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Effect of Dietary Betaine on Performance, Immunocompetence and Gut Contents Osmolarity of Broilers Challenged With a Mixed Coccidial Infection

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Abstract: This study was conducted to investigate the effect of dietary betaine on performance, humoral immunity, intestinal immune responses and gut contents osmolarity of broilers in coccidiosis condition. Three supplemental betaine levels (0, 0.6 or 1.2 g kg^{-1}) were fed to 189 mixed-sex broilers chicks which were randomly assigned to 9 floor cages in a completely random design with 3 replicates. To simulate a coccidiosis challenge, at day 28 of age the chickens were inoculated with a mixed culture of *Eimeria tenella* and *Eimeria acervulina* via the drinking water. Antibody response to Sheep Red Blood Cell (SRBC) was determined on day 21. The immunoglobulin A (IgA) content was quantified in sera and mucous membrane tissue of intestine. Ileum and cecum contents were measured for osmolarity at 21 and 42 days of age. The supplemented diets with 1.2 g kg^{-1} betaine improved average daily gain and feed conversion ratio in 21-42 and 7-42 days periods. Antibody response to SRBC was not affected by dietary treatments. Interestingly, sera IgA content was increased in birds subjected to coccidiosis-infection. The IgA content of both sera ($p<0.05$) and gut tissue ($p<0.01$) were increased by added betaine to diet. Variations in osmolarity and moisture of both ileum and cecum contents were similar and they were significantly ($p<0.001$ and $p<0.05$, respectively) decreased in day 42 measurement by betaine inclusion into the diet. Positive effects of dietary betaine on performance, immunity and digesta moisture and osmolarity redoubles the importance of adding betaine to diet of broilers especially, in stress conditions like coccidiosis-infection.

Key words: Betaine, coccidiosis, IgA, osmolarity, moisture of gut contents

INTRODUCTION

When the nutritional modulation of immune function is considered, betaine is one of the components which are involved in proper immune responses, as reviewed by Kidd (2004). In some researches, addition of betaine to the diet of coccidiosis-infected chickens could decrease the macroscopical and microscopical lesions of intestine (Tiihonen *et al*., 1997; Hess *et al*., 1998; Hamidi *et al*., 2009) but not in some others (Remus and Virtanen, 1996; Matthews *et al*., 1997). The action mechanism of betaine is not clearly known, but it is unlikely to be due to the direct effects on the parasites. It is possible that the role of betaine is related to its influence on antibody production or phagocytic activity because these cells are important in protection against coccidia (Yun *et al*., 2000a). The positive effects of

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betaine on the criteria of cellular immunity including phagocytosis, Nitric Oxide (NO) release, chemotaxis of monocytes toward chemotactic factors released by heterophils (Klasing *et al*., 2002; Warskulat *et al*., 1996) release of inflammatory cytokine (Zhang *et al*., 1996) intraepithelial lymphocytes and thickness of lamina propria (Hamidi *et al*., 2009) have been reported positive. Nevertheless, currently we do not have any knowledge of literature about the effect of dietary betaine on humoral immune responses especially in coccidiosis condition. Among the humoral responses to coccidiosis, the immunoglobulin A (IgA) seems to be most important, because it is an immunoglobulin against mucousal pathogens and has a fast effectiveness (Yun *et al*., 2000a; Wieland *et al*., 2006).

Birds maintain the intracellular volume of water that is crucial for homeostasis by osmoregulation. Inorganic ions (Na^+, K^+, Mg^{2+}, Cl^-, phosphate) and urea are limited osmotic effectors within the cells because they inhibit or disturb cellular enzymes, non-enzyme proteins and nucleic acids if allowed to reach high intracellular concentrations (Petronini *et al*., 1992, 1994). Thus, the most cells adapt to external osmotic stress by altering the intracellular concentration of low molecular weight organic solutes. It is because organic osmolytes (especially betaine) are highly compatible with enzyme function and altering their intracellular concentration dose not upset metabolism (Yancey *et al*., 1982; Dragolovich, 1994). The unique chemical properties of betaine provide it as an osmoprotectant which can modulate cell functions (especially in immune cells) by controlling the cell volume (Warskulat *et al*., 1996; Zhang *et al*., 1996). On the other hand, influence of betaine as an osmolyte can helps to improve function and stability of infected intestinal mucous (Allen *et al*., 1998; Allen and Fetterer, 2002). Previous researches showed that the addition of betaine into the drinking water of turkeys (Ferket, 1995) and also to broilers diet (Klasing *et al*., 2002) reduced their litter moisture and osmolarity of duodenal mucosa, respectively. But in the present research, it was of interest to examine the betaine for its effect on osmolarity and moisture of ileum and cecum contents of broilers in a stressful condition (coccidiosis). Moreover, we evaluated the betaine effects on stimulation of antibody and immunoglobulin A (IgA) production as humoral immune responses as well as on performance.

