

Effect of selenium nanoparticles and mannan oligosaccharide supplementation on muscle and lymphoid histomorphometry and morphometry of tibia bone in broilers reared under high stocking density

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Abstract: The current study investigated the potential of selenium-nanoparticles (SeNPs) and mannan-oligosaccharide (MOS) supplementation in ameliorating the high stocking density (HSD) stress on breast muscle, immune organs, and tibia bone in broilers. Three hundred and ninety two (392) day-old Ross chicks were divided in seven groups with eight replicates each ($n = 7$): as NSD [basal diet (BD) + normal stocking density: 10 chicks/m²], HSD (BD + high stocking density: 16 chicks/m²), Se-HSD [BD + Selenium (Se) 0.15 mg/kg of BD], MOS-HSD (BD + MOS 05 g/kg of BD), Se-MOS-HSD (BD + Se 0.15 mg/kg of BD + MOS 05 g/kg of BD), SeNP-HSD (BD + SeNP 0.15 mg/kg of BD), and SeNP-MOS-HSD (BD + SeNP 0.15 mg/kg of BD + MOS 05 g/kg of BD). HSD decreased ($p < 0.05$) breast muscle fibre diameter, density and area, pH at 0 h, pH at 24 h postslaughter, and water holding capacity (WHC), area of lymphoid tissue in caecal tonsil and bursa of Fabricius, tibia bone morphometric parameters, and Se and Copper (Cu) concentrations in bone, muscle, and serum in the HSD group compared to the NSD group. Breast muscle fibre diameter and density increased ($p < 0.05$) in the Se-MOS-HSD and SeNP-MOS-HSD groups and WHC only increased ($p < 0.05$) in the SeNP-MOS-HSD group. The area of lymphoid tissue in caecal tonsil and bursa of Fabricius increased ($p < 0.05$) in the SeNP-MOS-HSD group. The tibia bone weight, medullar canal diameter, tibio-tarsal index, weight/length index, and ash% improved ($p < 0.05$) in the SeNP-HSD and SeNP-MOS-HSD groups. Se and Cu concentration improved ($p < 0.05$) in bone, muscle, and serum of the Se-MOS-HSD, SeNP-HSD, and SeNP-MOS-HSD groups. We concluded that supplementation of SeNPs-MOS proved to be a more superior combination in mitigating HSD stress on breast muscle, lymphoid organs, and tibia bone.

Key words: Crowding stress, muscle fibre diameter, mannan oligosaccharides, tibia bone, lymph nodules, nanoselenium

1. Introduction

With the advancement of technology in poultry houses and the desire of the farming community for higher economic returns with higher meat production per unit area, broilers are raised at higher stocking density [1]. Stocking density is a complex phenomenon and can be stressful depending on various factors such as climatic conditions, floor or cage rearing, size, and physiological stage of broilers. Higher stocking density (HSD) is associated with depletion of lymphoid tissue in lymphoid organs [2], decreased water holding capacity (WHC) of breast muscle [3], and deteriorated bone health [4] in broilers. In tropical countries like Pakistan, the climatic condition is becoming harsher due to global warming and can influence broiler farming by augmenting crowding stress. To balance out economic benefit and stress at the

broiler level, stocking density should be managed through nutritional supplements such as prebiotics, probiotics, and essential minerals.

Selenium (Se) is an important micromineral and a natural antioxidant for the body supplied through dietary sources. Dietary selenium, when absorbed, is utilized for the synthesis of a group of selenoproteins in the body. Stress and Se availability are two factors regulating the synthesis and expression of selenoproteins in different body tissues [5]. Selenoprotein W plays an integral role in the development of immuno-competence of the bursa of Fabricius [6]. Similarly, Selenoprotein N and W protect breast muscle from drip loss and oxidative stress [7], and Selenoprotein DIO₂ is associated with tibia bone health [8]. Se potency to effectively perform an antioxidant role through these selenoproteins is dependent on the form,

