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The Effect of *In Ovo* Injection of Organic Manganese on the Hatchability of Broiler Breeder Hen Eggs and Productivity of Offspring Broiler Chickens

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Abstract

This study investigated the effects of *in ovo* injection of organic manganese on the hatchability of eggs of broiler breeder hens. It also evaluated the performance, carcass characteristics, blood biochemical parameters, liver enzymes, immune system response and intestinal microbiota in offspring broiler chickens. For the *in ovo* injection, 320 fertile eggs were selected from the Ross 308 breeder hens' flock at 55 weeks with the same average weight (65±1 g). Treatment 1: negative control group (no injection), treatment 2: positive control (injection of 0.272 mL of normal saline solution), treatment 3 (Mn20): injection of 0.272 mL of a solution containing 73.52 µg of organic manganese (20 µg for each egg), and treatment 4 (Mn30): injection of 0.272 mL solution containing 110.28 µg of organic manganese (30 µg for each egg). Hatched chicks were raised based on a completely random design, including four treatments and four replications with commercial diets based on corn-soybean meal. The results showed that *in ovo* injection of organic manganese led to an improvement in the hatchability compared to the positive control treatment, but the highest percentage belonged to the treatment without injection (negative control) ($P < 0.05$). In the whole period, no negative effects were observed on production performance, carcass characteristics and gut morphometry after injection ($P > 0.05$). Total cholesterol, triglycerides, low-density lipoprotein, high-density lipoprotein, atherogenic index, protein, albumin, globulin, uric acid, creatine kinase, erythrocytes and the average volume of red blood cells of chickens increased in the groups injected with organic manganese ($P < 0.05$) but alkaline phosphatase decreased ($P < 0.05$). Birds injected with organic manganese had the lowest leukocytes and heterophils and the highest lymphocytes ($P < 0.05$). In addition, a reduction of coliform and E*scherichia coli* populations and an increase of *Lactobacillus*, *Bifidobacterium* and lactic acid bacteria after *in ovo* injection of organic manganese was found ($P < 0.05$). In conclusion, *in ovo* injection of organic manganese, without having a positive effect on the hatchability, had both positive and negative effects on the biochemical parameters of the blood and the immune system. Moreover, it was effective in improving the chicken's cecal microbiota.

Introduction

Manganese is the fifth most abundant element on the planet. Manganese is one of the essential elements in the body in which it plays a role in many physiological processes as a catalyst and enzyme cofactor in the growth process and the metabolism of carbohydrates and fats. Consequently, manganese, as an enzyme cofactor, plays a vital role in many activities, such as proper cellular redox status, urea metabolism, neurotransmitter synthesis, autophagy processes etc. (Sobańska *et al*., 2021). It was the first deficient element in poultry nutrition and was recommended as a supplement in the diet (Creek, 1995; Shastak and Rodehutscord, 2015).

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Manganese is also structurally important in various enzymes such as manganese superoxide dismutase, glutamine synthetase, and phosphoenol pyruvate carboxykinase (Shastak and Rodehutscord, 2015; Zafar and Fatima, 2018). In chickens, due to the low content of manganese in the natural diet and the low absorption of this element from the intestine, it needs to be provided through diet (Conly *et al*., 2012; Tufarelli and Laudadio, 2017). Bone, having 3 to 4 μg of manganese per gram of tissue, is the richest source of manganese in the body. Liver tissue with 2 μg per gram is second after bone, followed by the pituitary and pineal glands (Pesti *et al*., 2005). The required amount of manganese depends on the species of the animal, growth stage, chemical composition of the manganese consumed, and the total diet of the poultry (Tufarelli and Laudadio, 2017). In fact, the requirements of poultry for manganese (per unit of feed weight) is due to the deficient absorption of manganese because of competition with calcium, phosphorus and common salts and its low retention in the body (excretion by bile), is higher than in mammals (Broom *et al*., 2021). The retention rate of manganese in young laying birds during 1-150 days varies between 0.5 and 2.5%. There is not enough of this element in the usual poultry feed (corn, wheat, peas, soy, etc.) (about 20 mg/kg of dry feed). Among cereals, wheat and its products are good sources of manganese, but corn is inferior (Olgun, 2017). Hay powder, dry brewer's dregs, rice bran, sprouted bran, and wheat bran are all good sources of manganese. Milk, corn and its products have very little manganese.

