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The effect of probiotic supplementation on meat and litter quality and leg health of broilers reared under high stocking density conditions

Rezpour, R.¹; Hosseinian, S. A.^{2*}; Shekarforoush, S. S.³; Basiri, S.³
and Haghathkhan, H.⁴

¹Ph.D. Student, Department of Clinical Science, School of Veterinary Medicine, Shiraz University, Shiraz, Iran; ²Avian Diseases Research Center, Department of Clinical Science, School of Veterinary Medicine, Shiraz University, Shiraz, Iran; ³Department of Food Hygiene and Public Health, School of Veterinary Medicine, Shiraz University, Shiraz, Iran; ⁴Ph.D. Student, Department of Food Hygiene and Public Health, School of Veterinary Medicine, Shiraz University, Shiraz, Iran

*Correspondence: S. A. Hosseinian, Avian Diseases Research Center, Department of Clinical Science, School of Veterinary Medicine, Shiraz University, Shiraz, Iran. E-mail: az.hosseinian@gmail.com

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Abstract

Background: High stocking density is one of the main factors that impair the performance and welfare of broilers. **Aims:** This study aimed to investigate the effects of *Bacillus* and *Lactobacillus* probiotics, either alone or in combination, on meat quality, peroxide value of fat tissue, leg health, and litter quality in broilers under high stocking density. **Methods:** Two hundred one-day-old Cobb 500 chicks were divided randomly into five treatments (4 replicates and ten birds per replicate): Control (normal stocking density, no probiotic), HD (high stocking density, no probiotic), HDpBacil (high stocking density, *Bacillus*-based probiotic), HDpLAB (high stocking density, *Lactobacillus*-based probiotic), HDpMix (high stocking density, combined probiotic containing *Bacillus* and *Lactobacillus*). Litter quality was assessed on days 21 and 42. Footpad dermatitis was also scored on days 14, 28, and 42. Gait score, peroxide value in fat tissue, and breast and thigh meat quality were measured on day 42. **Results:** The HD group had significantly the highest footpad dermatitis and gait scores and the lowest litter quality levels ($P<0.05$). The combined probiotic improved leg health and litter quality indices ($P<0.05$) and reduced the water holding capacity (WHC) and peroxide value ($P<0.05$). While, the highest peroxide value of abdominal fat and WHC of thigh meat were in the HD group ($P<0.05$). **Conclusion:** The combination of probiotics (*Bacillus* and *Lactobacillus*) can mitigate the adverse effects of high stocking density in broilers. It can be a beneficial feed additive in broilers against highly stressful conditions.

Key words: Broiler chicken, Leg health, Meat quality, Probiotic, Stocking density

Introduction

In response to the increasing global population and their demand for high-quality food, the poultry industry is aiming to intensive broiler production with the goal of reducing costs while maintaining bird welfare and production quality (Gržinić *et al.*, 2023). Stocking density (SD) refers to the number of birds or target body weight (kg) of birds per square meter (m^2) of rearing space (Zabir *et al.*, 2021). The UK Royal Society for the Prevention of Cruelty to Animals (RSPCA) Assured scheme (formerly known as Freedom Food) recommends 30 kg/m^2 (approximately 12.5 birds/ m^2) as the optimal stocking density for broilers weighing over 2 kg to ensure acceptable welfare (RSPCA, 2019).

High stocking density (HSD) or intensive rearing negatively impacts on the welfare, carcass characteristics, leg health, and litter quality of broilers

(Guinebretière *et al.*, 2024; Zhou *et al.*, 2024). Also, HSD leads to increase foot pad dermatitis (FPD) and lameness in broilers (Opengart *et al.*, 2018; Tahamtani *et al.*, 2018). Therefore, establishing an effective management strategy to improve leg health and litter quality at high stocking density is crucial and beneficial. Birds' health is improved by probiotics by balancing microbiota, improving intestinal health, and modulating physiological and immunological mechanisms throughout their body (Jha *et al.*, 2020; Yousaf *et al.*, 2022). *Bacillus* and *Lactobacillus* are the most used probiotics in chicken production (Ramlucken *et al.*, 2020). Since the used probiotic species and composition are main factors in their effectiveness, therefore, comparing the effects of different probiotic combinations is vital. Choosing the most effective probiotic mixture can be economically advantageous in enhancing the health and performance of broilers under stressful

conditions (Aliakbarpour *et al.*, 2012).

However, to our knowledge, there has been limited study into the comparative effects of *Bacillus* and *Lactobacillus* probiotics on broiler chickens under high stocking density conditions. Accordingly, the goal of this study was to investigate and compare the effects of administration of three different probiotic mixtures (multi-strain *Bacillus* and *Lactobacillus*, individually or in combination) via drinking water on meat quality, leg health, and litter quality indices in broiler chickens raised under high stocking density. Additionally, this study aimed to determine the most effective probiotic mixture for mitigating the negative consequences of high stocking density in broiler chickens.

Materials and Methods

Ethics statement

This experimental study was approved by the Shiraz University Research Ethics Committee (Approval No. 9730100/400) and performed at the Poultry research center, Faculty of Veterinary medicine, Shiraz University, Shiraz, Iran.

Birds and experimental design

A total of 200 day-old mixed-sex broiler chicks (Cobb 500) were obtained from a commercial hatchery company located in Shiraz, Fars. The birds were randomly divided into five equal groups, each consisting of four replicates with ten birds per replicate. The treatment groups were as follows: 1) Control: birds kept at normal stocking density with no probiotic administration; 2) High stocking density (HD): birds raised under high stocking density without probiotic treatment; 3) HD with *Bacillus* probiotics (HDpBacil): birds reared at HSD and administrated *Bacillus* probiotics via drinking water; 4) HD with *Lactobacillus* probiotics (HDpLAB): birds reared at HSD and received *Lactobacillus* probiotics via drinking water; 5) HD with mixed probiotics (HDpMix): birds housed at HSD and given a combination of *Bacillus* and *Lactobacillus* probiotics.

