



Research Article

Effects of *In-Ovo* Injection of Zinc-Methionine on Some Microbial and Parameters of Immunology of Broiler

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AAA conducted the study and wrote the manuscript. HHA and MKIA conducted farm experiment and laboratory work. All author have read and approved the final version of the manuscript.

Keywords

In ovo injection, Zinc-methionine, Hatching eggs, Broiler



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Abstract | This study was carried out to assess the influence of *in-ovo* injection of broiler embryo with Zinc-methionine, on some characteristics of biochemistry of blood serum in addition to total count of some bacteria in the gut of broiler. A 300 of hatching eggs of Ross broiler breeder flock were divided randomly into 4 treatment groups, 75 eggs in each group, which were: (control) non-injected, T2 injected with 60 ppm, T3 injected with 80 ppm and T4 injected with 100 ppm respectively. After hatching 45 chicks from were chosen each treatment and divided randomly into 3 replicates and reared until 35 day age. Results found that, there were significant ($P < 0.01$) effects of T3 and T4 in the total count of lactobacillus in the illume and jejunum, whereas T1 was significantly higher treatment in the *E. coli* at age of 21 and 35 day. Also T4 was the highest treatment in the lactobacillus at day 35 of the age of the illume and jejunum. For IgG there was significant ($P < 0.01$) effects of T4, also T3 and T4 were affected the levels of IgA significantly ($P < 0.01$), also IgM level was significant ($P < 0.01$) at higher concentration in the T2 in front of other treatments. Total protein was affected by the T2 and T3 which was higher in these treatments in comparative with other treatments significantly ($P < 0.01$). all treatments were at low level of glucose when comparing with T1 which was at higher concentration significantly ($P < 0.01$). in conclusion, zinc-methionine injection has enhance the immunity status of birds and improve the gut microbiota into positive way.

Novelty Statement | This study is the first to use zinc-methionine to enhance the intestinal microbiota of broilers in Iraq-Babylon.

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Introduction

The period of perinatal from last days of embryo to some days post hatching is vital period for the immune system and digestive tract to develop in the chicken. Composition of the egg and egg microenvironment only

the sources that can impact the fetus growth (Uni and Ferket, 2004). Therefore, this critical period consider to be critical for the growth of the immune system and the gastrointestinal tract of avian (Uyanga *et al.*, 2022; Salmanet *et al.*, 2024). In recent years, *in ovo* injection of hatching eggs has become a vital attention as it is regulating the growth in early stages and later development of chicken. Several studies have claimed that intervention of nutrition by early feeding (IOF) through *in-ovo* injection can enhance the immunity development, promote the health of gut of

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chicken which in turn will enhance the performance (Ma *et al.*, 2022). Zinc one of the additions that add to diet of chicken which is necessary mineral for poultry, functioning elaborately in enzymes systems and its involved in the synthesis of protein, metabolism of carbohydrate, and many essential biochemical processes (Sahoo and Mishra, 2014). Zinc required for normal growth, development of glandular and reproduction of chicken. it has a role on the skin hence it affect the quality of meat of broiler which is important nutrient for broiler (Nessrin *et al.*, 2014; Al-Saeedi *et al.*, 2022). The microelement zinc is necessary for the immune system, and it deficiency can affects different aspects of adaptive and innate immunity (Bonaventura *et al.*, 2015; Zaki and Al-Jebory, 2021). Wessels *et al.* (2021) found that using different source of zinc has improved the health states of broiler, enhance the immune titter and increased the relative weight of bursa fabricia and titer levels against Newcastle disease in poultry. There are many examples in human such as RNA and DNA polymerases, carbonic anhydrase, and alkaline phosphatase. Wu *et al.* (2018) have conducted a study on the effect of deficiency of methionine in broiler they claimed that low level of this amino acid has affected the levels of sIgA, IgM, IgA, and IgG in addition, count of B cell was reduced as well. For period of 6 weeks in the segments of gut duodenum and jejunum. It can say here, methionine has significant effects on the immunoglobulin. Hence, it affects the immunity in total. Mostafa and Sabeer (2011) showed that injecting fertile broiler eggs with methionine at level 5mg/egg has caused significant ($p < 0.01$) increment in the level of hemoglobin, red blood cell count, white blood count, packed cell volume, total protein, lymphatic cells, and titer levels against Newcastle and Infectious Bursal disease. They also claimed that, injection the eggs at day 19 of hatching was preponderance than injection at days 0, 7, and 14. Dong *et al.* (2023) found that adding methionine-zinc to the diet of chicken has altered the gut microbiota as they observed a high level of beneficial bacteria in the cecum. The Aim of current study was to investigate the effects of early feeding (*in-ovo* injection) of broiler embryo of chicken with zinc-methionine on some characteristics of biochemistry and immunology of blood serum in addition to total count of some bacteria in the gut of broiler chicken.

