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# The effect of dietary supplementation of organic trace minerals on performance, mineral retention, lymphoid organs and antibody titres of broilers

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## Abstract

Complete replacement of inorganic trace minerals (ITM) with proteinated organic trace minerals (PTM) at equal or lower inclusion rates was evaluated. One thousand and eight, one-d-old male chicks were divided into 24 pens containing 42 chicks, and randomly allocated to one of the following: T1: control group with ITM supplied at the standard commercial level in Ecuador; T2: PTM at 100% T1; T3: PTM at 66% TI; and T4: PTM at 33% T1. The 42-d experiment employed a three-phase feeding programme (1-14, 15-28 and 29-42 d). Restricted feeding was used to prevent the development of ascites associated with high altitude. At 21 and 42 d of age, Cu, Mn and Zn retention were measured in tibial bone, and Fe in whole blood. Lymphoid organ weights were at 21 and 42 d of age. Antibody titres were measured by ELISA at 42 d of age. Weight gain, feed consumption, feed conversion and mortality were similar among treatments, although were below breed expectations due to feed restriction practices. Blood Fe was not affected by treatment ($P$<0.05). At both 21 and 42 d of age, minerals in tibia differed ($P$<0.01), with Mn and Zn concentrations being significantly higher in all PTM groups, compared to ITM control at 42 d. However, at 21 d, Zn was higher for the ITM-fed birds. No differences in lymphoid organ (bursa, thymus, and spleen) weights were observed, or for Gumboro (infectious bursal disease), infections bronchitis virus and reovirus antibody titres. For Newcastle disease virus, T4 birds had significantly lower antibody titres compared to other treatment groups. In conclusion, replacement of inorganic minerals with a proteinated form organic minerals at lower inclusion rates had no negative impact on performance, lymphoid organ weight or antibody titres in broilers raised under commercial conditions of high altitude and restricted feeding.

**Keywords:** broilers, performance, organic minerals, retention, antibodies

## 1. Introduction

Although trace minerals represent a small percentage of the overall diet, they are essential nutrients involved in numerous metabolic and physiological processes and are required for all stages of poultry production. According to standard poultry requirements (NRC, 1994), iron (Fe) is a component of haemoglobin and cytochromes, while copper (Cu), manganese (Mn), selenium (Se), and zinc (Zn) function as essential factors in enzymes. Nutritional deficiency can result in lower production from loss of appetite and poor immune function (Underwood and Suttle, 1999). In contrast with other nutrients, trace mineral requirements for modern poultry and production systems are not well defined, and, in some cases, have been extrapolated from older data or from other avian species and age groups (Leeson and Caston, 2008). In addition, many of these older studies are not representative of commercial production systems as they have been conducted under research conditions using semi-purified diets. As a result, it is not uncommon to formulate commercial diets with trace mineral levels that are above the recommendations of the NRC (1994) or the Brazilian Tables for Poultry and Swine (Rostagno *et al*., 2017). This surplus of trace mineral supplementation in animal feed is likely used as a safety margin against deficiency (Power, 2004) and is enabled by the low cost of traditional, inorganic mineral sources, such as sulphates, carbonates, and oxides. The lower bioavailability associated with inorganic compared to organic trace mineral sources (Ao and Pierce, 2013; Lensing and Van der Klis, 2006), supplementation above actual requirements can lead to more minerals being excreted into the environment (Bao *et al.*, 2007; Nollet *et al.*, 2008). This poses a risk factor to the environment *via* bioaccumulation in soil and water systems.

Organic trace minerals are classified into different categories depending on the ligand in which the metal ion is bound to (e.g. amino acids, peptides, polysaccharides or organic acids). Metal proteinates or proteinated chelates are produced by the chelation of a soluble salt with amino acids and/or partially hydrolysed protein (AAFCO, 2000). During digestion inorganic trace minerals (ITM) tend to form insoluble complexes with other dietary constituents and interfere with nutrient absorption (DeWayne and Zunino, 1993; Shurson *et al.*, 2011). Several studies have shown that reducing the inclusion rates of dietary trace minerals using an organic proteinated source did not negatively affect performance and immunity (Abdallah *et al.*, 2009; Boruta *et al.,* 2007; Leeson and Caston, 2008; Lensing and Van der Klis, 2006) or carcass yield (Diaz and Carrion, 2013). In broilers, use of proteinated trace minerals (PTM) has been shown to improve feathering, reduce skin tearing (Tavares *et al.,* 2011) increase mineral absorption and reduce faecal excretion of dietary mineral (Ao and Pierce, 2013; Leeson and Caston, 2008).

