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Effects of Dietary Fermented Mealworm Larvae and Stocking Density on the Morphometric Characteristics and Mineral Contents of Tibia Bone of Broilers

Senay Sarica[1,a,*], Musa Yavuz[2,b], Elif Rabia Sanli[2,c], Hacer Baş-Ekici[3,d], Zeynep Yardim[4,e]

[1]*Department of Animal Science, Faculty of Agriculture, Tokat Gaziosmanpasa University, 60240 Tokat, Türkiye*
[2]*Department of Animal Science, Faculty of Agriculture, Isparta University of Applied Science, 32200 Isparta, Türkiye*
[3]*Department of Anatomy, Veterinary Faculty, Selcuk University, 42003 Konya, Türkiye*
[4]*Department of Animal Science, Faculty of Agriculture, Aydin Adnan Menderes University, 09070 Aydın, Türkiye*
[*]*Corresponding author*

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ABSTRACT

This study aimed to investigate the effects of the supplementation of defatted mealworm larvae meal fermented with probiotics to the diet of broilers reared under normal stocking density (NSD) and high stocking density (HSD) on the morphometric characteristics and mineral contents of tibia bone of broilers. A total of 450 one-d-old Ross 308 male broiler chicks were randomly distributed into six groups of similar mean weight, each containing five replicates. The experimental treatments were arranged as a 2 × 3 factorial design, incorporating two levels of stocking density (12 birds/m², designated as NSD, and 18 birds/m², designated as HSD) and three different diets in mash form: CONT- a corn-soybean meal-based diet containing no fermented defatted mealworm larvae meal (FDM) (0%); FDMLP- the diet obtained by supplementing defatted mealworm larvae meal (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 aggravated the morphometric parameters (weight, length, weight/length index, diameter of diaphysis and medullary canal, tibiotarsal index and breaking strenght) and decreased mineralization (ash, Ca and P contents) of the tibia of broilers, whereas the FDMLP and FDMLB diets improved tibia mineralization and morphology except its medullary canal diameter and ribusticity index of broilers due to the results of enhanced mineral absorption. In conclusion, the use of FDMLP and FDMLB as new antibacterial feed additives in broiler diets regardless of stocking density was able to improve tibia mineralization and morphology except its medullary canal diameter and ribusticity index of broilers due to the results of enhanced mineral absorption.

[a] *senaysarica2002@yahoo.com* *https://orcid.org/0000-0001-7927-5821*
[b] *musayavuz@isparta.edu.tr* *https://orcid.org/0000-0003-4936-1846*
[c] *elifsanli@isparta.edu.tr* *https://orcid.org/0000-0002-6862-6048*
[d] *vethacer.bas@gmail.com* *https://orcid.org/0000-0003-1941-1830*
[e] *zkacamakli@adu.edu.tr* *https://orcid.org/0000-0002-9235-3721*

Introduction

The demand for animal protein for the adequate and balanced nutrition of the rapidly increasing World and Turkey population is constantly increasing. In this respect, the broiler meat production is one of the major agricultural industries that considered as the main source of animal protein all over the World (Altaf et al., 2019). In recent decades, broiler producers are obliged to rear broilers under high stocking density (HSD) to decrease the production costs, produce more kilograms of broilers per unit area for a cheap, safe supply of meat and increase profitability (Khalil et al., 2021). However, HSD impairs broiler welfare especially during the later growing period when the body weight of broilers per m^2 is increased (Zhang et al., 2018). One of the main welfare concerns of HSD is the restriction in their movements and activities since broilers are reared only in a limited space (Buijs et al., 2012). This lack of exercise leads to development of bone weakness and leg deformation in broilers, as shown by the increased tibial dysplasia (Li et al., 2019) and decreased tibia breaking strength (Sun et al., 2018). HSD also negatively influences microflora and histomorphological structure of the small intestine of broilers (Kridtayopas et al., 2019; Sugiharto, 2022). As it is known, Ca and P are particularly significant for the development and maintenance of skeletal structure, as they are the essential macrominerals of bone tissue of avian organisms (Jabbar et al., 2024). In addition, vitamin D exerts beneficial effects on bone metabolism primarily by

stimulating the absorption of Ca and P absorption in the intestine and promoting their reabsorption in the kidneys, and regulating the bone formation (Zhang et al., 2021). However, HSD reduces the intestinal absorption and bioavailability of minerals such as Ca and P and vitamin D_3 that are necessary for bone especially tibia mineralization (Liu et al., 2020), and induces leg disorders in broilers (Li et al., 2019; Sun et al., 2018; Yan, 2016). Consequently, the increasing leg deformation of broilers reared under HSD increases the susceptibility to fracture during catching and transportation, which causes problems during processing and serious economic losses in the current broiler meat industry (Liu et al., 2020). It is estimated that the poultry industry incurs an annual economic loss of 0.016$ per broiler in US due to leg disorders (Cook, 2000).

Therefore, maximization of absorption and bioavailability of minerals such as Ca and P from the small intestine by the dietary supplementation of antimicrobial feed additives can be practical for preventing the leg disorders of broilers under HSD (Mohammed et al., 2021; Steczny & Kokoszyński, 2020). In this context, the dietary supplementation of antibiotic growth promoters (AGPs) as antibacterial feed additive to enhance their performance by improving the microbiota and histomorphological structure of the small intestine of broilers under HSD has been investigated in previous times (Hooge et al., 2003). Unfortunately, the use of AGPs for long periods in broiler diets has caused to the appearance of AGPs resistance and residual AGPs in broiler meat, which are harmful to human health and cause increasing public concern (Aslam et al., 2021). The use of AGPs in broiler diets was banned by the European Union in 2006 (Sugiharto, 2022). Likewise, the use of certain substances in animal diets has been prohibited following various amendments, culminating in the enactment of Law No. 25847 on June 16, 2005, Law No. 26056 on January 21, 2006, and Law No. 26511 on May 3, 2007 in Türkiye. These laws establish a complete ban on the use of growth-promoting antibiotics and hormones in all animal diets within Türkiye (Tuncer, 2007). The above-mentioned reasons has increased interest in the use of natural antibacterial feed additives such as probiotics, prebiotics and synbiotics etc. in diets of broilers reared under HSD (Sugiharto, 2022).

