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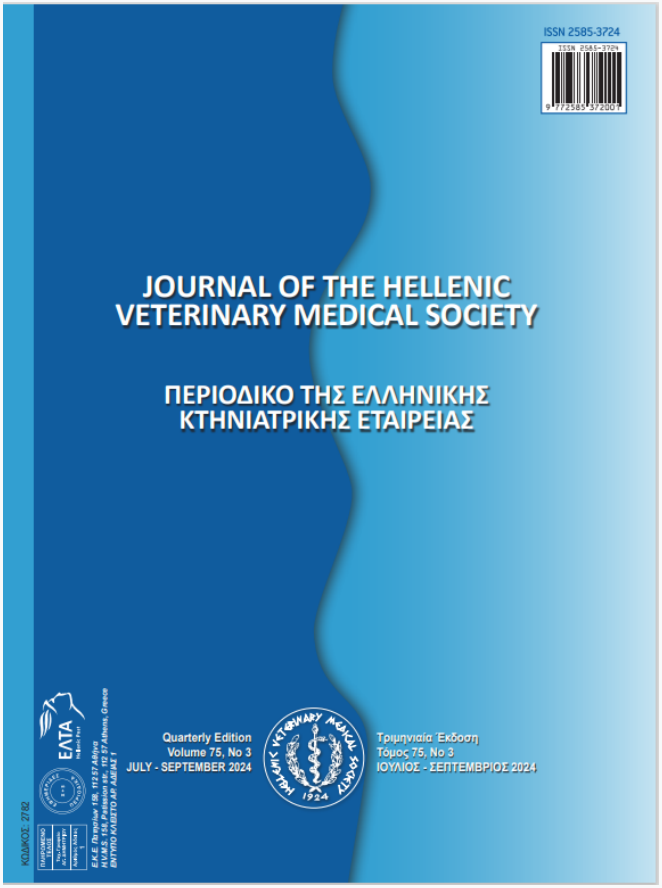

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Effects of dietary fermented mealworm larvae and stocking density on performance, blood stress indicator and intestine parameters of broilers

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ABSTRACT: The present study was conducted to evaluate the effects of the supplementation of defatted mealworm larvae meal fermented with probiotics as a new antibacterial feed additive to the diet of broilers reared under normal- (NSD) and high- (HSD) stocking density on growth performance, blood and slaughtering parameters, microorganism content and morphology of ileum and short-chain fatty acids content of ceca. A total of four hundred and fifty one-d-old Ross 308 male broiler chicks were randomly distributed into six groups of similar mean weight, each containing five replicates. Experimental treatments consisted of a 2 x 3 factorial arrangement with two levels of stocking density (12 birds/m^2 as NSD and 18 birds/m^2 as HSD) and three different mash diets: CONT- a corn-soybean meal-based diet containing no fermented defatted mealworm larvae meal (FDM) (0%); FDMLP- the diet obtained by supplementing DM fermented with *Lactobacillus plantarum* to the CONT diet (0.4%); FDMLB- the diet obtained by supplementing DM fermented with *Lactobacillus brevis* to the CONT diet (0.4%). HSD significantly decreased the growth performance except feed conversion ratio (FCR), carcass yields (CYs), *Lactobacillus* spp. content and villus height (VH) and villus surface area (VSA) of ileum and short-chain fatty acids (SCFAs) concentrations of ceca, but, increased the blood heterophil/lymphocyte (H/L) ratio and *Escherichia coli* content and crypt depth (CD) of ileum of broilers compared to NSD. The FDMLP and FDMLB diets significantly improved the FCR and increased final body weight (BW), BW gain, *Lactobacillus* spp. content and VH and VSA of ileum and SCFAs concentrations of ceca, however, reduced the blood H/L ratio and *Escherichia coli* content and CD of ileum of broilers when compared to the CONT diet. In conclusion, FDMLP and FDMLB can be utilized as new antibacterial feed additives in broiler diets regardless of stocking density.

Keywords: Broiler; fermented mealworm larvae; intestine; performance; stocking density

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INTRODUCTION

World population is predicted to increase by over a third, reaching over 9 billion people by the year 2050 (Boroojerdi and Rajabzadeh, 2021). In this respect, the expected growth of consumption of broiler meat as animal protein from 2010 to 2050 is estimated to be 173% (Boroojerdi and Rajabzadeh, 2021). In parallel with this, in recent decades, broiler producers are obliged to rear broilers under high stocking densities (HSDs) to decrease production costs, produce more kilograms of broiler chickens per unit area for a cheap, safe supply of meat and increase profitability (Khalil et al., 2021). In general, 18 to 30 kg/m^2, 30 to 37 kg/m^2 and >37 kg/m^2 (assuming 2.5 kg final BW on an average) are considered low, medium and high stocking density, respectively (Cengiz et al., 2015; Kridtayopas et al., 2019). However, HSD results in stress and the increase in corticosterone stress hormone level (Sugiharto, 2022; Meena et al., 2022) and negative consequences for broiler chickens, including impaired growth performance, health, welfare (Oddon et al., 2021; Sugiharto, 2022; Meena et al., 2022) and microflora and histomorphological structure of the small intestine of broilers (Goo et al., 2019; Kridtayopas et al., 2019; Sugiharto, 2022).

In this context, previous studies have investigated the dietary supplementation of antibiotic growth promoters (AGPs) as antibacterial feed additive to enhance performance by improving their deteriorated microflora and histomorphological structure of the small intestine of broilers reared under HSD (Hooge et al., 2003). Unfortunately, prolonged use of AGPs in broiler diets has been reported to cause the emergence of AGPs resistance (Shazali et al., 2014) and residual AGPs in broiler meat (Rafiq et al., 2022), which are harmful to human health and cause increasing public concern (Aslam et al., 2021). A ban on the use of AGPs in broiler diets by the European Union in 2006 due to the above-mentioned reasons has increased interest in the use of natural antibacterial feed additives such as probiotic, prebiotic, synbiotic etc. in diets of broilers reared under HSD (Sugiharto, 2022).

In addition to these, the use of dried mealworm larvae meal fermented with probiotics as a new antibacterial-based feed additive in broiler diets has recently come to the fore (Islam and Yang, 2017). Compared to animal-derived feed ingredients, insects as a novel feed ingredient have several advantages such as being able to convert organic residues into protein more efficiently, needing less space and water and having lower environmental impact and high nutritional values (Boroojerdi and Rajabzadeh, 2021; Lee et al., 2022; Sedgh-Gooya et al., 2022). Seven insect species including yellow mealworm (M) (*Tenebrio molitor*) have been approved by the European Union (EU) for use in aquaculture diets (Reg. 2017/893/UE). The European Commission Regulation (UE) 2021/1372 also allowed the use of proteins derived from insects for feeding chickens and pigs (Luparelli et al., 2022).

