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Article

Efficacy of *Saccharomyces cerevisiae* Fermentation Product and Probiotic Supplementation on Growth Performance, Gut Microbiome and Immunity of Broiler Chickens

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Abstract: Concern for global health security and the environment due to the emergence of antibiotic-resistant bacteria and antibiotic residues in meat and other livestock products has led many countries to restrict the use of antibiotics in animal feed. This experiment was performed to assess the impact of dietary supplementation with a probiotic (*Bacillus subtilis*) and a postbiotic (*Saccharomyces cerevisiae* fermentation product) on growth performance, carcass traits, blood haemato-biochemical profile, gut microbiome, gut morphology, and immune response in broilers as an alternative to antimicrobials in poultry production system to minimize the effect on global health security. A total of 324-day-old Ven Cobb 400 broiler chicks were randomly divided into three dietary groups, each containing 12 replicated pens (n = 12), .and each replicate contained nine broiler chickens. The dietary groups consisted of 1) a basal diet without any growth promoting (T_1), 2) the basal diet augmented with *Bacillus subtilis* at 200 g/MT feed (T_2), and 3) the basal diet supplemented with *Saccharomyces cerevisiae* fermentation product at 1.25 kg/MT feed (T_3). To calculate body weight gain, all birds and residual feed were weighed on a replicated basis over days 0, 7, 14, 21, 28, 35, and 42; mortality was recorded daily. Each replicate's mortality percentage was calculated and used to adjust BW, ADFI, and FCR calculations at the trial's end. At the end of the trial (42 d), two chickens from each replicate (24 chickens per group) were slaughtered for carcass traits, gut microbiome, and morphology measurements. Blood samples were collected for the haemato-biochemical profile on 35d and antibody titer on 28d and 35d.Feeding with SCFP (T_3 group) significantly improved average daily feed intake (ADFI) and average daily gain (ADG) of chickens compared to the T_1 (control) and T_2 (probiotic) groups from 1 to 14 days of age. FCR was significantly improved ($P<0.05$) in SCFP-fed birds (T3) relative to the control (T1)over the entire experimental period (1-42 days). Carcass traits and blood haemato-biochemical parameters remained unaffected ($P>0.05$) by any diets. However, cholesterol levels and concentrations of corticosterone were significantly lower ($P<0.05$) in SCFP-fed birds (T3) compared to those of the probiotic (T_2) and control (T_1) groups. Total *E. coli*, *Enterohaemorrhagic E. coli*, *ESBL-producing Enterobacteriaceae*, and *Salmonella* counts were significantly higher in the T_1group compared to T_3 and T_2 groups ($P<0.05$) and *Salmonella* counts were lower in T3 when compared to T2 ($P<0.05$). However, there was no significant difference in *Lactobacillus* count among treatment groups. PCR based characterization of ESBL-producing *Enterobacteriaceae* revealed maximum presence of $bla_{CTX\text{-}M\text{-}Type}$ (6/15, 40%), followed by $bla_{SHV\text{-}Type}$ (5/15, 33.3%) and $bla_{TEM\text{-}Type}$ (4/15, 26.6%). No variation in the occurrence pattern of ESBL/beta-lactamase associated genes in the studied ESBL- producing *Enterobacteriaceae* isolates was observed between the treatment groups. A significant increase in villi height and villi height to crypt depth ratio (VH: CD) ($P<0.05$) was observed in both T_3 and T_2 groups. Contrarily, villi widths did not vary across the dietary groups ($P>0.05$). The control group (T_1) displayed a notable deeper in crypts when compared to the T_3 and T_2 groups ($P<0.05$). On day 28, the SCFP-fed birds (T_3) and those fed the probiotics (T_2) exhibited a significant increase ($P<0.05$) in antibody

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titers against Newcastle disease virus and Infectious bursal disease virus. Increase in antibody titre against IBDV in birds with the addition of SCFP in the diet was detected for the first time. However, there were no significant differences observed on day 35. The phagocytic activity of neutrophils and lymphocytes in broiler chickens did not show any noticeable variations among the different groups at day 35(P>0.05).It can be concluded that *Saccharomyces cerevisiae* fermentation product and *Bacillus subtilis* probiotic could be viable alternatives to antimicrobials in poultry production considering beneficial impacts in broilers fed antibiotic-free diet.

Keywords: *Bacillus subtilis;* Broiler; Fermentation product; Gut microbiome; *Saccharomyces cerevisiae*

Introduction

One of the most profitable and productive agricultural industries is the poultry industry. Recent advancements in nutrition, genetics, housing management, chicken health and welfare have allowed it to flourish, resulting in a growing of egg production at 8.51% and broiler production at 7.52% [1]. The use of antibiotics as growth promoters has resulted in high levels of poultry output worldwide; these antibiotics have impacted chickens' intestinal flora and immune systems to aid in controlling disease [2,3].Concern for global health security and the environment due to the emergence of antibiotic-resistant bacteria and antibiotic residues in meat and other livestock products has led many countries to restrict the use of antibiotics in animal feed [4]. This has encouraged nutritionists and feed manufacturers around the world to search for alternatives to AGPs that can maintain efficient poultry production while ensuring that poultry meat and eggs are safe. Possible replacements for AGPs include feeding prebiotics, probiotics, synbiotics, enzymes, herbs, essential oils, acidifying feed with organic acids and postbiotics[5].