Each treatment group was housed separately in an environmentally controlled room for 42 days. Within each group, individual replicates were placed in separate pens that were furnished with fresh wood shavings to a depth of 7 cm and equipped with one manual trough feeder (120 cm long, 30 cm wide, and 23 cm high) and four automatic nipple drinkers. The treatment groups were reared under two stocking densities, calculated based on an estimated final body weight of 2.4 kg at 42 days of age. The control group was reared at a density of 12.5 birds/m² (equivalent to 30 kg of BW/m²), while the HD, HDpBacil, HDpLAB, and HDpMix groups were kept at a higher stocking density of 16 birds/m² (40 kg of BW/m²), as recommended by a previous study (Kridtayopas *et al.*, 2019). To ensure accurate comparisons, feeder and drinker space were adjusted equally in both stocking densities. During the current experiment, all environmental conditions (including

nutrition, temperature, humidity, lighting schedule, and ventilation) were maintained according to the Cobb 500 management guide (Cobb, 2022). The pens were equipped with gas heaters. The room temperature was initially set at 31-32°C for the first 48 h, then gradually reduced to 23-24°C by the end of the third week, and maintained at that level until the conclusion of the 42-day study. The birds were fed maize- and soybean meal-based pelleted diets across three feeding phases: the starter (days 1–10), the growth (days 11–25), and the finisher (days 26–42) as detailed in Table 1. These basal diets were formulated to meet the nutritional requirements of broilers, following the Cobb 500 guidelines (Cobb, 2022). Throughout the entire experiment, feed and water were provided *ad libitum*.

Table 1: Components of a basal diets (%), separated by growth phases including starter, grower and finisher periods

Ingredients (%)	Starter (1-10 d)	Grower (11-25 d)	Finisher (26-42 d)
Corn ground	53.47	58.08	61.39
Soybean meal 44%	37.28	33.27	29.30
Soybean oil degummed	3.02	3.08	4.08
Calcium carbonate 38%	1.73	1.44	1.36
Phosphate dicalcium	1.75	1.58	1.34
L-Lysine HCL	0.7	0.7	0.7
L- Threonine	0.10	0.10	0.10
DL-Methionine	0.20	0.15	0.15
L- Tryptophan	0.03	0.03	0.02
Glycine	0.04	0.04	0.03
Choline 50%	0.10	0.10	0.10
Sodium chloride	0.03	0.03	0.03
Mineral premix ¹	0.25	0.25	0.25
Vitamin premix ²	0.25	0.25	0.25
Calculated chemical composition			
ME (kcal/kg)	2900	3000	3100
Crude protein, %	22.0	20.5	19.0
Calcium, %	1.05	0.96	0.85
Available phosphorus, %	0.50	0.47	0.47
Sodium %	0.19	0.19	0.19
lysine %	1.31	1.25	1.14
methionine %	0.54	0.48	0.44
Met + Cys %	0.81	0.81	0.81
Threonine %	0.85	0.81	0.75
Tryptophan %	0.25	0.22	0.2

¹Provided per kilogram of diet: Se from sodium selenite: 0.25 mg, Zn from zinc oxide: 90 mg, Mn from manganese oxide: 120 mg, Fe from ferrous sulfate: 100 mg, Cu from copper sulfate: 20 mg, I from Potassium iodide: 10 mg, Co from Cobalt carbonate: 1,000 µg, and ² Provided per kilogram of the complete diet: vitamin A: 10,000 IU, vitamin D3: 5,000 IU, vitamin E: 40 IU, vitamin K3: 2 mg, vitamin B1: 3 mg, vitamin B2: 8 mg, vitamin B3: 0.25 mg, vitamin B5: 20 mg, vitamin B6: 5 mg, vitamin B12: 0.02 mg, folic acid: 1.5 mg, and biotin: 0.25 mg

Preparation of probiotic mixtures

Three different probiotic mixtures were used as follow: 1) multi-strain *Bacillus*: a probiotic blend consisting of four *Bacillus* strains, including *B. subtilis*, *B. licheniformis*, *B. coagulans*, and *B. indicus*, at an equal concentration of 1×10⁹ colony forming unit (CFU)/g; 2) multi-strain *Lactobacillus*: a blend of four *Lactobacillus* strains, including *L. acidophilus*, *L. plantarum*, *L. buchneri*, and *L. rhamnosus*, also at a concentration of 1×10⁹ CFU/g per strain; and 3) multi-species (mixed) probiotic: an equal combination of the two mixture mentioned above, comprising the eight

bacterial strains in total, as named mixed probiotic. The lyophilized probiotic bacteria were provided by Pardis Roshd Mehregan Company (Shiraz, Iran) and prepared daily. Probiotics types 1, 2, and 3 were provided for the HDpBacil, HDpLAB, and HDpMix groups, respectively, via drinking water. While freshwater without any probiotics was used for the control and HD groups. During the study, the probiotic mixtures were prepared daily and administered to the chickens at a dosage of 1 g/L of drinking water, giving a total of 4×10^9 CFU per bird per day.

Leg health measurements

At 14, 28, and 42 days of age, all broilers ($n=40$) in each treatment group had their footpad dermatitis scored macroscopically. Footpad lesions in birds were graded using a 5-point scale (0, no lesion; 1, deep lesion ≤ 0.5 cm; 2, deep lesion >0.5 cm to <1 cm; deep lesion >1 cm; 4, severe lesion in the footpad and lesion in the toe), as previously described (Louton *et al.*, 2022).

