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Unlocking the beneficial effects of multi-enzyme cocktail *Bacillus sonorensis* BD92 on commercial broiler growth performance and intestinal histology

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Abstract

Crude fiber (CF) is a vital component in poultry nutrition with a notable phytonutrient effectively indicating the presence of indigestible biomass in food due to the absence of digestive enzymes for CF in broilers. This study aimed to analyze the properties of a multi-enzyme cocktail (MEC) *Bacillus sonorensis* BD92 (BsBD92) comprised of xylanase, β-glucosidase, exo-glucanase, and endo-glucanase enzymes. Also, this study intended to look at the growth performance and intestinal histology of broilers in the starter and finisher phases by the addition of MEC BsBD92 to their diet. To evaluate the efficacy of MEC BsBD92, 140 one-day-old unsexed Cobb500 broiler chicks were randomly divided into seven groups receiving different diets. The characterization of exo-glucanase, xylanase, β-glucosidase, and endo-glucanase showed that their peak activities were observed at a temperature of 50.00 ˚C and a pH of 5.50. The 6.00% CF and 2.00 X MEC BsBD92 improved the intestinal morphology and feed conversion ratio, demonstrating a synergistic effect on growth performance. Whereas, increasing meat percentages to 61.06 and 65.09 g *per* 100 g body weight during the starter and finisher phases was also observed, respectively. The lipid profiles revealed significant variations in triglyceride and cholesterol levels. This study provides an innovative approach, considering not only lowering the feed cost using inexpensive fibrous feedstuffs but also improving the feed efficiency through supplementation of MEC BsBD92.

Introduction

Chicken has become the most popular meat, leading to significant growth in the poultry industry. Commercial feed enzymes are in high demand due to the growing market for chicken meat. These enzymes support the growth and nutrient uptake of chicken broilers. There is a trend to explore crude enzymes and non-commercial sources of feed as substitutes for commercial enzymes and diets in the poultry industry.[1,2] Poultry production greatly supports the meat industry in combating global protein demand.[2] The poultry industry must continuously innovate to improve the feed efficiency and lifespan of grill chickens due to the growing demand for chicken meat. The use of enzymes in chicken nutrition is a very targeted method being currently implemented.[2,3] Significant progress has been achieved in the past decade in improving the production, effectiveness, quality, thermostability, and specificity of commercial enzymes used as supplements in poultry diets. Similarly, adding crude enzymes to chicken feed has multiple benefits, such as reducing costs, improving nutrient digestion, and enhancing bird health. The effectiveness and safety of crude enzymes in feed regarding growth performance, specifically in relation to the immune system and internal organs, have not been comprehensively studied.[4,5]

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Dietary fiber (DF) consists of components, such as non-starch polysaccharides (NSPs), oligosaccharides, and lignin, being resistant to mammalian enzymatic digestion. It has factually been considered an anti-nutritional component and a diluting agent in poultry feed formulations. Its high concentration has adverse impacts on growth performance and nutrient retention. However, it has the capacity to undergo fermentation to a limited degree through the action of the gastrointestinal tract (GIT) microflora.[6] The DF can be categorized into insoluble and soluble, depending on its ability to dissolve in water. The inclusion of both types of DF in broiler diets has been found to have significant effects on various aspects, including intestinal structure, nutrient absorption, organ development, growth performance, and composition of the intestinal microbiota. Its inclusion, especially oligosaccharides in isolated form, has been shown to have prebiotic effects *e.g.*, improving the composition of gut bacteria and gut health, regulating immune function in the gut, nutrient utilization, and poultry performance, and promoting the formation of short chain fatty acids (SCFAs) within the GIT.[4,6] These NSPs-digesting enzymes and prebiotics can be employed in place of antibiotic growth boosters.[4] According to the meta-analysis, broiler performance, digestive tract development, and nutritional digestibility may all be enhanced by adjusting the ratio of DF portion in the feed given to the chickens.[7] Crude fiber (CF) is an important component of DF in poultry nutrition. It contains a significant phytonutrient, serving as an excellent indicator of the indigestible plant biomass being present in food and becomes digestible by GIT microflora.[6]

A significant amount (60.00 - 75.00%) of the total costs associated with processing poultry comes from poultry-related expenses. Cost-effective CF can be included in the feed to lower this expense. Animal nutrition, digestion, and metabolism can all be significantly impacted by feed concentrations of 10.00 - 30.00% CF. It may also have a direct or indirect impact on the development of GIT microflora.[8,9] On the other hand, its high concentration may encourage the development of dangerous bacteria, leading to digestive problems and decreased nutrient absorption.[9] It also decreases the abundance of beneficial lactic acid bacteria and bifidobacteria.[10] The exploration of cost-effective approaches for utilizing ingredients and additives, such as enzymes, probiotics, prebiotics, or unconventional ingredients, in high fiber feed has resulted in significant scientific progress.[3] Combining enzymes increases the amount of CF that can be metabolized in poultry feeds, benefitting the welfare of the birds by improving nutrient digestion, lowering lipid peroxidation, improving plasma biochemical characteristics, and boosting immunity. These innovations have significantly improved performance, nutritional aspects, and overall productivity in the poultry industry, being widely recognized in the scientific community.[3,5] The harmonious blending of these components reduces the need for expensive feed supplements, making poultry farming economically feasible at unprecedented levels.[3,11]

The selection of exogenous feed enzymes is crucial and highly dependent on the specific feed ingredients. It is critical to keep in mind that while an enzyme may signal excellent digestion in one diet, it might not necessarily do so in other feeds. The non-uniform chemical composition of CF may be responsible for this variation, as the distribution differs between different feeds.[12] Hence, the demand for novel enzymes capable of catalyzing multiple substrates arises. Accordingly, this study was conducted to examine the effect of dietary supplementation of *Bacillus sonorensis* BD92 (BsBD92) strain derived multi-enzyme cocktail (MEC BsBD92) on growth performance, serum biochemistry, and intestinal morphology in commercial broiler chickens.

