

## Dietary nano-dicalcium phosphate improves immune response and intestinal morphology of broiler chickens

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### Abstract

Various levels of substitution of nano-dicalcium phosphate ( $nCaHPO_4$ ) for the calcium and phosphorus supplied by limestone and monocalcium phosphate were evaluated, based on weight change, serum constituents, immune response and intestinal morphology of broiler chickens. The birds were assigned to five dietary treatments containing 0% (control), 40%, 60%, 80%, and 100% substitution levels of  $nCaHPO_4$  from days 0 to 42. Data were collected for blood measurement and intestinal morphology and analysed with one-way analysis of variance. Feeds substituted with 40%  $nCaHPO_4$  showed a higher mean value of final weight and better feed conversion. The results revealed similar haematological parameters across the treatment groups. At the same time,  $nCaHPO_4$  influenced most of the serum biochemical constituents, such as calcium, phosphorus, glucose, albumin, and total protein. High concentrations of total protein were observed for birds fed 60%  $nCaHPO_4$  compared with other treatments. The highest comparable means for albumin were observed in birds fed 40% and 60%  $nCaHPO_4$ . Cholesterol levels were increased when birds were fed 0% and 40% of diets. Elevated serum phosphorus concentration was observed when 0%, 40%, 60% and 100% substitution levels were fed, whereas 80% presented the least mean value. The results also showed that birds fed 40% nano-dicalcium phosphate had the highest villi length, which signified improved absorption of minerals. In conclusion, 40% and 60% levels of  $nCaHPO_4$  could be used to boost immune response and functional intestinal morphology of broiler chicken with no negative effect on haematological parameters.

**Keywords:** blood profile, intestinal morphology, nanoparticles, poultry

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### Introduction

Nanotechnology is gaining ground in veterinary and animal sciences by supplying minute scales that are vital for living organisms. The application of minerals in nano form showed improvement in feeding efficiency, minimizing losses from animal diseases, and converting animal by-products into value-added products (Chen & Yada, 2011). Nanomaterials have potential in a range of environmental applications owing to their extremely small particle size, large surface area, and high reactivity (Huang *et al.*, 2015). Research into the adoption of nanominerals in poultry production is necessary to grasp the advantages of nanomaterials and to curb food insecurity. McDonald *et al.* (2010) documented that various body processes require adequate amounts of minerals for proper growth and development. Minerals occur in body fluids and tissues as electrolytes concerned with the maintenance of osmotic pressure, acid-base balance, membrane permeability and transmission of nerve impulses.

The utilization rate of nanominerals is much higher than that of conventional inorganic and organic minerals because nanoparticles may enter the animal body through direct penetration (Talaat, 2017). Nanominerals can penetrate the small intestines and distribute into the blood, brain, lung, heart, kidney, spleen, liver, and intestine by absorption in the stomach (Hillyer & Albrecht, 2001) for immediate use and to rectify anomalies.

Modern commercial broilers are always faced with challenges, which include immunological stress, pathogen infection and environmental stress. These challenges instigate non-essential immune responses, resulting in inadequate production, host tissue damage and clinical diseases (Nie *et al.*, 2018). Immunological and oxidative stress can be caused by lipopolysaccharide intolerance and other factors. These might affect the physiological and biochemical processes of animals and interfere with their normal metabolism and functions, resulting in production losses (Nie *et al.*, 2018). To achieve optimum growth and production potential in modern broilers, poultry diets must be formulated to provide birds with all required nutrients in adequate amounts (Daghir, 2009). This study therefore set out to evaluate the influence in broiler diets of various levels of nano-dicalcium phosphate on blood parameters, immune response and intestinal morphology. Its results would assist farmers and animal nutritionists to establish the most efficient levels of  $nCaHPO_4$  during the production period.

## Materials and Methods

The experiment commenced after ethical approval by North-West University Animal Production Sciences Research Ethics Committee (NWU-AnimalProdREC) with approval number NWU0070118A5. The study was carried out on Rooigront Farm in North West, which is located 16 km south-east of the capital city, Mafikeng. The annual rainfall of this area is between 300 and 700 mm. Summer rainfall occurs between October and March, and winter brings dry sunny days and chilly nights. The geographical coordinates are 25 ° 55 ' 0 " S, 25 ° 48 ' 0 " E.

