

EFFICACY OF SUPPLEMENTING BACILLUS SUBTILIS, SACCHAROMYCES BOULARDII AND SACCHAROMYCES CEREVISIAE ON GROWTH PERFORMANCE AND GUT HEALTH OF BROILER CHICKENS

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Abstract

A feeding trial was conducted to evaluate how supplementing broiler diets with *Bacillus subtilis*, *Saccharomyces boulardii* and *Saccharomyces cerevisiae*, singly and in combination, affects growth performance, carcass traits, organ development and gut health. One hundred and twenty day-old Arbor Acres broiler chicks were allotted to five treatment groups of three replicates each: a control fed a basal diet alone, and four supplemented groups receiving the basal diet plus *Saccharomyces boulardii* (1×10^8 cfu/kg), *Saccharomyces cerevisiae* (1×10^8 cfu/kg), *Bacillus subtilis* (1×10^9 cfu/kg), or a combination of all three organisms (1×10^3 cfu/kg each). Birds fed the combined probiotic blend ate less feed yet reached the heaviest live body weight (2512 g) and the most efficient feed conversion ratio (1.51), against 2137 g and 1.82 for the control. The same group also produced the heaviest carcass (1725 g), the tallest jejunal villi (970 μ m) and the lowest intestinal counts of *Salmonella* and *E. coli*, alongside the highest *Lactobacillus* counts. Organ weights, intestinal lengths and most microbial parameters did not differ significantly among groups. These results suggest that a multi-strain probiotic blend gives a more consistent improvement in broiler growth and gut health than any single organism fed alone, and support its use as a practical alternative to antibiotic growth promoters in broiler production.

INTRODUCTION

Poultry meat is an affordable source of animal protein, and demand has increased broiler production. However, young chicks are susceptible to impaired growth, poor nutrient absorption and digestive disorders when harmful bacteria colonise the gut. Such disturbances can reduce weight gain and feed efficiency.

Antibiotics have therefore been widely included in poultry diets to control these problems.

Antibiotics are used in poultry feed to promote growth, treat infections and improve feed efficiency (Palamidi et al., 2016). Routine use may leave residues in meat and eggs and contribute to the spread of antimicrobial resistance between animals and humans.

Withdrawal periods may also be overlooked under commercial conditions. These concerns have led many countries to restrict or prohibit antibiotics as growth promoters in livestock feed (Khan & Naz, 2013). Consequently, attention has shifted to feed additives that can maintain bird health and production without these risks (Zorriehzahra et al., 2016). Probiotics are among the alternatives considered for this purpose (Froebel et al., 2020).

Research on probiotics in human and animal nutrition has increased during the past two decades (Reid et al., 2003; Huang et al., 2012). The joint FAO/WHO working group defined probiotics as live microorganisms that provide a health benefit when administered in adequate amounts (FAO/WHO, 2001). Their reported effects include support for mucosal defence, reduced oxidative stress and regulation of mucosal immune responses (Takahashi et al., 2004). Probiotic supplementation may also improve digestive enzyme activity and intestinal barrier function through interactions with the resident microbiota (Rajput & Li, 2012).

In poultry, probiotics have been examined for their effects on growth, productive efficiency, product composition and digestive health (Alagawany et al., 2018). Dietary supplementation with beneficial microorganisms has been associated with improved feed conversion and weight gain, as well as reduced colonisation by *Salmonella* and *Campylobacter* (Mountzouris et al., 2015). Early supplementation may be particularly relevant because the intestinal microbiota develops rapidly during the first days after hatching (Sivaramaiah et al., 2011). Probiotics have also been reported to support gut immunity and intestinal epithelial integrity, which may limit pathogen load (Liao & Nyachoti, 2017).

Bacillus species are used as probiotics because their spores tolerate a range of environmental conditions. The spore coat of *Bacillus subtilis* supports survival during storage, transport and feed pelleting, followed by germination under favourable intestinal conditions (Driks, 2002).

Some *Bacillus* organisms produce lactic acid, which can lower intestinal pH and restrict harmful bacteria (Rahimi et al., 2010). They also produce enzymes that help degrade complex feed components and may improve feed utilisation (Pourakbari et al., 2016). Protease, amylase and lipase production has been reported for *B. subtilis*, and supplementation has been associated with improved growth performance (Santoso et al., 2001). Its resistance to processing and storage stresses supports its use as a feed additive (Wu et al., 2011). *Bacillus* spores may also produce antimicrobial peptides and influence protective cytokines in the gut (Sumi et al., 2015).

Saccharomyces species are another group of probiotic microorganisms. *Saccharomyces boulardii* and *Saccharomyces cerevisiae* have been reported to stimulate immune responses in young birds without reducing growth performance (Manafi et al., 2018). *Saccharomyces boulardii* was isolated from lychee fruit in the 1920s and is now used as a probiotic yeast. Fermented, autolysed or enzymatically hydrolysed *S. cerevisiae* products contain nucleotides, B vitamins, amino acids and cell-wall polysaccharides, including beta-glucan and mannan. These components have been associated with intestinal repair, immune regulation and animal growth (Sauer et al., 2010; Superchi et al., 2012). Yeast-derived products have therefore been evaluated as alternatives to antibiotic growth promoters because they may improve nutrient digestibility and intestinal health and reduce intestinal inflammation (Fadl et al., 2020; Samarasinghe et al., 2004).

