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Effects of fermented soybean meal, probiotics and organic acids on intestinal morphology, pH and microbial count in broilers[#]

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Abstract

The study was conducted to compare the effects of fermented soybean meal (FSBM), probiotics and organic acids in the diet of broiler chicken on intestinal morphology, pH and microbial count. A total of 160, day-old Vencobb 430Y broiler chicks were randomly selected and divided into five treatments with four replicates of eight birds each. The dietary treatment groups were as follows: T_1 - basal diet with corn-soybean meal (control); T_2 - control diet with 100 per cent replacement of soybean meal by FSBM; T_3 - control diet supplemented with 0.05 per cent probiotics; T_4 - control diet supplemented with 0.1 per cent organic acids; T_5 - control diet supplemented with both probiotics and organic acids. The results indicated significant ($p<0.01$) improvement in the duodenal and jejunal morphology of birds in terms of villi height, crypt depth and their ratio in all the experimental groups. There was significant ($p<0.01$) reduction in ileal E. coli and coliform count in broilers of all the experimental groups compared to the control group. All the experimental diets significantly increased ($p<0.01$) the lactic acid bacteria count in ileum of broilers. Ileal pH was significantly ($p<0.01$) lowered in all the experimental groups compared to control group. In this study, the dietary inclusion of FSBM showed better improvement in intestinal morphology and microbial balance compared to control, probiotic and organic acids supplemented groups and at par with that of combination of probiotic and organic acids group.

Keywords: *Fermented soybean meal, intestinal morphology, probiotics, organic acids*

Probiotics are live microbial feed supplements that beneficially affect the host by improving its intestinal microbial balance with mechanism of competitive exclusion of pathogenic

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organisms and by stimulating the intestinal immune responses. Organic acids alter the gut microflora by direct killing through cell wall penetration or indirectly by altering the gut pH. Several studies reported that supplementation of probiotics or organic acids in broiler diet improved the growth performance and reduced the microbial counts in GIT, although these effects have not always been consistent.

In recent years, the fermentation of feed has been considered as a novel and effective biosafe nutritional strategy for substituting antibiotics in animal feed industry (Shi *et al.,* 2017). Fermented feeds are acidic in nature with lower pH value, contains high number of lactic acid bacteria (LAB) and high concentrations of lactic acid and acetic acid (Ashayerizadeh *et al*., 2018). It has been reported that dietary inclusion of fermented feeds in the diet of broilers improved the intestinal microbial balance by decreasing pathogenic bacteria and increasing LAB count and also improved the small intestinal morphology (Jazi *et al*., 2018). Soybean meal (SBM) is the commonly used protein source in poultry nutrition. It has been reported that fermentation process can improve the nutritional value of soybean meals by decreasing the anti-nutritional factors and improving the nutrient bioavailability, which may result in better usage of nutrients in birds (Shi *et al.*, 2017). Thus, in the present scenario of increasing price of SBM, the inclusion of fermented soybean meal (FSBM) in feed may produce advantageous effects on intestinal health and nutrient availability. Therefore, this study was conducted to compare the effects of FSBM, probiotics and organic acids in broiler diet on intestinal morphology, pH and microbial count.

Materials and methods

Experiment was conducted in the Department of Poultry Science, College of Veterinary and Animal Sciences, Mannuthy using 160, day-old commercial broiler chicks (Vencobb-430Y) from day-old to 42 days of age. All the chicks were wing banded, weighed individually and randomly allotted to five dietary treatment groups, each group consisting of four replicates of eight birds each, in a completely randomized design.

Preparation of FSBM

The freeze-dried cultures of *Lactobacillus acidophilus* (MTCC10307), *L. plantarum* (MTCC2621), *Bacillus subtilis* (MTCC441) and *Aspergillus oryzae* var. *brunneus* (MTCC8846) were purchased from Microbial Type Culture Collection and Gene Bank, CSIR - Institute of Microbial Technology, Sector- 39A, Chandigarh - 160036, India. Each kilogram of soybean meal was mixed with one litre of distilled water containing approximately 10^8 CFU/mL of *Lactobacillus acidophilus, L. plantarum, Bacillus subtilis* each and 10^6 spores/mL of *Aspergillus oryzae*, then incubated at 30°C for seven days in fermentation tank equipped with a one-way valve. Fermented product was dried at 50°C for two days, ground and kept at room temperature until it was mixed in the experimental diets.

