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The use of organic calcium derived from eggshell waste on physiological and intestinal conditions of broiler chickens

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ABSTRACT

The study aimed to investigate the effect of using eggshell waste or eggshell extract as organic calcium source in feed on the physiological conditions and intestines of broilers. A total of 392 one-day -old chicks were randomly distributed into four groups (10 birds each group), CONT (control diet containing 1% limestone as an inorganic calcium source), EGFL (feed containing 1% eggshell powder as an organic calcium source), EEG1 (feed containing 1% eggshell extracted with *Averrhoa bilimbi* L. fruit filtrate as an organic calcium source), and EEG05 (feed containing 0.5% eggshell extracted using *A. bilimbi* L. fruit filtrate). Body weight and feed intake were recorded weekly, whereas blood, intestinal content, and small intestinal segments were collected at day 35. Our results showed that during days 8-35, weight gain and feed consumption were lower (P<0.05) in EEG05 than in CONT and EGFL. Thymus relative weight tended (P=0.08) to be lower in EEG05 than in CONT, and gizzard was lower (P<0.05) in EGFL than in CONT and EEG05. Serum total triglyceride was significantly higher in CONT than in other groups. Moreover, serum high-density lipoprotein was higher in EEG1 and EEG05 than in CONT (P<0.05). furthermore, serum albumin were higher (P<0.05) in EEG1 than in CONT, EGFL and EEG05. Additionally, serum uric acid was higher (P<0.05) in EEG1 than in EGFL and EEG05. Among the groups, SGPT levels were lowest (P<0.05) in EEG05 birds. While there was no effect (P>0.05) of treatments on coliform and lactic acid bacteria in the ileum and caecum, jejunal crypt depth tended (P=0.09) to be lower in EEG1 and EEG05 than in CONT and EGFL broilers. Conclusively, using 1% eggshell powder or eggshell extract in feed as the substitute for limestone had no detrimental effect on broiler chickens' growth, physiological status and intestinal condition. Hence, eggshell powder or eggshell extracted with *A. bilimbi* L. fruit filtrate can be used as an organic calcium source to replace limestone.

Keywords: Acid, Broilers, Calcium, Eggshell, Limestone.

INTRODUCTION

Minerals are an essential component in broiler chicken feed. Determining the appropriate mineral levels in feeds is, therefore, one of the success factors in raising broiler chickens. One of the important minerals in broiler chicken feed is calcium. Calcium in broiler chicken feed functions to form bone, muscle contractions, and blood clotting (Kristianto *et al*., 2014). Organic

Eggshells are waste products produced by hatcheries, food industries, and households. As egg consumption and production expand globally, eggshell waste is expected to reach 30 million tones by 2030 (Mignardi *et al*., 2020). The nutrient content of egg shells consists of 0.81% moisture, 94.69% ash, 2.11% crude protein, 0.04% crude fat. Moreover, eggshell minerals include 34,200 mg calcium, 240 mg magnesium, 106 mg phosphor, 80 mg natrium, 60.20 mg kalium, 0.67 mg zinc and 11.47 mg iron (Shahnila *et al*. (2022). Compared to clam shells as a source of calcium, egg shells contain fewer heavy metals (Hassan *et al*., 2022). In previous research, Maranan *et al*. (2021) used eggshell powder as a substitute for limestone at 50%, 75%, and 100% in broiler feeds. They reported that the substitution of limestone with eggshell powder up to 50% in the feed did not have a negative impact, but the substitution of limestone at levels of 75% and 100% with eggshell powder had a negative impact on the growth and feed efficiency of broiler chickens. Furthermore, the use of eggshell powder reduced the broiler dressing percentage.

To avoid the negative impact of using eggshell powder as a source of calcium on broilers, a particular treatment may be needed, one of which is soaking in an acid solution. Soaking eggshells in acid typically reduces harmful components such as dangerous heavy metals and pathogenic microorganisms, eliminates fishy odors and increases organoleptic value (Wijinindyah *et al*., 2022). In this case, Wijinindyah *et al*. (2022) used tamarind fruit (*Tamarindus indica*) as a soaking medium. They reported that the tartaric acid, citric acid, and malic acid contained in *T. indica* reduced the water content in the eggshell, thereby extending the shelf life and minimizing contamination of the eggshell. Citric acid as a pretreatment medium for eggshells can increase the mineral content of eggshells, such as calcium, magnesium, phosphorus, and iron (Siddique *et al*. (2016).

Averrhoa bilimbi L. is a plant belonging to the Oxalidaceae family. The *A. bilimbi* fruit contains several organic acids, including citric acid, acetic acid, and oxalic acid, with citric acid as the largest component (Sugiharto, 2020). Based on its organic acid content, especially *citric acid*, the *A. bilimbi* fruit can be used as a soaking medium (solvent extraction) and enhancer to increase the availability and utility of the calcium in the eggshells. This study aimed to investigate the effect of using eggshell waste or eggshell extract as an organic calcium source in feed on the physiological conditions and intestinal health of broiler chickens.