Materials and Methods

This period of this study was form 25/2/2020 until 2/5/2020, at the hatchery of Jeflawe company for poultry/ Babil city/ Iraq, the first trial, meanwhile the second trial which was for rearing the hatched chicks was conducted at the farm. Three hundred hatching eggs were obtained from the Jeflawe company that were obtained from Turkish company (EGE-TAV A.S) which is a broiler parent stock eggs (ROSS 308). They have been weighed using SF-400 Electronic Kitchen scale. only eggs at weight of 53 ± 1 gram have been chosen, dirty and abnormal shape eggs were

excluded as well. These eggs were randomly distributed and injected (details below) for hatching. After hatching, chicks were divided randomly into four treatments 45 chicks each with 3 replicates 15 chicks each and reared for 35 days.

Solutions of eggs injection

Distilled water and zinc-methionine powder which was obtained from Zinpro company (USA) were used to prepare the solution of injection. Injecting chicks was done at day 17.5 of hatching, into amniotic sac after light examination (candling) for live and dead embryo.

Injection treatments

- Treat 1 not injected
- Treat 2 inject with 0.3 ML/ egg zinc-methionine solution at concentration 60 ppm
- Treat 3 inject with 0.3 ML/ egg zinc-methionine solution at concentration 80 ppm
- Treat 4 inject with 0.3 ML/egg zinc-methionine solution at concentration 100 ppm

Feed treatment

Chicks were fed on starter diet (23% protein and 3027 Kgl/kg feed) from day 1 till day 21 of age. Afterword, ration of grower (20% crude protein and 3195.3 Kcl/kg feed) until day 35. Feed and water were available *ad libitum* diets details in Table 1 according ross guide.

Table 1: Ingredient and chemical composition of the basal diets.

Grower %22-35	Starter % 1-21 day	Feed staff
40	30	Corn
24	28.25	wheat
24.8	31.75	Soybean protein (48 %)
5	5	* Protein
4.4	2.9	Sunflower oil
0.6	0.9	Lime
0.9	0.7	Diphosphate(DCP)
0.1	0.3	Nacl
0.2	0.2	Vitmens &menral concentrate
100	100	Total
20	23	Crude protein
3195.3	3027	Metabloate energy Kcl/kg feed
1.1	1.2	Lycen (%)
0.46	0.49	Methionine (%)
0.32	0.36	Cycten (%)
0.76	0.85	methionine + cycten (%)
0.49	0.45	Avilabe phosphor %
159.77	131.61	C/P ratio

*Premix content (Brocon-5 special) in each kg diet : 40% crude protein, 3.5% fat, 1% fiber, 6% calcium, 3%available phosphor, 3.25% lycein, 3.9% methionine + cycten, 2.2 % sodium, 2100 kcal/ kg meta energy, 2000 IU vit A, 40000 IU vit. D3, 500 mg vit E, 30 mg vit K3, 15mg vit B1+B2, 20 mg vit B6, 300mg vit B12, 10mg vit folic acid, 100 micrg butin, 1mg iron, 100mg copper, 1.2 mg mn, 800mg Zn 15mg I, 2mg Se, 6mg Co,

900mg BHT (antioxidant).