Although there are few studies that dietary probiotic or prebiotic supplementation did not influence histomorphological characteristics (Gutierrez-Fuentes et al., 2013; Javid et al., 2022; Mohammed et al., 2021; Steczny & Kokoszyński, 2020) of tibia in broilers, many studies have shown that the dietary supplementation of the natural antibacterial feed additives such as probiotics, prebiotics or synbiotics increased ash, calcium (Ca) and phosphorus (P) contents (Gutierrez-Fuentes et al., 2013; Ortiz et al., 2009; Ziaie et al., 2011) of tibia of broilers reared under normal stocking density.

In this context, dried mealworm larvae meal solid-state fermented with probiotics has been evaluated as a new antibacterial feed additive in broiler diet in a previous study (Islam & Yang, 2017). Compared with animal-derived feed ingredients, insects as a novel feed ingredient have advantages with their ability to convert organic residues into protein more efficiently, need less space and water, the lower environmental impact and high nutritional values (Lee et al., 2022; Sedgh-Gooya et al., 2022). Presently, insects such as mealworm larvae (M) are not only considered as a nutrient-rich feedstuff (Kwon et al., 2020) but also as an antibacterial feed additive due to their presence of antimicrobial peptides (AMPs) (Benzertiha et al., 2020; Elahi et al., 2022) and chitin (Islam & Yang, 2017; Gasco et al., 2018) for poultry nutrition. It is reported that the chitin amount of M is 4.30-8.91% (Hong et al., 2020). Consequently, chitin in M is partially degraded by the acidic chitinase in the proventriculus and gizzard of chicken to produce chitooligosaccharides, a prebiotic (Sedgh-Gooya et al., 2022) and thereby as a potential antibacterial feed additive for broilers or laying hens (Borrelli et al., 2017). However, the high chitin levels (>2.42 %) in M may impose negative effects on feed intake and protein availability and thereby worsen growth performance in broilers (Mulyono et al., 2019). As a result, both reducing the high chitin content and revealing the antimicrobial components of insects such M and black soldier fly larvae can be performed by solid-state fermentation (SSF) using specific microorganisms that are able to degrade chitin (Mulyono et al., 2019; Hadj Saadoun et al., 2020; Luparelli et al., 2022). Solid-state fermentation (SSF) is a form of microbial fermentation that takes place in conditions with minimal to no free water, since it simulates the natural environment of the selected microorganisms naturally adapted (Peng et al., 2022). Among probiotic bacteria species, Lactic acid bacteria (LAB) is the most used species for M solid-state fermentation (Islam & Yang, 2017). The SSF highlights the using possibility of the solid-state fermented M as an antibacterial feed additive for broilers (Islam & Yang, 2017; Hadj Saadoun et al., 2020; Luparelli et al., 2022).

To our knowledge, there is only a one-week study that was conducted to investigate whether M and super M meal solid-state fermented with only *Lactobacillus plantarum* could be used as antibacterial feed additives in broilers challenged with pathogen bacteria such as *Salmonella* and *Escherichia coli* (Islam & Yang, 2017). 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 on tibia morphometric parameters in broilers by improving the tibia bone mineralization due to their positive effects on microflora and histomorphological structure of the small intestine.

Therefore, the current experiment aimed to compare the effects of the addition 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 the morphometric characteristics and the ash, Ca and P contents of the tibia bone.

Materials and Methods

The Location of The Experiment

The experiment was conducted at the broiler chicken research unit within the Tokat Gaziosmanpasa University Agricultural Application and Research Center Directorate in Tokat, Türkiye.

Animal Care, Experimental Design and Diets

On the day of hatching, a total of 450 *Ross 308* healthy male broiler chicks, aged one-d, were acquired from a commercial hatchery in Türkiye. During the 6 wk experiment, the broiler chicks were kept on floor pens. Each floor pen was with a 10 cm-thick layer of fresh wood shavings. The temperature of the experimental room was maintained at 32ºC for the first wk and gradually reduced and remained at 21°C. A lighting schedule of 23L:1D was employed during overall experimental period. From hatching to 42 days of age, the experimental diets and drinking water were offered *ad libitum*. The present experiment was approved from the Animal Care and Use Committee of Tokat Gaziosmanpasa University, under process number 2019-HADYEK-47.

Experimental treatments consisted of a 2 x 3 factorial arrangement with two levels of stocking density (12 birds/m^2 as normal stocking density (NSD) and 18 birds/m^2 as high stocking density (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 defatted mealworm larvae meal (DM) fermented with *Lactobacillus brevis* to the CONT diet (0.4%). Each treatment comprised 5 replicates. 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, total sugar, calcium (Ca) and phosphorus (P) contents according to the methods of the AOAC (2007) at Ankara Food Control Laboratory (Ankara, Türkiye). All diets were formulated by taking into account the analyzed contents of the feed ingredients in accordance with phase feeding practices as the broiler chickens advanced in age and weight, aligned with the guidelines provided by the breeder (Ross 308, 2007). The feeding regimen was structured into three distinct phases: the starter phase, which extended from day 0 to day 10; the grower phase, which occured from day 11 to day 28; and the finisher phase was from day 29 to day 42. Ingredient composition and nutrition content of the control diet are showed in Table 1.

90-d-old mealworm larvae (M) (*Tenebrio molitor* L.) purchased from a commercial supplier and were freeze-dried. And then the 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). The freeze-dried M meal was defatted with petroleum ether using a soxhlet extractor under optimized conditions and then dried. Consequently, the fat content of DM was reduced from 23% to 6.6%, while its crude protein content increased from 44% to 76.2%.