Previous work indicates that combining *Saccharomyces boulardii* with *Bacillus subtilis* may improve growth performance and intestinal morphology. Reported responses include increased villus height and width, more goblet cells in the jejunum and ileum, and higher relative weights of the bursa of Fabricius and thymus than in birds receiving an unsupplemented diet. These observations suggest that the two organisms may affect different aspects of intestinal structure and immune

function. However, the response to adding *Saccharomyces cerevisiae* to this combination requires direct evaluation.

The gastrointestinal tract contains a complex microbial community whose collective genetic material exceeds that of the host bird (Wei et al., 2013). This microbiota contributes to energy metabolism, nutrient absorption, vitamin synthesis and immune regulation. Disturbance of its composition, termed dysbiosis, has been associated with several health disorders (Cisek & Binek, 2014). A stable intestinal microbial community may also restrict pathogen colonisation and regulate immune responses (Round & Mazmanian, 2009). In addition, microbial fermentation of carbohydrates and proteins contributes to digestive processes (Hamer et al., 2012). Because diet, medication and environmental stress can alter the microbiota, it is a relevant target for probiotic supplementation in poultry.

Bacillus subtilis, *Saccharomyces boulardii* and *Saccharomyces cerevisiae* have each been studied as individual probiotics, whereas evidence on their combined use remains limited. Their different reported activities may produce a response that is not obtained with a single organism. Therefore, this study compared *B. subtilis*, *S. boulardii* and *S. cerevisiae*, supplied individually or together, for their effects on broiler growth performance, carcass characteristics, antibody titres and intestinal health. The specific objectives were to compare their effects on growth performance and gut health.

Materials and Methods

The trial was carried out at the Poultry Husbandry Department, Faculty of Animal Husbandry and Veterinary Sciences, Sindh Agriculture University, Tandojam, and was designed to examine how the three probiotic organisms, alone and combined, influence gut health, resistance to common early-age bacterial infection, intestinal villi and continuity, intestinal length and the weight of visceral organs. One

hundred and twenty day-old broiler chicks were purchased from a local hatchery, weighed on arrival and transported to the research shed, which had been thoroughly cleaned and fumigated beforehand. The chicks were reared together on deep litter for the first week to allow them to settle in, and were then divided at seven days of age into five treatment groups of three replicates each, with ten birds per replicate.

Each group was housed on a deep-litter system with a full square foot of floor space allotted per bird. The shed had been disinfected, cleaned and lined with limestone left to dry for twenty-four hours before the chicks arrived, and temperature and humidity were kept within recommended ranges and logged throughout the trial. Brooding preparations began a day before the chicks arrived, with one brooder assigned to each group. Brooding temperature was held between 90°F and 95°F for the first week and then reduced by roughly five degrees each week until the house reached 70°F; a 100-watt bulb fitted to the centre of each brooder supplied heat, and a thermometer placed six to twelve inches away tracked the temperature in each pen. Litter was managed by first removing the coarse material from rice husk and sun-drying it for twelve hours before spreading a two- to four-inch layer on the floor; limestone was mixed into the husk and turned periodically to limit gas build-up and reduce the risk of infection at ground level. Birds were vaccinated according to the schedule normally followed by the department across the five weeks of the experiment.

The five dietary treatments were arranged as follows. Group A, the control, received the basal diet without any additive. Group B received the basal diet supplemented with *Saccharomyces boulardii* at 1×10^8 cfu per kilogram of feed. Group C received the basal diet supplemented with *Saccharomyces cerevisiae* at the same inclusion rate. Group D received the basal diet supplemented with *Bacillus subtilis* at 1×10^9 cfu per kilogram of feed. Group E received all three organisms together at 1×10^3 cfu per kilogram of feed each, added to the same basal

diet. All birds otherwise received identical feed and water and were managed under the same husbandry conditions throughout the trial.

Feed intake, water intake, feed conversion ratio, body weight and any clinical signs of illness, morbidity or mortality were recorded daily for every bird in the trial. Feed intake was calculated from the difference between feed offered and feed refused over each recording period, and water intake was tracked in the same way from the volume offered and remaining. Feed conversion ratio was worked out weekly as the ratio of feed consumed to weight gained. Morbidity and mortality were each expressed as a percentage of the birds affected relative to the total number in the group. Body weight was recorded using an electronic digital balance, with the initial weight taken at the start of the trial and the final weight taken at its close for each bird individually; weight gain was calculated as the difference between these two figures. Blood samples were drawn from three birds per breed group at twenty-five days of age, always before the morning feed, and stored until they could be analysed at the Central Veterinary Diagnostic Laboratory in Tandojam. Antibody titres were assessed by hemagglutination inhibition, using serial dilution of the sera against a standardised four haemagglutinating-unit antigen preparation to gauge how well maternal antibody protection had persisted in each group.