A total of 240 day-old broiler chicks were used. Nano-dicalcium phosphate ( $nCaHPO_4$ ) with a particle size of 40 nm was purchased from Mknano, Ontario, Canada. All diets were formulated in accordance with the requirements for broiler chickens (Applegate & Angel, 2014). Dietary treatments were created by replacing conventional monocalcium phosphate and limestone with  $nCaHPO_4$ . The starter, grower and finisher diets were formulated by a commercial feed manufacturer in Centurion, Gauteng, South Africa with replacement levels of 0%, 40%, 60%, 80%, and 100%  $nCaHPO_4$  (Table 1). The diets were isocaloric within a growth phase, but increased progressively in energy density from the starter phase to the finishing phase. The nutritional values of the diets are shown in Table 2. The five treatments were each replicated four times. Day-old chicks were weighed individually and distributed randomly in groups of twelve birds per replicate. In preparation for the arrival of the birds the house was cleaned thoroughly and disinfected and the drinkers and feeding troughs were ashed, clean and dried. Fresh water and feeds were given to the chickens daily ad libitum. Lights were switched on continuously for the first two weeks of the experiment. Fresh wood shavings were used for bedding and were spread to a depth of seven centimetres. The birds were housed in floor pens and the dietary treatments were randomly allocated to the pens.

Blood samples were collected at the end of the feeding trial (six weeks) from four randomly selected birds from each replicate. The wing vein of birds was punctured using the needle set and syringes of about 5 ml. EDTA vacutainer tubes were used to collect about 2 ml of plasma, and another 2 ml was collected in the red stoppered tubes for serum. The blood samples for haematology were placed in a cooler box with ice and transported to the laboratory for analysis. Red blood cells, haemoglobin, monocytes, eosinophil, and lymphocytes were determined with IDEXX LaserCyte. The blood chemistry analysis was carried out with IDEXX VetTest Chemistry Analyser and total protein, albumin, total cholesterol, calcium, inorganic phosphorus, globulin, alanine transaminase, and alkaline phosphatase were analysed.

To establish intestinal morphology, twelve birds per treatment were randomly selected and slaughtered. The small intestine tissue sample (duodenum, jejunum and ileum) fragments were obtained later and fixed in a mixture of 10% buffered formalin solution, processed with paraffin embedding, and stained with haematoxylin and eosin (H&E) (Anwar, 2013). Measurements of the cross-sections of the slides were then evaluated under a microscope to obtain the length of villi and width for the duodenum, jejunum and ileum using Zen software.

The GLM procedure of SAS (SAS Institute, Inc., Cary, North Carolina, USA) was used to analyse data for serum constituents and intestinal morphology using this linear model:

$$Y_{ij} = \mu + D_i + e_{ij}$$

where:  $Y_{ij}$  = a dependent variable recorded for the  $j$ th bird that was fed the  $i$ th diet ( $D_i$ ),

$\mu$  = the overall mean, and

$e_{ij}$  = the random error associated with an observation.

Collectively the errors were assumed to be normally and independently distributed. For all statistical tests, significance was declared at  $P < 0.05$ . Least square means were compared using the PDIF option of the LSMEANS statement.

**Table 1** Ingredients in starter, grower and finishing diets for broilers formulated with various levels of nano-dicalcium phosphate to replace limestone and monocalcium phosphate