While probiotics have many positive health benefits, their functionality and effectiveness are subject to debate. Recent findings suggest that for a variety of animal species, probiotics need to be tailored more specifically in order to maximize their beneficial effects. Furthermore, certain strains of probiotic bacteria were discovered to have antibiotic-resistance genes which can be passed on via plasmid transfer [6,7]. Additionally, studies showed that some probiotics can have a detrimental effect on the host by causing local inflammation in healthy hosts and exacerbating tissue inflammation in those with inflammatory bowel disease [8]. The ‘postbiotic ‘hasemerged which extends the scope of the probiotic concept beyond its inherent viability [9]. The term "postbiotic" refers to the soluble factors (stabilized bacteria, cellular products, or metabolic by-products) secreted by living microbes or released after microbial lysis [10]. These are mainly derived from *Lactobacillus*, *Bifidobacterium*, *Streptococcus*, faecal bacteria[11,12], and Saccharomyces cerevisiae yeast [13,14]. Recent research suggests that postbiotics offer various health benefits through immune system modulation (cell wall compounds may strengthen immunity), increased adhesion to intestinal cells (which restricts pathogen growth), and secretion of various metabolites [11,15]. Non-viable microorganisms or microbial cell extracts have an additional advantage in Probiotic-supplemented feed preparations as their viability may differ and dead cells may outnumber the live cells [16]. Moreover, these nonviable microbes and extracts can significantly reduce shelf life concerns while avoiding the risks related to microbial uptake and infection in consumer products [17].The aim of this study was to examine the effects of probiotic (*Bacillus subtilis*) and postbiotic (*Saccharomyces cerevisiae* fermentation product, SCFP) on the growth performance, immunity, gut health, and carcass characteristics of broiler chickensas an alternative to antimicrobials in poultry productionsystem to minimize the effect on global health security.

Materials and Methods

Broiler Chickens, Experimental Design And diets

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Three hundred twenty-four one-day-old mixed sex commercial broiler chickens (Vencobb 400, Venkys, Pune, India) were randomly divided into three dietary groups. Each group comprised of twelve replicated pens (n= 12) and each replicate contained nine broiler chickens. The dietary groups consisted of 1) a basal diet without any growth promoter (T_1); 2) the basal diet added with Probiotic -*Bacillus subtilis* (Zeus Biotech private limited, Mysore, India) at the rate of 200 g/ MT feed (T_2) 3) the basal diet added with Postbiotic- *Saccharomyces cerevisiae* fermentation product (SCFP; Diamond V Original XPC) at the rate of 1.25 kg/MT feed(T_3). The basal diet based on maize- soybean in mash form was formulated to meet or exceed nutritional requirements of broiler starter (day 1–14), grower (day 15–28) and finisher (day 29–42) chickens using a ration formulation software as per the commercial Vencobb400 broiler chicken recommendations [18]. The basal diet contained 22.19% of crude protein (CP) with 3000 kcal/kg of metabolizable energy (ME) for broiler starters (day 1–14), 20.80 % of CP with 3100 kcal/kg of ME for broiler growers (day 15–28), and 19.2% of CP with 3200 kcal/kg of ME for broiler finishers (day 29–39) (Tables 1 and 2).Premixes were prepared separately, which were added to the basal diet and mixed homogenously in a feed mixer to obtain the probiotic and postbiotic diets. The experimental diets were prepared every week. The blended mash feeds were packed in separate high density polyethylene bags with inner liner. The diets and water were provided ad libitum. The study protocol was approved by Institutional Animal Ethics Committee of West Bengal University of Animal and Fishery Sciences, Kolkata, India.

Table 1. Ingredient and nutrient compositionofbasal diets.

SL. No.	Ingredients (%)	Starter (1-14 d)	Grower (15-28 d)	Finisher (29-42 d)
1	Maize	57.289	59.381	62.519
2	Soyabean meal	37.247	34.035	30.003
3	Soybean oil	1.841	3.143	4.208
4	Dicalcium phosphate	1.503	1.375	1.261
5	Limestone phosphate	0.756	0.835	0.828
6	Salt	0.322	0.324	0.326
7	DL-methionine	0.314	0.260	0.231
8	L-lysineHCL	0.226	0.154	0.131
9	L-threonine	0.084	0.055	0.055
10	Toxin Binder[1]	0.050	0.050	0.050
11	Sodium bi-carbonate	0.100	0.100	0.100
12	Bio-Choline[2]	0.050	0.070	0.070
13	Trace mineral mixture[3]	0.100	0.100	0.100
14	Vitamin premix[4]	0.100	0.100	0.100
15	Antioxidant[5]	0.010	0.010	0.010
16	Phytase[6]	0.010	0.010	0.010
Nutrient composition				
1	Metabolizable energy (kcal/kg)[7]	3000.00	3100.00	3200.00
2	Crude protein (%)[8]	22.24	20.74	19.12

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3	Ether extract (%)[8]	4.37	5.73	6.81
4	Crude fiber (%)[8]	3.72	3.66	3.54
5	Calcium (%)[8]	0.93	0.90	0.86
6	Available phosphorus (%)[7]	0.45	0.42	0.39
7	Digestible lysine (%)[7]	1.22	1.09	0.98
8	Digestible methionine (%)[7]	0.60	0.53	0.49
9	Digestible methionine + cysteine (%)[7]	0.88	0.80	0.74
10	Digestible threonine (%)[7]	0.77	0.70	0.65

[1] Niltox™, Zeus Biotech Limited, Mysore, India. [2]BioCholine 60, Indian Herbs SpecialitiesPvt. Ltd. Solan, Himachal Pradesh, India [3]contains zinc 4.0%, manganese 4.0%, iron 1.5%, copper 0.8%, iodine 0.4%, selenium 300ppm, chromium 200ppm (Zenex animal health India Pvt. Ltd, Patiya, Ahmedabad, India) [4]contains vitamin E100g, vitamin A 40000000 IU, vitamin D_3 12000000IU, pantothenic acid 60g, vitamin K 8g, vitamin $B_1$120g, vitamin B_2 24g, vitamin B_6 10g, vitamin B_{12} 0.10g, biotin 0.40g, Folic acid 4g, niacin 100g (DSM Nutritional Products India Pvt. Ltd. Mahabubnagar, Telangana, India). [5]Endox, Kemin Industries, Inc., USA. [6] quantamblue, AB Vista, Pune, India. [7] Calculated values (based on the Asia South feed ingredients report 2016, EvonikPte Ltd, Singapore). [8] Analysed values (average of triplicate values).