MATERIALS AND METHODS

Diets, Birds and Experimental Design

This study was performed in the experimental farm of Isfahan University of Technology, Isfahan, Iran during September 2005 to April 2006. A total of 189 seven day-old mixed-sex broiler chicks (Ross 308) were randomly assigned to 9 floor cages. Chicks were fed a basal diet supplemented with 0, 0.6 or 1.2 g kg^{-1} betaine. A complete random design was used with 3 replicates of 21 birds per each dietary treatment during the 7 to 42 day period. The diets were formulated to meet National Research Council (1994) nutrient recommendations (Table 1). The chickens were fed starter diet *ad libitum* until day 21 and grower diet offered from day 21 to 42. Diets contained no coccidiostats.

Humoral Immune Assay

At day 16 of age, two chickens per pen were selected randomly for intraperitonal injection with a 1.0 mL of SRBC suspension diluted with Phosphate Buffer Saline (PBS), pH 7.4 by 5% v/v. Five days later, the same wing-banded birds were bled and antibody response was measured by microagglutination method (Wegmann and Smithies, 1966). Antibody titre was expressed as $\log_2$ of the reciprocal of the last dilution in which agglutination was occurred (Ambrose and Donner, 1973).

Table 1: Ingredients and calculated nutrient content of the basal starter and grower diets

Ingredients (%)	7 to 21 day	21 to 42 day
Corn	55.56	64.23
Soybean meal (44%)	34.04	29.39
Fish meal	3.50	1.00
Fatty acid	3.50	2.00
Dicalcium phosphate	1.01	1.12
Oystershell	1.39	1.39
D-L Methionine	0.14	0.03
Vitamin premix[A]	0.25	0.25
Mineral premix[B]	0.25	0.25
NaCl	0.24	0.22
Variable[C]	0.12	0.12
Energy (Kcal ME/kg) and calculated nutrient content (%)		
Energy (Kcal ME/kg)	2991	2990
Crude protein	21.52	18.67
Lysine	1.25	0.93
TSAA[D]	0.87	0.67
Calcium	0.93	0.81
Available phosphorus	0.42	0.37

A: Vitamin premix provides the following per kilogram: vitamin A 8800 IU; cholecalciferol 3300 IU; vitamin E 16.53 IU; vitamin B 0.023 mg; riboflavin 8 mg; niacin 33 mg; pantothenic acid 35 mg; menadione 1.5 mg; folic acid 0.8 mg; thiamin 3 mg; pyridoxine 2.7 mg; biotin 0.25 mg; ethoxyquin 125 mg; B: Mineral premix provides the following in mg kg^{-1}: Mn, 55; Zn, 50; Fe, 50; Cu, 5; Se, 0.1; I, 1.5. C: Variable amounts of betaine and washed builders sand. D: TSAA: Total sulfur amino acids

ELISA Experiments

A goat anti-chicken IgA ELISA kit (Bethyl Co., E30-130) was used to quantify the IgA content of sera and duodenal mucous membrane tissue. On d 21 and 42 of age, two birds per pen were slaughtered to get blood samples and then the blood serum was used for ELISA test. Also on d 42 a 3 to 4 cm in length section of duodenum (near the duodenal loop) was taken from those couple of birds. Samples were stored at -20°C till analysis time. The sections of duodenum were unfroze at 3 to 4°C then mucous membrane tissue was removed. About 0.4 g tissue was suspended in 1.6 mL cold PBS (pH 7.4) and its extract was used for ELISA determination of IgA. There were differences in water content of mentioned 0.4 g fresh tissues among the samples; therefore, to adjust data for this difference, the IgA amounts were expressed per g protein of fresh mucous membrane tissue. The protein measurement was based on the standard folin method using the spectrophotometer (Lowry *et al.*, 1951).

Parasites and Experimental Infection

At day 28 of age, all birds were inoculated orally with a mixed culture of *Eimeria tenella* and *Eimeria acervulina* to simulate a coccidiosis challenge by 8.5×10^3 and 7.5×10^3 oocysts per chicken, respectively.

