

## Influence of *In-ovo* Injection of Organic and Inorganic Salts of Copper, Zinc and Their Combination on Post-Hatch Growth, Immune Response, Organ Development and Gut Morphology of Two Strains of Broiler Chickens

By

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### Abstract

*In-ovo* feeding is an innovative strategy for delivering nutrients and bioactive compounds directly to the developing avian embryo to enhance post-hatch performance. This study evaluated the influence of *in-ovo* injection of organic and inorganic salts of copper (Cu) and zinc (Zn) and their combinations on post-hatch growth performance, cell-mediated and humoral immune responses, organ development and gut morphology of two broiler strains. A 2×7 factorial arrangement was used comprising two strains (Arbor Acre and Cobb 500) and seven *in-ovo* treatment groups (Control, organic Zn, organic Cu, inorganic Zn, inorganic Cu, organic Zn+Cu and inorganic Zn+Cu). Eggs were injected on day 18 of incubation with 0.1 ml of treatment solution per egg. Results revealed significant ( $P<0.05$ ) strain differences in growth performance, with Arbor Acre achieving higher final body weight (1,687.60 g/bird) and average daily weight gain (33.45 g/bird/day) compared to Cobb 500 (950.60 g/bird; 25.31 g/bird/day). *In-ovo* injection with inorganic Cu yielded the highest feed intake across treatments. Humoral immune response was significantly ( $P<0.05$ ) influenced by both strain and *in-ovo* treatment; organic Zn, organic Cu, organic Zn+Cu and inorganic Zn+Cu maintained antibody titres comparable to the control, while inorganic Zn and inorganic Cu individually resulted in markedly reduced titres, particularly in Cobb 500. Cell-mediated immunity was not significantly affected by strain or *in-ovo* treatment. Organ development parameters were similarly influenced by strain and mineral form. These findings demonstrate that *in-ovo* mineral supplementation outcomes are strain- and mineral-form-dependent, with organic chelated forms offering immunological advantages over inorganic sulphate salts in broiler production.

**Keywords:** Trace minerals, *In-ovo* feeding, Broiler strain, Growth performance, Immune response, Gut morphology

### INTRODUCTION

Trace minerals such as zinc (Zn) and copper (Cu) are indispensable micronutrients that play critical roles in the growth, immunity, and metabolic functions of broiler chickens. Zinc is a structural and catalytic component of over 300 enzymes and is essential for protein synthesis, cell proliferation, immune function, and intestinal integrity (NRC, 1994; Abd El-Hack *et al.* 2017). Copper, on the other hand, is involved in iron metabolism, connective tissue development, feathering, immune defence mechanisms, and functions as a

cofactor for various metalloenzymes including ceruloplasmin and superoxide dismutase (Obianwuna *et al.* 2024; Sözcü *et al.* 2025). Deficiencies in these trace elements during embryonic development can result in poor hatchability, retarded growth, immune suppression, and skeletal abnormalities in post-hatch chicks (Salmanzadeh, 2012; Ghane-Khoshkhejari *et al.*, 2024).

*In-ovo* feeding, the delivery of nutrients directly into the amniotic fluid or air cell of fertilized eggs at the late stages of incubation has emerged as a promising biotechnological



approach to enhance early embryonic nutrition and post-hatch performance. Pioneered by Uni and Ferket (2004), this technique exploits the natural ingestion of amniotic fluid by the embryo during the final days of incubation to deliver specific nutrients that may otherwise be limited in the egg yolk. The approach has been used to supplement carbohydrates (Foye *et al.*, 2006), vitamins, amino acids, and minerals directly to the developing embryo, with outcomes including improved intestinal development, enhanced early immunity, and superior post-hatch growth (Bhanja *et al.*, 2004; Bhanja *et al.*, 2009).

Trace minerals supplied to poultry may be in organic (chelated) or inorganic (sulphate salt) forms, and the bioavailability of these forms differs considerably. Organic trace minerals, in which the mineral is chemically bound to an organic ligand such as an amino acid or protein, are generally considered more bioavailable and less antagonistic to other nutrients compared to their inorganic counterparts (Obianwuna *et al.* 2024; Kishawy *et al.*, 2020). In broiler chickens, organic Zn and Cu supplementation via conventional feed has demonstrated improvements in growth performance, immune function, and carcass quality over inorganic forms (Ao *et al.*, 2009; Abdul-Majeed *et al.*, 2022). However, the comparative efficacy of organic versus inorganic forms of Zn and Cu when delivered through *in-ovo* injection remains inadequately characterized in the literature.