Because manganese is stored in bones, animal products containing bones usually have high levels of manganese (Pesti *et al*., 2005). Considering the doubling of the growth rate of poultry over the past years, the required amount of manganese is much higher than the amount recommended by NRC (1994), which is 60 mg/kg of dry matter for all growth stages of broiler chickens. However, the level of manganese in the tables of recommended nutrient requirements in the Ross 308 catalog has increased to 120 mg/kg, which can be attributed to genetic modification and the increased growth rate of current strains. Using the primary feeding strategy of eggs with the in ovo injection technique, we can approach our goals in improving poultry nutrition (Noy and Uni, 2010). *In ovo* injection provides an accessible source of nutrients for the growing embryos, which accelerates the growth and development of the intestine and in parallel increases the hatching weight of the birds, accelerates the growth rate before and after hatching, and improves the immune system performance. Improving and developing the gastrointestinal tract can lead to increased meat yield (Uni and Ferket, 2003; Kadam *et al*. 2008).

However, it has been determined that the absorption of manganese is via diffusion and is influenced by the level of manganese in the diet. Due to the lack of manganese in the diet and the parallel low bioavailability from this source, it seems that the use of new methods and technologies, such as *in ovo* injection, can help reduce possible manganese deficiencies in chickens (Oliveira *et al*., 2015). Since trace elements play an important role in many metabolic functions and are essential for growth and development (Świątkiewicz *et al*., 2014), they have been suggested as the most effective ingredients for the implementation of *in-ovo* feeding technology (Bhanja *et al*., 2015; Goel *et al*., 2016). These nutrients are important for the skeletal development of the fetus as they can improve bone health and growth (Torres and Korver, 2018). In addition, sources of dietary rare earth elements in organic form have better bioavailability than inorganic forms and are therefore needed in low amounts (Pereira *et al*., 2018). Sahr *et al.* (2020) believe that the *in ovo* injection of inorganic manganese leads to weight loss in chicken production, but no adverse effect on the health of chickens during the growth period was observed. Şentürk and Yıldız (2020) concluded that *in ovo* injection of manganese along with copper and zinc in organic form led to an increase in the hatchability of quails and further found that high levels of injection led to an increase in the weight of the heart, liver, and wing length. There are a limited number of studies on the effect of *in ovo* injection with organic manganese on the hatchability of breeder hen eggs and their broiler chicken offspring. Therefore, this experiment was conducted to fulfill this gap.

Materials and Methods

Experimental Conditions and *In Ovo* Injection of Manganese

Experiments were undertaken according to the scheme of Table 1. In total, 320 fertile eggs with an average weight of 65±1 g were collected from the Ross 308 broiler breeder hen flock at the age of 55 weeks. Eighty eggs were injected for each treatment (8 replications included ten fertile eggs). Incubation and hatching were carried out in the hatchery farm (Navid Morgh Gilan Co, Rasht, Iran) and private laboratory (ViroMed Co, Rasht, Iran). In the first 18 days, primary incubation was performed at 37.6°C and 56% relative humidity with six rotations. On the 10th day, according to the protocol recommended by Williams and Hopkins (2011) and Omidi *et al*. (2020), an *in ovo* injection was performed in the amniotic sac. Then, all the samples were placed in a multi-stage incubator (Jamesway incubator pt100, Canada) from 19^{th}-21^{st} days of incubation at a temperature of 37.0°C and 58.5% relative humidity and were incubated according to the protocol described by McQuoid (2000).

Table 1. Experimental treatment design and solution concentration of *in ovo* injection

Treatments	Name of test ingredient in the solution	Injected amounts of solution (mL)	Amounts of organic manganese in solution (µg)	Experimental name groups
1	-	0	0	Negative control
2	Soluble saline	0.272	0	Positive control
3	Organic manganese[1]	0.272	20	Mn20
4	Organic manganese[2]	0.272	30	Mn30

[1] In the third treatment, the concentration of organic manganese element is 73.52 µg per 0.272 milliliters of injection solution.

[2] In the fourth treatment, the concentration of organic manganese element is 110.28 µg per 0.272 milliliters of injection solution.

Bird Rearing Management

Among 320 hatched eggs, 160 chickens were reared in a private farm (Gilan province, Iran). Performance data were collected for 1-14 days old, 15-28 days old and 29-42 days old based on a completely randomized design with four treatments and four replications including ten birds. They were kept for 42 days in floor pens with dimensions of 1 m × 1 m and the management of chicken rearing in terms of temperature, light, drinking water and vaccination program was carried out based on the latest Aviagen recommendation. The pellet diets based on corn-soybean meal were provided *ad labium* (Table 2).