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organic, inorganic, or nanoform, of Se being supplemented in the diet. Due to higher bioavailability and surface activity and lower toxicity, nanoselenium is preferred over the organic and inorganic forms of Se. Nanoselenium has been supplemented in broilers and reported to reduce thermal and stocking density stresses [9, 10]. The research community is now looking for a superior combination of nanoparticles with pre- or probiotics to control the stressors in poultry farming. Mannan oligosaccharide (MOS) is a prebiotic and its supplementation is associated with improved gut health, beneficial microbial count, and nutrient absorption [11]. Although MOS has no nutritional value, its role in gut health and mineral absorption made it a very suitable combination with Selenium nanoparticles as a feed supplement. The potential of Se nanoparticles (SeNP) and MOS combination as a broiler stress reliever and performance enhancer is still unexplored. Owing to stress-relieving GIT localized and systemic effects of MOS and SeNP respectively, we hypothesized that a combination of SeNP and MOS will be a more robust strategy in managing the harmful effects of HSD on breast muscle, immune organs, and tibia bone of the broilers. Therefore, the objective of this study was to investigate the effects of the SeNP-MOS combination on meat quality, morphometric characteristics of the tibia bone, and lymphoid organs, and tissue mineral concentration in broilers raised under high stocking density.

2. Materials and methods

2.1. Synthesis of SeNP

The SeNP were synthesized according to the method described by Zhang et al. [12] with minor modifications. The chemical used in the process includes selenium dioxide, ascorbic acid (Sigma-Aldrich), and SODIUM HYDROXIDE (BDH). Briefly, Selenious acid solution (0.25 M) was prepared by dissolving selenium dioxide (Sigma-Aldrich) in deionized water. Afterward, Ascorbic acid (0.05 M) (Sigma-Aldrich) was added dropwise to the selenious acid solution under constant mechanical stirring to initiate the reaction. Sodium hydroxide solution (1 M) was added to the solution to increase its pH. The formation of SeNP was indicated by the change in colour from colourless to bright red. The solution was centrifuged to separate the SeNP which were washed with deionized water. The SeNPs were dried and stored in a clean vial for further use.

2.2. Experimental design

Three hundred and ninety two (392) day-old chicks (Ross-308) were weighed and assigned to seven groups (eight replicates/group and seven chicks/replicate) and allocated to stocking density of 10 chicks/m² (normal stocking density, NSD) or 16 chicks/m² (high stocking density HSD) in a completely randomized design (CRD). The chicks were reared on rice husk bedding in an environmentally

controlled experimental broiler house in University of Veterinary and Animal Sciences, Pattoki, Pakistan. Before chicks' arrival, the entire premises of broiler shed, drinkers, and utensils were thoroughly cleaned and experimental house was disinfected through fumigation. During the 1st week of the experiment, the temperature was maintained at 35 ± 1 °C and was gradually lowered to 26 ± 1 °C by the end of the 21st day. From 21st day onwards, till the end of trial, i.e. 42 days, it was maintained at 26 °C. The relative humidity was maintained at 65 ± 5% throughout the trial. The chicks were vaccinated as per guidelines given by poultry research institute, Punjab, Pakistan against infectious bronchitis (IB) and infectious bursal disease (IBD) on day 1. For Newcastle disease (ND), vaccination was done on day 5 and booster was given on day 15.

The broilers were fed ad libitum through a three-phase feeding regime (Table 1) according to the formulation recommended by the NRC (1994). The NSD and HSD groups were fed with a corn soya-based basal diet (BD). Additionally, five (05) HSD groups were supplemented with 0.15 mg selenium/kg BD from sodium selenite (Se-HSD group), 05 g MOS/kg BD (MOS-HSD group), 0.15 mg selenium from sodium selenite and 05 g MOS/kg BD (Se-MOS-HSD group), 0.15 mg SeNP/kg BD (SeNP-HSD group), 0.15 mg SeNP and 05 g MOS /kg of BD (SeNP-MOS-HSD group). The MOS and sodium selenite were purchased from local vendors. On day 42, two broilers from each replicate were slaughtered for sampling (16 broilers per group). After cervical dislocation, the integument and internal viscera of the birds were manually removed. Breast muscle was used for measuring pH and WHC and sampled for histomorphometric analysis. Similarly, caecal tonsil and bursa of Fabricius were collected for lymphoid tissue histomorphometry and right tibia was collected as drumstick for bone morphometry. This study was approved by the Ethical Review Committee of the University of Veterinary and Animal Sciences, Lahore, Pakistan, DR/1078, dated 11-10-2017.