Table 2. Effect of probiotic (*Bacillus subtilis*) andpostbiotic (*Saccharomyces cerevisiae* fermentation products) on final body weight (BW), Average daily gain (ADG), average daily feed intake (ADFI) and feed conversion ratio (FCR) and liveability of broiler chickens.

Attribute	**Treatment[1]**			**SEM[2]**	**P-Value**
	T1	**T2**	**T3**		
ADG (g/d)					
1-14 d	30.12[b]	30.35[b]	31.99[a]	0.232	0.001
15-28 d	76.19	76.10	75.11	0.377	0.446
29-42 d	85.71	87.84	89.52	1.699	0.669
1-42 d	64.01	64.76	65.54	0.565	0.554
Final BW (g)	2737.33	2770.02	2802.45	23.704	0.547
ADFI (g/d)					
1-14 d	33.74[b]	33.76[b]	35.65[a]	0.232	0.000
15-28 d	104.89[a]	102.57[b]	100.38[c]	0.431	0.000
29-42 d	154.47	153.03	153.36	1.367	0.090
1-42 d	97.70	96.45	96.46	0.533	0.561
FCR (g intake/g gain)					
1-14 d	1.12	1.11	1.12	0.058	0.922
15-28 d	1.38[a]	1.35[ab]	1.34[b]	0.007	0.059
29-42 d	1.81	1.76	1.73	0.022	0.293
1-42 d	1.53[a]	1.49[ab]	1.47[b]	0.008	0.015
Livability (%)	97.22	97.22	97.22	0.813	1.000

[ab]means bearing different superscripts in the same row differ significantly ($p \le 0.05$). [1] The control diet (T_1), control diet was supplemented with probiotic (*Bacillus subtilis*) at 200mg/MT feed (T_2), Postbiotic (*Saccharomyces cerevisiae* fermentation products) at 1.25 kg/MT feed (T_3). [2] SEM, standard error of means (n=12).

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Management and Rearing of Birds

The experimental house, feeding and watering troughs were properly cleaned and disinfected before arrivals of the chickens. The chickens were kept in floor pens (1.22 m × 0.76 m) that were separated by plastic wire netting. Rice husk and chopped paddy straw were utilized as litter, and sterile plastic feeders and water troughs were supplied in every pen. Continuous lighting was supplied to the chicks through compressed fluorescent lamps during the first two days of brooding, and this was then modified to have 23 hours of light interrupted by one hour of darkness each night. The experimental poultry house temperature was controlled by the heating elements throughout the experiment. The temperatures were gradually decreased from 32°C on day 1 to 24°C on day 22. Proper ventilation was ensured through the use of exhaust fans during the entire trial period. All the birds were vaccinated against Newcastle Disease virus (NDV) at 5 and 21 days of age, and Infectious Bursal Disease virus (IBDV) at 12 days of age.

Measurement of Performance Traits

Body weight (BW) of all chickens was measured on day 1, followed by weekly assessments and one final measurement taken in the morning on the last day of the growth trial. Average BW was calculated for each replicate. Weekly feed intake was determined by subtracting remaining feed from total feed offered per pen. Average daily feed intake (ADFI) was calculated for each replicate, dividing total feed intake per day in each pen by the total number of chickens present. Feed conversion ratio (FCR; grams of feed intake per gram of growth) was calculated based on cumulative feed intake and weight gain in each replicated pen. Mortality rate, if any, was recorded daily for each replicate with post-mortem examinations conducted to identify exact cause of death. At the end of the trial, mortality percentage for each replicate was calculated and used to adjust BW, ADFI and FCR calculations.

Slaughtering of Broiler Chickens and Measurements of Carcass Traits

At day 42, two birds (one male and one female with average BW close to the average BW of that replicate will be selected randomly) per replicate was slaughtered by cervical disarticulation for evaluation of carcass traits. Eviscerated carcass weight was measured after removal of skin, feather, head, shank, intestine and giblets, and different parts were weighed.

Collection of Blood Samples for Serum Biochemical Analyses

Blood samples for hematobiochemical and hormone assay were collected at day 35 after 12-hours of fasting. Two aliquots of blood samples were collected from wing vein of broiler chickens (2 birds were randomly chosen from each pen, total 24 birds per treatment).The first aliquot were placed in a tube containing anticoagulant (EDTA) for measurement of haemoglobin (Hb), total leukocyte counts (WBCs), and differential leukocyte counts, heterophil: lymphocyte (H: L) ratio at day 35 as per standard haematological procedure[19] Second aliquot of blood samples were collected without anticoagulant and serum were harvested and stored at -20^{0}C until analysis. Concentrations of serum metabolites (glucose, total protein, albumin, uric acid, triglycerides, cholesterol) were measured using commercial kits (DiaSys diagnostic India Pvt. Ltd., Mumbai, India). Concentrations of corticosterone were determined by commercial ELISA kit (DRGDiagnostics, Germany).