Bioavailability and direct animal responses to PTM vs ITM have been evaluated in various studies over the years (Ao *et al.*, 2009; Guo *et al.*, 2001; Jongbloed *et al.,* 2002; Ma *et al.,* 2014; Star *et al.,* 2012; Wang *et al.,* 2007; Yan and Waldroup, 2006). However, these studies were conducted under conditions of *ad libitum* feed intake. It is common to implement a restricted feeding programme when broilers are reared in high altitude environments, such as Ecuador, to prevent the development of ascites and mortality. Research is needed to understand the contributions that mineral source and levels make to broiler health and performance when raised in these environments with restricted feed intake.

The aim of this study was to evaluate the use of PTM at three different inclusion levels vs an ITM control in broilers raised under conditions of restricted feed intake and determine the effects on performance, mineral retention in blood and tissue, lymphoid organ weight and antibody titres.

## 2. Materials and methods

The trial was carried out under the Guide for the Care and Use of Laboratory Animals, Chapter 9: Poultry (NRC, 2011) under the Ethics programme of the Universidad Nacional Agraria La Molina, Peru.

### Diet formulation

Basal diets were formulated for starter (1-14 d), grower (15-28 d) and finisher (29-42 d) phases, based on maize and soybean meal to meet local nutritional standards for medium performance of broiler chickens (Rostagno *et al.,* 2005). Diets were formulated using Brill Formulation® (Feed Management Systems Inc., Hopkins, MN, USA) based on digestible amino acids and included commercial antibiotic growth promoters. Calcium and available phosphorus requirements were equivalent to average recommended industry values. Diets were fortified with complete vitamin mixes according to genetic line recommendations (Cobb-Vantress, 2015). Diet composition and calculated analysis are shown in Table 1. Trace mineral source and quantity varied according to the treatments. The control treatment (T1, inorganic trace minerals; ITM) provided 2.0, 1.7 and 1.5 kg/t of a premix (60 ppm Zn, 60 ppm Mn, 9 ppm Cu, 33.3 ppm Fe, 0.22 Se and 0.67 I) with each phase meeting the minimum requirements of Cobb-Vantress (2015). In the remaining treatment groups, supplemental ITM were entirely replaced by PTM. Treatments 2, 3 and 4 consisted of PTM (Bioplex TR-Se Poultry®, Alltech Inc., Nicholasville, KY, containing Cu, Fe, Mn and Zn as minerals chelated to small peptides and Se in form of selenium enriched yeast) corresponding to 100, 66 and 33% the level of the control treatment, respectively. A zero level of minerals was not incorporated as a treatment, as this would have led to severe welfare and health problems in the animals. Phytase was not added to treatment diets to avoid the effect of mineral release at different rates, which could not be quantified. This was an additional variable that the authors wanted to avoid.

To prevent the development of ascites in birds due to exposure of high geographical altitudes and lower oxygen pressure, mash diets were fed at 18-20% lower than genetic line recommendations (Cobb-Vantress, 2015).

### Housing, experimental design and management

A total of 1,008, one-d-old male Cobb second-quality chicks were obtained from a local hatchery. The experimental barn was located 2,500 metres above sea level and had a concrete floor bedded with rice husk for litter. Using a complete randomised block design, chicks were assigned into the four experimental treatment groups with six replicate floor pens of 42 chicks per replicate. Each floor pen had a density of 12.57 birds/$m^2$. Experimental treatments were blocked by pen location and initial body weight. Chicks

