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Research Article

Effect of Betaine Hydrochloride on Growth, Antioxidant Enzyme Status, Gut Health and Expression of Immunity-Related Genes in Broilers

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Abstract

This study investigated the effects of betaine hydrochloride on broiler chickens' antioxidant profile, gut health and immune system. Two hundred birds were divided into five groups of 40 birds and each group had 4 replicates of 10 birds. Group B0.0 served as the control and while B0.5, B1.0, B1.5 and B2.0 received 0.50, 1.00, 1.50 and 2.00g betaine hydrochloride/kg feed, respectively. The feed intake, body weight gain and feed conversion ratio were significantly ($p < 0.05$) improved by betaine supplementation in comparison to the control group with the highest effects noted in B2.0 group. Antioxidant enzyme activity was significantly higher ($p < 0.05$) in all betaine-supplemented groups in comparison to the control. In gut microflora count harmful bacteria (*E. coli* and *Salmonella*) decreased ($p < 0.05$), while beneficial *Lactobacilli* increased ($p < 0.05$), with B2.0 showing the best results. Notably, gut morphology was significantly improved by betaine supplementation, with increased villi length (VL) and villi height: crypt depth ratio, indicating enhanced nutrient absorption and gut health, with the most significant improvements observed in B2.0. Gene expression analysis revealed enhanced mRNA expression of TLR 2 and TLR 7, indicating improved immunity, and downregulation of TLR4, suggesting reduced inflammation. The most pronounced changes were observed in B2.0. Overall, the study suggests that 2.0 g/kg betaine hydrochloride supplementation (B2.0) has the most beneficial effects on antioxidant status, gut health, and immune system function in broiler chickens.

Keywords: Betaine; Antioxidant; Gut-Microflora; Gut-Morphology; Gene Expression

Introduction

Betaine, a naturally occurring compound found in various plant and animal products, has garnered attention for its potential benefits in poultry nutrition. Betaine is known to have two major functions in the body, as a methyl group donor and an organic osmolyte. As an important organic osmolyte, betaine can protect the cell against dehydration and increase water retention to maintain water balance which contributes towards maintaining both the bird's energy balance and feed intake and therefore, this spared energy may promote cell proliferation and growth of birds [1] which results into better FCR and body weight gain in poultry. Feeding betaine in poultry can significantly reduce reactive oxygen species (ROS), free radicals and improve the total antioxidant status of birds by its ability to reduce mitochondrial lipid peroxidation, scavenge free radicals and preserve optimum cellular functions [2]. It has also been suggested that betaine act as a natural antioxidant by restoring S-adenosyl methionine and have the ability to improve meat quality of broilers [3].

Previous researches has shown that due to its methyl donor and osmolytic properties, betaine supplementation can modulate gut microflora and improve gut morphometry in broilers by supporting beneficial intestinal microbes, balancing osmotic pressure, enhancing intestinal villus height and integrity, thereby improving nutrient digestibility and absorption [1,4,5]. Betaine's methyl group donating property promotes intestinal epithelial cell proliferation, while its osmolytic and antioxidant activities improve the intestinal environment and alleviate oxidative damage [6].

Additionally, betaine supply methyl group to the various biological methylation reactions including DNA methylation [7], thereby can exert epigenetic change and alter or modulate the expression of genes at the transcriptional levels. Building on these findings, this study aimed to investigate the impact of different levels of betaine supplementation on body growth and immunity related parameters like antioxidant enzyme activity, gut microflora, gut morphometry and mRNA gene expression of TLR's in broiler chickens.

Materials and Methods

Ethical approval

The animal experiment was conducted in accordance with guidelines approved by the Institutional Animal Ethics Committee, 12/CPCSEA Dated 12.04.2022 in the Department of Animal Nutrition, Lala Lajpat Rai University of Veterinary & Animal Sciences, Hisar.

Experimental design

A study of 6-week duration was conducted on two hundred commercial broiler chicks randomly distributed into five treatment groups with four replicates of ten birds each. The control group (B0.0) was offered only basal diet to fulfil the metabolizable energy (ME) and crude protein requirements of broilers and formulated as per BIS [8] while groups B0.5, B1.0, B1.5 and B2.0 were supplemented with 0.50, 1.00, 1.50 and 2.00 g betaine hydrochloride/kg basal feed. The ingredients used in formulating basal diet and their chemical composition analyzed as per AOAC [9] are presented in table 1.

