In ovo feeding of organic salts of zinc and copper: effects on growth performance and health status of two strains of broiler chickens

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Feeding of the embryo before hatch by *in ovo* administration of exogenous feed components has been reported to jump start growth and improve immune response in poultry. This study consisted of two experiments to determine the response of Arbor Acre strain of broiler chickens to *in ovo* injection of organic salts of zinc, copper and their combination in Experiment 1 while in Experiment 2, the response of Cobb 500 strain of broiler chickens to organic salts of zinc, copper and their combination was investigated. A total of 300 hatching eggs of Arbor Acre strain of broiler chickens were used for Experiment 1. After candling the eggs on the 14th day of incubation, 148 (59.2%) eggs were fertile. These eggs were distributed into four treatments: control (no *in ovo* supplementation), *in ovo* administration of organic zinc (80 µg/egg), organic copper (16 µg/egg) and combined organic zinc (80 µg/egg) and organic copper (16 µg/egg). In Experiment 2, 300 hatching eggs of Cobb 500 strain of broiler chickens were used. On 14th day of incubation, the eggs were candled and 232 (90.3%) eggs were fertile. The eggs were assigned into four treatment groups as in Experiment 1. *In ovo* injection of eggs was carried out on the 18th day of incubation in both experiments. Hatched chicks, 46 (31% hatchability); 191 (82% hatchability) in Experiments 1 and 2, respectively from each treatment were balanced for weight and the post-hatch response to *in ovo* injection of organic salts of zinc, copper and their combination was studied for 7 and 5 weeks in Experiments 1 and 2, respectively. Data collected were subjected to analysis of variance in a completely randomised design. Results in Experiment 1 indicated that hatchability was highest ($P \leq 0.05$) in eggs on *in ovo* injection of combination of organic zinc and copper while humoral immunity to sheep red blood cell was highest ($P \leq 0.05$) in birds from eggs on *in ovo* injection of organic zinc. Humoral immunity was improved ($P \leq 0.05$) by *in ovo* injection of copper while cell-mediated immunity was also improved ($P \leq 0.05$) by *in ovo* injection of organic zinc in Experiment 2. Growth performance was not significantly ($P > 0.05$) affected by the treatments in both Experiments 1 and 2. The haemoglobin and mean corpuscular haemoglobin were significantly ($P \leq 0.05$) influenced by *in ovo* injection of both organic zinc and copper on day 7 in Experiment 2. The study concluded that *in ovo* injection of organic salts of zinc at 80 µg/egg and/or copper at 16 µg/egg should be adopted to improve immune response in both Arbor Acre and Cobb 500 strains of broiler chickens.

Keywords: *In ovo* feeding, organic trace minerals, growth performance, Arbor Acre, Cobb 500, gut morphology, blood profile

Considering that the formation and early development of tissues occur in the embryonic phase, it is likely that there is a strong relationship between embryonic development and post-hatch performance of broiler chickens (Grodzik et al. 2013). Hence, a physiological and metabolic imbalance in the initial development of the birds during embryonic development could significantly affect the post-hatch growth performance.

Shafey et al. (2012) reported that the nutrients contained in the egg depend on the nutritional status of the hen. However, the rapid growth of the genetically improved bird can make the quantity of nutrients contained in the egg insufficient for the complete development of embryonic tissues (Grodzik et al. 2013) which may impair the initial performance of the birds (Kornasio et al. 2011; Kadam et al. 2013). In order to overcome these problems, *in ovo* feeding technique was developed to increase the availability of nutrients for the embryo. This technique provides a practical means of safely

0041–3216/2022/020146–165

introducing external nutrients into developing embryos (Foye et al. 2007; Kadam et al. 2008; Bello et al. 2014). Feeding the embryo before hatch by *in ovo* administration of external feed components was reported to cause a positive effect on hatchability, development of the digestive tract, body weight and nutritional status of the hatchling (Uni and Ferket 2004).

Copper and zinc are essential nutrients in broiler chicken diets and they play very important roles in the birds growth and development; cell proliferation and growth, tissue and bone development, immune development and response, reproduction, enzymes formation, gene regulation and protection against oxidative stress and damage (Shankar and Prasad 1998; Richards et al. 2007; Song et al. 2009). Zinc is noted to be responsible for normal growth and maintenance including other functions like bone development, feathering, enzyme structure and function as well as appetite regulation for all poultry (Batal et al. 2001). It was also reported to modulate the cell-mediated immune response at hatch (Bakyaraj et al. 2011). Copper is involved in blood proteins, iron metabolism and absorption, oxygen metabolism, collagen and elastin synthesis, bone formation, feather development and colouring (Chandra 1990; Scheideler 2008). Copper plays a significant role in vasculogenesis, angiogenesis, in the synthesis of haemoglobin and redox processes (Mroczek-Sosnowska et al. 2015). According to Goel et al. (2013) *in ovo* administration of 8 μg/egg of inorganic copper ($CuSO_4$) also enhances the immune response of broiler chickens.

A chelated mineral is the combination of metal ions with organic ligand or ligand complex such as amino acids, proteinate, polysaccharides or organic yeast (Dieck et al. 2003; Bao et al. 2010). Furthermore, chelated minerals are minerals that are protected from other minerals interaction when bound with chelating agents like amino acids or proteins (Pal and Gowda 2015). The supplementations of these chelated minerals are better in terms of absorption than other forms of mineral sources (Yi et al. 2007). Kidd et al. 2000, Abdallah et al. 2009 and Ao et al. 2009 showed that chelated trace mineral have greater bioavailability in broilers and are better absorbed and utilised than their inorganic counterparts.

The “Arbor Acres” product line is being steadily improved to ensure all products consistently add value to customer operations through established breed selection processes that use both traditional scientific techniques and the latest in technology (https://www.eu.aviagen.com). On the other hand, the Cobb 500 strain of broiler chicken is arguably the world’s most production efficient line of meat chickens. It has a very low feed conversion ratio, converts cheap low density feed very well, has consistent performance across climates, produces optimally with best uniformity and it has consistent processing results including white feather, yellow skin as carcass and about 96% liveability (https://www.cobb-vantress.com/en_US/products).

There is a dearth of information on the *in ovo* administration of chelated trace minerals as studies on trace mineral requirements in poultry production focused on the *in ovo* supplementation of inorganic trace minerals (Sogunle et al. 2018) and or dietary supplementation with trace minerals (Nollet et al. 2007, Jegede et al. 2011) in poultry without a comparison in the effects on different strains of broiler chickens. This study investigated the response of two strains of broiler chickens (Arbor Acre and Cobb 500) to *in ovo* injection of organic salts of zinc, copper and their combination.

Materials and methods

Description of the experiments

The study comprised of two experiments which were undertaken concurrently. In Experiment 1, the response of Arbor Acre strain of broiler chickens to *in ovo* injection of organic salts of zinc and copper and their combination was studied while the response of the Cobb 500 strain of broiler chickens to *in ovo* injection of organic salts of zinc and copper and their combination was studied in Experiment 2.

