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Effect of Probiotic *Bacillus clausii* on Production Parameters and Intestinal Histomorphology of Meat-Type Chicken

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Key words
***Bacillus clausii*, Gut health, Villus surface area, Broiler, Spore**

ABSTRACT

This study aimed to evaluate the effect of probiotic *Bacillus clausii* on performance parameters, the histomorphological architecture of the intestine, and its economic importance in broilers. For this purpose, 120 days old broiler chicks were randomly assigned to 4 groups comprised of group A (Control), group B (4×10^6spore @0.01 ml/L), group C (8×10^6 spore@0.02 ml/L) and group D (12×10^6spore@0.03 ml/L). Each group consisted of 3 replicates (n=10/replicate). The results revealed that water-supplemented *B. clausii* had no effect on daily feed intake in broiler chicks. However, higher ($p<0.05$) weight gain and improved FCR were found with the highest level of supplementation (0.03ml/L) of *B. clausii*. Histo-morphological results showed increased villus height, villus height: crypt depth, and villus surface area. Moreover, the supplementation of 12×10^6spore@0.03 ml/L also increased ($p<0.05$) the net profit due to increased production. In conclusion, the use of water-based supplemented *B. clausii* (12×10^6spore@0.03 ml/L) has a positive impact on production parameters and intestinal health in meat-type chicken.

INTRODUCTION

Antibiotics and antibiotic growth promoters are extensively used in poultry feed for therapeutic and prophylactic measures respectively to combat intestinal pathogens, improve poultry production parameters, decrease mortality, and prevention of various pathogenic diseases (Engberg *et al*., 2000; Waldroup *et al*., 1985). However, over extensive use of antibiotic drugs in poultry resulted in the risk of antibiotic residues in poultry meat and has increased the chance of antibiotic resistance against most pathogenic bacteria which is a major concern of public health (Laxminarayan *et al*., 2015). The use of antibiotics, antibiotics growth promoters, disinfectants, and pesticides in farmhouse chicken has developed the evolution of resistant strains of bacteria (Goldman, 2004). The antibiotic residues in poultry products (meat and eggs) have a direct influence on human health (Boerlin and Reid-Smith, 2008). In the presence of resistant bacteria, the therapeutic treatments of bacterial diseases might be unaffected or useless (Dale *et al*., 1992). Therefore, the use of antibiotics as a growth promoter in animal

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feedstuff has been strictly proscribed by the European Union since 2006. Now the tendency of antibiotic use in animal nutrition is diverting towards alternatives globally to avoid antimicrobial resistance (Rizzo *et al*., 2008). One such alternative is probiotics that are safe and used as growth promoters in poultry and animal nutrition for better performance and boosting immune status.

Probiotic means "for/in favor of life" in Greek (Ahmad and Ghoorchi, 2006). Probiotics can be better defined as mono or mixed culture of live microorganism, when run in suitable quantities they give beneficial effect to host health (FAO/WHO, 2002). Probiotics are reliable for the stimulation of enteric mucosal immunity. They boost protection against various toxins produced by pathogenic microbes and are also responsible for the enhanced production of digestive enzymes and vitamin B complexes (Walker and Duffy, 1998). Various Probiotics are used in poultry including *Bacillus*, *E. coli*, *Bifidobacterium, Enterococcus, Lactobacillus, Pediococcus, Lactococcus, Streptococcus* species, and yeast species (Fuller, 1992; Peric *et al*., 2010). Among these probiotics, bacillus has significant importance on account of spore production. Bacillus spp. spores or vegetative cells are more valuable owing to heat resistance and bile salt tolerance ability (Gilliland *et al*., 1984). Subspecies of *Bacillus* can survive and resist the acidic pH of the gastrointestinal tract (GIT) and can form colonies in the small intestine even in the presence of antibiotics (Duc *et al*., 2004). The German bacteriologist "Dieter Claus" discovered *Bacillus clausii* from the soil in 1995. It contributes similar characteristics like other species of *Bacillus*. The selection of *B. clausii* as a probiotic in animal/human spp. is based on some unique characteristics that include survival in higher pH, higher sodium chloride levels tolerance, and natural resistance to many antibiotics therapies (Jordan *et al*., 2015). *B. clausii* is an alkaliphilic that improves production parameters and produces various enzymes like high alkaline proteases and catalase. The alkaliphilic structural nature of *B. clausii* could be useful and helpful in curing and preventing various GIT disorders. The current study was designed to examine the effect of *B. clausii* supplementation on overall performances and duodenal histomorphology in broiler.

