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Research Article

Supplementation of *Bacillus subtilis* and lysine has positive impact on histo-morphometry of Jejunum and immune organs of broilers subjected to dexamethasone-induced stress

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Abstract

Different types of stresses affect the production and health of broilers. There is a great demand across the world to find out different solutions to overcome the detrimental effects of stress especially in poultry. The purpose of present study is to gauge the effects of probiotic supplementation alone as well as in combination with two doses of lysine in broilers subjected to dexamethasone-induced stress (DS) as a solution of above mentioned problem. For this experiment, a total of 336 day-old corncob broiler chicks were brought up for forty-two days and were attributed to seven groups on twenty-first day of experimentation. The groups were the negative control (basal diet only); positive control (basal diet + DS); probiotics (100mg/kg diet) + DS; Low lysine (5g/Kg diet) + DS; probiotics (100mg/Kg diet) + Low lysine + DS; High lysine (11g/Kg diet) + DS; and probiotics (100mg/ton diet) + High lysine + DS. To cause stress, dexamethasone was offered to the birds by drinking water (@ 3mg/L) daily from 21st to 28th day of their life. At the termination of the experiment (day 42), two birds from each replicate were slaughtered for tissue collection to conduct the jejunal and immune organs histomorphometry. The results demonstrated that the DS adversely affects jejunal and immune organs' health as compared to negatively controlled birds. However, the supplementation of *Bacillus subtilis* partially reversed the adverse effects of DS. Supplementation of *B. subtilis* + high lysine significantly improved the jejunal and immune organs' histomorphometry compared with the DS birds. Moreover, the performance and health attributes of this supplemented group were comparable with the control group. Probiotics *B. subtilis* + high lysine-supplemented birds outperformed the dexamethasone-induced stressed- supplemented birds. So this combination may be useful to increase the yield of domestic as well as commercial poultry farming. This study will encourage scientists to find out more solutions to the negative effects of stress affecting the birds and animals.

Keywords: *Bacillus subtilis*; Dexamethasone; Goblet cells; Histomorphometry; Lysine; Stress; Supplementation

Introduction

The poultry production has been launched in Pakistan since 1960 and this industry fulfilled the daily protein requirements of people to a large extent. 1.5 million Population of our country depends upon poultry for earning. The requirement of meat is increasing rapidly in Pakistan because of increase in population [1]. For the improvement of production and the gut health of broilers, the antibiotic growth promoters (AGPs) are widely used [2]. However, this may cause the anti-microbial resistance (AMR) [3] and eventually severe health issues in humans [4]. According to reports, the regular use of antibiotics in poultry may cause the fetal casualties and serious health complications in humans [5]. There is excessive requirement of effective replacement of the AGPs in the poultry feed. To tackle this issue, the researchers are searching for healthy growth promoters to improve growth performance and carcass yield of birds. Generally, the stress is considered as a factor, which leads to disruption of the homeostasis of animals as well as birds by disturbing the stable internal equilibrium of body [6]. Every type of stress causes the molecular and the cellular level changes; decline the health and the productivity. It disrupts the expression of the tight junction proteins of intestine leading to leaky gut and reduced absorptive area of intestine [7]. Many scientists have used dexamethasone to induce physiological stress in animals during experiments. Dexamethasone restrains the growth in broilers and the degree of restrain elevated with increased dose ($P<0.05$) [8]. It mimics the adverse effects of glucocorticoids that suppress the immunity and induce oxidative stress [8].

Probiotics are non-pathogenic normal microbes, which are inhabitants of intestinal micro-flora. They are beneficial for birds by balancing intestinal microbiota; inhibiting fast multiplication of the pathogens [9]. They activate the immune system of host by alerting it against any invasion [10]. Probiotics are now a days known as potential antibiotic substitutes because of their positive influence on the performance of birds as well as their security against diseases [11]. *Bacillus subtilis* is a probiotic that not only improves performance and the immunity [12] but also elevates fiber digestion and enzymatic activity [13] in birds reared under stress. Probiotics of genus Lactobacillus exhibit competitive inhibiting effect; produce the organic acids that lower the pH in crop which finally suppresses colonization of the pathogens in digestive system [14]. The protein diets are considered as the major part of the muscle tissues [15]. Muscles are mainly composed of proteins; amino acids which forms carcass. To get maximum carcass yield, proper formulation of diet for birds with adequate amount of amino acids is crucial. It is essential to fight against negative effects on feathering, locomotion, growth performance and immunity [16]. For poultry diet, the lysine is known as second limiting amino acid because it is essential for the formation of proteins by ideal protein concept (IPC) [17]. Lysine is effective in increasing body weight and breast yield [8]. Supplementation of lysine improves the morphology of intestine [18] like hyperactive cell-genesis, villus height and crypt depth [19]. According to our knowledge, a negligible quantity of work has been done on the combined effects of lysine and probiotics in the poultry field. That is why, a study was planned to determine the impact of lysine along with probiotics (*Bacillus subtilis*) supplementation against dexamethasone-induced stress in broilers. The effects of the supplementation were evaluated on the basis of jejunum and immune organs histomorphometry in dexamethasone-induced stressed broilers.