Broiler strain is another key determinant of production performance, as commercial strains such as Arbor Acre and Cobb 500 differ in their genetic potential for growth, feed utilization, immune competence, and adaptability to environmental stressors (Ebeid *et al.*, 2022; Huda *et al.*, 2026). Understanding how these strains respond differentially to *in-ovo* mineral supplementation is essential for developing strain-specific management protocols that optimize post-hatch outcomes. Whereas several studies have evaluated *in-ovo* injection of individual trace minerals in a single broiler strain (Ghane-Khoshkebijari *et al.*, 2024; Salmanzadeh, 2012; Das *et al.*, 2021), comparative studies across strains receiving both organic and inorganic forms of Cu and Zn and their combinations remain scarce.

In Nigeria and other tropical environments, where feed costs are high, disease pressure is significant, and broiler production is expanding rapidly, strategies that enhance the resilience and productivity of chicks from the outset of life are of considerable practical importance (Onagbesan *et al.*, 2023). *In-ovo* feeding of trace minerals offers an opportunity to prime the embryo nutritionally and immunologically prior to hatch, potentially reducing post-hatch mortality, improving early feed utilization, and enhancing overall flock performance. Against this background, this study was designed to evaluate the influence of *in-ovo* injection of organic and inorganic salts of Zn, Cu and their combinations on post-hatch growth performance, cell-mediated and humoral immune responses, organ development, and gut morphology of Arbor Acre and Cobb 500 broiler strains.

## Materials and Methods

### Description of the Experiments

The study comprised two experiments undertaken concurrently. In Experiment I, the response of Arbor Acre broiler chickens to *in-ovo* injection of organic and inorganic salts of zinc, copper and their combination was studied, while Experiment II evaluated the same parameters in Cobb 500 broiler chickens.

### 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 (Latitude 7°15' N; Longitude 3°26' E). The climate is humid with an average annual rainfall of 1,037 mm, average maximum temperature of 34.7°C and mean relative humidity of 83%.

### Source and Preparation of Test Ingredients

Chelated (organic) zinc and copper salts used for this study were obtained from Novus International, USA, while inorganic salts of zinc and copper were obtained from a reputable veterinary store within Ogun State, Nigeria. The compounds were dissolved in deionised water at concentrations of 80 µg organic chelated zinc and 80 µg inorganic zinc sulphate (ZnSO<sub>4</sub>·7H<sub>2</sub>O) per 100 ml, and 16 µg organic chelated copper and 16 µg inorganic copper sulphate (CuSO<sub>4</sub>·5H<sub>2</sub>O) per 100 ml of deionised water, respectively. Each egg received 0.1 ml of the treatment solution.

### Source and Management of Fertile Eggs

For each experiment, a total of 300 hatching eggs were procured from reputable hatcheries. The eggs were fumigated using potassium permanganate (KMnO<sub>4</sub>) and formalin at a ratio of 1:2 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 the air cell. Temperature (37.5–37.8°C) and humidity (60–65%) were automatically regulated. Egg turning was performed manually on an hourly basis to prevent developing embryos from adhering to the shell and to ensure uniform nutrient distribution.

In Experiment I, 250 eggs (83.3% settable) were set after sorting; candling at day 14 confirmed 154 fertile eggs (61.6% fertility), with 22 fertile eggs assigned per treatment group. In Experiment II, 257 eggs (85.7% settable) were set; 231 eggs (89.9% fertility) were confirmed fertile at day 14, with 33 fertile eggs per treatment group. The treatment groups are presented in Table 1.

On day 18 of incubation, fertile eggs in the *in-ovo* groups were injected with the respective nutrient solutions into the amnion using a 24-gauge hypodermic needle, following the method of Bhanja *et al.* (2004). The injection site was sterilized with 10% ethanol prior to injection at the broad end of the egg. Following injection, the site was sealed with sterile paraffin wax and the eggs transferred to the hatching compartment. The entire *in-ovo* injection process for each

treatment was completed within 30 minutes of removal from the incubator.