Experimental site

The hatchery phase of the experiments was carried out at the hatchery of the College of Animal Science and Livestock Production while the field trial was undertaken at the poultry unit of the Directorate of University Farms (DUFARMS), Federal University of Agriculture, Abeokuta, Nigeria located at Latitude 7^0 15' N, Longitude 3^0 26' E. The climate is humid with an average annual rainfall of 1037 mm, average maximum temperature of 34.7°C and mean relative humidity of 83%.

Source and management of fertile eggs

For each of the experiments, a total of 300 hatching eggs were procured from reputable hatcheries. The eggs were fumigated using potassium tetraoxomanganate VII ($KMNO_4$) and formalin at a ratio 1:2. The treatment lasted for 20 minutes in a closed chamber. The eggs were set in egg trays in a Chick Master® incubator with broad ends upward to prevent rupture of air cell. Temperature (37.5 - 37.8^{o}C) and humidity (60 - 65%) were automatically regulated. Egg turning was done manually on an hourly basis to prevent developing embryos from sticking to the shell and also to ensure uniform distribution of nutrients.

From these hatching eggs, 250 eggs (83.3% settable eggs) were set after sorting in Experiment 1; on candling at the 14th day, a total of 148 eggs (59.2% fertility) were fertile. Each of the *in ovo* treatment groups (control, organic salts of zinc, copper and their combination) contained 37 fertile eggs. In Experiment 2, 257 eggs (85.7% settable eggs) were set after sorting; on candling at the 14th day, a total of 232 eggs (90.3% fertility) were fertile. Each of the *in ovo* treatment groups (control, organic salts of zinc, copper and their combination) contained 58 fertile eggs. The treatment groups are shown in Table 1.

On the 18th day of incubation, the candled eggs (*in ovo* groups) were injected with nutrients (organic salts of zinc and copper) into amnion using a 24-gauge hypodermic needle (Bhanja et al. 2004). Before injection, the site was sterilised with 10% ethanol and the injection was done at the broad end of the egg. Following *in ovo* feeding, the injection site was sealed with sterile paraffin and the eggs transferred to the hatching compartment. The *in ovo* injection of each treatment was completed within 30 minutes of taking out from the incubator.

Source and preparation of test ingredients

Chelated zinc and copper salts used for this study were obtained from Novus International USA. They were dissolved in de-ionised water at 80 µg of organic zinc salt and 16 µg of organic copper salts into 0.5 ml of de-ionised water and administered at 0.1 ml/egg.

Table 1: Experimental layout

Treatment groups	*In ovo* injection
Group I	Control (no *in ovo* supplementation)
Group II	*In ovo* supplementation with 80 µg/egg of organic salts of zinc (Zn 351.80 µg. 0.5 ml^{-1} deionised water)
Group III	*In ovo* supplementation with 16 µg/egg of organic chelated copper (Cu 62.87 µg. 0.5 ml^{-1} deionised water)
Group IV	*In ovo* supplementation with 80 µg/egg of organic salts of zinc and 16 µg/egg copper

Post-hatch chick and management

In Experiment 1, a total of 46 chicks hatched (31.1% hatchability); 12 (32.4% hatchability) for the control; five (13.5% hatchability) from Zn-injected eggs; 14 (37.8% hatchability) from Cu-injected eggs and 15 (40.5% hatchability) from Zn*Cu-injected eggs. A total of 12 birds were allotted to each treatment. However, only the five chicks which were available were used from Zn-injected eggs.

In Experiment 2, a total of 191 chicks hatched (82.3% hatchability); 51 (87.9% hatchability) for the control; 49 (84.5% hatchability) from Zn-injected eggs; 42 (72.4% hatchability) from Cu-injected eggs and 49 (84.5% hatchability) from Zn*Cu-injected eggs. A total of 42 birds were allocated to each treatment.

The chicks were brooded for 2 weeks and fed commercial feeds containing 23% crude protein and 3200 Kcal/kg metabolisable energy at the starter phase, and 20% crude protein and 3000 Kcal/kg metabolisable energy at the finisher phase. They were served fresh clean water *ad libitum*. They were raised intensively in a deep litter system for 7 weeks in Experiment 1 and for 5 weeks (due to COVID-19 restriction on movement) in Experiment 2 with all necessary medications and vaccinations strictly adhered to.

Data collection

Hatching parameters

a. Egg weight

The weights of individual eggs were determined, using a sensitive weighing scale, before setting in the incubator.

b. Chick weight

The weights of the chicks at hatch were determined by weighing the hatched chicks on a sensitive weighing scale.

c. Chick to egg ratio

This was determined as the ratio of the chick weight to egg weight.

d. Percentage hatchability

This was calculated as:

% hatchability = No. of hatched chicks/ No. of viable (fertile) embryos * 100

Post-hatch growth performance parameters

Feed intake

The amount of feed given to the birds and the leftover was measured weekly to determine the feed intake.

Feed intake = Feed given – Feed leftover

Weight gain

The birds were weighed at the commencement of the experiments and subsequently on weekly basis.

Weight gain = Final weight – Initial weight

Average daily weight gain

Average daily weight gain = (Final weight Initial weight) / Number of days

Feed conversion ratio

This is the proportion of feed converted into flesh by the birds.

Feed conversion ratio = Total feed intake / Total weight gain

In vivo immune response of the bird

Cell-mediated immunity

At 21 days post-hatch, four birds per treatment, but one bird only from Zn-injected treatment in

Experiment 1, were selected and their cell-mediated immune response to phytohaemagglutinin type-P (PHA-P) was studied using the method of Corrier and Deloach (1990): 0.1 ml (concentration 1 mg/ml) of PHA-P was injected at 3rd and 4th inter-digital space of the right foot. The left foot served as the control, and it was injected with 0.1 ml phosphate-buffered saline. The foot web index was calculated as the difference between the swelling in the right and left feet before and after 24 hours of injection and expressed in millimetres.

The foot web/pad index was calculated as follows:

Cell-mediated immune response = (R2 – R1) – (L2 – L1)

R2 = thickness of right foot web after 24 hours of injection

R1 = thickness of the right foot web before injection

L2 = thickness of left foot web after 24 hours of injection

L1 = thickness of the left foot web before injection

Humoral immunity

The antibody response to the Sheep Red Blood Cell (SRBC) was studied at the 29th day post-hatch, wherein 1 ml of 1% SRBC was injected intravenously to the birds. The SRBC was washed thrice and centrifuged at 704 g for 10 minutes after each washing. After 5 days of SRBC immunisation, four birds per treatment, but one bird only from the Zn-injected treatment in Experiment 1, were selected and 2 mls of blood were collected from the brachial vein and the antibody titre was recorded by haemagglutination titre according to Vander Zipp (1983).