Table 1. Ingredient composition and nutrient 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 D_3; 50 mg vitamin E; 5 mg vitamin K_3; 3 mg vitamin B_1; 6 mg vitamin B_2; 5 mg vitamin B_6; 0.03 mg vitamin B_{12}; 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

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

Two probiotic bacteria (*Lactobacillus plantarum* and *Lactobacillus brevis*) (Leisner et al. 2008; Islam & Yang, 2017) 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 *Lactobacillus 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 & 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. Prior to fermentation, DM, distiller's dried grains with solubles (DDGS), defatted rice bran, and water were sterilized by autoclaving at 121°C for 15 min. In the first stage of the fermentation, DDGS (35%), defatted rice bran (35%), DM (30%) and distilled water (80%) 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 supplemented to the solid substrate medium in the fermenter and fermented at 38ºC for 48 h under anaerobic conditions. After an initial 48-hour fermentation period, a secondary fermentation was conducted using 1.0% *Saccharomyces cerevisiae* (baker's yeast), which had been activated for 1 hour at 37ºC in 250 ml of 0.1% peptone water (consisting of 10 g yeast and 90 ml peptone water) at 38ºC for an additional 48 hours 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). Upon completion of the total 96-h fermentation process, the fermented product was dried to a moisture content of less than 15% at 32ºC for 24 h using a drying oven. The same fermentation procedure was performed with *Lactobacillus brevis*. To determine microbial concentration, 1 gram of FDMLP or FDMLB was serially diluted in 9 ml of 0.85% sterile saline and thoroughly mixed. Total mesophilic aerobic bacteria counts were determined by plating serial 10-fold dilutions in triplicate onto Plate Count Agar (PCA) (Merck, Darmstadt, Germany), followed by incubation at 30°C for 48 h under aerobic conditions. Lactic acid bacteria (LAB) were enumerated by plating serial 10-fold dilutions in triplicate onto De Man, Rogosa, and Sharpe (MRS) agar (Merck, Darmstadt, Germany) and incubating at 39-40°C for 5 d under anaerobic conditions. Yeast and mold counts were assessed by plating serial 10-fold dilutions in triplicate onto Dichloran Rose Bengal Chloramphenicol (DRBC) agar (Merck, Darmstadt, Germany) and incubating at 25°C 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.

Measurements of The Tibia Morphometric 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 male broilers, representing the average body weights of each group, from each replicate, were selected (10 male broilers per treatment group) and slaughtered to measure of the tibia morphometric parameters on day 42**.** The individual left tibias were removed from a total of 60 broilers, labelled and frozen (-20°C) until analysis. The labelled tibias were thawed and immersed in boiling deioinized water for 10 min. The left tibias were de-fleshed by hand, defatted for 48 h in ethyl alcohol followed by a 48 h extraction in ethyl ether (Imari et al., 2020). The tibias were subsequently dried to a constant weight in a drying oven at 110°C for 12 h.

Table 3. The effects of the experimental treatments on the morphometric characteristics of the left tibia in 42-d-old broilers[1]

SDs, birds/m^2	DTs	Weight mg	Length mm	Weight/length index mg/mm	Diaphysis diameter mm	Medullary canal diameter mm	Tibiotarsal index	Ribusticity index	Breaking strength N
12	CONT	7940	97.538	81.413	7.977	4.373	46.864	4.896	305.00
12	FDMLP	8520	99.748	85.498	8.664	3.968	53.887	4.886	335.67
12	FDMLB	8200	99.560	82.434	8.242	4.432	45.815	4.869	316.60
18	CONT	7700	94.585	81.355	7.642	5.062	33.462	4.871	255.40
18	FDMLP	8340	98.574	84.581	8.646	4.745	43.029	4.863	295.60
18	FDMLB	8020	98.338	81.694	7.914	4.952	37.163	4.893	280.50
SEM		120.990	0.509	1.226	0.153	0.106	1.888	0.025	11.473
SDs									
12		8220^a	98.949^a	83.115^a	8.294^a	4.258^b	48.855^a	4.884	319.09^a
18		8020^b	97.166^b	82.543^b	8.007^b	4.920^a	37.885^b	4.876	277.17^b
SEM		169.398	0.623	1.821	0.207	0.121	2.031	0.038	16.444
DTs									
CONT		7820^b	96.061^b	81.384^c	7.809^c	4.717	40.163^c	4.884	280.20^b
FDMLP		8430^a	99.161^a	85.040^a	8.564^a	4.357	48.458^a	4.875	315.64^a
FDMLB		8110^a	98.949^a	82.064^b	8.078^b	4.692	41.489^b	4.881	298.55^a
SEM		207.469	0.763	2.230	0.254	0.142	2.390	0.047	18.217
P Value									
SDs		0.042	0.049	0.038	0.036	0.001	0.001	0.883	0.010
DTs		0.015	0.019	0.049	0.016	0.186	0.012	0.991	0.045
SDs × DTs		0.994	0.670	0.990	0.979	0.819	0.811	0.918	0.811

[a-c] Values in the same column not sharing a common superscript differ significantly; [1] Data are mean of 10 broilers from each treatment; SEM: Standard Error of Mean; SDs: Stocking Densities; DTs: Dietary Treatments; 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%).

Thereafter, tibias were individually weighed using an electronic balance with a precision of 0.001 g (Kern Germany, ABJ 220-4 M model) and their lenghts were determined using an digital caliper with an accuracy of 0.001 cm (Mitutoyo, Absolute Digimatic, Mitotoyo, Kruibeke, Belgium). The bones were scanned at a resolution of 600 dpi and images were then converted to digital media.

To calculate the cortical index (tibiotarsal index), diaphysis diameter of the tibiotarsus was measured with the ImageJ program (Doube et al., 2010). The diameter of the medullary canal was computed from the difference between internal and external diameter of the diaphysis (Midilli et al., 2015). The tibia weight/length index, cortical index and robusticity index were calculated according to the following formulas (Karaarslan & Nazligul, 2018):

$$TW/LI=TW/TL \quad (1)$$

TW/LI : The tibia weight/length index (mg/mm)
TW : The tibia weight
TL : The tibia length

$$CI= [(DD-MCD)/DD]\times100 \quad (2))$$

(Vahdatpour et al., 2014).