2.3. pH and WHC of breast muscle

The breast muscle from left side of two broilers per replicate (16 broilers per group) was collected. The pH of left side breast muscle was determined by pH meter (WTW-3110) having conventional meat probe electrode, which was inserted at least 1 cm deep into muscle. The pH was measured at the time of slaughtering (pH-0h). The muscle samples were placed in refrigerator at 4 °C for 24 h. The 2nd reading of pH was taken at 24 h postslaughtering (pH-24h). Additionally, 35–40 g of muscle tissue was sampled from left pectoral muscle and wrapped in labelled inflated plastic bags. This sample was used to calculate WHC using Honikel's gravimetric method [13]. The formula used for drip loss% for WHC calculation is: Drip loss% = $(W1 - W2/W1) \times 100$.

Table 1. Ingredients and nutritional value of basal diet (BD).

Ingredient%	Starter (1–14 days)	Grower (14–35 days)	Finisher (35–42 days)
Corn	57.63	58.382	61.8
Canola meal	12	12	8.5
Rape seed meal	3	3	3
Soybean meal Hi Pro	21	19.5	17.5
Poultry BP meal	2.55	3	5
Fish meal	2.2	2.5	2.5
Limestone	0.62	0.65	0.7
Mineral premix ¹	0.05	0.05	0.05
Vitamin premix ²	0.25	0.25	0.25
Sodium chloride	0.41	0.41	0.41
L-lysine HCl	0.16	0.13	0.16
DL-methionine	0.15	0.15	0.15
L-tryptophan	0.03	0.03	0.03
Calculated composition			
CP (%)	21.90	20	19.10
ME (kcal/kg)	3.05	3.20	3.20
Calcium (%)	0.95	0.95	0.88
Total phosphorus (%)	0.70	0.62	0.61
Available phosphorus (%)	0.45	0.41	0.38
Crude fibre (%)	2.63	2.52	2.48
Lysine digest (%)	1.34	1.15	1.07
Methionine digest (%)	0.59	0.54	0.54

¹Mineral premix (each kg contained): Se, 0.15 mg; Cu, 11.3 mg; Na, 0.18 g; Zn, 84 mg; Mn, 87 mg; Fe, 111.2 mg; K, 0.85 g; I, 0.1 mg; Mg, 18 g.

²Vitamin premix (each kg contained): vitamin B12 6.6 mg; vitamin E 18,371 IU; vitamin D3 1,543,220 IU; vitamin A 4,409,200 IU; niacin 18,371 mg; folic acid 699 mg; choline 191,066 mg; d-Biotin 220 mg; menadione 589 mg; riboflavin 2338 mg; pyridoxine 2866 mg; thiamine 1175 mg; d-pantothenic acid 8084 mg.

2.4 Breast muscle histomorphometry

The breast muscle from right side of two broilers per replicate (16 broilers per group) was collected and was used for histomorphometric analysis. A representative piece was cut perpendicular to muscle fibres long axis and was preserved in 10% neutral buffered formalin solution. Muscle sections were processed through paraffin embedding technique followed by haematoxylin and eosin staining technique for light microscopy [14]. For determination of muscle fibre diameter (μm), pictures of histological sections were captured through a camera (DP- 21, Olympus) at 10 \times objective lens under bright field microscope (Olympus BX-53). Muscle fibre diameter was calculated using a histomorphometry program as shown in Figure 1a (Progress capture Pro 2.7.7. Labomed USA).

Fibre diameter (μm) of eight muscle fibres from three different areas of the section was measured and presented as average muscle fibre diameter. Cross-sectional area of muscle fibre was measured from diameter of muscle fibre. For muscle fibre density, five areas on the section were captured by a camera (Meiji Techno HD- 1500T) attached to microscope (Olympus CX- 23) at 4 \times objective lens magnification. Muscle fibre density was calculated by a histomorphometry program (Progress capture Pro 2.7.7. Labomed USA) as shown in Figure 1b and presented as muscle fibre density/ mm^2 [15]. To calculate muscle fibre area/ mm^2 of captured section, values of muscle fibre area were first converted into mm^2 and then multiplied with the value of muscle fibre density and reported as area occupied by muscle fibres per 1 mm^2 of captured section.