Mealworm larvae (M) has been reported to be rich in crude protein (44-69%) and crude fat (23-47%) (Chen et al., 2023). Presently, insects such as M are not only considered as a nutrient-rich feedstuff (Kwon et al., 2020) but also as an antibacterial feed additive due to having antimicrobial peptides (AMPs) (Józefiak et al., 2018; Benzertiha et al., 2020; Hajati and Negarandeh, 2021; Elahi et al., 2022) and chitin (Islam and Yang, 2017; Gasco et al., 2018) for poultry nutrition. The chitin content of M has been reported to be 4.30-8.91% (Hong et al., 2020). Chitin in M is partially degraded by the acidic chitinase in the proventriculus and gizzard of chicken to produce chitooligosaccharides, a prebiotic (Hajati and Negarandeh, 2021; Lee et al., 2022). However, the high chitin levels (> 2.42%) in M may impose negative effects on feed intake and protein availability and thereby worsening growth performance in broilers (Mulyono et al., 2019).

Due to the reasons mentioned above, both the reduction of high chitin content and the emergence of antimicrobial components of insects such as M and black soldier fly larvae can be performed by solid-state fermentation (SSF) using specific microorganisms with chitinase enzyme activity that are able to degrade chitin (Mulyono et al., 2019; Hadj Saadoun et al., 2020; Luparelli et al., 2022). SSF is a microbial fermentation type that takes place in the absence or near absence of free water; because it stimulates the natural environment to which the selected microorganisms have naturally adapted (Peng et al., 2022). Among probiotic bacteria species, lactic acid bacteria (LAB) are the most used species for M solid-state fermentation (Islam and Yang, 2017). The SSF brings to the fore the possibility of using solid-state fermented M as new antibacterial feed additive by improving their nutritional value and creating functional feed additives (Hadj Saadoun et al., 2020; Luparelli et al., 2022).

However, to our best knowledge, there is only one study in the literature on the use of M and su-

per mealworm larvae meal solid-state fermented with *Lactobacillus plantarum* as antibacterial feed additives in diets of broilers challenged orally with *Salmonella* and *Escherichia coli* (*E. coli)* infection (Islam and Yang, 2017). In the above mentioned *in vivo* study, a one-week study was conducted to investigate whether M and super mealworm larvae meal solid-state fermented with only *Lactobacillus plantarum* could be used as antibacterial feed additives in broilers challenged with *Salmonella* and *Escherichia coli* (*E. coli)* infection. Unlike the above study, in the present research, we hypothesized that dietary supplementation of defatted M (DM) subjected to SSF with two different probiotics (*Lactobacillus plantarum* and *Lactobacillus brevis*) with chitinase activity as a new antibacterial feed additive could alleviate the detrimental effects of HSDs in broilers reared under HSD from hatching until 42 days of age. Therefore, the present study was conducted to compare the effects of the supplementation of DM solid-state fermented with two different probiotics with chitinase activity as a new antibacterial feed additive to diet of broilers reared under normal- and high-stocking density on growth performance, blood and slaughtering parameters, microorganism content and histomorphometric parameters of ileum and short-chain fatty acids content of ceca.

MATERIALS AND METHODS

Animal care

The complete protocol was reviewed and approved by the Animal Care and Use Committee of Tokat Gaziosmanpasa University (Process no. 2019-HADYEK-47).

Animals, diets and experimental diet

On the day of hatching, a total of four hundred and fifty one-d-old Ross 308 male broiler chicks were acquired from a commercial hatchery (Anadolu Ross, Ankara, Türkiye).

The broiler chicks were weighed, wing-banded and randomly distributed into six groups of similar mean weight, each containing five replicates. From hatching until 42 days of age, the chicks were kept on floor pens bedded with fresh wood shavings as litter. Temperature was kept at 32ºC for the first week, 28ºC for the second week and gradually reduced, and after 27 days of age, temperature was remained at 21°C. A fluorescent lighting schedule of 23 h light and 1 h dark was used during the experiment with an average light intensity of 20 lux. The diets in mash form and drinking water were provided *ad libitum*.

Experimental treatments consisted of a 2 x 3 factorial arrangement with two levels of stocking density (12 birds/m^2 as NSD and 18 birds/m^2 as HSD) (Kridtayopas et al., 2019) and three different mash diets: CONT- a corn-soybean meal-based diet containing no fermented defatted mealworm larvae meal (FDM) (0%); FDMLP- the diet obtained by supplementing DM fermented with *Lactobacillus plantarum* to the CONT diet (0.4%); FDMLB- the diet obtained by supplementing DM fermented with *Lactobacillus brevis* to the CONT diet (0.4%). Each treatment had 5 replicates. FDMLP and FDMLB were supplemented to an amount of corn and the mixture was added to diet. Diets were prepared weekly and stored in airtight containers. Prior to experimental diet formulation, feed ingredients and DM, FDMLP and FDMLB were analyzed for their dry matter, crude protein (CP), ether extract, crude ash, starch and total sugar contents according to the methods of the AOAC (2007) at Ankara Food Control Laboratory (Ankara, Türkiye). Metabolisable energy (ME) of feed ingredients and DM, FDMLP and FDMLB was calculated based on analysed values of feedstuffs according to the formula in the Official Gazette No. 29955 dated 21.01.2017 (Türkiye) as follows:

$$\text{Kcal / kg, ME} = \frac{\left[\left(0.1551 \times \text{crude protein } (\%) + 0.3431 \times \text{crude fat}(\%) + 0.1669 \times \text{starch } (\%) + 0.1301 \times \text{total sugar } (\text{as sucrose})(\%)\right]\right.}{4.184} \text{ x } 1000$$

All diets were formulated according to phase feeding practices as the broiler chickens advanced in age and weight, as recommended by the breeder (Ross 308, 2007); the starter phase lasted from day 0 to 10, the grower phase was from day 11 to 28 and the finisher phase was from day 29 to 42. Ingredient composition and nutrition content of the control diet are presented in Table 1.