Materials and Methods

Microbial culture. A wild-type strain of BsBD92 breaking down fibers being previously isolated from the microbiota of buffalo dung was used in this investigation.[13]

Characterization of MEC BsBD92. The MEC BsBD92 was produced using an optimized fermentation process at the National Institute for Biotechnology and Genetic Engineering College, Pakistan Institute of Engineering and Applied Sciences, Faisalabad, Pakistan, as described previously.[14] The optimum temperature was found by assessing enzyme activity throughout a range of 30.00 - 90.00 ˚C. Similarly, enzyme experiments utilizing a buffer with a pH range of 3.50 to 8.50 were used to determine the optimum pH level of the crude enzyme. The enzymatic activities were measured as described by Raza *et al*.[14]

pH and thermostability. The thermostability of the MEC BsBD92 was assessed by subjecting it to a range of temperatures (60.00 - 90.00 ˚C) for a duration of 60 min. Simultaneously, the pH stability was assessed by subjecting it to the incubation at different pH levels for a duration of 60 min. Enzyme activities were assessed following incubation at various temperatures (60.00 - 90.00 ˚C) and pH levels (3.50 - 8.50) under optimized conditions. The enzyme activities were assessed under optimal conditions of temperature and pH, without any prior incubation. The residual activity (%) was determined by setting the initial enzyme activity as 100% for each enzyme.[15,16]

Experimental design for application of MEC BsBD92. The institutional animal ethics committee gave approval and guidelines for conducting the experimental study (No. D331/2016). Investigating the growth performance of commercial broilers given a lab-made MEC BsBD92 supplemented with varying amounts of dietary CF (4.00, 6.00, and 8.00%) was the aim of the study. One-day-old, unsexed Cobb500 broiler chicks (n = 140) weighing

46.00 ± 1.00 g were taken from a commercial hatchery. Using a fully randomized design, they were dispersed in a factorial arrangement of 2 × 3 + 1, yielding seven groups with four replicates, each including five birds. Experimental birds were fed diets containing varied amounts of CF (4.00, 6.00, and 8.00%) plus the MEC BsBD92 in two different dosages (1.00 X MEC and 2.00 X MEC). The 1.00 X MEC BsBD92 contains 800 U of xylanase, 75.00 U of β-glucosidase, 118 U of exo-glucanase, and 250 U of endo-glucanase *per* kg of feed. While, 2.00 X MEC BsBD92 contains 500, 236, 150, and 1,600 U of endo-glucanase, exo-glucanase, β -glucosidase, and xylanase *per* kg, respectively. In this study, ST group was given 4.00% crude fiber (CF) in diet and served as standard control, group F6 was given 6.00% CF in diet, group F8 was given 8.00% CF in diet, group F6E1 was given 6.00% CF in diet along with 1X MEC, group F8E1 was given 8.00% CF in diet along with 1X MEC, group F6E2 was given 6.00% CF in diet along with 2X MEC and group F8E2 was given 8.00% CF in diet along with 2X MEC". In order to meet the nutritional requirements of chicks throughout the starter phase (1 - 21 days) and finisher phase (22 - 35 days), the diets (Table 1) used in this trial were developed in accordance with published studies.[15,17]

Performance parameters. To evaluate performance metrics, feed intake (FI) and body weight (BW) were evaluated in each group following a 3-hr feed withdrawal phase. As previously mentioned, the productivity index (PI) and feed conversion ratio (FCR) were computed.[18]

Sample collection for safety evaluation. Samples were obtained from a random selection of birds (n = 5) in each group at the end of starter and finisher phases of the feeding trials through the process of slaughtering. Blood samples were obtained to evaluate the safety of supplemented enzymes by analyzing serum biochemistry profile as described formerly.[15]

Intestinal histomorphometrical and histological examinations. The tissue samples (duodenum) underwent a dehydration procedure lasting 24 hr. Subsequently, the samples were embedded in paraffin for histological examination as described before.[15] The morphological analysis comprised the acquisition of images using an optical microscope (Eclipse Ci; Nikon, Chambersburg, USA) and NIS-Element D (version 5.01; NIS-Element, Melville, USA), a computer image analysis system.[19] The samples were subjected to the assessment of villi height (VH), villi width (VW), crypt width (CrW), and crypt depth (CrD). Ten well-oriented villi were selected from each cross-section of the intestine. The determination of the absorption surface area was conducted according to the methodology outlined by Kisielinski *et al*.[20]

Statistical analysis. For each parameter, the obtained data were analyzed by one way ANOVA using a completely randomized design. Tukey's test was utilized to compare the means at a significance level of 0.05 using Minitab Minitab (version 18.0; Minitab Inc., Boston, USA). Data are presented as mean ± SD.

Results

Characterization of MEC BsBD92. The results of the enzyme activity comparisons under varying temperatures and pH levels are shown in Figures 1A and 1B, respectively.

Table 1. Composition of experimental diets for broiler birds during starter (St) and growing (Gr) phase.

Ingredients	St1 (CF 4.00%)	St2 (CF 6.00%)	St3 (CF 8.00%)	Gr1 (CF 4.00%)	Gr2 (CF 6.00%)	Gr3 (CF 8.00%)
Corn	59.47	51.82	38.98	62.50	56.98	41.91
Wheat bran	0.00	7.93	10.95	0.00	8.80	11.94
Oats	0.00	0.69	9.36	0.00	0.00	11.60
Soybean meal	37.05	26.58	22.73	34.02	19.75	16.72
Sunflower meal	0.00	5.00	10.00	0.00	6.64	10.00
Cotton seed meal	0.00	5.00	5.00	0.00	5.00	5.00
Oil	0.50	0.00	0.00	0.65	0.00	0.00
Lysine	0.08	0.08	0.08	0.08	0.08	0.08
Methionine	0.25	0.25	0.25	0.24	0.24	0.24
DCP	1.18	1.18	1.18	1.06	1.06	1.06
Limestone	0.93	0.93	0.93	0.93	0.93	0.93
Salt	0.44	0.44	0.44	0.42	0.42	0.42
Mineral and vitamin premix	0.10	0.10	0.10	0.10	0.10	0.10
Total	100	100	100	100	100	100
Dry matter	90.50	89.41	89.45	89.75	89.43	89.54
Protein	21.00	21.00	21.00	19.00	19.00	19.00
Energy (kcal kg^{-1})	3,000	3,000	3,000	3,000	3,000	3,000
Lysine (%)	1.22	1.08	1.03	1.09	0.19	0.88
Methionine (%)	0.36	0.34	0.33	0.34	0.31	0.03
Calcium (%)	3.17	1.00	1.00	1.91	1.00	1.00
Phosphorus (%)	2.11	0.66	0.66	1.27	0.66	0.66
Crude fiber (%)	4.00	6.00	8.00	4.00	6.00	8.00

