Veterinary and Animal Science 32 (2026) 100637

Contents lists available at ScienceDirect

Veterinary and Animal Science

journal homepage: www.elsevier.com/locate/vas

# Dietary supplementation with a multi-strain *Bacillus subtilis* probiotic improves digestive efficiency, growth performance, and meat quality in densely stocked broilers

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ARTICLE INFO

*Keywords:*
Nutrient digestibility
Hematological indicators
Feed efficiency
Alternative growth promoters
Intensive poultry systems

ABSTRACT

*Bacillus subtilis* has attracted attention as a robust probiotic candidate to replace antibiotic growth promoters (AGPs) in poultry, owing to its spore-forming resilience and capacity to modulate gut function. However, inclusion levels and the efficacy of multi-strain *B. subtilis* mixtures remain underexplored, particularly at high stocking densities, when birds are more susceptible to physiological and environmental stressors. This study evaluated the effects of incremental levels of a multi-strain *B. subtilis* probiotic mixture on growth performance, nutrient digestibility, physiological responses, carcass traits, and meat quality of broiler chickens reared under intensive conditions. A total of 900 one-day-old Ross 308 broiler chicks were assigned to six dietary treatments in a completely randomized design, with 36 pens (13 birds/0.75 $m^2$) serving as experimental units. Diets comprised a basal diet without additives (PRB0), a positive control containing zinc bacitracin (POSC), and four diets supplemented with 0.3, 0.4, 0.5, or 0.6% of the *B. subtilis* probiotic mixture (PRB3 – PRB6). Diets were fed from the starter to the finisher phases. Probiotic supplementation influenced growth responses across the graded inclusion levels. Birds receiving the 0.5% inclusion level consistently exhibited the most favourable body weight gain and feed conversion efficiency compared with the other treatments. Probiotic supplementation enhanced the digestibility of organic matter, neutral detergent fibre, and gross energy, whereas crude protein digestibility exhibited a quadratic response. Haemato-biochemical profiles suggested improved physiological status in birds receiving mid-level probiotic inclusions (PRB3 – PRB5). Carcass yield and major meat quality traits were unaffected by dietary treatments, while minor effects on gut morphological indices were observed. Collectively, these findings suggest that a 0.5% inclusion level may be a promising probiotic supplementation strategy for broiler production under high stocking density.

## Introduction

Broiler production is a key agricultural subsector globally, providing an affordable source of dietary protein to support the needs of an expanding human population. Due to high production costs and increased demand for affordable poultry products, poultry producers have increased broiler production by raising stocking density. However, high stocking density can cause heat stress in chickens, which negatively affects the gastrointestinal tract, leading to poor growth and productivity (Son et al., 2022; Sumanu et al., 2022). Consequently, antibiotic growth promoters (AGPs) have been widely implemented to mitigate diseases and oxidative stress associated with high-density rearing conditions, while also enhancing feed efficiency, growth rates, and the overall health of broiler chickens (Nduku et al., 2025). However, growing concerns about antibiotic-resistant microorganisms and the accumulation of antibiotic residues in poultry products have led most countries to ban AGPs in food-producing animals, with exceptions remaining in some developing nations such as South Africa (Krysiak et al., 2021). Residues in broiler meat pose risks to consumers, including allergic reactions and disruption of the gut microbiota, which can impair

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https://doi.org/10.1016/j.vas.2026.100637

Available online 27 March 2026

immunity, digestion, and overall health (Abd El-Hack et al., 2022). However, implementing AGP bans in developing countries without effective replacements may negatively affect food security and reduce profitability for poultry producers (Laxminarayan et al., 2015; Hedman et al., 2020). Consequently, there is an urgent need to identify sustainable alternatives to AGPs, such as probiotics, that can support growth performance, meat quality, and product safety in broiler chickens reared under high stocking densities.

Probiotics are live microorganisms that confer health benefits to the host when administered in adequate amounts (Onyenweaku et al., 2016; Sumanu et al., 2024). Among these, *Bacillus subtilis* has garnered particular attention due to its spore-forming capacity, which enables it to withstand the industrial feed processing and the harsh conditions of the gastrointestinal tract (Sandvang et al., 2021). This inherent resilience suggests that combining multiple *B. subtilis* strains may enhance probiotic functionality through complementary modes of action. When using *B. subtilis* as an alternative to AGPs, determining an appropriate supplementation level is essential to ensure optimal bird health and performance while preventing potential negative effects associated with under- or over-supplementation. Although individual and combined *B. subtilis* strains have been evaluated for their effects on growth performance, nutrient digestibility, blood parameters, and meat quality in broilers raised under standard stocking densities, limited research has examined dose–response effects of a novel multi-strain *B. subtilis* probiotic under high-density rearing conditions. Therefore, this study investigated how incremental doses of a multi-strain *B. subtilis* probiotic influence growth performance, nutrient digestibility, haematological responses, and meat quality in densely stocked Ross 308 broiler chickens.

## Materials and methods

### *Ethical statement*

All animal care, handling, and sampling procedures were performed following the guidelines and regulations approved by the University of Mpumalanga's Research Ethics Committee – Animal Sciences (UMP REC-AS, approval no. UMP/Mlambo/1/2024) and North-West University's Animal Production Research Ethics Committee (NWU – ANIMPROD REC, approval no. NWU-00818-24-A5).

### *Experimental design*

A six-week feeding trial was conducted at Rooigrond Farm in the North West province of South Africa. The trial was conducted during February and April, during which temperatures in this area ranged from 10 to 36 °C. One-day-old Ross 308 chicks were purchased from Chicken Ranch, Centurion, South Africa. The four-strain novel *Bacillus* probiotic mixture (CPB 003, CPB 029, HP 1.6, and D 014 strains in equal proportions, with a final concentration of $1 \times 10^9$ CFU per gram) was developed and supplied by the Council for Scientific and Industrial Research (Pretoria, South Africa) and OptimusBio (Centurion, South Africa). Feed ingredients were bought from Nutroteq Pty (Ltd) (Centurion, South Africa) and SimpleGrow Agric Service Pty (Ltd) (Centurion, South Africa).

### *Feed formulation, experimental design, and bird management*

Each experimental diet was prepared (Table 1) for starter (0 – 14 days), grower (15 – 35 days), and finisher (36 – 42 days) phases to meet the nutritional requirements of Ross 308 broilers. The experimental diets comprised: 1. a basal broiler diet without AGP or *B. subtilis* probiotic mixture (PRB0); 2. the basal diet supplemented with 0.5% (w/w) zinc bacitracin (POSC); and four versions of the basal diet supplemented with *B.subtilis* probiotic mixture at 0.3 (PRB3), 0.4 (PRB4), 0.5 (PRB5), or 0.6% (PRB6). A total of 900 one-day-old Ross 308 male chicks (43.21 ± 0.372 g live weight) were randomly distributed into 36 replicate pens. Subsequently, six dietary treatments, each replicated six times, were randomly assigned to the pens in a completely randomized design. The recommended stocking density for broilers is 15 birds/m² (SAPA, 2012); however, this study employed a higher density of 13 birds per 0.75 m² (~17.3 birds/m²) to evaluate the stress-mitigating effects of the novel multi-strain *B. subtilis* probiotic. Sunflower husks were used as bedding in each pen. Feed and water were provided to birds *ad libitum*. The bedding was changed regularly to prevent a damp environment for the birds.