Collection of blood samples

With a 5 ml syringe fitted with a 24-gauge sterile hypodermic needle, 5 mls of blood was carefully drawn from the left brachial vein at the point of bifurcation on day 7 for birds on Experiments 1 and 2. About 2.5 ml of the blood was put into a sterilized bottle containing Ethylene diamine tetra acetic acid as anticoagulant, and mixed gently to prevent coagulation, while the rest of the blood was put into a sterilised bottle which had no anticoagulants for serum biochemical analysis according to the procedures of Jain (1986).

Determination of haematological parameters

Haematological parameters that were determined included: haemoglobin concentration, packed cell volume, red blood cell and white blood cell.

i. Haemoglobin concentration: for this analysis, the collected blood sample was diluted with Drabkin's solution, and was incubated for 10 minutes. Then the result was read under a spectrophometer (Mitruka and Rawnsley 1977).

ii. Haematocrit (packed cell volume): this was analysed by the use of a haematocrit centrifuge (Mitruka and Rawnsley 1977).

iii. Red blood cell (total erythrocyte count): the total red blood cell count was determined from an anticoagulated blood sample which was diluted with 0.99% NaCl and was mixed thoroughly (Baker and Silverton 1985). The diluted blood was mounted on a haemacytometer and the number of erythrocytes in a circumscribed volume of 0.01 m^3 was counted microscopically (Aiello 1998).

iv. White blood cell (total leucocyte count): the sample that was collected was counted under a microscope (Mitruka and Rawnsley 1977).

v. Neutrophils (differential leucocyte count) were estimated by counting 100 cells (leucocytes) under oil immersion through a microscope on a blood film that was prepared from fresh blood and was fixed with methanol for 3 minutes. The smears were stained with modified Wright's stain for poultry (Lucas and Jamroz 1974).

vi. Basophil, eosinophils, monocytes, neutrophils, lymphocyte were analysed through blood stains.

vii. White Blood Cell differential counts and Mean Corpuscular Haemoglobin Concentration were estimated using standard formulae (Howlett and Jaine, 2008).

viii. Mean Corpuscular Volume (MCV) was calculated as:
MCV = Hct x 10/RBC (range: 84 – 96 fl)

ix. Mean Corpuscular Haemoglobin (MCH) was calculated as:
MCH = Hb x 10/RBC (range: 26 – 36 pg)

x. Mean Corpuscular Haemoglobin Concentration (MCHC) was calculated as:
MCHC = Hb x 10/Hct (range: 32 – 36%)

Hb = haemoglobin concentration: RBC = red blood cell count: Hct = haematocrit

Determination of serum parameters

i. Total protein: serum total protein was determined spectro-photometrically according to the method of Tietz (1995) as described in the Randox(R) diagonistic kit manual.

ii. Serum albumin: this was determined spectro-photometrically according to the method of Tietz (1995) as described in the Randox(R) diagnostic kit manual.

iii. Cholesterol: this was determined spectro-photometrically according to the method of Tietz (1995).

iv. Alanine aminotransferase was determined spectro-photometrically using a commercial Randox kit.

Gut morphometry

On day 7 of Experiments 1 and 2, eight birds per treatment, except in the birds from Zn-injected eggs in Experiment 1 in which the remaining three birds were used, were sacrificed by cervical dislocation for gut development studies. Gut morphometry was done by recording the weights of gizzard, proventriculus, liver as well as the weight and length of the duodenum, jejunum, ileum and caecum.

Statistical analysis

Data collected were subjected to one-way analysis of variance in a completely randomised design. Significant differences ($P \leq 0.05$) among variable means were separated using Tukey test as contained in Minitab® version 17.1.0 (Minitab 2013).

Results

Effects of in ovo injection of organic salts of zinc, copper and their combination on hatching traits Arbor Acre and Cobb 500 strains of broiler chickens

The effects of *in ovo* injection of organic salts of zinc, copper and their combination on hatching traits of Arbor Acre and Cobb 500 strains of broiler chickens are presented in Figures 1a, 1b, 2a and 2b. For the Arbor Acre strain the highest hatchability of 40.5% was obtained in the eggs injected with combination of zinc and copper while the least hatchability of 13.5% was recorded in eggs on *in ovo* injection of zinc. Hatchabilities of 32.4 and 37.8% were recorded in eggs in the control group and organic copper, respectively. The highest chick weight of 43.7 g was obtained in chicks from eggs on the zinc-injected group; this was followed by chicks resulting from eggs injected with copper (40.8 g) and those in the control group (40.7 g).

In the Cobb 500 strain of broiler chickens, highest hatchability of 87.9% was obtained in eggs in the control while the least hatchability of 72.4% was recorded in eggs on *in ovo* injection of copper. Eggs injected with zinc and those injected with combination of zinc and copper both had hatchability of 84.5%. The highest chick weight of 50.5 g was obtained in chicks from eggs in the zinc-injected group followed by chicks from eggs injected with the combination of zinc and copper (49.6 g) and those in the control group (49.3 g). The chick:egg ratio ranged from 0.69 to 0.74, with least values recorded on *in ovo* injection of organic copper while the highest chick:egg ratio was obtained in organic zinc-injected group.

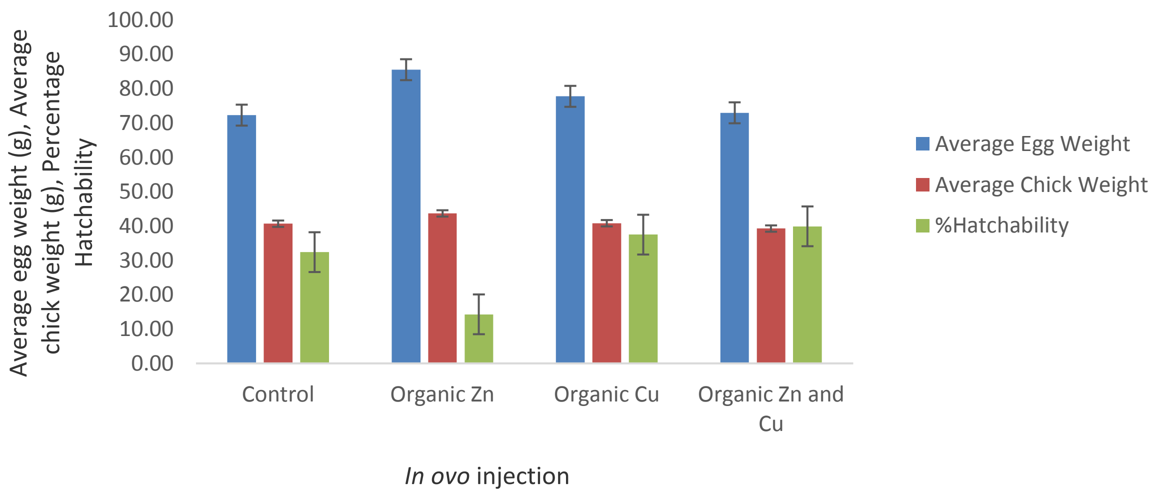

Figure 1a: Effect of *in ovo* injection of organic Zn, Cu and their combination of hatching traits of Arbor acre strain of broiler chicken. The bars refer to Sandard Error of Means