Mealworm larvae (M) (*Tenebrio molitor* L.) purchased from a commercial supplier in Antalya, Türkiye were grown on organic feed mainly consisting of wheat, wheat bran and carrot, without any contamination of animal origin products based on EC reg-

Table 1. Ingredient composition and nutrition content of the control diet (g/100 g, as-fed basis)

Item			
	Days		
	0-10	**11-28**	**29-42**
Ingredients			
Corn	57.30	58.99	64.00
Soybean Meal (44.8 % CP)	34.86	31.49	28.39
Fish Meal (65 % CP)	1.51	2.65	-
Vegetable Oil	1.92	3.35	3.82
Dicalcium Phosphate	2.20	1.85	2.10
Limestone	0.87	0.78	0.80
Salt	0.34	0.32	0.36
Vitamin Premix[1]	0.25	0.25	0.25
Trace Mineral Premix[2]	0.10	0.10	0.10
DL-Methionine	0.36	0.22	0.18
L-Lysine	0.22	-	-
L-Threonine	0.07	-	-
Calculated nutrient content			
Dry Matter	90.10	90.10	90.10
Crude Protein	23.00	22.00	19.00
ME (MJ/kg)	12.66	13.19	13.40
Ca	1.00	0.90	0.90
P available	0.50	0.45	0.45
Methionine+Cystine	1.09	0.94	0.80
Lysine	1.44	1.23	1.01
Na	0.16	0.16	0.16
Tryptophan	0.30	0.29	0.25
Threonine	0.93	0.84	0.72

[1] Vitamin premix/kg diet: 12 000 IU vitamin A; 1 500 IU vitamin D3; 50 mg vitamin E; 5 mg vitamin K3; 3 mg vitamin B1; 6 mg vitamin B2; 5 mg vitamin B6; 0.03 mg vitamin B12; 25 mg niacin; 12 mg Ca-D-pantothenate; 1 mg folic acid; 0.05 mg D-biotin; 2.5 mg apo-carotenoic acid ester; 400 mg choline chloride

[2] Trace Mineral Premix/kg diet: 80 mg Mn; 60 mg Fe; 60 mg Zn; 5 mg Cu; 0.2 mg Co; 1 mg I; 0.15 mg Se

ulation (no 1069/09). The 90 days-old M were not starved before being killed. They were freeze-dried overnight to remove moisture. Around 1 kg of freeze-dried M was ground into the meal using a miller. The M meal obtained was full-fat and produced from the larval stage of yellow meal worms. The crude protein content of M meal in the present study was increased by the chemical defatting process since protein may be utilized as substrates by microorganisms for SSF (Son et al., 2021). Defatting of freeze-dried M meal was performed by soxhlet device under optimized extraction conditions using petroleum ether to M meal ratio of 3:1 L/Kg, at 60°C for 4 h. After defatting, M meal was dried at 40°C for 3 h. As a result of this process, the high fat content of defatted M meal (DM) was reduced from 23% to 6.6% and its crude protein content was increased from 44% to 76.2%.

Two probiotic bacteria (*Lactobacillus plantarum* and *Lactobacillus brevis*) and *Saccharomyces cerevisiae* (baker's yeast) were used in the SSF of DM with probiotics. *Lactobacillus plantarum* strain and *Lactobacillus brevis* strain were isolated from Çeçil cheese and cheddar cheese, respectively. *Saccharomyces cerevisiae* (baker's yeast) was produced from sugar beet molasses. The probiotics with chitinase activity used in the fermentation were purchased from Neslihan Dikbaş Microorganism Culture Collection at the Agricultural Biotechnology Laboratories of Ataturk University. Chitinase enzyme activity of the purchased probiotics was analyzed in the Agricultural Biotechnology Laboratory of Ataturk University according to the method of Senol et al. (2014). According to the results of the analysis, the chitinase enzyme activities in terms of ammonium sulfate precipitation level were 15.00 U/L and 11.36 U/L for *Lactobacil-*

lus plantarum and *Lactobacillus brevis*, respectively. *Saccharomyces cerevisiae* (baker's yeast) was commercially supplied.

The fermentation of DM with two different probiotic bacteria was carried out by modifying the method of Islam and Yang (2017) in Semi-Solid Phase Fermenter (Infors-HT, Labfors AG, Bottmingen, Switzerland) in the laboratory of Isparta University of Applied Sciences, Agricultural Faculty, Department of Animal Science. Distiller's dried grains with solubles (DDGS) and defatted rice bran were used as solid media for probiotic strains during the fermentation process with DM. Before fermentation, DM, DDGS, defatted rice bran and water were autoclaved at 121°C for 15 min for sterilization. Before the fermentation, the dried DM was ground and used to prepare a mixture of 30% DM, 35% DDGS, 35% defatted rice bran and 80% distilled water for the fermentation process. In the first stage of the fermentation, DDGS, defatted rice bran, DM and distilled water were put into the fermenter and then carbon dioxide was added to create an anaerobic environment inside the fermenter. First, 100 ml of incubated *Lactobacillus plantarum* was added to the solid substrate medium in the fermenter and fermented at 38ºC for 48 h under anaerobic conditions. After 48 h, a second fermentation was performed with 1.0% *Saccharomyces cerevisiae* (baker's yeast) activated for 1 h at 37ºC in 250 ml 0.1% peptone water (10 g yeast + 90 ml peptone water) at 38ºC for another 48 h under anaerobic conditions. *Saccharomyces cerevisiae* during fermentation enhances the viability and growth of lactic acid bacteria (LAB), since it provides some nutrients, such as amino acids and vitamins to LAB (Menezes et al., 2018; Shi et al., 2020). After completion of a total of 96-h fermentation process, the fermented product was dried to less than 15% moisture at 32ºC for 24 h using a drying oven. The same fermentation procedure was performed with *Lactobacillus brevis*. To determine the microbial concentration, 1 g FDMLP or FDMLB was serially diluted with 9 ml of 0.85% sterile saline and thoroughly mixed. The counts of total mesophilic aerobic bacteria were then determined by plating serial 10-fold dilutions in triplicate into Plate Count Agar (PCA) and incubated at 30ºC for 48 h under aerobic conditions. The numbers of LAB were counted by plating serial 10-fold dilutions in triplicate into De-Man Rogosa and Sharp (MRS) agar and incubated at 39-40ºC for 5 d under anaerobic conditions. Enumeration of yeast and mold was conducted by plating serial 10-fold dilutions in triplicate into Dichloran Rose Bengal Chloramphenicol (DRBC) agar and incubated at 25ºC for 5 d under anaerobic conditions. After incubation, microbial colonies were immediately counted and expressed as log10 CFU/g. Nutrient composition and concentrations of microorganisms in DM, FDMLP and FDMLB are shown in Table 2. The amount of protein linked to acid detergent fiber (ADF) was determined (AOAC, 2007) and used to estimate the chitin contents of DM, FDMLP and FDMLB (Finke, 2007). The chitin contents of DM, FDMLP and FDMLB were found as 4.20%, 2.74% and 2.81%, respectively.

