MOLE: basal feed + 1.0% M. oleifera, WPC: basal feed + 1% whey protein concentrate, MOLE-WPC: basal feed + 0.5% M. oleifera + 0.5% whey protein concentrate, SEM : standard error mean, HDL: high density lipoprotein, LDL: low density lipoprotein, SGOT: serum glutamic oxaloacetic transaminase, SGPT: serum glutamic pyruvic transaminase, MDA: malondialdehyde, ACR: albumin to creatinine ratio

are presented in Table 2. The daily weight gain were higher on CONT and WPC group (P<0.05) than in MOLE groups, whereas MOLE-WPC not significantly different to other groups. The WPC group had a higher daily feed intake (P<0.05) compared to MOLE and MOLE-WPC groups, while MOLE-WPC has a lowest DFI compared to all groups. The treatments had no significant effects on broiler's feed efficiency.

Complete Blood Counts and Biochemical Parameters

The treatments had no significant effect on the complete blood counts of broilers as presented in Table 3. The data of blood biochemical parameters are listed in Table 4. MOLE and MOLE-WPC groups had a higher level of LDL ($P<0.05$) than in CONT group, and also WPC had no significant difference to another groups. The highest level of SGOT was observed in MOLE-WPC group ($P<0.05$), while CONT group was the lowest ($P<0.05$).

Digestive Tract's pH and Small Intestine Histomorphometry

The data of pH of duodenum, ileum and caecum are listed on Table 5, and there were no significant effects due to the treatments. However, all treatments had a significant impact on villi height and villi height to crypt depth ratio (VH/CD ratio) in duodenum (Table 6). The MOLE and MOLE-WPC groups had a higher duodenum villi height compared to CONT and WPC groups. The VH/CD ratio was higher ($P<0.05$) in MOLE and MOLE-WPC groups compared to CONT group but have no significant difference with WPC group. The treatments had no significant impact on jejunum and ileum histomorphometry.

Ileum and Caecum Microbiota

The data of ileum and caecum microbiota are presented in Table 7. The counts of ileum lactose negative bacteria (LNE) increased in WPC and MOLE-WPC groups ($P<0.05$) compared to MOLE group. While the ileum LNE counts in CONT group showed no significant difference to WPC and MOLE-WPC groups. The treatments in this study did not have the significant impact to ileum coliform and LAB, and all

Table 5. pH of digestive tracts

Items	CONT	MOLE	WPC	MOLE-WPC	SEM	P value
Duodenum	6.01	5.97	6.01	5.96	0.04	0.96
Ileum	5.56	5.16	5.85	5.81	0.11	0.13
Caecum	6.47	6.69	6.41	6.52	0.06	0.50

CONT: control (basal feed), MOLE: basal feed + 1.0% M. oleifera, WPC: basal feed + 1% whey protein concentrate, MOLE-WPC: basal feed + 0.5% M. oleifera + 0.5% whey protein concentrate, SEM : standard error mean

Table 6. Small intestine histomorphometri

Items	CONT	MOLE	WPC	MOLE-WPC	SEM	P value
Duodenum						
Villi height (VH)	902.11^{b}	1309.89^{a}	1049.51^{ab}	1290.36^{a}	63.36	0.05
Crypt depth (CD)	236.35	201.17	203.72	220.54	9.45	0.54
VH/CD ratio	3.82^{b}	6.55^{a}	5.36^{ab}	6.21^{a}	0.36	0.03
Jejunum						
Vili height (VH)	828.08	1106.43	1211.81	946.87	63.52	0.14
Crypt depth (CD)	164.52	176.25	202.86	187.97	9.94	0.58
VH/CD ratio	0.19	0.17	0.17	0.21	0.01	0.40
Ileum						
Vili height (VH)	590.11	689.01	648.92	559.43	39.38	0.63
Crypt depth (CD)	175.21	163.56	141.81	145.18	10.87	0.68
VH/CD ratio	3.40	4.50	4.69	4.06	0.24	0.26

^{a,b}Means marked with superscript letters in the same row are significantly different ($P<0.05$). CONT: control (basal feed), MOLE: basal feed + 1.0% M. oleifera, WPC: basal feed + 1% whey protein concentrate, MOLE-WPC: basal feed + 0.5% M. oleifera + 0.5% whey protein concentrate, SEM : standard error mean

bacterial numbers in caecum.