Studied parameters

Immunity status: Antibody titer was measured in the blood serum at age of bird 21 day using Elisa instrument according to guide of the kit according to [Voller et al. \(1977\)](#).

Biochemistry parameters

Level of glucose and cholesterol were determined in the blood serum by withdrawn blood from the birds after bird euthanized then the blood collected from wing vein directly into tubes that content anti-coagulant. Then serum was separated using centrifuge at speed 3000 rpm for 15 minutes afterword, they were measured in the Laboratory of Bioscience Department, Collage of Science, Bibel University. Glucose was measured using kit of Roche, Germany. According to [\(Coles, 1986\)](#). Meanwhile, cholesterol was determined using kit of Roche, Germany, according to [Franey and Elias \(1968\)](#). Triglyceride in the serum of blood by using enzymatic method according to [Fossati and Prencipe, \(1982\)](#). Meanwhile, total protein was measured using kit of Roche, Germany according to Biuret method [\(Kadhim et al., 2021\)](#).

Microbial total count

At ages 21 and 35 day three birds were chosen from each replicate and euthanized, intestinal tract was pooled and 2 grams samples were collected from *Jejunum* and *Ileum* into 5 ml tubes then stored in the cooled place for determination of microorganisms as follow:

Total count of Lactobacilli in the Jejunum and Ileum

One gram was taken from the content that stored in cold place in antiseptic conditions from all replicates, then serial dilution was done up to $(10)^{-10}$ using peptone water by pour-plate method. Which was done as follow: duplicate of one ml from each dilution was transport using Micropipette into plate then MRS agar pour on directly into this plate which was prepared and kept in water bath at 46°C. dilution and media were mixed well and left to solidified before incubated in at 37°C for 48 h up wright down in the incubator, afterword colonies were counted and calculated as follow:

$$\text{Total count} = \text{number of colonies} \times 1/\text{dilution}$$

Total count of *Coliform* in *Jejunum* and *Ileum*: Total count of *Coliform* in *Jejunum* and *ileum* was done in the same method of total count of lactobacilli except that 1 ml of dilution was transport into 15 ml of sterilized of MacConkey Agar plate and all the steps were follow as the same in the above.

Statistical analyses

Data were analysed using SAS software [\(SAS, 2012\)](#) which was carried out using completely randomized design (CRD) to study the effects of studied treatment on different characteristics treatment means were separated

by using a Duncan post hoc test and significant level was tested at $P < 0.01$.

Results and Discussion

Microbial count

[Table 2](#) show the effect of early feeding of Zinc-methionine on the total count of some of beneficial (Lactic Acid Bacteria) and pathogen bacteria (*E. coli*) in the jejunum and ileum at age 21 day. From this table appear that there was no significant ($P < 0.01$) differences among the treatments in total count of *E. coli* in the jejunum. Meanwhile there is a significant ($P < 0.01$) increment in the total count of *Lactobacilli* in the T4 compared with other treatments. Also T3 and T2 were highly significant ($P < 0.01$) than T1. Whereas, there was no significant ($P < 0.01$) difference between T3 and T4 and; T2 and T3. In the ileum it is clear that there is a significant ($P < 0.01$) prevalence of T1 compared to other treatments of the count of *E. coli* 5.22 ± 1.16 , 4.67 ± 1.54 , 4.13 ± 1.11 and 4.10 ± 1.20 for T1, T2, T3 and T4 respectively. Also, there is a significant ($P < 0.01$) increment of T2 to T3 and T4. Meanwhile there is no significant ($P < 0.01$) differences between T3 and T4. In the total count of Lactobacilli T4 and T3 were higher significantly ($P < 0.01$) than other treatments, T2 was significantly ($P < 0.01$) higher than T1, however there is differences between T3 and T4.

Table 2: Effect of early feeding with Zinc-methionine on the count of *E. coli*, and *Lactobacillus* in the jejunum and ileum of Broiler (Ross 308) at day 21.