Ingredients, g/kg	Nano-dicalcium phosphate level														
	0%			40%			60%			80%			100%		
	S	G	F	S	G	F	S	G	F	S	G	F	S	G	F
Yellow maize	595.3	634.2	651.6	598.9	636.8	653.9	600.7	638.1	654.8	602.4	639.3	655.8	604.7	640.9	657.0
Prime gluten			10.0			10.0			10.0			10.0			10.0
Extruded full fat soybean	120.5	120.0	120.0	113.9	120.0	120.0	110.7	120.0	120.0	107.4	120.0	120.0	103.2	120.0	120.0
Soybean	210.5	168.4	138.7	215.0	168.1	138.5	217.2	167.9	138.4	219.5	167.8	138.2	222.5	167.6	138.1
Sunflower	35.0	35.0	35.0	35.0	35.0	35.0	35.0	35.0	35.0	35.0	35.0	35.0	35.0	35.0	35.0
Hydroxyapatite				3.64	3.14	2.31	5.46	4.71	3.47	7.29	6.27	4.63	9.67	8.29	6.12
Limestone	12.52	11.93	11.14	10.76	10.42	10.03	9.88	9.67	9.47	9.00	8.91	8.91	7.85	7.94	8.20
Monocalcium phosphate	9.11	7.84	5.78	5.68	4.88	3.60	3.96	3.39	2.50	2.25	1.91	1.41			
Salt	1.79	1.83	2.01	1.79	1.83	2.01	1.78	1.83	2.01	1.78	1.83	2.01	1.78	1.83	2.01
Sodium bicarbonate	2.19	2.15	1.52	2.19	2.15	1.53	2.20	2.15	1.53	2.20	2.15	1.53	2.20	2.15	1.53
DL-Methionine	3.19	2.74	2.17	3.19	2.73	2.16	3.19	2.73	2.16	3.18	2.73	2.16	3.18	2.73	2.16
L-Threonine	4.06	3.66	2.74	4.07	3.66	2.75	4.07	3.66	2.75	4.06	3.66	2.75	4.07	3.66	2.75
Soybean oil mixer		6.76	14.34		5.88	13.68		5.44	13.35		5.00	13.02		4.43	12.60
Lignobond	2.00	2.00	2.00	2.00	2.00	2.00	2.00	2.00	2.00	2.00	2.00	2.00	2.00	2.00	2.00
Broiler premix	3.00	2.50	2.00	3.00	2.50	2.00	3.00	2.50	2.00	3.00	2.50	2.00	3.00	2.50	2.00
AxtraPhy phytase enzyme	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10
Salinomycin 12%	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50

S: starter diet, G: grower diet, F: finishing diet

**Table 2** Nutritional contents of starter, grower and finishing diets for broilers formulated with various levels of nano-dicalcium phosphate to replace limestone and monocalcium phosphate

Nutrients	Nano-dicalcium phosphate level														
	0%			40%			60%			80%			100%		
	S	G	F	S	G	F	S	G	F	S	G	F	S	G	F
Dry matter, g/kg	885.4	884.6	884.4	885.1	884.3	884.2	885.0	884.2	884.1	884.9	884.1	884.0	884.8	883.9	883.9
ME poultry, MJ/kg	12.6	12.9	13.3	12.6	12.9	13.3	12.6	12.9	13.3	12.6	12.9	13.3	12.6	12.9	13.3
Crude protein, g/kg	208.1	190.6	182.9	208.1	190.7	183.0	208.1	190.7	183.0	208.2	190.7	183.0	208.2	190.8	183.0
Lysine, g/kg	13.20	11.90	10.59	13.20	11.90	10.59	13.20	11.90	10.59	13.20	11.90	10.59	13.20	11.90	10.59
Methionine, g/kg	6.47	5.80	5.22	6.46	5.80	5.22	6.46	5.80	5.22	6.46	5.79	5.22	6.46	5.79	5.21
Sulfur amino acids, g/kg	9.80	8.91	8.28	9.80	8.91	8.28	9.80	8.91	8.28	9.80	8.91	8.28	9.80	8.91	8.28
Threonine, g/kg	8.83	7.95	7.50	8.83	7.95	7.50	8.83	7.95	7.50	8.83	7.95	7.50	8.82	7.95	7.50
Tryptophan, g/kg	2.48	2.23	2.08	2.48	2.23	2.08	2.48	2.23	2.08	2.48	2.23	2.08	2.48	2.23	2.08
Available phosphorus, g/kg	4.50	4.20	3.80	4.50	4.20	3.80	4.50	4.20	3.80	4.50	4.20	3.80	4.50	4.20	3.80
Calcium, g/kg	9.00	8.40	7.60	9.00	8.40	7.60	9.00	8.40	7.60	9.00	8.40	7.60	9.00	8.40	7.60
Chloride, g/kg	2.00	2.00	2.00	2.00	2.00	2.00	2.00	2.00	2.00	2.00	2.00	2.00	2.00	2.00	2.00
Sodium, g/kg	1.70	1.70	1.70	1.70	1.70	1.70	1.70	1.70	1.70	1.70	1.70	1.70	1.70	1.70	1.70
Total phosphorus, g/kg	5.93	5.49	4.95	5.90	5.47	4.94	5.89	5.47	4.94	5.88	5.46	4.93	5.87	5.45	4.92

