Italian Journal of Animal Science

ISSN: (Print) 1828-051X (Online) Journal homepage: http://www.tandfonline.com/loi/tjas20

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To cite this article: Jafar Gholami, Ali A.A. Qotbi, Alireza Seidavi, Adele Meluzzi, Siria Tavaniello & Giuseppe Maiorano (2015) Effects of in Ovo Administration of Betaine and Choline on Hatchability Results, Growth and Carcass Characteristics and Immune Response of Broiler Chickens, Italian Journal of Animal Science, 14:2, 3694, DOI: 10.4081/ijas.2015.3694

To link to this article: http://dx.doi.org/10.4081/ijas.2015.3694

PAPER

Effects of *in ovo* administration of betaine and choline on hatchability results, growth and carcass characteristics and immune response of broiler chickens

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Abstract

The effect of *in ovo* administration of different levels of betaine and choline on egg hatchability, immune response, growth and carcass traits of broiler chickens was studied. Four thousand hatching eggs from Ross 308 broiler breeder layers, weighed individually, were incubated for 21 days in a commercial hatchery. At 12th day of incubation, 3456 fertilized eggs were randomly divided into 8 experimental groups of 3 replicates each (144 eggs per replicate): negative control (NC) – not injected; positive control (PC) – injected with 0.5 mL deionized water; Bet 0.25 – injected with 0.5 mL deionized water+0.25 mg soluble betaine; Bet 0.375 – injected with 0.5 mL deionized water+0.375 mg soluble betaine; Bet 0.50 – injected with 0.5 mL deionized water+0.50 mg soluble betaine; Chol 0.25 – injected with 0.5 mL deionized water+0.25 mg soluble choline; Chol 0.375 – injected with 0.5 mL deionized water+0.375 mg soluble choline; Chol 0.50 – injected with 0.5 mL deionized water+0.50 mg soluble choline. Among the hatched chickens, 360 males were randomly chosen (45 for each group) and were grown up to 42nd day of age. The embryo mortality, pecked eggs, infected eggs and hatchability percentages were similar among the experimental groups. The betaine and choline treatments improved hatching weight and final weight of chickens, while reduced feed conversion ratio and abdominal fat percentage. No effect on carcass yield, and breast muscle, leg and wings percentages, as well as on immunoglobulin M (IgM), G (IgG), and total antibody (IgT) titers was observed. The treatments had little effect on internal organs.

Introduction

Betaine is a neutral chemical compound with a positively charged cationic functional group such as a quaternary ammonium or phosphonium cation (generally: onium ions) which carries no hydrogen atom and has a negatively charged functional group, such as a carboxylate group, which may not be adjacent to the cationic site. Hence a betaine may be a specific type of zwitterion. Historically the term was reserved for trimethylglycine only. The name reflects its origin and first isolation from sugar beet. Due to its chemical structure, various functions have been described for the betaine molecule (reviewed in Eklund *et al*., 2005). In biological systems, many naturally occurring betaines serve as organic osmolytes, substances synthesized or taken up from the environment by cells for protection against osmotic stress, drought, high salinity or high temperature. Intracellular accumulation of betaines, does not damage enzyme function, protein structure or membrane integrity, but permits water retention in cells, thus protecting from the effects of dehydration, and thereby facilitates the secretion of digestive enzymes (Kuznetsov and Shevyakova, 1997; Xing and Rajashekar, 2001; Eklund *et al*., 2005). If betaine stimulates cell proliferation in the intestinal tissue, the enlarged gut wall epithelium would provide an increased surface for nutrient absorption. However, the influence of betaine on intestinal muscle cell activity in calves seems to be dose-dependent with higher levels reducing muscle-cell activity, thus possibly decreasing the absorption capacity of the duodenum (Puchala *et al*., 1998). Effects of betaine as an osmotic active substance may be more pronounced in animals exposed to osmotic disorders such as coccidiosis in poultry (Augustine *et al*., 1997). In addition, betaine is important because of its role in the donation of methyl groups to homocysteine to form the essential amino acid methionine (Zeisel *et al*., 2003). In animal nutrition, betaine is widely discussed as a *carcass modifier* due to its lipotropic and growth-promoting effects (Eklund *et al*., 2005). However, inconsistency in the effects of betaine on growth performance and carcass composition in relation to the protein and energy content of the diet have been frequently reported and have been related to different factors (reviewed in Eklund *et al*., 2005).