**Table 1:** Groups of eggs for in-ovo injection

| Treatment Group   | In-ovo Injection                                                                                                                                      |
|-------------------|-------------------------------------------------------------------------------------------------------------------------------------------------------|
| Group I (Control) | Control — injected with 0.1 ml deionised water only                                                                                                   |
| Group II          | <i>In-ovo</i> supplementation with 80 µg.egg <sup>-1</sup> of organic chelated Zinc source (oZn)                                                      |
| Group III         | <i>In-ovo</i> supplementation with 16 µg.egg <sup>-1</sup> of organic chelated Copper source (oCu)                                                    |
| Group IV          | <i>In-ovo</i> supplementation with 80 µg.egg <sup>-1</sup> of inorganic Zinc Sulphate (IZn)                                                           |
| Group V           | <i>In-ovo</i> supplementation with 16 µg.egg <sup>-1</sup> of inorganic Copper Sulphate (ICu)                                                         |
| Group VI          | <i>In-ovo</i> supplementation with 80 µg.egg <sup>-1</sup> of organic chelated Zinc & 16 µg.egg <sup>-1</sup> of organic chelated Copper (OZn+Cu)     |
| Group VII         | <i>In-ovo</i> supplementation with 80 µg.egg <sup>-1</sup> of inorganic Zinc Sulphate & 16 µg.egg <sup>-1</sup> of inorganic Copper Sulphate (IZn+Cu) |

#### Post-Hatch Chick Management

In Experiment I, a total of 67 chicks hatched (43.5% hatchability): 13 from control (59.1%), 6 from oZn (27.3%), 15 from oCu (68.2%), 5 from IZn (22.7%), 6 from ICu (27.3%), 16 from OZn+Cu (72.7%), and 6 from IZn+Cu (27.3%). A total of 12 birds were allotted to control, oCu, and OZn+Cu groups respectively; only the available number of chicks was used in other treatment groups. In Experiment II, a total of 180 chicks hatched (77.9% hatchability): 33 from control (100%), 30 from oZn (90.9%), 24 from oCu (72.7%), 30 from IZn (90.9%), 21 from ICu (63.6%), 27 from OZn+Cu (81.8%), and 15 from IZn+Cu (45.5%). The resulting chicks were distributed into treatment groups based on the *in-ovo* injection assignment with three replicates of unequal numbers, and brooded for two weeks.

#### Experimental Diets and Design

Birds were fed commercial diets containing 23% crude protein and 3,200 kcal/kg metabolizable energy at the starter phase, and 20% crude protein and 3,000 kcal/kg metabolizable energy at the finisher phase. Fresh, clean water was provided ad libitum. Birds were raised intensively on a deep litter system for seven (7) weeks (Experiment I) and five (5) weeks (Experiment II, curtailed by COVID-19 movement restrictions), with all necessary medications and vaccinations strictly adhered to throughout. The experiment was designed in a 2×7 factorial arrangement comprising two broiler strains (Arbor Acre and Cobb 500) and seven *in-ovo* treatment groups.

#### Post-Hatch Growth Performance Parameters

**Feed intake:** The amount of feed offered to birds and refusals were measured weekly.

Feed intake = Feed given – Feed leftover.

**Weight gain:** Birds were weighed at the commencement of the experiment and weekly thereafter.

Weight gain = Final weight – Initial weight.

**Average daily weight gain:** Average daily weight gain = (Final weight – Initial weight) / Number of days.

**Feed conversion ratio:** Feed conversion ratio = Total feed intake / Total weight gain.

#### In Vivo Immune Response

**Humoral immunity:** Antibody response to Sheep Red Blood Cells (SRBC) was assessed at 29 days post-hatch. One millilitre of 1% washed SRBC (centrifuged at 704 g × 10 min after each of three washes) was injected intravenously per bird. Five days post-immunization, four birds per treatment (one bird per treatment in IZn group, Experiment I) were bled from the brachial vein (2 ml) and haemagglutination antibody titres were recorded according to Vander Zipp (1983)..

**Cell-mediated immunity:** At 21 days post-hatch, three birds per treatment (one bird per treatment in the IZn group of Experiment I) were selected for cell-mediated immune assessment using phytohaemagglutinin type-P (PHA-P) following the method of Corrier and Deloach (1990). Briefly, 0.1 ml of PHA-P (1 mg/ml) was injected into the 3rd and 4th inter-digital web of the right foot, while 0.1 ml phosphate-buffered saline was injected into the left foot as control. 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 index was calculated as: Cell-mediated immune response

$$\text{CMIR} = (R_2 - R_1) - (L_2 - L_1);$$

Where: R<sub>2</sub> = Thickness of the right foot web after 24 hours of injection

R<sub>1</sub> = Thickness of the right foot web before injection

L<sub>2</sub> = Thickness of the left foot web after 24 hours of injection

L<sub>1</sub> = Thickness of the left foot web before injection

#### Gut Morphometry

On day 7 of both experiments, eight birds per treatment (three birds from the IZn group, Experiment I, due to limited availability) were sacrificed by cervical dislocation for gut development studies. Organ weights were recorded for the gizzard, proventriculus, and liver. The weight and length of the duodenum, jejunum, ileum, and caecum were also measured.