**Table 1. Diet composition and calculated nutritional content.**

| Ingredient, % | Starter 0-14 d | Grower 15-28 d | Finisher 29-42 d |
|---|---|---|---|
| Yellow maize | 26.33 | 38.72 | 60.13 |
| Soybean meal | 37.30 | 32.60 | 29.70 |
| Rice | 25.00 | 18.00 | 0.00 |
| Crude palm oil | 5.40 | 5.10 | 5.00 |
| Limestone | 1.60 | 1.60 | 1.50 |
| Monocalcium phosphate | 2.10 | 1.80 | 1.60 |
| Vitamin & trace mineral premix[1] | 0.20 | 0.17 | 0.15 |
| Sodium chloride | 0.36 | 0.41 | 0.40 |
| DL methionine (99%) | 0.32 | 0.27 | 0.22 |
| L-lysine sulphate (50.7%) | 0.27 | 0.27 | 0.25 |
| L-threonine | 0.10 | 0.07 | 0.04 |
| Antifungal | 0.25 | 0.25 | 0.20 |
| Choline chloride (60%) | 0.15 | 0.15 | 0.15 |
| Anticoccidial | 0.06 | 0.06 | 0.06 |
| Mycotoxin adsorbent | 0.20 | 0.20 | 0.25 |
| Bacitracin methylene disalicylate | 0.05 | 0.05 | 0.06 |
| Antioxidant | 0.02 | 0.02 | 0.03 |
| Dextrose | 0.30 | 0.25 | 0.23 |
| | 100.00 | 100.00 | 100.00 |
| **Calculated analyses** | | | |
| ME, MJ/kg | 12.46 | 12.78 | 13.11 |
| Crude protein, % | 21.72 | 19.98 | 18.83 |
| dig. Lys., % | 1.24 | 1.13 | 1.03 |
| dig. Met., % | 0.61 | 0.55 | 0.48 |
| dig. total sulphur amino acids, % | 0.90 | 0.82 | 0.76 |
| dig. Thr., % | 0.81 | 0.73 | 0.67 |
| dig. Trp, % | 0.24 | 0.22 | 0.20 |
| Ether extract, % | 7.30 | 7.42 | 8.10 |
| Crude fibre, % | 2.89 | 3.00 | 3.37 |
| Calcium, % | 1.00 | 0.95 | 0,86 |
| Non-phytate P, % | 0.50 | 0.45 | 0.41 |
| Sodium, % | 0.22 | 0.18 | 0.18 |

[1] Vitamin and mineral premix per tonne of feed: vitamin A 7.3 MIU; vitamin D3 3.7 MIU; vitamin E 36.6 KIU; vitamin K 2.3 g; thiamin 2.3 g; riboflavin 5.5 g; pyridoxine 2.7 g; niacin 38 g; folic acid 1.3 g; cyanocobalamin 10.7 mg; pantothenic acid 10 g; biotin 86.7 mg. In the inorganic trace mineral (ITM) premix: Cu 9 g; I 0.67 g; Fe 33.3 g; Mn 60 g; Se 0.21 g; Zn 60 g. In the proteinated organic trace mineral (PTM) premix Cu, Fe, Mn, Se, and Zn were provided as Bioplex TR-Se at 100%, 66% or 33% the level of the ITM premix. Additional vitamin E fortification was supplement in Finisher diets.

were vaccinated against Marek's, IBD and NDV post-hatch. Vaccinations for Newcastle disease virus (NDV), infectious bursal disease (IBD), and infections bronchitis virus (IBV) were performed on d 8 and 21. Each pen was equipped with one tubular feeder and an automatic water supply. Temperature and natural ventilation were controlled by butane heaters and manually set curtains. Temperature was maintained at 32±1 °C during the first week, then was gradually reduced to 22±1 °C by the end of the fifth week. Management, health, and biosecurity measures were conducted as per conventional poultry practices.

### Measurements

Live body weight and feed intake were recorded in each pen on d 1, 14, 28 and 42. Chicks removed from the study for sexing, sampling, ascites, mortality and any other reason were recorded. As part of the restricted feeding programme, leftover feed was weighed and recorded daily before removal from the pens. The feed conversion ratio was calculated as feed intake divided by weight gain of live birds and adjusted for dead and culled birds (kg feed/kg of weight gain).

Tissue mineral concentrations and lymphoid organ measurements were conducted on d 21 and 42. One chick per repetition was randomly selected and euthanised via electrical stunning. Blood Fe concentration was estimated through blood haemoglobin measurement (Ma *et al.*, 2014). Blood samples (2 ml) were collected directly by making a cross-cut section of the right jugular vein. Blood was collected into heparinised tubes (Vacutainer® tubes; BD Inc., Oakville, ON, Canada) and samples were kept at 5 °C before transfer to the laboratory for analysis.

Two tibia bones were dissected from birds to determine the concentration of Mn, Cu, and Zn. Tibia samples were stored in plastic bags and frozen prior to transfer to a local laboratory, where they were boiled in deionised water for 10 min to remove all tissue. Bones with epiphyses attached were dried at 105 °C for 12 hours and then incinerated to 600 °C in a muffle furnace. Mineral concentration was determined by spectrometer ICP-AES (model iCAP6500 DUO, Thermo Scientific, Waltham, MA, USA).

The thymus, spleen and bursa were dissected from euthanised birds, and the weights of each organ were recorded (±0.1 g). The immune organ weight indices were calculated (organ weight (g) / BW (g) ×1000), according to Tanimura *et al.* (1995).