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Key words: Broiler chicken; Betaine and choline; *In ovo* injection; Performance; Immune response.

Acknowledgments: this manuscript is prepared from MSc thesis of first author at Department of Animal Science, Rasht Branch, Islamic Azad University, Rasht, Iran. We are grateful to the Rasht Branch, Islamic Azad University, Rasht, Iran for support.

Received for publication: 5 October 2014.
Accepted for publication: 13 January 2015.

Italian Journal of Animal Science 2015; 14:3694
doi:10.4081/ijas.2015.3694

Choline is a water-soluble essential nutrient. Despite being considered as one of the B-complex vitamins, choline does not answer the classical definition of a vitamin. It is required in relatively high amounts and does not have any coenzyme function. In biological tissues, most of the choline is present as bound form and a small proportion exists as free choline (Committee on Animal Nutrition, 1987). Choline has three essential metabolic functions (Chan, 1984): for the structural integrity of cell membranes, lipotropic agent in fat metabolism in liver and precursor for acetylcholine synthesis (the neurotransmitter agent for nerve impulses). Choline must be part of the human diet but dietary recommendations have discouraged people from eating certain high-choline foods, such as eggs and fatty meats (Institute of Medicine, 1998; Zeisel *et al*., 2003; Zeisel and Da Costa, 2009).

Johnston *et al*. (1997) presented applications of *in ovo* technology to improve poultry's protection against pathogens. During late embryogenesis in domestic fowl, solutions administered into the amnionic fluid (*i.e.*, amino acids, carbohydrates, prebiotics and synbiotics) may be taken up by the embryo, digested, and absorbed by the embryonic intestine prior to pipping (Uni *et al*., 2005; Foye *et al*., 2006; Bednarczyk *et al*., 2011), as well as affecting growth performance, carcass traits and meat quality (Maiorano *et al*., 2012) and

immune system development (Slawinska *et al.*, 2014). *In ovo* feeding of supplemental nutrients may help to overcome the constraint of limited egg nutrients (Foye *et al*., 2006).

The objective of this study was to determine the effects of *in ovo* administration of different levels of betaine and choline on egg hatchability, immune response, growth and carcass traits of broiler chickens.

Materials and methods

Hatchery management

Four thousand hatching eggs from Ross 308 broiler breeder layers (n=4000; 30 weeks old), weighed individually, were incubated for 21 days in a commercial incubator (PS500 Multi-Stage Controller; Jamesway Hatchery Company Inc., Toronto, Canada). The incubation conditions were: 37.7°C and 70-75% relative humidity from 1 to 18 days of incubation; 36.66°C and 75-82% relative humidity from 19 to 20 days of incubation; on the 21 day of incubation the temperature was decreased gradually (hour to hour) from 36.66 to 36.11°C.

On day 12 of incubation the eggs were removed from the incubator, candled and those unfertilized or with dead embryos were discarded. A total of 3456 fertilized eggs were washed and sanitized using iodine tincture prior to injection and were divided in 8 experimental groups of 3 replicates each (144 eggs per replicate).

Betaine (96%; Biochem, Lohne, Germany) and choline (67%; Miavit, Essen, Germany) were purchased, prepared a solution using deionized water and injected manually into the air chamber by using insulin syringes (Helal Iran Medical Devices Co; Soha, Karaj, Iran) at 12th day of incubation. The experimental groups were: negative control (NC): eggs not injected; positive control (PC): eggs injected with 0.5 mL deionized water; Bet 0.25: eggs injected with 0.5 mL deionized water+0.25 mg soluble betaine; Bet 0.375: eggs injected with 0.5 mL deionized water+0.375 mg soluble betaine; Bet 0.50: eggs injected with 0.5 mL deionized water+0.50 mg soluble betaine; Chol 0.25: eggs injected with 0.5 mL deionized water+0.25 mg soluble choline; Chol 0.375: eggs injected with 0.5 mL deionized water+0.375 mg soluble choline; Chol 0.50: eggs injected with 0.5 mL deionized water+0.50 mg soluble choline.

Immediately after the injection, the site was sealed with sterile paraffin and eggs were returned to the incubator.

At end of incubation, the number of hatched chicks was counted and individual body weight (BW) was measured. Percentage of mortality (12 to 21 day), pecked eggs, infected eggs (*e.g.* dirty eggs, thin layer eggs, exploded eggs due to contamination), hatchability, and chick weight/egg weight ratio were calculated.