CI : Cortical index (Tibiotarsal index)
DD : The diaphysis diameter
MCD : The medullary canal diameter

$$RI : TL/CRTW \quad (3)$$

(Karaarslan & Nazligul, 2018)

RI : Robusticity index
TL : The tibia length
CRTW : Cube root of the tibia weight

After the above-mentioned measurements, the tibia breaking strenght was measured by Warner-Bratzler method using ZWICK/ROELL Z 50 test equipment (Text Xpert Version 3.4) with a speed of 5 mm/min and recorded as Newton (N), the strength value that the bone can withstand at the moment of fracture (Aksit et al., 2017). Afterwards, the tibias were ashed in a muffle furnace at 600°C overnight. Tibia ash was determined as percentage of tibia dry matter weight (Ceylan et al., 2020). The ash of each tibia was analyzed to determine its Ca and P content by using inductively coupled plasma optical emission spectroscopy (ICP-OES; PerkinElmer, Optima 2l00 DV), following the procedure outlined by Leske & Coon (2002).

Statistical Analysis

In the analysis, the univariate general linear model was employed using SPSS version 17.0 (SPSSWIN, 2007) to evaluate the collected data. The model incorporated factors of stocking densities (SDs) and dietary treatments (DTs), along with their interaction effects. Significant differences among treatment means were assessed using Duncan's multiple range test (Duncan, 1955). Statistical significance was determined based on a threshold of $P < 0.05$.

Results and discussion

The Morphometric Characteristics of Tibia Bone

The effects of the experimental treatments on the morphometric characteristics of the left tibia in 42-d-old broilers were given in Table 3.

HSD significantly ($P<0.05$) decreased the weight, length and weight/length index of the tibia of broilers when compared with NSD (Table 3). These findings are consistent with those reported by Li et al. (2019), who observed that HSD significantly decreased both the weight and length of the tibia in broilers when compared to broilers raised under NSD. Contrary to these findings,

Karaarslan & Nazligul (2018) who stated that there are no significant differences between the length and the weight/length index of the tibia of broilers under reared NSD and HSD. The observed reduction in the weight and length of the tibia in broilers reared under HSD in the present study may be attributed to the restricted movement in a limited space, which likely resulted in reduced physical exercise, and thereby the increased tibia curvature by adversely affecting tibia proliferation, differentiation and formation compared with those of broilers under NSD (Li et al., 2019).

As shown in Table 3, HSD significantly ($P<0.05$) decreased diaphysis diameter, increased ($P<0.01$) medullary canal diameter and decreased ($P<0.01$) the tibiotarsal index when compared to NSD. These results may be derived from insufficient mobilization in a limited space of broilers (Li et al., 2019). Similarly, Aksit et al. (2017) observed that HSD significantly reduced the diaphysis diameter of the tibia in broilers compared to NSD.

However, the result related to tibiotarsal index in the present study is not consistent with the finding reported by Karaarslan & Nazligul (2018) who stated that there are no significant differences between the tibio-tarsal index of broilers under reared NSD and HSD.

As indicated in Table 3, HSD significantly ($P<0.05$) decreased breaking strength of the tibia of broilers compared to NSD. This finding is in agreement with the results reported by Sun et al. (2018) and Liu et al. (2020), who observed that HSD significantly reduces the breaking strength of the tibia in broilers. This unfavorable effect of HSD on breaking strength of the tibia of broilers may be the result of a lack of physical activity due to reduced space and unsynchronized development of muscular mass and skeletal system as SD increases, which leads to development of bone weakness, and thereby, the susceptibility to fracture of the tibia is higher (Vargas-Galicia et al., 2017). Contrary to our result, Aksit et al. (2017) and Sun et al. (2018) reported that breaking strength of the tibia of broilers was not significantly affected by SD.

These discrepancies between the results concerning the effect of SD on the morphometric characteristics of the tibia of broilers may be explained by the different experimental conditions such as bird strain, age, rearing systems (in cage or on litter), different numbers of birds per m^2 in pens and cages, environmental conditions, experimental period and type of feed additives (Li et al., 2019).

Moreover, the FDMLP and FDMLB diets exhibited a significant ($P < 0.05$) increase in the weight, length, and weight/length index of the tibia of broilers compared to those fed the CONT diet (see Table 3). The large tibia weight/length index of broilers in the present study showed that feeding with the FDMLP and FDMLB diets of broilers may have caused the denser tibia and thereby increase in tibia weight (Libouban et al., 2001).

The FDMLP and FDMLB diets significantly ($P<0.05$) increased the diaphysis diameter of the tibia and the tibiotarsal index of broilers compared to the CONT diet, although they did not affect the medullary canal diameter of the tibia of broilers (see Table 3). These results suggest that the supplementation of FDMLP and FDMLB to the diet enhanced the degree of mineralization and development of the bone. The FDMLP and FDMLB diets significantly ($P<0.05$) enhanced the breaking strength of the tibia in broilers compared to that of broilers fed the CONT diet.

Since there are no studies on the effects of dietary supplementation of FDMLP and FDMLB on the morphometric characteristics of the tibia of broilers, our results could not be compared with previous studies. However, the improvement in measured tibia parameters of broilers observed in the present study could be attributed to the increased bioavailability and absorption of minerals such as Ca and P for tibia mineralization by the increasing beneficial bacteria in the small intestine due 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 (Benzertiha et al., 2020; Mohammed et al., 2021) of FDMLP and FDMLB in the diet.

SDs, DTs and interaction between SDs and DTs did not significantly affect ribusticity index of the tibia of broilers (Table 3).

The Crude Ash, Ca and P Contents of Tibia Bone of Broilers

The effects of the experimental treatments on the ash, Ca and P contents of the left tibia in 42-d-old broilers were summarized in Table 4.