Immunology Parameters

The data listed in Table 8 show the treatment using MOLE and WPC or their combination did not affect the relative immune organ weight (bursa of Fabricius, spleen, thymus) and antibody titre against Newcastle disease and Avian Influenza in chicks.

Histopathologic Scoring of the Lymphoid Tissue and Small Intestines

Table 9 shows histopathologic scoring of the small intestines and spleen of broilers. The treatment with MOLE and WPC or their combination had a significance effect (P<0.05) on jejunum ileum and spleen lesion score. The WPC group had a lower lesion score (P<0.05) compared to CON, MOLE and MOLE-WPC groups. The WPC and MOLE-WPC groups had the lower ileum lesion score (P<0.05) compared to CONT group, but had no significance different with MOLE group. The spleen lesion score were greater (P<0.05) in MOLE-WPC and CONT groups compared to MOLE and WPC groups.

According to Figure 1-4, the visible changes in the duodenum were only mild damage in mucosal and muscularis layer. In jejunum (Figure 5-8), the figure of CONT group showed that there were severe erosion in mucosal layer and in MOLE group showed the moderate lesion in mucosal layer. While, the WPC group performed the normal mucosa and muscular layer. The figure MOLE-WPC group described the erosion in mucosal layer and moderate lesion in muscular layer. Figure 9-12 shows the broiler ileum lesion

Table 7. Ileum and caecum microbiota

Items (log CFU/mg)	CONT	MOLE	WPC	MOLE-WPC	SEM	P value
Ileum						
Coliform	1.10	1.01	3.37	3.37	0.47	0.34
LNE	2.02^{ab}	1.36^{b}	4.25^{a}	4.25^{a}	0.44	0.02
LAB	4.37	4.37	4.18	4.18	0.57	3.01
Caecum						
Coliform	3.46	3.47	3.42	4.42	0.42	0.82
LNE	2.51	3.37	3.91	2.68	0.42	0.64
LAB	8.14	7.95	7.95	7.91	0.07	0.61

^{a,b}Means marked with superscript letters in the same row are significantly different (P<0.05). CONT: control (basal feed), MOLE: basal feed + 1.0% M. oleifera, WPC: basal feed + 1% whey protein concentrate, MOLE-WPC: basal feed + 0.5% M. oleifera + 0.5% whey protein concentrate, SEM : standard error mean, CFU: colony forming unit, LNE: lactose-negative bacteria, LAB: lactic acid bacteria

Table 8. Immunology parameters

Items (% BW)	CONT	MOLE	WPC	MOLE-WPC	SEM	P value
Relative immune organ weight						
Bursa of Fabricius	0.04	0.04	0.03	0.04	0.002	0.26
Spleen	0.12	0.12	0.14	0.17	0.01	0.34
Thymus	0.29	0.23	0.20	0.25	0.01	0.30
Antibody titre						
ND	10.00	9.28	23.14	13.28	2.60	0.21
AI	1.71	2.57	13.42	12.14	2.60	0.24

CONT: control (basal feed), MOLE: basal feed + 1.0% M. oleifera, WPC: basal feed + 1% whey protein concentrate, MOLE-WPC: basal feed + 0.5% M. oleifera + 0.5% whey protein concentrate, SEM : standard error mean, GMT: geometric mean titre, ND: Newcastle Disease, AI: Avian Influenza

Table 9. Histopathologic scoring of the small intestines and spleen

Items	CONT	MOLE	WPC	MOLE-WPC	SEM	P value
Duodenum	10.86	15.71	17.50	13.93	0.12	0.38
Jejunum	18.43[a]	16.64[a]	6.29[b]	16.64[a]	0.13	<0.01
Ileum	20.57[a]	16.86[ab]	10.29[b]	10.29[b]	0.15	0.02
Spleen	10.40[b]	20.50[a]	20.50[a]	6.57[b]	0.14	<0.01

The data presented are the mean-rank. [a,b]Mean-rank marked with superscript letters in the same row are significantly different (P<0.05). CONT: control (basal feed), MOLE: basal feed + 1.0% M. oleifera, WPC: basal feed + 1% whey protein concentrate, MOLE-WPC: basal feed + 0.5% M. oleifera + 0.5% whey protein concentrate, SEM : standard error mean

score. The CONT group figure was described the moderate lesion in mucosal layer and no lesion in muscularis layer, but in MOLE, WPC and MOLE-WPC had showed the mild lesion in mucosal layer. Figure 13-16 were an overview of the broiler spleen lesion score. The CONT and MOLE-WPC groups performed a lesion score that were closed to normal with almost no microscopic organ changes, different from the MOLE and WPC groups which showed moderate-severe spleen lesion.