The determination of antibodies in serum from 42 d old birds was performed *via* ELISA (ELx800) using the IDEXX® enzyme immunoassay technology for IBD, IBV, NDV and REO disease (IDEXX Laboratories, Inc., Westbrook, ME, USA). Blood samples (2 ml) were collected from the brachial vein and temporarily stored at 5 °C before transfer to local laboratories for processing. The optical densities of the ELISA readings were transformed into antibody titres according to formulas recommended by IDEXX.

### Statistical analysis

The performance data were subjected to analysis of variance using a linear model with Statistix V.9 (Statistix, Tallahassee, FL, USA). Means of the response variables resulting in a significant F-test ($P$<0.05) were further compared using Tukey's test ($P$<0.05). The average values of mortality, blood and tibia mineral concentration and lymphoid organs were arcsine transformed before statistical analysis. Kruskal-Wallis test as non-parametric methods was used for analysing differences between disease antibody titres.

## 3. Results and discussion

### Performance

Overall, there were no differences in weight gain, feed consumption, feed conversion and mortality among treatment groups ($P$<0.05; Table 2). No statistical differences in feed intake were noted, as expected, in response to feed restrictions that were necessary to prevent metabolic disorders linked to high-altitude environments. This represented approximately 18% less feed consumption than what was recommended by the breed-specific guidelines (Cobb-Vantress, 2015). The average body weight at 42 d was 2.3 kg (considering initial live weight 45 g) with a feed conversion ratio of 1.66. This result was in line with recommendations for the Cobb genetic line for production at high altitudes (Bellido, 2015). Results with millions of birds up to 1000 metres above sea level (MASL) show body weights and feed conversion at 42 d of 2.642 kg and 1.75 respectively. However, in farms between 2,400 and 2,800 MASL the values of these parameters deteriorate to 2.564 and 1.89 respectively. Similarly, Osti *et al.* (2017) found that body weights of broilers grown between 2,000 and 3,000 MASL decreased by 15% and feed conversion by 5% compared to broilers raised below 1000 MASL. Only the data from the 15-28 d phase using 100% PTM and 66% PTM replacement showed significantly lower weight gain compared to the 33% PTM treatment ($P$<0.05), but not the ITM control. During the same period, birds fed 33% PTM showed the lowest feed conversion, although this was not significantly different from the ITM control.

In this trial, even feeding low levels of trace minerals as proteinates (33% PTM) resulted in normal growth, which was similar to birds supplemented with the higher level of ITM (control). Similar results were reported by Bao *et al. (*2007), who found that, even at lower supplemental levels, organic trace minerals were adequate in supporting optimum broiler performance. There were no additional benefits in growth and FCR for the higher PTM treatments.

Other studies did not observe any detrimental impact on performance when trace mineral inclusion rates were reduced in poultry diets formulated with PTM (Leeson and Caston, 2008). Peric *et al. (*2007) reported similar results in weight gain and feed conversion in Hubbard JV birds that were supplemented with reduced levels of PTM. Similarly, no negative effects to production were found in Ross 308 birds fed different supplementation levels of PTM ranging from 17% to 100% of the ITM levels (Nollet *et al.,* 2008), although higher final body weights were observed when these birds were supplemented with 100% inorganic or 100% proteinated trace minerals. A commercial broiler trial did not observe differences with lower levels of organic minerals (50% of Zn, 33.3% of Mn, 62.5% of Cu and 10% of Fe) compared to the inorganic control (Tavares *et al.,* 2011).

Although mortality was not different between treatments, cause of death was recorded throughout the experiment. Of the bird deaths recorded, 34% were due to ascites (17/50), 26% due to delayed or discarded birds (13/50), 14% due to sudden death in last weeks (7/50), 8% due to respiratory problems (4/50) and 18% for reasons such as omphalitis, *Escherichia coli*, curved legs, spondylolisthesis or poor vaccination (9/50).