Experimental diets

The five experimental treatments in the trial were as given below:

T_1- The standard broiler ration (SBR) formulated using maize and soybean meal as per BIS (2007) specifications

T_2- SBR ration with complete replacement of SBM by FSBM

T_3- SBR with probiotic mixture (Spectra-DFM^{GTH} containing 3.0×10^8 CFU/g of *Bacillus subtilis, B. licheniformis, B. amyloliquifaciens, Lactobacillus species, Pediococcus acidilactici* and *Enterococcus faecium*) at 0.5 g/kg

T_4- SBR with organic acid mixture (Acifed-FS -synergistic blend of essential organic acids of propionates and butyrates) at 1 g/kg

T_5- SBR with addition of probiotics 0.5 g/kg and organic acids 1 g/kg. Both probiotic and organic acid supplements were purchased from Neospark, Drugs and Chemicals Pvt. Ltd, Hyderabad.

Standard management practices were followed throughout the experiment. The feed and water were given *ad libitum* to the birds. The feed ingredients and the feed samples were subjected to proximate analysis as per AOAC

(2012). The chemical composition of fermented soybean meal and the ingredient composition of the experimental rations fed to the birds were presented in Table1 and Table 2.

Vitamin premix provided the following per kilogram of diet: Vitamin A 12500 IU, Vitamin D_3 2500 IU, Vitamin E 8 mg, Vitamin K 1.0 mg, Vitamin B_1 1.0 mg, Vitamin B_2 5 mg, Vitamin B_3 12 mg, Vitamin B_6 8.0 mg, Folic acid 0.6 mg, Vitamin B_{12} 15 mcg, Biotin 100 mcg, Calcium panthothenate 8 mg.

Trace mineral premix provided the following in milligrams per kilogram of diet: Manganese 100 mg, Zinc 85 mg, Iron 90 mg, Copper 15 mg, Iodine 1.8 mg, Selenium 0.45 mg, organic Chromium 0.15 mg.

Microbial analysis

Fresh intestinal content samples were collected from slaughtered birds at 6^{th} week of age and processed for microbiological analysis. To estimate the organisms, 0.1 ml of inoculum from selected dilution on eosin methylene blue (EMB) agar for *E. coli*, violet-red bile agar (VRBA) for coliform, Karl Friedrich (KF) Streptococcal agar for *Enterococcus faecalis* and modified de Man, Rogosa and Sharpe (MRS) agar for LAB was deposited on pre-prepared sterile solidified media and incubated at 37ºC for 24 to 48 hours. The number of organisms was expressed as $\log_{10}$ CFU per ml.

Table 1. Chemical composition of soybean meal on DM basis before and after fermentation, per cent

Item	Soybean meal	Fermented soybean meal	p-value
Dry matter	90.36±0.23	90.80±0.22	0.09
Crude protein	44.45^b±0.31	48.17^a±0.27	0.001
Crude fibre	5.36^a±0.39	4.31^b±0.12	0.001
Ether extract	1.86^a±0.07	1.43^b±0.03	0.008
Total ash	7.89±0.31	7.80±0.15	0.71
pH	6.30^a±0.87	4.33^b±0.12	0.001
LAB count ($\log_{10}$ CFU/ml)	5.45^b±0.22	9.54^a±0.03	0.001

