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# Viability of Probiotics in Balancing Intestinal Flora and Effecting Histological Changes of Crop and Caecal Tissues of Broilers

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**Abstract:** The present research was conducted on Hubbard Isa Starbro broilers to evaluate the dynamics of probiotics relating to balanced growth of intestinal flora and histological reactions and changes within the crop and cecal tissues. A total of eighty day-old broiler chicks were divided into four groups of equal numbers as group A (Probiotics fed group vaccinated), B (Probiotics fed group nonvaccinated), C (Conventional fed group vaccinated) and D (Conventional fed group nonvaccinated). Group C and D were taken as control birds fed with commercial ration and groups A and B as experimental birds were fed with commercial ration with the addition of 2 g probiotics (Protexin® Boost)/10 L drinking water upto 6th week of age. The effect of probiotics with regard to clearing bacterial infections and regulating intestinal flora was evaluated by determining the TVC and TLC of the crop and cecum samples of probiotics and conventional fed groups at the 2nd, 4th and 6th week of age. The result revealed competitive antagonism. The result of the study evidenced that probiotic organisms inhibited some nonbeneficial pathogens by occupying intestinal wall space. It has been demonstrated that broilers fed with probiotics had a tendency to display pronounced intestinal histological changes such as active impetus in cell mitosis and increased nuclear size of cells, than the controls. It is obviously found from this research work that probiotics supplementation promoted significant influence on intestinal microbial balance related with pronounced intestinal histological changes.

**Key words:** Probiotics, intestinal flora, histological changes, broiler

## INTRODUCTION

Over the years the word probiotic has been used in several different ways. Fuller[1] gave a unique definition of probiotics as a live microbial feed supplement which beneficially affects the host animal by improving its intestinal microbial balance. The stable flora which develops in the intestine helps the animal to resist infections, particularly in the gastrointestinal tract. The phenomenon has been described by various authors and given the names as bacterial antagonism[2], bacterial interference[3], barrier effect[4], colonization resistance[5] and competitive exclusion[6]. Fuller[7] observed that the avian lactobacilli reduced *E. coli* population in the gut and they firmly adhered to the epithelial lining of the crop of chickens. These lactobacilli produced acid metabolites that lowered the crop pH and ultimately limited the growth of *E. coli*. Ecological studies by Fuller and Turvey[8] on lactobacillus flora associated with the intestinal wall demonstrated that the crop was the reservoir of the bacillus for maintaining the bacterial balance in the intestines. Despite several reports on probiotic feeding, information with respect to their effects on gut flora interrelated with histological changes of crop and cecal tissues of broilers is meager. The present study was, therefore, undertaken to determine the effects of feeding probiotics on the dynamics of gut flora causing histological alteration with the consequence of changes in cell structure commensurating with adequate bacterial adhesion.

## MATERIALS AND METHODS

**Experimental birds:** Day old Hubbard Isa Starbro broiler chicks obtained from the reputed sale centre of Kazi Farms Limited, Mymensingh, Bangladesh were employed in this experimental study. A total of 80 day-old broiler chicks were divided into two main groups, group I (Probiotics fed group) and group II (conventional fed group) at the onset of the experimental study. All birds belonging to

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group I and II were further divided into 4 subgroups as group A (Probiotics fed group vaccinated), B (Probiotics fed group nonvaccinated), C (Conventional fed group vaccinated) and D (Conventional fed group nonvaccinated) on 6th day of age. The birds were reared under strict hygienic care and management practices throughout the entire period of study.

**Feeding and management:** Commercially available standard poultry feed (Quality Feeds Ltd., Dhaka, Bangladesh) was used throughout the experimental study. The broiler chicks were fed with standard broiler starter, broiler grower and broiler finisher ration as formulated by Quality feeds Ltd., Dhaka, Bangladesh. As per instruction probiotics were added to drinking water at a level of 2 g/10 L water and was given to birds belonging to group A and B for 5 days in a week. The remaining two groups such as group C and D were kept as control without adding probiotics in drinking water.