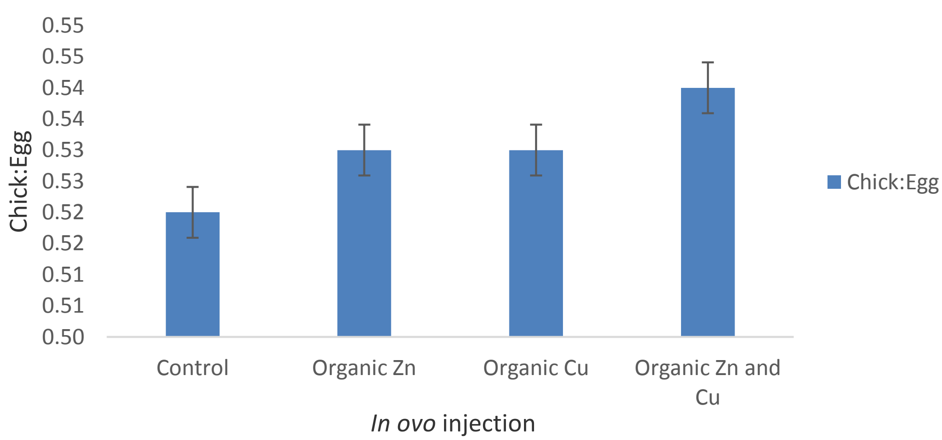

Figure 1b: Effect of *in ovo* injection of organic Zn, Cu and their combination of hatching traits of Arbor acre strain of broiler chickens. The bars refer to Standard Error of Means

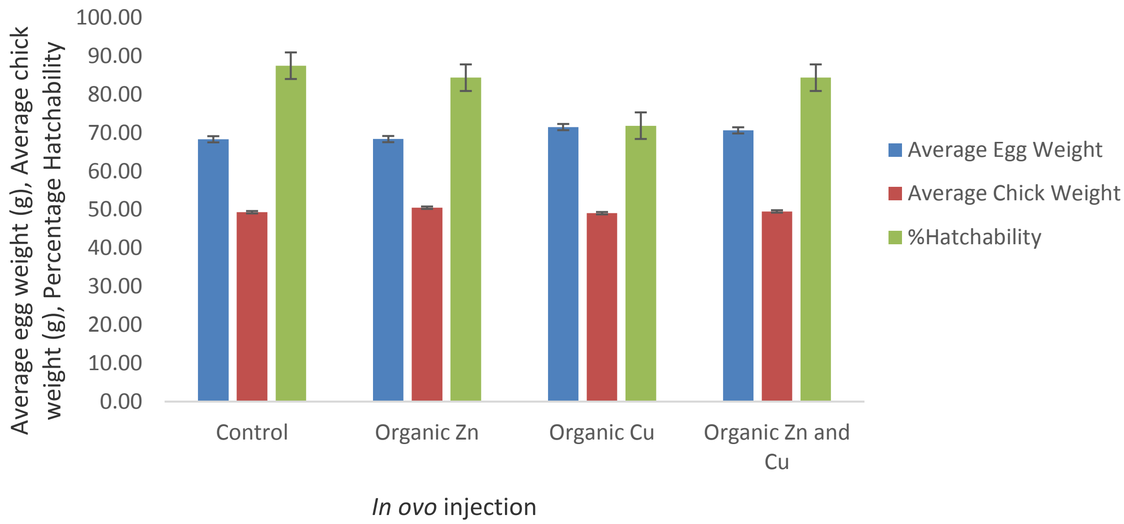

Figure 2 a: Effect of *in ovo* injection of organic Zn, Cu and their combination of hatching traits of Cobb500 strain of broiler chickens. The bars refer to Standard Error of Means

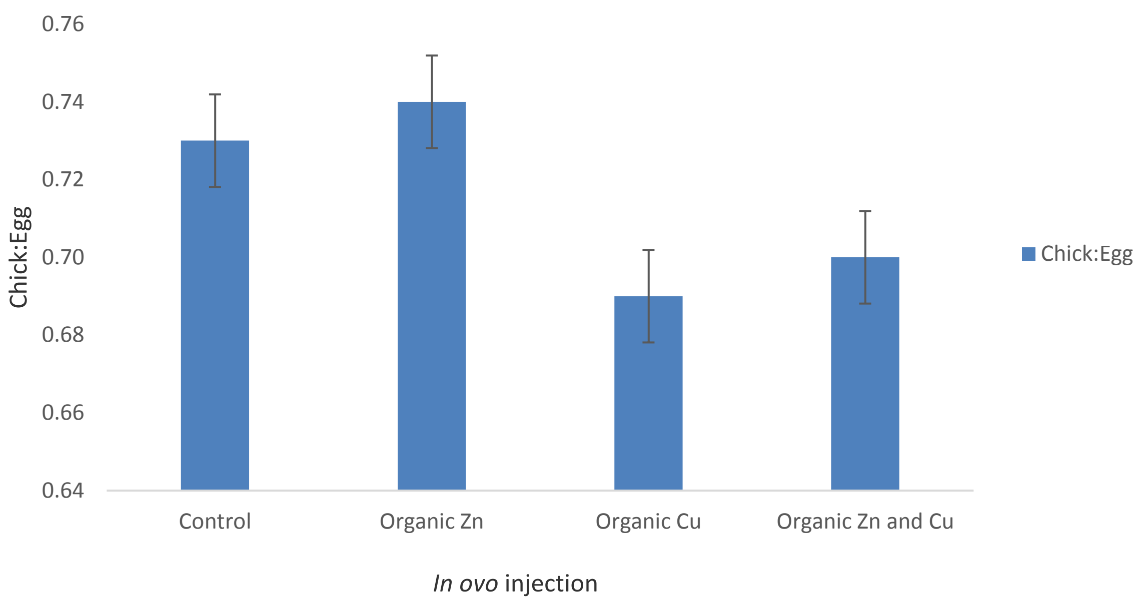

Figure 2b: Effect of *in ovo* injection of organic Zn, Cu and their combination on hatching traits of Cobb500 strain of broiler chickens. The bars refer to Standard Error of Means

Effects of in ovo injection of organic salts of zinc, copper and their combination on organ development and gut morphometry of Arbor Acre and Cobb 500 strains of broiler chickens at age 7 days

The effects of *in ovo* injection of organic salts of zinc, copper and their combination on organ development and gut morphometry of Arbor Acre and Cobb 500 strains of broiler chickens at age 7 days are presented in Table 2. There were no significant ($P > 0.05$) differences in all organs and gut morphometric parameters measured in both strains of broiler chickens.