At the end of the trial, surviving birds were slaughtered and dissected to record carcass weight, dressing percentage and the weight of the leg, chest, abdominal fat, intestine, gizzard, proventriculus, heart, liver, bursa of Fabricius, pancreas, spleen and thymus, along with the length of the duodenum, jejunum, ileum, caecum and colon. Any birds that died during the trial were subjected to post-mortem examination to check for gross lesions of the liver, kidney, proventriculus, gizzard and intestine, so that mortality could be traced to a probable cause

where possible. Jejunal tissue samples were also collected for histological examination of villus height and crypt depth, and samples of intestinal contents were cultured to quantify *Salmonella*, *Escherichia coli* and *Lactobacillus* populations.

The recorded variables covered growth performance, carcass and organ traits, intestinal structure, microbial counts and antibody titres. Growth variables included feed and water intake, feed conversion ratio, weight gain, morbidity and mortality. Carcass and organ measurements included carcass weight, dressing percentage and the relative weights of major visceral organs. Intestinal measurements comprised segment lengths, jejunal villus height and crypt depth. *Salmonella*, *E. coli* and *Lactobacillus* counts were used to assess intestinal microbiota, while antibody titres represented immune status. Measuring these variables in the same treatment groups allowed growth and carcass responses to be considered together with intestinal measurements.

Feed and water were offered ad libitum throughout the trial. Feeders and drinkers were checked and refilled at the same time each day. Litter condition, ammonia accumulation and general flock behaviour were observed daily, together with clinical signs. Data were analysed by one-way analysis of variance using SAS statistical software and are presented as means. When the analysis showed a significant treatment effect, means were separated by Tukey's test at $P < 0.05$.

Results

Feed intake (g)

Feed intake differed significantly among groups ($P = 0.0001$). Group A had the highest intake (3890.00 g), followed by groups E (3810.67 g) and B (3757.00 g); each belonged to a different statistical group. Groups D and C recorded the lowest values, 3712.67 and 3702.00 g, respectively, and did not differ significantly from each other.

Table 1. Feed intake (g)

Parameter	Group A (Control)	Group B (S. boulardii)	Group C (S. cerevisiae)	Group D (B. subtilis)	Group E (Combined)	P-value
Feed intake (g)	3890.00 ^a ± 26.45	3757.00 ^c ± 7.54	3702.00 ^d ± 7.21	3712.67 ^d ± 12.0	3810.67 ^b ± 10.06	0.0001

Values are mean ± SD (n = 3 replicates per group). Means within a row bearing different superscript letters differ significantly (P < 0.05); means sharing a common letter do not differ significantly.

Live body weight (g)

Live body weight differed significantly among treatments (P = 0.0008). Group E recorded the highest value (2512.00 g) and differed significantly from all other groups. Groups C, D, B and A recorded 2226.00, 2186.67, 2177.33

and 2137.33 g, respectively, with no significant differences among them. Thus, the individual probiotic treatments did not differ from the control, whereas the combined treatment produced a higher live body weight.

Table 2. Live body weight (g)

Parameter	Group A (Control)	Group B (S. boulardii)	Group C (S. cerevisiae)	Group D (B. subtilis)	Group E (Combined)	P-value
Live body weight (g)	2137.33 ^b ± 92.09	2177.33 ^b ± 6.11	2226.00 ^b ± 65.93	2186.67 ^b ± 87.50	2512.00 ^a ± 90.35	0.0008

Values are mean ± SD (n = 3 replicates per group). Means within a row bearing different superscript letters differ significantly (P < 0.05); means sharing a common letter do not differ significantly.

Feed conversion ratio

Feed conversion ratio differed significantly among treatments (P = 0.0012). Group A had the highest ratio (1.82), whereas group E had the lowest (1.51). Group C recorded 1.66 and did

not differ significantly from group E. Groups B and D recorded 1.72 and 1.69, respectively, and overlapped statistically with both higher- and lower-ratio groups.

Table 3. Feed conversion ratio

Parameter	Group A (Control)	Group B (S. boulardii)	Group C (S. cerevisiae)	Group D (B. subtilis)	Group E (Combined)	P-value
FCR	1.82 ^a ± 0.07	1.72 ^{ab} ± 0.05	1.66 ^{bc} ± 0.04	1.69 ^{ab} ± 0.07	1.51 ^c ± 0.05	0.0012

Values are mean ± SD (n = 3 replicates per group). Means within a row bearing different superscript letters differ significantly (P < 0.05); means sharing a common letter do not differ significantly.

Carcass weight (g)

Carcass weight differed significantly among treatments (P = 0.0002). Groups E and C

recorded the highest carcass weights, 1725.00 and 1715.33 g, respectively, and did not differ significantly from each other. Groups D and B

recorded 1631.00 and 1566.67 g, respectively,

while group A had the lowest value (1520.67 g).