Growth performance

During the 42-day experimental period, the growth performance of broilers was evaluated by recording their body weight gain (BWG), feed intake (FI) and feed conversion ratio (FCR). The body weight (BW) of broilers was recorded at the beginning of the experiment and on a weekly basis thereafter. FCR was cal-

Table 2. Nutrient composition and concentrations of microorganisms in DM, FDMLP and FDMLB

Item	DM	FDMLP	FDMLB
Microorganisms' concentrations (log10 cfu/g)			
Total Mesophilic Aerobic Bacteria	Not detected	3.92	4.29
Lactobacillus	Not detected	2.99	2.45
Yeast-Mold	Not detected	Not detected	Not detected
Nutrient Composition			
Dry Matter, %	95.70	92.00	89.81
Crude Protein, %	76.20	49.28	49.06
Crude Fat, %	6.60	8.14	9.40
Crude Ash, %	7.30	7.81	7.83
Starch, %	3.30	3.73	1.44
Total Sugar, %	0.50	0.36	0.36
Metabolisable Energy, Kcal/kg (for poultry)	3515	2650	2660

culated weekly as the amount of feed consumed per replicate pen of BWG. Throughout the experiment, broilers were handled according to the principles for the care of animals in experimentation (Ross 308, 2007). Mortality was recorded daily.

Slaughtering parameters

At the end of the experiment, the diets were withdrawn 6 h ago prior to slaughter (Xue et al., 2021). After 6 h feed withdrawal, two broilers from each replicates, whose BWs were similar to the group average, were selected from each treatment group (10 broilers per treatment) and a total of 60 broilers were slaughtered by severing the jugular vein to determine slaughtering parameters. A sodium pentobarbital injection (100 mg/kg) was applied as anesthesia to the experimental broilers before slaughter. The carcasses were immediately plucked, processed (removal of the head and feet), eviscerated (removal of the gastrointestinal tract), weighed and then chilled overnight in a refrigerator (+4ºC). The measurements performed included pre-slaughter BW, hot- and cold- carcass weights and weight of bursa of Fabricius. The weights of bursa of Fabricius and hot- and cold-carcass yields were calculated as a percentage of the pre-slaughter BW of broilers.

Hematological analysis

At 42 day of age, 1.5 milliliter of blood samples of 10 broilers slaughtered from each treatment group were collected from the jugular vein using a 3-ml syringe fitted with a 23-gauge needle 1.5 inches in length and placed into microtube with EDTA to estimate the Heterophil to Lymphocyte (H/L) ratio. The bleeding procedure was limited to 1 min or less to minimize the effects of handling stress. The blood samples were smeared on a glass slide for the determination of the H/L ratio. After drying, the smears were stained with May-Grunwald and Giemsa stains (Gross and Siegel, 1983). One-hundred leukocytes were counted on one slide of each broiler using a light microscope at *x* 1000 magnification. The H/L ratio was calculated by dividing the number of heterophils by the number of lymphocytes.

Determination of ileal bacterial populations

On day 42, the ileum of 10 broilers slaughtered from each of the treatment groups (from Meckel's diverticulum to the ileocecal junction) was removed individually. The ileum digesta were aseptically and individually collected in sterile 2-ml-tubes for ileal bacterial enumeration. One gram of ileum digesta was diluted 1:9 (wt/vol) with 0.85% sterile saline. The samples were serially diluted from 10^{-1} to 10^{-9} to determine the total aerobic bacteria concentration in the ileum and incubated on nutrient agar at 37°C for 48 h. The samples were diluted serially from 10^{-1} to 10^{-3} to determine *E. coli* concentration in the ileum and incubated on IMVIC (indole, methyl red, Voges-Proskauer, and citrate) agar at 37°C for 48 h. *Lactobacillus* spp. concentration in the ileum was determined by serially diluting the samples from 10^{-3} to 10^{-4} and anaerobically incubating them on MRS (De-Man, Rogosa and Sharpe) agar at 37°C for 72 h. After incubation, microbial colonies were immediately counted and expressed as log10 CFU/g (Anonymous, 1992; Nooreh et al., 2021).

Histomorphological measurements of ileum

On day 42, the ileum tissues of 10 broilers slaughtered from each treatment group were sent to the Department of Tissue Engineering, Hamidiye Institute of Health Sciences, University of Health Sciences under cold chain conditions containing dry ice for their histological study. First, the ileum tissue samples were flushed with saline solution to remove adherent intestinal contents and then fixed in 10% neutral buffered formaldehyde solution for 24 h (Calik et al., 2017). The fixed ileum tissues were dehydrated in graded ethanol solutions, cleared in xylol and embedded in paraffin. The paraffin blocks were then sectioned at a thickness of 5 μm with cryostat. Cross sections were prepared and stained with hematoxylin and eosin staining in order to determine the ileal histomorphometry. This AE2000 Motic inverted microscope, connected to an image analyzer, was also used to measure villus height (VH), villus width (VW), villus surface area (VSA) and crypt depth (CD) of ileum. For the measurement of ileum VH, VW and CD, cross-sections of 10 villi were randomly selected from each section. VH was measured as the distance from the apex of the villus to the junction of the villus and crypt. VW was determined by measuring the distance from the junction to the basement membrane of the epithelial cell at the bottom of the crypt at the bottom third of the length of the villus (base width of the ileum villi). CD was defined as the depth of the invagination between adjacent villi. Surface area was calculated using the formula = $(2\pi) \times (VW/2) \times (VH)$ in which VW = villus width and VH = villus height (Sakamoto et al., 2000).