The results revealed that the enzymes showed their highest activities at 50.00 ˚C and a pH of 5.50. The thermo- and pH stability of the enzymes were evaluated by subjecting them to the pre-incubation at various temperatures ranging from 60.00 - 90.00 ˚C and different pH levels. The residual activities were then determined and quantified, as depicted in Figures 1C and 1D, respectively.

Performance parameters. The growth performance characteristics of the birds in the experimental setting showed a gradual improvement upon the addition of MEC BsBD92. The difference was statistically significant ($p < 0.05$) regarding BW among the different treatment groups in the starter and finisher periods. The highest BW was observed in F6E2 group at starter phase (week three) and was followed by F8E2, F6E1, F8E1, ST, F6, and F8 groups, respectively. The same trend of BW was found at the finisher phase; the highest BW was consistently observed in the F6E2 group. The lowest BW was observed in F8 group (Table 2). To evaluate and contrast feed consumption, the average daily FI (g *per* bird daily) was calculated. This assay found a statistically significant difference regarding FI in all groups during whole experiments. In week 1, the highest FI was seen in F8 group, with a value of 22.31 ± 0.19 g *per* bird daily. Lower FIs were seen in F8E2, F8E1, F6E1, F6E2, F6, and ST groups. The F8 group had the highest FI at the end of the starter phase, while the ST group had the lowest one. Similarly, results showed the highest FI in F8 group, and F6, F8E1, and ST groups showed the least FI, respectively. The F6E2 group showed the least FI in CF supplementing MEC BsBD92 groups at every phase of growth (Table 3).

The FCR is a critical metric for evaluating growth performance. There is an inverse relationship between FCR and performance; with higher FCR values indicating lower performance, while lower FCR values correspond to higher performance. Significant differences in FCR were observed among the various treatment groups over the course of the five-week experimental period ($p < 0.05$). The results indicated that the birds in F6E2 group showed the lowest FCR at starter phase, with a value of 1.24 ± 0.004. This was followed by an FCR of 1.34 ± 0.012 in F8E2 group, 1.37 ± 0.00 in ST group, 1.40 ± 0.004 in F6E1 group, 1.50 ± 0.004 in F8E1 group, 1.55 ± 0.008 in F6 group, and 1.72 ± 0.009 in F8 group. At finisher phase, it was observed that F6E2 group showed the lowest FCR of 1.61 ± 0.004. The results also indicated that F6E2 group had the lowest FCR, even lower than ST group. This indicates that F6E2 group demonstrated superior performance in comparison with the other groups. The F6 (2.02 ± 0.012) and F8 (2.19 ± 0.008) groups (without MEC BsBD92 supplementing) showed the highest FCR at starter and finisher phases, showing the lowest performance (Table 4). The PI is a significant metric for assessing growth performance and the difference in PI of experimental groups was statistically significant ($p < 0.05$). The F6E2 group exhibited the highest PI value of 460.7 ± 1 .35, while the lowest PI value of 279.1 ± 1.04 was observed in F8 group at the starter phase (Fig. 2).

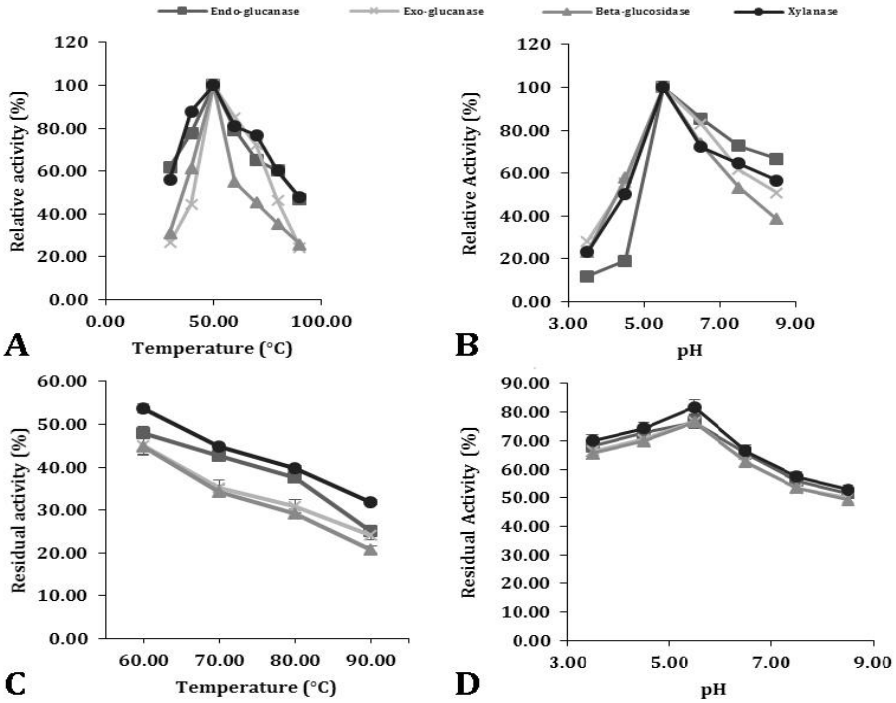

Fig. 1. Characterization of multi-enzyme cocktail BsBD92. Relative activities of different enzymes at **A)** different temperatures (30.00 - 90.00 ˚C; and **B)** different pH (3.50 - 8.50); residual activity after incubating **C)** at various temperatures and **D)** pH for 60 min are shown.