Table 4. Carcass weight (g)

Parameter	Group A (Control)	Group B (S. boulardii)	Group C (S. cerevisiae)	Group D (B. subtilis)	Group E (Combined)	P-value
Carcass weight (g)	1520.67 ^c ± 45.18	1566.67 ^{bc} ± 40.21	1715.33 ^a ± 50.29	1631.00 ^{ab} ± 18.03	1725.00 ^a ± 18.68	0.0002

Values are mean ± SD (n = 3 replicates per group). Means within a row bearing different superscript letters differ significantly (P < 0.05); means sharing a common letter do not differ significantly.

Dressing percentage (%)

Dressing percentage differed significantly among groups (P = 0.0013). Group E recorded the highest value (77.05%), followed by group D

(74.65%), which overlapped statistically with the higher and intermediate groups. Groups C and B recorded 71.95 and 71.17%, respectively, whereas group A had the lowest value (68.71%).

Table 5. Dressing percentage (%)

Parameter	Group A (Control)	Group B (S. boulardii)	Group C (S. cerevisiae)	Group D (B. subtilis)	Group E (Combined)	P-value
Dressing (%)	68.71 ^c ± 1.88	71.17 ^{bc} ± 1.02	71.95 ^{bc} ± 1.66	74.65 ^{ab} ± 2.68	77.05 ^a ± 0.22	0.0013

Values are mean ± SD (n = 3 replicates per group). Means within a row bearing different superscript letters differ significantly (P < 0.05); means sharing a common letter do not differ significantly.

Leg weight (%)

Leg weight did not differ significantly among groups (P = 0.0953). Values ranged from 17.44% in group D to 19.06% in group A. Groups E, C

and B recorded 18.78, 18.67 and 17.66%, respectively. All means belonged to the same statistical group.

Table 6. Leg weight (%)

Parameter	Group A (Control)	Group B (S. boulardii)	Group C (S. cerevisiae)	Group D (B. subtilis)	Group E (Combined)	P-value
Leg weight (%)	19.06 ^a ± 0.89	17.66 ^a ± 0.25	18.67 ^a ± 0.53	17.44 ^a ± 1.30	18.78 ^a ± 0.28	0.0953

Values are mean ± SD (n = 3 replicates per group). Means within a row bearing different superscript letters differ significantly (P < 0.05); means sharing a common letter do not differ significantly.

Chest weight (%)

Chest weight did not differ significantly across treatments (P = 0.0874). Group E carried the

heaviest breast yield numerically at 28.11 percent, followed by the control at 26.33 percent, group B at 25.88 percent and group C at 25.67 percent,

while group D was lowest at 22.71 percent. None of these five means was statistically separable

from the others, so this spread should not be read as a treatment effect.

Table 7. Chest weight (%)

Parameter	Group A (Control)	Group B (S. boulardii)	Group C (S. cerevisiae)	Group D (B. subtilis)	Group E (Combined)	P-value
Chest weight (%)	26.33 ^a ± 1.56	25.88 ^a ± 1.78	25.67 ^a ± 0.22	22.71 ^a ± 0.67	28.11 ^a ± 3.80	0.0874

Values are mean ± SD (n = 3 replicates per group). Means within a row bearing different superscript letters differ significantly (P < 0.05); means sharing a common letter do not differ significantly.

Abdominal fat weight (%)

Abdominal fat weight was essentially unaffected by treatment (P = 0.9292). The control and group E were tied for the highest value at 2.30 percent each, group B followed at 2.22 percent, group C

at 1.92 percent, and group D recorded the lowest value at 1.86 percent, but all five groups fell in the same statistical group and the spread was negligible.

Table 8. Abdominal fat weight (%)

Parameter	Group A (Control)	Group B (S. boulardii)	Group C (S. cerevisiae)	Group D (B. subtilis)	Group E (Combined)	P-value
Abdominal weight (%)	2.30 ^a ± 1.02	2.22 ^a ± 0.67	1.92 ^a ± 0.52	1.86 ^a ± 0.76	2.30 ^a ± 0.97	0.9292

Values are mean ± SD (n = 3 replicates per group). Means within a row bearing different superscript letters differ significantly (P < 0.05); means sharing a common letter do not differ significantly.

Intestine weight (%)

Intestine weight did not differ significantly among treatments (P = 0.0833). Group E

recorded 5.31%, followed by groups B (4.91%), A (4.75%), C (4.64%) and D (4.04%). None of the means differed significantly.

Table 9. Intestine weight (%)

Parameter	Group A (Control)	Group B (S. boulardii)	Group C (S. cerevisiae)	Group D (B. subtilis)	Group E (Combined)	P-value
Intestine weight (%)	4.75 ^a ± 0.43	4.91 ^a ± 0.18	4.64 ^a ± 0.18	4.04 ^a ± 0.84	5.31 ^a ± 0.41	0.0833

Values are mean ± SD (n = 3 replicates per group). Means within a row bearing different superscript letters differ significantly (P < 0.05); means sharing a common letter do not differ significantly.