Analyses of the short-chain fatty acids concentrations of ceca

On day 42, the ceca of 10 broilers slaughtered from each treatment group was removed individually. The cecal digesta were aseptically and individually collected in sterile 2-ml-tubes and then immediately stored at -20°C for the analysis of their short-chain fatty acids (SCFAs) contents. Frozen cecal digesta (0.5 g) were thawed at 4°C and diluted 4-fold with double-distilled water in steril screw-cap tubes. Cecal digesta were homogenized and centrifuged at 4000 *x g* for 15 min at 4°C. One milliliter of supernatant was then transferred to an Eppendorf tube and mixed with 0.2 mL ice-cold 25 % metaphosphoric acid solution to prevent the volatile fatty acids evaporation. The tubes were placed in an ice bath for 30 min and the samples were then centrifuged at 11 000 *x g* for 10 min at 4°C. The resulting supernatants were analyzed using a gas chromatograph (Shimadzu GC-2010, Shimadzu Co., Kyoto, Japan) coupled with a 30 m x 0.53 mm internal diameter column (Teknokroma TRB-FFAP, Teknokroma, Barcelona, Spain) and flame ionization detector to determine SCFAs concentrations in cecal digesta (Calik and Ergün, 2015). The injector-port and flame ionization detector temperatures were fixed at 230°C and 250°C, respectively. In the temperature program, the initial temperature was set at 120°C and held for 1 min after injection and then increased at 1°C/min to 160°C, where it was held for 4 min. Helium was used as the carrier gas. The injection volume was 1 µL and analyses were performed in duplicate. SCFAs represent the sum of acetic acid, propionic acid and butyric acid (Calik and Ergün, 2015).

Statistical analysis

Firstly, Kolmogorov-Smirnov and Shapiro-Wilk as tests of normality and variance homogeneity test were applied to raw data. All data obtained from the present study showed normal distribution. The univariate general linear model using the SPSS (17.0)® statistic package (SPSSWIN, 2007) was applied to the collected data with a model including stocking densities (SDs) and dietary treatments (DTs) and the interaction between SDs and DTs. Significant differences between treatment means were separated by Duncan's multiple range test (Duncan, 1955). All statements of significance were based on a P value of < 0.05.

RESULTS AND DISCUSSION

Growth performance

The effects of the experimental treatments on the final BW and BWG, FI and FCR of broilers from hatching to 42 day are shown in Table 3.

Table 3. Effects of the experimental treatments on the final BW and BWG, FI and FCR of broilers from hatching to 42 day[1]

Stocking Densities, birds/m²	Dietary Treatments	Final BW (g)	BWG (g)	FI (g)	FCR (g:g)
12	CONT	2695.59	2651.25	4566.34	1.72
12	FDMLP	2799.27	2754.95	4624.57	1.68
12	FDMLB	2762.66	2718.43	4596.65	1.69
18	CONT	2620.95	2576.54	4457.69	1.73
18	FDMLP	2688.31	2643.96	4448.46	1.68
18	FDMLB	2647.82	2603.47	4431.71	1.70
SEM		13.963	13.954	24.929	0.010
Stocking Densities (SDs)					
12		2752.51[a]	2708.21[a]	4595.86[a]	1.70
18		2652.36[b]	2607.99[b]	4445.96[b]	1.71
SEM		12.388	12.349	31.707	0.015
Dietary Treatments (DTs)					
CONT		2658.27[b]	2613.90[b]	4512.02	1.73[a]
FDMLP		2743.79[a]	2699.45[a]	4536.52	1.68[b]
FDMLB		2705.24[a]	2660.95[a]	4514.18	1.70[b]
SEM		15.173	15.124	38.833	0.018
P-value					
SDs		0.000	0.000	0.003	0.739
DTs		0.002	0.002	0.886	0.023
SDs x DTs Interaction		0.593	0.591	0.807	0.985

[a-b] Values in the same column not sharing a common superscript differ significantly (* P <0.05; ** P <0.01; *** P <0.001)
SEM: Standard Error of the Mean, [1] Data are means of five replicates per treatment group

HSD reduced the final BW (*P <0.001*), and BWG (*P <0.001*) and FI (*P <0.01*) of broilers during the period of 0 to 42 days compared to those of broilers under NSD (Table 3).

This may be related to the fact that broilers need more feeding space and physical access to feed and water due to the increase in their size with increasing age (Alqassem et al., 2018). HSD in old age restricts the movement of birds to a confined area of the pen (Khalil et al., 2021). As a result of this, broilers raised under HSD cannot access to feed and water (Cengiz et al., 2015; Rashidi et al., 2019). In addition, HSD leads to high environmental temperature in the microclimate of broilers. Thus, HSD decreases the airflow at the level of broilers, resulting in reduced dissipation of body heat to the air. Broilers grown under a high environmental temperature with poor air quality may suffer heat stress due to restricted heat exchange caused by crowding (Meena et al., 2022), which decreases FI and impairs the ability to meet the nutrient requirements for growth performance of broilers (Zhang et al., 2013). Furthermore, increasing glucocorticoid hormone level due to the crowding stress induces nervousness causes a decrease in feed consumption in broilers (Gomes et al., 2014). In addition, HSD also deteriorates the litter quality (Goo et al., 2019) increasing its moisture content, bacterial fermentation and ammonia volatilization, which leads to the suppression of broilers' growth performance (Khalil et al., 2021) by the significant damage to the intestinal histomorphology (Khalil et al., 2021) and disturbance to the intestinal microbiota (Cengiz et al., 2015). The above-mentioned effects of HSD led to the impairment of the health of broilers and worsen their growth performance. However, SDs did not influence FCR (*P >0.05*) of broilers during the period from days 0 to 42. This is consistent with the results reported by Kridtayopas et al. (2019), Li et al. (2019), Rashidi et al. (2019) and Li et al. (2022) who showed that there is no significant difference between FCR of broilers reared under HSD and NSD. However, other study undertaken by Houshmand et al. (2012) reported that NSD caused a significant improvement in FCR of broilers compared to HSD. These discrepancies between the results concerning the effect of SD on FCR of broilers may be explained by the different experimental conditions such as bird strain, type of feed additives and different numbers of birds per m^2 in pens and cages (Houshmand et al., 2012).

Moreover, feeding with the FDMLP and FDMLB diets increased the final BW (*P <0.01*), and BWG (*P <0.01*) and improved FCR (*P <0.05*) of broilers during the period of 0 to 42 days compared to those of broilers fed the CONT diet (Table 3). On the other hand, FI of broilers from 0 to 42 days was not affected by DTs. The improved growth performance of broilers observed in the present study may be related to the combined positive effects of probiotics (Cengiz et al., 2015), chitooligosaccharides as prebiotic and short chain fatty acids (Borrelli et al., 2017) as degradation products of chitin and AMPs (Józefiak and Engberg, 2017; Benzertiha et al., 2020) in the FDMLP and FDMLB diets due to their antibacterial properties on the microbiota composition and the digestive and absorptive capacity of the villus structures of the small intestine of broilers (Calik et al., 2017; Islam and Yang, 2017; Józefiak et al., 2018). In addition, *Lactobacillus* species in the FDMLP and FDMLB diets are known to secrete several enzymes that increase nutrient digestibility and absorption (Calik et al., 2017; Khalil et al., 2021). As a result of the above-mentioned positive effects of the FDMLP and FDMLB diets, the digestibility and absorption of nutrients in the small intestine and thereby the growth performance of broilers were improved (Islam and Yang, 2017).