Poultry house and birds

This experiment was performed under commercial condition in an air conditioned poultry house. Among the hatched chickens, 360 males were randomly chosen (45 birds for each experimental group equally shared in 3 cages at a density of 10 birds/m^2) and reared following the animal welfare recommendations of Ethic Committee of Rasht Branch, Islamic Azad University (Rasht, Iran), and care was taken to minimize the number of animals used. A heater was used and the temperature programmed was according to the instructions for Ross 308 broilers (Aviagen, Newbridge, Scotland, UK). Air humidity was kept at 55 to 65% in the early growing period by spraying water on the floor. The lighting program was 21L:3D and the light intensity was 20 lux.

Sanitation principles and health measures for raising chickens were applied. Drinkers were washed and cleaned daily. After each vaccination, 1:1000 multivitamin+electrolytes solution was mixed in the drinking water for 24 hours. Birds were vaccinated against avian influenza virus (1st day of age), Gumboro IBD virus (1st day of age), bronchitis vaccine IB H120 (1st and 10th days of age) and Newcastle disease virus (NDV), with vaccine B1 type (1st, 10th and 24th days of age) and Lasota (16th day of age).

The conditions of management of broiler chickens were the same in all groups. The broilers were fed *ad libitum* commercial diets (Table 1) according to their age, and water was provided *ad libitum*. Amounts of feed offered to each cage were recorded, and uneaten feed in

Table 1. Mean values of composition and chemical components of the diets.

	Period	
	1 to 21 d	22 to 42 d
Ingredient, g/kg		
Corn	563.0	628.5
Soybean meal	375.0	337.0
Soybean oil	16.0	0.0
Ca%22 P%18	18.0	10.0
Mineral oysters	10.0	11.0
NaCl	3.0	3.0
Sodium bicarbonate ($NaHCO_3$)	1.0	1.0
Lysine-hydro-chloride	1.5	0.7
DL-methionine	2.0	1.3
Vitamin mixture°	2.5	2.5
Mineral mixture#	2.5	2.5
Vitamin D_3	0.1	0.0
Vitamin E	1.4	1.0
Vitamin K	0.3	0.0
Vitamin A	0.3	0.0
Avizyme enzyme	0.5	0.5
Bio tox	0.6	0.0
Threonine	1.0	0.2
Phytase enzyme	0.1	0.1
Sel plex	0.1	0.1
L-carnitine	0.1	0.1
TechnoMos®	1.0	0.0
Natuzyme plus multienzyme	0.0	0.3
Nataphous enzyme	0.0	0.2
Nutrient analysis		
ME, kcal/kg	2950	3100
Protein, %	21.0	19.3
Lysine SID, %	1.21	1.12
Methionine SID, %	0.65	0.51
Calcium, %	1.0	0.7
Available phosphorus, %	0.5	0.4

SID, standardized ileal digestible. °It provided the following per kg of diet: vitamin A, 5000 U/g; vitamin D_3, 500 U/g; vitamin E, 3 mg/g; vitamin K_3, 1.5 mg/g; vitamin B_2, 1 mg/g. #It provided the following per kg of diet: calcium pantothenate, 4 mg/g; niacin, 15 mg/g; vitamin B_6, 13 mg/g; Cu, 3 mg/g; Zn, 15 mg/g; Mn, 20 mg/g; Fe, 10 mg/g; K, 0.3 mg/g.

each cage was weighed daily. Cumulative feed intake and feed conversion ratio (FCR) were calculated on a cage base.

Slaughter surveys

At 42 days of age, broilers (one bird for each replication), identified by numbered permanent wing bands, were weighed individually (after a fasting period of 12 h) and transported within 1.0 h (including careful catching and loading) to a commercial poultry slaughterhouse. After careful unloading and hanging in randomized order, the birds were electrically stunned and slaughtered (according to the Ethic Committee of Rasht Branch, Islamic Azad University, Rasht, Iran), carcass weight without giblets was recorded, and carcass yield percentage was calculated. Carcasses were dissected (leg, breast, wings; their percentages were calculated based on carcass weight) and, in addition, abdominal fat, gizzard, spleen, pancreas and liver were collected and their percentages were calculated based on carcass weight.