Gizzard weight (%)

Gizzard weight did not differ significantly among groups (P = 0.9097). Values were 2.30% in group

A, 2.25% in group E, 2.24% in group D, 2.16% in group C and 2.02% in group B. All means belonged to the same statistical group.

Table 10. Gizzard weight (%)

Parameter	Group A (Control)	Group B (S. bouldardii)	Group C (S. cerevisiae)	Group D (B. subtilis)	Group E (Combined)	P-value
Gizzard (%)	2.30 ^a ± 0.20	2.02 ^a ± 0.16	2.16 ^a ± 0.22	2.24 ^a ± 0.47	2.25 ^a ± 0.53	0.9097

Values are mean ± SD (n = 3 replicates per group). Means within a row bearing different superscript letters differ significantly (P < 0.05); means sharing a common letter do not differ significantly.

Pro-ventriculus weight (%)

Proventriculus weight did not differ significantly among treatments (P = 0.3926). Group D

recorded 0.47%, followed by groups B (0.44%), E (0.40%), A (0.38%) and C (0.34%).

Table 11. Pro-ventriculus weight (%)

Parameter	Group A (Control)	Group B (S. bouldardii)	Group C (S. cerevisiae)	Group D (B. subtilis)	Group E (Combined)	P-value
Pro-ventriculus (%)	0.38 ^a ± 0.11	0.44 ^a ± 0.04	0.34 ^a ± 0.06	0.47 ^a ± 0.01	0.40 ^a ± 0.07	0.3926

Values are mean ± SD (n = 3 replicates per group). Means within a row bearing different superscript letters differ significantly (P < 0.05); means sharing a common letter do not differ significantly.

Heart weight (%)

Heart weight was not significantly affected by treatment (P = 0.2422). Group B recorded the highest value at 0.59 percent, the control followed closely at 0.58 percent, group C at 0.55

percent and group E at 0.53 percent, while group D was lowest at 0.44 percent; the spread stayed within a tight range and none of the groups separated statistically.

Table 12. Heart weight (%)

Parameter	Group A (Control)	Group B (S. bouldardii)	Group C (S. cerevisiae)	Group D (B. subtilis)	Group E (Combined)	P-value
Heart weight (%)	0.58 ^a ± 0.11	0.59 ^a ± 0.02	0.55 ^a ± 0.09	0.44 ^a ± 0.08	0.53 ^a ± 0.00	0.2422

Values are mean ± SD (n = 3 replicates per group). Means within a row bearing different superscript letters differ significantly (P < 0.05); means sharing a common letter do not differ significantly.

Liver weight (%)

Liver weight showed no significant difference among treatments (P = 0.8807), the least variable of all the organ measurements recorded. Group E

recorded the highest value at 3.08 percent, group B followed at 3.05 percent, group D at 2.87 percent and the control at 2.82 percent, with group C lowest at 2.76 percent.

Table 13. Liver weight (%)

Parameter	Group A (Control)	Group B (S. boulardii)	Group C (S. cerevisiae)	Group D (B. subtilis)	Group E (Combined)	P-value
Liver weight (%)	2.82 ^a ± 0.25	3.05 ^a ± 0.43	2.76 ^a ± 0.08	2.87 ^a ± 0.64	3.08 ^a ± 0.42	0.8807

Values are mean ± SD (n = 3 replicates per group). Means within a row bearing different superscript letters differ significantly (P < 0.05); means sharing a common letter do not differ significantly.

Bursa of Fabricius weight (%)

Bursa of Fabricius weight did not differ significantly by treatment (P = 0.2528). Groups B and E were tied for the highest value at 0.08 percent each, the control followed at 0.07

percent, group C at 0.05 percent, and group D was lowest at 0.04 percent; values were low across the board and no group separated statistically from another.

Table 14. Bursa of Fabricius weight (%)

Parameter	Group A (Control)	Group B (S. boulardii)	Group C (S. cerevisiae)	Group D (B. subtilis)	Group E (Combined)	P-value
Bursa weight (%)	0.07 ^a ± 0.05	0.08 ^a ± 0.00	0.05 ^a ± 0.02	0.04 ^a ± 0.00	0.08 ^a ± 0.00	0.2528

Values are mean ± SD (n = 3 replicates per group). Means within a row bearing different superscript letters differ significantly (P < 0.05); means sharing a common letter do not differ significantly.

Spleen weight (%)

Spleen weight showed no significant treatment effect (P = 0.0766), although group D trended numerically highest at 0.16 percent, followed by

group C at 0.13 percent, group E at 0.11 percent, group B at 0.09 percent and the control lowest at 0.08 percent; the difference did not reach statistical significance.