Table 2: Effects of *in ovo* injection of organic salts of zinc, copper and their combination on organ development and gut morphometry of Arbor Acre and Cobb 500 strains of broiler chickens at age 7 days

	In ovo injection					
Parameters	Control	Zn	Cu	Zn*Cu	SEM	P-value
Arbor Acre strain						
Live weight (g/bird)	83.5	81.0	83.5	81.5	12.3	0.998
Organs						
Heart (%)	0.84	0.79	1.01	0.74	0.61	0.710
Liver (%)	3.55	3.35	4.45	3.12	0.61	0.514
Proventriculus (%)	1.05	0.91	1.20	1.04	0.24	0.862
Gizzard (%)	7.26	8.30	8.24	8.26	1.02	0.862
Gut						
Duodenum (%)	2.41	2.63	3.20	2.95	0.30	0.378
Duodenum length (cm/100 g)	13.6	16.3	16.8	16.8	2.04	0.660
Jejunum (%)	3.13	2.28	4.48	3.01	0.41	0.705
Jejunum (cm/100 g)	28.7	26.3	30.5	26.5	4.86	0.911
Ileum (%)	4.01	2.44	3.86	3.10	0.60	0.350
Ileum (cm/100 g)	30.4	28.7	31.7	29.2	3.27	0.912
Caecum (%)	1.30	1.08	1.18	0.95	0.20	0.672
Caecum (cm/100 g)	14.8	13.7	14.1	14.2	2.32	0.990
Colon (%)	0.86	0.43	0.54	0.38	0.13	0.192
Colon (cm/100 g)	4.77	4.12	4.50	3.25	0.82	0.506
Cobb 500 strain						
Live weight (g/bird)	141.5	142.0	162.0	145.3	12.1	0.613
Organs						
Heart (%)	1.00	0.77	0.96	0.91	0.06	0.149
Liver (%)	4.52	5.10	4.51	4.91	0.74	0.928
Proventriculus (%)	0.82	0.95	0.96	0.94	0.09	0.667
Gizzard (%)	6.94	7.50	8.25	6.83	0.38	0.088
Gut						
Duodenum (%)	1.73	4.81	1.71	1.67	0.15	0.900
Duodenum length (cm/100 g)	11.5	11.11	9.3	10.5	0.67	0.217
Jejunum (%)	3.09	2.98	3.19	2.97	0.26	0.907
Jejunum (cm/100 g)	22.8	22.7	19.8	24.7	2.61	0.535
Ileum (%)	2.68	2.72	2.55	2.70	0.09	0.503
Ileum (cm/100 g)	22.9	22.0	17.5	23.9	2.68	0.352
Caecum (%)	0.82	0.95	0.84	0.72	0.23	0.873
Caecum (cm/100 g)	10.7	10.3	9.0	9.9	1.52	0.860
Colon (%)	0.48	0.47	0.42	0.49	0.10	0.974
Colon (cm/100g)	2.98	2.68	2.14	2.92	0.24	0.129

Zn*Cu: combination of zinc and copper

SEM: standard error of mean; the SEMs stated apply to all treatments except Zn*Cu for Arbor acre

Effects of in ovo injection of organic salts of zinc, copper and their combination on humoral and cell-mediated immunity of Arbor Acre and Cobb 500 strains of broiler chickens

The effects of *in ovo* injection of organic salts of zinc, copper and their combination on humoral and cell-mediated immunity (after 24 hours of injecting phytohaemagglutinin type-P) of Arbor Acre and Cobb 500 strains of broiler chickens are shown in Figures 3a, 3b, 4a and 4b. In the Arbor Acre strain (Figure 3), chicks from the control had the highest cellular immunity of 0.47 mm after 24 hours of injection; this was followed by chicks from eggs on *in ovo* injection of organic zinc and copper with 0.40 and 0.36 mm, respectively. The lowest cellular immunity of 0.30 mm was obtained in the chicks from *in ovo* injection of the combination of organic zinc and copper. Humoral response to sheep red blood cell ranged from 5.78 mm to 6.63 mm. The lowest value was noted in birds in the control group while the highest value was recorded in birds from eggs on *in ovo* injection organic salt of zinc.

In the Cobb 500 strain (Figure 4), chicks from eggs in organic copper injected group had the highest cellular immunity of 0.30 mm after 24 hours of injection; this was followed by chicks from *in ovo* injection of organic salts of zinc and those on combination of zinc and copper with 0.40 and 0.36 mm, respectively. The lowest cellular immunity of 0.30 mm was obtained in the chicks from the control. Humoral response to sheep red blood cell ranged from 5.78 mm to 6.63 mm. The lowest value was recorded from birds in the control group while the highest value was recorded in birds injected with organic zinc.

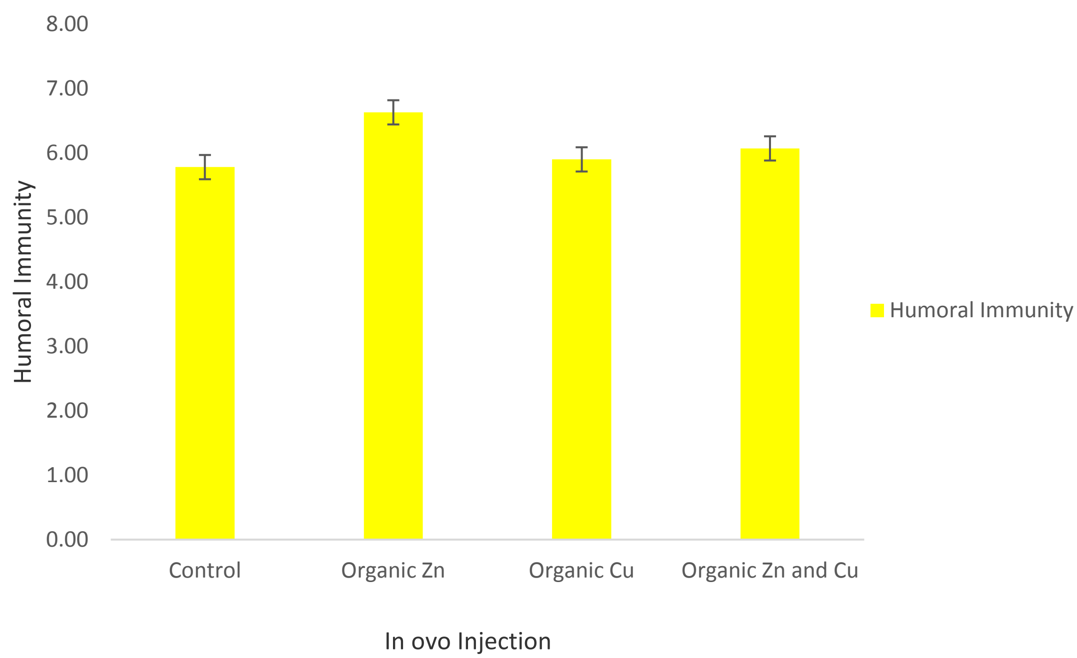

Figure 3a: Effect of *in ovo* injection of organic salts Zn, Cu and their combination on Cell-Mediated Immune Response and Humoral Immunity of Arbor acre strain of broiler chickens. The bars refer to Standard Error of Means