**Table 1**
Feed ingredient and nutrient composition (g/kg) of the basal diet across three feeding phases.

| Ingredients | Starter (0-14 d) | Grower (15-35 d) | Finisher (36-42 d) |
|---|---|---|---|
| Yellow Corn | 44.03 | 48.12 | 54.37 |
| Soybean meal (46% CP) | 41.71 | 37.94 | 32.67 |
| L-Lysine HCl (99% CP) | 0.21 | 0.14 | 0.17 |
| DL-Methionine (99% CP) | 0.42 | 0.45 | 0.35 |
| L-Threonine (98% CP) | 0.12 | 0.09 | 0.08 |
| Limestone Fine | 1.4 | 1.16 | 0.87 |
| Monocalcium phosphate | 2.17 | 1.77 | 1.47 |
| Salt fine | 0.24 | 0.26 | 0.25 |
| Sodium bicarbonate | 0.28 | 0.25 | 0.27 |
| Poultry vitamin and minerals premix* | 0.5 | 0.5 | 0.5 |
| Soy oil | 8.42 | 8.82 | 8.5 |
| *Calculated nutritional composition (%)* | | | |
| Crude Protein | 23 | 21.5 | 19.5 |
| Crude fat | 10.78 | 11.23 | 11.01 |
| Calcium | 0.95 | 0.8 | 0.65 |
| Available Phosphorus | 0.50 | 0.42 | 0.36 |
| AME Poultry | 2 975 | 3050 | 3100 |
| SID Methionine | 0.71 | 0.72 | 0.76 |
| SID Lysine | 1.32 | 1.18 | 1.08 |
| SID Tryptophan | 0.26 | 0.24 | 0.21 |
| SID Threonine | 0.88 | 0.79 | 0.72 |
| SID Isoleucine | 0.87 | 0.81 | 0.73 |
| SID Histidine | 0.54 | 0.5 | 0.46 |
| SID Valine | 0.93 | 0.87 | 0.79 |
| SID Leucine | 1.62 | 1.54 | 1.43 |
| SID Arginine | 1.42 | 1.32 | 1.17 |
| Linoleic acid | 5.58 | 5.81 | 5.68 |
| Sodium | 0.18 | 0.18 | 0.18 |
| Potassium | 1.02 | 0.95 | 0.86 |
| Chloride | 0.23 | 0.23 | 0.23 |
| SID Met + Cys | 1 | 0.92 | 0.86 |

Abbreviations: Poultry SID, standard ileal digestibility; AME, apparent metabolized energy;

* Vitamin and mineral premix contained: copper sulphate 8.0 mg; zinc sulphate 79 mg; ferrous sulphate 80 mg; niacin 30 mg; magnesium sulphate 100 mg; vitamin A 11,0 0 0 IU; potassium iodide 0.34 mg; pantothenic acid 10 mg; folic acid 0.7 mg; biotin 0.12 g; vitamin B6 5.1 mg; vitamin B1 2.5 mg; vitamin $B_2$ 4.5 mg; vitamin $D_3$ 2,500 IU; vitamin E 25 IU; vitamin $K_3$ 2.0 mg; and sodium selenite, 0.25 mg.

### *Nutrient digestibility*

Apparent nutrient digestibility was assessed using titanium dioxide ($TiO_2$) as an indigestible marker, following Short et al. (1996) with minor modifications. $TiO_2$ was included in the diets at a concentration of 5 g/kg. After slaughter, ileal digesta from each pen were collected, pooled, frozen (−20°C), and later oven-dried at 55°C. Feed and digesta samples were further dried at 105°C for dry matter determination and ashed at 550°C for 16 h. For $TiO_2$ quantification, ash samples were digested in diluted $H_2SO_4$ containing $Na_2SO_4$, heated for 20 h, cooled, diluted to volume, and allowed to settle. Absorbance was measured at 408 nm using a spectrophotometer. Titanium concentrations were determined against calibration standards prepared from a $TiO_2$ stock solution. Nutrient digestibility (ND) was calculated according to Myers et al. (2004):

$$ND(\%) = \left[1 - \left(\frac{TiO_2 \text{ in diet}}{TiO_2 \text{ in digesta}}\right)\left(\frac{\text{Nutrient in digesta}}{\text{Nutrient in diet}}\right)\right] \times 100$$

*Growth performance*

Day-old chicks were weighed on a platform scale and reweighed weekly to determine body weight gain (BWG). Feed offered to each pen was weighed at feeding, and refusals were collected and weighed before the next feeding to obtain average feed intake (AFI) as the difference between feed offered and feed refused, divided by the number of birds. Mortalities were recorded as they occurred, and carcass weights were used to adjust feed intake when calculating the feed conversion ratio (FCR). Body weight gain was calculated as the difference between final and initial body weight per bird, and FCR was obtained as the ratio of feed intake to body weight gain. These calculations followed the procedures described by Omoor et al. (2024).

*Blood parameters*

At day 40, blood was collected from two randomly selected birds per pen to assess haematological and serum biochemical parameters as described by Dlamini et al. (2023). Samples for haematology were drawn from the brachial vein into EDTA tubes and analyzed using an automated IDEXX LaserCyte Haematology Analyser to determine white blood cell counts, haemoglobin levels, red blood cell counts, and platelet indices. Blood for serum biochemistry was collected in clot-activator tubes, centrifuged at 412 × g for 15 minutes, and analysed using an IDEXX Catalyst One Chemistry Analyser for cholesterol, protein fractions, liver enzymes, mineral indices, glucose, amylase, and lipase.

*Slaughter procedure, carcass, and internal organs measurements*

At the end of the trial (day 42), six birds were randomly selected from each pen for slaughter and carcass evaluation. Thus, 216 chickens were transported in crates to the on-site abattoir at Rooigrond Farm and humanely slaughtered. After bleeding, birds were scalded and mechanically plucked, and their carcasses were grouped by experimental unit. Initial pH measurements were taken, after which carcasses were refrigerated overnight at 4°C, and cold carcass weight (CCW) was recorded. Twenty-four hours post-slaughter, carcass traits (drumstick, thigh, breast, and wings) and internal organs (proventriculus, gizzard, spleen, liver, pancreas, and intestines with contents) were weighed. All organ and carcass part weights were expressed relative to CCW (g/100 g).

*Breast and thigh meat quality attributes*

Meat pH and temperature were measured at 1 and 24 h post-slaughter in the central portions of the breast and thigh muscles using a calibrated pH–temperature probe. Meat colour (L*, a*, b*) was assessed on the surface of breast and thigh samples using a calibrated Minolta colorimeter, and hue and chroma were subsequently calculated following Kumalo et al. (2024). Breast meat tenderness was evaluated using a TA-XT Plus Texture Analyzer (Stable Micro Systems, Surrey, UK) fitted with a Meullenet–Owens Razor Shear Blade, and shear force values were recorded in Newtons. Drip loss (DL) was determined by weighing 5 – 10 g breast samples before and after a 48-h storage period at 4°C. Samples were suspended vertically by a thin string in 250 mL bottles to prevent contact with the container walls, and DL was expressed as a percentage according to Xiong et al. (2022). Water-holding capacity (WHC) was measured using the filter paper press method (Davoodi & Ehsani, 2020): 5 – 10 g breast samples were placed between filter papers and pressed with a 60 kg weight for 5 min; the proportional weight reduction was recorded. Cooking loss (CL) was assessed following Sari et al. (2021) with minor modifications. Breast samples (30 – 40 g) were sealed in plastic bags, steam-cooked in a water bath at 80°C for 60 min, cooled, and then reweighed to determine weight loss.