Additionally, the gait score was manually assessed and recorded in all broilers per treatment at the end of experiment (42 d). Gait scoring was performed using a 5-point gait score system (0: normal, dexterous, and agile; 1: slight abnormality, but difficult to define; 2: definitive and identifiable abnormality; 3: obvious abnormality, affects the ability to move; 4: severe abnormality, only takes a few steps; and 5: incapable of walking) according to the guidelines of the Welfare Quality, 2009.

Litter quality measurements

At days 21 and 42 of age, litter samples were collected from 5 locations within each pen (4 equidistant from each corner and one central, 30 cm away from drinkers), and then thoroughly pooled and subsequently, various indices (pH, temperature, moisture, and quality score) were measured on the collected litter samples. Litter pH was measured according to the study by Farhadi *et al.* (2016). Briefly, 10 g of the pooled litter sample was suspended in 100 ml deionized water (1:10 dilution) and left to rest for 1 h. Then, the pH value of the mixture was measured by an electronic pH meter (Basic 20, Crison, Spain). Measurements were performed in triplicate, and the mean was presented.

Litter moisture was determined according to a previously published method (AOAC, 2019) as a percentage of a litter weight loss before and after drying. Briefly, the appropriate pooled samples were weighed, transferred to a clean and dry Petri dish with a defined weight, and introduced to the oven maintained at 100°C until reaching to a constant weight. After cooling in a desiccator, the samples were reweighed, and the percentage of moisture in the samples was calculated. The litter quality score was visually evaluated per pen according to the five-point scoring system (score 1= friable, no capping or compaction whatsoever, 2= light capping, under a friable crumb surface, 3= surface capped and compacted, 4= surface wet and sticky, 5= litter depth wet and dough-like) (Guardia *et al.*, 2011). Also, litter temperature was recorded using a digital

thermometer, including whole litter depth in 5 sites across each pen, and the mean was used for statistical analysis.

Sampling and meat quality measurements

At 21 and 42 days old, four broilers from each treatment group were randomly chosen, weighed, and then humanely euthanized by cervical dislocation. Then, the breast and thigh meat were removed and transferred to the laboratory to measure meat quality indices. Meat sensory evaluations of the breast and thigh meat samples were conducted by thirty trained panelists using preprepared questionnaires, as reported by Berizi *et al.* (2016). The sensory attributes assessed in the meat samples included color, odor, texture, and overall acceptance, which were rated on a 4-point hedonic scale: 1= unacceptable, 2= fair, 3= acceptable, and 4= good, based on fresh samples (Khaledian *et al.*, 2021). In order to measure the meat color indicators, including lightness (L^*), redness (a^*), and yellowness (b^*), each meat sample was placed in a box with a white inner color, measuring $50 \times 50 \times 60$ cm, equipped with a light source. The surface of each sample was photographed by a camera at a distance of 30 cm from the sample. The angle between the camera lens and the sample surface, as well as the angle of the light source and the sample surface, were determined to be 90 and 20 degrees, respectively. After transferring the captured images to the computer system, the entire surface of each sample was selected. The indices L^* (brightness: 100 white and 0 black), a^* (red-green: positive for red and negative for green), and b^* (yellow-blue: positive for yellow and negative for blue) were recorded using Adobe Photoshop® software (Adobe Systems Inc., San Jose, CA, USA, version 24.7) in Lab mode, as similar to the study by Abbasvali *et al.* (2012). The texture profile analysis (TPA) of the breast and thigh meat samples was evaluated with a texture analyzer (Texture Pro CT V1.3 Build 15, Brookfield Engineering Labs, Inc., USA) using a 30 kg load cell. Samples were compressed twice to 50% of their height with a 4 mm diameter aluminum cylinder probe (TA44). The testing speeds were five mm/s in two cycles (Khaledian *et al.*, 2021). Texture profile parameters, including adhesiveness, cohesiveness, hardness, springiness, and chewiness, were calculated from the compression force versus time curves using Texture Exponent Lite software provided by the manufacturer, following the method described by Alvarez *et al.* (2002). All measurements were performed at room temperature ($23 \pm 2^\circ\text{C}$). Water holding capacity (WHC) of breast and thigh meat was determined using the press method by Abbasvali *et al.* (2012). In brief, 0.3 grams of meat sample were placed on Whatman filter paper No. 2 between two cover glasses and subjected to 1 kg of pressure for 20 min. The areas of the pressed meat and the wet area on the filter paper (in square centimeters) were measured using Adobe Photoshop® (CS6, version 24/7). The WHC was expressed as the ratio of the pressed meat area to the wet area. The fat content and moisture of the breast and thigh meat

samples were determined using standard methods, precisely the Soxhlet extraction method for fat and the oven air-drying method for moisture, as outlined by AOAC (2000).

Peroxide index analysis on fat tissue

On day 42, four broilers from each treatment group were selected and slaughtered using cervical dislocation method. Then, the abdominal fat tissue was collected and transferred to the laboratory for peroxide value (PV) analysis. PV, as a lipid oxidation indicator in abdominal fat tissue, was measured using methods by the Association of Official Analytical Chemists (AOAC, 2019) and Abeyrathne *et al.* (2021). Briefly, PV determination involves the oxidation of iodine in the presence of potassium iodide by the peroxides in the fat tissue samples, assuming all oxidizing substances present are peroxides. The PV is reported in milliequivalents of oxygen per kilogram of fat sample (mEqO₂/kg).

