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Effect of multi-strain probiotic feed supplement to diets containing fish meal on serum, immune parameters, histopathology and *Escherichia coli* count of commercial broiler chicken

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Abstract
Three hundred and fifty day old commercial broiler chicks were randomly allotted to 7 treatments with 10 replicates containing 5 chicks in each replicate. The treatments consist of corn-soya and fish meal-based control diet, Control diet supplemented with antibiotic (Bacitracin methylene disalicylate, @ 500g/ton) and probiotic at 100, 200, 400, 600 & 800 g/ ton diet. Fish meal (4%) was included in the diets as microbial challenge so as to assess the efficacy of the probiotic supplement. The total cholesterol concentration (mg/dl) in groups given graded levels of probiotic was significantly (P<0.05) lower in comparison to control and antibiotic groups. The total protein concentration (g/dl) was higher in groups fed diet supplemented (200g/ton, 400g/ton) to control group. The albumin concentration (g/dl) of antibiotic group was significantly (P<0.05) higher. The globulin concentration (g/dl) value of probiotic group (400g/ton) was significantly (P<0.05) increased. The humoral immune response to ND vaccine, the percent bursal, thymus and spleen weights were statistically similar among the treatments. *Escherichia coli* count was significantly (P<0.05) reduced in birds fed graded levels of probiotic. It can be concluded that probiotic at 400 g/ton may be supplemented which effectively improved total protein, globulin decreased cholesterol and reduced E coli count when compared to control and comparable to antibiotic but did not influence immune response.

Keywords: cholesterol, globulin, probiotic, fish meal, intestinal villi

1. Introduction
Probiotic is the live microbial feed supplement, which affects the host by improving its intestinal microbial balance by competitive exclusion of harmful pathogens in the gut. The use of sub-therapeutic levels of antibiotic as routine feed additive for growth promotion has been banned in many countries because of possible antibiotic residual effects and the development of drug resistant bacteria. This has lead to the development and application of many non antibiotic substance performance enhancers.

Gut micro biota stimulate the mucosal immune system, help to maintain intestinal homeostasis and play an important role in digestion and absorption (Hara and Shanahan, 2007; Dankowiakowska *et al*., 2013) [6, 4]. Probiotic compete with pathogenic bacteria for binding sites and nutrients, thus supporting a healthy gut microbial ecosystem (Mizak *et al*., 2012) [13]. *Bacillus*-based probiotics are resistant to heat and tolerant to acidic conditions, can survive desiccation as well as challenging storage conditions, which makes them ideal for in-feed applications (Bader *et al*., 2012) [1].

2. Materials and Methods
2.1 Birds and management
Three-hundred-and-fifty (350) day old commercial broiler chicks will be randomly allotted to seven (7) treatments with ten (10) replicates containing five (5) chicks in each replicate. Five birds were housed in each battery brooder cell (2' x 2') with an average floor space of 82 square inches or 205 sq. cm per bird. Feed and water were offered *ad lib* and the birds were raised under identical management conditions. Birds were immunized against New castle disease (ND) with Lasota vaccine on 7th (primary) and 28th (booster) days of age and Infectious bursal disease (intermediate–Georgia strain) vaccine on 14th (primary) and 21st (booster) days of age.

2.2 Experimental design
A growth trial was conducted in randomized block design, comprising of seven dietary treatments where in T_1 was basal diet + 4% fish meal, T_2 - Basal diet + 0.05% Antibiotic (Bacitracin methylene disalicylate, BMD) +4% fish meal, T_3 - basal diet +100billion cfu/ton of Probiotic+4% fish meal , T_4 - basal diet +200billion cfu/ton of Probiotic+4% fish meal, T_5 - basal diet + 400billion cfu/ton of Probiotic+4% fish meal, T_6 - basal diet + 600billion cfu/ton of Probiotic+4% fish meal, T 7- basal diet + 800billion cfu/ton of Probiotic+4% fish meal.

2.3 Serum Parameters
On 42nd day 3ml of blood was collected from one bird from each replicate from wing vein. Further, blood samples were centrifuged at 3000rpm for 5 minutes to separate the serum and serum was transferred to labeled 5ml eppendrof tubes which were stored at -20 ^{0}C until analysis. The serum is used for estimation of Cholesterol, Total protein, Albumen and Globulin by using spectrophotometer (Metstar MUV-61 PCS UV Double Beam) at 505nm with commercially available kits (Arkray Health care private limited).

2.4 Immune Parameters
2.4.1 Immune Organ Weight
One bird from each replicate was randomly selected, starved over night with free access to water, weighed and sacrificed by cervical dislocation on the next day. Relative weight of immune organs thymus, spleen and bursa were recorded.

2.4.2 Humoral Immune response to NDV
The humoral immunity was estimated in birds by measuring antibody titer to Newcastle disease (ND) vaccine (antibody production against ND virus). Broilers were vaccinated against ND by ocular route on 28th day of age with Lasota strain (ND Lasota Vac-500; Indovax Pvt. Ltd. Hyderabad, India) on 36th day of age, blood was collected from one bird per replicate and serum was separated. Haemagglutination inhibition (HI) activity of serum was estimated and the antibody titers (log_2) were measured following the standard procedure (Wegmann and Smithies, 1966) [25].