Table 2. Ingredients and nutrient composition of the basal diet of the experimental treatments

Items	Starter (1-14 days)	Grower (15-28 days)	Finisher (29-42 days)
Ingredients, (%)			
Corn grain	49.745	46.11	50.71
Soybean Meal (SBM)	37.00	35.50	31.00
Vegetable Oil	1.50	1.50	1.50
Di-Calcium-Phosphate	1.10	0.90	0.80
$CaCO_3$	1.25	1.20	1.20
Bentonite	0.00	1.50	1.50
NaCl	0.27	0.24	0.22
D, L-Methionine (liquid)	0.21	0.16	0.15
NaHCO3	0.06	0.08	0.11
Vitamin and Mineral Premix [1]	0.50	0.50	0.50
Diclazuril	0.03	0.03	0.00
Medermycin	0.00	0.00	0.06
DL-Methionine (powder)	0.08	0.05	0.04
Lysine	0.14	0.125	0.115
Threonine	0.06	0.05	0.04
Toxin binder	0.04	0.04	0.04
Phytase 1000	0.005	0.005	0.005
Endo-power multi-enzyme	0.01	0.01	0.01
Wheat flour	8.00	12.00	12.00
Calculated nutrient composition			
AME_n, Kcal/kg	2980	3000	3030
Crude Protein, %	21.00	20.00	18.50
Calcium, %	0.10	0.93	0.85
Available Phosphorus, %	0.48	0.47	0.45
Sodium, %	0.17	0.16	0.16
Chloride, %	0.20	0.20	0.12
Methionine, %	0.56	0.54	0.52
Threonine, %	1.98	0.87	0.85
Lysine, %	1.30	1.20	1.10
Arginine, %	1.68	1.54	1.51
Dietary cation-anion balance, mEq/kg	272.12	244.55	242.77

[1] The amount of vitamins and minerals per kg of the final diet: Vitamin A, 9000 IU; vitamin D3, 3000 IU; vitamin E, 18 IU; vitamin K_3, 3 mg; vitamin B_1 (Thiamine), 1.8 mg; vitamin B_2 (Riboflavin), 6 mg; vitamin B_6 (Pyridoxine), 3 mg; vitamin B_{12} (Cyanocobalamin), 0.012 mg; vitamin B_3 (Niacin), 30 mg; vitamin B_9 (Folic acid), 1 mg; vitamin H_3 (Biotin), 0.24mg; vitamin B_5 (Pantothenic acid), 10 mg; 500 mg; Choline,100 mg; Mn, 100 mg; Zinc, 80 mg; Iron, 10 mg; Cu, 1 mg; I,0.2 mg.

Sampling and Laboratory Measurements

Sampling of unhatched eggs (hatchery debris) was done to investigate the mortality rate of embryos and infertile or dead embryos on the 21st day of incubation. The unhatched eggs were categorized according to the number of pip eggs, exploded eggs, early rot, late rot, cracked eggs, and malformed embryos (Tullett, 2009). The percent hatchability was calculated according to the formula: number of chicks hatched/number of eggs laid in the hatchery) ×100 **(**Oliveira *et al*., 2015).

In addition to the percentage of chickens harvested, the hatched weight of chickens and their grading was recorded. During the post-hatch period, feed consumption and weight gain of the chicks were measured and the feed conversion ratio (FCR) was calculated. At 42nd days of age, two chickens were selected from each replication, weighed after 4 hours of starvation, and slaughtered and the weights of the carcass components were measured (Zaker-Esteghamati *et al*., 2021; Belali *et al*., 2021). The blood samples were collected from wing veins using 5 mL syringes and centrifugation at 3000 rpm (1765g). The samples were then transferred to the Viromed Laboratory (Rasht, Iran) for analysis. Total cholesterol, triglycerides, low-density lipoprotein, high-density lipoprotein, atherogenic index, very-low-density lipoprotein, total protein, albumin, globulin, alkaline phosphatase, uric acid, creatine kinase, hemoglobin, red blood cell, mean corpuscular of hemoglobin, mean corpuscular volume, mean corpuscular hemoglobin concentration, serum glutamic-oxaloacetic transaminase, aspartate aminotransferase, serum glutamic pyruvic transaminase, and alanine aminotransferase were analyzed using Pars Amzoon commercial kits. To evaluate humoral immunity, the birds were injected with 0.2 mL of sheep red blood cells (SRBC) in the wing vein on days 28 and 36 of the rearing period, and samples were taken on days 35 and 42 of rearing (Lerner *et al*., 1971). The amount of antibodies was obtained by the hemagglutination inhibition method (Seidavi *et al*., 2014).

On the 35th and 42nd days of rearing, two blood samples were taken from each experimental group. Serum titers of Newcastle disease virus and avian influenza virus were determined using the hemagglutination inhibition method (HI) according to the OIE standard (Shabani *et al*., 2015). On the 40th day of rearing, four birds were selected from each experimental group and 0.1 mL of PBS and phytohemagglutinin were injected into both chicken wings. After 18 hours, the thickness of the injection site was measured with a micrometer. It should be noted that the difference in the thickness after injection of the left wing (saline) and the thickness after injection of the right wing (PHA) were used as measurement criteria in the evaluation for cell safety (Feshanghchi *et al*., 2022). The values of heterophil (HE), monocyte (MO), and eosinophil (EO) were measured according to the method of Nosrati *et al*. (2017), Albarrak (2021), and Kim and Kang (2022), respectively.