Statistical analysis

The statistical design used for the present study was completely random. First, the normality of the data in each parameter was evaluated by the Kolmogorov-Smirnov test. Then, the data was analyzed using SPSS statistical software (version 20, IBM, USA). The quantitative normal data was analyzed by the one-way analysis of variance (ANOVA), followed by the Duncan *post hoc* test. Qualitative data, such as meat sensory index, gait score, footpad dermatitis score, and litter quality score, were analyzed using the Kruskal-Wallis. In addition, the non-parametric Mann-Whitney U test was also used for pair-wise comparisons between the different treatment groups. The data are expressed as mean and standard deviation (mean±SD) and the significance level was considered less than 0.05 (P<0.05). All graphs were drawn using GraphPad Prism software (version 9.0.0, Insight Partners, USA).

Results

Leg health indices

The effects of three probiotics on footpad dermatitis (FPD) and gait scores in broilers exposed to high stocking density stress are demonstrated in Figs. 1 and 2, respectively. On days 28 and 42, FPD scores were significantly higher in the HD group compared to the control group (Fig. 1, P<0.05). On day 42, both the HD and HDpLAB groups showed notably elevated FPD scores compared to the control group (P<0.05). The HDpBacil group exhibited a considerably lower FPD score compared to both HD and HDpLAB groups (P<0.05). No significant difference was between the HDpBacil group and the control group in FPD on day 42 (Fig. 1, P>0.05).

As shown in Fig. 2, the birds in the HD group had considerably higher gait scores than the control group on day 42 (P<0.05). The HDpMix group demonstrated a significant reduction in gait scores compared to the HD and HDpLAB groups (Fig. 2, P<0.05). Additionally, no

significant difference in gait scores was found between the control group, HDpBacil, and HDMix groups (P>0.05).

Litter quality

Table 2 demonstrated the effect of three distinct probiotic mixtures on litter quality in broilers exposed to high stocking density. On days 21 and 42, the HD group had considerably higher litter pH compared to the control group (P<0.05). The HDpMix group showed a clearly lower litter pH compared to the HD group on day 21. By day 42, all probiotic-treated groups (HDpBacil, HDpLAB, and HDpMix) had significantly lower litter pH than the HD group (P<0.05). There was no significant difference in litter PH between the control and probiotic-treated groups on day 42 (P>0.05).

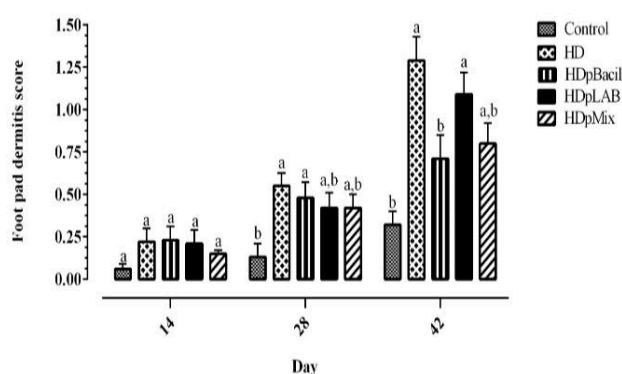


Fig. 1: Effects of three different probiotic mixtures on footpad dermatitis score in broilers exposed to high stocking density. Control: Broilers raised under normal stocking density, HD: Broilers raised under high stocking density, HDpBacil: Chickens under high stocking density and received multi-strain *Bacillus* probiotic, HDpLAB: Chickens under high stocking density and received multi-strain *Lactobacillus* probiotic, and HDpMix: Chickens under high stocking density and received mixture probiotic of multi-strain of *Bacillus* and *Lactobacillus*. Different letters (^{a,b}) indicate significant differences at P<0.05

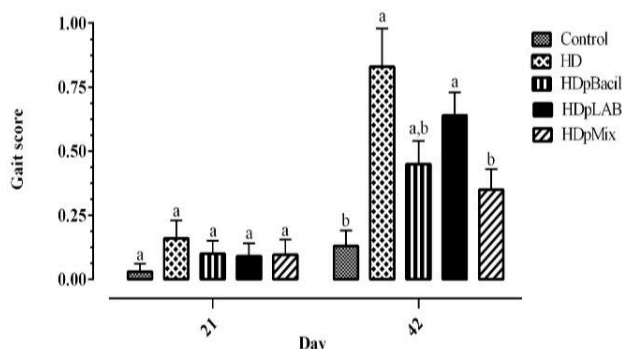


Fig. 2: Effects of three different probiotic mixtures on gait score in broilers exposed to high stocking density. Control: Broilers raised under normal stocking density, HD: Broilers raised under high stocking density, HDpBacil: Chickens under high stocking density received multi-strain *Bacillus* probiotic, HDpLAB: Chickens under high stocking density received multi-strain *Lactobacillus* probiotic, and HDpMix: Chickens under high stocking density received mixture probiotic of multi-strain of *Bacillus* and *Lactobacillus*. Different letters (^{a,b}) indicate significant differences at P<0.05

Table 2: Effects of three different probiotic mixtures on litter quality indices of broilers exposed to high stocking density on days 21 and 42