*Statistical analysis*

Levene's test and the NORMAL option in the Univariate Procedure statement were used to assess homogeneity of variance and normality, respectively. Dietary differences were analyzed using one-way ANOVA according to the statistical model $Y_{ij} = \mu + D_i + E_{ij}$, where $Y_{ij}$ = dependent variable, $\mu$ = population mean, $D_i$ = effect of diets, and $E_{ij}$ = random error associated with observation *ij,* assumed to be normally and independently distributed. Mean differences were compared using the probability of difference (PDIFF). To evaluate potential dose–response patterns associated with increasing levels of the *Bacillus subtilis* probiotic mixture (0, 0.3, 0.4, 0.5, and 0.6%), orthogonal polynomial contrasts were performed to test for linear and quadratic trends. Regression analysis was used only to describe the direction of significant trends across probiotic inclusion levels and not as the primary basis for treatment inference. The following non-linear equation was used to fit response data: $y = ax^2 + bx + c$, where $y$ = response variable, *a, b*, and *c* = coefficients of the quadratic equation, and $x$ = dietary levels of the multi-strain *B. subtilis* probiotic. The $x$ value for the most favourable response was determined as: $x = \frac{-b}{2a}$. Regression results were interpreted cautiously and were used only to describe trends across probiotic inclusion levels. Conclusions regarding treatment effects were based primarily on GLM comparisons among dietary treatments. All statistical analyses were performed using SAS (2013), and significance was declared at $P < 0.05$.

## Results

Treatment effects were first evaluated using one-way ANOVA. Polynomial contrasts were subsequently used to identify linear or quadratic trends across increasing levels of probiotic inclusion.

*Nutrient digestibility*

Dietary treatments had a significant impact on nutrient digestibility (Table 2). Organic matter digestibility (OMD) differed among treatments ($p = 0.003$), with POSC exhibiting the lowest values, whereas all other diets were comparable. A negative quadratic response to incremental levels of the probiotic was also detected for OMD. Neutral detergent fibre digestibility (NDFD) followed a similar pattern: probiotic-supplemented diets showed higher and comparable digestibility, while PRB0 and POSC resulted in the lowest values ($p < .0001$), with a negative quadratic effect. Ether extract digestibility (EED) and crude protein digestibility (CPD) did not differ significantly among treatments ($p = 0.153$; $p = 0.084$), although CPD exhibited a negative quadratic response to increasing probiotic inclusion. Gross energy digestibility (GED) varied among treatments ($p = 0.003$); POSC again produced the lowest values, PRB0 was intermediate, and all probiotic-supplemented diets were similar. Additionally, GED increased linearly with increasing probiotic dosage.

*Mortality and growth performance*

The inclusion of *B.subtilis* probiotic mixture in the broiler diet showed no significant effect on the mortality of Ross 308 broilers. Table 3 shows that there were no significant dietary effects on the growth performance metrics in the starter phase. Significant dietary effects were observed on BWG and FCR during the grower phase, with diet PRB5 promoting the highest BWG ($p = 0.017$) and the lowest FCR, whereas diet PRB6 showed the lowest gain and the highest FCR. Weight

**Table 2**
Impact of dietary supplementation with a novel multi-strain *B. subtilis* probiotic mixture on nutrient digestibility (g/kg DM) in Ross 308 male broilers (n = 900).

| | [1]Diets | | | | | | | Significance | | |
|---|---|---|---|---|---|---|---|---|---|---|
| [2]Parameters | POSC | PRB0 | PRB3 | PRB4 | PRB5 | PRB6 | [3]SEM | ANOVA | Linear | Quadratic |
| OMD | 68.03$^{b}$ | 71.16$^{a}$ | 73.47$^{a}$ | 73.90$^{a}$ | 72.81$^{a}$ | 72.29$^{a}$ | 0.641 | 0.003 | 0.129 | 0.014 |
| NDFD | 55.35$^{b}$ | 58.47$^{b}$ | 70.50$^{a}$ | 68.37$^{a}$ | 70.63$^{a}$ | 68.47$^{a}$ | 1.026 | <.0001 | <.0001 | 0.001 |
| EED | 61.31 | 72.13 | 66.65 | 68.97 | 69.09 | 62.66 | 2.936 | 0.153 | 0.113 | 0.747 |
| CPD | 88.27 | 87.49 | 90.29 | 89.71 | 89.01 | 88.87 | 0.620 | 0.084 | 0.145 | 0.020 |
| GED | 70.03$^{b}$ | 72.88$^{ab}$ | 74.39$^{a}$ | 75.69$^{a}$ | 75.04$^{a}$ | 75.05$^{a}$ | 0.799 | 0.003 | 0.033 | 0.304 |

$^{a,\ b}$ Common superscripts within a row indicate that the means do not differ ($p > 0.05$).

[1] Diets: POSC = a basal broiler diet with 0.5 g/kg zinc bacitracin but without the novel *B.subtilis* probiotic mixture; PRB0 = a basal broiler diet without AGP or the novel *B. subtilis* probiotic mixture; PRB3 = a basal broiler diet supplemented with 0.3% of the novel *B.subtilis* probiotic mixture; PRB4 = a basal broiler diet supplemented with 0.4% of the novel *B.subtilis* probiotic mixture; PRB5 = a basal broiler diet supplemented with 0.5% of the novel *B.subtilis* probiotic mixture; PRB6 = a basal broiler diet supplemented with 0.6% of the novel *B.subtilis* probiotic mixture.

[2] Parameters: OMD, organic matter digestibility; NDFD, neutral detergent fiber digestibility; EED, ether extract digestibility; CPD, crude protein digestibility; GED, gross energy digestibility.

[3] SEM: standard error of means. ANOVA: Analysis of variance.

**Table 3**
Effects of dietary supplementation with incremental levels of a novel Bacillus subtilis probiotic mixture on growth performance of Ross 308 male broilers (n = 900).

| | [1]Diets | | | | | | | *P* value | | |
|---|---|---|---|---|---|---|---|---|---|---|
| [2]Parameters | POSC | PRB0 | PRB3 | PRB4 | PRB5 | PRB6 | [3]SEM | ANOVA | Linear | Quadratic |
| Starter (0 - 14 days) | | | | | | | | | | |
| FI (g/bird) | 426.9 | 438.7 | 431.3 | 430.8 | 430.3 | 439.0 | 3.862 | 0.186 | 0.539 | 0.032 |
| BWG (g/bird) | 249.5 | 240.3 | 245.0 | 238.5 | 249.0 | 246.2 | 7.03 | 0.836 | 0.751 | 0.482 |
| FCR | 1.782 | 1.765 | 1.767 | 1.812 | 1.736 | 1.790 | 0.047 | 0.911 | 0.870 | 0.918 |
| Grower (15 – 35 days) | | | | | | | | | | |
| FI (g/bird) | 1162 | 1174 | 1205 | 1189 | 1155 | 1179 | 13.57 | 0.147 | 0.920 | 0.627 |
| BWG (g/bird) | 712.8$^{ab}$ | 750.5$^{ab}$ | 723.3$^{ab}$ | 760.0$^{ab}$ | 783.3$^{a}$ | 699.0$^{b}$ | 17.45 | 0.017 | 0.388 | 0.075 |
| FCR | 1.552$^{bc}$ | 1.650$^{abc}$ | 1.669$^{ab}$ | 1.523$^{c}$ | 1.518$^{c}$ | 1.696$^{a}$ | 0.033 | 0.001 | 0.499 | 0.109 |
| Finisher (36 – 42 days) | | | | | | | | | | |
| FI (g/bird) | 1618 | 1618 | 1640 | 1636 | 1664 | 1648 | 23.44 | 0.713 | 0.213 | 0.881 |
| BWG (g/bird) | 1233 | 1256 | 1295 | 1220 | 1293 | 1256 | 22.15 | 0.123 | 0.422 | 0.495 |
| FCR | 1.288 | 1.315 | 1.269 | 1.343 | 1.287 | 1.314 | 0.024 | 0.330 | 0.984 | 0.592 |
| Cumulative (0 – 42 days) | | | | | | | | | | |
| FI (g/bird) | 3207 | 3231 | 3276 | 3222 | 3283 | 3267 | 33.47 | 0.490 | 0.404 | 0.938 |
| BWG (g/bird) | 2195 | 2246 | 2263 | 2218 | 2325 | 2201 | 31.88 | 0.066 | 0.324 | 0.185 |
| FCR | 1.428$^{ab}$ | 1.474$^{ab}$ | 1.448$^{ab}$ | 1.453$^{ab}$ | 1.412$^{b}$ | 1.485$^{a}$ | 0.016 | 0.027 | 0.528 | 0.081 |

$^{a,\ b}$ Common superscripts within a row indicate that the means do not differ ($p > 0.05$).