DISCUSSION

The results of this study demonstrated that dietary administration of MOLE had a detrimental effect on broiler chicken growth. Alabi et al. (2020) have previously shown that administering MOLE to broiler chicks decreased their feed intake and growth performance of broilers, which is in accordance with our findings. The latter authors pointed out that the antinutritional components in MOLE were responsible for the decrease in feed intake and growth in chicks given MOLE. In this regard, these antinutritional substances, such as tannins and saponin, may obstruct the digestive enzyme activities and compromise nutrient absorption (Abd El-Hack et al., 2022). The corresponding results were also documented by Cui et al. (2018), in which dietary inclusion of *Moringa oleifera* leaves meal impaired growth performance of broiler chickens. They found that *Moringa oleifera* leaves had an effect on reducing broiler abdominal fat content. Such reduced abdominal fat content may therefore reduced the final body weight gain of broilers (Cui et al., 2018). In fact, the latter circumstance was also noted in the current investigation, where feeding MOLE to broilers reduced the proportion of abdominal fat content (data not shown) and hence broiler final body weight.

The use of WPC in feed had no impact on broiler daily weight gain. This finding was in line with Pineda-Quiroga et al. (2017) that the use of WPC had no effect on the performance of broiler chickens. In contrast, Kanza et al. (2013) and Greenhalgh et al. (2022) reported that production parameters such as feed consumption, body weight and body weight gain increased with feeding whey protein. Several studies have reported that giving whey protein (due to high content of protein) can interfere with kidney function which then affects the growth of broilers (Ko et al., 2017; Ashour et al., 2019). Other studies further suggested that whey protein may modulate lipid metabolism in adipocytes, such as plasma release triacylglycerol and intestinal fat absorption (Boscaini et al., 2020). This is thought to exert minimal fat deposition in the body, resulting in decreased absolute body weight gain in chicks. In addition of this study, the protein content of the basal feeds were formulated to meet the nutritional requirement of broilers. In this respect, the addition of WPC in the basal feeds resulted in higher protein intake than the need of broilers (exceeding requirements). Such conditions are very likely to interfere with kidney function, and thereby attenuated the growth-promoting effect of WPC. However, this inference must be interpreted with caution because

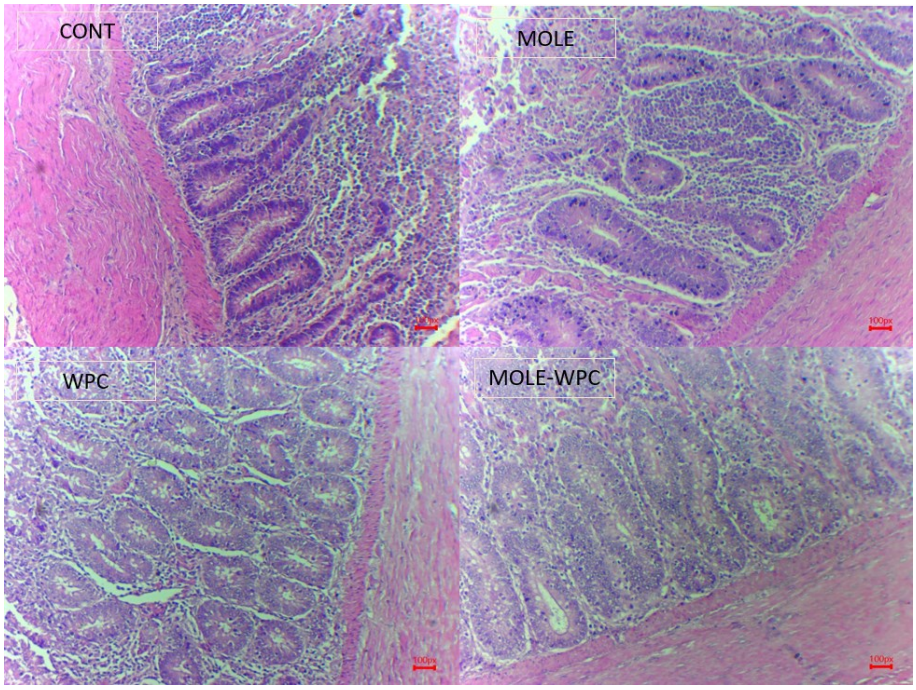

Figure 1-4. Duodenum microscopic photograph of 42 days old broiler chickens. CONT (mild lesion in mucosal and no lesion in muscular layer), MOLE (mild lesion in mucosal layer), WPC (mild lesion in mucosal layer), MOLE-WPC (mild lesion in mucosal layer). H&E staining, 40×.