Indices	Day	Treatment group (mean±SD)					P-value
		Control	HD	HDpLAB	HDpBacil	HDpMix	
pH	21	7.30±0.50 ^b	8.86±0.56 ^a	8.40±0.10 ^{a, b}	8.13±0.35 ^{a, b}	7.57±0.53 ^b	0.002
	42	8.36±0.51 ^b	9.45±0.10 ^a	7.17±0.60 ^b	6.95±0.56 ^b	6.95±0.05 ^b	0.000
Temperature (°C)	21	25.01±1.73	26.33±2.52	27.03±1.00	26.67±2.52	27.66±2.51	0.760
	42	25.67±1.15	28.67±0.58	28.33±2.08	29.67±0.58	30.01±1.73	0.060
Quality score	21	1.25±0.50 ^b	3.64±0.31 ^a	2.75±0.48 ^a	2.76±0.51 ^a	1.25±0.38 ^b	0.000
	42	1.50±0.57 ^b	4.75±0.47 ^a	3.51±0.58 ^a	3.75±0.49 ^a	2.00±0.82 ^b	0.000
Moisture (%)	21	22.64±1.15 ^{a, b}	29.57±4.04 ^a	26.66±2.08 ^{a, b}	24.33±3.51 ^{a, b}	20.77±4.04 ^b	0.021
	42	36.02±5.22 ^b	60.32±5.51 ^a	42.67±3.06 ^b	47.33±2.89 ^b	40.00±3.46 ^b	0.000

Control: Broilers raised under normal stocking density, HD: Broilers raised under high stocking density, HDpLAB: Chickens under high stocking density and received multi-strain *Lactobacillus* probiotic, HDpBacil: Chickens under high stocking density and received multi-strain *Bacillus* probiotic, HDpMix: Chickens under high stocking density and received mixture probiotic of multi-strain of *Bacillus* and *Lactobacillus*, litter quality was evaluated according to the five-point scoring system (score 1= friable, no capping or compaction whatsoever, 2= light capping, under a friable crumb surface, 3= surface capped and compacted, 4= surface wet and sticky, 5= litter depth wet and dough-like). Different letters (^{a, b}) indicate significant differences at P<0.05

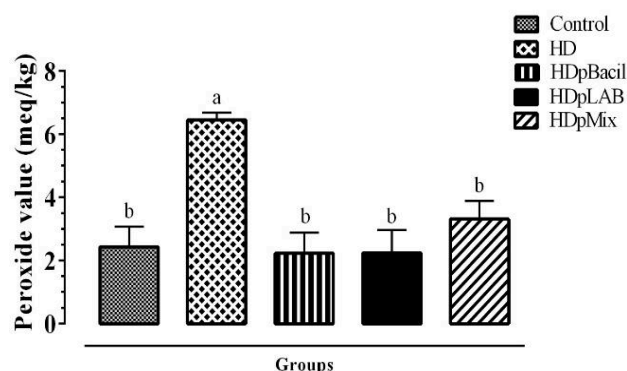


Fig. 3: Effects of three different probiotic mixtures on peroxide value of abdominal fat tissue in broilers exposed to high stocking density. Control: Broilers raised under normal stocking density, HSD: Broilers raised under high stocking density, HDpBacil: Chickens under high stocking density and received multi-strain *Bacillus* probiotic, HDpLAB: Chickens under high stocking density and received multi-strain *Lactobacillus* probiotic, and HDpMix: Chickens under high stocking density and received mixture probiotic of multi-strain of *Bacillus* and *Lactobacillus*. Different letters (^{a, b}) indicate significant differences at P<0.05

As presented in Table 2, the litter quality scores were also meaningfully higher in the HD group compared to the control group on days 21 and 42 (P<0.05). Among all HD groups, the HDpMix group showed the best litter quality scores on both studied days (P<0.05). As presented in Table 2, the moisture content of litter increased significantly in the HSD group compared to the other treatment groups, particularly on day 42. Probiotic administration in the HDpBacil, HDpLAB, and HDpMix groups effectively reduced the litter moisture compared to the HD group (P<0.05). No significant differences in litter temperature were observed among the different treatment groups (Table 2, P>0.05).

Peroxide value in fat tissue

The effects of three different probiotic formulations on the peroxide value in the abdominal fat tissue of broilers raised under HSD demonstrated in Fig. 3. The HSD group showed a clearly higher PV compared to the control group (P<0.05). The peroxide value in birds in the HDpBacil, HDpLAB, and HDpMix groups decreased noticeably compared to those in the HD group (P<0.05). There was no significant difference in PV levels between other treatment groups (P>0.05).

Meat quality indices

The effects of three different probiotic compounds on breast and thigh meat quality in broilers subjected to high stocking density are presented in Tables 3 and 4, respectively. The results showed no statistically significant differences among treatment groups in terms of sensory properties, texture profile parameters, or color values (L*, a*, and b*) for both breast and thigh meat (P>0.05).

In thigh meat, the HD group had a significantly higher WHC than the control (Fig. 4B, P<0.05), while WHC levels in the HDpBacil and HDpLAB groups were significantly reduced compared to the HD group (P<0.05). However, the birds that received *Bacillus*-based probiotic (HDpBacil group) had a noticeably lower WHC in breast meat compared to the HD group (Fig. 4A, P<0.05). In thigh meat, the HD group had a markedly higher WHC compared to the control groups (Fig. 4B, P<0.05). As seen in Fig. 4B, the birds in the HDpBacil and HDpLAB groups had significantly lower WHC compared to the HD group (P<0.05).

Figure 5 shows the effects of the probiotic treatments on crude fat and moisture content in breast and thigh meat in broilers exposed to high stocking density. No significant differences were found among the groups for either moisture or fat percentage in both meat types (P>0.05).