To assess the intestinal microbiota, a sterile microtube was used to sample the contents of the cecum, which were then subjected to a 9-step serial dilution with a peptone-water solution (Merck, 1.07228.0500). The diluted homogenate in 0.1 mL amounts was spread with a sampler on microbial culture medium and incubated as Hosseintabar et al. (2013) recommended. Microbial culture of *Lactobacillus*, *Escherichia coli*, coliform and Bifidobacterium and the total population of lactic acid bacteria, respectively in microbial culture media including Rogosa agar (Merck, 1.05413.0500), E.M.B agar (Merck, 1.01347.0500), MacConkey agar (Merck, 1.10426.0500), Tos propionate agar (Merck, 100043) and Nutrient agar (Merck, 105450) were prepared according to the manufacturer's recommendations. The incubation temperature and time were chosen according to the Omidi *et al*. (2020). The data were expressed as $\log_{10}$ colony forming units (CFU). Data normality was checked by the Shapiro-Wilk method. The homogeneity test of the variance of the experimental treatments was done using Bartlett's test using SAS 9.1 software. Duncan's method was used to compare the means obtained at 0.05 level.

Results and Discussion

The results of traits related to the incubation period are shown in Table 3. The hatchability percentage in the treatment without injection (negative control) was the highest ($P < 0.05$). Treatments injected with organic manganese (Mn20 and Mn30) had a higher hatchability percentage compared to the positive control group ($P < 0.05$). The chickens' weight after the injection of organic manganese (20 µg) was the highest and similar to the group without injection (negative control) ($P < 0.05$). Sahr *et al*. (2020) encountered a decrease in the hatchability percentage and chick weight after *in ovo* injection of inorganic manganese, which contrasts with the results of this research. It seems that the inorganic form, unlike the organic form, has a negative effect on the hatchability and weight, and in fact, the organic form has less toxicity in the perinatal period and does not interfere with other inorganic elements (Watts, 1990).

Table 3. The results of traits related to the incubation period

Items	Treatments[1]				*SEM*	P-value
	Negative control	Positive control	Mn20	Mn30		
Hatchability (%)	96.25[a]	58.75[c]	76.25[b]	66.25[bc]	4.75	0.001
Chick weight (g)	46.63[a]	44.31[b]	45.69[a]	45.66[b]	0.42	0.001

[1] Negative control: Without injection, Positive control: Injection of 0.272 mL of normal saline solution, Mn20: Positive control + 20 µg organic Mn, Mn30: Positive control + 30 µg organic Mn.
[a,b,c] Means within each row with different superscripts differ significantly at $P < 0.05$.

Visual evaluations showed that grade 1 chicks that are suitable for sale are included in the negative control (93.75%), positive control (51.25%), Mn20 (61.25%) and Mn30 (51.25%), respectively (Figure 1). Grade 2 chicks (cull chicks) were respectively in negative control (2.5%), positive control (5%), Mn20 (15%) and Mn30 (15%). The unhatched eggs in the negative control, positive control, Mn20 and Mn30 were 3, 33, 20 and 27, respectively. On days 11 to 17, the number of feathers in the negative control, positive control, Mn20 and Mn30 treatments were counted as 1, 27, 7 and 11, respectively. On the 18th to 19th days, the number of unhatched eggs (dead-in-shell) for the negative control, positive control, Mn20, and Mn30 treatments was observed as 1, 3, 6, and 7, respectively. The diagnosed parameters of pip eggs (i.e., the first break in the eggshell by the hatching bird) for the Mn20 and Mn30 treatments were 4 and 6, respectively. Three, one and one infected eggs were observed in positive control, Mn20 and Mn30 treatments, respectively. Early rot, exploded eggs, cracked eggs, and malformed embryos were not observed among the experimental treatments.

The results of the traits related to the performance of broiler chickens are shown in Table 4. No negative effects on production performance (feed consumption, body weight, and FCR) were observed after *in ovo* injection in the starter, grower, finisher and the whole period ($P > 0.05$). Sahr *et al*. (2020) reported that with *in ovo* injection of inorganic manganese, the production performance of chickens was similar to the control group without any negative effects, and despite the difference in the form of manganese, the results were consistent with the current research.