#### Statistical Analysis

Data were subjected to one-way analysis of variance in a factorial arrangement. Significant differences (P≤0.05) among treatment means were separated using Tukey's test as

contained in Minitab® statistical software version 17.1.0 (Minitab, 2013).

## Results and Discussion

### Growth Performance

The effects of broiler strain and *in-ovo* injection of organic and inorganic salts of Zn, Cu and their combination on growth performance are presented in Table 2. Significant ( $P<0.05$ ) differences were observed between strains for all growth performance parameters measured. Arbor Acre recorded significantly higher final body weight (1,687.60 vs. 950.60 g/bird) and average daily weight gain (33.45 vs. 25.31 g/bird/day) but also higher feed intake (91.37 vs. 59.22 g/bird/day) and a less favourable feed conversion ratio (2.73 vs. 2.37) compared to Cobb 500. These strain differences are consistent with established reports of inherent genetic variation in growth potential between commercial broiler lines (Huda *et al.*, 2026; Ebeid *et al.*, 2022), and may also reflect differences in feed utilization efficiency and metabolic rate between the two strains. The superior FCR of Cobb 500 suggests more efficient nutrient partitioning toward body mass accretion relative to feed consumed, despite lower absolute body weight.

Regarding *in-ovo* treatment effects, final body weight and daily weight gain were not significantly affected by the *in-ovo* injection treatment per se, suggesting that the nutritional conditioning achieved through *in-ovo* mineral delivery did not

translate into uniform improvements in post-hatch growth across all groups. However, feed intake differed significantly ( $P<0.05$ ) among treatment groups, with inorganic Cu-injected birds recording the highest feed intake (94.94 g/bird/day), followed by inorganic Zn+Cu (85.04 g/bird/day) and organic Zn (76.56 g/bird/day). The control group recorded the lowest feed intake (62.31 g/bird/day). This stimulatory effect of *in-ovo* Cu on feed intake may be attributed to the role of copper in appetite regulation and gastrointestinal development, consistent with findings of Sözcü *et al.* (2025), who reported that copper influences digestive enzyme secretion and mucosal absorptive capacity.

The strain  $\times$  *in-ovo* interaction was significant ( $P<0.05$ ) for initial body weight, final body weight, average daily weight gain, and feed intake, confirming that the two strains responded differently to the same *in-ovo* treatments. Within the Arbor Acre strain, inorganic Cu produced the highest final body weight (2,100 g/bird) and daily gain (41.95 g/bird/day), while within Cobb 500, the inorganic Cu and organic Zn+Cu groups recorded numerically higher final weights (1,013.90 and 1,025.00 g/bird, respectively) compared to other Cobb 500 treatments. These findings suggest that Arbor Acre may be more responsive to the anabolic stimulus provided by *in-ovo* Cu supplementation, possibly due to strain-specific differences in copper transport, hepatic metabolism, or tissue responsiveness to copper-dependent enzyme systems (Abdul-Majeed *et al.*, 2022).

**Table 2:** Effect of strain and *in-ovo* injection of organic and inorganic salts of Zn, Cu and their combination on growth performance of broiler chickens