2.5 Histopathology
Ten birds from each dietary treatment were slaughtered on 42nd day and tissue pieces of small intestine were collected from the birds and fixed in 10% neutral buffered formalin (NBF) for histopathology. The small representative pieces of fixed tissues were cut and subjected to overnight washing under running tap water. The tissues were then dehydrated in ascending grades of alcohol, cleared in xylol and embedded in paraffin at 55-56 °C. The paraffin blocks were cut into thin sections of 5 micron thickness by microtome. The cut sections were lifted on grease free glass slides precoated with Mayer's egg albumin and were kept in incubator overnight at 37 °C for drying. The slides were stained with routine Haematoxylin and Eosin (H and E) stain (Culling, 2013) [3] and the stained sections were mounted with DPX mountant and kept ready for microscopic examination.

2.6 *Escherichia coli* count
One bird from each replicate was slaughtered on 42nd day and intestines were dissected at Meckel's diverticulum. Approximately 1g of ileal digesta was collected aseptically from each bird into a test tube and suspended in 9ml of sterile (0.9%) sodium chloride saline. Serial dilutions of each sample were prepared and plated on Nutrient agar by surface spread method to study total viable colony counts. Visible microbial colonies were counted and expressed as $1og_{10}$ value (Yang *et al*, 2008) [26].

2.7 Statistical analysis
The data was analysed using General Linear Model procedure of Statistical Package for Social Sciences (SPSS) 15th version and means were compared using Duncan's multiple range test (Duncan, 1955) [5] and significance was considered at $P<0.05$.

3. Results and Discussion
3.1 Serum Parameters
The serum cholesterol concentration (mg/dl) in groups given graded levels of probiotic was significantly ($P<0.05$) lower in comparison to control and antibiotic groups. The total protein concentration (g/dl), albumin (g/dl) and globulin (g/dl) in groups fed with graded levels of probiotic showed slight improvement in comparison to control and antibiotic groups but there is no significant ($P>0.05$) difference among different dietary treatments. The above findings are in line with Panda *et al.* (2006) [6], Shanumuga Priya and Saravana Babu (2013) [22], Shirisha *et al.* (2017) [23] who observed that the total cholesterol was decreased, while total protein was increased, at the inclusion of probiotics in broiler diets. The decrease in cholesterol level could be related to de-conjugating of bile salts (Klaver and Van der Meer, 1993) [12] as well as inhibit the activity of acetyl-Co A carboxylase and 3-hydroxyl-3-methyl glutaryl coenzyme A reductase which are the rate limiting enzymes in cholesterogenesis by means of bacteria of probiotic (Salehizadeh *et al.*, 2019) [18], as a result they are absorbed less from the intestine and excreted more in the faeces. On contrary, Shahir *et al.*, (2014) [21], reported that supplementing broiler diet with probiotic did not have any effect on total protein, albumen and globulin.

Table 1: Effect of dietary inclusion of multi strain probiotic at graded level on the Serum biochemical profile in broiler chicken at 42 d.

Diets	Levels (g/ton)	Cholesterol (mg/dl)	Protein (g/dl)	Albumin (g/dl)	Globulin (g/dl)
Control	0	210.22^{a}	4.714^{bc}	2.267^{bc}	2.302^{bc}
Antibiotic	500	198.08^{ab}	5.978^{a}	2.858^{a}	3.120^{abc}
Probiotic	100	176.57^{bc}	5.550^{ab}	2.412^{bc}	2.994^{abc}
Probiotic	200	167.66^{bc}	5.880^{a}	2.447^{b}	3.432^{ab}
Probiotic	400	158.09^{c}	6.122^{a}	2.555^{ab}	3.854^{a}
Probiotic	600	151.64^{c}	4.588^{bc}	2.024^{c}	2.564^{bc}
Probiotic	800	150.34^{c}	4.349^{c}	2.368^{bc}	1.981^{c}
N		10	10	10	10
P Value		0.001	0.004	0.002	0.020
SEM		4.669	0.162	0.054	0.160

Means bearing different superscripts within a column are significantly ($P<0.05$) different.

3.2 Immune Parameters
3.2.1 Immune Organ weight
The percent bursal, thymus and spleen weights were statistically similar among the treatments. This result is in consistent with Panda *et al.* (2006) [6] who observed lack of difference in spleen and bursa in probiotic supplemented group. On contrary, Rama Rao *et al.* (2004) [17], Shirisha *et al.* (2017) [23] reported the higher lymphoid organ (bursa, spleen) weights in probiotic supplemented group.

Table 2: Effect of dietary inclusion of multi strain probiotic at graded levels to diets containing fish meal on relative immune organ weight of broiler chicken at 42 d.