Immune organs weight. The study assessed the immune system maturity in each of the experimental groups by measuring the relative weight (g *per* 100 g BW) of immunological organs, such as the pancreas, thymus, and bursa of Fabricius. It was observed in all experiments that there existed a statistically insignificant disparity in the weight of the bursa and thymus. Table 5 shows that there was a statistically significant change ($p < 0.05$) in the pancreatic relative weight. During the finisher phase, F8 group had the highest pancreas weight (0.28 ± 0.005 g *per* 100 g BW). In contrast, the ST group exhibited the lowest weight, measured as 0.21 ± 0.006 g *per* 100 g BW. Similarly, the F6E2 group also displayed a low weight of 0.22 ± 0.004 g *per* 100 g BW. The study was adhered to the established scientific protocol.

Safety evaluation. The impacts of MEC BsBD92 and dietary CF on serum chemistry parameters, like liver and renal function tests, and lipid profile have been assessed.

Table 2. Body weight of broilers from different experimental groups supplemented with multi-enzyme cocktail BsBD92 on weekly basis.

Groups	Day 7	Day 14	Day 21	Day 28	Day 35
ST	153.50 ± 13.14^{c}	404.00 ± 34.40^{b}	905.00 ± 64.30^{c}	1,578.00 ± 99.41^{bc}	2,310.70 ± 168.15^{bcd}
F6	153.00 ± 14.52^{c}	398.50 ± 35.95^{b}	870.50 ± 72.83^{cd}	1,497.30 ± 97.80^{c}	2,236.30 ± 189.93^{cd}
F8	152.50 ± 14.79^{c}	365.50 ± 41.28^{c}	816.00 ± 50.33^{d}	1,394.00 ± 72.00^{d}	2,139.30 ± 111.92^{d}
F6E1	162.50 ± 13.73^{bc}	426.50 ± 27.79^{ab}	930.50 ± 62.00^{bc}	1,592.70 ± 99.02^{bc}	2,362.30 ± 144.15^{bc}
F8E1	164.00 ± 14.62^{bc}	424.00 ± 19.59^{ab}	925.50 ± 69.96^{bc}	1,564.00 ± 94.50^{bc}	2,335.30 ± 58.17^{cd}
F6E2	174.50 ± 13.59^{ab}	451.00 ± 26.81^{a}	1,013.00 ± 67.97^{a}	1,711.30 ± 82.12^{a}	2,591.70 ± 180.38^{a}
F8E2	179.00 ± 13.74^{a}	450.00 ± 30.00^{a}	974.50 ± 61.27^{ab}	1,614.00 ± 79.06^{ab}	2,456.70 ± 172.96^{ab}

ST group: 4.00% crude fiber (CF) in diet, F6 group: 6.00% CF in diet, F8 group: 8.00% CF in diet, F6E1 group: 6.00% CF in diet along with 1X MEC, F8E1 group: 8.00% CF in diet along with 1X MEC, F6E2 group: 6.00% CF in diet along with 2X MEC, and F8E2 group: 8.00% CF in diet along with 2X MEC.

$^{a-d}$ Different letters indicate significant differences in each row ($p < 0.05$).

Table 3. Feed intake of broilers given various levels of dietary crude fibers and multi-enzyme cocktail BsBD92 for 5 weeks of experiment.

Groups	Day 7	Day 14	Day 21	Day 28	Day 35
ST	17.67 ± 0.25^{e}	45.56 ± 0.44^{de}	98.53 ± 0.33^{e}	155.11 ± 1.19^{c}	187.48 ± 1.31^{e}
F6	18.00 ± 0.07^{e}	48.86 ± 0.43^{b}	104.51 ± 0.55^{c}	162.97 ± 0.73^{a}	213.70 ± 1.31^{b}
F8	22.31 ± 0.19^{a}	47.87 ± 0.37^{bc}	110.90 ± 0.60^{a}	165.14 ± 0.67^{a}	233.18 ± 0.86^{a}
F6E1	19.41 ± 0.07^{c}	48.14 ± 0.17^{b}	101.28 ± 0.33^{d}	156.39 ± 1.17^{bc}	200.93 ± 0.51^{d}
F8E1	20.85 ± 0.21^{b}	50.39 ± 0.46^{a}	107.70 ± 0.33^{b}	159.32 ± 0.85^{b}	213.40 ± 2.26^{b}
F6E2	18.80 ± 0.17^{d}	44.37 ± 0.18^{e}	99.82 ± 0.37^{de}	145.31 ± 0.47^{d}	201.82 ± 0.59^{d}
F8E2	21.24 ± 0.15^{b}	46.71 ± 0.48^{cd}	100.65 ± 0.93^{d}	142.21 ± 1.13^{d}	207.85 ± 1.50^{c}

ST group: 4.00% crude fiber (CF) in diet, F6 group: 6.00% CF in diet, F8 group: 8.00% CF in diet, F6E1 group: 6.00% CF in diet along with 1X MEC, F8E1 group: 8.00% CF in diet along with 1X MEC, F6E2 group: 6.00% CF in diet along with 2X MEC, and F8E2 group: 8.00% CF in diet along with 2X MEC.

$^{a-e}$ Different letters indicate significant differences in each row ($p < 0.05$).