The birds were housed in deep litter system from day-old to 42 days of age following standard management practices. The birds were fed starter, grower and finisher diets for 1 to 7, 8 to 21 and 22 to 42 days of age, respectively. Feed and water were provided *ad-lib*. All the birds were vaccinated against Ranikhet disease on 4th day and IBD on 13th day of age. Feed intake, residue left per replicate and body weight of birds were measured at the beginning and end of each phase of growth throughout the experiment to calculate the feed consumption, body weight gain and feed conversion ratio of birds.

Ingredients (Kg/100kg)	Starter (0-7d)	Grower (8-21d)	Finisher (22-42d)
Maize	50.00	52.64	58.20
Soybean Meal	25.00	22.36	16.80
Fishmeal	6.00	6.00	6.00
Groundnut cake	10.00	10.00	10.00
Vegetable Oil	4.67	5.48	5.9
Mineral Mixture	2.00	2.00	2.00
Common salt	0.5	0.5	0.5
Agemix[2]			
Veldot[3]			
Choline (60%)			
Lysine (98%)			
DL-Met (98%)			
Chlortetracycline			
Total	100.00	100.00	100.00
Analysed nutrients (% DMB)			
Crude Protein	22.79	21.81	20.10
Ether Extract	7.78	8.38	8.61
Crude Fibre	4.89	4.45	4.15
ME (Kcal/Kg)	3010.67	3104.64	3195.56
Calculated nutrients (% DMB)			
Dig. Lysine	1.19	1.13	1.05
Dig. meth + cys	0.78	0.76	0.71
Dig. Thre	0.79	0.75	0.68

Table 1: Composition (% DMB) of basal Diets.

[1]Each kg of mineral mixture contained 300g Ca; 90g P, 4g Mg, 4g Zn, 0.; 1.0g I, and 0.5g Cu.

[2]Each 5kg Agemix (produced by Neospark Drugs and Chemicals Pvt. Ltd., India) contains Vitamin A: 12.5MIU; Vitamin D3: 2.5MIU; Vitamin E:8.0g; Vitamin K3: 1.0g; Vitamin B1: 800mg; Vitamin B2: 5.0g; Vitamin B6: 1.6g; Vitamin B12: 20.0mg; Niacin: 12.0g; Calcium d-Pantothenate: 8.0g; Folic Acid: 800.0mg; Biotin: 6.0mg; Choline Chloride: 300.0g; Calcium: 760.0g; Copper: 15.0g; Iodine: 1.0g; Iron: 60.0g; Manganese: 80.0g; Zinc: 80.0g and Selenium: 300.0mg).

[3]Veldot (produced by Venky India Ltd.) contains Dinitro-O-Toluamide, a coccidiostat.

At the end of the feeding trial (42 days), about 2 ml blood sample was collected from one bird per replicate via brachial wing vein puncture into vacutainer tubes containing EDTA for blood antioxidant enzyme status and cytokine mRNA expression of TLRs.

Cross sections of ileum (1-2 mm thickness) were fixed in 10% neutral buffered formalin for 24 hours, then sectioned (5 µm), stained and examined under microscope for morphometric analysis of villi length, width and depth. For microbial count of ileal content samples were collected aseptically, weighed (1g) and transferred to vials containing 0.9% normal saline solution (1:1). After homogenisation and making serial dilutions upto six times, 0.1ml of diluted sample were poured and spread uniformly on different agar mediums, incubated and further studied for different bacterial assay.

Results and Discussion

During the starter phase of this experiment, no significant differences ($p > 0.05$) were observed in the mean feed intake values among all groups. However, as the experiment progressed, feed intake was significantly enhanced by betaine supplementation in comparison to control, particularly in the group receiving 2.0 g/kg betaine (B2.0) exhibiting highest mean feed intake values during the grower, finisher and overall trial period (Table 2).

These findings are consistent with previous research by Amer et al. [10], who reported that betaine supplementation (2 g/kg diet) significantly increased ($p < 0.05$) feed intake in broilers. The methyl group donor and organic osmolyte properties of betaine are thought to contribute to its growth-promoting effects in animals.