[1] Diets: POSC = a basal broiler diet with 0.5 g/kg zinc bacitracin but without the novel *B.subtilis* probiotic mixture; PRB0 = a basal broiler diet without AGP or the novel *B. subtilis* probiotic mixture; PRB3 = a basal broiler diet supplemented with 0.3% of the novel *B.subtilis* probiotic mixture; PRB4 = a basal broiler diet supplemented with 0.4% of the novel *B.subtilis* probiotic mixture; PRB5 = a basal broiler diet supplemented with 0.5% of the novel *B.subtilis* probiotic mixture; PRB6 = a basal broiler diet supplemented with 0.6% of the novel *B.subtilis* probiotic mixture.

[2] Parameters: FI, feed intake; BWG, body weight gain; FCR, feed conversion ratio.

[3] SEM: standard error of means. ANOVA: Analysis of variance.

**Table 4**
Effects of dietary supplementation with incremental levels of a novel *Bacillus subtilis* probiotic mixture on haematological parameters in Ross 308 male broilers (n = 900).

| | [1]Diet | | | | | | *P* value | | |
|---|---|---|---|---|---|---|---|---|---|
| [2]Parameters | POSC | PRB0 | PRB3 | PRB4 | PRB5 | PRB6 | ANOVA | Linear | Quadratic |
| RBC (M/μL) | 0.515±0.130$^{b}$ | 0.603±0.130$^{b}$ | 1.257±0.184$^{a}$ | 0.760±0.130$^{b}$ | 0.805±0.160$^{b}$ | 0.460±0.130$^{b}$ | 0.018 | 0.333 | 0.018 |
| Platelet (K/μL) | 1395±238.1$^{ab}$ | 1205±238.1$^{ab}$ | 248.0±336.7$^{b}$ | 4092±238.1$^{a}$ | 426.9±291.6$^{b}$ | 595.8±238.1$^{b}$ | 0.018 | 0.160 | 0.260 |
| HGB (g/dL) | 10.45±0.949 | 12.02±0.949 | 9.550±0.949 | 11.33±0.949 | 11.47±0.949 | 8.783±0.949 | 0.162 | 0.012 | 0.8779 |
| WBC(K/μL) | 57.71±11.60$^{ab}$ | 101.9±11.60$^{a}$ | 23.36±11.60$^{b}$ | 19.91±11.60$^{b}$ | 40.43±11.60$^{b}$ | 20.37±11.60$^{b}$ | 0.001 | <.001 | 0.007 |
| LYM (%) | 0.886±0.126$^{ab}$ | 0.972±0.126$^{ab}$ | 0.766±0.126$^{ab}$ | 0.511±0.126$^{b}$ | 0.628±0.126$^{b}$ | 1.194±0.126$^{a}$ | 0.009 | 0.989 | 0.002 |
| NEU (%) | 0.387±0.081$^{ab}$ | 0.329±0.081$^{ab}$ | 0.493±0.081$^{ab}$ | 0.716±0.081$^{a}$ | 0.472±0.081$^{ab}$ | 0.191±0.081$^{b}$ | 0.003 | 0.853 | 0.001 |
| MONO (%) | 0.433±0.086 | 0.424±0.086 | 0.382±0.086 | 0.523±0.086 | 0.446±0.086 | 0.247±0.086 | 0.361 | 0.424 | 0.181 |
| EOS (%) | 0.239±0.042 | 0.202±0.042 | 0.288±0.042 | 0.218±0.042 | 0.230±0.042 | 0.120±0.042 | 0.149 | 0.291 | 0.018 |
| PDW (%) | 0.399±0.062$^{a}$ | 0.393±0.062$^{ab}$ | 0.164±0.062$^{ab}$ | 0.159±0.062$^{b}$ | 0.246±0.062$^{ab}$ | 0.399±0.062$^{a}$ | 0.011 | 0.628 | 0.001 |

$^{a,\ b}$ Common superscripts within a row indicate that the means do not differ ($p > 0.05$).

[3]SEM: standard error of means. ANOVA: Analysis of variance.

[1] Diets: POSC = a basal broiler diet with 0.5 g/kg zinc bacitracin but without the novel *B.subtilis* probiotic mixture; PRB0 = a basal broiler diet without AGP or the novel *B. subtilis* probiotic mixture; PRB3 = a basal broiler diet supplemented with 0.3% of the novel *B.subtilis* probiotic mixture; PRB4 = a basal broiler diet supplemented with 0.4% of the novel *B.subtilis* probiotic mixture; PRB5 = a basal broiler diet supplemented with 0.5% of the novel *B.subtilis* probiotic mixture; PRB6 = a basal broiler diet supplemented with 0.6% of the novel *B.subtilis* probiotic mixture.

[2] Parameters: RBC, red blood cells; PLT, platelet; HGB, hemoglobin; WBC, white blood cells; LYM, lymphocytes; NEU, neutrophils; MONO, monocytes; EOS, eosinophils; PDW, platelet distribution width.

gains in birds on diets PRB0, POSC, PRB4, and PRB3 were statistically similar ($p > 0.05$). FCR for PRB4 did not differ significantly from that for PRB5, whereas PRB0, POSC, and PRB3 showed intermediate FCR values. No dietary treatment effects were observed in the finisher phase and overall growth performance metrics. However, cumulative FCR was significantly affected by dietary treatments, with diet PRB6 (1.485) recording the highest ($p = 0.027$) FCR and PRB5 (1.412) the lowest, while PRB0, POSC, PRB3, and PRB4 were statistically similar.

### Blood parameters

Dietary treatments significantly influenced all haematological parameters except monocytes (Table 4). Red blood cell (RBC) counts were highest ($p = 0.018$) in birds fed PRB3 and lowest in those receiving PRB6. Platelet counts were higher ($p = 0.018$) in PRB4-fed broilers than in PRB3, PRB5, and PRB6, while the control group showed intermediate values. Haemoglobin concentrations did not differ among diets ($p = 0.162$). White blood cell (WBC) counts differed significantly across treatments, with PRB0 birds exhibiting the highest values ($p = 0.001$) and PRB4 the lowest. Lymphocyte percentages were significantly affected by dietary treatments ($p = 0.009$), with the highest values in PRB6 and the lowest in PRB4. The neutrophil percentage differed significantly among dietary treatments ($p = 0.003$), with PRB4 producing the highest values and PRB6 producing the lowest. Although eosinophil counts did not differ significantly across diets ($p = 0.149$). Platelet distribution width (PDW) was affected by diet ($p = 0.011$), with POSC and PRB6 showing the highest values, PRB4 the lowest, and other treatments remaining statistically similar. Table 5 shows that supplementation with the novel multi-strain *B. subtilis* probiotic had no effect ($p = 0.142$) on any of the measured serum biochemical components in broiler chickens. However, increasing the levels of the *B. subtilis* probiotic induced a positive quadratic response in phosphate [$R^2 = 0.364$; $p = 0.029$] and calcium [$R^2 = 0.480$; $p = 0.013$].

### Carcass and internal organs

Table 6 indicates that dietary treatments had no significant effects on carcass traits, final body weight, CCW, or dressing percentage. However, relative duodenal weight differed among treatments ($p = 0.020$), with PRB0 and POSC producing the highest values and the PRB5 diet the lowest. Jejunal length was also influenced by diet ($p < 0.001$), while PRB0, POSC, PRB4, and PRB5 were similar.