| Factors          |                       | Initial W (g/b)    | Final W (g/b)          | W Gain (g/b/d)      | Feed Intake (g/b/d)  | FCR               |
|------------------|-----------------------|--------------------|------------------------|---------------------|----------------------|-------------------|
| Strain           | Arbor Acre            | 48.74 <sup>b</sup> | 1687.60 <sup>a</sup>   | 33.45 <sup>a</sup>  | 91.37 <sup>a</sup>   | 2.73 <sup>a</sup> |
|                  | Cobb 500              | 64.91 <sup>a</sup> | 950.60 <sup>b</sup>    | 25.31 <sup>b</sup>  | 59.22 <sup>b</sup>   | 2.37 <sup>b</sup> |
|                  | P-Value               | <0.005             | <0.005                 | <0.005              | <0.005               | 0.033             |
|                  | SEM                   | 0.601              | 43.230                 | 1.077               | 1.660                | 0.108             |
| In-ovo injection | Control               | 55.11 <sup>b</sup> | 1184.50                | 26.57               | 62.31 <sup>d</sup>   | 2.33              |
|                  | Organic Zn            | 56.19 <sup>b</sup> | 1319.60                | 29.15               | 76.56 <sup>bc</sup>  | 2.59              |
|                  | Organic Cu            | 54.95 <sup>b</sup> | 1233.20                | 27.36               | 70.20 <sup>cd</sup>  | 2.65              |
|                  | Inorganic Zn          | 55.88 <sup>b</sup> | 1202.10                | 26.81               | 67.04 <sup>cd</sup>  | 2.49              |
|                  | Inorganic Cu          | 53.20 <sup>b</sup> | 1556.90                | 34.57               | 94.94 <sup>a</sup>   | 2.66              |
|                  | Organic Zn+Cu         | 66.22 <sup>a</sup> | 1387.50                | 30.88               | 70.97 <sup>cd</sup>  | 2.32              |
|                  | Inorganic Zn+Cu       | 56.20 <sup>b</sup> | 1350.00                | 30.29               | 85.04 <sup>ab</sup>  | 2.83              |
|                  | P-Value               | <0.005             | 0.095                  | 0.180               | <0.005               | 0.490             |
|                  | SEM                   | 1.130              | 81.971                 | 2.033               | 3.130                | 0.203             |
| Strain× In-ovo   | Arbor Acre× Control   | 44.67 <sup>b</sup> | 1440.00 <sup>abc</sup> | 28.48 <sup>ab</sup> | 76.78 <sup>cde</sup> | 2.71              |
|                  | Arbor Acre× Org Zn    | 48.75 <sup>b</sup> | 1750.00 <sup>a</sup>   | 34.72 <sup>ab</sup> | 101.29 <sup>ab</sup> | 2.97              |
|                  | Arbor Acre× Org Cu    | 44.73 <sup>b</sup> | 1590.00 <sup>ab</sup>  | 31.54 <sup>ab</sup> | 81.54 <sup>bcd</sup> | 2.62              |
|                  | Arbor Acre× Inorg Zn  | 44.00 <sup>b</sup> | 1500.00 <sup>abc</sup> | 29.71 <sup>ab</sup> | 81.17 <sup>bcd</sup> | 2.73              |
|                  | Arbor Acre× Inorg Cu  | 44.50 <sup>b</sup> | 2100.00 <sup>a</sup>   | 41.95 <sup>a</sup>  | 129.96 <sup>a</sup>  | 3.10              |
|                  | Arbor Acre× Org Zn+Cu | 67.38 <sup>a</sup> | 1750.00 <sup>a</sup>   | 34.34 <sup>ab</sup> | 82.32 <sup>bc</sup>  | 2.40              |

|  |                         |                    |                       |                     |                      |       |
|--|-------------------------|--------------------|-----------------------|---------------------|----------------------|-------|
|  | Arbor Acre× Inorg Zn+Cu | 47.13 <sup>b</sup> | 1683.00 <sup>a</sup>  | 33.39 <sup>ab</sup> | 86.51 <sup>bc</sup>  | 2.59  |
|  | Cobb 500×Control        | 65.56 <sup>a</sup> | 928.90 <sup>c</sup>   | 24.67 <sup>ab</sup> | 47.85 <sup>f</sup>   | 1.95  |
|  | Cobb 500×Org Zn         | 63.63 <sup>a</sup> | 889.10 <sup>c</sup>   | 23.59 <sup>b</sup>  | 51.83 <sup>f</sup>   | 2.22  |
|  | Cobb 500×Org Cu         | 65.17 <sup>a</sup> | 876.50 <sup>c</sup>   | 23.18 <sup>b</sup>  | 58.86 <sup>ef</sup>  | 2.68  |
|  | Cobb 500×Inorg Zn       | 67.76 <sup>a</sup> | 904.20 <sup>c</sup>   | 23.90 <sup>ab</sup> | 52.90 <sup>f</sup>   | 2.24  |
|  | Cobb 500×Inorg Cu       | 61.90 <sup>a</sup> | 1013.90 <sup>bc</sup> | 27.20 <sup>ab</sup> | 59.92 <sup>def</sup> | 2.22  |
|  | Cobb 500×Org Zn+Cu      | 65.07 <sup>a</sup> | 1025.00 <sup>bc</sup> | 27.43 <sup>ab</sup> | 59.62 <sup>def</sup> | 2.34  |
|  | Cobb 500×Inorg Zn+Cu    | 65.28 <sup>a</sup> | 1016.70 <sup>bc</sup> | 27.18 <sup>ab</sup> | 83.52 <sup>bc</sup>  | 3.07  |
|  | P-Value                 | <0.005             | 0.035                 | 0.025               | <0.005               | 0.193 |
|  | SEM                     | 1.580              | 114.20                | 2.883               | 4.357                | 0.283 |

a,b,c,d,e,f means in the same column within each factor with different superscripts differ significantly ( $P < 0.05$ ). FCR = Feed Conversion Ratio; W = Weight; b = bird; d = day.