Materials and Methods

Experimental birds & dietary supplementation

For the experiment, 336 day-old broiler chicks were up brought for nearly forty-two days in poultry experiment station of the faculty of animal husbandry and veterinary sciences, Sindh agriculture university, Tandojam. In this experiment, all the procedures including bird care as well as slaughtering were performed after approval of the Institutional Ethical Committee, Sindh Agriculture University,

Tandojam, under Reference Number 493. They were managed under the protocol [20] and were properly vaccinated according to the protocol adopted locally. The broilers were attributed to seven groups on twenty-first day of experimentation. The groups were Negative control, Positive control, DS+P @ 100mg/Kg d, DS+L @ 5g/g d, DS+P @ 100mg/Kg d+L @ 5g/Kg d, DS+L @11g/Kg d, DS+P @ 100mg/Kg d+L@11g/g d. The supplements were added to the basal diet (Table 1 & 2). To cause stress, dexamethasone was offered to all groups of birds other than negative control by drinking water (@ 3mg/L) daily from 21st to 28th day of their life [21].

Table 1. Composition of starter basal diet (1-21 days)

Materials	Quantity (%)
Corn	57
Wheat Bran	2.9
Canola	6
Rape Seed	4
Soya Bean	22
APC	3
Limestone	1.35
MCP	1.15
Salt	0.19
Soda	0.13
Lysine Sulphate	0.7
Methionine	0.31
Threonine	0.16
Isoleucine	0.11
Poultry Oil	0.5
Premix	0.5

Table 2. Composition of the grower (22-35 days) and finisher (36-42 days) diets

Grower diet	Quantity	Finisher diet	Quantity
Materials	**% age**	**Materials**	**% age**
Corn	55	Corn	53
Wheat Bran	1	Safflower Seed	5
Canola	6	Wheat Bran	1
Rape Seed	6	Canola	4
Soya Bean	22	Rape Seed	6
APC	4.5	Soya Bean	22
Limestone	1.15	APC	4.5
MCP	0.92	Limestone	1.15
Salt	0.14	MCP	0.74
Soda	0.13	Salt	0.15
Lysine Sulphate	0.47	Soda	0.13
Methionine	0.23	Lysine Sulphate	0.39
Threonine	0.08	Methionine	0.24
Isoleucine	0.02	Threonine	0.11
Poultry Oil	1.86	Isoleucine	0.05
Premix	0.5	Valine	0.04
		Poultry Oil	1
		Premix	0.5

Histo- morphometric study

On the closure of study, a pair of broilers from a replicate was taken randomly to kill through cervical dislocation. From jejunum, a few segments of 5 cm each were separated; by distal portion of the duodenal loop up to the Meckel's diverticulum. They were splashed with normal saline, fixed in formaline solution (10%) and dehydrated. After embedding, these tissues were sectioned (5-7µm) by a microtome. Mounting the tissues on slides, they were stained by H&E method. Whereas, some slides were stained for the identification of goblet cells by AB-PAS (Alcian Blue Periodic Acid Schiff) [22]. By the commercial programmed (Prog Res® 2.1.1) Capture Camera Control Software, histomorphometry was done. The measurements from 5 villi (with intact lamina propria) were taken and their average was calculated for record; height of villus, width of villus, crypt depth, lamina propria thickness, muscularis mucosa thickness as well as the thickness of muscular externa [23]. Height of villus (µm) was calibrated taken by top of the villus upto villus-crypt junction, while crypt depth in µm taken from the depth of the invagination among the villi that are adjacent. Surface area of the villus (µm²) was calibrates by; $(2\pi) \times (VW/2) \times (VL)$ [24].

For caecal tonsils; height, the width, number and the area of 5 lymphatic nodules were calibrated from 3 microscopic sections (stained by H&E) randomly and average was noted. Area of the lymphatic nodule by; (NL) x (NW), while NL is the length of nodule and NW is the width of nodule. For the bursa of fabricius; height, width, number and the area of the lymphatic follicle were calibrated from 3 microscopic sections. Lymphatic follicular area was measured by; (FW) x (FL), while FW is the width of follicle and FL is the length of follicle [24].