**Table 2. Weight gain, feed consumption, feed conversion and mortality (%) of chickens fed with inorganic (ITM) or proteinated (PTM) trace mineral sources.[1,2]**

| Age | ITM | 100% PTM | 66% PTM | 33% PTM | SEM[3] | *P*-value | $R^2$ |
|---|---|---|---|---|---|---|---|
| **Weight gain (g)** | | | | | | | |
| 0-14 d | 297 | 298 | 291 | 294 | 3.38 | 0.495 | 0.26 |
| 15-28 d | $831^{ab}$ | $813^{b}$ | $813^{b}$ | $859^{a}$ | 9.16 | 0.010 | 0.62 |
| 29-42 d | 1,119 | 1,131 | 1,143 | 1,118 | 17.49 | 0.726 | 0.32 |
| 0-42 d | 2,247 | 2,243 | 2,247 | 2,270 | 20.37 | 0.776 | 0.41 |
| **Feed intake (g)** | | | | | | | |
| 0-14 d | 390 | 400 | 387 | 392 | 4.90 | 0.284 | 0.35 |
| 15-28 d | 1,248 | 1,242 | 1,229 | 1,256 | 8.84 | 0.225 | 0.60 |
| 29-42 d | 2,182 | 2,192 | 2,183 | 2,184 | 16.90 | 0.975 | 0.27 |
| 0-42 d | 3,820 | 3,834 | 3,798 | 3,831 | 25.35 | 0.747 | 0.43 |
| **Feed conversion ratio** | | | | | | | |
| 0-14 d | 1.31 | 1.34 | 1.33 | 1.33 | 0.01 | 0.385 | 0.33 |
| 15-28 d | $1.50^{ab}$ | $1.53^{a}$ | $1.51^{ab}$ | $1.46^{b}$ | 0.02 | 0.049 | 0.61 |
| 29-42 d | 1.95 | 1.94 | 1.91 | 1.96 | 0.02 | 0.562 | 0.35 |
| 0-42 d | 1.70 | 1.71 | 1.69 | 1.69 | 0.00 | 0.135 | 0.69 |
| **Mortality (%)** | | | | | | | |
| 0-14 d | 1.67 | 0.83 | 0.83 | 1.67 | 0.58 | 0.572 | 0.40 |
| 15-28 d | 3.75 | 2.92 | 1.25 | 1.67 | 1.09 | 0.377 | 0.37 |
| 29-42 d | 1.67 | 1.25 | 1.25 | 2.08 | 0.74 | 0.829 | 0.33 |
| 0-42 d | 7.08 | 5.00 | 3.33 | 5.42 | 0.92 | 0.064 | 0.64 |

[1] ITM = 100% inorganic trace minerals. 100% PTM= PTM supplied as BioplexTR-Se® at 100% ITM level. 66% PTM= PTM supplied as BioplexTR-Se at 66% ITM level. 33% PTM= PTM supplied as BioplexTR-Se at 33% ITM level.

[2] Means in the same row with different superscript letter are statistically different ($P<0.05$).

[3] SEM = standard error of the mean.

## Mineral retention

Blood iron concentrations were not affected by treatments ($P<0.05$) at 21 or 42 d of age (Table 3). In general, blood Fe concentrations were higher at 42 d than at 21 d. Increased rate of absorption and adaptation of birds raised in high altitudes offered a likely explanation of these results (Druyan, 2012; Hernandez, 1987; Zhang *et al.*, 2007), as this could influence the amount of haemoglobin, and hence iron, present in blood. Significant differences ($P<0.01$) in the retention of Cu, Mn and Zn in the tibia at 21 and 42 d of age were observed (Table 3). At 21 d, the 100% and 66% PTM treatment groups had significantly higher tibia Cu concentrations than the other treatments, with the highest tibia Cu concentration obtained with 66% PTM ($P<0.01$). In contrast, at 42 d of age the highest tibia Cu concentrations were observed in the 100% ITM and 100% PTM treatment groups ($P<0.01$). Reports of organic trace mineral retention in poultry are variable. Similar to the present study, Aksu *et al.* (2011) reported no difference in tibia Cu concentration Cu in 42 d old broilers when inorganic and organic minerals were supplemented at the same level (8 mg Cu/kg feed), but observed reduced tibial Cu when organic Cu supplementation was reduced by two thirds in the diet. Bao *et al.* (2007) did not observe differences in tibial Cu concentration in 29-d old broilers fed different sources of Cu ranging from 2-8 mg Cu/kg feed. Conversely, some reports showed no influence on mineral retention in different tissues when using low mineral doses supplied as PTM, compared to typical industry inclusion rates (Vieira, 2015).

At 21 d of age, tibia Cu concentrations were higher than at 42 d of age, possibly due to the fact that enzymes containing Cu are involved in the formation of connective tissue and bone mineralisation (Church and Pond, 1996). The highest levels of tibial Cu agreed with the work of Gheisari *et al.* (2010), who used organic chelates. However, it differed from findings reported by Bao *et al.* (2007), who reported no significant differences in tibia copper concentrations between inorganic and organic treatment groups, and El-Husseiny *et al.* (2012), who found the highest tibia Cu concentration when using inorganic trace minerals.