Mean values bearing different superscripts within a row differ significantly

Table 2. Ingredient composition of experimental rations, per cent

Ingredients	Pre starter (0-7 days)		Starter (8-21 days)		Finisher (22-42 days)	
	SBM diets (T_1, T_3, T_4, T_5)	FSBM diet (T_2)	SBM diets (T_1, T_3 T_4, T_5)	FSBM diet (T_2)	SBM diets (T_1, T_3 T_4, T_5)	FSBM diet (T_2)
Yellow maize	51. 23	56.30	52.70	57.23	56.52	60.51
SBM	42.00	-	39.20	-	34.13	-
FSBM	-	37.50	-	35.10	-	30.56
Rice bran oil	2.80	2.14	4.20	3.60	5.30	4.82
Dicalcium phosphate	1.80	1.83	1.83	1.90	2.06	2.13
Calcite	1.40	1.40	1.40	1.40	1.30	1.26
Salt	0.30	0.30	0.30	0.30	0.30	0.30
Sodium bicarbonate	0.15	0.15	0.15	0.15	0.15	0.15
L-Lysine	0.23	0.24	0.20	0.20	0.07	0.08
DL -Methionine	0.17	0.17	0.20	0.20	0.20	0.20
Total	100	100	100	100	100	100
Vitamin premix	0.05	0.05	0.05	0.05	0.05	0.05
Trace mineral mix	0.1	0.1	0.1	0.1	0.1	0.1

Additional supplements (g /100 kg feed) Liver tonic- 25 g, Choline chloride and toxin binder -100 g, Anticoccidial 50 g

Intestinal morphology and pH estimation

Intestinal segments of approximately 3 cm length of duodenum, jejunum and ileum were excised and fixed in 10 per cent neutral-buffered formalin for 48 hours. Cross sections were prepared according to method of Gridley (1960) and examined. pH of ileal content was measured according to the protocol of Izat *et al.* (1990).

Data collected on various parameters were analysed using SPSS (version 24.0).

Results and discussion

Microbial fermentation had a positive effect on the chemical composition of SBM. The fermentation increased the crude protein content, while it decreased the crude fibre and ether extract content of SBM. An increase in crude protein content of FSBM could be due to the formation of microbial protein and reduction in the non-protein compounds like fibre and carbohydrates during fermentation as reported by Jazi *et al.* (2017). The reduction in crude fibre of FSBM might be due to the synthesis of enzymes such as cellulase, xylanase, glucanase by bacterial species and fungus used for fermentation (Ashayerizadeh *et al*., 2018). The low ether extract values in FSBM might be due to microbial lipase activity of *Bacillus subtilis* (Terlabie *et al*., 2006).

The fermentation process also significantly ($p<0.01$) decreased the pH value and increased ($p<0.01$) the lactic acid bacteria (LAB) count in soybean meal. Decreased pH value of FSBM might be due to the production of organic acids and some other short chain fatty acids during the fermentation by the microorganisms. Present findings are in line with those of Ashayerizadeh *et al*. (2018) and Jazi *et al.* (2019), who also reported that use of probiotic organisms for the fermentation of plant protein by-products, significantly decreased the pH value and increased the LAB count.

Intestinal morphological analysis

Table 3 summarize the effects of experimental treatments on intestinal morphometric analysis. The villus height and villus height to crypt depth ratio (VH:CD) are important indicators of intestinal digestion and absorption capacity (Shirani *et al*., 2019). Increased villus height indicates the increase in surface area for absorption of digested nutrients. The depth of crypt is related to cell replacement rate and faster replacement rate requires energy and protein which leads to decrease in the growth and development of other tissues and organs. Thus, a decrease in depth of crypt will enhance the growth rate of birds (Markovic *et al*., 2009).

The dietary inclusion of FSBM significantly ($p<0.01$) improved the duodenal, jejunal and ileal villi height, decreased the crypt depth and also increased VH:CD ratio in duodenum and jejunum. These results are in accordance with Soumeh *et al.* (2019) who observed the improved jejunal and ileal morphology in broilers with dietary inclusion of FSBM. The improved intestinal morphology with FSBM diet might be due to improvement in intestinal microflora balance in favour of beneficial microbes like LAB (Jazi *et al.*, 2018) and reduction in antinutritional factors of SBM during fermentation, such as antigenic proteins (Feng *et al*., 2007). The metabolic activity of microorganisms during fermentation process can increase enzymes and other compounds like peptides, amino acids and organic acids which enhance the gut function (Jazi *et al.,* 2019).