Enumeration of Pre-Caecal Bacterial Count

The caecal contents of the intestine were aseptically collected from the chickens after the slaughter at day 42 andwere placed into sterile sample collection bag (HiMedia, India). The samples were processed on same day for bacteriological study. Population of total bacteria, pathogenic/zoonotic *E. coli* (Enterohaemorrhagic*E. coli*), antimicrobial resistant bacteria (*ESBL*-producing *Enterobacteriaceae*), *Salmonella,* and *Lactobacillus*in caecal content were determined as per standard procedure [20]. One gram of the caecal content was serially diluted to 10 fold with sterile phosphate buffer saline (PBS), 10 µLwas placed on sorbitol-MacConkey agar (for

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Enterohaemorrhagic*E. coli*, HiMedia, India), ESBL agar [for ESBL-producing *Enterobacteriaceae (KESC group), HiMedia, India), Xylose Lysine Deoxycholate agar* (for *Salmonella*, HiMedia, India), *Lactobacillus* agar (HiMedia, India) and were incubated at 37°C for 24 or 48 hours, and the characteristic colonies for each bacterial population were enumerated in a digital colony counter(HiMedia, India) and the numbers were expressed as Log10 colony forming units (CFU) per gram of sample. The selected isolates (n=15, 5 isolates from each treatment group) of ESBL-producing *Enterobacteriaceae (KESC group) were further characterized with PCR for presence of ESBL/beta-lactamase variants (bla*$_{CTM\text{-}Type}$, *bla*$_{SHV\text{-}Type}$, *bla*$_{TEM\text{-}Type}$) [21].

Morphological Study of Small Intestine

At day42, 24 chickens from each dietary treatment were slaughtered and small intestinal tissue samples were collected for measurement of the height and width of the intestinal villus and crypt depth. The small intestine was removed and 2–3 cm sections of duodenum, jejunum (between the entry of bile duct and Mackel's diverticulum) and ileum were removed and rinsed with sterile PBS. The cross-sectional lengths of 1 cm were fixed in buffered formaldehyde solution (100 mL/L; pH 7.2), followed by embedding in paraffin wax. The slides with the tissue sections were then stained with Delafield's Hematoxyline and Eosin, and mounted on distreneplasticiser xylene (DPX) as per the standard histopathological procedures [22]. All the measurements were made using an ocular micrometer (under a microscope fitted with a stage micrometer) and image analysis software (Biowizard 4.2, Dewinter Optical and New Delhi, India). The criterion for villus selection (twelve villi per section) was based on the presence of intact lamina propria. The villus height (VH) [in micrometre (μm)] was measured from the tip of the villus to the villus-crypt junction, while crypt depth (CD) was defined as the depth of the invagination between two villi. At least three sections with ten observations were viewed for each sample and the values [in micrometre (μm)] were averaged to constitute a single observation.

Measurement of Antibody Titre against Newcastle Disease virus and Infectious Bursal Disease virus

The humoral immune response was examined by measuring the antibody titre after vaccination against Newcastle disease virus (NDV) and Infectious bursal disease (IBDV). Live lentogenicB1 strain (eye drop, 0.2 mL; Venkateswaara hatcheries Private Limited, India) and live lentogenicLaSota strain (0.2 mL; Venkateswaara hatcheries Private Limited, India), IBD live intermediate plus type (0.2 mL; Venkateswaara hatcheries Private Limited, India) vaccines were administered on day 5,21 and 14, respectively. Blood samples (2 mL) were collected on day 28 and 35 from the wing vein (two birds randomly selected from each replicate pen), immediately transferred to the centrifuge tubes without anticoagulant, and serum was harvested by centrifuging the whole blood. The NDV and IBDV antibody titres were detected by an ELISA kit (IDEXX Laboratories Inc., USA). The optical density (OD) for each of the samples was determined in duplicates, and the mean OD values thus obtained were used to interpret the results. Cell-mediated immunity was measured by *in vitro* phagocytic activity of neutrophils and lymphocyte proliferation response at day 35[23].

Chemical Analysis of Feeds Samples

Feed samples were analyzed[24] for dry matter (DM; method 934.01), CP (method 968.06; Kelplus, Pelican Equipments, Chennai, India), crude fibre (CF; Foss Fiber Cap 2021 Fiber Analysis System, Foss Analytical, Hilleroed, Denmark) and ether extract (EE; method 920.39; Socsplus, Pelican Equipments, Chennai, India) and calcium content following the method of Talapatra *et al.,* [25]. The AIA contents [25]of the diets were analysed according to the method described by Furuichi and Takahashi [26]

Statistical Analysis

The data were analysed by one-way analysis of variance (ANOVA) using SPSS [27] in a completely randomised design with a model containing treatment as the main effect and pen as an

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experimental unit for body weight gain, feed intake and FCR, while individual bird will be treated as experimental unit for other parameters. Mortality data fulfilled the homogeneity criteria and thus were not transformed for statistical analyses. Probability values of $P \leq 0.05$ were declared as significant and the values of $P \leq 0.01$ were declared as trend. When treatment effect was significant, the differences among the treatment means were detected using Tukey's test.

Results

Average Daily Gain, Feed Intake and Feed Efficiency

The average daily gain (ADG) of chickens increased significantly ($P < 0.05$) in the T_3 group compared to the T_1 and T_2 groups from 1 to 14 days of age (Table 2). No significant differences among treatment groups were found in the rest of the experimental period or over the entire experiment period (1–42 d). The average daily feed intake (ADFI) of chickens was significantly higher ($P < 0.05$) in T3 groups compared with that of the T_1 and T_2 groups from 1 to 14 days of age. Subsequently, ADFI for the T_1 group was significantly greater than those for both the T_2 and T_3 groups from 15-28days of age. However, there were no significant differences between treatment groups from 29-42 days or over the entire experiment period (1–42 d). No significant differences in FCR were noted during the starter (1-14 days) and finisher (29-42 days) phase. During the grower phase (15-28 days), FCR was tended to lower ($P<0.07$) in SCFP-fed birds (T_3) compared to T_1 and T_2. The FCR during the overall period (1-42 days) for the T_3 group was improved ($P<0.05$) in comparison to the T_1, while the T_2 group was not different from T_1 and T_3.