Diets	Levels (g/ton)	Relative Weights		
		Spleen	Thymus	Bursa
Control	0	0.134	0.340	0.123
Antibiotic	500	0.151	0.359	0.129
Probiotic	100	0.150	0.356	0.127
Probiotic	200	0.157	0.363	0.139
Probiotic	400	0.194	0.363	0.142
Probiotic	600	0.243	0.308	0.142
Probiotic	800	0.259	0.348	0.120
N		10	10	10
P Value		0.569	0.555	0.956
SEM		0.020	0.834	0.006

3.2.2 Humoral Immune response to NDV

There was no significant (P>0.05) difference in the antibody titers against ND vaccine for humoral immune response between the treatments. This result is in agreement with findings of Kabir *et al.* (2004) [8]; Nayebor *et al.* (2007) [14] who found no significant difference in ND titer. On contrary, Khaksefidi and Ghoorchi (2006) [9], Cross *et al.* (2003), reported significantly higher antibody production against NDV in birds supplemented with Probiotic containing *B. subtilis*. The discrepancies between earlier and present findings could be due to specificity of the strain, experimental conditions and level of dietary feed additive (Khan *et al.*, 2013) [10].

Table 3: Effect of dietary inclusion of multi strain probiotic at graded levels to diets containing fish meal on immune response in broiler chicken up to 42 d.

Diets	Levels (g/ton)	ND titers (log_2)
Control	0	2.400
Antibiotic	500	2.401
Probiotic	100	2.900
Probiotic	200	3.300
Probiotic	400	3.600
Probiotic	600	3.700
Probiotic	800	3.300
		10
		0.204
		0.170

3.3 Histopathology

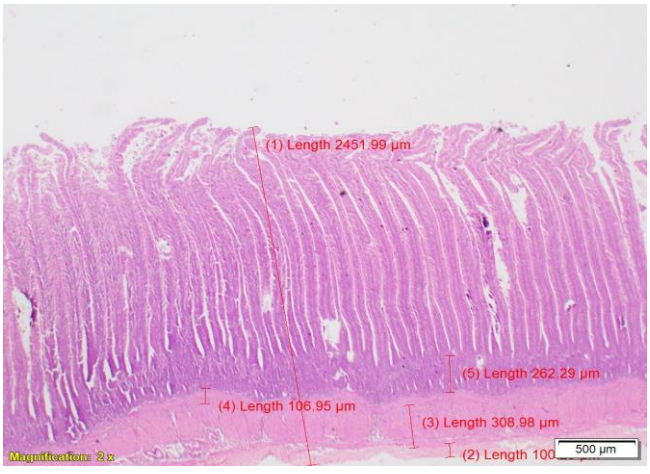

Fig 1: Haematoxylin and eosin (H&E) staining of intestine at 42 d of age in Broiler chickens supplemented with probiotic (400g/ton) diet.
Note: Scale bar, 500 µm & 200 µm.

Uniform shape, size and length of villi. Inner muscles is thin outer muscularis is thick and decrease in sub mucosa regenerating crypts. Serosa and muscularis are showing numerous capillaries.

3.4 *Escherichia coli* Count

E. coli count was significantly (P<0.05) reduced in antibiotic and graded levels of probiotic groups compared to control group. The above findings are in agreement with Rama Rao *et al.* (2004) [17]; Knap *et al.* (2011) [17]; Jeong and Kim (2014), Shirisha *et al.* (2017) [23], Nguyen *et al.* (2019) [15]. Santoso *et al.* (1995) [19] who reported that supplementation of probiotics significantly (P<0.05) reduced the mean log 10 values of *E. coli* counts in the intestinal excreta of broiler when compared to control. The reduced *E. coli* count might be due to anti-microbial activity of *Bacillus subtilis* by establishing favourable bacterial population through competitive exclusion and antagonism. In contrast with present study, some of the authors reported that addition of probiotics had no significant difference on total bacterial count (Sen *et al.*, 2012).

Table 4: Effect of dietary inclusion of multi strain probiotic at graded levels to diets containing fish meal on *Escherichia coli* (log $_{10}$ of cfu/ml count) in the intestine of the broiler chicken at 42 d.

Diets	Levels (g/ton)	*Escherichia coli* (log $_{10}$ of cfu/ml count) in ileal digesta
Control	0	6.04^{a}
Antibiotic	500	5.40^{bc}
Probiotic	100	5.64^{ab}
Probiotic	200	5.36^{bc}
Probiotic	400	5.32^{c}
Probiotic	600	5.40^{bc}
Probiotic	800	5.36^{bc}
		10
		0.001
		0.054

Means bearing different superscripts within a column are significantly (P<0.05) different.

4. Conclusion

It can be concluded that probiotic at 400 g/ton may be supplemented which effectively sanitized upper gastro intestinal tract from *E. coli* and maintained suitable environment in the intestine and stimulated morphological growth of intestinal villi, supported higher nutrient absorption leads to enhanced breast meat yield there by increased body weight gain at 42 d of age, better than antibiotics.

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