Osmolarity and Moisture of Gut Contents

Ileum and cecum contents were collected from two chickens per pen on day 21 and 42 of age. Ileum sections were removed from 6 cm down stream of Meckel's diverticulum to 6 cm up stream of ileocecal valve and its contents were collected. Both cecums were sampled. Some of each sample was dried at 75°C to measure moisture content. The rest of each sample was diluted with deionized water and then their extracts were used to osmolarity quantification by digital osmometer (vapor pressure osmometer, 5520 Vescore, USA). Data was adjusted for added water.

Statistical Analysis

Data was analyzed using the GLM procedures of SAS Institute (SAS, 1999). The comparisons were made among the means using Duncan's multiple range tests (Duncan, 1955) procedure. The means differences were considered significant at a probability $p<0.05$.

RESULTS

Performance

The Average Daily Gain (ADG) was not affected by dietary betaine at 7-21 day experimental period (Table 2). Addition of 1.2 g kg^{-1} betaine to the chicken's diets significantly increased ADG in 21-42 and 7-42 day periods compared to unsupplemented group ($p<0.05$; $p<0.001$, respectively). The lower dietary betaine level (0.6 g kg^{-1}) could not significantly influence ADG to differ from unsupplemented control group. Despite of the improvements in ADG of chickens in both periods, the overall Body Weight (BW) of birds (at 21 or 42 d of age) did not significantly differ among the treatments. As shown in Table 2, Feed Conversion Ratio (FCR) was improved by the highest level of supplemental betaine both in 21-42 and 7-42 day experimental periods ($p<0.001$; $p<0.01$, respectively). Nevertheless, introduction of 0.6 g kg^{-1} betaine into the diets could not additionally affect FRC compared to that observed in control group.

Immune Responses

Dietary supplemental betaine had no significant effect on antibody response to SRBC, which evaluated in serum at day 21 of life (Table 3).

The comparison of average levels of IgA in serum between d 21 and 42 showed that the level of immunoglobulin was elevated with age and pathogen exposure, which its overall mean was 183 μg mL^{-1} on day 21 and 821 μg mL^{-1} on day 42. As shown in Table 3, IgA content of serum on d 21 of age was significantly elevated by 0.6 g kg^{-1} betaine supplementation ($p<0.01$) and was maximized by 1.2 g kg^{-1} level of supplemental betaine. This measurement on d 42 showed the higher IgA level in both supplemented group compared to unsupplemented control group. Furthermore, the amount of intestinal IgA, measured in mucous membrane tissue, on d 42 of age was highly significantly ($p<0.001$) affected by betaine supplementation (Table 3).

Osmolarity and Moisture of Gut Contents

The osmolarity and moisture of both ileum and cecum contents on day 21 of age were not significantly affected by treatments (Table 4). However, osmolarity of ileum and cecum

Table 2: Effect of dietary betaine supplementation on performance parameters of coccidian-challenged broilers during starter and grower stages

	7-21 day			21-42 day			7-42 day				
Betaine	ADG	FI		ADG	FI		ADG	FI		Day 21 BW	Day 42 BW
(g kg^{-1})	----(g day^{-1})-----		FCR	-----(g day^{-1})----		FCR	---(g day^{-1})---		FCR	-------(g)------	
0	28.5^{a}	45.3^{a}	1.60^{a}	43.0^{b}	111.0^{a}	2.53^{a}	36.2^{b}	81.8^{a}	2.23^{a}	546^{a}	1420^{a}
0.6	30.1^{a}	46.8^{a}	1.56^{a}	44.9^{ab}	109.6^{a}	2.35^{b}	38.5^{ab}	82.5^{a}	2.13^{ab}	568^{a}	1447^{a}
1.2	28.3^{a}	45.3^{a}	1.59^{a}	46.7^{a}	107.2^{a}	2.33^{b}	40.4^{a}	81.4^{a}	2.07^{b}	543^{a}	1443^{a}
SEM	0.9	1.3	0.02	0.6	1.2	0.04	0.78	1.0	0.03	14	15
Probability>F	NS	NS	NS	*	NS	***	*	NS	**	NS	NS

A: SEM: Standard error of means, ADG: Average daily gain, FI: Feed intake, FCR: Feed conversion ratio, BW: Body weight. B: $^{a-b}$Values within variables with no common superscripts differ significantly ($p<0.05$). C: NS: Not Significant, *: $p<0.05$, **: $p<0.01$, ***: $p<0.001$

Table 3: Effect of dietary inclusion of betaine on antibody titre and IgA content of sera and intestinal tissue in broilers subjected to ciccidia infection