At 21 d of age, the 33% PTM treatment birds had significantly lower tibia Mn concentrations compared to the other treatments. However, at 42 d of age, all PTM groups had greater deposition of Mn compared to the

**Table 3. Iron concentrations in blood (g/dl) and copper, manganese and zinc in the tibia (mg/kg) of chickens fed with inorganic (ITM) or proteinated (PTM) trace mineral sources.[1,2]**

| Age | 21 days | | | | 42 days | | | |
|---|---|---|---|---|---|---|---|---|
| Treatments[3] | Iron | Copper | Manganese | Zinc | Iron | Copper | Manganese | Zinc |
| ITM | 22.04 | 0.90[c] | 1.72[a] | 60.97[a] | 58.47 | 0.55[a] | 2.50[d] | 81.85[c] |
| 100% PTM | 22.2 | 1.23[b] | 1.76[a] | 42.53[d] | 65.52 | 0.55[ab] | 2.58[c] | 82.99[b] |
| 66% PTM | 20.95 | 2.22[a] | 1.76[a] | 48.49[c] | 48.09 | 0.50[b] | 2.67[b] | 83.47[b] |
| 33% PTM | 23.17 | 0.83[c] | 1.38[b] | 52.02[b] | 47.45 | 0.46[c] | 2.76[a] | 87.36[a] |
| *P*-value | 0.8659 | <0.0001 | 0.00001 | <0.0001 | 0.2517 | 0.0002 | <0.0001 | <0.0001 |

[1] Six male chickens per treatment group.
[2] Means in the same column with different superscript letter are statistically different ($P<0.05$)
[3] ITM = 100% inorganic trace minerals. 100% PTM = PTM supplied as BioplexTR-Se® at 100% ITM level. 66% PTM = PTM supplied as BioplexTR-Se at 66% ITM level. 33% PTM = PTM supplied as BioplexTR-Se at 33% ITM level.

ITM control, with the 33% PTM treatment resulting in the highest tibia Mn concentrations. This was in contrast to Bao *et al.* (2007), who reported the highest concentrations of tibia Mn in broilers fed diets supplemented with high levels of inorganic or organic Mn (80 mg/kg feed) at 29 d old. El-Husseiny *et al.* (2012) reported higher tibia Mn concentrations in 35 d old broilers supplemented 16 vs 8 mg organic Mn per kg feed. Other authors, such as Zhao *et al.* (2010) and Gheisari *et al.* (2010), found no significant differences in Mn tibial concentrations when comparing PTM to ITM supplementation in 52 and 49 d old broilers, when organic Mn was reduced or at the same level as the inorganic Mn.

For Zn at 21 d of age, the ITM group had the highest level of tibial zinc ($P<0.05$). However, at 42 d, all PTM treatments showed higher tibia Zn concentrations, with the 33% PTM treatment being the highest. Mwangi, *et al.* (2017) and Sunder *et al.* (2008) did not find statistical differences in FCR and body weight gain, and indicated that lower amounts of Zn (25 mg/kg and 29 mg/kg respectively) were adequate to support optimal growth during the 21 d post-hatch period, despite the NRC (1994) Zn recommendation of 40 mg/kg of Zn for broiler chicks. In addition, Rossi *et al.* (2007) and Vieira *et al.* (2013) did not find any effect on bird performance when feeding diets without Zn supplementation.

Differences in trace mineral absorption are due to bioavailability (digestion and absorption) and can be affected by intestinal pH, dietary interactions and mineral source. Blood and tissue trace mineral concentrations are not necessarily in direct proportion to intake. Mucins, present in the aqueous layer of the intestinal lumen, contain sulphatomucins and sialomucins that confer a negative charge, which can bind positively charged ions and allows nutrients to reach the villi for absorption. This phenomenon is directly proportional to the rate of ligand exchange, i.e. there is a competition between ions for binding, so that, at some point, this mechanism can become saturated and excess will be eliminated in faeces (Power, 2004). This is an exclusive consideration for organic minerals with high bioavailability. For inorganic minerals, mineral-to-mineral antagonism, intestinal pH and other anti-nutritional factors, such as polyphenols and metallic binders, should be considered (Church and Pond, 1996). Use of exogenous enzymes adds complexity to the issue of minimising dietary trace mineral levels. For example, it can be expected that phytase addition will influence trace mineral bioavailability through breaking the bonds between phytic acid and minerals such as Zn, Mn, and Cu (Banks *et al.*, 2004). Interestingly Aoyagi and Baker (1995) suggested that phytase can reduce Cu utilisation from soybean meal by 50%, ascribing this situation to a higher release of Zn which, itself, competes with and reduces Cu absorption. It is important to note that the current study diet did not include a phytase enzyme.