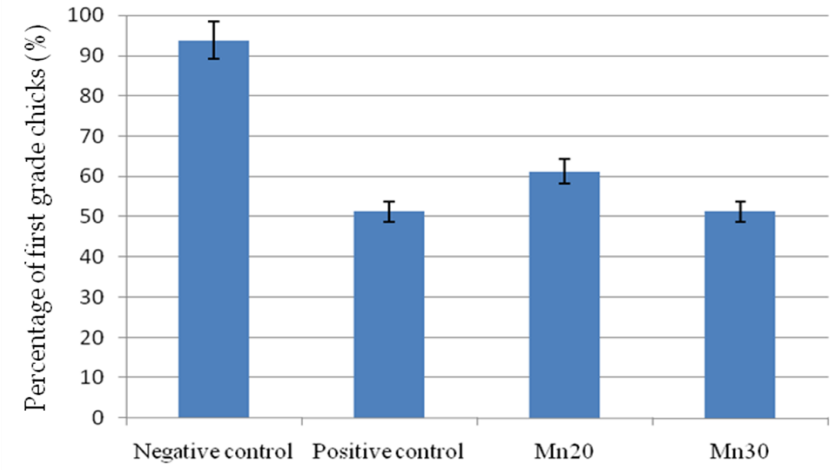

Figure 1. Percentage of first-grade chickens for sale in difference treatments

Table 5 shows that the characteristics of the carcasses of the chickens after the *in ovo* injection of organic manganese were similar to the control group and no negative effects were encountered on the relevant traits ($P > 0.05$). The results of the intestinal traits of broiler chickens are also shown in Table 6. After the *in ovo* injection of organic manganese, the traits related to the intestines of the production chickens were not negatively affected and were similar to the control group ($P > 0.05$). Sahr *et al*. (2020) also did not observe any difference in the treatments injected with inorganic manganese for carcass components and gastrointestinal tract traits, which was in line with the results of the present study. However, an increase in heart weight was noticeable compared to the control group, which was not observed in the present study. Further, Şentürk and Yıldız (2020) observed an increase in the weight of the heart and liver with the *in ovo* injection of a mixture of organic forms of manganese, copper and zinc on quail, which conflicted with the results of this research. Geng *et al*. (2022) reported that *in ovo* injection of manganese under normal and stress conditions has no negative effect on embryo growth and broiler performance.

Table 4. Growth performance (mean ±*SEM*) of broilers received organic Mn in their embryonic period via *in ovo* injection

Items	Treatments[1]				*SEM*	P-value
	Negative control	Positive control	Mn20	Mn30		
Feed consumption (g)						
1-14 d	524.25	543.75	495.25	502.75	23.19	0.472
15-28 d	1655.97	1600.00	1647.90	1653.60	31.34	0.568
29-42 d	2697.72	2606.50	2544.97	2749.67	286.53	0.950
1-42 d	4878.10	4750.30	4688.10	4906.00	285.54	0.936
Weight Gain (g)						
1-14 d	457.38	454.78	431.20	418.10	16.39	0.318
15-28 d	1022.17	1033.25	1066.37	1078.82	17.29	0.124
29-42 d	1379.67	1310.350	1266.57	1409.32	169.15	0.929
1-42 d	2859.20	2798.40	2764.20	2906.30	169.84	0.941
FCR (g/g)						
1-14d	1.15	1.19	1.15	1.20	0.02	0.340
15-28 d	1.62	1.55	1.54	1.53	0.02	0.124
29-42 d	1.96	1.99	2.01	1.95	0.08	0.637
1-42 d	1.70	1.69	1.69	1.68	0.007	0.423

[1] Negative control: Without injection, Positive control: Injection of 0.272 mL of normal saline solution, Mn20: Positive control + 20 µg organic Mn, Mn30: Positive control + 30 µg organic Mn.
[a,b,c] Means within each row with different superscripts differ significantly at $P < 0.05$.

Table 5. Carcass characteristics (mean ±*SEM*) of broilers when injected their parent as *in ovo* with manganese

Items	Treatments[1]				*SEM*	P-value
	Negative control	Positive control	Mn20	Mn30		
Live body weight (g)	3115.50	2809.00	2829.00	3162.50	207.87	0.51
Defeather body weight (g)	2766.50	2473.00	2560.00	2917.80	175.16	0.31
Full abdomen carcass weight (g)	2572.50	2191.50	2300.00	2617.50	162.90	0.23
Empty abdomen carcass weight (g)	2074.00	1884.00	1961.00	2241.00	133.12	0.30
Relative weight of head (%)	1.95	2.13	1.83	1.82	0.11	0.20
Relative weight of breast (%)	27.05	27.46	29.05	29.06	1.24	0.56
Relative weight of drumsticks (thighs) (%)	18.82	19.88	20.08	20.69	0.48	0.19
Relative weight of wings (%)	5.81	6.49	6.43	6.50	0.35	0.47
Relative weight of abdominal fat (%)	0.85	0.62	0.75	1.00	0.15	0.40
Relative weight of pancreas (%)	0.15	0.18	0.19	0.18	0.02	0.63
Relative weight of gizzard (%)	0.95	0.88	0.97	0.88	0.05	0.64
Relative weight of heart (%)	0.48	0.49	0.46	0.49	0.05	0.98
Relative weight of back thoracic vertebrae (%)	10.89	10.06	9.53	10.73	0.39	0.10
Relative weight of neck (%)	3.26	3.48	3.82	3.74	0.24	0.37
Relative weight of proventriculus (%)	0.31	0.41	0.40	0.31	0.04	0.32
Relative weight of crop (%)	0.30	0.22	0.26	0.25	0.03	0.48