MATERIALS AND METHODS

The study was conducted in the Department of Poultry Science, Faculty of Animal Husbandry and Veterinary Sciences, The University of Agriculture Peshawar, Pakistan.

Study design and dietary plan

The study was conducted on 120 days old broiler chicks. The birds were reared according to standard managemental conditions on the wood-shaving floor for 35 days. Before the entry of the birds, the shed was thoroughly washed, cleaned, and disinfected. On day 1st, temperature and relative humidity (RH) were maintained at 95°F and 70 %, respectively. The temperature was gradually decreased by 5°F per week until it reached 70-75^{o}F and RH 65% on day 21. Birds were immunized according to standard vaccination protocol against new castle disease (ND), infectious bursa disease (IBD), and infectious bronchitis (IB) (Giambrone and Clay, 1986). Immediately after the arrival, the chicks were weighed and assigned to 4 groups group A (Control), group B (4×10^6spore @0.01 ml/L), group C (8×10^6 spore@0.02 ml/L), and group D (12×10^6spore@0.03 ml/L). Each group was comprised of 3 replicates (n=10/replicate). The birds were fed on a corn-based basal diet as a starter and grower (Table I) *ad-libitum* and they had free approached freshwater.

Table I. Diet composition of broiler starter and grower feed and calculated analysis.

Ingredient (%)	Starter	Grower
Corn	40.15	57.57
Rice broken	15	---
Guar meal	1.00	---
Sunflower meal	12.00	13.00
Rice polish	---	4.00
Rapeseed meal	5.00	7.60
Wheat bran	1.34	---
Canola meal	9.00	5.00
Soy meal	11.54	9.60
Molasses	2.00	---
Sodium chloride	0.21	0.21
Di-calcium phosphate	1.33	1.49
Sodium bicarbonate	0.03	0.065
DL-Methionine	0.10	0.12
L-Lysine	0.30	0.35
Vit-mineral premix*	1.00	1.00
Nutrient composition		
Calculated metabolisable energy (kcal.kg)	2750	2850
Crude protein (%)	19.6	18.5
Crude fibre (%)	6.05	6.35
Crude fat (%)	2.16	2.35
Dry matter (%)	87	88
Total ash (%)	5.77	5.40

*Vitamin mineral premix (each kg contained): retinol, 200,000 IU; tocopherol, 1072 IU; ascorbic acid, 26000 IU; cholecalciferol, 80,000 IU; thiamine, 11666 IU; menadione, 11,333 IU; pyridoxine, 33333 IU; niacin, 5,36,000 IU; folic acid, 13600 IU; riboflavin, 54,000 IU ; methylcobalamin, 223 IU; biotin, 1340 IU; Ca, 195 g; Mg, 6 g; Fe, 2,000 mg; Na, 18 g; Zn, 1,200 mg; K, 70 g; Mn, 1,200 mg; Cu, 400 mg; I, 40 mg, Co, 20 mg and Se, 8 mg

Performance parameters

Feed was offered with known quantity on daily basis. Feed refused was deducted from feed offered to get daily feed intake. Total feed intake was calculated at the end of the trial. Weight gain was measured on weekly basis by subtracting the initial weight from the final weight at the end of every week. The feed conversion ratio (FCR) was calculated as described by Shah *et al*. (2018) and Shuaib *et al*. (2021). For the histological study, three birds were randomly selected from each replicate on day 35 of the experiment. The birds were euthanized and a 3cm long segment from the duodenum was collected, cleaned from digesta with normal saline (0.9%), and preserved in neutral buffered formalin solution (Shah *et al*., 2019; Shuaib *et al*., 2022). The tissue samples were processed in graded series of alcohol and cleared with xylene through the paraffin embedding technique (Bancroft *et al*., 2013). Three non-serial sections of 5µm thickness from tissue sample were obtained through rotary Microtome (AEM 450 Amos Scientific, Australia). The microscopic sections were stained with Hematoxylin and Eosin (H & E) stains (Shah *et al*., 2020). Three non-serial microscopic sections of the duodenum were analyzed through a commercial program (Prog Res 21.1 Capture Prog Camera Control Software) at 40X. Fifteen well-defined villi from three microscopic sections were selected for villus height, villus width, and crypt depth and their average was considered as the final value. The villus surface area (VSA) was calculated according to the formula; (2 π) (VW/2) (VL) where VW is villus width and VL villus length or height (Khan *et al*., 2016). All the experimental chicks were closely observed for any clinical signs of illness, if any over there. Mortality was recorded and necropsy procedures were followed to know the possible cause of death.