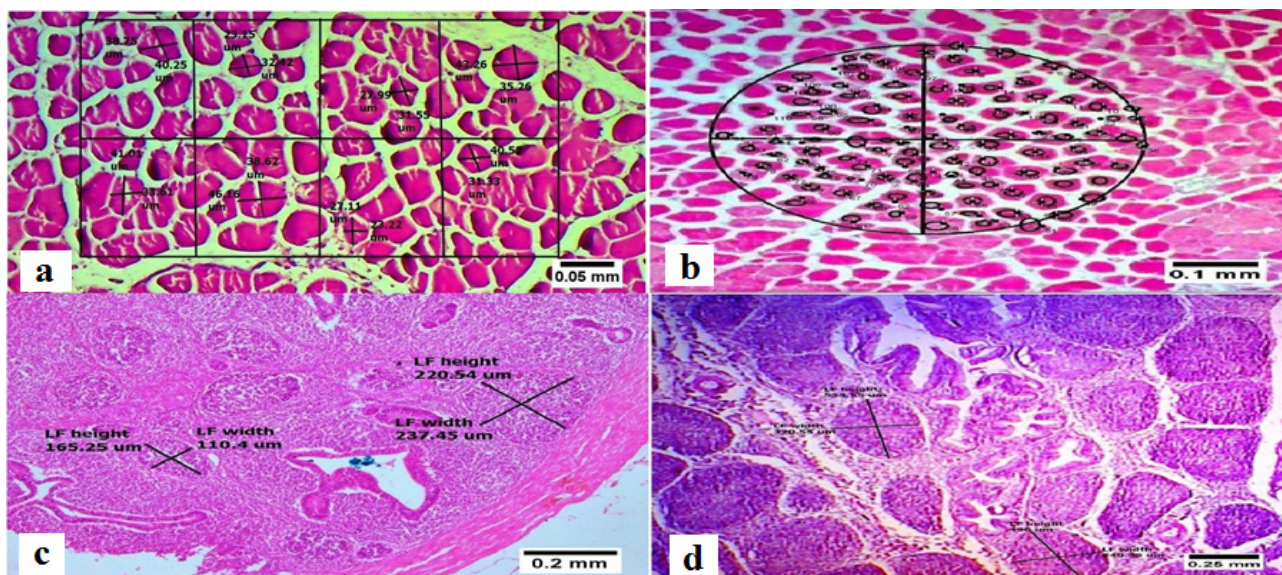


Figure 1. A combination of micrographs showing histomorphometric analysis. **a:** micrograph of breast muscle, showing method used for measuring muscle fibre diameter (10 \times , H&E). **b:** micrograph of breast muscle, showing counting method used for muscle fibre density 4 \times , H&E). **c:** micrograph of caecal tonsil, showing lymphoid follicles (LF) and lines indicating their height and width (10 \times , H&E). **d:** micrograph of bursa of Fabricius, showing lymphoid nodules (LN) and lines indicating their height and width (4 \times , H&E).

To calculate connective tissue space/mm² of the captured section, the value of muscle fibre area/mm² of captured section was subtracted from 1 and presented as area occupied by connective tissue component per 1 mm² of captured section.

2.5. Immune organ histomorphometry

The samples of bursa of Fabricius and caecal tonsils were collected from two broilers per replicate (16 broilers per group) and preserved in 10% neutral buffered formalin solution. Subsequently, samples of both organs were processed through paraffin embedding technique followed by haematoxylin and eosin staining technique [14] for light microscopy. In caecal tonsil, the height (NH) and width (NW) of lymphatic nodules were measured in three microscopic fields of each section captured through the camera (Meiji Techno HD-1500T) attached to the microscope (Olympus CX-23) at 10 \times objective lens (Figure 1c). The nodular area was measured using the formula = (NH) \times (NL). In bursa of Fabricius, the lymph follicles height (FH), width (FW), and area was measured in three microscopic fields of each section captured through the camera (Meiji Techno HD1500T) attached to the microscope (Olympus CX-23) at 4 \times objective lens (Figure 1d). Area of the nodule was measured using formula = (FH) \times (FL).

2.6. Bone morphometric and geometric parameters

The right tibia was separated as drumstick with intact flesh from two broilers per replicate (16 broilers per group). Each bone was labelled and immersed in boiling

water (100 $^{\circ}$ C) for 10 min. Thereafter, the tibiae were cooled at room temperature, defleshed by hand, and patellae were removed. Bones were air dried for 24 h at room temperature. The tibia weight, length, bone outer diameter, and medullary canal diameter were measured based on methods described by Mutuş et al. [16]. The tibio-tarsal index and robusticity index were calculated as described by Kocabagli [17]. The bone weight/length index was also measured with the method described by Mutuş et al. [16]. For bone ash determination, bone fragments were dried in a hot air oven at 105 $^{\circ}$ C for 24 h, burnt in a muffle furnace at 600 $^{\circ}$ C for 6 h. The percentage bone ash was measured relative to dry tibia weight [16].