HSD significantly decreased the ash ($P<0.05$) and Ca contents ($P<0.05$) and P content ($P<0.001$) of defatted tibia bone in 42-d-old broilers compared to those of broiler reared under NSD (Table 4). This might be explained by decreasing duodenal Ca (calcium-binding protein D28k transcription) and P (type IIb sodium-phosphate co-transporter mRNA) absorption in broilers reared under HSD (Sun et al., 2018). The low Ca content of the tibia also may arise due to the lower serum parathyroid hormone (PTH) concentration in broilers reared under HSD, because PTH is able to absorb bone and increase osteoclasts activity, allowing the broilers to complete the process of bone Ca activation (Wang et al., 2020). The decreasing tibia ash, Ca and P contents of broilers might be attributed to the increase in the synthesis of corticosterone stress hormone in the adrenal glands due to HSD. Excess corticosterone stress hormone level inhibits the osteoblast osteoblastogenesis (Henneicke et al., 2014) and reduced bone mineral density such as Ca and P contents (Kang et al., 2016). Moreover, HSD caused intestinal injury such as reduced villus height and villus surface area in broilers by increasing heat stress, by which heat stress hampers intestinal absorption of Ca and P (Yan et al., 2019). These findings in the present study are partially in line with the previous findings in the litter-floor rearing system, which reported that tibia ash and phosphorus contents of broilers on day 42 were decreased, but, their tibia Ca content was not affected by HSD (Liu et al., 2020).

These results in the present study differed from the previous study that pointed out no effect of SD on tibia ash, Ca and P contents of broilers reared in the litter-floor system (Vargas-Galicia et al., 2017; Sun et al., 2018). Differences in feeding environments (including temperature, relative humaditiy, etc.), age, broiler strain and feedstuffs in diets might be the cause for this discrepancy among studies.

Table 4. The effects of the experimental treatments on the ash, Ca and P contents of the left tibia in 42-d-old broilers[1]

SDs, birds/m^2	DTs	Ash, %	Ca, %	P, %
12	CONT	38.95	30.02	8.65
12	FDMLP	40.47	31.10	8.95
12	FDMLB	39.28	30.45	8.91
18	CONT	37.80	29.68	6.53
18	FDMLP	38.87	29.79	7.09
18	FDMLB	38.81	29.70	6.64
SEM		0.332	0.237	0.215
SDs				
12		39.57^a	30.53^a	8.84^a
18		38.49^b	29.72^b	6.75^b
SEM		0.462	0.337	0.132
DTs				
CONT		38.38^b	29.85^b	7.59^b
FDMLP		39.67^a	30.45^a	8.02^a
FDMLB		39.05^{ab}	30.08^{ab}	7.78^{ab}
SEM		0.566	0.413	0.162
P Value				
SDs		0.011	0.011	0.000
DTs		0.029	0.006	0.018
SDs x DTs		0.781	0.715	0.667

$^{a-b}$ Values in the same column not sharing a common superscript differ significantly; [1] Data are mean of 10 broilers from each treatment; SEM: Standard Error of Mean; Ash percent (as a percent of dry bone weight): Ca and P percent (as a percent of dry ash weight) of defatted tibia bone on d 42.; SDs: Stocking Densities; DTs: Dietary Treatments; 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%).

As shown in Table 4, only the FDMLP diet significantly increased the ash ($P<0.05$), Ca ($P<0.01$) and P ($P<0.05$) contents of the tibia in 42-d-old broilers compared to those of broilers fed the CONT and FDMLB diets. The improvement in the tibia ash, Ca and P contents of broilers in the present study could be related to the increased resorption of Ca and P by the beneficial bacteria in the small intestine due 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 (Benzertiha et al., 2020; Mohammed et al., 2021) of FDMLP and FDMLB in the diet, which, in turn, increases availability of serum Ca and P for bone formatting and/or remodeling (Mohammed et al., 2021). Previous studies have evidenced that synbiotics improve intestinal integrity and thereby increased absorption and bioavailability of Ca and P for bone mineralization (Yan et al., 2019).

Conclusions

Overall, the results gathered in this study indicate that HSD results in reducing tibia morphology and mineralization, whereas the use of FDMLP and FDMLB as new antibacterial feed additives in broiler diets regardless of stocking density was able to improve tibia mineralization and morphology except its medullary canal diameter and ribusticity index of broilers due to the results of enhanced mineral absorption. Nevertheless, further research is needed to investigate the effects of dietary supplementation of FDMLP and FDMLB on the expression of parathyroid hormone-related protein in the tibial growth plate and the gene expressions of calcium-binding protein-D28k (CaBP-D28k) and sodium-dependent phosphate transporter IIb (NaPi-IIb) in the small intestine of broilers. Further studies should also focus on elucidating the molecular components of FDMLP and FDMLB responsible for their antibacterial activity and clarifying their mechanisms of action to alleviate the adverse effects of HSD on tibial health in broilers.

Declarations

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Declaration of Competing Interest

The authors declare no conflict of interest.

References

Akşit, M., Helva, I. B., & Yardım, Z. K. (2017). Etlik piliçlerde farklı yerleşim sıklığı ve kesikli aydınlatmanın verim ve tibia kemiği özellikleri üzerine etkisi. Hayvansal Üretim, 58(2), 45-71. https://doi.org/10.29185/hayuretim.357146

Altaf, M. T., Mahmud, A., Mehmood, S., & Saime, S. (2019). Effects of supplemented growth promoters on performance and intestinal morphology in broilers reared under different stocking densities. Brazilian Journal of Poultry Science, 21(4), 1-6. https://doi.org/10.1590/1806-9061-2019-1073

AOAC. (2007). Association of Official Analytical Chemists. 18th edition. W.D.C., USA.