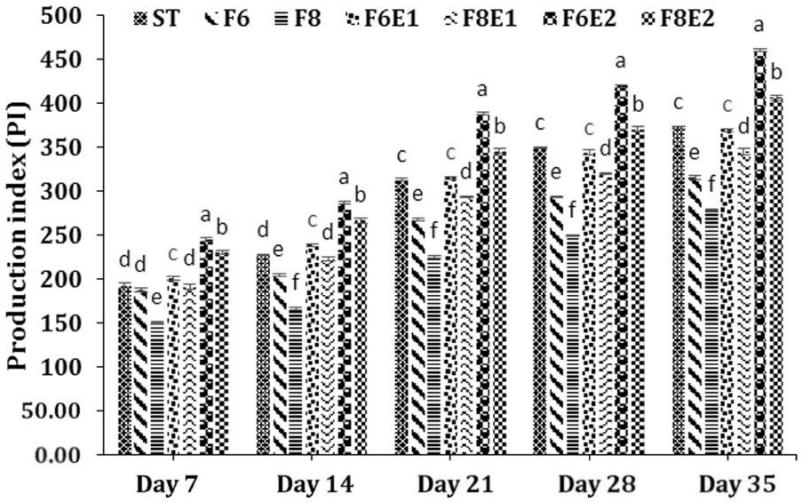

Fig. 2. The production index (Mean ± SD) of broiler chicks during a five-week trial that were given varying amounts of dietary crude fibers and the multi-enzyme cocktail BsBD92. ST group: 4.00% crude fiber (CF) in diet, F6 group: 6.00% CF in diet, F8 group: 8.00% CF in diet, F6E1 group: 6.00% CF in diet along with 1X MEC, F8E1 group: 8.00% CF in diet along with 1X MEC, F6E2 group: 6.00% CF in diet along with 2X MEC, and F8E2 group: 8.00% CF in diet along with 2X MEC.

$^{a-f}$ Different letters indicate significant differences among the groups ($p < 0.05$).

Liver and renal function tests. The results of liver function tests (alanine transaminase [ALT]/glutamate pyruvate alanine aminotransferase) showed a significant variation among the experimental groups. However, it is noteworthy that the observed values fell within the normal range. The F8 group showed the highest ALT level of 32.20 ± 1.166 U L^{-1}, while F6E1 group showed the lowest ALT level of 22.20 ± 1.326 U L^{-1} after the starter phase, and the same order was also observed at the finisher phase. The experimental groups exhibited non-significant fluctuations in the results of aspartate transaminase (AST)/glutamate oxaloacetate aspartate aminotransferase as shown in Table 5. The findings of this assay demonstrated that the MEC BsBD92 CF does not elicit any detrimental impact on liver function. Renal function tests were also conducted to assess the safety profile of the supplements administered. The creatinine and urea levels were all within the normal range for each experimental group. The statistical analysis findings, being displayed in Table 5, revealed no discernible differences in these values across the groups.

Lipid profile. As shown in Figures 3A and 3B, the lipid profile analysis showed statistically significant differences ($p < 0.05$) in the levels of CHO (mg dL^{-1}) and TG (mg dL^{-1}) among the different groups. The TG and CHO levels were determined to be within the normal range, with F6 and G8 groups having significantly lower amounts. The highest TG and CHO levels were observed in the ST group, while the lowest TG and CHO levels were observed in the F8 group at starter and finisher phases.

Histological examination and intestinal histomorphometry. A significant difference was found among the experimental groups after five weeks of the experiment. The experiment showed significant differences in the measurements of VW, VH, CrD, and CrW. The F6 experimental group, with a 6.00% CF concentration, showed moderate villi shortening and thickening. In contrast, the F8 group with 8.00% CF concentration showed abnormal villi shortening and thickening, resulting in reduced absorption area. The F6E1 group showed a slight increment in VH and VW; F8E1 group showed alterations in the reduction of villus length. Significant improvement in villi was observed in F6E2 and F8E2 groups, as evidenced by the data presented in Figure 4. A statistically significant difference ($p < 0.05$) was observed in the maximum absorption area (μm^2) across the various groups (Fig. 5).

Table 4. Feed conversion ratios (mean ± SD) of broilers given varying amounts of dietary crude fibers and multi-enzyme cocktail BsBD92.

Groups	Day 7	Day 14	Day 21	Day 28	Day 35
ST	1.14 ± 0.01^{cd}	1.27 ± 0.01^{c}	1.37 ± 0.00^{e}	1.61 ± 0.01^{e}	1.78 ± 0.01^{e}
F6	1.16 ± 0.00^{c}	1.39 ± 0.01^{b}	1.55 ± 0.00^{b}	1.82 ± 0.00^{b}	2.02 ± 0.01^{b}
F8	1.45 ± 0.01^{a}	1.57 ± 0.01^{a}	1.72 ± 0.00^{a}	2.00 ± 0.00^{a}	2.19 ± 0.00^{a}
F6E1	1.15 ± 0.00^{c}	1.27 ± 0.00^{c}	1.40 ± 0.00^{d}	1.65 ± 0.01^{d}	1.83 ± 0.00^{d}
F8E1	1.22 ± 0.01^{b}	1.35 ± 0.01^{b}	1.50 ± 0.00^{c}	1.74 ± 0.00^{c}	1.94 ± 0.02^{c}
F6E2	1.01 ± 0.00^{e}	1.12 ± 0.00^{d}	1.24 ± 0.00^{g}	1.45 ± 0.00^{g}	1.61 ± 0.00^{g}
F8E2	1.11 ± 0.00^{d}	1.20 ± 0.01^{e}	1.34 ± 0.01^{f}	1.55 ± 0.01^{f}	1.73 ± 0.01^{f}

ST group: 4.00% crude fiber (CF) in diet, F6 group: 600% CF in diet, F8 group: 8.00% CF in diet, F6E1 group: 6.00% CF in diet along with 1X MEC, F8E1 group: 8.00% CF in diet along with 1X MEC, F6E2 group: 6.00% CF in diet along with 2X MEC, and F8E2 group: 8.00% CF in diet along with 2X MEC.

[a-g] The values in each column sharing similar superscripts show non-significant difference ($p < 0.05$).