Carcass Traits

No statistically significant differences ($P > 0.05$) were observed in slaughter BW, eviscerated carcass weight, dressing percentage, breast, frame, thigh, drumstick, wing, neck, gizzard, liver, heart, spleen, bursa and abdominal fat weight in grams across the various treatment groups (Table-3).

Table 3. Effect of probiotic (*Bacillus subtilis*) andpostbiotic (*Saccharomyces cerevisiae* fermentation products) on carcass traits in broiler chickens at day 42.

Carcass traits	**Treatment[1]**			**SEM[2]**	**P-Value**
	T1	**T2**	**T3**		
Slaughter body weight (g)	2718.92	2757.08	2784.58	24.096	0.549
Eviscerated carcass weight (g)	1829.83	1867.58	1874.92	16.685	0.510
Dressing Percentage	67.34	67.72	67.37	0.251	0.802
Breast (g)	731.58	739.75	754.83	12.849	0.766
Frame (g)	315.00	334.25	325.92	5.548	0.375
Thigh (g)	279.33	283.00	284.33	6.226	0.947
Drumstick (g)	254.83	272.33	268.50	5.832	0.449
Wing (g)	143.67	145.83	151.92	3.707	0.655
Neck (g)	74.71	78.83	80.21	2.204	0.584
Gizzard (g)	57.56	56.21	54.98	0.775	0.408
Liver (g)	44.92	42.20	43.43	0.702	0.294
Heart (g)	11.56	11.46	11.18	0.152	0.581
Spleen (g)	2.78	2.74	2.62	0.078	0.712
Bursa (g)	1.52	1.46	1.48	0.055	0.894
Abdominal fat (g)	38.58	40.44	42.29	0.887	0.237

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[1] The control diet (T_1), control diet was supplemented with probiotic (*Bacillus subtilis*) at 200mg/MT feed (T_2), Postbiotic (*Saccharomyces cerevisiae* fermentation products) at 1.25 kg/MT feed (T_3). [2] SEM, standard error of means (n=12).

Blood Biochemical Profile

The concentration of glucose, total protein, albumin, triglyceride and uric acid in serum did not vary significantly ($P > 0.05$) based on dietary treatments in this study (Table 4). However, cholesterol concentrations were significantly higher ($P < 0.05$) in the T_1 and T_2 groups compared to the T_3 group.

Table 4. Effect of probiotic (*Bacillus subtilis*) andpostbiotic (*Saccharomyces cerevisiae* fermentation products) on blood biochemical profile (35 d) serum cortisol concentration in broiler chickens.

Attribute	**Treatment[1]**			**SEM[2]**	**P-Value**
	T1	**T2**	**T3**		
Glucose (mg/dl)	137.03	136.49	138.38	2.991	0.967
Total Protein (mg/dl)	2.86	2.88	2.67	0.060	0.309
Albumin (mg/dl)	1.77	1.79	1.72	0.065	0.912
Cholesterol (mg/dl)	118.09[a]	120.41[a]	90.01[b]	3.868	0.001
Triglyceride (mg/dl)	145.97	142.68	139.55	3.311	0.742
Uric Acid (mg/dl)	2.85	3.30	3.17	0101	0.181
Corticosterone(nmol/L)					
28 d	2.615	2.837	2.200	0.128	0.117
35d	2.027[a]	1.840[a]	1.049[b]	0.122	0.001

[ab]Means bearing different superscripts in the same row differ significantly ($p \leq 0.05$). [1] The control diet (T_1), control diet was supplemented with probiotic (*Bacillus subtilis*) at 200mg/MT feed (T_2), Postbiotic (*Saccharomyces cerevisiae* fermentation products) at 1.25 kg/MT feed (T_3). [2] SEM, standard error of means (n=12).

Blood Haematological Profile

The blood haematological profile of broilers in different experimental groups has been presented in Table 5. No statistically significant differences ($P > 0.05$) were observed in haemoglobin, total leukocyte count (TLC), difference leukocyte count- heterophil, eosinophil, basophil, lymphocyte, monocyte and ratio of heterophil and lymphocyte across the various treatment groups.

Table 5. Effect of probiotic (*Bacillus subtilis*) andpostbiotic (*Saccharomyces cerevisiae* fermentation products) on blood haematological profile in broiler chickens at day 35.

Attribute	**Treatment[1]**			**SEM[2]**	**P-Value**
	T1	**T2**	**T3**		
Haemoglobin (g/dl)	13.45	13.72	13.85	0.520	0.953
Total leukocyte count (nX10^3 /µL)	22.28	21.82	21.85	0.367	0.859
Heterophil (%)	33.80	33.56	32.34	0.681	0.657
Eosinophil (%)	1.80	1.55	1.58	0.228	0.890
Basophil (%)	1.91	1.24	1.65	0.235	0.516
Lymphocyte (%)	59.28	60.56	60.98	0.738	0.632
Monocyte (%)	3.22	3.09	3.46	0.268	0.857
Heterophil : lymphocyte	0.58	0.56	0.54	0.017	0.598

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[1] The control diet (T_1), control diet was supplemented with probiotic (*Bacillus subtilis*) at 200mg/MT feed (T_2), Postbiotic (*Saccharomyces cerevisiae* fermentation products) at 1.25 kg/MT feed (T_3). [2] SEM, standard error of means (n=12).