S: starter diet, G: grower diet, F: finishing diet

## Results and Discussion

Initial weight, average daily gain and average feed intake were not ( $P > 0.05$ ) influenced by supplementation with various levels of nCaHPO<sub>4</sub> (Table 3). However, final weight gain (kg) and feed conversion ratio of birds were ( $P < 0.05$ ) affected by the treatments. Birds fed 40% nCaHPO<sub>4</sub> had the highest final weight, which was greater than those not supplemented with nCaHPO<sub>4</sub>. Birds fed the other diets had final weights that were intermediate between these extremes. Birds fed 40% nCaHPO<sub>4</sub> were most efficient, whereas those fed 80% nCaHPO<sub>4</sub> had the greatest feed conversion ratio.

**Table 3** Effect of various dietary levels of levels of nano-dicalcium phosphate on growth performance of broiler chickens

Performance trait	Nano-dicalcium phosphate level					SE
	0%	40%	60%	80%	100%	
Initial weight	241.20	242.45	243.93	231.20	244.78	5.10
Final weight	1592.25 <sup>b</sup>	1715.10 <sup>a</sup>	1628.00 <sup>ab</sup>	1626.85 <sup>ab</sup>	1638.78 <sup>ab</sup>	5.35
Average daily gain	48.26	52.58	49.43	49.85	49.78	0.72
Daily feed intake	68.75	64.48	70.03	73.10	70.08	1.49
Feed conversion ratio	1.43 <sup>ab</sup>	1.23 <sup>b</sup>	1.40 <sup>ab</sup>	1.48 <sup>a</sup>	1.40 <sup>ab</sup>	0.03

<sup>a,b</sup> Within a row, values with a common superscript were not different with probability  $P = 0.05$

The levels of nCaHPO<sub>4</sub> provided in the diets had no detectable effects ( $P > 0.05$ ) on the haematological profile of the broiler chickens (Table 4).

**Table 4** Influence of various dietary levels of nano-dicalcium phosphate on haematology of broiler chickens

Value	Nano-dicalcium phosphate level					SE	P-value	Reference ranges
	0%	40%	60%	80%	100%			
HGB, g/dL	6.74	6.55	6.10	8.85	6.33	0.49	0.084	0.09 - 8.05 <sup>1</sup>
RBC, × 10 <sup>6</sup> /μL	2.27	1.83	2.50	2.00	2.43	0.11	0.411	0.04 - 2.69 <sup>1</sup>
MONO, %	7.20	6.33	9.00	7.50	13.33	1.03	0.325	0.90 - 10.51 <sup>1</sup>
EOS, %	2.28	2.40	3.63	2.60	3.00	0.28	0.575	1.50 - 3.40 <sup>2</sup>
LYM, %	79.50	83.50	79.05	73.98	61.73	3.23	0.116	3.49 - 52.50 <sup>1</sup>

HGB: haemoglobin, RBC: red blood cell count, MONO: monocytes, EOS: eosinophils, LYM: lymphocytes  
Sources: <sup>1</sup>Kuttappan *et al.* (2013), <sup>2</sup>Abdi-Hachesoo *et al.* (2011)

With the exception of alkaline phosphatase, the serum biochemical constituents were influenced ( $P < 0.05$ ) by various levels of nCaHPO<sub>4</sub> (Table 5). Total protein concentration in the serum was increased for birds fed the 60% nCaHPO<sub>4</sub> diet compared with the other treatments. Serum albumin was lower in birds fed the 0%, 80%, and 100% levels of nCaHPO<sub>4</sub> and higher in those provided the 40% and 60% diets. The birds that were fed the 0%, 40%, 60%, and 80% nCaHPO<sub>4</sub> diets had higher globulin levels than those fed the 100% diet. Birds fed the 60% nCaHPO<sub>4</sub> diet had higher levels of alanine transaminase in the serum, followed by the 0% and 100% groups and those fed the 40% and 80% diets had the lowest values. Cholesterol levels were higher when the 0% and 40% diets were fed compared with those from bird fed diets containing higher levels of nCaHPO<sub>4</sub>. Similar high mean values of serum calcium and phosphorus concentrations were observed when 0%, 40%, 60%, and 100% diets were fed compared with 80% nCaHPO<sub>4</sub>.