MATERIALS AND METHODS

Ethical Approval

The Animal Ethical Committee of the Faculty of Animal and Agricultural Sciences, Universitas Diponegoro approved the current experiment (approval number: 59-07b/A-16/KEP-FPP).

Preparations of Eggshell Powder and Eggshell Extract

The eggshell was collected from a hatchery close to the university. After being rinsed with running water, the eggshell was sun-dried and ground into flour with a hammer mill. The *A. bilimbi* L. fruit was picked from the campus garden. To make the fruit filtrate, the fruit was washed with running water, drained, blended (using an electronic blender), and then filtered using a filter cloth. To make eggshell extract, sterilized (autoclave) eggshell powder, *A. bilimbi* L. fruit filtrate, and sugarcane molasses were mixed in an anaerobic jar at a ratio of 1:1:0.01 (g:mL:g). After homogenization, the jar was firmly closed (anaerobic) and incubated for two days. The mixture was sun-dried before being stored at room temperature until the *in vivo* study.

Broiler Trial

A total of 392-day-old chicks (Cobb strain) purchased from the local hatchery were employed in this current study. The chicks were raised in a broiler house (opened-house system) and used husks as litter. From arrival to day 7, the chicks were provided with commercial prestarter feed containing 14% moisture, 8% ash, 20% crude protein, 5% crude fat and 5% crude fiber (according to the feed label). From day 8-35 the chicks were provided with the treatment diets as presented in Tables 1 and 2. The chicks were distributed to four treatment groups with seven replicates, each containing 14 chicks. The experimental groups were CONT (control diet containing 1% limestone as an inorganic calcium source), EGFL (feed containing 1% eggshell powder as an organic calcium source), EEG1 (feed containing 1% eggshell extracted with *A. bilimbi* L. fruit filtrate as an organic calcium source), and EEG05 (feed containing 0.5% eggshell extracted using *A. bilimbi* L. fruit filtrate).

For the entire rearing period, broiler chickens were given experimental feeds and drinking water *ad libitum*. All chickens were vaccinated against Newcastle disease virus (ND), Infectious Bronchitis (IB), and Infectious Bursal Disease (IBD) to avoid infections during the rearing period. In detail, the chicks were given the Medivac ND-IB vaccine by eye drops on day 4, the Medivac ND Lasota vaccine *via* drinking water on day 12, and the IBD vaccine *via* drinking water on day 19.

Data Collection and Laboratory Analyses

Body weight and feed consumption were recorded during the starter and finisher phases. The feed conversion ratio (FCR) was determined by the amount of feed divided by the broiler's body weight gain. Feed efficiency was obtained from the increase in body weight divided by the amount of feed given multiplied by 100%. The performance index was determined by calculating the survival rate multiplied by the increase in body weight and then divided by the FCR, which has been multiplied by the rearing period. The survival rate was obtained from 100 minus the depletion or mortality rate.

One chick representing the average body weight of chicks in each replication was taken to obtain a blood sample at day 35. Blood samples were taken *via* the wing vein with a sterile 3 mL syringe. A total of 2 mL of blood was placed in a non-ethylenediaminetetraacetic (EDTA) blood tube for blood serum analysis and 1 mL in an EDTA blood tube for complete blood profile analysis. The complete blood profile of broilers was analyzed using Prima fully auto hematology analyzer, manufactured by PT. Prima Alkesindo Nusantara, Jakarta, Indonesia. To prepare blood serum, the non-EDTA blood was first centrifuged at 3,000 rpm for 10 min. The serum was placed in an Eppendorf tube and stored in the

Table 1. Compositions of starter feeds (days 8-21)

Ingredients (%)	CONT	EGFL	EEG1	EEG05
Yellow corn	53.5	53.5	53.5	54.45
Palm oil	2.32	2.32	2.32	2.00
Soybean meal	40.13	40.13	40.13	40.0
DL-methionine	0.19	0.19	0.19	0.19
Bentonite	0.75	0.75	0.75	0.75
Limestone	1.00	-	-	-
Eggshell powder	-	1.00	-	-
Eggshell extract	-	-	1.00	0.50
Monocalcium phosphate	1.30	1.30	1.30	1.30
Premix[1]	0.34	0.34	0.34	0.34
Chlorine chloride	0.07	0.07	0.07	0.07
Salt	0.40	0.40	0.40	0.40
Chemical elements:				
ME[2] (kcal/kg)	2,900	2,900	2,900	2,903
Crude protein	22.0	22.0	22.0	22.0
Crude fibre	5.47	5.47	5.47	5.52
Ca	1.14	1.14	1.14	0.94
P (available)	0.57	0.57	0.57	0.57