Table 15. Spleen weight (%)

Parameter	Group A (Control)	Group B (S. boulardii)	Group C (S. cerevisiae)	Group D (B. subtilis)	Group E (Combined)	P-value
Spleen (%)	0.08 ^a ± 0.00	0.09 ^a ± 0.04	0.13 ^a ± 0.04	0.16 ^a ± 0.02	0.11 ^a ± 0.02	0.0766

Values are mean ± SD (n = 3 replicates per group). Means within a row bearing different superscript letters differ significantly (P < 0.05); means sharing a common letter do not differ significantly.

Thymus weight (%)

Thymus weight differed significantly among treatments (P = 0.0046). Group E recorded the highest value (0.37%) and differed significantly

from groups A and B. Group C recorded 0.34% and overlapped with the higher- and lower-ranked groups. Groups D, B and A recorded 0.28, 0.22 and 0.16%, respectively.

Table 16. Thymus weight (%)

Parameter	Group A (Control)	Group B (S. boulardii)	Group C (S. cerevisiae)	Group D (B. subtilis)	Group E (Combined)	P-value
Thymus (%)	0.16 ^c ± 0.09	0.22 ^{bc} ± 0.07	0.34 ^{ab} ± 0.07	0.28 ^{abc} ± 0.07	0.37 ^a ± 0.02	0.0046

Values are mean ± SD (n = 3 replicates per group). Means within a row bearing different superscript letters differ significantly (P < 0.05); means sharing a common letter do not differ significantly.

Intestinal Length

Duodenal length did not differ significantly among groups (P = 0.2123). Groups C and D

recorded 30.67 cm, followed by groups E (30.00 cm), B (28.33 cm) and A (27.67 cm).

Table 17. Duodenal length (cm)

Parameter	Group A (Control)	Group B (S. boulardii)	Group C (S. cerevisiae)	Group D (B. subtilis)	Group E (Combined)	P-value
D.D length	27.67 ^a ± 0.57	28.33 ^a ± 2.51	30.67 ^a ± 2.08	30.67 ^a ± 2.08	30.00 ^a ± 1.00	0.2123

Values are mean ± SD (n = 3 replicates per group). Means within a row bearing different superscript letters differ significantly (P < 0.05); means sharing a common letter do not differ significantly.

Jejunal length (cm)

Jejunal length did not differ significantly among treatments (P = 0.1397). Values were 76.33 cm in

group E, 70.00 cm in group D, 69.00 cm in group A, 67.00 cm in group B and 65.43 cm in group C.

Table 18. Jejunal length (cm)

Parameter	Group A (Control)	Group B (S. boulardii)	Group C (S. cerevisiae)	Group D (B. subtilis)	Group E (Combined)	P-value
Jejunum length	69.00 ^a ± 1.00	67.00 ^a ± 5.00	65.43 ^a ± 2.20	70.00 ^a ± 1.00	76.33 ^a ± 9.29	0.1397

Values are mean ± SD (n = 3 replicates per group). Means within a row bearing different superscript letters differ significantly (P < 0.05); means sharing a common letter do not differ significantly.

Ileal length (cm)

Ileal length did not differ significantly by treatment (P = 0.3674). Group E recorded the

longest ileum at 67.00 cm, group C followed at 65.33 cm, the control at 64.33 cm and group B at 60.67 cm, with group D shortest at 59.33 cm.

Table 19. Ileal length (cm)

Parameter	Group A (Control)	Group B (S. bouldarii)	Group C (S. cerevisiae)	Group D (B. subtilis)	Group E (Combined)	P-value
Ileum length	64.33 ^a ± 2.08	60.67 ^a ± 1.15	65.33 ^a ± 8.73	59.33 ^a ± 4.60	67.00 ^a ± 5.19	0.3674

Values are mean ± SD (n = 3 replicates per group). Means within a row bearing different superscript letters differ significantly (P < 0.05); means sharing a common letter do not differ significantly.

Total small intestine length (cm)

Total small intestine length showed no significant treatment effect (P = 0.2817). Group E was again numerically longest at 173.33 cm, group C

followed at 161.43 cm, the control at 161.00 cm and group D at 160.00 cm, with group B shortest at 156.00 cm.

Table 20. Total small intestine length (cm)

Parameter	Group A (Control)	Group B (S. bouldarii)	Group C (S. cerevisiae)	Group D (B. subtilis)	Group E (Combined)	P-value
Total Small intestine	161.00 ^a ± 2.00	156.00 ^a ± 5.29	161.43 ^a ± 12.82	160.00 ^a ± 7.21	173.33 ^a ± 13.50	0.2817

Values are mean ± SD (n = 3 replicates per group). Means within a row bearing different superscript letters differ significantly (P < 0.05); means sharing a common letter do not differ significantly.

Caecal length (cm)

Caecal length did not differ significantly among groups (P = 0.2291). Group E recorded the longest caecum at 35.67 cm, the control followed

at 34.67 cm, group D at 34.26 cm and group C at 32.33 cm, with group B shortest at 31.67 cm, staying within a comparatively narrow range.