**Table 5** Effect of various levels of nano-dicalcium phosphate on serum constituents of boiler chickens

Constituents	Nano-dicalcium phosphate level					SE	P-value	Reference ranges
	0%	40%	60%	80%	100%			
TP, g/L	82.33 <sup>b</sup>	81.67 <sup>b</sup>	115.67 <sup>a</sup>	86.00 <sup>b</sup>	71.33 <sup>b</sup>	5.34	0.047	23.8 - 52.2 <sup>1</sup>
ALB, g/L	22.33 <sup>b</sup>	25.00 <sup>ab</sup>	33.00 <sup>a</sup>	20.67 <sup>b</sup>	23.67 <sup>b</sup>	1.54	0.035	11.7 - 52.2 <sup>1</sup>
GLU, g/ L	59.00 <sup>ab</sup>	56.00 <sup>ab</sup>	81.00 <sup>a</sup>	51.33 <sup>ab</sup>	48.00 <sup>b</sup>	4.76	0.032	11 - 27.75 <sup>2</sup>
ALT, IU/ L	69.00 <sup>bc</sup>	58.67 <sup>cd</sup>	92.33 <sup>a</sup>	52.50 <sup>d</sup>	75.33 <sup>b</sup>	4.16	0.043	19 to 50 <sup>3</sup>
ALKP, U/L	1893.0	1597.7	1739.7	1947.0	1761.7	64.6	0.002	568 - 8,831 <sup>1</sup>
CHOL, mmol/L	5.08 <sup>a</sup>	4.95 <sup>a</sup>	3.80 <sup>b</sup>	4.06 <sup>b</sup>	4.01 <sup>b</sup>	0.18	0.023	2.25 - 4.97 <sup>1</sup>
P, mg/ mL	5.13 <sup>ab</sup>	4.64 <sup>ab</sup>	5.80 <sup>a</sup>	4.55 <sup>b</sup>	4.88 <sup>ab</sup>	0.18	0.031	3.59 - 11.40 <sup>1</sup>
Ca, mg/ mL	3.20 <sup>ab</sup>	2.99 <sup>ab</sup>	4.00 <sup>a</sup>	2.54 <sup>b</sup>	2.78 <sup>ab</sup>	0.19	0.031	4.70 - 16.25 <sup>1</sup>

<sup>a,b,c,d</sup> Within a row, values with a common superscript were not different with probability  $P=0.05$

TP: total protein, ALB: albumin, GLU: globulin, ALT: alanine transaminase, ALKP: alkaline phosphatase, CHOL: cholesterol, P: phosphorus, Ca: calcium

Sources: <sup>1</sup>Meluzzi *et al.* (1992), <sup>2</sup>Abdi-Hachesoo *et al.* (2011), <sup>3</sup>Lumeij (1997)

Table 6 indicates the length and width of the ileum, duodenum and jejunum of broilers fed various levels of nano-dicalcium phosphate. All parameters were ( $P < 0.05$ ) influenced by nCaHPO<sub>4</sub>. Birds fed 40% generally had larger intestinal tracts than those fed 0%, 60%, 80%, and 100%. The length of the duodenum was increased when 100% nCaHPO<sub>4</sub> was fed to the birds, with the 80% diet producing a shorter duodenum. Substitution levels of 40% and 60% nCaHPO<sub>4</sub> increased the length of the jejunum compared with the 0%, 80% and 100% diets. The ileum width was reduced when 0% and 80% diets were fed compared with 40%, 60% and 100% nCaHPO<sub>4</sub>. The width of the duodenum was unchanged by the dietary treatments.