[1]The following nutrients are provided per kilogram 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 vitamin A, 250,000 IU vitamin D_3, 1,350 g pantothenic acid, 1,875 g vitamin E, 250 g vitamin K_3, 250 g vitamin B_1, 750 g vitamin B_2, 500 g vitamin B_6, 2,500 mg vitamin B_{12}, 5,000 g niacin, 125 g folic acid and 2,500 mg biotin

[2]ME (metabolizable energy) was calculated according to formula: 40.81 {0.87 (crude protein + 2.25 crude fat + nitrogen free extract) + 2.5}

CONT: control diet containing 1% limestone as an inorganic calcium source, EGFL: feed containing 1% eggshell powder as an organic calcium source, EEG1: feed containing 1% eggshell extracted with *A. bilimbi* L. fruit filtrate as an organic calcium source, EEG05: feed containing 0.5% eggshell extracted using *A. bilimbi* L. fruit filtrate.

Table 2. Compositions of finisher feeds (days 22-35)

Ingredients (%)	CONT	EGFL	EEG1	EEG05
Yellow corn	58.54	58.54	58.54	59.75
Palm oil	2.96	2.96	2.96	2.51
Soybean meal	34.7	34.7	34.7	34.44
DL-methionine	0.19	0.19	0.19	0.19
Bentonite	0.75	0.75	0.75	0.75
Limestone	1.00	-	-	-
Eggshell powder	-	1.00	-	-
Eggshell extract	-	-	1.00	0.50
Monocalcium phosphate	1.05	1.05	1.05	1.05
Premix[1]	0.34	0.34	0.34	0.34
Chlorine chloride	0.07	0.07	0.07	0.07
Salt	0.40	0.40	0.40	0.40
Chemical elements:				
ME[2] (kcal/kg)	3,000	3,000	3,000	3,000
Crude protein	20.0	20.0	20.0	20.0
Crude fibre	5.51	5.51	5.51	5.58
Ca	1.07	1.07	1.07	0.87
P (available)	0.53	0.53	0.53	0.54

[1]The following nutrients are provided per kilogram 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 vitamin A, 250,000 IU vitamin D_3, 1,350 g pantothenic acid, 1,875 g vitamin E, 250 g vitamin K_3, 250 g vitamin B_1, 750 g vitamin B_2, 500 g vitamin B_6, 2,500 mg vitamin B_{12}, 5,000 g niacin, 125 g folic acid and 2,500 mg biotin

[2]ME (metabolizable energy) was calculated according to formula: 40.81 {0.87 (crude protein + 2.25 crude fat + nitrogen-free extract) + 2.5}

CONT: control diet containing 1% limestone as an inorganic calcium source, EGFL: feed containing 1% eggshell powder as an organic calcium source, EEG1: feed containing 1% eggshell extracted with *A. bilimbi* L. fruit filtrate as an organic calcium source, EEG05: feed containing 0.5% eggshell extracted using *A. bilimbi* L. fruit filtrate.

freezer (-10°C) until analysis.

Serum was tested for total protein, albumin, uric acid, creatinine, serum glutamic oxaloacetic (SGOT), serum glutamic pyruvic transaminase (SGPT), high-density lipoprotein (LDL), low-density lipoprotein (LDL), triglycerides, and total cholesterol. Enzyme-based colorimetric techniques were used to measure serum lipid profiles (total triglycerides, total cholesterol, LDL, and HDL) and serum levels of creatinine and uric acid. Using spectrophotometric/ photometric techniques, the levels of serum total protein, albumin, SGOT, and SGPT were determined. The total protein value was subtracted from the serum albumin value to determine the globulin concentration. All biochemical analyses of serum samples were carried out following the manufacturer's instructions (DiaSys Diagnostic System GmbH, Holzheim, Germany).

The chickens were euthanized, and the intestines and internal organs were removed. Digesta from the ileum and cecum was collected to determine the counts of selected bacteria in the intestine. Segments (~2 cm) of the duodenum, jejunum, and ileum were obtained and placed in 10% buffered formalin (Leica Biosystems Richmond, Inc., Richmond, USA) for the measurement of small intestinal morphology (villous height and crypt depth). The internal organs were then weighed (empty condition). The total plate count method was employed to determine the bacterial counts in the ileal and cecal contents. On MacConkey agar (Merck KGaA, Darmstadt, Germany), the counts of coliforms and lactose-negative *Enterobacteriaceae* (LNE) were counted as red and colorless colonies following a 24-hours aerobic incubation at 38°C. The total count of lactose-negative *Enterobacteriaceae* and coliforms was used to calculate the number of *Enterobacteriaceae*. Lactic acid bacteria (LAB) were counted on de Man, Rogosa, and Sharpe (MRS; Merck KGaA) agar after 48 hours of anaerobic incubation at 38°C. Hematoxylin and eosin-stained 5-µm sections of the duodenum, jejunum, or ileum were used to examine small intestinal segments histologically. An optical microscope fitted with a digital camera (Leica Microsystems GmbH, Wetzlar, Germany) was used to measure each segment's villous height and crypt depth. The mean values of crypt depth and villous height were determined for each sample using five measurements.