Immunity traits

According to the method described by Pourhossein *et al*. (2015), sheep red blood cells (SRBC) suspension [5% phosphate-buffered saline (PBS)] was injected into the breast muscle of 48 birds (6 per each group) ageing 21 and 35 days. Blood samples were withdrawn from the brachial vein and serum was collected after centrifugation at 1500× g for 15 min. In serum total antibody titers to SRBC were determined by hemagglutination assay at 28 and 42 days. In U-bottom microtiter plates, two-fold serial dilutions of heat-inactivated (at 56°C) serum were made with PBS (0.01 mol/L; pH 7.4) for total antibody, or PBS with 1.4% 2-mercaptoethanol for immunoglobulin G (IgG) antibody. Antibody titers, IgG, immunoglobulin M (IgM) and total antibody (IgT), were recorded as $\log_2$ of the highest dilution of serum that agglutinated an equal volume of a 0.5% SRBC suspension in PBS. The IgM titer was determined by the difference between IgT and IgG titers.

Statistical analyses

One way analysis of variance (ANOVA) was performed for all variables considered in the study (SPSS, 2010). Scheffé's test was applied to compare the mean values among the experimental groups. Antibody titers were logarithmically transformed prior to analysis to achieve homogeneity of variance and were expressed as $\log_2$.

Results and discussion

According to the available information, no research has yet been conducted to study the effect of choline *in ovo* administered and few information are available on the effect of betaine *in ovo* administered (Kadam *et al*., 2013) on egg hatchability, immune response, growth and carcass traits of broiler chickens.

The embryo mortality (total and for each period of observation) and pecked eggs percentages were found to be similar among the groups (P>0.05). In general, the percentage of infected eggs (ranging from 0.46 to 1.38%) was affected by treatment (P=0.02), however, no significant differences (P>0.05) were found among the experimental groups; the lower values were observed in NC and Chol 0.25 groups (Table 2).

Hatchability performance are presented in Table 3. In general, hatchability percentage, ranging from 93.05 to 94.90%, was affected by treatment (P=0.043), but were not found significantly differences among the experimental groups; the hatchability value shown from the Chol 0.25 group was slight higher (P>0.05) in comparison with those of the other treated groups and similar to that in NC group. Differently, hatching weight of chicken was higher in Bet 0.375, Bet 0.5, Chol 0.25, Chol 0.375 and Chol 0.5 (P>0.05) compared to control groups (NC and PC) (P<0.01); the group receiving the lowest dose of choline (Chol 0.25) showed the highest BW value (39.25 g) that differed significantly from those receiving either 0.25 mg (P<0.01) or 0.375 mg (P<0.05) of betaine. In addition, chicks of NC group (36.81 g) were lighter than those of PC and Bet 0.25 groups (P<0.01), while the chicks of these latter groups had similar BW. Our results partially contrast with the findings of Kadam *et al*. (2013), who reported that 100 mg of betaine/egg (on day 18 of incubation) gave the best hatchability, 85%, in comparison with other experimental groups (10 mg, 50 mg and 200 mg on day 18 of incubation), however this value was lower than that measured in the control (untreated) group (93%); while, in agreement with the results of present study, hatching weight was highest in the group that received 100 mg of betaine/egg compared with the control group. The chick BW/egg weight percentage was higher in Bet 0.5, Chol 0.25, Chol 0.375 and Chol 0.5 groups (P>0.05) compared to NC, PC and Bet 0.25 groups (P<0.001); while Bet 0.375 value ratio percentage was higher than those of NC and PC groups (P<0.01), as well as the value of Bet 0.25 group compared to that of NC group (P<0.05).

Table 2. Average value (%) for mortality (12 to 21 d), pecked eggs and infected eggs during the incubation.

Group	Mortality				Pecked eggs	Infected eggs
	12 to 17 d	18 to 19 d	20 to 21 d	12 to 21 d		
NC	2.08	0.69	0.69	3.46	0.69	0.46
PC	2.77	1.38	0.69	4.84	0.69	1.38
Bet 0.25	2.77	1.38	0.69	4.84	0.69	0.69
Bet 0.375	2.77	1.38	1.38	5.53	0.69	0.69
Bet 0.5	2.77	1.38	0.69	4.84	0.69	1.38
Chol 0.25	1.38	1.38	1.38	4.14	0.69	0.46
Chol 0.375	1.38	1.38	1.38	4.14	0.69	1.38
Chol 0.5	2.07	1.38	1.38	4.83	0.69	1.38
SEM	0.17	0.08	0.10	0.20	0.07	0.10
P	0.074	0.237	0.081	0.259	1.00	0.02

NC, negative control (eggs not injected); PC, positive control (eggs injected with 0.5 mL deionized water); Bet 0.25, eggs injected with 0.5 mL deionized water+0.25 mg soluble betaine; Bet 0.375, eggs injected with 0.5 mL deionized water+0.375 mg soluble betaine; Bet 0.50, eggs injected with 0.5 mL deionized water+0.50 mg soluble betaine; Chol 0.25, eggs injected with 0.5 mL deionized water+0.25 mg soluble choline; Chol 0.375, eggs injected with 0.5 mL deionized water+0.375 mg soluble choline; Chol 0.50, eggs injected with 0.5 mL deionized water+0.50 mg soluble choline.