Table 21. Caecal length (cm)

Parameter	Group A (Control)	Group B (S. bouldarii)	Group C (S. cerevisiae)	Group D (B. subtilis)	Group E (Combined)	P-value
Ceacal	34.67 ^a ± 1.15	31.67 ^a ± 1.52	32.33 ^a ± 4.04	34.26 ^a ± 1.55	35.67 ^a ± 1.52	0.2291

Values are mean ± SD (n = 3 replicates per group). Means within a row bearing different superscript letters differ significantly (P < 0.05); means sharing a common letter do not differ significantly.

Colon length (cm)

Colon length was the one intestinal segment that responded significantly to treatment (P = 0.0033). Group E had the longest colon at 8.86 cm, statistically indistinguishable from group B at 8.56 cm; these two groups were the longest and did not differ from each other. Group C followed

at 7.16 cm and group D at 6.63 cm, both in an overlapping middle range, while the control had the shortest colon at 5.85 cm and was statistically distinct from groups B and E. A longer colon in the supplemented groups is consistent with the more developed gut structure seen elsewhere in this trial.

Table 22. Colon length (cm)

Parameter	Group A (Control)	Group B (S. boulardii)	Group C (S. cerevisiae)	Group D (B. subtilis)	Group E (Combined)	P-value
Colon	5.85 ^c ± 0.23	8.56 ^{ab} ± 0.51	7.16 ^{abc} ± 0.76	6.63 ^{bc} ± 1.18	8.86 ^a ± 0.80	0.0033

Values are mean ± SD (n = 3 replicates per group). Means within a row bearing different superscript letters differ significantly (P < 0.05); means sharing a common letter do not differ significantly.

Total large intestine length (cm)

Total large intestine length did not differ significantly by treatment (P = 0.2256). Group E recorded the longest total large intestine at 44.53

cm, group D followed at 40.90 cm, the control at 40.53 cm and group B at 40.23 cm, with group C shortest at 39.50 cm.

Table 23. Total large intestine length (cm)

Parameter	Group A (Control)	Group B (S. boulardii)	Group C (S. cerevisiae)	Group D (B. subtilis)	Group E (Combined)	P-value
Total large Intestine length	40.53 ^a ± 1.28	40.23 ^a ± 1.96	39.50 ^a ± 3.96	40.90 ^a ± 2.70	44.53 ^a ± 2.33	0.2256

Values are mean ± SD (n = 3 replicates per group). Means within a row bearing different superscript letters differ significantly (P < 0.05); means sharing a common letter do not differ significantly.

Jejunal Histomorphology (µm)

Villus height and crypt depth differed significantly among treatments (P = 0.0001 for each). Villus height increased from 554.29 µm in group A to 970.00 µm in group E. Group D recorded 909.00 µm, whereas groups C and B

recorded 759.43 and 746.26 µm, respectively, and did not differ significantly from each other. Crypt depth was 97.58, 106.58, 113.30, 123.10 and 132.64 µm in groups A, B, C, D and E, respectively. Each group differed significantly from the others for crypt depth.

Table 24. Jejunal histomorphology (µm)

Parameter	Group A (Control)	Group B (S. boulardii)	Group C (S. cerevisiae)	Group D (B. subtilis)	Group E (Combined)	P-value
Villus height	554.29 ^d ± 3.86	746.26 ^c ± 2.92	759.43 ^c ± 2.67	909.00 ^b ± 4.58	970 ^a ± 6.83	0.0001
Crypt depth	97.58 ^e ± 1.61	106.58 ^d ± 1.68	113.30 ^c ± 1.90	123.10 ^b ± 1.69	132.64 ^a ± 1.73	0.0001

Values are mean ± SD (n = 3 replicates per group). Means within a row bearing different superscript letters differ significantly (P < 0.05); means sharing a common letter do not differ significantly.

Intestinal Microbiota

Intestinal microbial counts ($\log_{10}$ cfu/g)

Treatment significantly affected Salmonella, E. coli and Lactobacillus counts ($P = 0.0001$ for each), and all groups differed significantly for each organism. Salmonella counts decreased from 4.37 $\log_{10}$ cfu/g in group A to 3.21, 2.69, 2.01 and 1.32 $\log_{10}$ cfu/g in groups B, C, D and E, respectively. E. coli counts followed the same

order, declining from 5.83 $\log_{10}$ cfu/g in group A to 4.85, 4.63, 3.42 and 2.91 $\log_{10}$ cfu/g in groups B, C, D and E. Lactobacillus counts increased from 7.13 $\log_{10}$ cfu/g in group A to 7.64, 7.87, 8.13 and 8.39 $\log_{10}$ cfu/g in groups B, C, D and E, respectively. Group E therefore had the lowest Salmonella and E. coli counts and the highest Lactobacillus count.