Probiotic and organic acid supplementation also improved ($p<0.01$) the morphology in term of villus height, crypt depth and their ratio in duodenum and jejunum. Similarly, Paul *et al*. (2007) with calcium propionate (3 mg/kg) supplementation and Sen *et al*. (2012) with *B. subtilis* (3 g/kg) supplementation also reported the improvement in villus height in duodenum, jejunum and ileum of broilers.

Broilers in the probiotic and organic acid combination group (T_5) had significantly ($p<0.01$) higher villus height and VH:CD ratio in jejunum when compared to birds supplemented with probiotic or organic acid alone, indicating the synergistic effects. In support to this study, Rodjan *et al.* (2018) observed chicks fed with combination of *Bacillus* and organic acid

Table 3. Effect of dietary treatments on intestinal morphology of broilers at six weeks of age

Parameters	Treatment					p- value
	T1 SBR (control)	T2 FSBM based ration	T3 SBR+ probiotic	T4 SBR+organic acid	T5 SBR+probiotic + organic acid	
V Villus height (µm)						
Duodenum	889.44[c] ± 38.77	1012.82[b] ± 29.14	1013.95[b] ± 31.83	1102.34[ab] ± 22.00	1134.43[a] ± 41.37	0.001
Jejunum	828.60[d] ± 30.37	1164.73[b] ± 32.24	968.73[c] ± 40.83	947.64[c] ± 28.97	1378.06[a] ± 29.78	0.001
Ileum	917.91[b] ± 24.45	1093.34[a] ± 51.94	1064.60[a] ± 47.11	1078.80[a] ± 49.65	1176.05[a] ± 45.47	0.004
Crypt depth (µm)						
Duodenum	137.96[a] ± 5.49	103.14[bc] ± 6.22	100.15[c] ± 4.00	119.93[b] ± 6.22	114.16[bc] ± 5.43	0.001
Jejunum	142.79[a] ± 4.31	123.13[b] ± 4.04	119.68[b] ± 6.91	119.61[b] ± 5.28	125.51[b] ± 4.46	0.01
Ileum	113.65 ± 3.98	119.38 ± 5.19	123.99 ± 7.42	124.89 ± 4.56	120.70 ± 6.32	0.61
Villus height to crypt depth ratio						
Duodenum	6.69[b] ± 0.34	10.69[a] ± 0.62	10.34[a] ± 0.43	9.56[a] ± 0.58	10.64[a] ± 0.66	0.001
Jejunum	5.94[d] ± 0.28	9.63[b] ± 0.30	7.92[c] ± 0.61	8.38[c] ± 0.49	11.33[a] ± 0.40	0.001
Ileum	8.40 ± 0.39	9.04 ± 0.70	9.21 ± 0.69	8.87 ± 0.45	10.41 ± 0.60	0.10

Mean values bearing different superscript within a row differ significantly ($p<0.05$)

mixture in the diet had significantly taller villi in intestine than chicks fed on probiotic or organic acid alone. The improved intestinal morphology in experimental groups (T_2 to T_5) might be due to their effects on intestinal microbiota balance and microbial metabolites especially butyrate, which influence the gut epithelial cell proliferation and increase the villus height (Jazi *et al*., 2018).

Enumeration of bacteria and pH

The effect of experimental diets on microbial population and pH of the ileal content in broilers are presented in Table 4.

Dietary inclusion of FSBM in broilers significantly ($p<0.01$) increased the ileal LAB count and decreased ($p<0.05$) the *E. coli* and coliform count in present study. These findings are in accordance with Jazi *et al*. (2018) and Soumeh *et al.* (2019) who reported increased LAB count and decreased coliform count in crop and ileum of broilers fed with corn-FSBM based diet.