Gut Microbiome

Dietary treatments did not have a notable impact on the counts of *Lactobacillus spp*. in the caecaldigesta (Table 6). The count of *Enterohaemorrhagic E. coli* and total *E. coli* were significantly higher (P<0.05) in the T_1 group compared to the T_2 and T_3 groups. Additionally, the count of ESBL-producing *Enterobacteriaceae* (KESC group) and *Salmonella* were significantly higher (p<0.01) in the T_1 group when compared to T_2 and T_3 groups.*Salmonella* was also lower in T2 when compared to T1 (p<0.05). PCR based characterization of ESBL- producing *Enterobacteriaceae* revealed maximum presence of *bla*$_{CTX\text{-}M\text{-}Type}$ (6/15, 40%), followed by *bla*$_{SHV\text{-}Type}$ (5/15, 33.3%) and *bla*$_{TEM\text{-}Type}$ (4/15, 26.6%). No variation in the occurrence pattern of ESBL/beta-lactamase associated genes in the studied ESBL-producing *Enterobacteriaceae* isolates was observed between the treatment groups.

Table 6. Effect of probiotic (*Bacillus subtilis*) andpostbiotic (*Saccharomyces cerevisiae* fermentation products) on viable bacteria numbers (log_{10} CFU/g) in caecal content in broiler chickens at day 42.

Attribute	**Treatment[1]**			**SEM[2]**	**P-Value**
	T1	**T2**	**T3**		
Lactobacillus	5.898	5.928	5.890	0.008	0.108
Total *E. coli*	7.377^a	7.136^b	7.058^b	0.051	0.024
Enterohaemorrhagic E. coli	3.882^a	3.245^b	3.140^b	0.661	0.000
ESBL producing Enterobacteriaceae	3.109^a	2.833^b	2.298^c	0.0664	0.000
Salmonella	7.526^a	7.045^b	6.813^c	0.061	0.000

[abc]Means bearing different superscripts in the same row differ significantly (p ≤ 0.05). [1] The control diet (T_1), control diet was supplemented with probiotic (*Bacillus subtilis*) at 200mg/MT feed (T_2), Postbiotic (*Saccharomyces cerevisiae* fermentation products) at 1.25 kg/MT feed (T_3). [2] SEM, standard error of means (n=12).

Gut Morphology

The VH in duodenum was significantly increased (P<0.05) in the T_3 and T_2 groups than T_1group (Table 7). The VH in jejunum was significantly increased (P<0.05) in the T_3 and T_2 groups than T_1 group. The VH in ileum was higher (p<0.05) inT_3 group than T_2 groups than T_1 group. The VW in duodenum, jejunum and ileum was similar among the treatments (P > 0.05). The CD in duodenum was increased (p<0.05) in T_1 group than T_3 and T_2 groups. The CD in jejunum was increased (p<0.05) in T_1 group than T_3 and T_2 groups. The CD of ileum was increased (p<0.05) in the T_1 and T_2 groups than T_3similar among the treatments (P > 0.05). The VH/CD ratio in duodenum and jejunum was increased (P <0.05) in the T_3 and T_2groups than T_1. The VH/ CD ratio in ileum was higher (P <0.05) in the T_3 group than T_1 and T_2 groups.

Table 7. Effect of probiotic (*Bacillus subtilis*) andpostbiotic (*Saccharomyces cerevisiae* fermentation products) on gut morphology in broiler chickens at day 42.

Attribute	**Treatment[1]**			**SEM[2]**	**P-Value**
	T1	**T2**	**T3**		
Duodenum					
Villi height (VH; µm)	814.33^b	991.25^a	1049^a	37.409	0.023
Villi width (VW; µm)	92.67	89.83	84.75	3.158	0.598
Crypt depth (CD; µm)	99.83^a	80.00^b	79.58^b	2.991	0.004

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VH/CD ratio	8.33^{b}	12.74^{a}	13.76^{a}	0.704	0.002
Jejunum					
Villi height (VH; μm)	818.58^{b}	948.67^{a}	967.75^{a}	26.797	0.042
Villi width (VW; μm)	95.42	99.42	100.58	2.285	0.633
Crypt depth (CD; μm)	98.58^{a}	82.75^{ab}	78^{b}	3.635	0.049
VH/CD ratio	8.86^{b}	12.00^{a}	12.68^{a}	0.528	0.004
Ileum					
Villi height (VH; μm)	857.75^{b}	967.08^{ab}	1035.50^{a}	29.147	0.036
Villi width (VW; μm)	105.67	106	106.67	3.539	0.994
Crypt depth (CD; μm)	90.83^{a}	87.25^{a}	74.75^{b}	2.436	0.014
VH/CD ratio	9.65^{b}	11.24^{b}	14.03^{a}	0.497	0.000

^{ab}Means bearing different superscripts in the same row differ significantly ($p \leq 0.05$). [1] The control diet (T_1), control diet was supplemented with probiotic (*Bacillus subtilis*) at 200mg/MT feed (T_2), Postbiotic (*Saccharomyces cerevisiae* fermentation products) at 1.25 kg/MT feed (T_3). [2] SEM, standard error of means (n=12).

Immune Response

On day 28, antibody titres against the IBD vaccine were significantly higher ($P < 0.05$) in the T_3 groups compared to the T_2 and T_1 groups (Table 8). However, on Day 35 there were no significant differences ($P > 0.05$) among the dietary treatment groups regarding antibody titres against this vaccine. Moreover, on Day 28 antibody titres against the ND vaccine were also greater in T_2 and T_3 groups ($P < 0.05$) than T_1 group. No significant differences were detected between the dietary treatment groups on day 35 for this vaccine also (p>0.05). There were no significant differences among the treatment groups for *in-vitro* phagocytic activity of neutrophils and lymphocytes.