Economics parameter

Economic was evaluated based on the basis of production cost, gross return and net return. Production cost include feed, vaccine, chicks, medications and probiotic cost. Gross return was based on live bird sell price. Net return was evaluated by subtraction of production cost from live bird weight sell price.

Statistical analysis

The data were analyzed through statistical Package Scientific Analysis System (SAS) 2010 using one-way ANOVA. The data were presented as Mean ± standard error. The post hoc test Tuckey's was used to find significance among groups. The level of significance was considered as $p<0.05$.

RESULTS

The feed intake results are presented in Figure 1 which revealed that there was no significant effect of water-supplemented *B. clausii* on feed intake in broilers. The effects of water-based supplementation of *B. clausii* on weight gain, FCR, and intestinal histomorphology of broiler chickens is presented in Tables II. The highest ($p<0.05$) weight gain was observed in *B. clausii* supplemented group D while the lowest weight gain was recorded in the control and B groups. Improved and lower ($p<0.05$) FCR was recorded in group D and control group compared to other groups. Water-based *B. clausii* supplementation in broilers significantly ($p<0.05$) altered villi architecture. Histo-morphological study of villi showed an increase ($p<0.05$) in the villus height, width, villus height to crypt depth ratio, and villus surface area in *B. clausii* supplemented group D as compared to other treatment groups while the crypt depth significantly ($p<0.05$) decreased in group D as compared to other groups. Water-based supplementation of *B. clausii* in broiler chicks did not affect the production cost of broiler chicks up to the marketed age as described in Figure 2. However, gross return and net profit were affected ($p<0.05$) by the use of probiotics in drinking water. The highest ($p<0.05$) gross return was noted in *B. clausii* supplemented group D. Similarly, net profit was observed higher ($p<0.05$) in group D compared to all groups. Mortality was recorded non significance among the experimental groups.

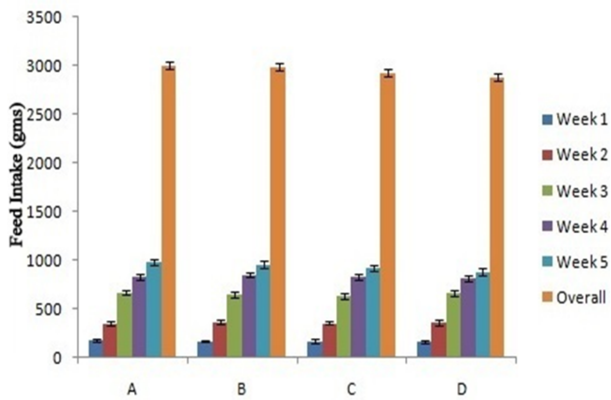

Fig. 1. Effect of *Bacillus clausii* on feed intake (g) of broiler chicken.

DISCUSSION

The current study evidences no significant decrease in feed intake. We did not find any relevant data regarding *B. clausii* effect on feed intake in broilers in the literature

Table II. Effect of *Bacillus clausii* on weight gain, feed conversion ratio and intestinal morphology in broiler (Mean±SE).