Statistical Analysis

An analysis of variance (ANOVA) was performed on the collected data using SPSS version 16.0. Duncan's multiple analysis was employed when there was a significant effect of the treatment ($P \leq 0.05$). Tendency was considered when $0.05 \leq P < 0.10$.

RESULTS

Productive Performance and Relative Internal Organ Weights of Broiler Chickens

The data on weight gain, feed intake, FCR, feed efficiency, and performance index of broiler chickens are presented in Table 3. There was no difference ($P > 0.05$) in broiler chickens' weight gain, feed intake, FCR, feed efficiency, and performance index across the dietary treatment groups at the measurement at days 8-21. Feed intake was lower ($P < 0.05$) in EEG05 than that of CONT and EGFL, but was not different from that of EEG1 birds at days 22-35. During the measurement at days 8-35, weight gain and feed consumption were lower ($P < 0.05$) in EEG05 than that of CONT and EGFL, but were not different from that of EEG1 broilers.

The relative weight of the thymus tended ($P = 0.08$) to be lower in EEG05 than that of CONT birds. The relative weight of the gizzard was lower ($P < 0.05$) in EGFL compared to that of CONT and EEG05, but did not differ from that of EEG1 birds. The relative weight of other internal organs such as the spleen, *Bursa of Fabricius*, small intestine, caecum, heart, liver, proventriculus, pancreas, and abdominal fat were not different among the dietary treatment groups (Table 4).

Blood Profile and Serum Biochemistry of Broiler Chickens

The data on complete blood counts of broilers are presented in Table 5. There was no significant ($P > 0.05$) influence of the dietary treatments on the complete blood counts of broiler chickens.

Total triglyceride in the serum was higher ($P < 0.05$) in CONT than in other broiler treatment groups. The levels of HDL were higher ($P < 0.05$) in EEG1 and EEG05 compared to that in CONT, but did not differ from EGFL group. Serum albumin levels were higher ($P < 0.05$) in EEG1 than in

Table 3. Productive performance of broiler chickens

Measured parameters	CONT	EGFL	EEG1	EEG05	SEM	P value
Days 8-21						
Weight gain (g)	633	641	629	619	9.20	0.99
Feed intake (g)	881	809	821	812	15.1	0.29
FCR	1.40	1.26	1.30	1.31	0.02	0.17
Feed efficiency (%)	71.9	79.9	77.0	76.6	1.30	0.18
Performance index	355	360	353	348	5.17	0.88
Days 22-35						
Weight gain (g)	1017	1021	999	921	17.2	0.13
Feed intake (g)	1843^{a}	1740^{a}	1716^{ab}	1632^{b}	24.4	0.01
FCR	1.81	1.71	1.73	1.77	0.02	0.44
Feed efficiency (%)	55.4	58.6	58.0	56.6	0.78	0.48
Performance index	378	401	395	347	10.9	0.29
Days 8-35						
Weight gain (g)	1453^{a}	1413^{a}	1392^{ab}	1323^{b}	16.6	0.03
Feed intake (g)	2486^{a}	2430^{a}	2382^{ab}	2229^{b}	33.3	0.03
FCR	1.71	1.74	1.71	1.69	0.01	0.49
Feed efficiency (%)	58.6	58.6	58.5	59.4	0.44	0.89
Performance index	367	381	374	347	7.13	0.38

[a,b]Means marked with superscript letter in the same row are significantly different ($P \leq 0.05$) CONT: control diet containing 1% limestone as an inorganic calcium source, EGFL: feed containing 1% eggshell powder as an organic calcium source, EEG1: feed containing 1% eggshell extracted with *A. bilimbi* L. fruit filtrate as an organic calcium source, EEG05: feed containing 0.5% eggshell extracted using *A. bilimbi* L. fruit filtrate, FCR: feed conversion ratio, SEM: standard error of the means.