Probiotic supplementation reduced ($p<0.05$) the *E. coli* and coliform count and also improved ($p<0.01$) the LAB count in ileum of broilers. Similarly, Hung *et al.* (2012) with *B. coagulans* at (0.10, 0.20 and 0.25 g/kg) supplementation in duodenum and Zhang *et al*. (2014) with multi-probiotic mixture at (1×10^5 CFU/kg) supplementation also reported improved intestinal microbial balance in broilers. In contrast, Salehimanesh *et al.* (2016) could not observe any significant effect on microbial population in ileum of broilers by *Lactobacillus* spp. (0.9 g/kg) supplementation. Several factors like animal-to-animal variation, strain of yeast or bacteria used and experimental procedures may contribute to the variation of results (Hassanein and Soliman, 2010).

The present finding of organic acid supplementation in this study are in accordance with that of Rodjan *et al.* (2018) who reported

Table 4. Effect of experimental treatments on ileal microbial count (log_{10} CFU/ml) and pH of broilers

Attribute	Treatment					p-value
	T1 SBR (control)	T2 FSBM based ration	T3 SBR+ probiotic	T4 SBR+ organic acid	T5 SBR+ probiotic+ organic acid	
E. coli count	4.47^{a} ±0.07	3.11^{b} ±0.33	3.13^{b} ±0.25	3.61^{b} ±0.10	3.54^{b} ±0.15	0.01
Coliform count	5.27^{a} ±0.39	2.80^{b} ±0.32	3.30^{b} ±0.95	3.45^{b} ±0.36	3.59^{b} ±0.23	0.001
E. faecalis count	3.79 ±0.12	2.96 ±0.15	3.34 ±0.37	3.10 ±0.10	3.24 ±0.15	0.09
LAB count	6.51^{b} ±0.17	8.75^{a} ±0.30	8.20^{a} ±0.07	6.59^{b} ±0.43	7.99^{a} ±0.55	0.001
Ileal pH	6.75^{a} ± 0.03	6.28^{c} ± 0.03	6.43^{b} ± 0.04	6.30^{c} ± 0.02	6.40^{b} ± 0.01	0.001

Mean values bearing different superscript within a row differ significantly ($p<0.05$)

that addition of organic acid mixture in the diet of broilers significantly ($p<0.05$) reduced the *E. coli* count, but not showed any effect on LAB count in ileum and caecum of broilers when compared to control group. Similarly, Thirumeignanam *et al*. (2006) also reported the reduction in total bacterial count in crop, duodenal and caecum of broilers with a mixture of propionic and butyric acid supplementation in the diet.

Specific regions in the gastrointestinal tract have specific pH which establishes the microbial population in that area thereby affecting the digestibility and absorptive capacity. Normally the beneficial microorganisms can thrive in acidic pH. However, the pathogenic organisms can survive only in neutral or slightly alkaline pH. All the experimental diets (T_2 to T_5) significantly ($p<0.01$) lowered the pH in ileum of broilers when compared to control group. The present results are in accordance with Jazi *et al.* (2018) and Soumeh *et al.* (2019) who reported lower pH values in intestine of broilers fed with FSBM included diet when compared to control diet fed birds. The lower ileal pH in FSBM supplemented group might be due to organic acids in the fermented feed which were produced during the fermentation process. Similarly, Fonseca *et al*. (2010) observed decreased pH value in crop of broilers with *Lactobacillus* supplementation in the diet. The decreased pH in probiotic group might be because of beneficial bacteria (LAB), which will produce organic acids like lactic acid and acetic acid and lowers the pH in GIT (Dhama *et al.,* 2011). Thirumeignanam *et al*. (2006) reported that dietary supplementation of propionic and butyric acid mixture reduced the pH in intestine of broilers.

Conclusion

The dietary inclusion of FSBM in broilers could bring about improvement in intestinal morphology and microbial balance compared to control, probiotic, and organic acids supplemented groups. The results were at par when compared with that of combination of probiotic and organic acids group. Therefore, the inclusion of FSBM in broilers diet can be recommended to improve the gut health and performance of broilers as alternative to probiotics and organic acids.

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Conflict of interest

The authors report no conflict of interest.

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