Aslam, B., Khurshid, M., Arshad, M. I., Muzammil, S., Rasool, M., Yasmeen, N., Shah, T., Chaudhry, T.H., Rasool, M.H., Shahid, A., Xueshan, X., & Baloch, Z. (2021). Antibiotic resistance: One health one world outlook. Frontiers in Cellular and Infection Microbiology, 11, Article 771510. https://doi.org/10.3389/fcimb.2021.771510

Benzertiha, A., Kierończyk, B., Kołodziejski, P., Pruszyńska-Oszmałek, E., Rawski, M., Józefiak, D., & Józefiak, A. (2020). *Tenebrio molitor* and *Zophobas morio* full-fat meals as functional feed additives affect broiler chickens' growth performance and immune system traits. Poultry Science, 99(1), 196-206. https://doi.org/10.3382/ps/pez450

Borrelli, L., Coretti, L., Dipineto, L., Bovera, F., Menna, F., Chiariotti, L., Nizza, A., Lembo, F., & Fioretti, A. (2017). Insect-based diet, a promising nutritional source, modulates gut microbiota composition and SCFAs production in laying hens. Scientific Reports, 7, Article 16269. https://doi.org/10.1038/s41598-017-16560-6

Buijs, S., Van Poucke, E., Van Dongen, S., Lens, L., Baert, J., & Tuyttens, F. A. M. (2012). The influence of stocking density on broiler chicken bone quality and fluctuating asymmetry. Poultry Science, 91, 1759-1767. https://doi.org/10.3382/ps.2011-01859

Cengiz, O., Koksal, B. H., Tatli, O., Sevim, O., Ahsan, U., Uner, A. G., Ulutas, P.A., Beyaz, D., Buyukyoruk, S., Yakan, A., & Onol, A. G. (2015). Effect of dietary probiotic and high stocking density on the performance, carcass yield, gut microflora and stress indicators of broilers. Poultry Science, 94(10), 2395-2403. https://doi.org/10.3382/ps/pev194

Ceylan, N., Koca, S., Yavas, I., Cenesiz, A., Kahraman, N., & Ozlu, S. (2020). Response of modern broiler chickens to dietary calcium and phosphorus levels below recommendations. Italian Journal of Animal Science, 19(1), 1244-1252. https://doi.org/10.1080/1828051X.2020.1819171

Doube, M., Kłosowski, M. M., Arganda-Carreras, I., Cordelières, F. P., Dougherty, R. P., Jackson, J., Schmid, B., Hutchinson, J.R., & Shefelbine, S. J. (2010). BoneJ: Free and extensible bone image analysis in ImageJ. Bone, 47, 1076-1079. https://doi.org/10.1016/j.bone.2010.08.023

Duncan, D. B. (1955). Multiple range test and multiple F tests. Biometrics, 11, 1-42. https://doi.org/10.2307/3001478

Elahi, U., Xu, C. C., Wang, J., Lin, J., Wu, S. G., Zhang, H. J., & Qi, G. H. (2022). Insect meal as a feed ingredient for poultry. Animal Bioscience, 35(2), 332-346. https://doi.org/10.5713/ab.21.0435

Finke, M. D. (2007). An estimate of chitin in raw whole insects. Zoo Biology, 26(2), 105-115. https://doi.org/10.1002/zoo.20123

Gasco, L., Finke, M. D., & Van Huis, A. (2018). Can diets containing insects promote animal health? Journal of Insects as Food and Feed, 4(1), 1-4. https://doi.org/10.3920/JIFF2018.x001

Gutierrez-Fuentes, C. G., Zuñiga-Orozco, L. A., Vicente, J. L., Hernandez-Velasco, X., Menconi, A., Kuttappan, V. A., Kallapura, G., Latorre, J., Layton, S., Hargis, B.M., & Téllez, G. (2013). Effect of a lactic acid bacteria based probiotic, Floramax-B11® on performance, bone qualities and morphometric analysis of broiler chickens: An economic analysis. International Journal of Poultry Science, 12(6), 322-327. https://doi.org/10.3923/ijps.2013.322.327

Hadj Saadoun, J., Luparelli, A. V., Caligiani, A., Macavei, L. I., Maistrello, L., Neviani, E., Galaverna, G., Sforza, S., & Lazzi, C. (2020). Antimicrobial biomasses from lactic acid fermentation of black soldier fly prepupae and related by-products. Microorganisms, 8(11), Article 1785. https://doi.org/10.3390/microorganisms8111785

Henneicke, H., Gasparini, S. J., Brennan-Speranza, T. C., Zhou, H., & Seibel, M. J. (2014). Glucocorticoids and bone: Local effects and systemic implications. Trends in Endocrinology & Metabolism, 25, 197-211. https://doi.org/10.1016/j.tem.2013.12.006

Hong, J., Han, T., & Kim, Y. Y. (2020). Mealworm (*Tenebrio molitor* larvae) as an alternative protein source for monogastric animal: A review. Animals, 10(11), Article 2068. https://doi.org/10.3390/ani10112068

Hooge, D. M., Sims, M. D., Sefton, A. E., Spring, P., & Connolly, A. (2003). Effect of dietary mannan oligosaccharide with or without bacitracin or virginiamycin, on live performance of broiler chickens at relatively high stocking density on new litter. Journal of Applied Poultry Research, 12(4), 461-467. https://doi.org/10.1093/japr/12.4.461

Imari, Z. K., Hassanabadi, A., & Moghaddam, H. N. (2020). Response of broiler chickens to calcium and phosphorus restriction: Effects on growth performance, carcass traits, tibia characteristics and total tract retention of nutrients. Journal of Animal Science, 19(1), 929-939. https://doi.org/10.1080/1828051X.2020.1808101

Islam, Md. M., & Jang, C. J. (2017). Efficacy of mealworm and super mealworm larvae probiotics as an alternative to antibiotics challenged orally with *Salmonella* and *E. coli* infection in broiler chicks. Poultry Science, 96(1), 27-34. https://doi.org/10.3382/ps/pew220

Jabbar, A., Tahir, M., Naz, S., Alrefaei, A.F., Sultan, A., Abdelrahaman, S., & Selvaggi, M. (2024). Enhancing calcium and phosphorus utilization in broiler chickens through cholecalciferol in sequential calcium-phosphorus-deficient diets. Journal of Applied Animal Research, 52(1), 2337177. https://doi.org/10.1080/09712119.2024.2337177

Javid, M. A., Masood, S., Zaneb, H., Rehman, H. U., Nazir, M. M., Waqas, M. Y., Asif, M., Basit, M.A., Abbas, G., & Sufyan, A. B. (2022). Effect of dietary growth promoter supplementations on the serum biochemistry and morphometric characteristics of tibia bone in broiler chicken. Brazilian Journal of Poultry Science, 24(4), 1-5. https://doi.org/10.1590/1806-9061-2021-1454