Table 4. Relative internal organ weight of broiler chickens

Measured parameters (% live BW)	CONT	EGFL	EEG1	EEG05	SEM	P value
Spleen	0.08	0.08	0.09	0.06	<0.01	0.12
Thymus	0.16	0.13	0.13	0.10	0.01	0.08
Bursa of Fabricius	0.04	0.04	0.04	0.03	<0.01	0.42
Duodenum	0.55	0.64	0.56	0.66	0.02	0.93
Jejunum	1.17	1.22	1.31	1.16	0.03	0.57
Ileum	0.91	1.03	0.97	0.93	0.04	0.78
Caecum	0.32	0.37	0.31	028	0.01	0.10
Heart	0.41	0.37	0.41	0.41	0.01	0.33
Liver	2.35	2.10	2.33	2.06	0.06	0.20
Proventriculus	0.43	0.42	0.45	0.43	0.01	0.87
Gizzard	1.61^{a}	1.39^{b}	1.51^{ab}	1.60^{a}	0.03	0.05
Pancreas	0.27	0.26	0.26	0.27	0.09	0.89
Abdominal fat	0.87	0.86	0.98	0.90	0.07	0.94

^{a,b}Means marked with superscript letter in the same row are significantly different (P≤0.05)

CONT: control diet containing 1% limestone as an inorganic calcium source, EGFL: feed containing 1% eggshell powder as an organic calcium source, EEG1: feed containing 1% eggshell extracted with *A. bilimbi* L. fruit filtrate as an organic calcium source, EEG05: feed containing 0.5% eggshell extracted using *A. bilimbi* L. fruit filtrate, BW: body weight, SEM: standard error of the means.

Table 5. Complete blood counts of broiler chickens

Measured parameters	CONT	EGFL	EEG1	EEG05	SEM	P value
Erythrocytes (10^{12}/L)	2.09	2.26	2.06	1.90	0.06	0.27
Haemoglobin (g/dL)	7.39	8.11	7.71	7.13	0.19	0.28
Haematocrits (%)	35.9	38.3	35.9	32.5	1.10	0.33
MCV (fl)	171	170	174	172	0.61	0.24
MCH (pg)	35.7	36.5	37.5	38.5	0.68	0.54
MCHC (g/dL)	20.4	20.7	20.9	22.1	0.42	0.54
RDW-SD (10^{-15} L)	51.5	49.9	50.5	48.3	0.88	0.99
RDW-CV (%)	10.4	10.1	10.0	9.73	0.18	0.72
MPV (10^{-15} L)	7.23	6.33	6.41	6.61	0.19	0.99
PDW (%)	6.90	5.13	5.00	5.31	0.40	0.33
Leukocytes (10^{9}/L)	64.8	68.1	65.6	64.1	1.82	0.89
Heterophils (10^{9}/L)	3.21	4.26	3.60	4.11	0.33	0.71
Lymphocytes (10^{9}/L)	61.6	63.9	62.0	59.9	1.76	0.90
Thrombocytes (10^{9}/L)	70.7	85.1	76.0	71.0	8.18	0.93

CONT: control diet containing 1% limestone as an inorganic calcium source, EGFL: feed containing 1% eggshell powder as an organic calcium source, EEG1: feed containing 1% eggshell extracted with *A. bilimbi* L. fruit filtrate as an organic calcium source, EEG05: feed containing 0.5% eggshell extracted using *A. bilimbi* L. fruit filtrate, MCH: mean corpuscular haemoglobin, MCV: mean corpuscular volume, MCHC: mean corpuscular haemoglobin concentration, RDW-CV: red blood cell distribution width-coefficient variation, RDW-SD: red blood cell distribution width-standard deviation, PDW: platelet distribution width, MPV: mean platelet volume, SEM: standard error of the means.

CONT, EGFL and EEG05 broilers. Uric acid levels were higher (P<0.05) in the serum of EE-G1 than in EGFL and EEG05, but did not differ from that of CONT broilers. Among the treatment groups, the levels of SGPT were lowest (P<0.05) in EEG05 birds. The serum levels of total cholesterol, LDL, HDL, total protein, globulin, creatinine, and SGOT did not vary (P>0.05) among the dietary treatment groups (Tabel 6).

Bacterial Populations and Morphology of the Intestine of Broiler chickens

Dietary treatments did not exert a substantial effect (P>0.05) on the numbers of coliform and lactic acid bacteria in the ileum and caecum of broiler chickens (Table 7). Data on the intestinal villous height and crypt depth of broiler chickens are presented in Table 8. Villi height, crypt depth, and villous height to crypt depth ratio of duodenum, jejunum, and ileum or broilers did not differ (P>0.05) among the treatment diets. Yet, there was a tendency (P=0.09) for the jejunal crypt depth to be lower in EEG1 and EEG05