Treat-ments	Average $\pm$ standard error			
	Jejunum		Ileum	
	<i>E. coli</i>	<i>Lactobacilli</i>	<i>E. coli</i>	<i>Lactobacilli</i>
T1	5.13 ± 1.55	$5.25^c \pm 1.13$	$5.22^a \pm 1.16$	$8.32^c \pm 0.90$
T2	5.42 ± 1.00	$5.89^b \pm 1.37$	$4.67^b \pm 1.54$	$8.67^b \pm 0.75$
T3	5.63 ± 1.23	$6.14^{ab} \pm 1.18$	$4.13^c \pm 1.11$	$9.00^a \pm 0.95$
T4	5.11 ± 1.80	$6.32^a \pm 1.10$	$4.10^c \pm 1.20$	$9.23^a \pm 0.50$
Significant	N. S	**	**	**

^{a-c} Means with different superscripts within the same column are significantly different at $p < 0.05$, ** N.S non-significant. T1, T2, T3, T4 are control (no injection) and treatments of injection with zinc-methionine (60, 80, 100 ppm), respectively.

At age 35 day [Table 3](#) explain the effects of treatments on the total count of lactobacilli as beneficial bacteria and *E. coli* as a pathogenic bacteria in the jejunum. In this table T1 was decreased compared to all other treatments. Also, total count of *E. coli* was decreased significantly ($P < 0.01$) in the T3 and T4 compared to T2, but there is no significant ($P < 0.01$) differences between T3 and T4. Total count of lactobacilli T4 was higher treatment among all other treatment and T2 and T3 were higher than T1 significantly ($P < 0.01$).

In the ileum results showed in Table 3 that there was a significant ($P<0.01$) decreasing in the total count of *E. coli* in comparison with other treatments, T3 and T4 were higher than T2, meanwhile there was not significant differences between T3 and T4. Total count of Lactobacilli T4 was the higher treatment among all other significantly ($P<0.01$), also T2 and T3 were higher than T1. Researches refer that high levels of amino acids in the intestinal tract of bird have an inhibition effects against pathogen, Chang *et al.* (2022a) found that adding the Zinc-methionine to the diet of bird decrease the count of *E. coli* and increase the lactobacillus and *Bifedobacteria*. Also reported that using lysen and methionine in the poultry feed have improve the defence of gut against *Clostridium perfringens*, *Escherichia coli* (Konieczka, 2022). The mode of action of zinc is unclear yet but it may be because it necessary for many activates of bacteria such as proliferation of cell, transcription, DNA replication, synthesis of protein, in which influence the growth and reproduction of bacteria (Xia *et al.*, 2021).

Table 3: Effect of early feeding with Zinc-methionine on the count of *E. coli*, and *Lactobacillus* in the jejunum and ileum of Broiler (Ross 308) at day 35.

Treat-ments	Average $\pm$ standard error			
	Jejunum		Ileum	
	<i>E. coli</i>	<i>Lactobacilli</i>	<i>E. coli</i>	<i>Lactobacilli</i>
T1	4.98 $\pm$ 0.71	8.15 $\pm$ 0.27	2.49 $\pm$ 0.50	8.00 $\pm$ 1.18
T2	4.0 $\pm$ 0.25	9.55 $\pm$ 0.15	2.57 $\pm$ 0.91	9.24 $\pm$ 1.65
T3	4.16 $\pm$ 0.20	9.63 $\pm$ 0.35	2.11 $\pm$ 0.25	9.22 $\pm$ 1.80
T4	4.20 $\pm$ 0.41	9.76 $\pm$ 0.63	2.08 $\pm$ 0.55	9.87 $\pm$ 2.10
Significant	**	**	**	**

a-c Means with different superscripts within the same column are significantly different at $p<0.05$, ** N.S non-significant. T1, T2, T3, T4 are control (no injection) and treatments of injection with zinc-methionine (60,80,100 ppm), respectively.