**Protexin® Boost:** Protexin® Boost marketed by Novartis (Bangladesh) Limited and manufactured by Probiotics International Limited, UK is a product containing nine strains of variable organisms namely *Lactobacillus plantarum*, *Lactobacillus bulgaricus*, *Lactobacillus acidophilus*, *Lactobacillus rhamnosus*, *Bifidobacterium bifidum*, *Streptococcus thermophilus*, *Enterococcus faecium*, *Aspergillus oryzae* and *Candida pintolopessi*.

**Immunization:** Birds were vaccinated against Baby Chick Ranikhet Disease by BCRDV (Livestock Research Institute, Mohakhali, Dhaka, Bangladesh) and Gumboro disease by Bur-706 vaccine (Merial, France) as per schedule of manufacturer.

**Enumeration of Total Viable Count (TVC) and Total Lactobacillus Count (TLC):** Probiotic fed birds were sacrificed, dressed and their crops and caeca were obtained aseptically. To enumearte the TVC and TLC of gut contents and to undertake histological studies a total of 6 birds of 2nd week of age and 7 birds each of 4th and 6th weeks of age from each group were selected for this purpose. To perform this study, portions of crop and cecum with their contents were obtained aseptically using a sterile scalpel and forceps. These portions were homogenized uniformly in a blender. The total viable bacterial count of homogenized samples was determined as per recommendation of International Organization for Standardization[9]. The results of the total bacterial count were expressed as the number of organism of colony forming units per gram (CFU/g) of crop and cecum samples. For the determination of total lactobacillus count the procedures of sampling, dilution and streaking were similar to those followed in total viable bacterial count. Only in case of lactobacillus count, MRS (de Man, Rogosa and Sharpe) agar (HiMedia Laboratories Limited, Mumbai, India) was used. The calculation for TLC was similar to that of total viable count.

**Cultural and biochemical examination of bacterial isolates obtained from samples:** The quantitation of bacteria in crop and cecum samples was done according to the standard method[10] and ISO[9]. The examination followed detail study of cultural characteristic including colony formation, staining reactions and biochemical properties. In order to find out different types of microorganisms in crop and cecum samples different kinds of bacterial colonies were isolated in pure culture and identified as per instruction of Cowan[11].

**Preparation of samples for histological studies:** For histological studies, the portions of crop and cecum were collected and fixed in the Bouin's fluid. The tissues were dehydrated in the graded alcohol, cleared in xylene, embedded in paraffin and finally the sections were cut at 6 micron thickness by rotary microtome (Model 820, USA). The sections so prepared were stained with standard Hematoxylin and Eosin (H and E) method[12] and few sections were stained by Gram's staining technique.

**Statistical analysis:** The data on Total Viable Count (TVC) and Total Lactobacillus Count (TLC) obtained from the bacteriological examination of crop and cecum samples of broilers were analyzed in Completely Randomized Design (CRD) using computer package MSTAT-C [13].

## RESULTS

**Total Viable Count (TVC):** The values of TVC obtained from crop samples of probiotics fed group (vaccinated and non-vaccinated) at the 2nd, 4th and 6th week of age were log 8.40 and log 8.36; log 8.19 and log 8.11; log 7.98 and log 7.81, respectively and log 8.41 and log 8.44; log 8.20 and log 8.22; log 8.27 and log 8.27 for conventional fed group (vaccinated and non-vaccinated), respectively (Table 1a and b). While the values of TVC obtained from cecum samples of probiotics fed group (vaccinated and non-vaccinated) at the 2nd, 4th and 6th week of age were log 7.98 and log 8.10; log 8.06 and log 8.04; log 6.90 and log 8.06, respectively and log 8.13 and log 8.16; log 8.15 and log 8.24; log 8.29 and log 8.22 for conventional fed group (vaccinated and non-vaccinated), respectively (Table 1a and b).