Table 6. Serum biochemistry of broiler chickens

Measured parameters	CONT	EGFL	EEG1	EEG05	SEM	P value
Total cholesterol (mg/dL)	96.8	99.8	101	92.0	3.56	0.82
Total triglyceride (mg/dL)	87.0^{a}	58.6^{b}	51.0^{b}	51.7^{b}	5.49	0.05
LDL (mg/dL)	27.4	30.9	25.4	23.2	2.87	0.83
HDL (mg/dL)	52.0^{b}	56.1^{ab}	65.7^{a}	58.4^{a}	1.83	0.05
Total protein (g/dL)	2.73	2.59	3.01	2.61	0.08	0.27
Albumin (g/dL)	1.12^{b}	1.13^{b}	1.26^{a}	1.09^{b}	0.02	0.01
Globulin (g/dL)	1.61	1.47	1.75	1.52	0.07	0.58
Uric acid (mg/dL)	6.67^{ab}	5.80^{b}	7.57^{a}	6.142^{b}	0.22	0.02
Creatinine (mg/dL)	0.06	0.07	0.07	0.05	0.01	0.65
SGOT (U/L)	220	221	229	231	6.88	0.93
SGPT (U/L)	7.51^{a}	5.71^{b}	6.03^{ab}	3.59^{c}	0.36	<0.01

^{a,b}Means marked with superscript letter in the same row are significantly different (P≤0.05) CONT: control diet containing 1% limestone as an inorganic calcium source, EGFL: feed containing 1% eggshell powder as an organic calcium source, EEG1: feed containing 1% eggshell extracted with *A. bilimbi* L. fruit filtrate as an organic calcium source, EEG05: feed containing 0.5% eggshell extracted using *A. bilimbi* L. fruit filtrate, LDL: low-density lipoprotein, HDL: high-density lipoprotein, SGOT: serum glutamic oxaloacetic transaminase, SGPT: serum glutamic pyruvic transaminase, SEM: standard error of the means.

Table 7. Selected bacteria count in the ileum and caecum of broiler chickens

Measured parameters	CONT	EGFL	EEG1	EEG05	SEM	P value
Ileum (Log cfu/g)						
LAB	3.85	4.37	3.68	3.81	0.37	0.93
Coliform	2.03	1.47	<1.00	<1.00	0.18	0.13
LNE	1.34	<1.00	<1.00	<1.00	0.08	0.41
Enterobacteriaceae	1.75	1.48	<1.00	<1.00	0.14	0.12
LAB/coliform	2.36	3.67	3.68	3.81	0.39	0.53
Caecum (Log cfu/g)						
LAB	7.71	7.77	7.35	7.62	0.15	0.79
Coliform	3.10	3.96	2.89	2.49	0.24	0.16
LNE	1.46	1.78	1.32	<1.00	0.15	0.34
Enterobacteriaceae	3.41	3.97	2.89	2.50	0.24	0.15
LAB/coliform	2.97	1.99	3.83	4.10	0.41	0.28

^{a,b}Means marked with superscript letter in the same row are significantly different (P=0.05)
CONT: control diet containing 1% limestone as an inorganic calcium source, EGFL: feed containing 1% eggshell powder as an organic calcium source, EEG1: feed containing 1% eggshell extracted with *A. bilimbi* L. fruit filtrate as an organic calcium source, EEG05: feed containing 0.5% eggshell extracted using *A. bilimbi* L. fruit filtrate, LAB: lactic acid bacteria, LNE: Lactose-negative *Enterobacteriaceae*, SEM: standard error of the means, "<": suggests that some of the observations from which the mean was derived had values lower than the limits of detection. The detection level was applied and used to perform the calculations when the colonies could not be counted on the plates. As a result, the true mean value is lower than the stated value.

compared to CONT and EGFL broilers.

DISCUSSION

Our current study showed that on days 8-21, organic calcium derived from eggshell waste resulted in productive parameters corresponding to using limestone as an inorganic calcium source. At the finisher phase (days 22-35) and throughout the study period (days 8-35) the use of eggshell powder or eggshell extract at 1% of feeds as the substitute for limestone (1% of feed) had no adverse effect on the productive performance of broilers. This may suggest that eggshell powder or eggshell extract can replace limestone as a calcium source for broiler chickens. This finding was in line with Novack *et al.* (2023), revealing that egg shells (industrial egg residue) can totally substitute limestone as an inorganic calcium source in broiler feeds. Yet, with respect to the level in feed, the use of eggshell extract at 0.5% of feed compromised feed intake and weight gain of broilers during finisher and for the entire period of dietary treatment, as compared to control. Previously we expected that soaking (extraction) of eggshell powder using organic acids would result in increased mineral content of eggshells, particularly calcium (Siddique *et al*., 2016). On this basis, we formulated the feed with 0.5% eggshell extracted using *A. bilimbi* L. fruit filtrate. Yet, considering that the soaking or extraction of eggshell powder using *A. bilimbi* L. fruit filtrate resulted in a minor increase in calcium content (32.28% *vs.* 33.1%% for eggshell powder and eggshell extract, respectively), the inclusion of eggshell extract at 0.5% of feed may cause inadequate calcium supply for the EEG05 chicks. Note that calcium is crucial for digestive enzyme activities (such as amylase, lipase, and trypsin) and intestinal development of broiler (Xing *et al*., 2020). Therefore, the low calcium supply may imply a lower feed intake and, hence the growth rate of the broiler in the current study.