Józefiak, A., & Engberg, R. M. (2017). Insect proteins as a potential source of antimicrobial peptides in livestock production: A review. Journal of Animal and Feed Sciences, 26(2), 87-99. https://doi.org/10.22358/jafs/69998/2017

Kang, H. K., Park, S. B., Kim, S. H., & Kim, C. H. (2016). Effects of stock density on the laying performance, blood parameter, corticosterone, litter quality, gas emission and bone mineral density of laying hens in floor pens. Poultry Science, 95, 2764-2770. https://doi.org/10.3382/ps/pew264

Karaarslan, S., & Nazligul, A. (2018). Effects of lighting, stocking density and access to perches on leg health variables as welfare indicators in broiler chickens. Livestock Science, 218, 31-36. https://doi.org/10.1016/j.livsci.2018.10.008

Khalil, F., Ibrahim, R. R., Emeash, H., & Hassan, A. (2021). Probiotic supplementation alleviated stress and improved performance, meat quality sensory acceptability and microbiological status of broilers. Journal of Advanced Veterinary Research, 11(2), 93-101.

Kridtayopas, C., Rakangtong, C., Bunchasak, C., & Loongyai, W. (2019). Effect of prebiotic and synbiotic supplementation in diet on growth performance, small intestinal morphology, stress, and bacterial population under high stocking density condition of broiler chickens. Poultry Science, 98(10), 4595-4605. https://doi.org/10.3382/ps/pez152

Kwon, G. T., Yuk, H. G., Lee, S. J., Chung, Y. H., Jang, H. S., Yoo, J. S., Cho, K.H., Kong, H., & Shin, D. (2020). Mealworm larvae (*Tenebrio molitor* L.) exuviae as a novel prebiotic material for BALB/c mouse gut microbiota. Food Science and Biotechnology, 29(4), 531-537. https://doi.org/10.1007/s10068-019-00699-1

Lee, J. H., Kim, T. K., Cha, Ji. Y., Jang, H. W., Yong, H. I., & Choi, Y. S. (2022). How to develop strategies to use insects as animal feed: Digestibility, functionality, safety and regulation. Journal of Animal Science and Technology, 64(3), 409-431. https://doi.org/10.5187/jast.2022.e27

Leisner, J.L., Vogensen, F.K., Kollmann, J., Aideh, B., Vandamme, P., Vancanneyt, M., & Ingmer, H. (2008). α-chitinase activity among lactic acid bacteria. Systematic and Applied Microbiology, 31(2008), 151-156. https://doi:10.1016/j.syapm.2008.03.003

Leske, K., & Coon, C. (2002). The development of feedstuff retainable phosphorus values for broilers. Poultry Science, 81, 1681-1693. https://doi.org/10.1093/ps/81.11.1681

Li, X. M., Zhang, M. H., Liu, S. M., Feng, J. H., Ma, D. D., Liu, Q. X., Zhou, Y., Wang, X.J., & Xing, S. (2019). Effects of stocking density on growth performance, growth regulatory factors and endocrine hormones in broilers under appropriate environments. Poultry Science, 98(12), 6611-6617. https://doi.org/10.3382/ps/pez505

Libouban, H., Moreau, M. F., Legrand, E., Baslé, M. F., Audran, M., & Cappard, D. (2001). Comparison insight dual x-ray absorptiometry (DxA), histomorphometry, ash weight, and morphometric indices for bone evaluation in an animal model (the orchidectomized rat) of male osteoporosis. Calcified Tissue International, 68, 31-37. https://doi.org/10.1007/BF02685000

Liu, H., Bai, S. P., Zhang, K. Y., Ding, X. M., Wang, J. P., Zeng, Q. F., Peng, H.W., Bai, J., Xuan, Y., & Su, Z. W. (2020). Effects of stocking density on the performance, tibia mineralization and the expression of hypothalamic appetite genes in broiler chickens. Annals of Animal Science, 21(3), 1103-1117. https://doi.org/10.2478/aoas-2020-0110

Luparelli, A. V., Hadj Saadoun, J., Lolli, V., Lazzi, C., Sforza, S., & Caligiani, A. (2022). Dynamic changes in molecular composition of black soldier fly prepupae and derived biomasses with microbial fermentation. Food Chemistry X, 14, Article 100327. https://doi.org/10.1016/j.fochx.2022.100327

Midilli, M., Salman, M., Muglali, O. H., Cenesiz, S., Ormanci, N., Pakdil, M., & Gurcan, I. S. (2015). The effects of different zinc sources and microbial phytase supplementation on the tibial bone properties, strength and Zn mineralization in broilers fed with diet low phosphorus. Kafkas University Veterinary Faculty Journal, 21(4), 607-614. https://doi.org/10.9775/kvfd.2015.13001

Mohammed, A. A., Zaki, R. S., Negm, E. A., Mahmoud, M. A., & Cheng, H. W. (2021). Effects of dietary supplementation of a probiotic (*Bacillus subtilis*) on bone mass and meat quality of broiler chickens. Poultry Science, 100, 1-11. https://doi.org/10.1016/j.psj.2020.11.073

Mulyono, M., Yunianto, V. D., Suthama, N., & Sunarti, D. (2019). The effect of fermentation time and Trichoderma levels on digestibility and chemical components of black soldier fly (*Hermetia illucens*) larvae. Livestock Research for Rural Development, 31(10), 1-4.

Ortiz, L. T., Rodríguez, M. L., Alzueta, C., Rebolé, A., & Treviño, J. (2009). Effect of inulin on growth performance, intestinal tract sizes, mineral retention and tibial bone mineralisation in broiler chickens. British Poultry Science, 50, 325-332. https://doi.org/10.1080/00071660902806962

Peng, W., Talpur, M. Z., Zeng, Y., Xie, P., Li, J., Wang, S., Wang, L., Zhu, X., Gao, P., Jiang, Q., Shu, G., & Zhang, H. (2022). Influence of fermented feed additive on gut morphology, immune status and microbiota in broilers. BMC Veterinary Research, 18(218), 1-16. https://doi.org/10.1186/s12917-022-03322-4

Sahraei, M., Abarghani, A., Ghanbari, A., & Karami, R. (2019). The effects of dietary synbiotic supplementation on tibia bone morphology and production efficiency in broiler chickens. In Proceedings of the 2nd International and 6th National Iranian Congress on Organic vs. Conventional Agriculture, August 2019, Ardabil, Iran (pp. 1-7).