**Table 6** Effect of various levels of nano-dicalcium phosphate in broiler diets on intestinal morphology

Measurement	Nano-dicalcium phosphate level					SE
	0%	40%	60%	80%	100%	
Ileum length, $\mu\text{m}$	253.50 <sup>b</sup>	428.12 <sup>a</sup>	325.70 <sup>b</sup>	295.26 <sup>b</sup>	277.56 <sup>b</sup>	18.94
Duodenum length, $\mu\text{m}$	370.86 <sup>bc</sup>	498.76 <sup>ab</sup>	366.83 <sup>bc</sup>	326.39 <sup>c</sup>	507.39 <sup>a</sup>	25.13
Jejunum length, $\mu\text{m}$	450.99 <sup>b</sup>	637.88 <sup>a</sup>	598.43 <sup>a</sup>	390.70 <sup>b</sup>	394.73 <sup>b</sup>	32.61
Ileum width, $\mu\text{m}$	42.50 <sup>b</sup>	74.46 <sup>a</sup>	55.81 <sup>ab</sup>	42.84 <sup>b</sup>	65.16 <sup>ab</sup>	4.52
Duodenum width, $\mu\text{m}$	54.46	79.95	53.63	68.09	72.34	4.60
Jejunum width, $\mu\text{m}$	68.20 <sup>b</sup>	102.05 <sup>a</sup>	68.02 <sup>b</sup>	60.85 <sup>b</sup>	71.36 <sup>ab</sup>	5.39

<sup>a,b,c</sup> Within a row, values with a common superscript were not different with probability  $P=0.05$

The current study is in line with that of Hassan *et al.* (2016), in which they observed that various levels of nCaHPO<sub>4</sub> enhanced bodyweight gain and feed conversion of broiler chicks compared with conventional dicalcium phosphate. In a related study, Ahmadi & Famadi (2018) observed similar results for supplementation with nano-selenium.

The increasing levels of nCaHPO<sub>4</sub> produced no detectable effects on the haematology of the chickens, and all values fell within the normal ranges for healthy chickens (Kuttappan *et al.*, 2013; Abdi-Hachesoo *et al.*, 2011). Thus, these dietary modifications were deemed to have no negative effects that might cause anaemia, infection and negative implications in the birds (Obikaonu *et al.*, 2011). The results of the present study agree with those observed by Vijayakumar and Balakrishnan (2015), who reported that calcium phosphate nanoparticles did not exert a significant influence on haemoglobin, packed cell volume, total erythrocyte count, total leucocyte count, and differential count of white blood cells compared with the

control group fed with conventional dicalcium phosphate. Thus supplementation of calcium phosphate nanoparticles instead of conventional dicalcium phosphate had no effect on the health of the birds, which reinforced the safe use of nanomineral supplements.

Non-significantly different alkaline phosphatase values were within the normal range of 568–8831 U/L (Meluzzi *et al.*, 1992). The significantly higher total protein levels in the current study were above the normal range (23.8 - 52.2 g/L) reported by Meluzzi *et al.* (1992). However, they were similar to those observed by Rezende *et al.* (2017), who reported higher serum protein concentration in female birds. Fallah *et al.* (2018) reported no significant influence of calcium and protein concentrations in broilers at 21 days old. They added that increasing calcium concentration from 4.0 g/kg to 5.5 g/kg raised serum phosphate to a maximum concentration at a later stage. Serum proteins are produced in the liver and their functions include blood maintenance through the colloidal osmotic effect, metabolism regulation, and body defence against foreign agents (Tóthová *et al.*, 2019). Blood plasma proteins play vital roles in the transportation of minerals and hormones (Piotrowska *et al.*, 2011; Rezende *et al.*, 2017). Higher levels of total proteins in the current study indicated that nCaHPO<sub>4</sub> was available in the blood system. Therefore, it would enhance the production of fibrinogen, which is one of the coagulation factors. The results of the current study for increased levels of total protein may be explained by Tóthová *et al.* (2019), who reported that transitions in nutrition, and variations in diet composition and feed consumption during growth alter the metabolic rate and physical condition of growing chickens, which may be associated with changes in total protein in the serum.