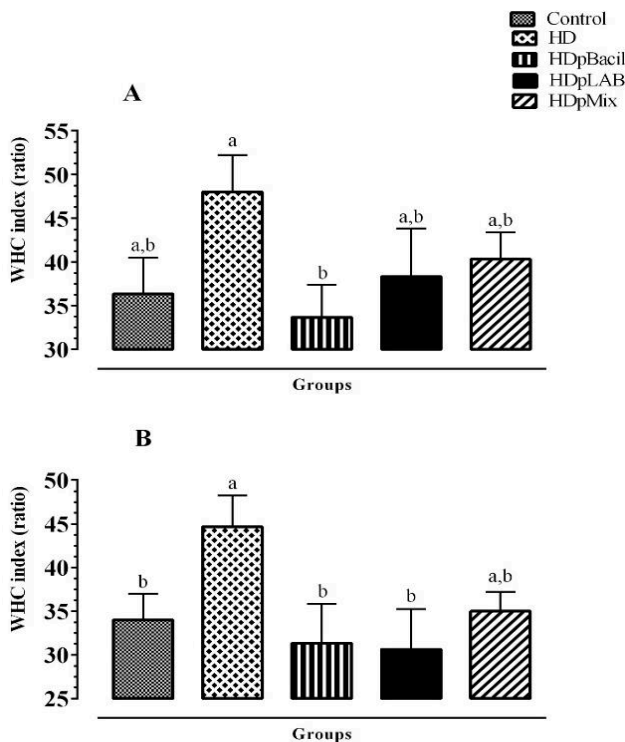


Fig. 4: Effects of three different probiotic mixtures on water holding capacity (WHC) of (A) breast and (B) thigh meat in broilers exposed to high stocking density. Control: Broilers raised under normal stocking density, HD: Broilers raised under high stocking density, HDpBacil: Chickens under high stocking density and received multi-strain *Bacillus* probiotic, HDpLAB: Chickens under high stocking density and received multi-strain *Lactobacillus* probiotic, and HDpMix: HSD broilers received mixture probiotic of multi-strain of *Bacillus* and *Lactobacillus*. Different letters (a, b) indicate significant differences at P<0.05

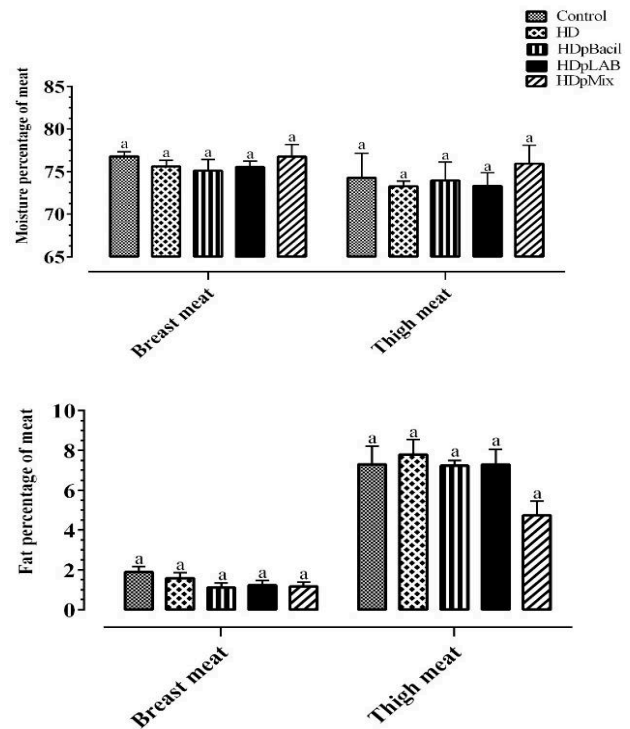


Fig. 5: Effects of three different probiotic mixtures on chemical traits of breast and thigh meat in broilers exposed to high stocking density. Control: Broilers raised under normal stocking density, HD: Broilers raised under high stocking density, HDpBacil: Chickens under high stocking density and received multi-strain *Bacillus* probiotic, HDpLAB: Chickens under high stocking density and received multi-strain *Lactobacillus* probiotic, and HDpMix: Chickens under high stocking density and received mixture probiotic of multi-strain of *Bacillus* and *Lactobacillus*. All groups share the same letter (a), indicating no significant difference at P<0.05

Table 3: Effects of three different probiotic mixtures on breast meat quality of broilers exposed to high stocking density

Items	Indices	Treatment group (mean±SD)					P-value
		Control	HD	HDpLAB	HDpBacil	HDpMix	
Sensory indices	Color	3.45±0.82	3.40±0.94	3.70±0.73	3.80±0.70	3.55±0.76	0.47
	Odor	3.65±0.59	3.60±0.60	3.55±0.69	3.75±0.44	3.65±0.59	0.86
	Texture	3.70±0.57	3.40±0.59	3.55±0.76	3.36±0.67	3.70±0.57	0.53
	Overall acceptance	3.70±0.57	3.40±0.68	3.40±0.68	3.50±0.69	3.65±0.59	0.44
Texture profile analysis	Hardness (N)	17.18±4.89	14.99±2.83	26.36±4.47	20.06±4.25	20.65±12.47	0.36
	Adhesiveness (mJ)	0.10±0.02	0.19±0.05	0.15±0.05	0.12±0.04	0.16±0.03	0.09
	Cohesiveness	0.66±0.01 ^{a,b}	0.64±0.04 ^{a,b}	0.63±0.04 ^b	0.72±0.03 ^a	0.70±0.02 ^{a,b}	0.02
	Springiness (mm)	12.21±0.08	11.91±0.29	12.00±0.30	12.71±0.53	12.96±0.33	0.04
	Chewiness	139.01±41.42	115.10±27.29	200.04±30.58	182.34±33.06	182.80±104.84	0.37
Color indices	L	56.00±6.00	56.67±1.53	56.33±3.21	51.67±4.04	54.33±4.04	0.34
	a	19.67±3.51	20.33±2.52	19.33±2.89	24.00±4.58	19.67±2.08	0.23
	b	10.67±3.51	13.33±2.52	12.33±2.31	13.67±6.66	8.67±2.52	0.66