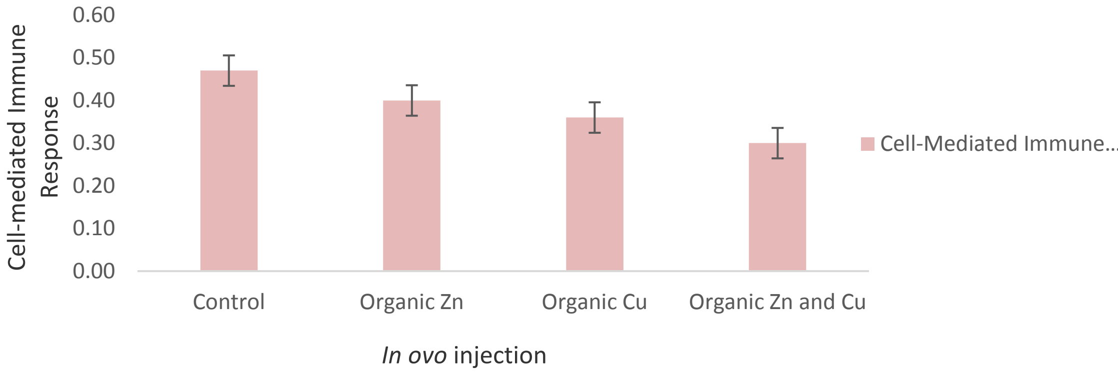

Figure 3b: Effect of *in ovo* injection of organic salts, Zn, Cu and their combination on Cell-Mediated Immune Response and Humoral Immunity of Arbor acre strain of broiler chickens. The bars refer to Standard Error of Means

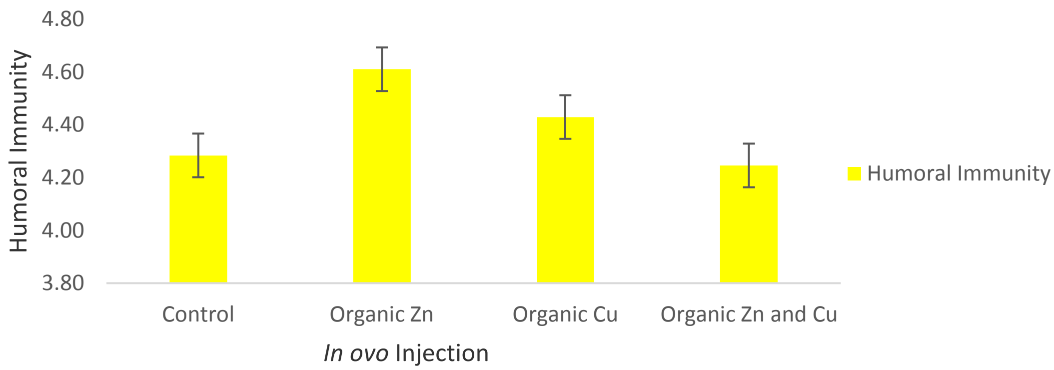

Figure 4a: Effect of *in ovo* injection of organic salts Zn, Cu and thei combination on Cell-Mediated Immune Response and Humoral Immunity of Cobb500 Strain of broiler chickens. The bars refer to Standard Error of Means

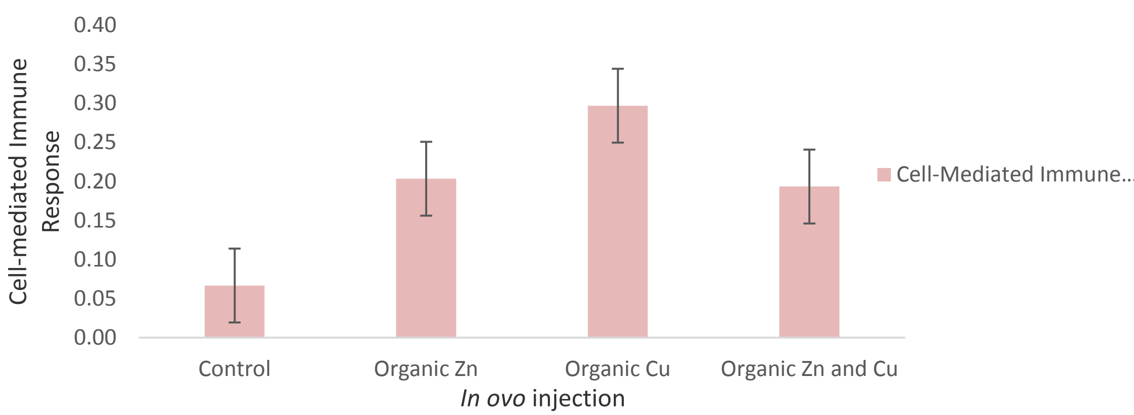

Figure 4b: Effect of *in ovo* injection of organic salts Zn, Cu and their combination on cell-Mediated Immune Response and Humoral Immunity of Cobb500 strain of broiler chickens. The bars refer to Standard Error of Means

Effects of in ovo injection of organic salts of zinc, copper and their combination on growth performance of Arbor Acre and Cobb 500 strains of broiler chickens

In Table 3, the effects *in ovo* injection of organic salts of zinc, copper and their combination on growth performance indices of Arbor Acre and Cobb 500 strains of broiler chickens are presented with no significant (P > 0.05) effects on all growth performance parameters measured.

Table 3: Effects of *in ovo* injection of organic salts of zinc, copper and their combinations on growth performance of Arbor Acre and Cobb 500 strains of broiler chickens

	In ovo injection					
Parameters	Control	Zn	Cu	Zn*Cu	SEM	p-value
Arbor Acre strain						
Initial weight (g/bird)	44.7	48.8	44.7	67.4	12.2	0.152
Final weight (g/bird)	1440	1750	1590	1750	171	0.573
Daily weight gain (g/bird)	28.5	34.7	31.5	34.3	3.48	0.601
Daily feed intake (g/bird)	76.8	101.3	81.5	82.3	7.18	0.222
Feed conversion ratio	2.71	2.97	2.62	2.40	0.31	0.666
Cobb 500 strain						
Initial weight (g/bird)	65.6	63.6	65.2	65.1	0.75	0.345
Final weight (g/bird)	929	889	877	904	85	0.974
Daily weight gain (g/bird)	24.7	23.6	23.2	24.0	2.44	0.976
Daily feed intake (g/bird)	47.9	51.8	58.9	52.9	1.88	0.857
Feed conversion ratio	1.95	2.22	2.68	2.24	0.27	0.351

Zn*Cu: combination of zinc and copper
SEM: standard error of mean; the SEMs stated apply to all treatments except Zn*Cu for Arbor acre

Effects of in ovo injection of organic salts of zinc, copper and their combination on haematological parameters of Arbor Acre and Cobb 500 strains of broiler chickens at age 7 days

The effects of *in ovo* injection of organic salts of zinc, copper and their combination on haematological parameters of Arbor Acre and Cobb 500 strains of broiler chickens at day 7 are presented in Table 4. There were no significant (P > 0.05) differences in all the haematological parameters determined for Arbor Acre strain of broiler chickens except in monocytes. Chicks from eggs on *in ovo* injection of zinc had reduced (P ≤ 0.05) monocytes when compared to the control. In Cobb 500 strain of broiler chickens, the *in ovo* injection of combination of zinc and copper significantly (P ≤ 0.05) increased the mean corpuscular volume when compared to birds from other treatment groups.