2.7. Mineral analysis

One gram of dried bone, 1 g of left breast muscle, and 1 mL of serum were acid digested according to the method described by Saeed et al. [18]. The final residue of each sample was diluted with deionized water to make a final volume of 50 mL. Se and Cu concentrations in each sample of the aforementioned tissues were measured using atomic absorption spectrophotometer (Model AA 6501, Shimadzu Ltd. Japan).

2.8. Statistical analyses

The data were analysed using one-way analysis of variance (ANOVA) using IBM SPSS Statistics 20.0 and presented as mean along with pooled standard error of the mean. Mean separation among groups was done with Tukey's test (SPSS v. 20.0) (19). The significance level was set at $p < 0.05$.

3. Results

The effect of SeNP-MOS supplementation on breast muscle pH at 0 h and 24 h postslaughter and WHC of broilers reared under high stocking density is presented in Table 2. The breast muscle pH at 0 h, pH at 24 h postslaughter, and WHC were lower ($p < 0.05$) in the HSD group compared to the NSD group. Supplementation of SeNP-MOS combination increased ($p < 0.05$) WHC in HSD broilers in SeNP-MOS-HSD group compared to HSD group and this increase in WHC was comparable to NSD group. The pH at both 0 h and 24 h postslaughter did not improve ($p > 0.05$) in any of the supplemented HSD broilers.

The effect of SeNP-MOS supplementation on breast muscle histomorphometry of broilers reared under high stocking density is presented in Table 3. The muscle fibre diameter, area, and muscle fibre density were decreased ($p < 0.05$) in the HSD group compared to the NSD group. The supplementation of both Se-MOS and SeNP-MOS combinations increased ($p < 0.05$) muscle fibre diameter and muscle fibre area in the Se-MOS-HSD and SeNP-MOS-HSD groups and this increase was comparable with the NSD group. The muscle fibre density did not increase

($p > 0.05$) in any of the supplemented HSD broilers. The muscle fibre area/mm² of the captured section and connective tissue spaces area/mm² did not vary ($p > 0.05$) significantly among all groups.

The effect of SeNP-MOS supplementation on caecal tonsil and bursa of Fabricius histomorphometry of broilers reared under high stocking density is presented in Table 4. The caecal tonsil lymphoid nodule area and bursa of Fabricius lymphoid follicular area were lower ($p < 0.05$) in the HSD group compared to the NSD group. The lymphoid follicular area only in bursa of Fabricius increased ($p < 0.05$) in the Se-MOS-HSD and SeNP-MOS-HSD groups compared to the HSD group and this increase was comparable to the NSD group.

The effect of SeNP-MOS supplementation on tibia bone morphometric and geometric parameters of broilers reared under high stocking density is presented in Table 5. All bone morphometric and geometric parameters, except bone length, was lower ($p < 0.05$) in the HSD group compared to the NSD group. The supplementation of SeNP and SeNP-MOS improved ($p < 0.05$) tibia bone weight, medullar canal diameter, tibio-tarsal index, and

Table 2. Effect of selenium nanoparticles-MOS on histomorphometric characteristics of breast muscle in 42-day-old broiler chickens reared under higher stocking density.

Parameters	Control (NSD)	Control (HSD)	Se- 0.15 + HSD	MOS + HSD	Se- 0.15 – MOS + HSD	SeNP + HSD	SeNP -MOS + HSD	p-value
MF-diameter (µm)	51.86 ± 1.16 ^a	47.57 ± 1.16 ^c	48.75 ± 0.56 ^{bc}	47.87 ± 0.60 ^c	50.24 ± 0.59 ^{ab}	49.04 ± 0.49 ^{bc}	51.47 ± 0.52 ^a	<0.001
MF-area (µm ²)	2127.83 ± 94.38 ^a	1792.83 ± 84.98 ^c	1869.99 ± 42.25 ^{bc}	1804.10 ± 4.71 ^{bc}	1986.33 ± 46.84 ^{ab}	1891.47 ± 37.94 ^{bc}	2084.14 ± 43.07 ^a	<0.001
MF-density (Number/mm ²)	384.69 ± 16.86 ^b	434.31 ± 10.97 ^a	432.88 ± 4.31 ^a	433.13 ± 4.60 ^a	422.13 ± 9.95 ^a	428.06 ± 5.28 ^a	412.38 ± 5.34 ^a	0.001
Area of MF/mm ²	0.80 ± 0.021	0.77 ± 0.034	0.81 ± 0.021	0.78 ± 0.019	0.84 ± 0.026	0.81 ± 0.018	0.86 ± 0.011	0.082
Area of CT/mm ²	0.20 ± 0.021	0.23 ± 0.034	0.19 ± 0.021	0.22 ± 0.019	0.16 ± 0.026	0.19 ± 0.018	0.14 ± 0.011	0.082

^{a-d} within the same row, means with different superscripts are significantly different ($p < 0.05$).