### Lymphoid organs

The bursa of Fabricius, thymus, and spleen are the primary lymphoid organs in avian species. Any changes in the size and weight of these organs can dramatically affect lymphocyte proliferation and subsequent immune responses (Park *et al.*, 2013). Results of bursa (B), thymus (T), spleen (S), and their relationships to body weight (BW) and the interrelationships between B/T and B/S are shown in Table 4. These parameters showed no significant differences among treatments at either 21 or 42 d ($P<0.05$).

These results were in agreement with several other studies that found no significant differences in the relative bursa (Gheisari *et al.*, 2010; Sunder *et al.*, 2013) and spleen (Gheisari *et al.*, 2010) weights due to trace mineral source or organic Mn supplementation. However, organic Mn

**Table 4. Lymphoid organs[1] indices (g/kg body weight; g/g lymphoid organs) of chickens fed with inorganic (ITM) or proteinated (PTM) trace mineral sources.[2]**

| Age | 21 days | | | | | 42 days | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Treatments[3] | B/BW | T/BW | S/BW | B/T | B/S | B/BW | T/BW | S/BW | B/T | B/S |
| ITM | 1.88 | 3.73 | 0.74 | 0.54 | 2.75 | 1.36 | 1.97 | 0.97 | 0.67 | 1.45 |
| 100% PTM | 1.84 | 2.88 | 0.94 | 0.64 | 2.03 | 1.86 | 2.48 | 0.94 | 0.80 | 2.00 |
| 66% PTM | 1.86 | 3.53 | 0.77 | 0.56 | 2.63 | 1.74 | 2.48 | 1.04 | 0.81 | 2.00 |
| 33% PTM | 2.32 | 3.65 | 1.06 | 0.66 | 2.39 | 1.21 | 2.71 | 1.08 | 0.46 | 1.23 |
| *P*-value | 0.420 | 0.336 | 0.210 | 0.613 | 0.593 | 0.197 | 0.421 | 0.9525 | 0.3035 | 0.172 |

[1] B = bursa of Fabricius; T = Thymus; S = Spleen; B/T = bursa:thymus ratio; B/S = bursa:spleen ratio. B/BW index was multiplied by 1000 according to Alamsyah *et al.*, 1993; Tanimura *et al.*, 1995. Six male chickens per treatment group.
[2] Means in the same column with different superscript letter are statistically different (*P*<0.05).
[3] ITM = 100% inorganic trace minerals. 100% PTM = PTM supplied as BioplexTR-Se® at 100% ITM level. 66% PTM = PTM supplied as BioplexTR-Se at 66% ITM level. 33% PTM = PTM supplied as BioplexTR-Se at 33% ITM level.

supplementation level was shown to affect spleen weight of 35-d-old broilers (Sunder *et al.,* 2013).

The weight or size of the bursa is considered an indicator of immunocompetence in the bird as it is the site of B-lymphocyte development and differentiation (Cheema *et al.,* 2003; Qureshi *et al.,* 1998), and a higher B/BW index is considered favourable. Some methods multiply this index by 1000 (Alamsyah *et al.,* 1993; Tanimura *et al.,* 1995) or by 100 (Cazaban *et al.,* 2015; Wehner, 1999), and values between 1.1-1.3 or 0.11-0.13 respectively from 7 to 42 d of age are expected as a minimum. In this trial, none of the treatments resulted in an average index below 1.0.

The thymus provides a specific site for T-lymphocyte development and differentiation, which is essential for cell-mediated immunity and modulation of the response (Owen, 1977). Results from this trial showed no differences in T/BW and B/T ratio among treatments (*P*<0.05). Perozo-Marin *et al. (*2004) reported a B/T ratio as 0.53±0.21 at 21 d of age, similar to the B/T ratio in this study. The size of the thymus is a sensitive indicator of health status, as the bursa of Fabricius, thymus and spleen can atrophy in the presence of glucocorticoids and corticosterone in stressful situations (Park *et al.*, 2013).

The spleen is a part of the lymphatic system and its main functions are to capture circulating antigens in blood, activate macrophages and trigger the production of unspecified plasma cells. In this trial, there were no differences between treatments in spleen/BW (S/BW) and bursa/spleen (B/S) ratios (*P*<0.05). Pulido *et al. (*2001) stated that a B/S ratio greater than two may be considered a suitable indicator of immunocompetence. In this study, at 21 d all treatments resulted in B/S greater than two, whereas, at 42 d, only the 100% PTM and 66% PTM resulted in B/S of 2. The ITM and 33% PTM B/S values at 42 d were in agreement with Perozo-Marin *et al. (*2004) who reported coefficients lower than two after 35 d of age.