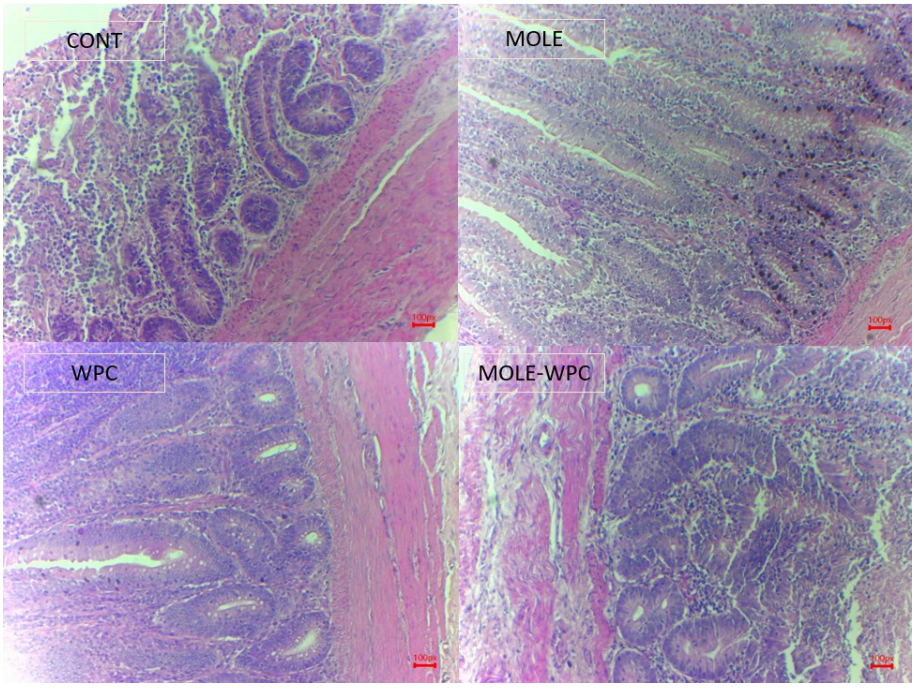

Figure 5-8. Jejunum microscopic photograph of 42 days old broiler chickens. CONT (severe erosion in mucosal layer), MOLE (moderate lesion in mucosal layer), WPC (normal mucosa and no lesion in muscular layer), MOLE-WPC (erosion in mucosal and moderate lesion muscular layer). H&E staining, 40×.