Parameters	Weeks	Groups				P. value
		A	B	C	D	
Weight gain(g)	1	108.0±1.52	111.0±3.78	121.0±3.78	118.6±2.60	0.06
	2	218.3±1.20^{c}	224.6±3.17^{cb}	230.0±2.08^{b}	242.3±1.85^{a}	0.01
	3	298.0±2.00^{b}	297.6±5.78^{b}	310.0±3.21^{b}	350.0±5.77^{a}	0.02
	4	378.6±4.66^{c}	381.6±4.40^{c}	403.3±4.25^{b}	447.0±4.04^{a}	0.01
	5	491.0±2.08^{b}	492.0±4.72^{b}	531.0±6.35^{a}	522.3±1.76^{a}	0.02
	Overall	1494±1.00^{c}	1507±10.11^{c}	1595.3±13.2^{b}	1680.3±5.78^{a}	0.01
FCR	1	1.61±0.05^{a}	1.51±0.04^{a}	1.55±0.03^{a}	1.37±0.07^{b}	0.03
	2	1.59±0.01^{ab}	1.63±0.01^{a}	1.54±0.01^{b}	1.49±0.01^{c}	0.01
	3	2.24±0.02^{a}	2.18±0.03^{a}	2.04±0.02^{b}	1.89±0.01^{c}	0.02
	4	2.19±0.06^{a}	2.23±0.03^{a}	2.06±0.01^{b}	1.83±0.02^{c}	0.01
	5	1.99±0.02^{a}	1.94±0.02^{a}	1.72±0.01^{b}	1.68±0.01^{b}	0.04
	Overall	2.01±0.02^{a}	1.98±0.01^{a}	1.83±0.01^{b}	1.71±0.00^{c}	0.03
VH (mm)	------	1.11±0.01^{b}	1.11±0.01^{b}	1.16±0.02^{b}	1.37±0.05^{a}	0.01
VW (mm)	------	0.08±0.01^{c}	0.10±0.01^{c}	0.14±0.02^{b}	0.20±0.01^{a}	0.02
CD (mm)	------	0.25±0.02^{a}	0.20±0.02^{ab}	0.23±0.01^{a}	0.18±0.01^{b}	0.03
VSA (mm)^{2}	------	0.27±0.01^{c}	0.35±0.01^{c}	0.51±0.03^{b}	0.86±0.02^{a}	0.01
(VH/CD)	------	4.44±0.42^{b}	5.55±0.34^{b}	5.04±0.67^{b}	7.61±0.40^{a}	0.04

Different superscripts with means in row are significantly different at P<0.05. FCR, feed conversion ratio; VH, villus height; VW, villus width; CD, crypt depth; VSA, villus surface area.

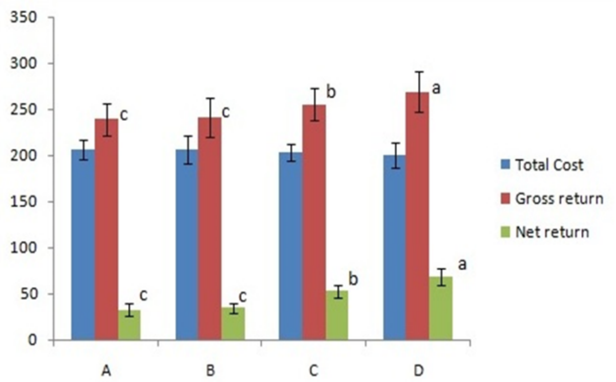

Fig. 2. Effect of *Bacillus clausii* on economics of broiler chicken. Different superscripts on bars presents significance among groups at p<0.05.

to compare our results. However, Upadhaya *et al.* (2019) reported that the inclusion of *B. subtilis* in broiler ration showed no apparent effect on daily feed intake. Similarly, Zhen *et al.* (2018) also documented that a diet supplemented with *Bacillus coagulans* in broiler chicks did not alter the feed intake in *Salmonella* challenged broilers. The same results through the use of *B. subtilis* spores in broilers-induced *Salmonella* infection were reported by Hayashi *et al.* (2018). The present study results are supported by the findings of Cheng-liang *et al.* (2018). They reported that dietary *Bacillus* spp. did not alter the mean feed intake in broiler chicks. The studies of Park and Kim (2014) and Salim *et al.* (2013) were in line with our findings that dietary *Bacillus* can improve the productive parameters of broiler chicks. The contradiction in these studies' results might be contributed to various factors like probiotic administration dose, animal age, diet composition, and feed formulation. *B. clausii* @ 0.03ml/L of water significantly improved body weight gain as compared to other treatment groups. This increase in body weight gain might be due to enhancement in nutrient digestibility through increased secretion of endogenous enzymes in the gastro-intestinal tract (GIT) by *B. clausii* (Wang and Gu, 2010). The current results were also supported by studies of Cartman *et al.* (2008) and Gu *et al.* (2015) which reported that *Bacillus* spp. augment some exogenous enzymes (amylase, protease, and lipase) and promote some unknown growth factors that cause fermentation in the gut that modulate gut histomorphology. The results of the current study were also similar to the statement of Zhen *et al.* (2018) who documented that supplementation of meat-type bird's ration with *Bacillus* spp. significantly improved weight gain. In the current study, *B. clausii* supplementation @ 0.03ml/L markedly improved the FCR. The improvement might be attributable to an increase in weight gain in the said group. No relevant literature is available on the use of *B. clausii* in broiler production to which the results may be compared. However, different strains of *Bacillus* were used as probiotics in broiler ration on different aspects. The results of these studies are consistent with the current study. Upadhaya *et al*. (2019) narrated that feed inclusion of *B. subtilis* in broiler ration significantly affects FCR and promotes the gut health of birds. Cheng-liang *et al*. (2018) documented that feed added *Bacillus* spp. caused the best FCR in meat-type birds. Zhen *et al*. (2018) also concluded research results that feed additive (probiotics) in broiler chicks at any level of supplementation has significant effects on the feed conversion ratio in broiler chicks by balancing the internal intestinal micro-flora, which reduced the pathogenic load and enhanced beneficial bacterial population. As per the economics of the study, it was found that water supplementation *B. clausii* had a significant effect on the economics of the broiler chicks. Group D which was supplemented with water-based probiotics @ 0.03ml/L increased the gross and net return, this increase in economics might be contributed to the beneficial effect of probiotics on significant feed utilization and conversion into weight in broiler chicks (Djordje *et al*., 2014). The existing literature regarding the other probiotic use and its effect on economics on broiler production provides evidence of significance to which the findings of the current study can be compared. The results of the present study regarding the total cost of broiler production agreed with studies of Araujo *et al*. (2019) and Patel *et al*. (2015). They documented that the cost of the production of broiler chicks did not alter with the use of probiotics in its ration. Present investigations are similar to the findings of Patel *et al*. (2015). They concluded that in broiler production, supplementation of probiotics significantly improved the gross and net return. The results of the present investigation are supported by findings of Djordje *et al*. (2014), who reported that feed-added probiotics significantly increased economic parameters (gross and net return). The studies of Anjum *et al*. (2005) and Sultan *et al*. (2006) also support the findings of the current study that feed-added probiotics in broiler ration result in profitable revenue (body weight gain).