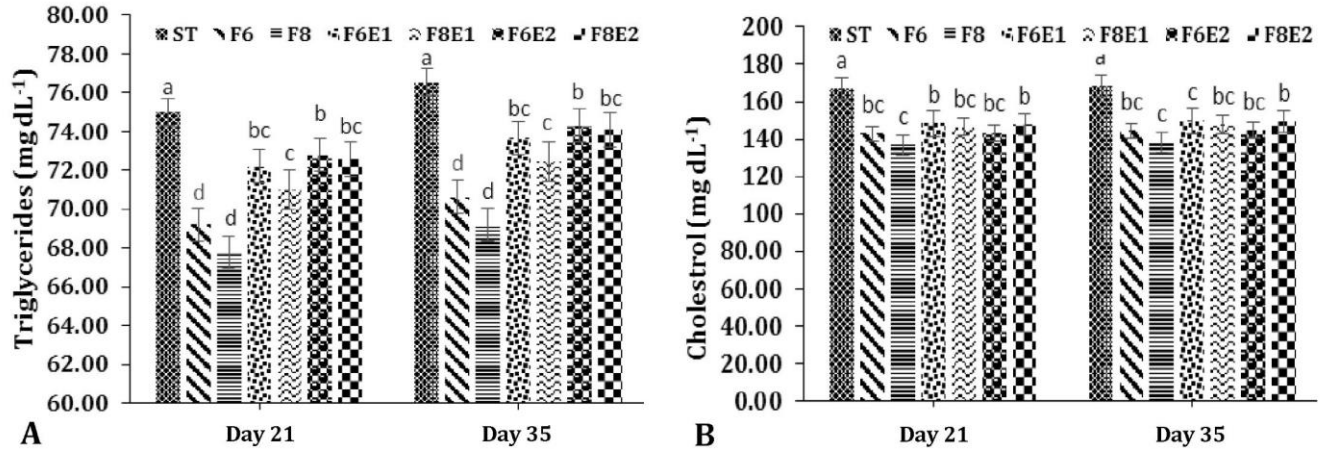

Fig. 3. Lipid profile analysis including **A)** triglycerides and **B)** cholesterol. ST group: 4.00% crude fiber (CF) in diet, F6 group: 6.00% CF in diet, F8 group: 8.00% CF in diet, F6E1 group: 6.00% CF in diet along with 1X MEC, F8E1 group: 8.00% CF in diet along with 1X MEC, F6E2 group: 6.00% CF in diet along with 2X MEC, and F8E2 group: 8.00% CF in diet along with 2X MEC.

[abc] Different letters indicate significant differences among the groups ($p < 0.05$).

Table 5. Weight of different organs and levels of some biochemical parameter of broilers supplemented with varying amounts of dietary crude fibers and the multi-enzyme cocktail BsBD92 at growing (21 days) and finisher (35 days) phases. Data are presented as mean ± SD.

Parameters	Age (Days)	ST	F6	F8	F6E1	F8E1	F6E2	F8E2
Bursa of Fabricius weight (g)	21	0.23 ± 0.00^{a}	0.22 ± 0.00^{b}	0.21 ± 0.00^{b}	0.21 ± 0.00^{b}	0.21 ± 0.00^{b}	0.21 ± 0.00^{b}	0.21 ± 0.00^{b}
	35	0.05 ± 0.00^{a}	0.05 ± 0.00^{a}	0.05 ± 0.00^{a}	0.05 ± 0.00^{a}	0.05 ± 0.00^{a}	0.05 ± 0.00^{a}	0.05 ± 0.00^{a}
Pancreas weight (g)	21	0.25 ± 0.00^{cd}	0.27 ± 0.00^{ab}	0.29 ± 0.00^{a}	0.24 ± 0.00^{d}	0.26 ± 0.00^{bc}	0.25 ± 0.00^{cd}	0.27 ± 0.00^{ab}
	35	0.21 ± 0.00^{d}	0.23 ± 0.00^{bc}	0.28 ± 0.00^{a}	0.23 ± 0.00^{c}	0.24 ± 0.00^{b}	0.22 ± 0.00^{c}	0.24 ± 0.00^{bc}
Thymus weight (g)	21	0.23 ± 0.00^{a}	0.23 ± 0.00^{a}	0.23 ± 0.00^{a}	0.23 ± 0.00^{a}	0.23 ± 0.00^{a}	0.23 ± 0.00^{a}	0.23 ± 0.00^{a}
	35	0.22 ± 0.00^{bc}	0.22 ± 0.00 ^{abc}	0.22 ± 0.00^{abc}	0.21 ± 0.00^{c}	0.22 ± 0.00^{bc}	0.24 ± 0.00^{a}	0.23 ± 0.00^{ab}
ALT (U L^{-1})	21	25.00 ± 0.89^{c}	28.00 ± 0.63^{b}	32.20 ± 1.16^{a}	22.20 ± 1.32^{d}	25.00 ± 0.89^{c}	24.80 ± 0.74^{c}	26.80 ± 0.74^{bc}
	35	25.77 ± 0.92^{c}	28.86 ± 0.65^{b}	33.19 ± 1.20^{a}	22.88 ± 1.36^{d}	25.77 ± 0.92^{c}	25.56 ± 0.77^{c}	27.62 ± 0.77^{bc}
AST (U L^{-1})	21	79.6 ± 0.48^{a}	80.00 ± 0.63^{a}	80.00 ± 0.63^{a}	80.00 ± 0.63^{a}	80.00 ± 0.63^{a}	79.60 ± 0.48^{a}	79.40 ± 0.48^{a}
	35	82.06 ± 0.50^{a}	82.47 ± 0.65^{a}	82.47 ± 0.65^{a}	82.47 ± 0.65^{a}	82.47 ± 0.65^{a}	82.06 ± 0.50^{a}	81.85 ± 0.50^{a}
Creatinine (mg dL^{-1})	21	0.36 ± 0.10^{a}	0.34 ± 0.05^{a}	0.34 ± 0.05^{a}	0.32 ± 0.04^{a}	0.3 ± 0.06^{a}	0.34 ± 0.08^{a}	0.38 ± 0.15^{a}
	35	0.36 ± 0.10^{a}	0.34 ± 0.05^{a}	0.34 ± 0.05^{a}	0.32 ± 0.04^{a}	0.3 ± 0.06^{a}	0.34 ± 0.08^{a}	0.38 ± 0.15^{a}
Urea (mg dL^{-1})	21	3.40 ± 0.49^{a}	3.80 ± 0.75^{a}	3.80 ± 0.75^{a}	3.60 ± 0.49^{a}	3.80 ± 0.75^{a}	3.40 ± 0.49^{a}	3.80 ± 0.75^{a}
	35	3.43 ± 0.49^{a}	3.83 ± 0.76^{a}	3.83 ± 0.76^{a}	3.63 ± 0.49^{a}	3.83 ± 0.76^{a}	3.43 ± 0.49^{a}	3.83 ± 0.76^{a}