No significant interaction (*P >0.05*) between SDs and DTs for the final BW, and BWG, FI and FCR of broilers from 0 to 42 days of broilers was observed (Table 3).

Slaughtering parameters

The effects of the experimental treatments on the relative weight (RW) of bursa of Fabricius and hot- and cold-carcass yields of broilers on day 42 are given in Table 4.

The RW of the bursa of Fabricius of broilers was not significantly affected by DTs and SDs x DTs interaction (*P >0.05*) (Table 4). However, HSD decreased the RW of bursa of Fabricius (*P <0.05*) of broilers compared to that of broilers reared under NSD. The finding related to the RW of the bursa of Fabricius of broilers is in agreement with the result reported by Alqassem et al. (2018) found that the RW of the bursa of Fabricius of broilers was significantly decreased by HSD compared to NSD. In the present study, HSD might have been significantly decreased immunity of broilers by its reduction of the RW of the bursa of Fabricius compared to NSD.

Furthermore, this result concurs with the finding

Table 4. Effects of the experimental treatments on the relative weight (RW) of Bursa of Fabricius and hot- and cold-carcass yields of broilers at 42 day, %[1]

Stocking Densities, birds/m²	Dietary Treatments	Bursa of Fabricius	Hot Carcass Yield	Cold Carcass Yield
12	CONT	0.19	73.52	72.22
12	FDMLP	0.21	73.96	73.01
12	FDMLB	0.20	74.23	72.99
18	CONT	0.17	72.88	72.07
18	FDMLP	0.17	73.21	72.46
18	FDMLB	0.18	73.50	72.26
SEM		0.006	0.158	0.155
Stocking Densities (SDs)				
12		0.20[a]	73.90[a]	72.74[a]
18		0.17[b]	73.20[b]	72.26[b]
SEM		0.008	0.209	0.218
Dietary Treatments (DTs)				
CONT		0.18	73.20	72.14
FDMLP		0.19	73.58	72.73
FDMLB		0.19	73.86	72.63
SEM		0.010	0.256	0.256
P-value				
SDs		0.035	0.030	0.014
DTs		0.916	0.249	0.274
SDs x DTs		0.811	0.988	0.737

[a-b] Values in the same column not sharing a common superscript differ significantly (*P <0.05)
SEM: Standard Error of the Mean, [1] Data are means of 10 individual chickens per treatment group

reported by Islam and Yang (2017) who reported that the dietary supplementation of a mealworm larvae-based probiotic as an antibacterial feed additive did not have any significant effect on the weight of the bursa of Fabricius compared to the negative CONT diet. Similar finding was obtained by Benzertiha et al. (2020) who indicated that there were no significant differences in the RWs of the bursa of Fabricius of broilers fed diets supplemented with the full-fat *tenebrio molitor* meal as a feed additive compared to the negative control diet. Inconsistency among results may be because of the nutritive value and properties of the *tenebrio molitor* meal used, which can be influenced by the species, the life stage (adult, larva or pupa) and the rearing substrate of *tenebrio molitor* and methods applied in obtaining *tenebrio molitor* meal (Sànchez-Muros et al., 2014).

HSD also decreased the hot- (P *<0.05*) and cold-carcass yields (P *<0.05*) of broilers compared to those of broilers reared under NSD (Table 4). The results of the carcass yield (CY) obtained in the present study were similar to the finding of Khalil et al. (2021), who recorded that increasing SD decreased carcass yield of broilers. This decrease in the relative carcass yield of broilers occurred with increasing SD in the present study may be linked to the reduction in their final BW (Cengiz et al., 2015; Khalil et al., 2021). However, contrasting results were reported by Cengiz et al. (2015) and Alqassem et al. (2018) who observed that SD did not influence the relative carcass yield of broilers. The inconsistency in results may arise due to the different experimental conditions such as bird strain, type of feed additives and different numbers of birds per m^2 in pens and cages.

On the other hand, the hot- and cold-carcass yields of broilers were not influenced by DTs (Table 4).

No significant interaction (P *>0.05*) was detected between SDs and DTs for the hot- and cold-carcass yields of broilers (Table 4).

Mortality

In broilers fed the CONT diet, one broiler reared under NSD and two broilers reared under HSD died. The result of the Chi-square test revealed no significant difference (Chi-Square value=3) between the experimental groups in terms of mortality of broilers.

Blood heterophil/lymphocyte ratio

The effects of the experimental treatments on the blood heterophil/lymphocyte (H/L) ratio of broilers are summarized in Table 5.

HSD increased (*P <0.001*) the blood H/L ratio of broilers compared to that of broilers reared under NSD. HSD is a critical stressor in intensive broiler production, because it is associated with an increase in stress hormones, which result in turbulences in blood H/L ratio (Meena et al., 2022). The increased blood H/L ratio caused by HSD indicated the stressful effect of overcrowding on broilers. This result is in agreement with the finding reported by Kridtayopas et al. (2019) who stated that HSD increased the blood H/L ratio of broilers when compared to NSD. However, this data disagrees with the results reported by Cengiz et al. (2015) who found no significant difference between NSD and HSD in terms of the blood H/L ratio of broilers.

Moreover, the blood H/L ratio was lower (*P <0.001*) in broilers fed the FDMLP and FDMLB diets than those of broilers fed the CONT diet. It is assumed that feeding the FDMLP and FDMLB diets may recover enteric nervous system abnormalities caused by stress via the increase of the beneficial microorganism content in the small intestine of broilers (Kridtayopas et al., 2019). As a result of the stress-reducing effects of the FDMLP and FDMLB diets due to their above-mentioned mechanism of action, the blood H/L ratios of broilers reared under both NSD and HSD were reduced.

In addition, there was a significant interaction (*P <0.001*) between SDs and DTs in terms of blood H/L of broilers. The blood H/L ratios of broilers reared under both NSD and HSD were decreased (*P <0.001*) by the FDMLP and FDMLB diets compared to the CONT diet.

Microorganism content of ileum

The effects of the experimental treatments on the microorganism content of ileum of broilers at 42 day are summarized in Table 6.