### Breast and thigh meat quality attributes

Experimental diets significantly affected several meat quality attributes (Table 7). For breast meat colour, PRB0 produced the highest redness values ($p = 0.043$), whereas no dietary effects were detected for thigh colour or for breast and thigh pH ($p = 0.080$). Shear force values differed among treatments ($p = 0.024$), with PRB0 meat being the toughest and PRB4 the most tender. A positive quadratic response was observed for shear force [$R^2 = 0.337$; $p = 0.012$]. The WHC also exhibited a positive quadratic pattern [$R^2 = 0.337$; $p = 0.012$], with PRB0 and PRB5 showing the highest WHC and PRB4 the lowest ($p = 0.011$). The DL was significantly influenced by PRB4 diet ($p = 0.001$), while POSC and PRB6 had the lowest ($p = <.0001$). The CL differed among treatments, with POSC and PRB3 producing the highest CL values and PRB5 and PRB0 the lowest ($p = <.0001$).

## Discussion

### Nutrient digestibility

Supplementing broiler diets with the *B. subtilis* probiotic mixture improved the digestibility of OM, NDF, and GE, indicating enhanced nutrient utilisation. These effects are consistent with reports that probiotics stimulate digestive enzyme activity and promote efficient nutrient absorption (Mountzouris et al., 2010; Jha et al., 2020). These effects are consistent with reports that probiotics stimulate digestive enzyme activity and promote efficient nutrient absorption (Jin et al., 1997; He et al., 2019; Idowu et al., 2025). The superior digestibility observed at the 0.5% inclusion level aligns with the improved growth performance in this group, suggesting a direct link between nutrient use efficiency and production outcomes. The higher NDF digestibility in probiotic-fed broilers, compared with PRB0 and POSC birds, further highlights the ability of *B. subtilis* to enhance fibre breakdown, an effect not achieved with zinc bacitracin. Crude protein digestibility tended to decline at the highest probiotic inclusion level (0.6%), suggesting that excessive supplementation may reduce protein utilization efficiency. Similar observations by Mountzouris et al. (2010) suggest that excessive

**Table 5**
Impact of dietary supplementation with incremental levels of a novel multi-strain *Bacillus subtilis* probiotic mixture on serum biochemical parameters in Ross 308 male broilers (n = 900).

| | [1]Diet | | | | | | | P value | | |
|---|---|---|---|---|---|---|---|---|---|---|
| [2]Parameters | POSC | PRB0 | PRB3 | PRB4 | PRB5 | PRB6 | [3]SEM | ANOVA | Linear | Quadratic |
| Glucose (mg/dL) | 119.4 | 96.25 | 112.7 | 104.2 | 96.33 | 91.00 | 17.98 | 0.774 | 0.401 | 0.979 |
| Phosphate (mg/dL) | 7.720 | 8.525 | 7.450 | 7.600 | 8.583 | 8.567 | 0.548 | 0.287 | 0.268 | 0.029 |
| Calcium (mg/dL) | 10.48 | 10.70 | 10.45 | 10.46 | 10.67 | 10.55 | 0.227 | 0.916 | 0.975 | 0.013 |
| TP (g/dL) | 3.920 | 3.625 | 4.250 | 3.400 | 3.900 | 4.433 | 0.490 | 0.553 | 0.841 | 0.487 |
| GGT (U/L) | 7.620 | 4.125 | 8.850 | 10.40 | 3.167 | 11.50 | 3.849 | 0.420 | 0.533 | 0.904 |
| TBL (mg/dL) | 24.28 | 57.88 | 15.90 | 0.200 | 0.283 | 0.333 | 17.69 | 0.142 | 0.161 | 0.887 |
| Cholesterol (mg/dL) | 189.4 | 197.8 | 135.3 | 115.6 | 115.5 | 125.5 | 35.26 | 0.280 | 0.345 | 0.880 |
| Globulins (g/dL) | 2.420 | 2.733 | 2.733 | 1.860 | 2.233 | 2.767 | 0.482 | 0.459 | 0.624 | 0.687 |
| Albumin (g/dL) | 1.540 | 4.425 | 1.483 | 1.540 | 1.650 | 1.633 | 0.974 | 0.222 | 0.301 | 0.096 |
| ALB/GLOB | 0.900 | 0.567 | 0.533 | 3.280 | 0.950 | 0.600 | 1.157 | 0.255 | 0.440 | 0.994 |
| ALT (U/L) | 14.00 | 315.5 | 13.25 | 16.00 | 14.33 | 13.33 | 224.2 | 0.733 | 0.323 | 0.343 |
| ALKP (U/L) | 621.5 | 720.3 | 673.8 | 1233 | 693.8 | 1213 | 234.7 | 0.152 | 0.899 | 0.226 |
| Amylase (U/L) | 259.2 | 174.5 | 323.2 | 292.0 | 247.8 | 279.8 | 46.26 | 0.258 | 0.195 | 0.492 |
| Lipase (U/L) | 144.5 | 132.5 | 190.4 | 155.6 | 152.2 | 163.0 | 32.99 | 0.630 | 0.251 | 0.856 |

[1] Diets: POSC = a basal broiler diet with 0.5 g/kg zinc bacitracin but without the novel *B.subtilis* probiotic mixture; PRB0 = a basal broiler diet without AGP or the novel *B. subtilis* probiotic mixture; PRB3 = a basal broiler diet supplemented with 0.3% of the novel *B.subtilis* probiotic mixture; PRB4 = a basal broiler diet supplemented with 0.4% of the novel *B.subtilis* probiotic mixture; PRB5 = a basal broiler diet supplemented with 0.5% of the novel *B.subtilis* probiotic mixture; PRB6 = a basal broiler diet supplemented with 0.6% of the novel *B.subtilis* probiotic mixture.

[2] Parameters: TP, total protein; TBL, total bilirubin; GGT, gamma-glutamyl transferase; ALB/GLOB, albumin to globulin ratio; ALT, alanine aminotransferase; ALKP, alkaline phosphatase.

[3] SEM: standard error of means. ANOVA: Analysis of variance.

**Table 6**
Impact of dietary supplementation with incremental levels of a novel multi-strain Bacillus subtilis probiotic mixture on carcass traits and internal organs (g/100 g CCW) of Ross 308 male broilers (n = 900).