## Humoral Immune Response

The humoral immune response (HI titre) of both broiler strains to *in-ovo* mineral injection is illustrated in Figure 1. Arbor Acre consistently demonstrated higher antibody titres to SRBC across all treatment groups compared to Cobb 500, reflecting inherent genetic differences in B-lymphocyte activity and bursal development between the two strains (Kishawy *et al.*, 2020). Among treatments, organic Zn (6.63 in Arbor Acre), organic Zn+Cu (6.07) and organic Cu (5.90) produced the highest titres, while inorganic Zn-injected Cobb 500 birds and inorganic Cu-injected Arbor Acre birds yielded zero titres, suggesting complete immunosuppression in these strain × treatment combinations. The depressive effect of individual inorganic mineral salts on humoral immunity may reflect an antagonistic interaction between inorganic mineral forms and lymphocyte function. High concentrations of inorganic zinc and copper have been reported to exhibit immunotoxic effects, potentially disrupting B-cell activation, antibody synthesis, and complement activity when presented in a pro-oxidant inorganic form to the developing embryo (Ao *et al.*, 2009; Abdul-Majeed *et al.*, 2022). In contrast, organic chelated forms may be less disruptive to embryonic immune programming due to their lower pro-oxidant potential and controlled mineral release.

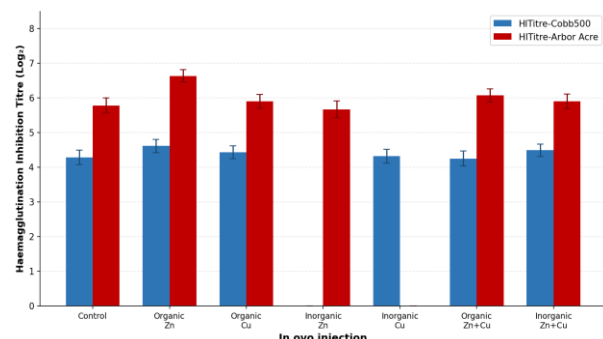


Figure 1: Effect of *in-ovo* injection of Organic and Inorganic Zn, Cu and Their combination on Humoral Immune Response (HI Titre) of Cobb 500 and Arbor Acre broiler chickens

## Cell-Mediated Immune Response

.. The effects of strain and *in-ovo* treatment on cell-mediated immunity (CMI), expressed as foot web swelling index (mm) at 24 hours post-PHA-P injection, are depicted in Figure 2.

Neither strain ( $P > 0.05$ ) nor *in-ovo* treatment ( $P > 0.05$ ) significantly influenced CMI, although Arbor Acre birds tended to show numerically higher responses than Cobb 500 in the control and organic Zn groups (0.48 vs. 0.07 mm and 0.39 vs. 0.20 mm, respectively). Organic Cu-injected birds showed the highest pooled CMI response (0.33 mm), while inorganic Zn+Cu yielded the lowest (0.11 mm). The lack of significant treatment effects on CMI may be attributed to the relatively small sample sizes used for immune assessment, particularly in Experiment I, where the limited hatchability of certain treatment groups constrained the number of birds available for evaluation. Furthermore, the PHA-P assay at 21 days of age may represent a period when the T-cell-mediated response is still maturing and may not fully reflect *in-ovo*-induced immunological differences. Das *et al.* (2021) reported that *in-ovo* supplementation with micronutrients can enhance cell-mediated responses, but the magnitude of response is often dependent on the dose, timing, and form of supplementation. The numerically superior CMI observed in organic Cu-supplemented birds may reflect the role of copper in T-lymphocyte proliferation and natural killer cell activity, as reported by Sözcü *et al.* (2025).