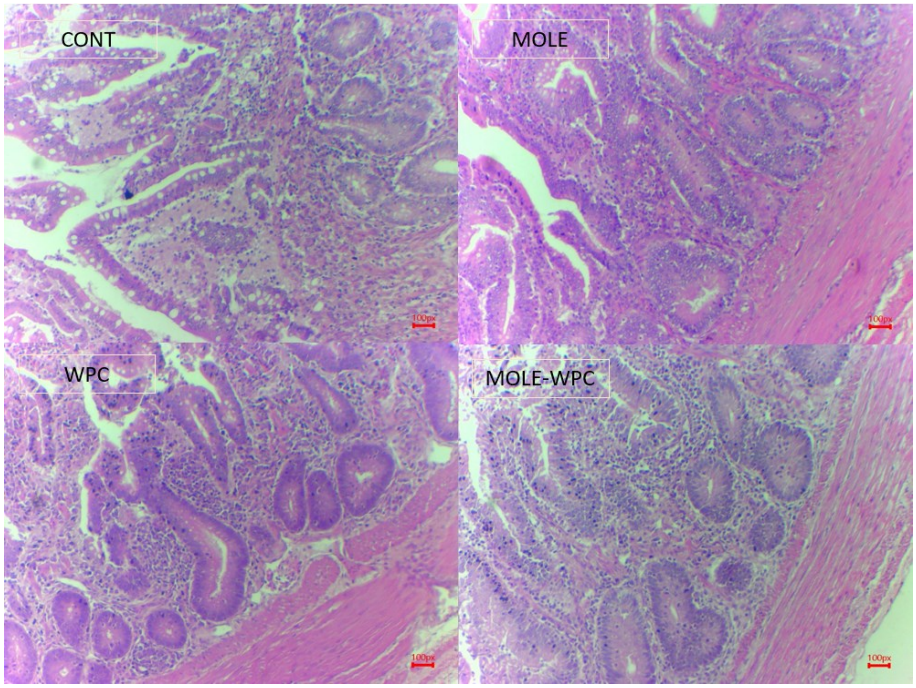

Figure 9-12. Ileum microscopic photograph of 42 days old broiler chickens. CONT (moderate lesion in mucosal and no lesion in muscular layer), MOLE (mild lesion in mucosal layer), WPC (mild lesion in mucosal layer), MOLE-WPC (mild lesion in mucosal layer). H&E staining, 40×.

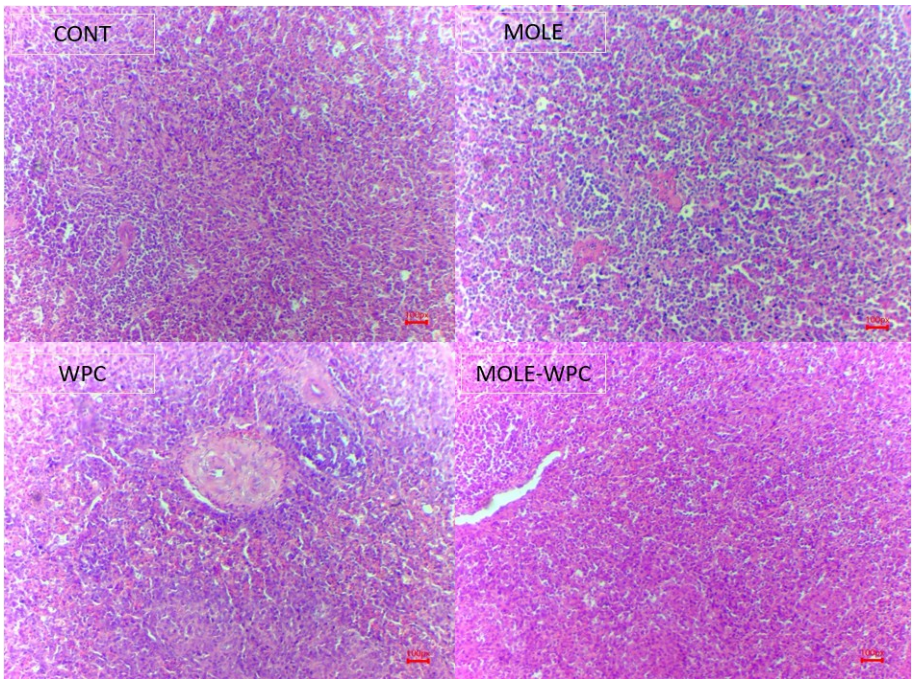

Figure 13-16. Spleen microscopic photograph of 42 days old broiler chickens. CONT (mild lesion in reticular cells colony), MOLE (moderate lesion in red pulp), WPC (moderate lesion in red pulp), MOLE-WPC (close to normal). H&E staining, 40×.

indicators of renal impairment, reflected in uric acid and creatinine levels, did not differ among treatment groups.

In this study, feed supplementation with MOLE and WPC, or their combination had no significant effect on complete blood counts of broilers. This was in line with Egbu et al. (2022) and Castillo et al. (2017) showing no effect of *M. oleifera* supplementation on the values of haemoglobin, MCH, MCHC, MCV, platelets and leukocytes of broiler and quail. Supplementing WPC in this treatment also did not altered the blood parameters of broilers. This finding was in contrary to the previous study (Kanza et al., 2013) that found the increase of platelets and white blood cells due to whey protein supplementation in broilers. Blood profile is the best signal for broiler health status that could helps to identify whether there is acute or chronic immune disorders and infective or non-infective disease occurred. This current results might therefore indicates that the bioactive components in whey were less-able to affects the blood profile of broilers.