| | [1]Diets | | | | | | | P value | | |
|---|---|---|---|---|---|---|---|---|---|---|
| [2]Parameters | POSC | PRB0 | PRB3 | PRB4 | PRB5 | PRB6 | [3]SEM | ANOVA | Linear | Quadratic |
| FBW (g) | 2289 | 2238 | 2306 | 2260 | 2368 | 2243 | 31.83 | 0.066 | 0.939 | 0.671 |
| CCW (g) | 1373 | 1348 | 1315 | 1304 | 1428 | 1273 | 42.06 | 0.451 | 0.827 | 0.888 |
| Dressing (%) | 59.60 | 57.22 | 56.54 | 58.37 | 60.30 | 57.30 | 1.351 | 0.337 | 0.850 | 0.689 |
| Breast | 17.08 | 18.86 | 18.22 | 17.32 | 16.11 | 17.44 | 1.134 | 0.626 | 0.127 | 0.806 |
| Wing | 5.765 | 5.385 | 5.861 | 5.935 | 5.296 | 5.859 | 0.205 | 0.148 | 0.347 | 0.328 |
| Thigh | 8.223 | 7.562 | 8.064 | 8.120 | 7.557 | 7.965 | 0.309 | 0.513 | 0.506 | 0.340 |
| Drumstick | 7.066 | 6.991 | 7.254 | 7.143 | 6.727 | 7.121 | 0.257 | 0.777 | 0.810 | 0.625 |
| Proventriculus | 0.583 | 0.627 | 0.657 | 0.686 | 0.639 | 0.651 | 0.027 | 0.179 | 0.437 | 0.247 |
| Gizzard | 2.670 | 2.716 | 2.858 | 2.954 | 2.826 | 2.841 | 0.098 | 0.382 | 0.203 | 0.254 |
| Spleen | 0.179 | 0.163 | 0.158 | 0.167 | 0.189 | 0.187 | 0.018 | 0.773 | 0.060 | 0.477 |
| Liver | 3.010 | 3.122 | 2.910 | 3.094 | 2.964 | 3.053 | 0.126 | 0.841 | 0.891 | 0.416 |
| Pancreas | 0.373 | 0.358 | 0.372 | 0.338 | 0.324 | 0.385 | 0.025 | 0.500 | 0.869 | 0.611 |
| Duodenum | 1.648[a] | 1.646[a] | 1.472[ab] | 1.420[ab] | 1.385[b] | 1.468[ab] | 0.063 | 0.020 | 0.061 | 0.225 |
| Jejunum | 2.997 | 3.233 | 2.958 | 3.201 | 2.911 | 3.198 | 0.183 | 0.693 | 0.958 | 0.504 |
| Ileum | 2.149 | 2.391 | 2.257 | 2.276 | 2.178 | 2.289 | 0.109 | 0.678 | 0.423 | 0.473 |
| SIW | 6.794 | 7.269 | 6.687 | 6.897 | 6.474 | 6.956 | 0.303 | 0.569 | 0.537 | 0.385 |
| Caeca | 0.897 | 0.907 | 1.025 | 1.013 | 0.930 | 0.964 | 0.072 | 0.722 | 0.786 | 0.549 |
| Duodenum (cm) | 39.55 | 42.01 | 40.28 | 39.85 | 40.38 | 41.17 | 1.264 | 0.737 | 0.307 | 0.120 |
| Jejunum (cm) | 90.89[ab] | 92.31[ab] | 85.67[b] | 91.04[ab] | 98.22[ab] | 99.86[a] | 3.252 | 0.046 | 0.025 | 0.027 |
| Ileum (cm) | 98.2 | 100.6 | 97.3 | 97.6 | 101.4 | 101.8 | 2.899 | 0.748 | 0.951 | 0.133 |
| Caeca (cm) | 22.38 | 23.30 | 23.49 | 23.64 | 22.76 | 23.48 | 0.445 | 0.244 | 0.573 | 0.791 |

[a, b] Common superscripts within a row indicate that the means do not differ ($p > 0.05$).
[1] Diets: POSC = a basal broiler diet with 0.5 g/kg zinc bacitracin but without the novel *B.subtilis* probiotic mixture; PRB0 = a basal broiler diet without AGP or the novel *B. subtilis* probiotic mixture; PRB3 = a basal broiler diet supplemented with 0.3% of the novel *B.subtilis* probiotic mixture; PRB4 = a basal broiler diet supplemented with 0.4% of the novel *B.subtilis* probiotic mixture; PRB5 = a basal broiler diet supplemented with 0.5% of the novel *B.subtilis* probiotic mixture; PRB6 = a basal broiler diet supplemented with 0.6% of the novel *B.subtilis* probiotic mixture.
[2] Parameters: FBW, final body weight; CCW, cold carcass weight; SIW, small intestines weight.
[3] SEM: standard error of means. ANOVA: Analysis of variance.

**Table 7**
Effects of a novel multi-strain Bacillus subtilis probiotic mixture supplemented in a basal broiler diet on meat quality attributes in Ross 308 male broiler chickens (n = 900).

| | [1]Diets | | | | | | | P value | | |
|---|---|---|---|---|---|---|---|---|---|---|
| [2]Parameters | POSC | PRB0 | PRB3 | PRB4 | PRB5 | PRB6 | [3]SEM | ANOVA | Linear | Quadratic |
| Thigh measurements | | | | | | | | | | |
| *L** | 47.49 | 47.66 | 47.54 | 48.48 | 48.46 | 47.30 | 0.440 | 0.265 | 0.894 | 0.093 |
| *a** | 4.862 | 4.787 | 4.937 | 4.640 | 4.696 | 4.576 | 0.166 | 0.640 | 0.183 | 0.746 |
| *b** | 4.378 | 4.141 | 4.371 | 4.292 | 4.599 | 4.394 | 0.263 | 0.896 | 0.328 | 0.585 |
| Chroma | 6.571 | 6.346 | 6.611 | 6.330 | 6.580 | 6.352 | 0.243 | 0.904 | 0.978 | 0.588 |
| Hue | 0.729 | 0.710 | 0.719 | 0.747 | 0.775 | 0.763 | 0.030 | 0.612 | 0.063 | 0.680 |
| pH 1hr | 6.156 | 6.072 | 6.097 | 6.054 | 6.052 | 6.038 | 0.061 | 0.774 | 0.625 | 0.665 |
| pH 24hrs | 6.232 | 6.042 | 6.132 | 6.037 | 5.939 | 6.119 | 0.074 | 0.131 | 0.971 | 0.998 |
| Breast measurements | | | | | | | | | | |
| *L** | 48.06 | 46.83 | 47.44 | 46.72 | 47.02 | 45.72 | 1.121 | 0.752 | 0.257 | 0.306 |
| *a** | 3.116[a] | 3.196[a] | 2.564[b] | 2.907[b] | 3.039[b] | 2.717[b] | 0.150 | 0.043 | 0.390 | 0.538 |
| *b** | 4.647 | 4.691 | 4.937 | 4.986 | 5.017 | 5.134 | 0.236 | 0.655 | 0.152 | 0.727 |
| Chroma | 5.617 | 5.687 | 5.576 | 5.803 | 5.881 | 5.814 | 0.201 | 0.870 | 0.343 | 0.930 |
| Hue | 0.973 | 0.975 | 1.091 | 1.036 | 1.024 | 1.082 | 0.034 | 0.080 | 0.202 | 0.595 |
| pH 1hr | 5.839 | 5.937 | 5.954 | 5.815 | 5.834 | 5.864 | 0.069 | 0.628 | 0.254 | 0.982 |
| pH 24hrs | 5.850 | 5.929 | 5.854 | 5.843 | 5.774 | 5.843 | 0.042 | 0.270 | 0.029 | 0.416 |
| SF (N) | 3.773[ab] | 4.626[a] | 3.706[ab] | 3.370[b] | 3.857[ab] | 3.907[ab] | 0.237 | 0.024 | 0.018 | 0.012 |
| WHC (%) | 84.21[ab] | 89.57[a] | 82.92[ab] | 76.06[b] | 87.83[a] | 82.92[ab] | 2.478 | 0.011 | 0.141 | 0.064 |
| DL (%) | 7.650[c] | 8.497[bc] | 14.11[ab] | 14.57[a] | 12.33[abc] | 6.789[c] | 1.385 | 0.001 | 0.957 | <.0001 |
| CL (%) | 32.80[a] | 27.43[b] | 32.58[a] | 30.02[ab] | 27.04[b] | 29.32[ab] | 0.867 | <.0001 | 0.684 | 0.011 |

[a, b, c] Common superscripts within a row indicate that the means do not differ ($p > 0.05$).
[1] Diets: POSC = a basal broiler diet with 0.5 g/kg zinc bacitracin but without the novel *B.subtilis* probiotic mixture; PRB0 = a basal broiler diet without AGP or the novel *B. subtilis* probiotic mixture; PRB3 = a basal broiler diet supplemented with 0.3% of the novel *B.subtilis* probiotic mixture; PRB4 = a basal broiler diet supplemented with 0.4% of the novel *B.subtilis* probiotic mixture; PRB5 = a basal broiler diet supplemented with 0.5% of the novel *B.subtilis* probiotic mixture; PRB6 = a basal broiler diet supplemented with 0.6% of the novel *B.subtilis* probiotic mixture.
[2] Parameters: *L**, lightness; *a**, redness; *b**, yellowness; SF, shear force; WHC, water holding capacity; DL, drip loss; CL, cooking loss.
[3] SEM: standard error of means. ANOVA: Analysis of variance.

probiotic supplementation may increase microbial competition for amino acids, reducing the fraction available to the host (Jha et al., 2020). Overall, the findings suggest that moderate inclusion levels of the novel multi-strain *B. subtilis* probiotic enhance nutrient liberation and absorption, supporting its potential as an effective alternative to AGPs in intensive broiler production.