Statistical analysis

The data was analyzed statistically by the One Way Analysis of Variance technique (ANOVA) in a completely randomized design. The means of the treatments were compared by Tukey's test [25] with the significant level at $P<0.05$.

Results

How the supplementation of probiotics (*B. subtilis*) and lysine affects the histo-morphology of jejunum, the cecal tonsils and the histochemistry of the goblet cells of chickens under dexamethasone stress have been represented (Table 3). In the jejunum, the histo-morphometric measurements showed that the villus height, villus width, crypt depth, villus surface area, lamina propria thickness, muscularis mucosa thickness and thickness of muscularis externa were in the similar pattern among groups of experimental birds. These values were the lowest in positive control group followed by DS-Pro group and the DS-L (5g/Kg diet) group. Whereas these values were the highest in DS- Pro- L (11g/Kg diet) group followed by DS- L (11g/Kg diet) group and the DS-Pro- L (5g/Kg diet) ($P<0.05$). The villus height to crypt depth ratio depicted a different pattern i.e. opposite to the above one ($P<0.05$).

The effect of lysine and probiotics on the goblet cell histochemistry & the number of intraepithelial lymphocytes in the jejunum of broiler chickens has been introduced (Table 4). The count of all the three types (acidic, mixed and total) of goblet cells in the jejunum depicted same pattern. They were the lowest in positive control group followed by DS-Pro group and the DS-L (5g/Kg diet) group while these values were highest in DS- Pro- L (11g/Kg diet) group followed by DS- L (11g/Kg diet) group and the DS-Pro- L (5g/Kg diet) group ($P<0.05$). The intra-epithelial lymphocytes' number was found to be the lowest in positive control group followed by DS-Pro group and the DS-L

(5g/Kg diet) group while these values were highest in DS- Pro- L (11g/Kg diet) group followed by DS- L (11g/Kg diet) group and the DS-Pro- L (5g/Kg diet) group (P<0.05). The parameters of caecal tonsils and that of bursa of fabricius; LLN, WLN and ALN were noted to be the lowest in the positive control group followed by DS-Pro and DS-L (5g/Kg diet) groups (Table 5). Whereas these were the highest in DS- Pro- L (11g/Kg diet) group followed by DS- L (11g/Kg diet) group and the DS-Pro- L (5g/Kg diet) group. Number of lymphatic nodules of both organs were the highest in the positive control group and these were the lowest in DS- Pro- L (11g/Kg diet) group followed by DS- L (11g/Kg diet) group and the DS-Pro- L (5g/Kg diet) group (P<0.05).

Table 3. Effects of lysine and probiotics (*Bacillus subtilis*) on the Jejunal histo-morphology of broiler chickens under dexamethasone-induced stress

GROUPS									
Parameters	**Negative Control**	**Positive Control**	**DS+ Probiotic**	**DS+ Lysine (5g/Kg diet)**	**DS+ Probiotic+ Lysine (5g/Kg diet)**	**DS+ Lysine (11g/Kg diet)**	**DS+ Probiotic + Lysine (11g/Kg diet)**	**SEM**	**P-Value**
VH (µm)	757^{b}	588^{d}	710^{c}	710^{c}	740^{b}	740^{b}	800^{a}	9.81	<0.001
VW (µm)	93^{a}	49^{c}	67^{b}	67^{b}	80^{ab}	81^{ab}	94^{a}	9.9	<0.001
CD (µm)	130^{ab}	82^{c}	110^{b}	111^{b}	121^{ab}	118^{b}	141^{a}	12.18	<0.001
VH: CD	5.86^{ab}	6.93^{a}	6.52^{ab}	6.52^{ab}	6.14^{ab}	6.29^{ab}	5.75^{b}	0.64	<0.02
VSA (mm²)	0.22^{ab}	0.089^{d}	0.149^{c}	0.15^{c}	0.18^{bc}	0.18^{b}	0.23^{a}	0.022	<0.001
LPT (µm)	51^{ab}	23^{c}	41^{bc}	42^{bc}	54^{ab}	55^{ab}	67^{a}	13.51	<0.001
MMT (µm)	31^{a}	16^{b}	29^{a}	30^{a}	30^{a}	31^{a}	33^{a}	5.68	<0.001
MET (µm)	123^{ab}	93^{c}	94^{c}	95^{bc}	104^{bc}	105^{bc}	147^{a}	15.99	<0.001