The effect of zinc on total count of *E. coli* in the ileum at day 35 is presented in Table 3. Total cunt of *E. coli* were significantly ($p<0.01$) higher for T1 compared with other treatments and for T2 compared with T3 and T4 while there is no significant differences between T3 and T4. In meantime, total count of beneficial bacteria was significantly ($p<0.01$) higher for T4 compared with other studied treatments, also treatments T2 and T3 were higher than the control. Researchers claim that amino acids in the intestine of birds have an inhibition effects against pathogens (Montout *et al.*, 2021). Also as mentioned researchers found that high concentrations methionine supplementation caused the reduction in the count of *C. profrengens*, in which may give an illustration to the reduction of *E.coli* in this study. Microbial community in ilium and jejunum was balanced toward increment in Lactobacilli and degradation in *E. coli* which may affected by addition of Zinc. In addition, deficiency of zinc has

an effects on the balance of microbiota in the gut and its availability and absorption will increase when its attached with amino acids and protein on other hand, it is found that low level of zinc in the gastrointestinal can affect the health of whole digestive system and increase the inflammation (Spenser *et al.*, 2015).

Also the improvement in the gut microbiota may be a results of reduction in the stress by the this addition especially at the end of hatching (day 19) because of increasing of temperature in the hatchery and also the stress of transporting the eggs from the incubator to the hatchery.

Parameters of immunity and biochemistry

Table 4 observe the effects of early feeding of zinc-meth on some of immunological globulins and biochemical, as it can see that treats have a significant ($p>0.01$) effects of treatment T4 on the level of IgG compared to other treatments. Also T3 was higher than treatment T1 and T2. However there was no significant effect between T1 and T2. The effect of treatments T3 and T4 on the level of IgA they were preponderate significantly ($p>0.01$) against T1 and T2 as well. T1 was higher than T2. In the level of IgM T2 was the highest treatment among all other treatments significantly ($p>0.01$) and T4 against T1. This results come in line with the findings of Chang *et al.* (2022) as they found that feeding zinc-methionine have enhance the levels of IgG and IgM in the gut of poultry.

Table 4: Effect of *in-ovo* injection with zinc-methionine on the immune-globulins of broiler (Ross 308).

Treatments	Average $\pm$ standard error		
	IgG mg/ml	IgA mg/ml	IgM mg/ml
T1	2.09 $\pm$ 1.50	2.17 $\pm$ 1.10	2.41 $\pm$ 0.32
T2	2.10 $\pm$ 1.70	1.94 $\pm$ 1.13	4.21 $\pm$ 0.28
T3	2.40 $\pm$ 1.25	3.13 $\pm$ 1.21	3.55 $\pm$ 1.10
T4	2.57 $\pm$ 1.30	3.15 $\pm$ 1.18	3.84 $\pm$ 0.50
Significant	**	*	**

a-c Means with different superscripts within the same column are significantly different at $p<0.05$, ** N.S non-significant. T1, T2, T3, T4 are control (no injection) and treatments of injection with zinc-methionine (60,80,100 ppm), respectively.

Table 5 shows the effects of treatments on some blood biochemistry characteristics, it can notice that levels of total protein in the serum of T2 (6.12 $\pm$ 2.18) and T3 were significantly ($p>0.05$) higher than other treatments T1 and T4. For levels of glucose in the serum, T1 was significantly ($p>0.05$) the highest treatment (240.51 $\pm$ 6.13) among other treatments. Also T4 and T3 were higher than T2. Meanwhile this study found that there were no effects of treatments on the levels of cholesterol and triglyceride.

The reason behind high significant levels of immuno-

globulins in the treatments of injection of zinc-methionine may be because of zinc contribution in the synthesis and metabolize of proteins (Katayama, 2019), also may be due to the activity of zinc to protect metalloproteins like growth hormone for example and insulin and semi-insulin factor (IGF-1) in which it contribute to protect the growth and development of body and increase of immunoglobulins proteins synthesis as these hormones act to regulate glucose absorbance of cellular immunity (Midilli *et al.*, 2014).

Table 5: Effect of *in-ovo* injection with zinc-methionine on the blood biochemical parameters of Broiler (Ross 308).