[1] Negative control: Without injection, Positive control: Injection of 0.272 mL of normal saline solution, Mn20: Positive control + 20 µg organic Mn, Mn30: Positive control + 30 µg organic Mn.
[a,b,c] Means within each row with different superscripts differ significantly at $P < 0.05$.

The results of the traits related to the biochemical parameters of the broilers blood are shown in Table 7. The *in ovo* injection of organic manganese led to an increase in TC, TG, LDL, HDL, atherogenic index (AI), TP, Alb, Glb, UA, CK, RBC and MCV, compared to the control group ($P < 0.05$). Blood AP decreased after *in ovo* injection of organic manganese ($P < 0.05$). Liver enzymes were the highest and lowest in the group injected with 20 and 30 µg of organic manganese, respectively ($P < 0.05$). Sahr *et al*. (2020) did not observe significant differences in blood parameters after inorganic manganese *in ovo* injection. It seems that organic manganese is ineffective in improving blood lipid profile, but it does increase blood proteins with a decrease of alkaline phosphatase, indicating the chickens' health and lack of physiological pressure on them after *in ovo* injection.

Table 6. Gut morphometry (mean ±*SEM*) of broilers when injected their parent as *in ovo* with manganese

Items	Treatments[1]				*SEM*	P-value
	Negative control	Positive control	Mn20	Mn30		
Relative weight of duodenum (%)	0.41	0.43	0.50	0.39		
Duodenum length (mm)	274.75	309.00	310.50	282.75	33.84	0.83
Duodenum width (mm)	6.78	8.37	8.56	8.55	0.93	0.49
Duodenum diameter (mm)	11.40	12.03	11.93	11.56	0.47	0.76
Relative weight of jejunum (%)	0.92	0.91	1.04	0.82	0.10	0.49
Jejunum length (mm)	820.00	821.75	803.25	873.75	59.69	0.85
Jejunum width (mm)	9.01	8.20	7.91	8.08	0.45	0.36
Jejunum diameter (mm)	12.10	11.10	282.40	11.30	135.76	0.42
Relative weight of ileum (%)	0.69	0.80	0.73	0.68	0.07	0.66
Ileum length (mm)	892.80	853.30	686.80	921.50	107.53	0.44
Ileum width (mm)	7.21	7.47	7.83	8.10	8.47	0.40
Ileum diameter (mm)	10.36	11.08	10.23	10.38	0.61	0.75
Relative weight of colon (%)	0.09	0.12	0.11	0.09	0.01	0.36
Colon length (mm)	81.25	87.50	84.25	84.00	6.35	0.92
Colon length (mm)	8.62	8.74	8.99	8.73	0.38	0.91
Colon diameter (mm)	11.07	11.78	11.33	11.21	0.51	0.78
Relative weight of right cecum (%)	0.13	0.13	0.15	0.16	0.24	0.46
Right cecum length (mm)	197.75	196.25	206.50	209.25	13.54	0.87
Right cecum width (mm)	7.73	7.29	7.71	8.00	0.48	0.77
Right cecum diameter (mm)	9.37	9.55	10.13	9.62	0.95	0.94
Relative weight of left cecum (%)	0.12	0.15	0.16	0.17	0.01	0.13
Left cecum length (mm)	198.75	205.00	191.00	208.50	8.67	0.52
Left cecum width (mm)	7.49	7.56	7.55	8.28	0.43	0.55
Left cecum diameter (mm)	10.47	9.35	9.51	10.61	0.63	0.41

Negative control: Without injection, Positive control: Injection of 0.272 mL of normal saline solution, Mn20: Positive control + 20 µg organic Mn, Mn30: Positive control + 30 µg organic Mn.

[a,b,c] Means within each row with different superscripts differ significantly at $P < 0.05$.