Scholz-Ahrens, K. E., Ade, P., Marten, B., Weber, P., Timm, W., Acil, Y., Gluer, C.C., & Shrezenmeir, J. (2007). Prebiotics, probiotics and synbiotics affect mineral absorption, bone mineral content and bone structure. The Journal of Nutrition, 137, 838S-846S. https://doi.org/10.1093/jn/137.3.838S

Sedgh-Gooya, S., Torki, M., Darbernami, M., Khamisabadi, H., & Abdolmohamadi, A. (2022). Growth performance and intestinal morphometric features of broiler chickens fed on dietary inclusion of yellow mealworm (*Tenebrio molitor*) larvae powder. Veterinary Medicine and Science, 8(5), 2050-2058. https://doi.org/10.1002/vms3.881

Senol, M., Nadaroglu, H., Dikbas, N., & Kotan, R. (2014). Purification of chitinase enzymes from *Bacillus subtilis* bacteria TV-125, investigation of kinetic properties and antifungal activity against *Fusarium culmorum*. Annals of Clinical Microbiology and Antimicrobials, 13(35), 1-7. https://doi.org/10.1186/s12941-014-0035-3

Son, Y. J., Hwang, I. K., Nho, C. W., Kim, S. M., & Kim, S. H. (2021). Determination of carbohydrate composition in mealworm (*Tenebrio molitor* L.) larvae and characterization of mealworm chitin and chitosan. Foods, 10(640), 1-15. https://doi.org/10.3390/foods10030640

SPSSWIN. (2007). SPSS for Windows 6.1.4. Istanbul, Turkey.

Steczny, K., & Kokoszyński, D. (2020). Effect of probiotic preparations (EM) and sex on morphometric characteristics of the digestive system and leg bones and caecal microflora in broiler chickens. Journal of Applied Animal Science, 48(1), 45-50. https://doi.org/10.1080/09712119.2020.1718680

Sugiharto, S. (2022). Dietary strategies to alleviate high-stocking-density-induced stress in broiler chickens-a comprehensive review. Archives Animal Breeding, 65(1), 21-36. https://doi.org/10.5194/aab-65-21-2022

Sun, Z. W., Fan, Q. H., Wang, X. X., Guo, Y. M., Yang, H. J., & Dong, X. (2018). High stocking density alters bone-related calcium and phosphorus metabolism by changing intestinal absorption in broiler chickens. Poultry Science, 97, 219-226. https://doi.org/10.3382/ps/pex294

Tuncer, H.I. (2007). To banned usage of hormones, antibiotics, anticoccidials and drugs in compound animal feed (A Review). Journal of Lalahan Animal Research Institute, 47(1), 29-37.

Vahdatpour, T., Ebrahimnezhad, Y., & Vahdatpour, S. (2014). Effects of dietary functional additives on characteristics and minerals of tibia bone and blood parameters of Japanese quails (*Coturnix coturnix japonica*). International Journal of Plant Animal and Environmental Sciences, 4, 690-695.

Vargas-Galicia, A. J., Sosa-Montes, E., Rodríguez-Ortega, L. T., Pro-Martinez, A., Ruiz-Feria, C. A., González-Cerón, F., Gallegos-Sánchez, J., Arreola-Enríquez, J., & Bautista-Ortega, J. (2017). Effect of litter material and stocking density on bone and tendon strength and productive performance in broilers. Canadian Journal of Animal Science, 97, 673-682. https://doi.org/10.1139/cjas-2016-0246

Wang, J., Qiu, L., Gong, H., Celi, P., Yan, L., Ding, X., Bai, S., Zeng, Q., Mao, X., Xu, S., Wu, C., & Zhang, K. (2020). Effect of dietary 25-hydroxycholecalciferol supplementation and high stocking density on performance, egg quality and tibia quality in laying hens. Poultry Science, 99, 2608-2615. https://doi.org/10.1016/j.psj.2019.12.054

Yan, F. (2016). The effects of dietary probiotic inclusion on skeletal health of poultry and its possible mechanisms. Open Access Dissertations, 1033.

Yan, F. F., Mohammed, A. A., Murugesan, G. R., & Cheng, H. W. (2019). Effects of a dietary synbiotic inclusion on bone health in broilers subjected to cyclic heat stress episodes. Poultry Science, 98, 1083-1089. https://doi.org/10.3382/ps/pey508

Xue, G., Cheng, S., Yin, J., Zhang, R., Su, Y., Li, X., Li, J., & Bao, J. (2021). Influence of pre-slaughter fasting time on weight loss, meat quality and carcass contamination in broilers. Animal Bioscience, 34(6), 1070-1077. https://doi.org/10.5713/ajas.20.0560

Zhang, Y. R., Zhang, L. S., Wang, Z., Liu, Y., Li, F. H., Yuan, J. M., & Xia, Z. F. (2018). Effects of stocking density on growth performance, meat quality and tibia development of Pekin ducks. Animal Science Journal, 89, 925-930. https://doi.org/10.1111/asj.12997

Zhang, H., Majdeddin, M., Gaublomme, D., Taminiau, B., Boone, M., Elewaut, D., Daube, G., Josipovic, I., Zhang, K., & Michiels, J. (2021). 25-hydroxycholecalciferol reverses heat induced alterations in bone quality in finisher broilers associated with effects on intestinal integrity and inflammation. Journal of Animal Science and Biotechnology, 12(1), 104. https://doi.org/10.1186/s40104-021-00627-6

Ziaie, H., Bashtani, M., Karimi Torshizi, M. A., Naeeimipour, H., Frhangfar, S. H., & Zeinali, A. (2011). Effect of antibiotic and its alternatives on morphometric characteristics, mineral content and bone strength of tibia in Ross broiler chickens. Global Veterinaria, 7(4), 315-322.