*Growth performance*

Dietary supplementation with *B. subtilis* probiotics is widely recognised for its ability to enhance gut health by supporting the growth and metabolic activity of beneficial microbes, stabilising intestinal pH, and improving digestive enzyme secretion (Jiang et al., 2021). These mechanisms collectively enhance nutrient digestion and absorption, which, under normal rearing conditions, translate into improved growth performance. Given that high stocking density compromises gut integrity, immunity, and nutrient utilisation, the present study evaluated whether a multi-strain *B. subtilis* probiotic could mitigate these stress-related impairments and improve broiler productivity. In the current trial, probiotic supplementation did not significantly alter overall feed intake (FI) relative to the control diet; however, the pattern of responses across inclusion levels during the starter phase revealed an interesting trend. Birds receiving 0.4% and 0.5% inclusion levels consumed less feed than those on either lower or higher doses. This decline in intake at the mid doses is indicative of enhanced nutrient use efficiency, as reflected by the superior BWG and FCR recorded for PRB5. Improved digestive function, reflected by enhanced nutrient liberation and absorption, reduced the birds' metabolic demand for feed while still supporting optimal growth. This interpretation is consistent with observations by Liu et al. (2020), who reported reduced FI but improved BWG in broilers supplemented with *B. subtilis* at $5 \times 10^8$ CFU/g.

The reduced performance observed at the highest probiotic level (0.6%) suggests that over-supplementation may impair growth. Excessive probiotic inclusion has been linked to a lowered gut pH, altered microbial composition, and increased competition between the host and microbes for nutrients (Mohamed et al., 2022). Furthermore, high probiotic loads may accelerate the passage rate of digesta, reducing gut retention time and limiting nutrient absorption (Ravindran & Abdollahi, 2021; Singh & Kim, 2021). These mechanisms align with the observed reduction in BWG among PRB6 birds despite their increased FI, indicating inefficient conversion of ingested feed into tissue accretion. The improvement in FCR at the 0.4% and 0.5% inclusion levels further underscores the positive influence of moderate *B. subtilis* supplementation on digestive efficiency. Birds in the PRB5 group achieved higher BWG with proportionally lower FI, suggesting that this inclusion level optimises the balance between microbial activity and nutrient availability. The enhancement of feed efficiency at an optimal dosage is consistent with findings from Yu et al. (2024) and Upadhaya et al. (2019), who reported improved BWG and FCR with *B. subtilis* supplementation at comparable levels.

Literature reveals heterogeneity in growth performance outcomes, with some studies showing improvements in body weight gain (Biswas et al., 2023; Mohamed et al., 2022), while others report limited or no effects (Oladokun & Adewole, 2023; Hashemitabar & Hosseinian, 2024). These inconsistencies likely stem from variations in probiotic strain characteristics, viability, and dose, as well as the route of delivery (feed vs. water), bird genotype, diet composition, stocking density, and overall farm hygiene (Jeong & Kim, 2014; Zaghari et al., 2020). Importantly, the present results indicate that probiotic efficacy varies with inclusion level: the highest inclusion level failed to improve performance and, in some cases, exerted negative effects. Taken together, the results indicate that the 0.5% inclusion level produced the most favourable growth performance among the tested treatments under high-density rearing conditions. Exceeding this threshold may impose metabolic or physiological burdens that compromise performance. This variation with inclusion level has practical implications for industry adoption, emphasising the need for careful optimisation to ensure that probiotics function as effective and economically viable alternatives to antibiotic growth promoters.

*Blood parameters*

Haematological indices provide insight into the physiological and metabolic status of birds, including their response to nutritional interventions and environmental stressors (Dlamini et al., 2023). In this study, the elevated RBC counts observed in PRB3-fed broilers suggest enhanced oxygen-carrying capacity, potentially reflecting improved nutrient absorption and overall physiological status. Higher red blood cell levels can support more efficient oxygen transport to tissues, thereby promoting metabolic activity, growth, and overall performance. Similar stimulatory effects of *B. subtilis* on erythropoiesis have been reported in previous studies (Wang et al., 2017; Fairushin et al., 2022; El-Sayed et al., 2024). In contrast, the marked reduction in RBC counts at the highest inclusion level (PRB6) indicates that excessive probiotic supplementation may impair erythropoietic activity. This may be linked to disrupted gut integrity or altered mineral absorption, particularly of iron, resulting from excessive microbial fermentation at very high probiotic dosages (Ogbuewu et al., 2022). Although haemoglobin concentrations did not differ significantly among treatments, the gradual reduction observed at higher inclusion levels suggests that excessive probiotic supplementation may interfere with haemoglobin synthesis by competing for minerals or altering gut pH (Abdel-Hafeez et al., 2016). Together, these findings indicate that moderate supplementation levels support healthier hematological profiles, whereas excessive inclusion may have adverse effects.

White blood cell responses offer additional insight into stress and immune activation. Birds in the PRB0 group exhibited WBC counts above the normal physiological range (2.5 – 19.5 K/μL; Nwaigwe et al., 2020), indicating heightened stress under high stocking density. In contrast, WBC counts in PRB4 birds fell within the normal range, suggesting attenuation of systemic immune activation. This is likely mediated by improved gut barrier integrity and reduced translocation of opportunistic pathogens, benefits commonly associated with probiotic supplementation (Mountzouris et al., 2007; Li et al., 2018; Yousefi et al., 2019; Yosi & Metzler-Zebeli, 2023). The elevated neutrophil percentage in PRB4 further supports enhanced innate immune responsiveness, which is advantageous under high-density conditions where pathogen exposure and stress are elevated (Ibrahim et al., 2018). Platelet distribution width, a marker of inflammatory activity (Smith & Weyrich, 2011), was also influenced by dietary treatment. Lower PDW values in PRB3 and PRB5 indicate reduced inflammatory stress, consistent with probiotic-mediated modulation of intestinal health. Although serum biochemical indices were not significantly affected, the positive quadratic responses in calcium and phosphate concentrations suggest improved mineral metabolism at low-to-moderate probiotic doses, likely due to enhanced nutrient absorption associated with a more favourable gut microbial profile (Awad et al., 2009; Sohail et al., 2010). Overall, the haematological and mineral responses indicate that moderate inclusion levels of the multi-strain *B. subtilis* probiotic (0.3 – 0.5%) improved immune modulation, reduced physiological stress, and supported healthier metabolic profiles in broilers reared at high stocking densities. Excessive supplementation (0.6%) was, however, detrimental, reinforcing the importance of dosage optimisation to maximise probiotic efficacy.

*Carcass traits and internal organs*

Carcass traits and internal organ measurements are important indicators of how dietary interventions influence nutrient utilisation, gut morphology, and overall productive efficiency in broilers. In this study, most carcass traits were unaffected by dietary treatments, suggesting that *B. subtilis* supplementation did not compromise overall carcass yield. However, significant effects were observed in the duodenum weight and jejunum length. The reduced duodenum weight in PRB5 birds aligns with the improved FCR observed in this group, suggesting more efficient digestion and nutrient absorption at this probiotic inclusion level. A smaller duodenum may indicate a reduced digestive workload, allowing more dietary energy to be allocated to muscle accretion rather than to maintaining gut tissue (Inayah et al., 2022).