Table 1a: Occurrence of TVC and TLC in crop and cecum of probiotics fed broilers at the 2nd, 4th and 6th week of age

| | Probiotics Fed group (PFG) | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 2nd week | | | | 4th week | | | | 6th week | | | |
| | A | | B | | A | | B | | A | | B | |
| Parameters | Crop | Cecum | Crop | Cecum | Crop | Cecum | Crop | Cecum | Crop | Cecum | Crop | Cecum |
| TVC | 8.40 | 7.98 | 8.36 | 8.10 | 8.19 | 8.06 | 8.11 | 8.04 | 7.98 | 6.90 | 7.81 | 8.06 |
| TLC | 7.93 | 7.40 | 7.81 | 7.78 | 7.95 | 7.91 | 7.98 | 7.98 | 7.93 | 6.88 | 7.78 | 8.02 |
| Ratio of TVC and TLC | 1: 0.94 | 1: 0.93 | 1: 0.93 | 1: 0.96 | 1: 0.97 | 1: 0.98 | 1: 0.98 | 1: 0.992 | 1: 0.993 | 1: 0.995 | 1: 0.995 | 1: 0.995 |

Table 1b: Occurrence of TVC and TLC in crop and cecum of conventional fed broilers at the 2nd, 4th and 6th week of age

| | Conventional Fed group (CFG) | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 2nd week | | | | 4th week | | | | 6th week | | | |
| | C | | D | | C | | D | | C | | D | |
| Parameters | Crop | Cecum | Crop | Cecum | Crop | Cecum | Crop | Cecum | Crop | Cecum | Crop | Cecum |
| TVC | 8.41 | 8.13 | 8.44 | 8.16 | 8.20 | 8.15 | 8.22 | 8.24 | 8.27 | 8.29 | 8.27 | 8.22 |
| TLC | 3.08 | 3.20 | 3.40 | 3.30 | 3.51 | 3.40 | 3.54 | 3.65 | 3.54 | 3.48 | 3.48 | 3.40 |
| Ratio of TVC and TLC | 1: 0.37 | 1: 0.39 | 1: 0.40 | 1: 0.40 | 1: 0.43 | 1: 0.42 | 1: 0.43 | 1: 0.44 | 1: 0.43 | 1: 0.42 | 1: 0.42 | 1: 0.41 |

All counts are expressed in logarithms, TVC: Total Viable Count, TLC: Total Lactobacillus Count, A and C: Vaccinated birds, B and D: Nonvaccinated birds

Table 2: Frequency distribution of bacterial flora isolated from crop and cecum samples

| | Number of isolates | | Percentages (%) | |
|---|---|---|---|---|
| Name of isolates | PFG (A+B) | CFG (C+D) | PFG (A+B) | CFG (C+D) |
| *Lactobacillus* sp | 93 | 36 | 83.04 | 18.37 |
| *Staphylococcus* sp | 6 | 34 | 5.36 | 17.35 |
| *Streptococcus* sp | 5 | 30 | 4.46 | 15.31 |
| *Bacillus* sp | 5 | 28 | 4.46 | 14.29 |
| *Escherichia coli* | 3 | 45 | 2.68 | 22.96 |
| *Micrococcus* sp | 0 | 8 | 0.00 | 4.08 |
| *Salmonella* sp | 0 | 7 | 0.00 | 3.57 |
| *Proteus* sp | 0 | 5 | 0.00 | 2.55 |
| Others (Unidentified) | 0 | 3 | 0.00 | 1.53 |
| Total: | 112 | 196 | 100.00 | 100.00 |

PFG : Probiotics Fed group, A : Vaccinated birds,
B : Nonvaccinated birds, CFG : Conventional Fed group,
C : Vaccinated birds, D : Nonvaccinated birds

**Total Lactobacillus Count (TLC):** The TLC obtained from crop samples of probiotics fed group (vaccinated and non-vaccinated) at the 2nd, 4th and 6th week of age were log 7.93 and log 7.81; log 7.95 and log 7.98; log 7.93 and log 7.78, respectively and log 3.08 and log 3.40; log 3.51 and log 3.54; log 3.54 and log 3.48 for conventional fed group (vaccinated and non-vaccinated), respectively (Table 1a and b). On the other hand, the TLC obtained from cecum samples of probiotics fed group (vaccinated and non-vaccinated) were log 7.40 and log 7.78; log 7.91 and log 7.98; log 6.88 and log 8.02, respectively and log 3.20 and log 3.30; log 3.40 and log 3.65; log 3.48 and log 3.40 for conventional fed group (vaccinated and non-vaccinated), respectively (Table 1a and b).