The experimental treatments did not significantly influence the total aerobic bacteria content of ileum of broilers (*P >0.05*) (Table 6). HSD increased *E. coli* content (*P <0.01*) and decreased *Lactobacillus* spp. content (*P <0.01*) of ileum of broilers compared to NSD (Table 6). This result concurs with the findings

Table 5. Effects of experimental treatments on the blood heterophil/lymphocyte (H/L) ratio of broilers[1]

Stocking Densities, birds/m^2	Dietary Treatments	H/L ratio
12	CONT	0.38^{A}
12	FDMLP	0.26^{B}
12	FDMLB	0.28^{B}
18	CONT	0.49^{A}
18	FDMLP	0.35^{B}
18	FDMLB	0.35^{B}
SEM		0.016
Stocking Densities (SDs)		
12		0.31^{b}
18		0.40^{a}
SEM		0.017
Dietary Treatments (DTs)		
CONT		0.44^{a}
FDMLP		0.31^{b}
FDMLB		0.32^{b}
SEM		0.020
P-value		
SDs		0.000
DTs		0.000
SDs x DTs Interaction		0.000

$^{a-b}$ Values in the same column not sharing a common superscript differ significantly (***P <0.001)
$^{A-B}$ Capital letters on the right show the interaction between SDs and DTs
SEM: Standard Error of the Mean, ^{1} Data are means of 10 individual chickens per treatment group

Table 6. Effects of experimental treatments on the microorganism content of ileum of broilers on day 42, log10 CFU/g[1]

Stocking Densities, birds/m²	Dietary Treatments	Total aerobic bacteria	*E. coli*	*Lactobacillus* spp.
12	CONT	6.26	3.14	6.15
12	FDMLP	6.47	2.42	6.98
12	FDMLB	6.43	2.46	6.86
18	CONT	6.17	3.65	5.99
18	FDMLP	6.26	3.41	6.21
18	FDMLB	6.37	3.49	6.05
SEM		0.064	0.135	0.114
Stocking Densities (SDs)				
12		6.39	2.67^{b}	6.66^{a}
18		6.27	3.52^{a}	6.09^{b}
SEM		0.089	0.121	0.133
Dietary Treatments (DTs)				
CONT		6.21	3.39^{a}	6.07^{b}
FDMLP		6.37	2.91^{b}	6.60^{a}
FDMLB		6.40	2.98^{b}	6.46^{a}
SEM		0.109	0.158	0.163
P-value				
SDs		0.417	0.001	0.006
DTs		0.570	0.015	0.009
SDs x DTs Interaction		0.872	0.531	0.327

$^{a-b}$ Values in the same column not sharing a common superscript differ significantly (*P <0.05; **P <0.01)
SEM: Standard Error of the Mean, [1] Data are means of 10 individual chickens per treatment group

reported by Cengiz et al. (2015) and Kridtayopas et al. (2019) who stated that HSD significantly decreased the *Lactobacillus* spp. content and increased *E. coli* content of ileum broilers compared to low SD or NSD. On the contrary, Zhang et al. (2013) pointed out that there are no significant differences between HSD and NSD in terms of *E. coli* and *Lactobacillus* spp. contents of ileum of broilers. This may be attributed to stress and worsening litter microbial population due to HSD that results in the overgrowth of pathogenic bacteria and depresses the growth of beneficial bacteria, which shifts the bacteria of the small intestine to a state of dysbiosis (Kridtayopas et al., 2019).

Besides, feeding the FDMLP and FDMLB diets decreased *E. coli* content (P <0.05), but, increased *Lactobacillus* spp. content (P <0.01) of ileum of broilers compared to those of broilers fed the CONT diet (Table 6). This may be related to the combined effects of probiotics (Cengiz et al., 2015), chitooligosaccharides as prebiotic and short chain fatty acids as degradation products of chitin (Borrelli et al., 2017; Kwon et al., 2020) and AMPs (Józefiak and Engberg, 2017; Benzertiha et al., 2020) in the FDMLP and FDMLB diets, which have the beneficial functions on microbial balance in the small intestine of broilers by enhancing beneficial bacteria such as *Lactobacillus* spp. and inhibiting pathogenic bacteria such as *E. coli* in their small intestine (Józefiak et al., 2018; Kridtayopas et al., 2019; Benzertiha et al., 2020).

There was no significant interaction (P >0.05) between SDs and DTs in terms of *E. coli* and *Lactobacillus* spp. contents of ileum of broilers (Table 6).

Histomorphometric parameters of ileum

The effects of the experimental treatments on the histomorphometric parameters of ileum of broilers on day 42 are given in Table 7.

HSD significantly reduced VH (P <0.01) and VSA (P <0.01) while increasing CD (P <0.05) of ileum of broilers compared to NSD (Table 7). The finding related to VH of ileum is partially in line with the results of Kridtayopas et al. (2019) who reported that HSD significantly reduced VH, on the other hand, did not influence CD of ileum of broilers when compared to NSD. Accordingly, Shakeri et al. (2014) stated that HSD significantly decreased VH of ileum of broilers compared to NSD. The stress from HSD may induce dysfunction in the mucosal tight junction (Song et al., 2014) and the microbiota of the small intestine. As a

Table 7. Effects of experimental treatments on the histomorphometric parameters of ileum of broilers at 42 day[1]

Stocking Densities, birds/m²	Dietary Treatments	Villus Height (VH), µm	Crypt Depth (CD), µm	Villus Surface Area (VSA), µm²
12	CONT	850.38	120.00	106447.59
12	FDMLP	944.02	100.00	155876.03
12	FDMLB	898.32	105.00	123149.35
18	CONT	753.18	134.00	72076.43
18	FDMLP	849.25	110.00	93719.72
18	FDMLB	816.32	112.00	88753.11
SEM		24.102	4.212	7147.19
Stocking Densities (SDs)				
12		897.57^{a}	108.33^{b}	128490.99^{a}
18		806.25^{b}	118.67^{a}	84849.75^{b}
SEM		33.092	5.532	7385.67
Dietary Treatments (DTs)				
CONT		801.78^{b}	127.00^{a}	89262.01^{c}
FDMLP		896.63^{a}	105.00^{b}	124797.88^{a}
FDMLB		857.32^{a}	108.50^{b}	105951.23^{b}
SEM		37.523	6.510	9045.56
P-value				
SDs		0.007	0.021	0.001
DTs		0.026	0.007	0.044
SDs x DTs Interaction		0.990	0.934	0.510

$^{a-c}$ Values in the same column not sharing a common superscript differ significantly (*P <0.05; **P <0.01)
SEM: Standard Error of the Mean, [1] Data are means of 10 individual chickens per treatment group

result of these, the intestinal structure and epithelial development are negatively affected (Kridtayopas et al., 2019). On the contrary, Li et al. (2017) reported that there are no significant differences between NSD and HSD in terms of VH, CD and VH/CD of ileum of broilers on day 42.