Growth performance, FCR and carcass traits are presented in Table 4. Compared with NC group, other groups had higher final BW, more evident in the Chol 0.25 and Chol 0.375 groups (P<0.01), while PC, Bet 0.25, Bet 0.375, Bet 0.5 and Chol 0.5 chickens had intermediate values of BW (P<0.05). In addition, chickens from Bet 0.5 group were lighter compared with those of the Chol 0.375 group (P<0.05). The FCR, ranging from 1.69 to 1.96, was found higher in NC group than in Chol 0.25 and Chol 0.375 (P<0.01), and PC, Bet 0.25, Bet 0.375, Bet 0.5 and Chol 0.5 (P<0.05); FCR was also higher in Bet 0.25 group than in Chol 0.375 group (P<0.05).

The results of this study disagree with the findings reported by Kadam *et al*. (2013), who found not significantly influence of betaine on BW and FCR between hatch and 21 day, suggesting that administering betaine at 100 mg/egg would not adversely affect hatchability but did not significantly improve subsequent growth and feed efficiency. Conversely, Matthews *et al*. (1997) reported that betaine in the diet decreased ADG and feed intake in uninfected chicks but increased weight gain and feed intake in coccidiosis-infected chicks. In a later study, Matthews and Southern (2000) confirmed that betaine did not consistently affect growth performance in chicks. Improvements in feed conversion ratio ranging between 2.8 and 7.9% in laying hens (Zou *et al*., 2002) were reported when betaine was added to the diet. This may be explained by a more efficient utilization of dietary protein for lean accretion which is supported by reduced blood urea-N levels, increased N retention and reduced requirement for metabolisable energy (Eklund *et al*., 2005).

The dietary requirement for choline in young chickens and turkey poults as a growth promoter was demonstrated by several authors (reviewed in Ruiz *et al*., 1983). Conversely, in a study conducted by Blair *et al*. (1986) has been observed that choline supplementation did not increase weight gain or feed efficiency for pullets from 8 to 20 weeks of age. However, the dietary requirement for choline in laying hens has been studied mainly in relation to its role in egg production as well as in the prevention of fatty liver.

Carcass yield, breast muscle, leg and wings percentages were similar among the groups (P>0.05). Differently, abdominal fat percentage was influenced by treatment. In fact, compared with NC and PC broilers, those of groups receiving Bet 0.375, Bet 0.5, and Chol 0.25 had lower (P<0.01) abdominal fat percentage; moreover, 0.375 and 0.5 Bet groups had lower (P<0.05) abdominal fat percentage than 0.25

Table 3. Average value for hatchability performance.

Group	Hatchability, %	Chick weight, g	Chick weight/egg weight, %
NC	94.90	36.81^{A}	64.42^{Aa}
PC	93.05	37.74^{B}	64.9^{A}
Bet 0.25	93.98	37.97^{BD}	65.77^{ACb}
Bet 0.375	93.05	38.50^{CDa}	66.75^{BCD}
Bet 0.5	93.05	39.03^{C}	67.47^{D}
Chol 0.25	94.44	39.25^{CEb}	67.35^{D}
Chol 0.375	93.75	38.93^{C}	67.69^{D}
Chol 0.5	93.29	38.75^{C}	67.78^{D}
SEM	1.19	0.16	0.26
P	0.043	0.000	0.000

NC, negative control (eggs not injected); PC, positive control (eggs injected with 0.5 mL deionized water); Bet 0.25, eggs injected with 0.5 mL deionized water+0.25 mg soluble betaine; Bet 0.375, eggs injected with 0.5 mL deionized water+0.375 mg soluble betaine; Bet 0.50, eggs injected with 0.5 mL deionized water+0.50 mg soluble betaine; Chol 0.25, eggs injected with 0.5 mL deionized water+0.25 mg soluble choline; Chol 0.375, eggs injected with 0.5 mL deionized water+0.375 mg soluble choline; Chol 0.50, eggs injected with 0.5 mL deionized water+0.50 mg soluble choline. $^{A-E}$Means in a column with different superscripts are different (P<0.01); ^{a,b}means in a column with different superscripts are different (P<0.05).