ST group: 4.00% crude fiber (CF) in diet, F6 group: 600% CF in diet, F8 group: 8.00% CF in diet, F6E1 group: 6.00% CF in diet along with 1X MEC, F8E1 group: 8.00% CF in diet along with 1X MEC, F6E2 group: 6.00% CF in diet along with 2X MEC, and F8E2 group: 8.00% CF in diet along with 2X MEC. ALT: Alanine transaminase; AST: Aspartate transaminase

[a-d] The values in rows with different superscripts indicate statistical significance ($p < 0.05$).

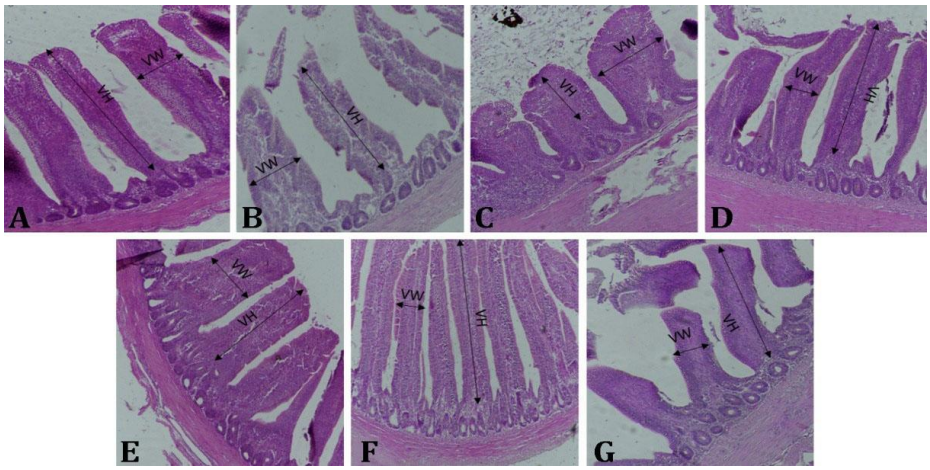

Fig. 4. Histomorphometry of broiler chicken duodenum in experimental groups given varying amounts of dietary crude fibers and the multi-enzyme cocktail BsBD92. **A)** ST group: 4.00% crude fiber (CF) in diet, **B)** F6 group: 6.00% CF in diet, **C)** F8 group: 8.00% CF in diet, **D)** F6E1 group: 6.00% CF in diet along with 1X MEC, **E)** F8E1 group: 8.00% CF in diet along with 1X MEC, **F)** F6E2 group: 6.00% CF in diet along with 2X MEC, and G) F8E2 group: 8.00% CF in diet along with 2X MEC. VH: Villus height; VW: Villus width (Hematoxylin and Eosin staining, 200×).

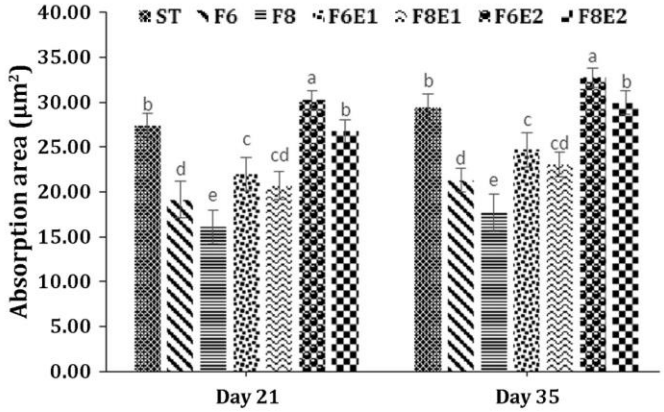

Fig. 5. The intestinal absorption area of broiler chicks given varying amounts of dietary crude fibers and the multi-enzyme cocktail BsBD92. ST group: 4.00% crude fiber (CF) in diet, F6 group: 600% CF in diet, F8 group: 8.00% CF in diet, F6E1 group: 6.00% CF in diet along with 1X MEC, F8E1 group: 8.00% CF in diet along with 1X MEC, F6E2 group: 6.00% CF in diet along with 2X MEC, and F8E2 group: 8.00% CF in diet along with 2X MEC.

Discussion

The results of the present study revealed that the enzymes showed their highest activities at 50.00 ˚C, indicating a preference for this temperature range for catalytic efficiency and their potential application in various industrial processes; these findings are in line with the literature.[21,22] Further, MEC BsBD92 exhibited the highest levels of activity at a pH of 5.50, within a pH range spanning from 3.50 to 8.50. This finding suggests that the enzymes are most efficient and effective at catalyzing their respective reactions within this specific pH window. The observed pH dependence of enzyme activity is consistent with previous studies in the field, which have also reported optimal enzyme performance at slightly acidic conditions.[23,24] This pH range encompasses both acidic and slightly alkaline conditions, indicating the versatility and adaptability of the enzymes to function across a broad pH level in digestive tract and intestine which are slightly acidic.[25,26]