Similar studies have also demonstrated that the addition of 2 g/kg betaine to broiler diets significantly increases ($p < 0.05$) average daily feed intake [11-14]. However, some studies have reported no significant effects of betaine supplementation on feed intake in broilers [15,16].

Attributes	B 0.0	B 0.5	B 1.0	B 1.5	B 2.0
Feed Intake (g)					
Starter (0-7d)	145.35^{a} ± 4.34	144.67^{a} ± 5.65	144.54^{a} ± 3.25	145.43^{a} ± 7.89	146.46^{a} ± 2.34
Grower (8-21d)	840.67^{a} ± 6.54	847.78^{a} ± 5.67	867.92^{b} ± 8.56	885.64^{b} ± 9.43	930.31^{c} ± 6.87
Finisher (22-42d)	2504.36^{a} ± 19.67	2547.49^{b} ± 17.64	2538.63^{b} ± 20.23	2569.75^{b} ± 18.35	2607.66^{c} ± 17.98
Overall (0-42d)	3490.38^{a} ± 16.37	3539.94^{b} ± 28.45	3551.09^{b} ± 34.24	3600.82^{c} ± 18.41	3684.43^{d} ± 24.08
Body Weight Gain (g)					
Starter (0-7d)	118.45^{a} ± 1.23	119.65^{ab} ± 1.32	120.54^{b} ± 3.24	120.46^{b} ± 2.43	122.56^{c} ± 1.65
Grower (8-21d)	462.76^{a} ± 15.12	478.82^{ab} ± 8.43	518.42^{b} ± 11.32	580.23^{c} ± 12.76	627.21^{c} ± 9.32
Finisher (22-42d)	1453.53^{a} ± 13.24	1518.89^{b} ± 17.32	1545.85^{bc} ± 15.65	1556.80^{bc} ± 21.42	1583.90^{c} ± 18.19
Overall (0-42d)	2034.74^{a} ± 11.42	2117.36^{b} ± 17.35	2184.81^{bc} ± 24.68	2257.49^{c} ± 27.31	2333.67^{d} ± 19.37
Feed conversion ratio					
Starter (0-7d)	1.22^{a} ± 0.02	1.20^{a} ± 0.01	1.19^{a} ± 0.02	1.20^{a} ± 0.04	1.19^{a} ± 0.03
Grower (8-21d)	1.81^{c} ± 0.03	1.77^{c} ± 0.03	1.67^{b} ± 0.02	1.52^{a} ± 0.05	1.48^{a} ± 0.04
Finisher (22-42d)	1.72^{c} ± 0.02	1.67^{b} ± 0.01	1.64^{a} ± 0.03	1.65^{a} ± 0.03	1.64^{a} ± 0.05
Overall (0-42d)	1.71^{c} ± 0.02	1.67^{bc} ± 0.03	1.63^{b} ± 0.03	1.59^{a} ± 0.01	1.57^{a} ± 0.01

Table 2: Feed intake (g), BWG (g) and FCR of experimental birds.

Means bearing different superscripts in a row differ significantly ($p < 0.05$).

The mean body weight gain was increased significantly in betaine supplemented groups compared to the control (B0.0). The group supplemented with 2g betaine per kg feed (B2.0) showed the highest increment followed by B1.5, B1.0, and B0.5 (Table 3). These findings are consistent with a more or less similar experiment (B0, B-1.0, B-1.5 and B2.0 groups supplemented betaine @ 0.0, 1.0, 1.5 and 2.0 g/kg feed, respectively), which demonstrated that total weight gain in betaine supplemented groups was increased significantly ($P < 0.05$) in comparison to control [11]. Similarly, another study reported that broilers fed betaine-hydrochloride supplemented diets (1.3 and 2 g/kg) exhibited improved body weight and weight gain ($p < 0.05$) compared to controls during a 38-day period [17].

The observed increase in body weight gain may be attributed to betaine's potential to enhance nutrient digestibility and energy-protein metabolism, mediated by its methyl group donor function [1].

However, our results diverge from those of other studies, which reported no significant effects of betaine supplementation on body weight gain in broilers [18,19].