Betaine (g kg^{-1})	Antibody response to SRBC[A]	Sera IgA on day 21 (μg mL^{-1})	Sera IgA on day 42 (μg mL^{-1})	Intestinal[B] IgA On d 42 (μg g^{-1} p)
0	3.50^{a}	132	626^{b}	22.26^{b}
0.6	3.83^{a}	200^{b}	882^{a}	31.37^{a}
1.2	3.83^{a}	217^{a}	954^{a}	33.92^{a}
SEM	0.16	9.7	38.8	1.38
Probability > F	NS	*	*	**

A: Data for antibody response to sheep red blood cells (SRBC) are as $\log_2$ of the reciprocal of the last dilution in which there was agglutination. B: Amount of immunoglobulin A (IgA) was measured in mucous membrane tissue of intestine and expressed per g protein of fresh tissue. Values within variables with no common superscripts differ significantly ($p<0.05$). C: NS: Not Significant, *: $p<0.05$, **: $p<0.01$, ***: $p<0.001$

Table 4: Effect of dietary betaine supplementation on osmolarity and moisture of ileum and secume contents in broilers subjected to ciccidia infection

	Osmolarity on day 21		Osmolarity on day 42		Moisture on day 21		Moisture on day 42	
Betaine (g kg^{-1})	Ileum	Cecum	Ileum	Cecum	Ileum	Cecum	Ileum	Cecum
	(mOsm kg^{-1})				(kg kg^{-1} DM)			
0	459^{a}	428^{a}	499^{a}	488^{a}	4.400^{a}	3.3100^{a}	5.6433^{a}	3.7233^{a}
0.6	424^{a}	399^{a}	443^{b}	388^{b}	3.5167^{a}	2.6100^{a}	3.9200^{b}	2.8867^{ab}
1.2	420^{a}	423^{a}	408^{b}	369^{b}	3.7033^{a}	2.8667^{a}	3.6300^{b}	2.3267^{b}
SEM	7.7	7.6	12.2	14.6	0.375	0.205	0.370	0.259
Probability>F	NS	NS	***	***	NS	NS	*	*

DM: Dry mater. Values within variables with no common superscripts differ significantly ($p<0.05$). C: NS: Not Significant, *: $p<0.05$, **: $p<0.01$, ***: $p<0.001$

contents on day 42 of age were diminished by both levels of dietary supplemental betaine ($p<0.001$). As shown in Table 4 the moisture of ileum contents on day 42, was observably declined by addition of 0.6 or 1.2 g kg^{-1} betaine to the chick's diets ($p<0.05$). Also, the moisture of cecum contents in that day were significantly ($p<0.05$) reduced by 1.2 g kg^{-1} supplemental betaine, whereas addition of 0.6 g kg^{-1} betaine to the diets did not induce significant difference from unsupplemented group.

DISCUSSION

The presented results indicated that betaine had no significant effect on ADG and FCR in 7-21 day period, when the chickens were not infected. However, dietary betaine supplementation had a significant effect on ADG without affecting feed intake (FI) in 21-42 d period, when chickens were infected. The elevation of ADG without increase in FI led to improvement of FCR. Although, chickens that received 1.2 g kg^{-1} supplemental betaine had higher ADG, their BW at final day did not differ from control chickens. This improvement in FCR might impart, due to the protective effect of betaine on intestinal epithelium against coccidiosis lesions (Kidd *et al*., 1997). The protective effect of betaine can cause to stabilize the epithelial cells and gut mucous. Besides, the less damage to epithelia, the more absorptive compartment. Therefore, gastrointestinal functions (digestion and absorption) would be improved. Consequently the food can be used more efficiently. Our histological observations on intestinal sections (Hamidi *et al*., 2009) confirm this opinion in which there was a decline of intestinal lesions scores when 1.2 g kg^{-1} betaine had been added to chicken diets. Also, it has been shown that dietary betaine increases digestibility of nutrients such as methionine (Augustine and Danforth, 1999) carotenoids, lysine, protein and fat (Remus and Virtanen, 1996). In general, betaine may affect FCR by improving the nutrients digestibility and absorption ability of gut tract.

The second assumable effector for FCR improvement, observed in presented study, might be the function of betaine as an osmolyte. Betaine assists Na^+-K^+ ATPas pumps of the cell membrane, which leads to sparing observable amounts of energy (Haussinger, 1998). Presented results indicate that supplemental betaine decreased the osmolarity and moisture of gut contents in coccidiosis-infected chickens. On the other hand, the diarrhea -a typical symptom of coccidiosis infection- could induce subclinical acidosis which affects metabolic reactions and consequently the efficiency (Scott *et al.*, 1982). Thus, another assumable hypothetical- but less deducible from our results- mechanism for FCR improvement due to dietary betaine is by partially reducing diarrhea and consequently assisting the metabolism to be more efficient.