Table 25. Intestinal microbial counts ($\log_{10}$ cfu/g)

Parameter	Group A (Control)	Group B (S. boulardii)	Group C (S. cerevisiae)	Group D (B. subtilis)	Group E (Combined)	P-value
Salmonella	4.37 ^e $\pm$ 0.10	3.21 ^d $\pm$ 0.02	2.69 ^c $\pm$ 0.07	2.01 ^b $\pm$ 0.01	1.32 ^a $\pm$ 0.03	0.0001
E. coli	5.83 ^e $\pm$ 0.02	4.85 ^d $\pm$ 0.03	4.63 ^c $\pm$ 0.03	3.42 ^b $\pm$ 0.02	2.91 ^a $\pm$ 0.01	0.0001
Lactobacillus	7.13 ^e $\pm$ 0.20	7.64 ^d $\pm$ 0.22	7.87 ^c $\pm$ 0.25	8.13 ^b $\pm$ 0.21	8.39 ^a $\pm$ 0.10	0.0001

Values are mean $\pm$ SD (n = 3 replicates per group). Means within a row bearing different superscript letters differ significantly ($P < 0.05$); means sharing a common letter do not differ significantly.

Discussion

Probiotics may help maintain the balance of intestinal bacteria (Lutful Kabir, 2009). Antagonism and competitive exclusion can limit the attachment and invasion of pathogenic bacteria (Wine et al., 2009). Changes in microbial composition may subsequently affect immunity, digestion and nutrient absorption (Tarradas et al., 2020). Bacillus subtilis is suitable for this purpose because its spores tolerate the low pH and bile salts of the gastrointestinal tract and may survive brief exposure to temperatures above 110°C (Sivaramaiah et al., 2011). Bacillus spores also produce antimicrobial peptides and may increase protective cytokines, including interleukin-1 and interferon-gamma (Sumi et al., 2015). These properties provide possible explanations for the performance responses reported after B. subtilis supplementation.

The combined probiotic treatment produced the highest live body weight and carcass weight and the lowest feed conversion ratio. The response agrees with Li et al. (2011), who reported improved performance after B. subtilis supplementation in broilers. In the present study, treatment effects were significant for feed intake, live body weight and feed conversion ratio, although not every supplemented group differed from the control. Zhang et al. (2013) also reported higher average daily gain after Bacillus supplementation. Zeng et al. (2021) observed a clearer response in average daily gain between 1 and 42 days than during the early growth phase. Amerah et al. (2013) found that B. subtilis improved feed conversion ratio and associated this response with extracellular digestive enzymes and other metabolites produced by Bacillus. Changes in gut microbial composition may also contribute to these effects (Guo et al., 2020). In

contrast, Lee et al. (2010) reported little effect of probiotics on cumulative broiler growth. Differences among studies may be related to probiotic strain, dose and rearing conditions.

Dressing percentage and individual cut yields are relevant measures of broiler carcass performance (Ahmat et al., 2021). Group E had a higher dressing percentage than the control, although several treatment means overlapped statistically. Sarangi et al. (2016) similarly found no meaningful increase in carcass yield following probiotic supplementation. In contrast, Salehizadeh et al. (2019) reported heavier carcasses and individual cuts after *B. subtilis* supplementation. Variation among studies may be associated with differences in probiotic strain, dose, formulation, bird age or hygiene conditions (Zhang et al., 2012). Abudabos et al. (2019) also reported improved villus structure and intestinal integrity after *B. subtilis* supplementation, consistent with the jejunal response observed in the present trial.

The increases in villus height and crypt depth were accompanied by lower *Salmonella* and *E. coli* counts and a higher *Lactobacillus* count in group E. Increased villus height relative to crypt depth has also been reported after probiotic supplementation under an experimentally induced intestinal challenge (Zhang et al., 2021). Taller villi increase the absorptive surface of the small intestine and may support nutrient uptake and growth (Mohammadagheri et al., 2016). Related *Bacillus* species have also improved small-intestinal morphology compared with commercial antibiotic treatment (Lin et al., 2019). The structural and microbial responses observed here may therefore have contributed to the lower feed conversion ratio and higher live body weight in group E, although the present study did not directly test this mechanism.

Conclusion

Supplementation with *Bacillus subtilis*, *Saccharomyces boulardii* and *Saccharomyces cerevisiae* affected feed intake, live body weight, feed conversion ratio and carcass weight. Most

organ weights and intestinal lengths did not differ significantly among treatments. Significant differences were recorded for thymus weight, colon length, jejunal villus height, crypt depth and the counts of *Salmonella*, *E. coli* and *Lactobacillus*. The combined treatment generally produced the most favourable growth, intestinal morphology and microbial responses among the treatments evaluated.

Recommendation

Under the conditions of this trial, the combined supplementation of *Bacillus subtilis*, *Saccharomyces boulardii* and *Saccharomyces cerevisiae* may be used to improve broiler growth performance and selected indicators of gut health. Further studies should determine the inclusion rates that provide consistent responses under commercial production conditions.

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Author Contributions

Inam ullah: Plan the study, executed trial, analyzed the data, written the draft. **Imdad Ali Leghari:** Conceptualization, Supervised the study, reviewed and edited the manuscript

Data Availability Statement

The supporting primary data is available from corresponding author and can be provided upon the reasonable request

Conflict of interest

The study was conducted without financial relationships and there is no any potential conflicting interest among the authors

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