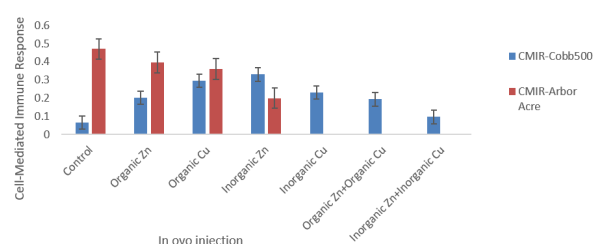


Figure 2 : Effect of *in-ovo* injection of Organic and Inorganic Zn, Cu and Their combination on Cell-mediated Immune response of Cobb500 and Arbor Acre broiler chickens

## Organ Development and Carcass Characteristics

The effects of strain and *in-ovo* treatment on organ development and carcass characteristics are presented in Table 3. Significant strain effects were observed for several organ development parameters. Arbor Acre birds demonstrated heavier absolute organ weights consistent with their higher body weight, while Cobb 500 birds showed numerically different organ proportions relative to body weight. The interaction between strain and *in-ovo* treatment significantly ( $P < 0.05$ ) influenced organ development outcomes, suggesting that the form and combination of minerals delivered *in-ovo*

interact with genetic background to shape early organ morphogenesis.

Zinc plays a well-documented role in intestinal morphology through its involvement in cell proliferation, brush border enzyme activity, and mucosal repair (NRC, 1994; Abd El-Hack *et al.* 2017). The superior organ development observed in organically supplemented groups may reflect enhanced zinc-dependent enterocyte maturation and intestinal villi elongation in the pre-hatch period, which is consistent with

findings of Foye *et al.* (2006), who reported that *in-ovo* nutrient delivery accelerated gastrointestinal tract development. Copper, similarly, influences the structural integrity of connective tissue in organ walls through its role in lysyl oxidase activity and collagen cross-linking (Sözcü *et al.* 2025). The numerical variation in gizzard, proventriculus, and duodenal weights across treatment groups may therefore reflect differential stimulation of these copper-dependent developmental pathways.

**Table 5:** Effect of strain and *in-ovo* injection of organic and inorganic salts of Zn, Cu and their combination on organ development and gut morphometry parameters of broiler chickens

| Factors                 |                         | Liver (% BW)      | Heart (% BW) | Proventriculus (% BW) | Gizzard (% BW)    | Duodenum Wt (% BW) | Duodenum Length (cm/100g) |
|-------------------------|-------------------------|-------------------|--------------|-----------------------|-------------------|--------------------|---------------------------|
| Strain                  | Arbor Acre              | 3.12 <sup>a</sup> | 0.65         | 0.45 <sup>a</sup>     | 2.10 <sup>a</sup> | 0.72 <sup>a</sup>  | 5.84 <sup>a</sup>         |
|                         | Cobb 500                | 2.87 <sup>b</sup> | 0.62         | 0.38 <sup>b</sup>     | 1.85 <sup>b</sup> | 0.59 <sup>b</sup>  | 5.12 <sup>b</sup>         |
|                         | P-Value                 | <0.05             | 0.412        | <0.05                 | <0.05             | <0.05              | <0.05                     |
|                         | SEM                     | 0.082             | 0.021        | 0.024                 | 0.091             | 0.043              | 0.102                     |
| <i>In-ovo</i> injection | Control                 | 2.95              | 0.61         | 0.39                  | 1.89              | 0.63               | 5.44                      |
|                         | Organic Zn              | 3.08              | 0.65         | 0.43                  | 2.04 <sup>a</sup> | 0.68               | 5.62                      |
|                         | Organic Cu              | 3.10              | 0.64         | 0.44                  | 2.07 <sup>a</sup> | 0.67               | 5.58                      |
|                         | Inorganic Zn            | 2.91              | 0.60         | 0.38                  | 1.87              | 0.61               | 5.33                      |
|                         | Inorganic Cu            | 3.02              | 0.63         | 0.41                  | 1.99              | 0.65               | 5.52                      |
|                         | Organic Zn+Cu           | 3.15 <sup>a</sup> | 0.66         | 0.45                  | 2.10 <sup>a</sup> | 0.70               | 5.71                      |
|                         | Inorganic Zn+Cu         | 2.88              | 0.63         | 0.39                  | 1.88              | 0.64               | 5.42                      |
|                         | P-Value                 | 0.072             | 0.358        | 0.166                 | <0.05             | 0.273              | 0.184                     |
|                         | SEM                     | 0.118             | 0.031        | 0.035                 | 0.130             | 0.063              | 0.152                     |
|                         | Strain× <i>In-ovo</i>   |                   |              |                       |                   |                    |                           |
| Strain× <i>In-ovo</i>   | Arbor Acre× Control     | 3.18              | 0.64         | 0.43                  | 2.08              | 0.71               | 5.87                      |
|                         | Arbor Acre× Org Zn      | 3.31 <sup>a</sup> | 0.67         | 0.46                  | 2.22              | 0.76               | 6.05                      |
|                         | Arbor Acre× Org Cu      | 3.28 <sup>a</sup> | 0.67         | 0.48                  | 2.19              | 0.75               | 5.97                      |
|                         | Arbor Acre× Inorg Zn    | 3.05              | 0.63         | 0.41                  | 2.01              | 0.68               | 5.75                      |
|                         | Arbor Acre× Inorg Cu    | 3.14              | 0.65         | 0.45                  | 2.14              | 0.73               | 5.90                      |
|                         | Arbor Acre× Org Zn+Cu   | 3.35 <sup>a</sup> | 0.68         | 0.49                  | 2.27              | 0.79               | 6.15                      |
|                         | Arbor Acre× Inorg Zn+Cu | 3.04              | 0.64         | 0.42                  | 2.03              | 0.68               | 5.76                      |
|                         | Cobb 500×Control        | 2.72              | 0.58         | 0.35                  | 1.70 <sup>b</sup> | 0.55               | 5.01                      |
|                         | Cobb 500×Org Zn         | 2.85              | 0.63         | 0.40                  | 1.86              | 0.60               | 5.19                      |
|                         | Cobb 500×Org Cu         | 2.92              | 0.61         | 0.40                  | 1.95              | 0.59               | 5.19                      |
|                         | Cobb 500×Inorg Zn       | 2.77              | 0.57         | 0.35                  | 1.73 <sup>b</sup> | 0.54               | 4.91                      |
|                         | Cobb 500×Inorg Cu       | 2.90              | 0.61         | 0.37                  | 1.84              | 0.57               | 5.14                      |
|                         | Cobb 500×Org Zn+Cu      | 2.95              | 0.64         | 0.41                  | 1.93              | 0.61               | 5.27                      |
|                         | Cobb 500×Inorg Zn+Cu    | 2.72              | 0.62         | 0.36                  | 1.73 <sup>b</sup> | 0.60               | 5.08                      |
|                         | P-Value                 | 0.043             | 0.321        | 0.210                 | 0.031             | 0.189              | 0.152                     |
|                         | SEM                     | 0.165             | 0.043        | 0.050                 | 0.183             | 0.088              | 0.215                     |