Our study also revealed that MEC with CF diet had significant impact on the digestive system and revealed a notable disparity in the BW variable across various treatment groups throughout the duration of the study. The current results are consistent with the reported data, where the use of a carefully designed enzyme mixture in bird food has resulted in a notable increment of nutrient digestion, reduced lipid peroxidation, enhanced plasma biochemical traits, and boosted immune response and BW.[3,9,27] The improved digestion efficiency of nutrients leads to decreased FI, as the bird obtains more nutrients from a smaller amount of feed. This observation implies that these birds lack the enzymes needed to metabolize certain dietary components. These findings are also in line with reported data. [3,5,11] It can be inferred that MEC BsBD92 supplementation may have demonstrated a beneficial effect on the gut stress and inflammation, breakdown of anti-nutritional factors of CF, and enhancement of overall gut health. This phenomenon results in enhanced availability and absorption of nutrients.[3,5,11,27] However, it is of utmost importance to recognize that the exact response to enzyme supplementation can vary depending on the bird species, nutritional composition of the diet, and specific type and dosage of enzymes administered. To accurately determine the underlying factors influencing the observed low FCR, high PI, and enhanced FI when incorporating MEC into the diet, it is crucial to conduct thorough scientific research, considering the distinct circumstances of the study.[3]

Further, bursa and thymus weights differences were not statistically significant. This finding suggests that chickens may be able to tolerate a CF diet without suffering any side effects through a process of adaptation. The same effects of multi-enzyme supplementation on the liver, spleen, thymus, bursa, and other internal organs have been documented in the literature.[3,28] The intestinal weight of MEC supplemented groups (F6E1, F8E1, F6E2, and F8E2) was significantly lower than non-supplemented groups (F6 and F8); the intestine length in MEC supplemented groups was significantly higher than non-supplemented groups. The mechanism of length increase is not well defined, because enzymatic supplementation has been suggested for optimal growth performance, including low FI and FCR, better meat quality, gut health, lower intestinal weight, and high PI levels, due to the improvement in nutrient digestibility and utilization.[3] Therefore, it is proposed that it may indirectly involve in intestinal length or physical structure by improving gut health. [29] It is also suggested that the increase of the intestine length is an adaptive mechanism to the augmented need for exogenous enzymes.[30] Few studies have reported that the enzyme supplementation of DF exerts a profound influence on the dimensions and weight of intestine. These enzymatic supplementations have elicited a substantial augmentation in the longitudinal extension of the GIT, specifically from its initial point to the anatomical region known as a cecum.[30,31]

Our study demonstrated that the inclusion of MEC in the diet did not result in any adverse effects on liver function. This study is in line with the dietary effect by lipase, showed low levels of ALT and AST in serum after supplementation.[32] Similarly, another study has reported the identical specific impact of an enzyme mixture on the liver, resulting in a reduction of ALT and AST.[33] However, the levels may increase slightly due to the muscle trauma instead of liver damage.[34] No discernible alterations were also observed in the concentrations of creatinine and urea, pivotal biomarkers employed for the evaluation of renal functionality. So, these results showed no impairments in renal function by MEC supplementation. The empirical evidence suggests a notable augmentation in the process of protein anabolism.[35] The MEC-treated groups (especially F6E2) showed marginally diminished levels (non-significantly) of creatinine and urea concentrations in comparison with the F6 and F8 groups at the starter and finisher phases. The present observations align with prior investigations that have demonstrated an augmentation in protein anabolism, leading to a reduction of uric acid and amino acids in serum.[35]

It is a suitable indication for determining how poultry reacts to dietary changes and food components. The lipid profile, including TG and CHO (mg dL^{-1}), showed increased levels among the MEC groups compared to the F6 and F8 groups without MEC at the starter and finisher phases. This phenomenon may be explained by decreased lipid metabolism and absorption from the colon's posterior side, which may be brought on by a fibrous layer obstructing the flow of bile salts.[36] This result is consistent with earlier research on the addition of multi-enzyme to feed, showed an increase in plasma total protein, globulin,

and blood CHO. The improvement in bile salt function and the chyme's emulsifying abilities, raising total CHO in the blood, may be responsible for the rise in blood lipid profile after the addition of enzymes combination.[35]

The impact of CF diet on the intestine manifested as a fascinating phenomenon, characterized by the remarkable transformation of the villi. The effect of CF on intestine is villi shortening and thickening as the percentage content increased (6.00 to 8.00% CF in F6 and F8 groups) and it reduced absorption areas. The addition of MEC was directly proportional to the increase in VH, VW, CrD, and CrW. However, the ideal composition of MEC supplementation was 6.00% CF plus 2X MEC in F6E2 group considering all tested assays and hence, showed the highest absorption areas. The CF is an indigestible part of feed and its high concentration has negative impact on overall villus morphology (VH, VW, CrD, and CrW) of small intestine, reducing the surface area of nutrients absorption. However, the MEC in diet reduces this negative impact by increasing its digestibility and absorption even at high level. This increment in villus and crypt structure and size is referenced as gut health, growth performance, feed efficiency, and immune function.[5] Therefore, enzyme supplementation can be a beneficial strategy for improving the health and performance of poultry, especially when they are fed diets that are high in CF. Another possibility is improvement of villus and crypt structure by GIT microbial ecology which is controlled by types of supplemented enzymes.[9,37] An additional benefit of the intricate symbiosis between chickens and their GIT microbiota is the remarkable process of NSPs fermentation, leading to the SCFAs production. The SCFAs play a crucial role in strengthening of the mucosal lining structural integrity. They also ensure the balanced regulation of immune processes, ensuring a state of equilibrium, and aid in the maturation of the bird's immune system.[38] This study suggests that MEC does not show any discernible toxic effects on the feed, and hepatic and renal systems. Furthermore, it appears that the hepatic and renal systems possess inherent mechanisms enabling them to safeguard themselves against any potential toxic effects that may arise from exposure to MEC. The present investigation has received validation from existing literature, demonstrating that the supplementation of various doses of multi-enzymes does not exert a statistically significant impact on the biochemical composition of blood serum, internal organs of the body, or indicators of liver and renal functionality.[28]

The complex fibrous substrates making up poultry feed must be broken down by a mixture of enzymes. The findings revealed that adding MEC BsBD92 to the chicken feed has a good effect on carcass traits, growth performance, and intestinal morphology without having a detrimental effect on safety measures. In order to increase production, it could be a useful addition to poultry diets. To validate these results and evaluate their applicability to commercial chicken production, more studies and field experiments could be required.

Acknowledgments

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Conflict of interest

The authors declare no conflict of interest.

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