**Ratio of TVC and TLC:** The ratio of TVC and TLC obtained from the bacteriological examination of crop samples of probiotics fed group (vaccinated and non-vaccinated ) at the 2nd, 4th and 6th week of age were 1:0.94 and 1: 0.93; 1: 0.97 and 1: 0.98; 1: 0.99 and 1: 0.995, respectively and 1: 0.37 and 1: 0.40; 1:0.43 and 1:0.43; 1:0.43 and 1: 0.42 for conventional fed group (vaccinated and nonvaccinated), respectively (Table 1a and b). On the other hand, the ratio of TVC and TLC obtained from the bacteriological examination of cecum samples of probiotics fed group (vaccinated and nonvaccinated) at the 2nd, 4th and 6th week of age were 1:0.93 and 1:0.96; 1:0.98 and 1:0.99; 1:0.995 and 1:0.995, respectively and 1:0.39 and 1:0.40; 1:0.42 and 1:0.44; 1:0.42 and 1:0.41 for conventional fed group (vaccinated and nonvaccinated), respectively (Table 1a and b).

**Frequency distribution of different microorganisms:** Percentage distribution of selected bacteria present in crop and cecum samples of probiotics fed broilers are presented in Table 2 which revealed *Lactobacilli* 83.04%, *Staphylococci* 5.36%, *Streptococci* 4.46%, *Bacilli* 4.46% and *Escherichia coli* 2.68%. On the other hand, percentage distribution of selected bacteria found in crop and cecum samples of conventional fed broilers are presented in Table 2. These microbes were *Escherichia coli* 22.96%, *Lactobacilli* 18.37%, *Staphylococci* 17.35%, *Streptococci* 15.31%, *Bacilli* 14.29%, *Micrococci* 4.08%, *Salmonella* 3.57%, *Proteus* 2.55% and others 1.53% (Table 2).

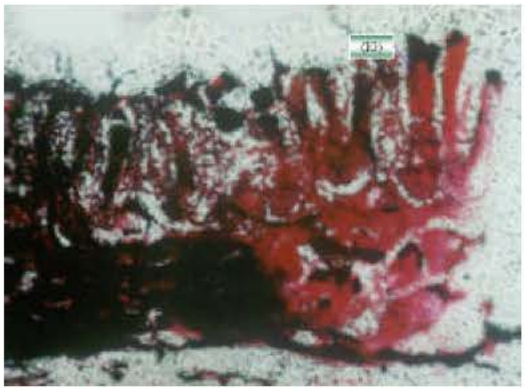

Fig. 1a: Photomicrograph of section of cecum of vaccinated probiotics fed broilers showing increased length of the Intestinal Glands (IG), (×83, H and E)

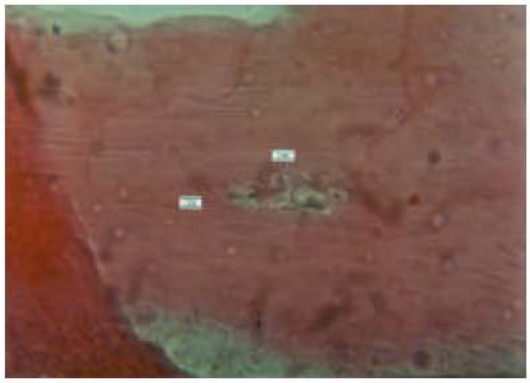

Fig. 2: Photomicrograph of section of crop tissues of vaccinated probiotics fed broilers showing active Cell Mitosis (CM) in the Submucosa (SM), (×83, H and E)