There was a tendency for the thymus' relative weight to be lower in EEG05 than that of CONT birds in the present study. The study confirmed that calcium adequacy is positively correlated with the development of lymphoid organs in chickens (Xing *et al*., 2020). In this case, adequate calcium contributes to improving microbial populations (increasing *Lactobacillus* and *Bifidobacterium* populations and decreasing *Escherichia coli* and *Salmonella* populations) in the digestive tract so that it indirectly has a positive effect on the development of lymphoid tissues and organs in poultry (Xing *et al*., 2020). Li *et al*. (2022) supported this inference and reported that the improved bacterial community was attributed to the enhanced thymus index of broiler chickens.

Except for gizzard, the use of eggshell powder or eggshell extract had no substantial effects on the relative internal organ weight of broilers. This finding suggested that eggshell powder or eggshell extract can be used as a substitute for limestone in broiler feeds. In line with our results, Suthama *et al*. (2021) reported no difference in the relative weight of broiler chickens' internal organs (i.e., *Bursa of Fabricius* and spleen). Regarding the gizzard, the relative weight of such organs was lower in EGFL than that of other birds. In this study, the eggshell powder and extract were fine powder, while limestone was coarse particles. Indeed, coarse materials have been reported to increase the gizzard development of poultry and, on the other hand, fine materials showed a negative effect on the development of gizzard (Naderinejad *et al*., 2016). In this regard, the coarse limestone seemed to increase the development of the gizzard, whereas the fine eggshell powder negatively affected the gizzard development. In relation to the eggshell extract, the organic acid content (derived from *A. bilimbi* L. fruit filtrate) of eggshell extract was most likely to increase the relative weight of gizzard. This inference was supported by Sultan *et al*. (2015), who revealed that organic acid increased the gizzard weight in Japanese quails. Likewise, Martínez *et al*. (2021) showed that organic acids increased the relative weight of gizzard in neonatal broilers. Yet, this inference should be taken cautiously as there was no notable difference in gizzard relative weight between EEG1 and EGFL birds.

Data in the present study demonstrated no variations in complete blood counts of broilers receiving either limestone (as an inorganic calcium source), eggshell powder or eggshell extract (as organic calcium source). Considering that calcium is essential in hematopoiesis (Paredes-Gamero *et al*., 2012), this finding, therefore, confirmed that the use of eggshell powder or eggshell extract as the substitute for limestone did not exert any adverse effects on the hematopoiesis and blood profile of broiler chickens.

Our current data showed that using eggshell powder or eggshell extract as organic calcium source for broilers reduced total triglyceride in blood serum. It was demonstrated that feeding organic calcium increased the calcium bioavailability of the chicks (Henry and Pesty, 2002). Indeed, calcium in the body may react with fat in broilers' digestive tract, leading to the formation of calcium soaps, which are then excreted. Our finding agreed with human study showing that high-calcium feed was attributed to the higher fat excretion resulting in decreased serum triglyceride levels (Boon *et al*., 2007). It was apparent in this study that serum HDL levels were higher in the birds receiving 1% or 0.5% eggshell extracted with *A. bilimbi* L. fruit filtrate when compared with the birds provided with limestone as an inorganic calcium source. HDL is a good cholesterol that returns fats and cholesterol from the blood to the liver for excretion. In the current study, the lower blood triglyceride levels in the chicks receiving eggshell extract corresponded with the higher HDL blood levels in the respective chicks. The reason for the increase in HDL levels in the serum of broiler chickens that received eggshell extract as an organic calcium source is unknown. It can be seen in the study of Pratama *et al*. (2021) that administering *A. bilimbi* L. fruit filtrate increased HDL levels in the serum of broilers. As explained by the latter authors, the organic acid and LAB content in the *A. bilimbi* L. filtrate used for soaking medium (solvent extraction) in the present investigation might have been responsible for the increased HDL level in the blood of broiler chickens.

It has been shown in the current study that dietary inclusion of 1% eggshell extracted with *A. bilimbi* L. fruit filtrate as an organic calcium source resulted in higher levels of albumin in the serum of broilers, when compared with other birds. The exact rationale for this phenomenon remains unclear, but it was most likely that organic acids in eggshell extract may contribute to the increased albumin content in serum. This inference was supported by the study of Capcarova *et al*. (2014), which revealed that dietary treatment with citric and acetic acids increased the albumin levels in the blood of broilers. The latter authors further explain that the increase in serum albumin content was associated with the improved absorption of amino acids in acidic conditions that may consequently increase protein synthesis. Yet, the increased serum albumin level was not observed in the EEG05 birds. As the level of eggshell extract in feed was lower than that of EEG1, the content of organic acids was possibly insufficient to lead to the acidic condition in the gastrointestinal tract of broilers. Yet, this assumption should be taken carefully as we did not measure the pH values of the gastrointestinal tract of the broiler in our current study.