Values are presented as Means ± SEM, a-e different superscripts significantly differ (P<0.05) in a row
DS: Dexamethasone stress; VH: villus height; CD: Crypt depth; VSA: villus surface area; VW: villus width; LPT: lamina propria thickness; MMT: muscularis mucosa thickness; MET: muscularis externa thickness

Table 4. Effects of lysine and probiotics (*Bacillus subtilis*) on the goblet cell histochemistry & the no. of Intraepithelial lymphocytes in the jejunum of broiler under dexamethasone-induced stress

GROUPS									
Cell type	**NC**	**PC**	**DS+P**	**DS+L (5g/Kg d)**	**DS+P+ L (5g/Kg d)**	**DS+L (11g/Kg d)**	**DS+P+ L(11g/Kg d)**	**SEM**	**P-Value**
Acidic Goblet Cells	173^{c}	104^{d}	187^{b}	187^{b}	189^{b}	189^{b}	202^{a}	2	<0.001
Mixed Goblet Cells	36^{c}	31^{d}	41^{b}	41^{b}	41^{b}	41^{b}	56^{a}	2	<0.001
Total Goblet Cells	209^{c}	135^{d}	228^{b}	228^{b}	230^{b}	230^{b}	259^{a}	3	<0.001
Intra epithelial lymphocytes	69^{bc}	53^{d}	66^{c}	67^{bc}	71^{abc}	72^{ab}	76^{a}	3	<0.001

Values are presented as Means ± SEM, a-e different superscripts significantly differ (P<0.05) in a row.
NC: Negative control; PC: Positive control; DS: Dexamethasone stress; P: Probiotic; L: Lysine; d: diet;
LLN; length of Lymphatic nodule: WLN; width of Lymphatic nodule: ALN; area of Lymphatic nodule: NLN; number of Lymphatic nodules

Table 5. Effects of lysine and probiotics (*Bacillus subtilis*) on the immune organs histo-morphometry of the broiler chickens under dexamethasone-induced stress

GROUPS									
Parameters	**NC**	**PC**	**DS-P**	**DS-L (5g/Kg d)**	**DS-P- L (5g/Kg d)**	**DS- L (11g/Kg d)**	**DS- P-L(11g/Kg d)**	**SEM**	**P-Value**
Caecal Tonsil									
LLN(mm)	121^{a}	93^{d}	93.86^{cd}	94.29^{bcd}	99^{b}	98.571^{bc}	121.143^{a}	2.91	<0.001
WLN(mm)	80.13^{a}	41.336^{c}	48.731^{b}	48.719^{b}	51.67^{b}	51.73^{b}	81.76^{a}	3.04	<0.001
ALN(mm^2)	9695^{a}	3842.2^{c}	4536.6^{b}	4589^{b}	5119^{b}	5099^{b}	9834^{a}	359	<0.001
NLN/mm^2	2.714^{b}	6^{a}	4.286^{ab}	4.286^{ab}	4.143^{ab}	4.143^{ab}	2.714^{b}	1.13	<0.001
Bursa of fabricius									
LLN(mm)	0.43571^{b}	0.25143^{e}	0.29857^{d}	0.30143^{d}	0.35000^{c}	0.35286^{c}	0.47000^{a}	0.0193	<0.001
WLN(mm)	0.22571^{a}	0.14429^{b}	0.14429^{b}	0.14571^{b}	0.17000^{b}	0.17000^{b}	0.2200^{a}	0.02	<0.001
ALN(mm^2)	0.09429^{a}	0.03143^{d}	0.03714^{d}	0.03857^{cd}	0.05429^{bc}	0.05714^{b}	0.09857^{a}	0.007	<0.001
NLN/mm^2	11.286^{c}	19.143^{a}	17.429^{ab}	17.857^{ab}	16.143^{b}	16.286^{b}	12.429^{c}	1.62	<0.001

Values are presented as Means ± SEM, a-e different superscripts significantly differ (P<0.05) in a row.
NC: Negative control; PC: Positive control; DS: Dexamethasone stress; P: Probiotic; L: Lysine; d: diet;
LLN; length of Lymphatic nodule: WLN; width of Lymphatic nodule: ALN; area of Lymphatic nodule: NLN; number of Lymphatic nodules