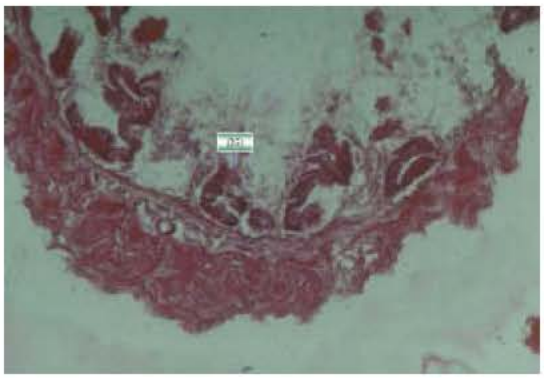

Fig. 1b: Photomicrograph of section of cecum of vaccinated conventional fed broilers showing usual length of the Intestinal Glands (IG), (×83, H and E)

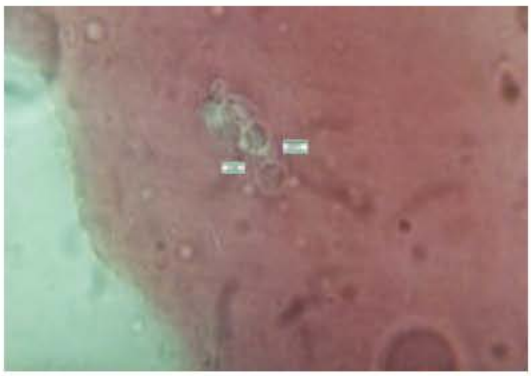

Fig. 3: Photomicrograph of section of crop tissues of vaccinated probiotics fed broilers showing Increased Nuclear Size (INS) of cells in the Submucosa (SM), (×830, H and E)

**Histological study:** Bacteria associated with the intestinal wall could be seen in the sections of the crop and cecum samples of probiotics fed broilers (vaccinated and nonvaccinated). These samples were obtained on the 2nd, 4th and 6th week of age. In the crop section adequate bacterial adhesion on the epithelial layer was evidenced. These bacteria were Gram positive and rod to coccoid shape in morphology. Huge mass of aggregation of rod and coccoid shaped bacteria in the submucosa of crop of probiotics fed broilers was demonstrated. In the cecum aggregation of bacteria was seen adjacent to the submucosa. This layer contained mainly the Gram positive rods with only occasional Gram positive cocci. The length of the intestinal glands of cecum of probiotics fed broilers as recorded was found to be greater than the conventional fed broilers (Fig. 1a and b). Pronounced cell mitosis and increase of nuclear size were particularly identified in the submucosa of crop of probiotics fed broilers, while these characteristics were not consistently marked in the conventional fed broilers (Fig. 2 and 3).

## DISCUSSION

The total viable bacteria in crop and cecum samples of probiotics fed birds during the course of the experiment clearly indicated the promotion of defensive mechanism. The development of this status has helped to reduce the nonbeneficial bacteria. The beneficial effect of probiotics was similarly evidenced by Singh *et al.*[14] who found that the microbial counts tended to decrease with introduction of probiotics in feed. In another study of Shoeib and Madian[15] it was demonstrated also that the addition of biogen at a rate of

2 g $kg^{-1}$ diet as a probiotic was superior to pronifer in reducing the total bacterial count and *E. coli* count.