In the present study, the uric acid level was higher in EEG1 compared to other treatment groups. Many studies have confirmed uric acid to be a powerful antioxidant in poultry; hence, reduced blood uric acid levels may be associated with increased oxidative stress (Simoyi *et al*., 2002; Stinefelt *et al*., 2005). Owing to this fact, the increased serum uric acid concentration in EEG1 seemed to be associated with the improved antioxidative status of the respective chicks. In this study, the increase in serum uric acid was in line with the increased albumin levels in the serum of broilers. Previously, Capcarova *et al*. (2014) suggested that serum albumin is an important circulating antioxidant in the body, which shows direct protective effects against free radicals. Taking all these facts together, the dietary inclusion of 1% eggshell extracted with *A. bilimbi* L. fruit filtrate as an organic calcium source could positively affect broilers' antioxidative status. In this case it was very likely that organic acids originating from the *A. bilimbi* L. fruit filtrate had a major role in the antioxidant status in the chicken's body. In line with this, Khan *et al*. (2022) reported improvements in antioxidant status in broiler chickens given organic acids in the feed.

It has commonly been suggested that high levels of SGOT and SGPT in the serum may indicate a problem in the liver. In this current study, SGOT and SGPT serum levels did not differ between birds in control and other treatment groups. Indeed, the levels of SGPT are notably lower in birds receiving eggshell powder or eggshell extracted using *A. bilimbi* L. fruit filtrate as the source of organic calcium. This may indicate that substituting the limestone as an inorganic calcium source with eggshell powder or eggshell extract as an organic calcium source did not adversely affect liver health.

Data in the current study did not show any substantial effect of dietary inclusion of 1% eggshell powder and 1% and 0.5% eggshell extracted with *A. bilimbi* L. fruit filtrate on the numbers of lactic acid bacteria and coliform bacteria in the ileum and caecum of broilers. Regarding the calcium source in broiler feeds, the study of Xing et al. (2020) reported that the source of calcium may affect the composition of bacteria in the intestines of broilers. They revealed that, as compared to calcium derived from active dicalcium phosphate, calcium derived from scallop shell powder decreased the numbers of *Lactobacillus* and *Bifidobacterium*, while increasing the numbers of *Escherichia coli* and *Salmonella*. Unlike the study mentioned above, data in the current study did not show any substantial effect of calcium source on the selected bacteria numbers in the ileum and caecum of broilers. Yet, feeding

1% and 0.5% eggshell extracted with *A. bilimbi* L. fruit filtrate as an organic calcium source resulted in a numerical decrease in the numbers of coliform bacteria in the ileum of broilers. It was most likely that organic acids in the eggshell extract exhibited antibacterial activities, resulting in lower counts of pathogenic bacteria in the broiler intestine (Sugiharto, 2016).

Data from our current study indicated that jejunal crypt depth tended to be lower in broilers fed eggshells extracted with *A. bilimbi* L. fruit filtrate as an organic calcium source compared to those of fed limestone. More significant amounts of damaged enterocytes are typically correlated with deeper crypts. Here, stem cells in the crypt give rise to progenitor cells, which multiply quickly, move up the villus, and eventually differentiate into secretory and absorptive enterocytes. To replace the damaged enterocytes, the replicating pool of enterocytes is increased by increasing the crypt depth (Tan *et al*., 2014). The deeper crypt may be a sign of more damaged villus enterocytes, which would reduce absorption capacity. It is still unclear why EEG1 and EEG05's crypt depth is lower than that of CONT and EGFL. However, given that there was no discernible difference in crypt depth between CONT and EGFL birds, it appeared that the organic acid derived from the *A. bilimbi* L. fruit filtrate was responsible for the lower crypt depth in EEG1 and EEG05. According to Sugiharto (2016), organic acids may prevent damaged enterocytes in broilers by improving the microbial composition of the intestine and reducing pathogenic bacteria. Hence, the migration of progenitor cells from the crypt to the villus may be reduced.

CONCLUSION

Using 1% eggshell powder or eggshell extract in feed as the substitute for limestone had no detrimental effect on broiler chickens' growth, physiological status, and intestinal condition. Hence, eggshell powder or eggshell extracted with *A. bilimbi* L. fruit filtrate can be used as an organic calcium source to replace limestone. However, compared to using 1% limestone, 0.5% eggshell extract as an organic calcium source did not produce the same results.

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