Discussion

The intestinal microbiota plays a fundamental role to improve animal health and the product safety. When the count of pathogenic bacteria in gut decreases, it improves ability of the epithelial cells of villus to regenerate enhancing the absorptive ability of intestine [26]. The intestinal mucosa is majorly responsible for digestive and absorptive process of the nutrients that controls growth activities in the animals [17]. In this study, the histological parameters of jejunum and number of goblet cells, number of intra-epithelial lymphocytes as well as the cecal tonsils were taken under consideration. It was revealed that by addition of probiotics and two different levels; 5g/Kg diet and 11g/Kg diet of lysine showed encouraging outcomes; histo-morphological parameters under stress as compared to that of positive control group of birds. It was indicated that with the increase in length and width of the villi, the surface area also increases [27]. Elevated epithelial cells' turnover leads to longer villi, which is manifestation of more surface area and eventually increased absorption rate and healthy intestine [28]. The current outcomes found to be in line by the study, which depicts that due to supplementation with amino acids like lysine maximized the surface area of villi and ultimately more absorption of bile acids [29]. Increased ratio of villus height to the crypt depth bears direct correlation to the increased turnover of epithelial cells and the activated mitosis of cells [23]. More depth of villus crypt stimulated the cell yield and the villus regeneration [30]. Lamina propria wideness indicates that the health of gut is improved. Lamina propria holds dendritic cells that activate immune response to stimulate defense action against the infection. The elevated immune response fastens the motility of gut by regulating mucin production [31]. In the current study, the lamina propria thickness was significantly high for treated group i.e. DS-Pro- L (11g/Kg diet). Supplementation of birds with probiotic and lysine was effective and could affect positively the crypt depth, number of cells and height of villi [18]. Deeper crypts might be due to the normal sloughing of cells; enhancing the demand for speedy turnover of villus cells in intestine [30]. Goblet cells are the part of jejunal epithelium that consists of

glycoproteins in the form of the mucin. These are significant element of the innate system of immunity at gut, which produce a layer of mucin. Mucin is accountable for the protection, the lubrication and the transport of luminal contents among the epithelial lining **[32, 33]**. The types of goblet cells are the acidic, mixed and the total ones. Acidic cells produce mucin that protects the intestine from pathogens and the neutral mucin of mixed cells facilitate the movement of digesta due to being less viscus **[34]**. The goblet cells arranged among columnar cells of intestine are responsible for mucosal resistance by production of mucilage that controls immunity **[17]**. In the present study, all three types of the goblet cells in jejunum boosted in number in treated groups gradually from DS-Probiotic to DS- Pro- L (11g/Kg diet). It indicates clearly that height and width of villi as well as number of goblet cells has significantly improved positively due to supplementation of lysine and *Bacillus subtilis* making efficient digestibility.

Gut associated lymphoid tissue (GALT) produce invulnerability against the systematic as well as the local pathogens **[35]**. Caecal tonsil is the largest gut associated lymphoid tissue in avian with accumulation of 45.7 % of the lymphoid nodules **[36]**. The caecal tonsils are a source of immunity in birds against infectious diseases like Newcastle disease and Avian Influenza **[37]**. In this consideration, the amplified length, width, surface area and the net count of lymphatic nodules in the caecal tonsils in the jejunum were noted in treated groups gradually from DS-Probiotic to DS-Pro- L (11g/Kg diet). This indicates the immune-stimulatory effect of supplementation of probiotic and lysine on GALT. The elevated morphometric calibration of the lymphatic nodules in DS- Pro- L (11g/Kg diet) was noted that is supportive in producing interaction between the immune cells and the microorganisms. It may develop higher antigen presentation and more antibody production. The increased area of bursal follicles may be due to the improved lymphocyte concentration of bursa of fabricius **[38]**. Similarly, it was seen that the supplementation with a mixture of yeast and probiotics elevated the lymphocyte concentration of lymphoid organs **[39]**. In probiotics supplemented group, the increase in weight of lymphoid organ can be linked to increase in immune cells and lymphoid follicle number **[40, 41]**. The conclusion of this study indicates that the combination of probiotics and lysine at the dose of 11g/Kg of diet not only improved the jejunal health by ameliorating microbial microarchitecture as well as more cellular count, along with noticeable immunomodulatory impact but also covered the negative effects of stress in broilers.

Conclusion

In this experiment, the most improved impact of supplementation of probiotic along with lysine (11g/ Kg diet) on the jejunal and immune organs histomorphology in stressed broiler chickens was observed. Thus, the use of probiotics (*Bacillus subtilis*) and lysine in combination might be helpful for the broilers reared commercially.

Authors' contributions

Designed this study: JA Gandahi & MG Shah, Headed the laboratory analysis: NH Kalhoro, Conducted the experiment and wrote down the manuscript: T Farooq, Helped in analysis of data: GM Lochi, SA Haadi.

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