The recovery of TLC obtained from the crop and cecum samples of probiotics and conventional fed broilers were found always on the increase in probiotics fed broilers as compared to conventional fed broilers at the 2nd, 4th and 6th weeks of age. This result indicates an important characteristic of a good probiotic. The capacity to survive and metabolize in the gut reflects that there exits a balance of beneficial and nonbeneficial bacteria, as the result shows that the probiotic fed birds performed to its maximum efficiency. Fuller[7], Canalli *et al.*[16], Mahajan *et al.*[17] and Li *et al.*[18] reported the beneficial effect of probiotics. In their study, the reduction in number of *E. coli* of the intestinal caecal contents could be due to inhibitor produced in the crop being carried into small intestine or due to locally synthesized inhibitor. The authors stated that faecal coliforms decreased significantly in the intestine of chickens given probiotic (100 g $t^{-1}$) compared with control. In another study, Endo and Nakano[19] indicated that the probiotic decreased the number of Enterobacteriaceae (*E. coli* and *Salmonella*) in the caeca. Ko *et al.*[20] analogously evidenced inverse relationship between *Lactobacillus* and *E. coli*. They demonstrated a decrease in the coliform bacteria population in the cecum of the broilers fed diets with 0.1 and 0.3% probiotics.

Evidently from the ratio of TVC and TLC obtained from the bacteriological examination of crop and cecum samples of probiotics and conventional fed broilers (vaccinated and non-vaccination) it was revealed that the values of TLC were found always on the increase in probiotics fed broilers as compared to conventional fed broilers at the 2nd, 4th and 6th week of age. The present research suggests that vaccination of broilers has no influence on the propagation of intestinal microflora. The present findings more or less support the views of Tarakanov *et al.*[21] who demonstrated that the number of hemolytic bacteria in the crop and cecum was 7 times lower in the probiotic fed experimental broilers compared with the control group. They further indicated that number of *Escherichia coli* got reduced by 37% and *Salmonella* by 2 times in the experimental broilers, whereas intestinal count of amylolytic and lactobacillus increased.

It is noteworthy that *Micrococci*, *Salmonella*, *Proteus* and others unidentified were not found in crop and cecum samples of probiotics fed broilers. In these studies *Lactobacilli* showed the highest percentage of occurrence. Next to *Lactobacilli*, *Staphylococci* ranked the second position, *Streptococci* and *Bacilli* secured the third position and *Escherichia coli* obtained the fourth position. Soerjadi *et al.*[22] evidenced in their study that *Lactobacilli* reduced the number of *Salmonellae* adhering to the crop mucosa by 1 to 2 logarithms. Tarakanov *et al.*[21] observed that number of *Escherichia coli* was reduced by 37% and *Salmonella* by 2 times in the experimental broilers, whereas, intestinal count of amylolytic and lactobacillus increased. The count of coliforms and enterococci decreased during feeding of the acidophilus milk was reported by Patidar and Prajapati[23]. They also found that on average, feeding of *L. acidophilus* $I_4$ and $C_2$ caused to lower coliform count, while $I_4$ and $V_3$ reduced enterococci count. The presence of high percentage of pathogenic *Escherichia coli*, *Staphylococci*, *Streptococci* and *Bacilli* in addition to the presence of *Micrococci*, *Salmonella* and *Proteus* are alarming for poultry industry. Many researchers are also of the same view that the presence of the above organisms could be potential hazard not only to human health but also to establishment of poultry industry.

Bacteria associated with the intestinal wall could be seen in the sections of the crop and cecum samples of probiotics fed broilers (vaccinated and nonvaccinated). Fuller *et al.*[8] obtained the similar result. They reported that the main sites of adhesion of bacteria were crop, ileum and cecum. The present findings also correspond with the findings of Fuller[7] who led to the opinion that avian lactobacilli adhered to chicken crop epithelial cells could have formed a property of symbiotic relationship with the chicken internal environment and thus helped to regulate the composition of its intestinal microflora. The present results of histological changes support the findings of Samanya and Yamauchi[24]. They indicated that birds who were fed dietary *B. subtilis* var. natto for 28 days had a tendency to display greater growth performance and pronounced intestinal histologies, such as prominent villus height, extended cell area and consistent cell mitosis, than the controls.

In conclusion, the present research confirms that probiotics act as bioregulators of the intestinal microflora. Beneficial organisms enter a system prior to the establishment of nonbeneficial flora. The study further revealed that the intake of probiotics for 42 days have stimulated to bring histological changes in the intestinal wall. This constituted more active impetus in cell mitosis and greatly increased nuclear size than controls.

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