In addition, the FDMLP and FDMLB diets significantly increased VH (P <0.05) and VSA (P <0.05) whereas decreasing CD (P <0.01) of ileum of broilers on day 42. Our result related to VH of ileum is consistent with Calik et al. (2017) who reported that ileal VH of broilers was increased by dietary synbiotics on day 42. The improvement in the ileum morphology of broilers fed the FDMLP and FDMLB diets in the current study is possibly due to the combined antibacterial properties of their probiotic, prebiotic, SCFA and AMP contents (Kridtayopas et al., 2019). Chitin in the FDMLP and FDMLB diets was degraded to SCFAs in ceca of broilers and thereby increased the formation of the short chain organic acids such as lactic acid in the small intestine. The increase in the acidity in the small intestine of broilers, which decrease the growth and intestinal colonization of many pathogenic bacteria and infectious processes, ultimately reduces inflammatory processes at the intestinal mucosa, which increase VH (Altaf et al., 2019). Moreover, the FDMLP and FDMLB diets may stimulate the goblet cells and mucus secretion that works as intestinal barriers, thus protects the small intestine from pathogenic bacterial infection and pathogenic toxins due to their probiotic, prebiotic, SCFA and AMP contents. As a result of these, the morphology of the small intestine and thereby nutrient utilization of broilers are improved (Kridtayopas et al., 2019).

However, there was no significant interaction (P >0.05) between SDs and DTs in terms of VH, VSA and CD of ileum of broilers (Table 7).

Short-chain fatty acids concentrations of ceca

The effects of the experimental treatments on the short-chain fatty acids (SCFAs) concentrations of ceca of broilers on day 42 are presented in Table 8.

In the present study, HSD significantly reduced the concentrations of acetate (P <0.01), butyrate (P <0.01) and propionate (P <0.001) in ceca of broilers compared to NSD. This finding is in line with the results of Li et al. (2017) who stated that HSD significantly decreased the butyrate and total SCFAs

Table 8. Effects of experimental treatments on the SCFAs concentrations (μmol/g of digesta) of ceca of broilers on day 42[1]

Stocking Densities, birds/m²	Dietary Treatments	Acetate	Butyrate	Propionate	Total SCFAs
12	CONT	42.133	6.831	11.044	60.008
12	FDMLP	80.916	16.398	23.414	120.728
12	FDMLB	61.311	10.898	18.250	90.458
18	CONT	31.998	6.255	5.573	44.031
18	FDMLP	38.329	7.840	6.696	54.341
18	FDMLB	34.762	6.460	8.172	47.713
SEM		4.465	0.900	1.489	6.617
Stocking Densities (SDs)					
12		61.453^{a}	11.376^{a}	17.569^{a}	90.398^{a}
18		35.030^{b}	6.852^{b}	6.814^{b}	48.695^{b}
SEM		4.873	0.964	1.384	6.752
Dietary Treatments (DTs)					
CONT		37.066^{c}	6.543^{c}	8.309^{c}	52.020^{c}
FDMLP		59.623^{a}	12.119^{a}	15.055^{a}	87.535^{a}
FDMLB		48.037^{b}	8.679^{b}	13.211^{b}	69.086^{b}
SEM		5.968	1.181	1.695	8.270
P-value					
SDs		0.001	0.004	0.000	0.000
DTs		0.042	0.014	0.020	0.025
SDs x DTs Interaction		0.197	0.079	0.156	0.134

$^{a-c}$ Values in the same column not sharing a common superscript differ significantly (*P <0.05; **P <0.01; ***P <0.001)
SEM: Standard Error of the Mean, [1] Data are means of 10 individual chickens per treatment group

concentrations of ileum of broilers at 42 day of age. As a result, the reduction of the SCFAs concentrations in ceca of broilers cannot disturb the oxidation-reduction of coenzymes and prevent harmful bacterial growth and metabolism (Li et al., 2017).

Moreover, the acetate (P <0.05), butyrate (P <0.05) and propionate (P <0.05) concentrations of ceca of broilers were increased by feeding the FDMLP and FDMLB diets compared to those of broilers fed the CONT diet (Table 8).

Unfortunately, no information is available in the literature on the effects of feeding the FDMLP and FDMLB diets on the SCFAs production in ceca of broilers. Among SCFAs, butyrate is considered as the primary energy source for enterocytes and plays an important role in cellular differentiation, proliferation and repair of the intestinal mucosa (Calik and Ergün, 2015). Consequently, the results of the present study clearly suggest that the FDMLP and FDMLB diets due to their fermentable chitin, as a potential prebiotic, improve intestinal histomorphology in correlation with the increased levels of cecal butyrate (Calik and Ergün, 2015; Borrelli et al., 2017). The present study indicated that the increase of SCFAs concentrations in ceca of broilers fed the FDMLP and FDMLB diets contribute to lower pH of the intestinal digesta, which corresponds to an increased beneficial bacterial population in their intestinal digesta of broilers (Calik and Ergün, 2015; Li et al., 2017).

No significant interaction (P >0.05) was observed between SDs and DTs in terms of acetate, butyrate and propionate concentrations of ceca of broilers on day 42 (P >0.05) (Table 8).

CONCLUSION

The present study showed that HSD negatively affected growth performance except FCR, carcass yields, blood H/L ratio, microorganism content and histomorphometric parameters of ileum and SCFAs concentrations of ceca of broilers. However, feeding the FDMLP and FDMLB diets positively influence growth performance except FI, blood H/L ratio, microorganism content and histomorphometric parameters of ileum and SCFAs concentrations of ceca of broilers reared under HSD and NSD. In conclusion, FDMLP and FDMLB can be utilized as new antibacterial feed additives in broiler diets regardless of stocking density. Nevertheless, there is a need for

further studies to ascertain the effects of dietary supplementation of FDMLP and FDMLB in combination as antibacterial feed additive in order to alleviate the detrimental effects of HSD in broilers reared under HSD, which is a global problem. Further studies are also necessary to deepen knowledge about the molecular components of FDMLP and FDMLB for the antibacterial activity and to highlight their mechanism of action to alleviate the detrimental effects of HSDs in broilers reared under HSD.

CONFLICT OF INTEREST

We declare no conflict of interest with any financial organization regarding the material discussed in the manuscript.

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