Low-density lipoprotein values were substantially increased by MOLE or MOLE-WPC supplementation. This finding was not in line with AbouSekken (2015) and Alnidawi *et al*. (2016) who reported a decrease in LDL values due to *M. oleifera* leaf supplementation in broiler feed. Detailed explanation of these differences was not known with certainty until now. In this study, MOLE was extracted from *M. oleifera* leaves which were added with maltodextrin as a filler. Maltodextrin has a long carbohydrate chain along with 2-3% glucose and 5-7% maltose. In general, the body digests maltodextrin as a simple carbohydrate which can then be easily converted into instant energy (Parikh et al., 2014). Bai et al. (2019), reported that the high content of simple carbohydrates in feed can increase or stimulate LDL transcytosis resulting in higher LDL level. Based on the facts above, it was possible that the increased LDL level in the serum of broiler chickens receiving MOLE or MOLE-WPC was due to maltodextrin stimulation on LDL transcytosis. SGOT is an indicator of liver health, in which high levels of SGOT indicate impaired liver function in broiler chickens. When compared to the control group, the SGOT levels in the treatment groups were not significantly different in this present study. This showed that the administration of MOLE and MOLE-WPC did not have a negative impact on the liver health of broiler chickens.

Small intestinal morphology is one of the factors that can determine the utilization of nutrients and indicators of physiological status of broiler chickens. In this study, MOLE and MOLE-WPC increased the VH and the ratio of VH to CD of the duodenum. Nkukwana et al. (2015) and Khan et al. (2017) reported an increase in VH in broiler chickens treated with *Moringa oleifera* leaf meal. Higher villi in the duodenum indicate an increase in the absorption area thereby improving nutrient absorption by the chickens (Muhammadsadeghi *et al*., 2019). Furthermore, Mahfuz and Piao (2019) and Khan et al. (2017) reported that enlargement of the absorption area is usually accompanied by an increase in the number of goblet cells which act as the mucosal immune system in poultry. In terms of the MOLE treatment, the content of short-chain carbohydrates and polyphenols in MOLE can be the main cause of increased intestinal VH (Nkukwana et al., 2015; Das et al., 2020). Likewise, Kamboh and Zhu (2014) suggested that polyphenol compounds can stimulate epithelial cell mitosis which results in higher VH. In general, the VH/CD of duodenum can indicate the digestibility and absorption of the small intestine, in which a higher VH/CD reflects a strong ability to digest and absorb nutrients (Tian et al., 2021).

In this study, the dietary inclusion of MOLE reduced the numbers of lactose negative enterobacteria (LNE) in the ileum compared to that of WPC and MOLE-WPC. Aside from being a source of antioxidants, MOLE is known to show antimicrobial activity (Gupta et al., 2018). In this case, Oluduro (2012) showed that *M. oleifera* contains methyl N-4-(α-L-rhamnopyranosyloxy)

benzyl carbamate and 4-(α-D-glucopyranosyl-1→4-α-L-rhamnomyranosyloxy) benzyl thiocarboxamide which can act as antimicrobials. According to this study, chickens in the WPC and MOLE-WPC groups had a higher population of LNE in the ileum compared to the MOLE group. In this case, the protein in WPC has a potential role in promoting the growth of intestinal microflora. Whey can also act as a substrate for enterobacterial growth due to the presence of lactose, vitamins and minerals (You et al., 2017; Geiger et al., 2016).

In the current investigation, the dietary administration of MOLE, WPC, or both had no discernible effect on the development of immune organs and humoral immunity, as indicated by the bursa Fabricius, spleen, thymus weight, and ND-AI antibody titre. Eladia and Ampode (2021) noted the same findings, in which administering of *M. oleifera* to broiler chicks had no impact on their immune status of broilers. In this study, data on spleen histopathologic scoring lesion showed that MOLE-WPC group have a better condition compared to MOLE and WPC groups. It indicated that bioactive compounds in MOLE and WPC seemed to act as immune cells protectors in broiler spleen. Dietary supplementation of WPC and combination MOLE and WPC improved the integrity of jejunum and ileum of broilers. In this case, WPC and MOLE-WPC chicks had better gut lesion score compared to CONT and MOLE groups. This results may be due to biophenols and cysteine-rich protein contents in WPC which can induce the integrity of gut cell wall (Xiao et al., 2020; Solak and Akin, 2012).

CONCLUSION

In conclusion, dietary supplementation of MOLE, WPC or their combination improved the gut integrity and immune organ, but had no appreciable impact on production performance and physiological status in broilers.

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CONFLICT OF INTEREST

All authors declare that there has no conflict of interests.

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