Conversely, the greater duodenum weight in PRB0 likely reflects increased digestive effort in birds receiving no functional feed additives, consistent with their poorer feed efficiency. Jejunal morphology varied with probiotic inclusion level, with PRB6 birds exhibiting the longest jejunum and PRB3 birds the shortest. A higher jejunal length at the 0.6% inclusion level may reflect morphological adaptation to maximize nutrient extraction when the diet composition or nutrient availability becomes suboptimal (Naeem & Bourassa, 2025). Probiotics are known to influence intestinal architecture, including villus height and crypt dynamics (Wijoyo & Nindria, 2024), and the present results support the concept that probiotic dosage can induce structural changes in the gastrointestinal tract. However, the longest jejunum was observed in PRB6 birds, which also demonstrated the poorest growth performance and the highest FCR. This pattern suggests that morphological elongation of the jejunum at excessive probiotic levels may be a compensatory rather than a beneficial response. High probiotic inclusion may dilute the nutrient density of the diet or alter intestinal fermentation patterns (Oikeh et al., 2019), triggering additional feed intake attempts that are insufficient to fully compensate for reduced nutrient availability (Leeson et al., 1991; Taylor et al., 2021). Maintaining a larger intestinal surface area also increases metabolic costs associated with peristalsis, enzyme production, and tissue maintenance (Clauss et al., 2016), diverting energy away from muscle growth (Yosi & Metzler-Zebeli, 2023). This is consistent with the lower BWG and impaired FCR observed in PRB6 birds.

The current findings align with those of Reis et al. (2017), who reported reduced duodenum mass and increased jejunum length in broilers supplemented with *B. subtilis*. While moderate supplementation enhances digestive efficiency, excessive inclusion appears to shift energy allocation toward maintaining gut tissue rather than supporting carcass yield. From a production perspective, this shift is undesirable, as reduced carcass weight diminishes the marketable output and overall profitability. Collectively, the results indicate that lower to moderate doses of *B. subtilis* (0.3 – 0.5%) optimise gut morphology and nutrient use efficiency, whereas excessive supplementation (0.6%) induces maladaptive intestinal enlargement and compromises growth. These findings reinforce the importance of dosage optimisation to maximise carcass output under intensive production conditions.

### *Breast and thigh meat quality attributes*

Meat quality is commonly assessed through pH, colour, tenderness, water-holding capacity (WHC), drip loss (DL), and cooking loss (CL) (Guo et al., 2020; Tang et al., 2021). In this study, supplementation with*B. subtilis* did not significantly affect the pH of breast or thigh muscles, although the slightly lower pH values observed at higher inclusion levels suggest a modest shift toward more acidic meat. The pH values remained within the normal physiological range (5.2 – 6.2; Ristic & Damme, 2013), indicating that the inclusion of probiotics did not compromise post-mortem muscle metabolism. As pH influences the redox state of myoglobin and, consequently, meat colour (Fletcher, 2002; Zhang et al., 2024), the reduced redness in probiotic-fed birds reflects more desirable consumer-preferred colour characteristics compared with the higher redness observed in untreated groups. This improvement may be attributed to the antioxidant-enhancing properties of *B. subtilis*, which help stabilise myoglobin and limit oxidative discoloration (Al-Owaimer et al., 2014). Water-related traits are also critical determinants of meat quality, influencing juiciness, texture, and nutritional value (Yang et al., 2016; Ismail & Huda, 2024). Birds receiving PRB5 exhibited increased WHC and reduced DL and CL, mirroring improvements reported in previous studies (Park & Kim, 2014; Mohammed et al., 2021; Wang et al., 2024). Enhanced WHC is often associated with better muscle membrane integrity and reduced protein oxidation, suggesting that *B. subtilis* may help preserve structural proteins through its antioxidant effects (Cramer et al., 2018). Similarly, the reduced DL and CL in both PRB5 and POSC parallel findings by Attia et al. (2023), indicating that *B. subtilis* can achieve water-retention benefits comparable to those of zinc bacitracin.

Shear force values tended to decrease as probiotic inclusion increased, but remained within the normal range for broilers (Bowker & Zhuang, 2019), suggesting improved tenderness without compromising muscle structure. This may be linked to enhanced proteolytic activity or improved muscle fibre integrity mediated indirectly through gut microbiota–muscle axis interactions, as proposed by Herich et al. (2025). Although some studies have reported no significant effects of *B. subtilis* on meat quality (Upadhaya et al., 2019), variation in strain composition, dosage, environmental conditions, and probiotic viability likely accounts for such discrepancies. In the present study, supplementation at moderate levels (0.4 – 0.5%) consistently improved key meat quality parameters, supporting the broader literature that *Bacillus*-based probiotics enhance sensory and physicochemical attributes of broiler meat (Zhou et al., 2015; Bai et al., 2017; Tang et al., 2021). Collectively, these findings indicate that the multi-strain *B. subtilis* blend, particularly at the 0.5% inclusion level, improves meat colour, tenderness, and water-holding capacity, thereby enhancing overall meat quality in broilers reared under high stocking density.

## Conclusions

Supplementation with a multi-strain *Bacillus subtilis* probiotic improved digestive efficiency, physiological status, and growth performance of broilers reared under high stocking density. The 0.5% inclusion level produced the most favourable responses, including improved body weight gain, feed conversion efficiency, and enhanced digestibility of organic matter, neutral detergent fibre, and gross energy, while also supporting desirable meat quality attributes. In contrast, the highest inclusion level (0.6%) appeared to induce maladaptive intestinal responses and reduced performance, highlighting the importance of dose optimisation. However, the study did not evaluate gut microbiota dynamics or digestive enzyme activity and was conducted under a single production environment, limiting mechanistic interpretation. Future studies integrating microbiome and enzymatic analyses are warranted.

## Declaration of generative AI and AI-assisted technologies in the writing process

During the preparation of this work, the authors utilized ChatGPT to enhance readability and language use in certain sections of the paper. After using this tool/service, the authors reviewed and edited the content as needed and take full responsibility for the publication's content.

## Ethical statement

All experimental procedures involving animals were conducted in accordance with internationally accepted standards for the care and use of animals in research and were approved by the University of Mpumalanga's Research Ethics Committee – Animal Sciences (UMP REC-AS; approval no. UMP/Mlambo/1/2024) and the North-West University Animal Production Research Ethics Committee (NWU–ANIMPROD REC; approval no. NWU-00818-24-A5).

Birds were handled and managed to minimize stress and discomfort throughout the experimental period. All sampling and slaughter procedures were performed humanely in accordance with institutional and national animal welfare guidelines.

## CRediT authorship contribution statement

**Zamile Mahlangu:** Writing – review & editing, Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Caven Mguvane Mnisi:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Software, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Victory Osirimade Sumanu:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Project administration, Formal analysis, Data curation. **Ghaneshree Moonsamy:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Resources, Project administration, Funding acquisition, Formal analysis, Conceptualization. **Omolola Esther Fayemi:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Project administration, Investigation, Funding acquisition, Conceptualization. **Rajesh Jha:** Writing – review & editing, Writing – original draft, Visualization, Validation, Resources, Methodology, Investigation, Conceptualization. **Victor Mlambo:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Software, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization.

## Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

## Acknowledgements

We acknowledge the Council for Scientific and Industrial Research (South Africa) and OptimusBio for the donation of probiotics. This research was funded by the National Research Foundation grant (CPRR23032387169).

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