The use of *B. clausii* supplementation in broiler chicks also affected intestinal histo-morphology. Broiler chicks supplemented @ 0.03ml/L of *B. clausii* modified histomorphological architecture of duodenal villi (height, width, crypt depth, villus height to crypt depth ratio, and surface area as compared to the control group. This improvement in villi status might be due to the beneficial effects of probiotics on GIT by increasing the digestive and absorptive area of the intestine which subsequently improves nutrient utilization and absorption (Caspary, 1992) as proved in our study. Moreover, probiotic has been reported for activating cell mitosis and inducing GIT epithelial cell proliferation which would be the cause of the increase in the villi status (Samanya and Yamauchi, 2002). Current study results are in agreement with the findings of Al-Baadani *et al*. (2016). He documented that broiler chicks fed with probiotics significantly improve the villus height/length, surface area, and health as compared to antibiotics-treated groups. Previous studies of Bai *et al*. (2017) and Jayaraman *et al*. (2013) described that feed-added probiotics in broiler ration significantly increased the villi length and surface area. Studies conducted by Abdel-Raheem *et al*. (2012) and Sen *et al*. (2012) also support our present study findings by documenting that feed-added *Bacillus* spp. in meat-type birds significantly improve the ileum and jejunum villus height and health. The findings of Mongkol and Yamauchi (2002) are also parallel to the present findings. They reported that the use of probiotics in meat-type birds significantly affected the duodenal villus surface by protruded cell clusters, cell protuberances, and depressed blood ammonia concentration. The results of the current study are in agreement with the findings of Peric *et al*. (2010). He documented that the use of probiotics in diet brought marked changes in the morphological structure of the intestine by increasing the villi height and surface area which would be the cause of providing a large area for absorption.

CONCLUSION

The results of the present designed study revealed that probiotic *B. clausii* has a positive impact on the performance parameters including weight gain, feed conversion ratio as well as intestinal health. Hence, the probiotic *B. clausii* @ 0.03ml/L of water may be used in poultry for better production performance which will result in better economic returns.

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IRB approval

The experimental work was approved by the Advanced Studies and Research Board (ASRB) (No. 491LM/FAHVS dated 09/06/2022), The University of Agriculture, Peshawar, KP, Pakistan.

Ethical statement

The study was approved by the Ethical Review Committee of the Faculty of Animal Husbandry and Veterinary Sciences, The University of Agriculture, Peshawar, Pakistan. All measures were taken to minimize the pain and discomfort of birds during the conduction of this experiment.

Statement of conflict of interest

The authors have declared no conflict of interest.

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