Table 4. Average value for growth, feed conversion ratio and carcass traits.

Group	Final BW, g	FCR	Carcass yield, %	Breast muscle, %	Leg, %	Wings, %	Abdominal fat, %
NC	2330.0^{Aa}	1.96^{Aa}	63.98	22.05	21.66	6.33	0.59^{Bb}
PC	2608.7^{bc}	1.78^{bc}	67.19	25.30	21.83	6.52	0.59^{Bb}
Bet 0.25	2528.7^{b}	1.83^{b}	68.64	24.96	22.32	6.38	0.41^{b}
Bet 0.375	2597.0^{bc}	1.78^{bc}	67.77	26.09	22.04	6.58	0.16^{Aa}
Bet 0.5	2614.7^{bc}	1.78^{bc}	68.52	25.30	21.32	6.46	0.20^{Aa}
Chol 0.25	2666.3^{B}	1.75^{B}	67.86	25.63	22.18	6.85	0.32^{A}
Chol 0.375	2727.7^{Bc}	1.69^{Bc}	66.30	25.11	22.58	7.28	0.41^{b}
Chol 0.5	2611.3^{bc}	1.78^{bc}	67.48	25.58	22.84	7.16	0.45^{b}
SEM	24.90	0.02	0.64	037	0.15	0.09	0.03
P	0.000	0.000	0.742	0.171	0.237	0.081	0.000

BW, body weight; FCR, feed conversion ratio; NC, negative control (eggs not injected); PC, positive control (eggs injected with 0.5 mL deionized water); Bet 0.25, eggs injected with 0.5 mL deionized water+0.25 mg soluble betaine; Bet 0.375, eggs injected with 0.5 mL deionized water+0.375 mg soluble betaine; Bet 0.50, eggs injected with 0.5 mL deionized water+0.50 mg soluble betaine; Chol 0.25, eggs injected with 0.5 mL deionized water+0.25 mg soluble choline; Chol 0.375, eggs injected with 0.5 mL deionized water+0.375 mg soluble choline; Chol 0.50, eggs injected with 0.5 mL deionized water+0.50 mg soluble choline. ^{A,B}Means in a column with different superscripts are different (P<0.01); $^{a-c}$means in a column with different superscripts are different (P<0.05).

Bet group, and 0.375 and 0.5 Chol groups.

Studies on pigs reported that betaine added to the finisher diet of pigs increases lean (Casarin *et al*., 1997; Cromwell *et al*., 1999) and decreases fat (Matthews *et al*., 2001; Fernandez-Figares *et al*., 2002) in the carcass. In agreement with early studies, Coma *et al*. (1995) and Eklund *et al*. (2005) suggested that supplemental betaine can reduce protein turnover rate resulting in higher N retention which, in turn, has a positive effect on carcass leanness.

The reduction of fat with the choline supplementation was found by Ruiz *et al*. (1983), who reported that the liver lipid content was significantly higher in extra heavy birds fed the basal diet with no supplemental choline when compared with the average liver lipid content of extra heavy birds fed diets supplemented with choline.

Internal organs percentages are presented in Table 5. The percentage of heart (ranging from 0.46 to 0.63%), gizzard (ranging from 1.33 to 1.51%) and liver (ranging from 1.88 to 2.35%) were not affected by the treatment; while spleen percentage was higher in Chol 0.375 group (+0.05% point) compared with Bet 0.375 group ($P<0.05$), and pancreas percentage was higher in Chol 0.375 group (+0.11% point) compared with PC group ($P<0.05$). With respect to a higher percentage of lymphoid organ (spleen), recorded in chicks of Chol 0.375 group, it is concluded that the active components of Chol 0.375 induce a positive effect on this organ.

Immunoglobulins M, G, and total antibody titers values against SRBC, at 28th and 42nd day of age, were not different among the experimental groups (Table 6), demonstrating that the state of activity of the immune system is not affected by betaine and choline injected at different concentrations. Tsiagbe *et al*. (1987) observed that choline supplemented in chicken diet did not affect either humoral or cellular immune responses either alone or in combination with methionine. Conversely, Swain and Johri (2000) observed a significant response to choline addition in the antibody titers of chicks.