Treat- ments	Average $\pm$ standard error			
	Total protein g/100 ml	Glucose mg/100 ml	Cholesterol mg/100 ml	T.G mg/100 ml
T1	5.63 ^b $\pm$ 1.00 ^b	240.51 ^a $\pm$ 6.13 ^a	200.00 $\pm$ 2.00	82.67 $\pm$ 3.14
T2	6.12 ^a $\pm$ 2.18 ^a	196.31 ^c $\pm$ 5.16 ^c	199.60 $\pm$ 2.25	82.74 $\pm$ 3.25
T3	6.13 ^a $\pm$ 2.43	221.90 ^b $\pm$ 5.00	203.21 $\pm$ 2.53	80.17 $\pm$ 3.75
T4	5.95 ^{ab} $\pm$ 2.14	223.41 ^b $\pm$ 5.71	195.91 $\pm$ 2.50	81.22 $\pm$ 3.26
Signif- icant	**	*	N.S	N.S

a-c Means with Different superscripts within the same column are significantly different at $p < 0.05$, ** N. S non-significant. T1, T2, T3, T4 are control (no injection) and treatments of injection with zinc-methionine (60,80,100 ppm), respectively.

A possible explanation for roles of Zinc in health performance of chicken, is that through improvement of activity of metabolism of whole body as it concenter as a part of many enzymes that involved in the metabolism of carbohydrates, fat, protein, and nucleic acids. Furthermore, Zn has is vital for the functions of hormone, such as growth and pancreatic hormones and has an antioxidant properties (Ogbuewu and Mbajorgu, 2023).

Also zinc is one of requirements of natural synthesis of protein in the body and protect it through antioxidant in the cellular membrane (Midilli *et al.*, 2014). Therefore high level of immunoglobulins in this study when adding zinc-methionine may be because of the role of methionine in the enhancement of immunity.

The possible explanation for the high levels of total protein in the blood may be due to methionine bound zinc which improve it absorption by embryos and because of the roles of zinc in the activity of enzymes and in the synthesis of protein. Liu *et al.* (2011) claim that zinc-methionine use as indicator for bioavailability of zinc in the animal, because of zinc absorbance will increased when it be as complex compound with amino acids or proteins.

Methionine is essential for protein synthesis and for methylation reaction of DNA, which in turn can improve

the immunity status of birds.

Therefore, high level of glucose in the T1 compering with control treatments of zinc-methionine injection, may be because of oxidation) that accrued in this treatment. Research found that level of glucose increase in blood of birds as a results of increment in hormone that acts as enzyme catalyst which in turn decomposition the glycogen (adrenaline noradrenaline and glucagon) by the Glycogenolysis (Midilli *et al.*, 2014), consequently, adrenal cortex gland will increase secretion of Corticosterone which in turn will produce sugar from secondary sources lipid and protein subsequently raise the sugar level in the blood (Al-Jebory *et al.*, 2024; Salman *et al.*, 2024).

Conclusions and Recommendations

In conclusion it can be say that, from this study that, injection of Zinc-methionine in the amenutic sic of the chicken embryo has improve the characteristics of microbial and immunity for the hatch chicks and it could use in other birds.

As this study shown enhancement of immunity and positive modification of gut microbiota when embryo of chicken injected with Zinc-methionine we recommend that this injection should be used in the commercial hatcheries. Also more studies need to be done on other parameters and to investigate the effects of this injection on the heat stress as it a big challenge in our countries.

Declarations

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IRB approval

The experimental procedures in the research work of the manuscript followed the guidelines of the Research Ethics Committee of a college of Agriculture, Al-Qasim Green University, and complied with relevant Iraqi legislations with approved no. (Agri.No.25.1.20).

Ethics approval

The experimental procedures were approved by Agricultural College- AlQasim Green University, that complied with the Practice for the Care and Use of Animals for Scientific Purposes (with NU.AP 10.1.2020).

Declaration of generative AI and AI-assisted technologies in the writing process

No Generative AI and AI-assisted technologies were used in the writing process.

Conflict of interest

The authors have declared no conflict of interest.

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