The present study demonstrated a notable improvement in feed conversion efficiency (FCE) with betaine supplementation. Although feed conversion ratio (FCR) values were statistically similar across all groups during the starter phase, they subsequently decreased significantly in betaine-supplemented groups compared to the control (B0.0). Over the entire experimental period, the lowest FCR was recorded in group B2.0, followed closely by group B1.5, with both groups exhibiting statistically similar values that differed significantly ($p < 0.05$) from the remaining groups (Table 3). These findings are consistent with various previous researches, which has consistently demonstrated that betaine supplementation at levels of 1.0-2.0 g/kg significantly improves feed conversion ratio (FCR) compared to control groups [11,20-22].

However, in contrast to the present study, some investigations have also found minimal or no effects of betaine on FCR in broiler birds [18,19].

Antioxidant enzymes

Betaine supplementation significantly ($p < 0.05$) enhanced the activities of antioxidant enzymes, including catalase, superoxide dismutase (SOD), and glutathione peroxidase (GPx), in the blood of broilers (Table 4). Notably, the highest catalase enzyme activity was observed in the group B2.0, whereas the highest SOD and GPx activities were recorded in the group B1.5. Although the mean values of treatment groups B1.5 (1.50 g/kg) and B2.0 (2.00 g/kg) were statistically similar ($p > 0.05$) for all three antioxidant enzymes, the overall trend suggests a positive correlation between betaine supplementation and antioxidant enzyme activity.

Consistent with these findings, Yang., *et al*. demonstrated that betaine supplementation (1.0g/kg feed) significantly ($p < 0.05$) increased the activities of GPx, SOD, and glutathione (GSH) level, as well as total antioxidant capacity (T-AOC), in the breast muscle of broiler birds. Although the activity of catalase was increased non-significantly ($p > 0.05$) in betaine-supplemented groups, the overall antioxidant effect of betaine was evident [16].

The antioxidant properties of betaine are thought to occur through its ability to restore S-adenosyl methionine, which contributes to an enhancement in the supply of substrate needed for the synthesis of GSH [3]. GSH plays a crucial role in protecting cells from reactive metabolites and reactive oxygen species. By boosting antioxidant defenses, betaine supplementation may help mitigate oxidative stress and promote overall health and well-being in broilers.

Treatment	B0.0	B0.5	B1.0	B1.5	B2.0
Antioxidant enzyme activities					
Catalase(U/g Hb)	$22.14^{a} \pm 0.25$	$23.78^{b} \pm 0.32$	$24.18^{b} \pm 0.34$	$25.84^{c} \pm 0.28$	$26.24^{c} \pm 0.29$
SOD (U/g Hb)	$25.12^{a} \pm 0.24$	$26.92^{b} \pm 0.26$	$26.78^{b} \pm 0.27$	$27.88^{c} \pm 0.23$	$27.48^{bc} \pm 0.31$
GPx (U/ml)	$1.36^{a} \pm 0.11$	$1.94^{b} \pm 0.08$	$2.18^{b} \pm 0.09$	$2.92^{c} \pm 0.10$	$2.75^{c} \pm 0.07$
Gut microbial count					
E. coli (log cfu/g)	$6.43^{c} \pm 0.07$	$5.49^{b} \pm 0.06$	$4.69^{a} \pm 0.06$	$4.40^{a} \pm 0.13$	$4.38^{a} \pm 0.17$
LAB (log cfu/g)	$5.63^{a} \pm 0.16$	$6.16^{b} \pm 0.15$	$6.30^{bc} \pm 0.09$	$6.49^{c} \pm 0.11$	$6.67^{c} \pm 0.12$
Salmonella (log cfu/g)	$3.71^{c} \pm 0.04$	$2.92^{b} \pm 0.03$	$2.65^{b} \pm 0.07$	$2.19^{a} \pm 0.04$	$2.05^{a} \pm 0.03$
Gut morphometry					
Villi height (µ)	$1264.88^{a} \pm 9.24$	$1331.36^{b} \pm 8.46$	$1486.46^{c} \pm 9.38$	$1507.35^{cd} \pm 8.30$	$1520.14^{d} \pm 15.80$
Villi width (µ)	$386.60^{b} \pm 4.24$	$364.21^{ab} \pm 6.56$	$347.78^{a} \pm 9.59$	$352.48^{a} \pm 4.54$	$346.44^{a} \pm 5.32$
Crypt depth (µ)	$307.60^{a} \pm 4.26$	$279.05^{ab} \pm 5.14$	$263.52^{b} \pm 5.35$	$264.60^{b} \pm 6.90$	$262.99^{b} \pm 6.61$
Villi Height: Crypt depth	$4.11^{b} \pm 0.05$	$4.77^{ab} \pm 0.06$	$5.64^{a} \pm 0.08$	$5.69^{a} \pm 0.10$	$5.78^{a} \pm 0.20$