Conclusions

According to the results of this trial, it can be concluded that the embryo mortality, pecked eggs, infected eggs and hatchability percentages were found to be similar among the experimental groups. However, seems that the injection of 0.25 mg of choline could reduce the infected eggs and could produce a better hatchability in comparison with the administration of betaine and other doses of choline. Differently, hatching weight and chick BW/egg weight were higher in treated groups compared with NC group. The betaine and choline treatments improved final BW of the birds and reduced FCR and abdominal fat percentage; while, carcass yield, and breast muscle, leg and wings percentages were found to be similar among the experimental groups. The betaine and choline treatments had little effect on internal organs. The IgM, IgG and IgT titers values against SRBC, at 28th and 42nd day of age were not different among the experimental groups. However, further research is needed to increase knowledge regarding the effect of *in ovo* betaine and choline administration on the performance of offspring broiler chickens.

Table 5. Average values for internal organs.

Group	Hearth, %	Gizzard, %	Spleen, %	Pancreas, %	Liver, %
NC	0.56	1.41	0.13	0.29	1.88
PC	0.53	1.33	0.13	0.20^{a}	2.25
Bet 0.25	0.54	1.37	0.11	0.27	2.06
Bet 0.375	0.46	1.47	0.09^{a}	0.28	2.35
Bet 0.5	0.50	1.51	0.11	0.28	2.14
Chol 0.25	0.56	1.40	0.10	0.24	2.07
Chol 0.375	0.63	1.45	0.14^{b}	0.31^{b}	2.28
Chol 0.5	0.60	1.48	0.13	0.26	2.24
SEM	0.02	0.03	0.01	0.01	0.06
P	0.104	0.749	0.006	0.014	0.610

NC, negative control (eggs not injected); PC, positive control (eggs injected with 0.5 mL deionized water); Bet 0.25, eggs injected with 0.5 mL deionized water+0.25 mg soluble betaine; Bet 0.375, eggs injected with 0.5 mL deionized water+0.375 mg soluble betaine; Bet 0.50, eggs injected with 0.5 mL deionized water+0.50 mg soluble betaine; Chol 0.25, eggs injected with 0.5 mL deionized water+0.25 mg soluble choline; Chol 0.375, eggs injected with 0.5 mL deionized water+0.375 mg soluble choline; Chol 0.50, eggs injected with 0.5 mL deionized water+0.50 mg soluble choline. ^{a,b}Means in a column with different superscripts are different ($P<0.05$).

Table 6. Average values for immune response after injection of sheep red blood cell (log2).

Group	28th day of age			42nd day of age		
	IgM against SRBC	IgG against SRBC	IgT against SRBC	IgM against SRBC	IgG against SRBC	IgT against SRBC
NC	2.00	2.00	4.00	1.00	1.00	2.00
PC	1.33	1.67	3.00	1.00	1.00	2.00
Bet 0.25	1.67	1.67	3.33	1.00	1.33	2.33
Bet 0.375	2.33	3.33	5.67	0.67	1.33	2.00
Bet 0.5	2.33	2.67	5.00	1.00	1.33	2.33
Chol 0.25	2.33	3.00	5.33	1.33	1.33	2.67
Chol 0.375	2.00	3.00	5.00	1.33	1.33	2.67
Chol 0.5	2.00	2.00	4.00	1.33	1.33	2.67
SEM	0.16	0.21	0.31	0.08	0.09	0.11
P	0.794	0.261	0.265	0.466	0.950	0.559

NC, negative control (eggs not injected); PC, positive control (eggs injected with 0.5 mL deionized water); Bet 0.25, eggs injected with 0.5 mL deionized water+0.25 mg soluble betaine; Bet 0.375, eggs injected with 0.5 mL deionized water+0.375 mg soluble betaine; Bet 0.50, eggs injected with 0.5 mL deionized water+0.50 mg soluble betaine; Chol 0.25, eggs injected with 0.5 mL deionized water+0.25 mg soluble choline; Chol 0.375, eggs injected with 0.5 mL deionized water+0.375 mg soluble choline; Chol 0.50, eggs injected with 0.5 mL deionized water+0.50 mg soluble choline; IgM, immunoglobulin M antibody; TSRBC, sheep red blood cell; IgG, immunoglobulin G antibody; IgT, total antibody.

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