Table 7. Blood constitutes (mean ±*SEM*) of broilers when injected their parent as *in ovo* with manganese

Items [2]	Treatments[1]				*SEM*	P-value
	Negative control	Positive control	Mn20	Mn30		
TC (mg/dL)	116.75[b]	114.00[b]	142.75[a]	144.25[a]	2.85	0.001
TG (mg/dL)	50.92[c]	58.32[c]	69.25[b]	78.67[a]	2.42	0.001
LDL (mg/dL)	48.90[c]	44.10[c]	63.57[a]	57.47[b]	1.88	0.001
HDL (mg/dL)	52.90[b]	55.92[b]	58.20[b]	65.85[a]	2.17	0.001
AI (LDL/HDL)	0.93[b]	0.79[b]	1.09[a]	0.87[b]	0.05	0.009
VLDL (mg/dL)	10.17[c]	11.65[c]	13.82[b]	15.72[a]	0.48	0.001
TP (g/dL)	2.81[b]	3.68[a]	3.42[a]	3.49[a]	0.15	0.008
Alb (g/dL)	1.28[b]	1.61[a]	1.53[a]	1.55[a]	0.05	0.005
Glb (g/dL)	1.52[b]	2.07[a]	1.89[a]	1.94[a]	0.09	0.01
AP (U/L)	9035[a]	7710[b]	5327[c]	6707[b]	361.14	0.001
UA (mg/dL)	3.56[b]	3.74[b]	3.82[b]	4.79[a]	0.21	0.007
CK (U/L)	16973[c]	26613[b]	31875[a]	24650[b]	903.29	0.001
HGB (g/dL)	17.32[b]	17.77[b]	19.17[a]	18.99[a]	0.37	0.01
RBC (10*6/µL)	2536750[b]	2579500[b]	2771250[a]	2781000[a]	56780.00	0.01
MCH (pg)	68.20	68.92	69.22	68.25	0.35	0.17
MCV (fL)	143.25[b]	144.75[ab]	146.00[a]	146.25[a]	0.73	0.05
MCHC (g/dL)	47.40[a]	47.39[a]	47.34[a]	46.63[b]	0.22	0.08
SGOT (AST) (U/L)	326.25[bc]	372.50[ab]	414.50[a]	308.75[c]	15.63	0.001
SGPT (ALT) (U/L)	69.25[b]	74.75[b]	83.75[a]	56.75[c]	2.62	0.001

[1] Negative control: Without injection, Positive control: Injection of 0.272 mL of normal saline solution, Mn20: Positive control + 20 µg organic Mn, Mn30: Positive control + 30 µg organic Mn.

[2] TC: total cholesterol, TG: triglycerides, LDL: low-density lipoprotein, HDL: high-density lipoprotein, AI: atherogenic index, VLDL: very-low-density lipoprotein, TP: total protein, Alb: albumin, Glb: globulin, AP: alkaline phosphatase, UA: uric acid, CK: creatine kinase, HGB: hemoglobin, RBC: red blood cell, MCH: mean corpuscular of hemoglobin, MCV: mean corpuscular volume, MCHC: mean corpuscular hemoglobin concentration, SGOT: serum glutamic-oxaloacetic transaminase, AST: aspartate aminotransferase, SGPT: serum glutamic pyruvic transaminase, ALT: alanine aminotransferase.

[a,b,c] Means within each row with different superscripts differ significantly at $P < 0.05$.

The results of the traits related to the immune system of broiler chickens are shown in Table 8. The groups injected with organic manganese have the lowest leukocytes and heterophils, while the lymphocyte percentage increases significantly ($P < 0.05$). Heterophile/lymphocyte decreased with *in ovo* injection with manganese significantly ($P < 0.05$). Sahr *et al*. (2020) did not observe any decrease in the function of the immune system after the *in ovo* injection of inorganic manganese and only observed an increase in the percentage of basophils, which contrasts with the results of the present study. Geng *et al*. (2022) reported the improvement of antioxidant capacity after *in ovo* injection of manganese in normal and stressed conditions of chickens, which conflicts with the results of the present research. The high level of lymphocytes and their role in fighting harmful bacteria and increasing the production of antibodies can be considered an advantage of the current research. However, an excessive increase led to chicken sensitivity and increased inflammatory reactions. On the other hand, a decrease in leukocytes and heterophils shows the inherent vulnerability of the produced chickens, so it is necessary to strengthen and stimulate the immune system through nutrition during the rearing period. Virden *et al*. (2004) believe that feeding organic manganese improves antibody response against infections like bronchitis and Newcastle disease in breeder hens, and this effectiveness will be more noticeable in conditions of stress and tension.