Table 3: Antioxidant enzyme activities and gut microbial count of broiler birds under different dietary treatments. Means bearing different superscripts in a column differ significantly ($p < 0.05$).

Gut microflora

The findings of this study demonstrate that betaine supplementation has a beneficial impact on the gut microbiome of broiler birds. Notably, a significant ($p < 0.05$) reduction in the count of harmful E. coli and Salmonella spp. (log cfu/g) was observed in the ileum of birds in the betaine-treated groups compared to the control group. The highest reduction was seen in the group supplemented with 2.0 g betaine per kg feed (B2.0) (Table 5). Conversely, the count of beneficial lactobacilli (log cfu/g) was significantly increased, maximum in B2.0, suggesting a shift in the balance of the gut microbiome towards a more beneficial composition as the dose of betaine was increased. These findings are consistent with a previous study, which reported that betaine supplementation (1.0g/kg) significantly ($p < 0.05$) increased the beneficial lactobacilli bacteria count in the gut of broilers compared to the control group [23].

The increase in beneficial microbes in the gut may be attributed to the osmoprotective effect of betaine and its buffering capacity, which helps regulate gut pH. Additionally, betaine stimulates mucin production from goblet cells by upregulating the MUC2 gene [24], thereby increasing the amount of mucin available to protect the gut epithelium from harmful pathogens and improve gut lining.

Mucin plays a crucial role in adhering beneficial bacteria to the gut epithelium, promoting their colonization while selectively excluding harmful pathogens. The interaction between mucin and bacteria is complex and selective, allowing the gut to distinguish between commensal and pathogenic microbiota by the presence of specific molecular patterns from microorganisms (eg, pattern recognition receptors) [25,26].

Furthermore, beneficial bacteria can protect against pathogen invasion by increasing mucus production and occupying binding sites available on mucins, thereby impeding pathogen adhesion [25,27]. Betaine supplementation can also reduce the fear response of heat-stressed broiler chickens and consequently decrease tonic immobility duration [28]. Thus by enhancing gut motility, betaine ensures the proper movement of food and beneficial bacteria throughout the GIT while checks the growth of harmful bacterial clumps.

Gut morphometry

The gut morphology analysis of this study revealed that betaine supplementation significantly ($p < 0.05$) enhanced the villi height of the ileum in treated groups compared to the control group (Table 6). The villi height-to-crypt depth ratio, a key indicator of gut health, was also significantly improved in the betaine-supplemented groups (B1.0, B1.5, and B2.0) compared to the control group, with the highest improvement observed in the B2.0 group.

These findings are consistent with previous studies, which have demonstrated that betaine supplementation can positively impact gut morphology. For instance, a study by Yousefi., *et al.* reported that betaine supplementation (1g/kg) resulted in significantly greater duodenal villus height and villus surface area, as well as an improved jejunum villus height-to-crypt depth ratio [29]. Similarly, Sun et al. found that dietary betaine supplementation (0.5 g/kg feed) increased the villus height and mucosal thickness of the ileum, also villus width was increased linearly ($p < 0.05$) with increasing levels (0.5, 1, 2 g/kg) of betaine supplementation [22].

The beneficial effects of betaine on gut morphology can be attributed to its multifaceted properties, including its role as a methyl group donor, osmolyte, and antioxidant. Betaine's methyl group donating property may promote the proliferation of intestinal epithelial cells, while its osmolytic property can improve the intestinal environment. Additionally, betaine's antioxidant activity can help alleviate intestinal oxidative damage induced by heat stress [4,6].