<sup>a,b</sup> Means in the same column within each factor with different superscripts differ significantly (P<0.05). BW = Body weight.

## Conclusion

This study demonstrates that both broiler strain and the form of trace mineral (organic vs. inorganic) delivered via *in-ovo* injection are critical determinants of post-hatch growth performance, immune competence and gut morphological development. Arbor Acre broiler chickens consistently outperformed Cobb 500 in absolute growth parameters, while Cobb 500 showed superior feed conversion efficiency. Organic chelated forms of Zn and Cu, particularly their combination, supported significantly higher humoral immune responses compared to inorganic sulphate forms, with individual inorganic forms causing profound immunosuppression in strain-specific combinations. Cell-mediated immunity was numerically but not significantly affected by treatments, suggesting larger sample sizes are needed in future studies for adequate statistical resolution. The significant strain  $\times$  *in-ovo* interaction across most parameters underscores the necessity of strain-specific mineral supplementation protocols in commercial hatchery management.

These findings advocate for the preferential adoption of organic chelated forms of Zn and Cu in *in-ovo* supplementation programmes aimed at improving immunological priming, while further research should determine optimal dosage–response relationships and evaluate longer-term production outcomes including carcass characteristics, meat quality and egg production traits in layer-type birds.

## Recommendations

Based on the findings of this study, the following recommendations are made:

1. Hatchery managers should prioritize organic chelated forms of Zn and Cu over inorganic sulphate salts for *in-ovo* trace mineral supplementation, given their superior immunological outcomes and avoidance of immunosuppressive effects observed with individual inorganic forms.
2. Strain-specific protocols for *in-ovo* mineral supplementation should be developed, as the significant strain  $\times$  treatment interaction demonstrated in this study indicates that a single mineral form or combination may not be equally efficacious across different broiler genotypes.
3. Future studies should employ larger flock sizes per treatment group and extend the evaluation period to include carcass quality, histomorphometric analysis of intestinal villi and complete blood count parameters to comprehensively characterize the physiological impact of *in-ovo* mineral delivery.
4. The combination of organic Zn and organic Cu (OZn+Cu) is recommended as the most balanced *in-ovo* supplementation strategy, given its consistent

positive effects on both growth performance and humoral immune response in both Arbor Acre and Cobb 500 broiler strains.

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