Effects of in ovo injection of organic salts of zinc, copper and their combination on serum biochemical indices of Arbor Acre and Cobb 500 strains of broiler chickens at age 7 days

Table 5 shows serum biochemical indices of Arbor Acre and Cobb 500 strains of broiler chickens on *in ovo* injection of organic salts of zinc, copper and their combination at age 7 days. Only aspartate aminotransferase (ALT) was significantly (P ≤ 0.05) influenced by the *in ovo* injection of organic zinc, copper and its combination. Chicks from eggs on *in ovo* injection of the combination of zinc and copper had a reduced ALT with similar effect observed among other treatments. In Cobb 500 strain of broiler chickens, significant (P≤ 0.05) difference was obtained in low density lipoproteins (LDL) of birds from eggs on *in ovo* injection of Cu. There were no significant (P > 0.05) differences in the other serum biochemical indices recorded at day 7.

Table 4: Effects of *in ovo* injection of organic zinc, copper and their combination on haematological parameters of Arbor Acre and Cobb 500 strains of broiler chickens at age 7 days

	In ovo injection					
Parameters	Control	Zn	Cu	Zn*Cu	SEM	P-value
Arbor Acre strain						
PCV (%)	34.5	35.5	33.5	30.5	2.50	0.578
Hb (g/dl)	11.1	11.7	10.6	11.4	0.80	0.799
RBC ($\times10^6$/mm^3)	3.15	3.20	2.80	2.80	0.35	0.776
WBC ($\times10^6$/mm^3)	13.3	13.9	12.8	14.4	1.39	0.859
Heterophils (%)	31.0	32.5	29.0	27.5	3.79	0.801
Lymphocytes (%)	64.5	65.0	66.5	68.5	3.67	0.864
Eosinophils (%)	1.50	1.00	1.50	1.50	0.66	0.929
Basophils (%)	0.50	0.50	1.00	0.50	0.43	0.803
Monocytes (%)	2.50[a]	1.00[b]	2.00[ab]	2.00[ab]	0.25	0.043
MCV (fl)	111	112	120	110	7.91	0.804
MCH (g/dl)	35.4	36.8	38.06	40.88	3.03	0.649
MCHC (g/dl)	32.0	32.9	31.8	37.2	1.08	0.067
Cobb 500 strain						
PCV (%)	32.5	32.0	30.0	31.5	2.94	0.934
Hb (g/dl)	11.1	10.9	10.9	10.5	0.77	0.954
RBC ($\times10^6$/mm^3)	2.70	2.85	2.45	2.40	0.56	0.237
WBC ($\times10^6$/mm^3)	12.0	11.6	10.9	11.5	1.05	0.852
Heterophils (%)	26.5	28.5	29.0	28.0	4.32	0.977
Lymphocytes (%)	69.0	68.0	69.0	69.5	4.39	0.995
Eosinophils (%)	1.50[a]	1.00[ab]	0.00[b]	1.00[ab]	0.25	0.053
Basophils (%)	1.50	1.00	0.50	1.00	0.79	0.847
Monocytes (%)	1.50	1.50	1.00	0.50	0.66	0.689
MCV (fl)	120.4[ab]	112.5[b]	122.1[ab]	131.2[a]	2.45	0.026
MCH (pg)	41.3	38.2	44.3	43.7	1.10	0.053
MCHC (g/dl)	34.3	33.4	36.3	33.3	0.89	0.235

[a,b]Means on the same row having different superscripts are significantly (P ≤ 0.05) different
Zn*Cu: combination of zinc and copper
SEM: standard error of mean; the SEMs stated apply to all treatments except Zn*Cu for Arbor acre
PCV: packed cell volume, Hb: Haemoglobin concentration, RBC: red blood cell, WBC: white blood cell, MCV: mean corpuscular volume, MCH: mean corpuscular haemoglobin, MCHC: mean corpuscular haemoglobin concentration

Table 5: Effects of *in ovo* injection of organic zinc, copper and their combination on serum biochemical indices of Arbor Acre and Cobb 500 strains broiler chickens at age 7 days

	In ovo injection					
Parameters	Control	Zn	Cu	Zn*Cu	SEM	P-value
Arbor Acre strain						
Total protein (g/dl)	6.90	3.15	5.15	3.90	1.23	0.293
Albumin (g/dl)	4.20	2.20	3.70	2.25	9.01	0.360
Globulin (g/dl)	2.70	1.15	1.45	1.65	0.41	0.179
Cholesterol (g/dl)	92.50	107.55	99.90	77.35	13.30	0.502
Triglyceride (g/dl)	75.66	91.81	87.15	67.97	7.90	0.273
LDL (g/dl)	19.45	24.00	20.97	16.95	3.26	0.548
VLDL (g/dl)	15.13	18.36	17.43	13.59	1.58	0.273
HDL (g/dl)	57.47	65.19	61.51	46.81	9.38	0.591
AST (U/l)	79.50	57.00	74.00	61.00	13.90	0.656
ALT (U/l)	17.00^{a}	13.50^{ab}	15.00^{ab}	12.50^{ab}	0.79	0.025
Cobb 500 strain						
Total protein (g/dl)	3.35	2.15	3.25	2.20	0.66	0.491
Albumin (g/dl)	2.05	1.25	2.20	1.40	0.30	0.200
Globulin (g/dl)	2.30	0.85	1.050	0.80	0.31	0.075
Cholesterol (g/dl)	118.15	112.60	114.65	107.00	3.11	0.224
Triglyceride (g/dl)	76.00	95.20	76.70	84.80	8.68	0.458
LDL (g/dl)	34.45^{ab}	31.10^{b}	35.40^{a}	31.50^{b}	0.59	0.016
VLDL (g/dl)	15.20	19.04	15.34	16.96	1.74	0.458
HDL (g/dl)	68.50	62.46	63.91	58.54	3.41	0.352
AST (U/l)	57.5	47.0	55.0	48.5	3.79	0.291
ALT (U/l)	18.0	12.0	11.0	12.5	2.19	0.249

^{a,b}Means on the same row having different superscripts are significantly ($P \leq 0.05$) different, Zn*Cu: combination of zinc and copper

SEM: standard error of mean; the SEMs stated apply to all treatments except Zn*Cu for Arbor acre

LDL: low density lipoprotein, VLDL: very low density lipoprotein, HDL: high density lipoprotein, AST: alanine transaminase, ALT: aspartate aminotransferase