Control: Broilers raised under normal stocking density, HD: Broilers raised under high stocking density, HDpLAB: Chickens under high stocking density and received multi-strain *Lactobacillus* probiotic, HDpBacil: Chickens under high stocking density and received multi-strain *Bacillus* probiotic, and HDpMix: Chickens under high stocking density and received mixture probiotic of multi-strain of *Bacillus* and *Lactobacillus*, Sensory indices assessed by a 4-point hedonic scale (1 = unacceptable, 2 = fair, 3 = acceptable, and 4 = good). Different letters (a, b) indicate significant differences at P<0.05

Table 4: Effects of three different probiotic mixture on thigh meat quality of broilers exposed to high stocking density

Items	Indices	Treatment group (mean±SD)					P-value
		Control	HD	HDpLAB	HDpBacil	HDpMix	
Sensory indices	Color	3.47±0.70	3.50±0.69	3.35±0.93	3.50±0.61	3.30±0.73	0.87
	Odor	3.80±0.52	3.65±0.49	3.63±0.60	3.75±0.44	3.65±0.67	0.83

	Texture	3.85±0.37	3.55±0.69	3.65±0.49	3.70±0.57	3.85±0.37	0.27
	Overall acceptance	3.80±0.41	3.45±0.69	3.50±0.61	3.65±0.59	3.65±0.59	0.34
Texture profile analysis	Hardness (N)	12.68±0.05	16.41±7.86	10.47±3.74	12.51±2.61	12.86±2.94	0.61
	Adhesiveness (mJ)	0.17±0.30	0.21±0.09	0.27±0.07	0.26±0.06	0.28±0.02	0.27
	Cohesiveness	0.63±0.09	0.69±0.03	0.73±0.0	0.75±0.09	0.72±0.02	0.24
	Springiness (mm)	8.31±0.42	8.54±0.57	8.42±0.65	8.67±0.40	8.52±0.35	0.91
	Chewiness	67.97±24.60	96.36±46.80	66.86±36.08	79.25±3.05	79.37±20.31	0.73
Color indices	<i>L</i>	52.67±0.21	55.67±7.77	50.33±5.03	53.67±0.51	54.33±10.69	0.92
	<i>a</i>	21.33±6.66	19.33±4.16	17.67±0.58	19.67±0.58	19.33±3.51	0.73
	<i>b</i>	9.33±4.93 ^{ab}	11.33±4.93 ^a	3.33±1.15 ^b	9.00±3.61 ^{ab}	5.33±0.58 ^{ab}	0.03

Control: Broilers raised under normal stocking density, HD: Broilers raised under high stocking density, HDpLAB: Chickens under high stocking density and received multi-strain *Lactobacillus* probiotic, HDpBacil: Chickens under high stocking density and received multi-strain *Bacillus* probiotic, and HDpMix: Chickens under high stocking density and received mixture probiotic of multi-strain of *Bacillus* and *Lactobacillus*, Different letters (^{a, b}) indicate significant differences at P<0.05

Discussion

The findings of this study showed that HSD negatively impacts footpad health, as evidenced by a significant increase in FPD score in birds in the HD group compared to those in the control group on days 28 and 42 (P<0.05). On day 42, broilers in the HD group exhibited more severe footpad lesions than those in other treatment groups. This condition is likely due to increased excreta and moisture levels in the litter under HSD, which degrade litter quality and lead to contact pododermatitis, a key contributor to FPD (De Jong *et al.*, 2014; Zabir *et al.*, 2021). FPD is a painful condition and an important indicator of poor flock welfare, negatively impacting on growth, productivity, and behavior in broiler chickens (Alabi *et al.*, 2023). Moreover, HSD negatively impacted broiler leg health in this study, as reflected by significantly higher gait scores in the HD group, three times greater than those of the control group (P<0.05). Previous studies stated that HSD caused poor gait scores and lameness in birds due to limited rearing space and reduced physical activity (Christofolletti *et al.*, 2016; Shynkaruk *et al.*, 2023).

In the current study, both *Bacillus* and combined probiotics significantly reduced the FPD score in broilers compared to the HD group (P<0.05). The *Bacillus* and combined probiotics also noticeably lowered gait scores on day 42 compared to the HD group, with significant effects observed only in the group receiving the combined probiotic (P<0.05). The FPD and gait scores in the HDpBacil and HDpMix groups were not markedly different from those in the control group, indicating notable improvements.

The findings suggest that the *Bacillus* probiotic, whether used alone or in combination with *Lactobacillus*, is more effective at improving leg health in broilers under HSD than the *Lactobacillus* alone. Previous studies documented that probiotics could enhance bone health and gait score by improving gut microbiota balance and mineral and vitamin absorptions (Sobczak *et al.*, 2018; Yan *et al.*, 2018; Mohammed *et al.*, 2021).

The effectiveness of probiotics depends on bacterial species and their dosages (AlFatah, 2020). In this study, *Bacillus* probiotics had a greater impact than *Lactobacillus* probiotics, likely due to their greater stability, resistance to pH changes, and better

colonization in the lower gastrointestinal tract (Khalid *et al.*, 2022). It has also been established that multi-species probiotics are more effective than single species due to synergistic interactions between strains or higher effective doses. The combined probiotics showed greater benefits for leg health than the *Lactobacillus* probiotic alone. It can be concluded that the combination of *Bacillus* and *Lactobacillus* probiotics can improve leg health and overall poultry performance, making them a promising strategy for reducing leg disorders in broiler production.