Despite of the above theme, present results about the effect of supplemental betaine on performance and feed efficiency are not confirmed by several reports including Esteve-Garcia and Mack (2000), Matthews and Southern (2000) and Klasing *et al.* (2002) who have reported no improvement in body weight gain and FCR with betaine supplementation. However, in agreement with us, Matthews *et al.* (1997) reported that betaine increased ADG of coccidiosis-infected chicks. Similarly, Schutte *et al.* (1997) observed that feed conversion efficiency was significantly improved by adding 0.4 g kg^{-1} betaine to the practical diets. Furthermore, Waldensted *et al.* (1999a) reported that supplementation of infected chick diet with 1 g kg^{-1} betaine improved BW at 22, 29 and 35 day of age. But when betaine with Narasin was added to the diets it didn't have this effect. In another research, Waldensted *et al.* (1999b) reported that coccidio-infected chicks which received 15% betaine with 66 mg kg^{-1} salinomycin had higher BW and lower FCR comparing to controls and groups which received only betaine or Salinomycin.

The effects of betaine on cellular immune responses have been properly studied. Supplemental betaine has been reported to induce the followings: increase of phagocytosis, release of NO [Phagocytosis and NO release are critical effectors function in defense against parasites including coccidian and NO stimulates macrophages to phagocyte (Ovington *et al.*, 1995)] chemotaxis of monocytes toward chemotactic factors released by heterophils (Klasing *et al.*, 2002; Warskulat *et al.*, 1996) release of inflammatory cytokine (Zhang *et al.*, 1996) increase in number of intraepithelial lymphocytes and altering thickness of lamina propria (Hamidi *et al.*, 2009). Nevertheless, we do not have any knowledge of literature about the effect of dietary betaine on humoral immune responses. As previously stated in results, two humoral immune criteria were evaluated which just one of them (IgA content of both sera and intestinal tissue) was affected by dietary betaine supplementation.

Recently, attention has been paid to passive immunoprophylaxis by orally administering the IgA that it could benefits especially for young broilers because of its fast effectiveness (Wieland *et al.*, 2006). This function reveals the importance of IgA role in protection against parasite antigens. However, the other immune response (antibody response to SRBC) was not affected by treatments. The evaluation of this humoral immune response, as well as other ones, in variety of conditions in future researches would more clarify the influence of dietary betaine on immunocompetence. The responses would be influenced by conditions like diet components, parasitic infection (be or not to be and its kind) and the age of birds.

General comparison of immunoglobulin A levels between 21 and 42 days of age indicated that the concentration of this immunoglobulin was elevated by coccidial infection, although, some of this increment may have been induced by growth of chickens. Yun *et al.* (2000b) have reported similar results for variation of Eimeria-specific antibodies (IgA, IgM and IgG) in the circulating and intestinal secretions. Yun *et al.* (2000a) in the

review of intestinal immune responses to coccidiosis, suggested that the level of IgA in bile may not correlate with its level in infected areas of intestine. Hence, in this study, IgA amounts were evaluated in sera and intestinal tissue.

Comparison of osmolarity and moisture of gut contents between day 21 and 42 reveals that betaine had no statistically significant influence on these measurements at day 21, although, there were some numerical differences, but at day 42 it had. With consideration of this fact that on day 21 birds were not infected but in final day they were, this idea comes to us that betaine might has more effectiveness in stressful conditions. A researcher (Ferket, 1995) had added betaine to drinking water of turkeys showing symptoms of diarrhea for 24 h reported decline of litter moisture from 46 to 27% within following 5 days. This researcher, also, reported that this betaine treatment was effective in stopping diarrhea in 96% of male turkey flocks over the age of 70 days, but it was less effective in younger flocks (<60%). Furthermore, Klasing *et al.* (2002) reported that dietary betaine decreased osmolarity of the duodenal mucosa. The almost similar results have been reported by Tiihonen *et al.* (1997) as well. The importance of this effect of betaine is because diarrhea in poultry is of practical concern, which increases litter moisture and consequently increases atmospheric ammonia and odour emission. High litter moisture increases the susceptibility of a flock to pathogens and lesions, which are induced by wet litters (e.g., Hock Scabs and Breast Burn).

CONCLUSION

An interesting conclusion of this research was that diet supplementation with betaine resulted in more IgA production in sera and intestinal tissue. This effect together with positive effect on performance and reduction of osmolarity and moisture of gut contents redoubles the importance of adding betaine in broilers diet especially in stressful conditions like coccidiosis infection. Nonetheless, further researches would reveal more aspects of its effects.

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