Albumin concentrations were similar to those reported by Meluzzi *et al.* (1992) and Rezende *et al.* (2017). On the other hand, globulin concentration (48.00 - 81.00 g/L) was above normal range (11 - 27.75 g/L) recorded by Abdi-Hachesoo *et al.* (2011). Serum albumin can increase gradually with age, reaching the highest level on days 32 and 46 of fattening (Tóthová *et al.*, 2019). This could be attributed to maintaining the metabolic balance during rapid growth. In a similar study, Piotrowska *et al.* (2011) reported the highest value of 17.1g/L for albumin in broiler serum. Al-Beitawi *et al.* (2016) reported that total protein, albumin and globulin (mg/dl) increased significantly at 28 days old when 2% nano-clay minerals were added in broiler feeds. They added that at 36 days old, total protein and globulin concentration were increased when 1% and 2% nano-clay minerals were added compared with other dietary treatments. Cholesterol levels (3.80 - 5.08 mmol/L) in this current study were within the range of 2.25 - 4.97 mmol/l reported by Meluzzi *et al.* (1992). This indicated that an increase in nCaHPO<sub>4</sub> in the feed could help maintain normal levels of cholesterol concentration in the blood. Meanwhile, a similar study (Rezende *et al.*, 2017) showed that the cholesterol levels were higher in broiler females than males. Age, diet, and reproduction activity could also influence cholesterol levels. Findings by Al-Beitawi *et al.* (2016) showed that cholesterol levels, high-density lipoproteins, and triglycerides in the serum of chickens were influenced significantly by the addition of 2% nano-clay minerals once a week or once in two weeks in broiler diets.

In the present study, the patterns of serum calcium and phosphorus were inexplicable with the 60% treatment producing significantly higher levels of both minerals than the 80% treatment. However, neither of these levels were significantly different from those observed at the 0%, 40% and 100% levels and all values were within the normal ranges. Serum calcium concentrations in this study were lower compared with 4.70 - 16.25 mg/ml reported by Meluzzi *et al.* (1992). However, the phosphorus concentrations in this study were comparable with the 4.55 - 580 mg/mL range reported by Meluzzi *et al.* (1992) for clinically healthy birds. The observed difference in serum calcium levels could be attributed to variations because of sex, reproductive organs, high temperatures, age, and forms of calcium phosphate employed in this present study (Dutta *et al.*, 2013). Ansar *et al.* (2004) also reported a significant difference on serum phosphorus level in chicks fed a control diet. The findings of the current study are not in agreement with those observed by Vijayakumar and Balakrishnan (2015), who reported no significant influence among treatments when calcium phosphate nanoparticles were supplemented in broiler diets. Silva *et al.* (2007) were of the opinion that variations in mineral components of blood usually differ with age owing to physiological changes that happen during each developmental phase.

The histology results from this study indicated a significant increase in the length and width of the ileum, jejunum, and duodenum of birds fed various levels of nCaHPO<sub>4</sub>. The increase in villi length and width in the current study could be attributed to improved absorption and utilization because of properties of nanoparticle size. The greater surface area and absorption capacity are associated with longer villi, which results in mature enterocytes on the villus tips, whereas poor nutrient absorption and digestion is an indicator of shorter villi which results in decrease absorptive area and less mature enterocytes (Paiva *et al.*, 2014). Abdulla *et al.* (2016) reported that birds fed 6% soybean and palm oil in combination with levels of calcium, 1%, and 1.25% had greater villi height for duodenum and jejunum compared with those fed linseed oils with 1.5% calcium. They also reported a [thannon-significant difference for the ileum height for all treatments. Pohl *et al.* (2012), in agreement with the current study, observed a significant increase on the duodenal villus height when 10% calcium formate was included in diets of broilers when compared with broilers exclusively

fed calcium carbonate as a calcium source. However, these authors observed a non-significant influence on the villi width of duodenum because of the dietary treatments. Sawors *et al.* (2013) and Al-Beitawi (2016) observed that nano-clay minerals could exert a positive effect on tissue development.

## Conclusion

Replacement of limestone and monocalcium phosphate with  $n\text{CaHPO}_4$  might improve the health status of broilers, as indicated by serum constituents and their increased level of intestinal development. Dicalcium phosphate was more effective and efficient when used in nano form (40% - 60%) than conventional particles, which could be the result of high availability, minute particle size and bioactivity of the nanoparticles. Nano-dicalcium phosphate substituted in diets at levels of 40% and 50% could improve broiler immunity and intestinal morphology.

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## Authors' Contributions

AOY and LEM were in charge of project design and implementation, and writing the manuscript. MDM was in charge of field experimentation and draft write up, and MDM and TOA formatted and proofread the manuscript and rechecked the data analysis methods. AOY and LEM were responsible for data analysis and interpretation.

## Conflict of Interest Declaration

The authors declare that there was no conflict of interest in this research

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