Table 8. Immunity (mean ±*SEM*) of broilers when injected their parent as *in ovo* with manganese

Items [2]	Treatments[1]				*SEM*	P-value
	Negative control	Positive control	Mn20	Mn30		
Leukocytes (µ/L)	16400^{a}	111950^{b}	9900^{c}	9900^{c}	416.83	0.001
Heterophile (%)	45.75^{a}	41.25^{b}	30.75^{c}	29.500^{c}	0.97	0.001
Lymphocyte (%)	51.20^{b}	55.25^{b}	65.50^{a}	67.50^{a}	1.33	0.001
Heterophile/ lymphocyte	0.89 ^{a}	0.74 ^{a}	0.46 ^{b}	0.43 ^{b}	0.008	0.001
Monocytes (%)	2.75	3.00	3.50	3.00	0.40	0.61
AI within 21 days (log_2)	5.50	6.250	5.50	5.25	0.27	0.10
AI within 28 days (log_2)	5.00	5.75	5.75	6.00	0.17	0.19
AN after first injection within 7 days (log_2)	3.25	3.50	4.00	3.00	0.76	0.81
AN after second injection within 7 days (log_2)	5.25	5.25	4.25	4.50	0.33	0.12
AIBV after first injection within 7 days (log_2)	815.00	576.00	494.80	694.00	124.64	0.33
AIBV after second injection within 7 days (log_2)	9.65	8.77	8.87	9.37	0.41	0.42
Relative weight of thymus (%)	0.14	0.18	01.8	0.09	0.04	0.44
Relative weight of liver (%)	2.21	2.18	2.27	2.40	0.13	0.64
Relative weight of spleen (%)	0.08	0.13	0.10	0.07	0.01	0.18
Relative weight of bursa of Fabricius (%)	0.11	0.09	0.09	0.10	0.01	0.95

[1] Negative control: Without injection, Positive control: Injection of 0.272 mL of normal saline solution, Mn20: Positive control + 20 µg organic Mn, Mn30: Positive control + 30 µg organic Mn.
[2] AI: Antibody titer against Influenza; AN: Antibody titer against Newcastle; AIBV: Antibody titer against infectious bronchitis virus (IBV).
[a,b,c] Means within each row with different superscripts differ significantly at $P < 0.05$.

The results of the traits related to the microbiota of the intestinal cecum of broiler chickens are shown in Table 9. The results indicate that the *in ovo* injection of organic manganese leads to a significant decrease in coliform and *Escherichia coli* in the intestinal cecum ($P < 0.05$). On the other hand, the population of *Lactobacillus*, *Bifidobacterium* spp. and lactic acid bacteria increased significantly in the groups injected with organic manganese ($P < 0.05$). The authors did not find any report on the role of manganese on the cecum microbiota. The present research results, which showed an increase of gram-positive bacteria and a decrease of gram-negative bacteria, indicate that the state of intestinal microbiota of birds after *in ovo* injection should be without problems.

Table 9. Cecal microbiota (log_{10} CFU/g) of broilers when injected their parent as *in ovo* with manganese

Items	Treatments[1]				*SEM*	P-value
	Negative control	Positive control	Mn20	Mn30		
Coliform	4.992751^{b}	5.012429 ^{a}	4.092361 ^{d}	4.495333 ^{c}	1.52	0.001
Lactobacillus	3.879082 ^{c}	2.296665 ^{d}	3.924383 ^{a}	3.912554 ^{b}	1.63	0.001
Escherichia coli	5.15592 ^{a}	5.015001 ^{b}	4.199824 ^{c}	3.592288 ^{d}	1.66	0.001
Bifidobacterium	4.107363 ^{d}	3.07050 ^{d}	5.058257 ^{b}	5.180044 ^{a}	1.58	0.001
Lactic acid bacteria	3.874743 ^{b}	3.818424 ^{c}	4.051991 ^{a}	3.865977 ^{a}	1.71	0.001

[1] Negative control: Without injection, Positive control: Injection of 0.272 mL of normal saline solution, Mn20: Positive control + 20 µg organic Mn, Mn30: Positive control + 30 µg organic Mn.
[a,b,c,d] Means within each row with different superscripts differ significantly at $P < 0.05$.

In summary, the results of the present study showed that experimental groups with organic manganese injection with *in ovo* technology had a lower hatchability than those without injection. However, no negative effects on production performance, carcass characteristics and gastrointestinal microbiota were observed in the injected samples. The increase in blood lipid and protein parameters and the subsequent decrease in alkaline phosphatase indicates the complex effects of this element on the biochemical status of the blood and its indirect role in the health of the flock. The immune system was also affected after the injection of organic manganese and increased the response to inflammatory stimuli. Perhaps the most notable effect of organic manganese *in ovo* injection was the improvement of the microbiota of the caecum of the birds, which showed an increase in gram-positive bacteria and a decrease in gram-negative bacteria. With these somewhat limited studies of organic manganese *in ovo* feeding on several functional and clinical parameters of birds, more studies using different doses should be carried out in the future to understand the full potential of the favorable effects of manganese in poultry nutrition.

Declarations

i. Funding

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ii. Conflicts of interest/Competing interests

There are no conflicts of interest.

iii. Ethics approval

The study was approved by the research committees of the authors' institution.

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