Table 8. Effect of probiotic (*Bacillus subtilis*) andpostbiotic (*Saccharomyces cerevisiae* fermentation products) on antibody titre (log_{10}) against Infectious bursal disease virus (IBDV) and Newcastle disease virus (NDV), phagocytic activity of neutrophil (as expressed in optical density at 450 nm) and lymphocytes (stimulation index) in broiler chickens at 35 day.

Attribute	**Treatment[1]**			**SEM[2]**	**P-Value**
	T1	**T2**	**T3**		
Antibody titre					
IBDV-28 d	2.719^{b}	2.808^{ab}	3.041^{a}	0.052	0.028
IBDV-35 d	2.757	3.009	2.871	0.066	0.307
NDV-28 d	2.608^{b}	2.985^{a}	2.865^{a}	0.051	0.006
NDV-35 d	2.401	2.576	2.556	0.061	0.453
In vitro phagocytic activity					
Neutrophil	0.567	0.515	0.544	0.012	0.227
Lymphocyte	1.124	1.145	1.133	0.028	0.958

^{ab}Means bearing different superscripts in the same row differ significantly ($p \leq 0.05$). [1] The control diet (T_1), control diet was supplemented with probiotic (*Bacillus subtilis*) at 200mg/MT feed (T_2), Postbiotic (*Saccharomyces cerevisiae* fermentation products) at 1.25 kg/MT feed (T_3). [2] SEM, standard error of means (n=12).

Discussion

The solution-based approach to increase poultry production, to reduce production cost and to decrease negative environmental impact is the priority for the poultry researchers. As modern poultry production system is associated with numerous stressors like change of feed, high stocking

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density, processing in the hatchery which reduces bird immunity and increases bacterial pathogen colonization affecting not only the bird health and growth, but also compromises the food safety concerns [28].Use of antibiotics in sub-therapeutic dose in poultry feed was considered as one of such approach to control gut pathogens. Currently non-therapeutic use of antibiotics in poultry is facing reduced social acceptance as it may generate antimicrobial resistant commensals compromising the food safety and quality issues. European Union and United States-FDA banned the non-therapeutic use of antibiotics in livestock and poultry since long [29,30], but cessation of non-therapeutic antibiotic usage in poultry farming was correlated with reduced growth and increased mortality of the birds due to bacterial infections such as colibacillosis, salmonellosis and necrotic enteritis [31].Replacement of antibiotics with a suitable alternative without hampering the growth, immunity and health of the birds is a pressing research question. *Saccharomyces cerevisiae* is considered as the most promising candidate either as a probiotic (live yeast form) or as prebiotic in the poultry diet which showed remarkable improvement of growth performance, modulation of bird immune system, repairing the gastrointestinal tract and reducing the gut pathogen colonization[32]. However, the research gap exists whether different methods of extraction affect the efficacy of *Saccharomyces cerevisiae* as postbiotic. So, the present study was conducted to evaluate the effects of postbiotic (*Saccharomyces cerevisiae* fermentation product, SCFP) along with a probiotic (*Bacillus subtilis*) on the growth performance, immunity, gut health, and carcass characteristics of broiler chickens.

Feeding with SCFP (T_3 group) significantly improved average daily feed intake (ADFI) and average daily gain (ADG) of chickens compared to the T_1 (control) and T_2 (probiotic) groups from 1 to 14 days of age. Similarly, feeding with yeast hydrolysate significantly improved ADFI, ADG, and body weight during starter and grower phase of the experimental birds than the control groups [32,33]. It could be explained with the increased villi height associated with better absorption of nutrients, increasing the secretion of auxiliary digestive enzymes and anti-inflammatory effects of yeast hydrolysate in animals [34,35]. In contrast, few studies [36,37] reported improvement of body weight gain during later phase (after 21 days) of the growth with the feeding of yeast hydrolysate,associated with presence of gut microbiota secreting short chain fatty acids (SCFA) and improved metabolic activities. Although not evaluated, but the findings of present study could be correlated with the presence of SCFA-forming beneficial gut microbiota during starter and grower phase of the growth. Significant improvement of FCR in SCFP-fed birds (T_3) than the control (T_1) groups across the entire period of the experiment (1-42 days) is supported with the earlier findings [36,37].The meta-analysis of the findings [38] suggested inclusion of yeast or yeast products (less than 10 gram/Kg of diet) could improve growth and FCR of the birds.

The absence of statistically significant differences in slaughter body weight, eviscerated carcass weight, dressing percentage, weight of breast, frame, thigh, drumstick, wing, neck, gizzard, liver, heart, spleen, and bursa between the treatment groups is corroborative with the earlier studies[33,39]. Addition of probiotics in the diet helps in detoxification process which might be the reason for normal size of the liver in the treatment groups [40].

Dietary addition of SCFP in the experimental birds did not alter the concentration of glucose, total protein, albumin, triglyceride and uric acid in serum which confirmed the absence of adverse side effects in the studied birds [33]. In supportive with earlier reports [41,42], the present study also confirmed significant reduction of blood cholesterol concentration in SCFP-treated birds than the control or probiotic fed groups. Lower serum concentration of cholesterol in the birds is associated with production of eggs with low egg cholesterol level having market demands specially in the health-conscious consumers[43].

The present study revealed that dietary supplementation of SCFP had no significant effect on haemoglobin, total leukocyte count, difference leukocyte count and ratio of heterophil and lymphocyte which was also observed in a previous study in which dietary supplementation of *Saccharomyces cerevisiae* with *Nigella sativa* did not find any significant effect on blood biochemical profile in broiler chickens [44].