In contrast, a study by Sakomura., *et al*. [30] found that betaine supplementation did not affect the morphometrics of the crypts and villi in the intestines of heat-stressed broilers. However, the present study's findings suggest that betaine supplementation can positively impact gut morphology, potentially by increasing water binding capacity and promoting changes in the structure of the gut epithelium [4].

Furthermore, the gut microbiota may also play a role in the intestinal development and microstructure morphology [31]. Betaine may be involved in modulating the gut microbiota, thereby promoting intestinal development and health. However, the underlying mechanisms require further investigation to be fully elucidated.

mRNA gene expression

The incorporation of betaine in the diet enhanced the relative mRNA expression of Toll-like receptor (TLR) cell markers in the serum of broilers, thereby confirming its potential to stimulate T cell immune function in the serum (Table 4). Notably, TLR2, which recognizes gram-positive bacteria through lipoteichoic acid content in the cell wall [32], exhibited more than two-fold increase in relative mRNA expression in the B1.5 and B2.0 groups when compared with the control (B0.0). This upregulation may be attributed to the enhanced populations of Lactobacillus species as the diet incorporating or promoting selective strains of lactic acid species have potential to improve the gut mRNA levels of TLR 2.

In contrast, the relative mRNA expression of TLR4 showed a downregulation pattern in response to betaine supplementation, which can be linked to the reduced number of gram-negative bacteria, such as Coliform, belonging to the family Enterobacteriaceae. The TLR4 receptor plays a crucial role in recognizing lipopolysaccharides of gram-negative bacteria [33].

The mRNA expression of TLR7 exhibited an upregulation pattern with increasing doses of betaine in different dietary treatment groups. TLR7 recognizes antiviral compounds and single-stranded viral RNA, suggesting that betaine may modify the T cell immune response to viruses in the serum of broiler chicks.

Studies have shown that betaine supplementation increases mRNA expressions of some other immunity related genes (Nrf 2, GPX, CAT, SOD, γ-GCLc, HO-1, NQO1 and GSH) also, indicating improved antioxidant capacity and immune function [16,34] thus proving betaine as a potential immune booster feed supplement in broilers diet.

Target gene	Treatment	C_T Mean	C_T SD	ΔC_T Mean	$\Delta\Delta C_T$ Mean	R. Q
TLR-2	B 0.0	23.58	0.08	4.04	0	1
	B 0.5	23.25	0.11	3.80	-0.24	1.18
	B 1.0	22.94	0.13	3.52	-0.52	1.43
	B 1.5	22.48	0.12	3.00	-1.04	2.05
	B 2.0	22.14	0.14	2.58	-1.46	2.75
TLR-4	B 0.0	22.56	0.12	3.02	0	1
	B 0.5	22.83	0.13	3.38	0.36	0.77
	B 1.0	23.13	0.11	3.71	0.69	0.61
	B 1.5	23.28	0.13	3.80	0.78	0.58
	B 2.0	23.71	0.15	4.15	1.13	0.45
TLR-7	B 0.0	24.75	0.16	5.21	0	1
	B 0.5	24.54	0.18	5.09	-0.12	1.08
	B 1.0	24.25	0.15	4.83	-0.38	1.30
	B 1.5	24.14	0.21	4.66	-0.55	1.46
	B 2.0	23.85	0.23	4.29	-.092	1.89
β-Actin	B 0.0	19.54	0.21			
	B 0.5	19.45	0.19			
	B 1.0	19.42	0.15			
	B 1.5	19.48	0.21			
	B 2.0	19.56	0.14			

Table 4: Relative quantification expression analysis of the toll like receptors (TLR 2, TLR 4 and TLR 7) with reference to the endogenous reference gene β actin.

Conclusion

This study conclusively demonstrates that supplementing broiler chicken with 2.0 g/kg feed of betaine hydrochloride yields the most pronounced benefits, significantly enhancing the growth, antioxidant status, gut health, and immune system function. These findings underscore the potential of betaine as a valuable feed additive for promoting overall health and well-being in broiler chickens. Overall, the study suggests that betaine supplementation can be a useful strategy to enhance the efficiency and productivity of broiler production systems.

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