In the current study, birds in the HD group showed significantly higher litter moisture and quality scores, indicating the negative effect of overcrowding on litter conditions (P<0.05). Alabi *et al.* (2023) showed that optimal litter moisture in broiler farms should range between 25% and 30%. However, on day 42, the HD group exhibited a litter moisture level of 60.32%, considerably higher than that of the control group (36.02%). These findings align with previous studies, such as Cengiz *et al.* (2018), which reported that HSD leads to increased litter moisture and poorer footpad health. Poor litter quality plays a key role in the occurrence and severity of FPD (Singh *et al.*, 2021; Kuter *et al.*, 2023). Additionally, wet litter and low litter quality under HSD condition fosters bacterial activity that increases ammonia production and litter pH levels (Bist *et al.*, 2023).

On day 42, the litter pH in the control group was 8.36, within the typical range of 8.0 to 9.0 as reported by Alabi *et al.* (2023). In contrast, the HD group had a substantially higher litter pH of 9.45 (P<0.05). Consistent with previous researches (Alabi *et al.*, 2023; Kwon *et al.*, 2024), we find high stocking density leads to poor litter quality, elevated moisture, and increased pH. Therefore, developing an effective strategy to enhance litter quality and reduce pododermatitis is crucial for broiler chickens' welfare in high-density production systems.

All three probiotic treatments notably decreased litter pH and moisture levels compared to the HD group. As shown in Table 2, the combined probiotic also improved litter quality score in broilers kept under HSD condition. The gut microbiome plays an important role in litter quality by influencing the composition of litter-associated microbes and reducing intestinal pH (Tang *et al.*, 2021; Such *et al.*, 2023; Horyanto *et al.*, 2024).

Based on the findings of this study, a mixed probiotic containing *Bacillus* and *Lactobacillus* positively impacted litter quality in broilers under HSD. Therefore, combined probiotics are recommended as dietary supplements to support broiler welfare in stressful rearing conditions.

In the current experiment, HSD led to a significant increase in peroxide value in the abdominal fat tissue in the HD group compared to the control group ($P < 0.05$). HSD is a known environmental stressor for broilers that promotes the generation of reactive oxygen species (ROS), leading to oxidative stress and lipid oxidation (Thema *et al.*, 2022). Lipid oxidation in fat tissue results in the formation of lipid peroxides and other oxidative products, which contribute to elevated PV levels in tissue (Domínguez *et al.*, 2019). These results are consistent with earlier studies by Humam *et al.* (2020) and Pečjak *et al.* (2022), which also showed increased lipid oxidation in broilers exposed to stress. The current findings further showed that administering *Bacillus* and *Lactobacillus* probiotics, either alone or in combination, significantly reduced oxidative stress, as evidenced by lower PV levels in abdominal fat ($P < 0.05$).

These findings support previous research indicating probiotics had antioxidant properties, mitigating oxidative stress and reducing lipid oxidation in poultry tissues (Obianwuna *et al.*, 2023). In this study, all probiotic treatments were equally effective in reducing oxidative stress, with no significant difference observed between *Bacillus* and *Lactobacillus* strains. Therefore, it can be concluded that both single-species and multi-species probiotics containing *Bacillus* and *Lactobacillus* can serve as effective feed additives to protect broilers from oxidative stress during the rearing period.

This study also found a significant increase in WHC in the thigh meat of birds in the HD group compared to the control group ($P < 0.05$). Although WHC in the breast meat was also higher in the HD group, the difference was not statistically significant ($P > 0.05$). These findings align with earlier studies suggesting that stress in broilers triggers the release of corticosterone, which promotes protein breakdown and muscle catabolism that increase WHC in meat (Furukawa *et al.*, 2021; Son *et al.*, 2022).

Additionally, this study showed that probiotic administration, particularly *Bacillus*-based probiotic, significantly reduced WHC in both thigh and breast meat in the HDpBacil group compared to the HD group. This supports findings from Cramer *et al.* (2018), who found that probiotics can enhance meat WHC. However, the impact of probiotics on WHC varies across studies, likely due to differences in experimental design, probiotic strains, bird age, and duration of probiotic use (Hossain *et al.*, 2015). In summary, high stocking density negatively affected meat quality by raising WHC, while *Bacillus* probiotic treatment helped reverse this effect by lowering WHC in broiler meat.

In this investigation, neither increased stocking density nor probiotic supplementation had any clear effect on meat quality parameters, including color, sensory attributes, texture profile, or chemical

composition. These findings are consistent with those of Thema *et al.* (2022), who also reported no effect of stocking density on chicken meat color. However, they contrast with results from Khalil *et al.* (2021), who reported that probiotics enhanced the sensory qualities of meat.

Similarly, the current results align with study by Cengiz *et al.* (2015), which reported no significant influence of stocking density or probiotic supplementation on the chemical composition of meat. The reasons for the lack of observed effects in the present study remain unclear. However, they may be related to variations in experimental conditions such as sample size, breed, sex, age at slaughter, gut microbiota composition, as well as differences in probiotic strains, dosages, administration methods, and treatment duration (Mallott and Amato, 2021; Härer and Rennison, 2023).

The results showed that probiotic supplementation, especially multi-strain *Bacillus* probiotics, significantly attenuated the negative impacts of HSD on abdominal fat peroxide levels, litter quality, thigh meat, and leg health in broilers. Additionally, the mixed probiotics of *Bacillus* and *Lactobacillus* improved gait scores and litter quality in broilers under HSD. In conclusion, the mixed probiotics seemed to perform more effectively compared to single species probiotics that can be recommended as a feed additive to support leg health and litter quality in broilers raised under environmental stress.

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Conflict of interest

The authors declare that they have no conflict of interest.

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