### Antibody titres

Antibody titres at 42 d of age are shown in Table 5. The IBD, IBV and REO titres were similar between treatments (*P*<0.05). For NDV, the ITM, 100% PTM and 66% PTM groups had the highest antibody titre values (*P*<0.05).

Several other researchers have reported that PTM supplementation may have beneficial effects on intestinal development, immune system status and liveability. In a recent study, Echeverry *et al.* (2016) found decreased intestinal goblet cells, lower numbers of ileum germinal centres and higher uric acid plasma concentrations compared to a control and bacitracin methylene disalicylate fed birds, which suggested enhanced health status. Aksu *et al.* (2010) found that supplementation with lower levels of

**Table 5. IDEXX® index antibody titres[1] of 42-day-old broilers fed with inorganic (ITM) or proteinated (PTM) trace mineral sources.[2]**

| Treatments[3] | Infectious bursal disease | Infections bronchitis virus |
|---|---|---|
| ITM | 183 (45, 149) | 239 (134, 84) |
| 100% PTM | 254 (19, 179) | 180 (74, 84) |
| 66% PTM | 494 (148, 123) | 302 (178, 75) |
| 33% PTM | 279 (66, 134) | 176 (107, 121) |
| *P*-value | 0.2028 | 0.2090 |

[1] Antibody titres reported as arithmetic mean (geometric mean, %coefficient of variation). Twenty male chickens per treatment group.
[2] Means in the same column with different superscript letter are statistically different (P<0.05).
[3] ITM = 100% inorganic trace minerals. 100% PTM = PTM supplied as BioplexTR-Se® at 100% ITM level. 66% PTM = PTM supplied as BioplexTR-Se at 66% ITM level. 33% PTM = PTM supplied as BioplexTR-Se at 33% ITM level.

organically-complexed Cu, Zn, and Mn, instead of ITM, in broiler diets had no negative effects on antioxidant defence systems. The results from Abdallah *et al.* (2009) indicated that increasing levels of PTM from 50% to 100% had no significant effect on sheep red blood cell antibody titres, and were higher compared with those from birds fed ITM.

Trace minerals, such as Fe, Zn, Mn and Cu, are essential co-factors in key metabolic processes, as they play a role in regulating cellular pathways and can influence the viability of potential pathogens in the mucosa of the gastrointestinal tract. Therefore, bioavailability and bio-efficacy are contextual dependents, and it is necessary to consider their specific function (nutritional vs regulatory vs antimicrobial) when comparing nutrient sources (Klasing and Iseri, 2013). For example, Yang *et al.* (2011) found that additional supplementation of individual salts (inorganic sulphates of Cu, Fe, Zn, and Mn) in standard diets did not improve the growth performance or immune function of broilers. In contrast, organic chelated trace minerals offer benefits above those of inorganic trace mineral sources. Maletto and Cagliero (1993) demonstrated that amino acid chelates enhanced protein synthesis, and led to more efficient use of dietary carbohydrates and proteins *via* mineral-dependent enzymatic activities in the intestinal lumen. In this case, the higher bioavailability of trace minerals in the chelated form can activate enzymes to improve metabolism and performance in broilers.

Considering the economic and global importance of broiler production, more information is required to achieve an in-depth understanding of the effects of feeding low levels of PTM on immune response and antioxidant defence systems under different environmental challenges. The capacity of the immune response depends on several biological factors, such as age, individual physiology, nutritional status, underlying pathogenesis and environmental management.

## 4. Conclusions

This study identified the limitations, interactions and adequate levels of proteinates and how they could be used to optimise future commercial broiler performance. The results indicated it is possible to use lower levels of PTM without compromising growth performance, compared to 100% ITM diets formulated for high altitude production systems. Reduced levels (33% of control) of trace mineral supplementation with proteinates did not negatively affect broiler performance when raised under conditions of restricted intake and high altitude. Replacing high levels of ITM with one or two thirds of the control levels as organic trace minerals maintained an adequate immune response of broilers under high altitude rearing conditions.

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## Conflict of interest

The authors have no known conflicts of interest. All named authors have contributed equally and read and approved the manuscript. All authors have accepted the order of authors listed in the manuscript. S. Elliott is an employee of Alltech Inc, USA.

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