Effect of SCFP dietary supplementation on poultry gut microbiome revealed significant reduction of total *E. coli*, pathogenic *E. coli* (EHEC) and *Salmonella* in comparison to the probiotic-fed

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group and control birds. Reduction of *E. coli* and *Salmonella* colonization was also observed in earlier studies in the birds fed with the yeast products which could be explained by exclusion of the pathogens due to competition for carbon source in the gut, binding of the pathogens with surface of yeast produced functional carbohydrates instead of intestinal receptors which prevent activation of pro-inflammatory cytokines based signaling pathways and production of enzymes to disintegrate bacterial toxins [28,45]. *Saccharomyces cerevisiae* was found more effective against Gram-negative pathogens such as *E. coli* and *Salmonella* due to its capacity to disintegrate the bacterial outer membrane, found only in Gram-negative bacteria, causing increased permeability and depolarization of the cytoplasmic membrane [46]. Agglutination of pathogens expressing mannose specific type-1 fimbriae (such as *E. coli* and *Salmonella*) by the yeasts is another possible mechanism [47].

Dietary treatments did not have a notable impact on the counts of *Lactobacillus* in the caecaldigesta. Similarly feeding with dried yeast culture [44] and other prebiotics [48] did not reveal significant modulation on *Lactobacillus* count in broiler chickens. *Lactobacillus* itself can act as probiotic by preventing colonization of gut pathogens and the lactic acid produced by the lactobacilli is used by butyric acid producers increasing the digestibility of the birds[49]. Hence in the present study maintenance of lactobacilli in the treatment groups *at par* to the control group could be found to be beneficial.

One of the noteworthy findings of the present study is significant reduction of antimicrobial resistant pathogens (ESBL-producing *Enterobacteriaceae*) in the treatment groups in comparison to the control group. *Bacillus subtilis* probiotic strains earlier showed*in-vitro* antimicrobial effect against ESBL-producing *E. coli*, although failed to prevent gut colonization of ESBL-bacteria when studied *in-vivo*[50]. There is no report on efficacy SCFP on ESBL-producing *Enterobacteriaceae* to compare the present finding. The present study revealed maximum occurrence of $bla_{CTM\text{-}Type}$ followed by $bla_{SHV\text{-}Type}$ and $bla_{TEM\text{-}Type}$in the studied birds which is supportive with earlier studies. The CTX-M is considered as the major ESBL determinant in apparently healthy poultry, whereas the SHV and TEMdeterminants are predominant in poultry with subclinical infections (Olsen et al. 2014).

The villi height in duodenum, jejunum and ileum was significantly increased in the birds supplemented with SCFP and probiotic than control group which confirms the earlier observations[32,51]. Whereas the ratio between villi height and crypt depth was significantly increased in ileum of SCFP fed group than the birds supplemented with probiotic and the control group. *Saccharomyces cerevisiae* has trophic effect on illeal and jejunal villi than the duodenum as detected in the present study which is consistent with earlier observations [52]. The ileum is the primary site for amino acid absorption and longer ileal villi implies higher nutritional utilization reflected in better growth performance.

On day 28, antibody titres against both the IBD and NDV vaccine were significantly higher in the SCFP (T_3) groups compared to the probiotic (T_2) and control (T_1) groups. The oligosaccharides present in the yeast hydrolysate can activate the macrophages and the cytokines are released to generate the acquired immune response[33]. Like the mammals, the immune response in birds after vaccination is characterized with the generation of IgM first (up to day 30 post vaccination) followed by IgY[53]. The previous study explored dietary supplementation of yeast products promote the production of IgM in the birds vaccinated against NDV [37] which is the reason for higher antibody titre in T3 group than the others on the day 28. Whereas the effect of yeast supplementation on generation of IgY is still unclear and it might explain the absence of variations in all the groups in antibody titre on day 35. However, significantly higher antibody titre against IBDV in the birds fed with SCFP was not detected earlier.

The present study could not find modulation of cell mediated immune response in the studied birds which was more pronounced in challenge studies specially with intracellular pathogens (for example, *Coccidia*) fed with yeast hydrolysate and was also dependent on the dosage of the yeast products [54].

Conclusions

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Feeding with SCFP significantly improved average daily feed intake and average daily gain of chickens compared to the control and probiotic groups from 1 to 14 days of age. FCR was significantly improved in SCFP-fed birds relative to the control over the entire experimental period. Cholesterol levels and concentrations of corticosterone were significantly reduced with dietary supplementation of SCFP. *E. coli*, *Enterohaemorrhagic E. coli*, *ESBL-producing Enterobacteriaceae*, and *Salmonella* counts were significantly reduced in SCFP-fed group than control/probiotic groups. PCR based characterization of ESBL- producing *Enterobacteriaceae* revealed maximum presence of $bla_{CTX\text{-}M\text{-}Type}$. No variation in the occurrence pattern of ESBL/beta-lactamase associated genes in the studied ESBL-producing *Enterobacteriaceae* isolates was observed between the treatment groups. Significant increase in villi height and villi height to crypt depth ratio was observed in both SCFP-fed and probiotic-fed groups. On day 28, the SCFP-fed birds and those fed with probiotics exhibited a significant increase in antibody titres against Newcastle disease virus and Infectious bursal disease virus. Increase in antibody titre against IBDV in birds with the addition of SCFP in the diet was detected for the first time. It can be concluded that *Saccharomyces cerevisiae* fermentation product and *Bacillus subtilis* probiotic could be viable alternatives to antimicrobials in poultry production considering beneficial impacts in broilers fed antibiotic-free diet.

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