Discussion

In ovo feeding, which involves the injection of exogenous nutrients into developing embryo enhances enteric development during embryogenesis and translates to improved performance of hatched chicks (Foye et al. 2007). Variations had been reported on results relating to *in ovo* injection of nutrients on hatching traits and post-hatch performance of poultry species. Results in Experiment 1 showed that hatchability was least in chicks administered *in ovo* injection of zinc. This result is at variance with an earlier report (Batal et al. 2001) that zinc is essential for embryonic development of all poultry species. This result also contradicts the findings of Sogunle et al. (2018) who reported that *in ovo* injection of inorganic zinc increased hatchability. The variations observed in this study and that of Sogunle et al. (2018) could be attributable to the differences in trace elements and strains of the broiler chickens used. In addition, sources of eggs and storage conditions prior to collection and setting could be responsible for the discrepancies in the results obtained. In Experiment 2, eggs in the control group had the highest hatchability with comparably improved hatchability noted in eggs injected with organic salts of zinc and the combination of organic salts of zinc and copper. The relatively improved hatchability in Zn-injected eggs is similar to the findings of Batal et al. (2001) and Sogunle et al. (2018) who affirmed zinc as a crucial trace mineral for embryonic development. Results obtained in Experiments 1 and 2 showed that *in ovo* administration of the combination of organic salts of zinc and copper did not negatively affect hatchability. This means that there was no negative interaction between the minerals. This is in agreement with the study of Pal and Gowda (2015) who reported that minerals are protected from other mineral interaction when bound with chelating agents like amino acids or proteins. However, there had been varying hatchability results with *in ovo* administration in broiler chickens; decreased hatchability (McGruder et al. 2011); increased hatchability (Bottje et al. 2010, Sahr et al. 2020) and no effect (Zhai et al. 2011).

Results obtained from organ development and gut morphometric at day 7 in both experiments showed no significant difference in all the parameters measured. These results contradicted the findings at day 7 as reported by Uni et al. (2005), that *in ovo* feeding results in the improvement of the development of gastrointestinal tracts.

Results obtained in both experiments showed that *in ovo* injection of organic zinc improved humoral immunity of Arbor Acre and Cobb 500 strains of broiler chickens. This improvement is attributable to the role of zinc in enhancing the physiological functions of the immune cells. Zinc plays an essential role in a wide array of processes including cell proliferation and animal growth, immune development and response, reproduction, gene regulation and defence against oxidative stress and damage (Shankar and Prasad 1998, Underwood and Suttle 1999, Fraker et al. 2000, Blanchard et al. 2001, Ibs and Rink 2003, Song et al. 2009). Zinc also modulates the inflammatory response (Peterson et al. 2008), development of immune organs and functionality of immune cells (Rink and Haase 2007). The metalloenzymes of which zinc is grouped, play an important role in the bird's immune response and in hormone production (O'Dell 1981). The results obtained are in agreement with findings on *in ovo* injection of lysine (Lotan et al. 1980), arginine (Kidd et al. 2001) and 8 µg/egg of inorganic copper ($CuSO_4$) (Goel et al. 2013), which were found to enhance the immune response of broiler chickens. However, this result contradicts the findings of Sogunle et al. (2018) who reported non-significance in the growth of immune organs and response to PHA-P or SRBC in broiler chickens subjected to *in ovo* injection of zinc, selenium, copper and their combinations.

The results obtained on the influence of *in ovo* injection of organic zinc, copper and their combination on growth performance in Experiments 1 and 2 showed no significant difference in the growth parameter indices. This insignificant result corroborates the report of Sahr et al. (2020) that *in ovo* administration of inorganic salts of zinc and copper do not significantly influence any of the growth performance indices. On the contrary, Sogunle et al. (2018) reported significant difference in the weight gain of broiler chickens on *in ovo* injection of inorganic salts of zinc, copper and selenium.

Literature is limited on the influence of *in ovo* feeding of organic salts of zinc and copper on haematological indices of broiler chickens. However, at age 7 days in Experiment 1, reduced monocytes was observed in birds from eggs on *in ovo* injection of organic zinc when compared with other treatment groups; though the least value of 1.0% was observed in birds from eggs on *in ovo* injection of organic zinc, the value was within the (0.06-5.00%) reference monocytes values for healthy chickens as reported by Mitruka and Rawnsley (1977) and Riddell (2001).

In Experiment 2, it was observed that eosinophil was reduced in the birds from egg in the copper injected group at age 7 days. Mean corpuscular volume (MCV) was also reduced in birds from eggs on *in ovo* injection of organic zinc at day 7 of age but the values were still within the normal range as reported by Mitruka and Rawnsley 1997, Pampori and Igbal 2007 for chickens. MCV has been identified to perform immune regulatory functions both in health and disease (Jacobsen et al. 2012).

Serum biochemical indices are important indicators for detecting organ diseases in domestic animals (Igbal et al. 2012) and the amount of available protein in the diets. In Experiment 1, Alanine aminotransferase (ALT) was significantly affected at age 7 days. ALT activity has been used as an indicator of liver function and elevated levels are monitored in liver malfunction (Murray et al. 2000). Reduced values were observed in all the *in ovo* injected groups compared to the control group with the least value observed in birds from eggs injected with the combination of organic salts of zinc and copper. However, the values were still within the normal range for chicken (12.00 U/L-23.33 U/L) as reported by Obikaonu et al. (2011). In Experiment 2 none of the serum biochemical indices were significantly affected at both starter and finisher phases. Variations and similarities in blood relative to previous findings in literature can be attributed to factors like age, sex and nutrition (Etim et al. 2014). The fact that most values of the haematological and serum induces of Arbor Acre and Cobb 500 strains of broiler chickens fell within the standard reported for healthy chickens suggest that *in ovo* injection of organic salts of zinc, copper and their combination did not have any detrimental effect on the biological process of haematopoiesis in both strains of broiler chicken used in this study.

Conclusion

From this study, it can be concluded that:

i. *In ovo* injection of organic salt of zinc resulted in a reduced hatchability in Arbor Acre strain of broiler chickens
ii. *In ovo* injection of the combination of both zinc, copper and their combination did not have any negative effect on hatchability in both strains of broiler chickens used in Experiments 1 and 2.
iii. Organ development and gut morphometric parameters in the two strains of broiler chickens were not significantly influenced by the *in ovo* injection of organic salts of zinc, copper and their combination.
iv. *In ovo* injection of organic salt of zinc improved humoral immunity in Arbor Acre and Cobb 500 strains of broiler chickens.
v. *In ovo* injection of organic salts of zinc, copper and their combination did not significantly influence the growth performance of Arbor Acre and Cobb 500 strains of broiler chickens used in this study.

Recommendations

i. To improve the humoral immune response in Arbor Acre and Cobb 500 strains of broiler chickens, *in ovo* injection of organic salt of zinc at 18^{th} day of embryonic development is recommended.
ii. Further studies should be carried out using *in ovo* techniques in administrating other nutrients that have been reported to enhance poultry production when administered through feed and water.

Conflict of interest

The authors hereby declare that there is no conflict of interest.

Acknowledgement

The authors wish to gratefully acknowledge the grants-in-aid received from the Centre of Excellence in Agricultural Development and Sustainable Environment, Federal University of Agriculture, Abeokuta for the research.

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