Values represent means ± standard error mean of eight replicates (16 broilers/per group).

NSD: Normal stocking density (10 birds/m²), HSD: High stocking density (16 birds/m²), Se: Selenium, SeNP: Selenium nanoparticles, MOS: Mannan oligosaccharide, MF: Muscle fibre, CT: Connective tissue.

Table 3. Effect of selenium nanoparticles-MOS on breast muscle pH and water holding capacity in 42-day-old broiler chickens reared under higher stocking density.

Parameters	Control (NSD)	Control (HSD)	Se- 0.15 + HSD	MOS + HSD	Se- 0.15 – MOS + HSD	SeNP + HSD	SeNP – MOS + HSD	p-value
pH-0h	6.55 ± 0.018 ^a	6.44 ± 0.015 ^b	6.45 ± 0.029 ^{ab}	6.43 ± 0.022 ^b	6.47 ± 0.032 ^{ab}	6.47 ± 0.019 ^{ab}	6.48 ± 0.027 ^{ab}	0.040
pH-24h	6.07 ± 0.037 ^a	5.88 ± 0.032 ^b	5.98 ± 0.043 ^{ab}	5.95 ± 0.041 ^{ab}	5.96 ± 0.039 ^{ab}	6.00 ± 0.029 ^{ab}	5.97 ± 0.042 ^{ab}	0.034
WHC (drip loss %)	1.58 ± 0.11 ^b	2.18 ± 0.13 ^a	1.91 ± 0.08 ^{ab}	1.93 ± 0.12 ^{ab}	1.80 ± 0.10 ^{ab}	1.79 ± 0.07 ^{ab}	1.67 ± 0.07 ^b	0.001

^{a-b} within the same row, means with different superscripts are significantly different ($p < 0.05$)

Values represent means ± standard error mean of eight replicates (16 broilers/per group)

NSD: Normal stocking density (10 birds/m²), HSD: High stocking density (16 birds/m²), Se: Selenium, SeNP: Selenium nanoparticles, MOS: Mannan oligosaccharide, WHC: Water holding capacity.

Table 4. Effect of selenium nanoparticles-MOS on histomorphometric parameters of caecal tonsil and bursa of Fabricius in 42-day-old broiler chickens reared under higher stocking density.

Parameters	Control (NSD)	Control (HSD)	Se- 0.15 + HSD	MOS + HSD	Se- 0.15 - MOS + HSD	SeNP + HSD	SeNP - MOS + HSD	p-value
Caecal Tonsil								
Lymphoid nodule height (µm)	189.31 ± 7.60	159.25 ± 8.10	163.44 ± 6.68	166.25 ± 10.56	181.56 ± 7.42	172.69 ± 8.18	181.56 ± 7.78	0.06
Lymphoid nodule width (µm)	209.63 ± 8.88	181.06 ± 10.08	183.63 ± 7.68	195.13 ± 8.16	201.25 ± 10.39	186.13 ± 7.34	200.63 ± 9.27	0.26
Lymphoid nodule area (mm ²)	0.041 ± 0.002 ^a	0.029 ± 0.002 ^b	0.029 ± 0.001 ^b	0.032 ± 0.002 ^b	0.037 ± 0.002 ^{ab}	0.032 ± 0.001 ^b	0.036 ± 0.002 ^{ab}	<0.001
Bursa of Fabricius								
Lymphoid follicle height(µm)	300.94 ± 11.69	240.56 ± 14.51	271.56 ± 14.22	262.06 ± 15.12	269.13 ± 14.20	262.00 ± 17.87	288.44 ± 16.10	0.17
Lymphoid follicle width (µm)	574.06 ± 31.10	481.56 ± 36.07	516.81 ± 31.56	524.44 ± 38.56	592.25 ± 31.01	547.75 ± 30.67	593.56 ± 20.19	0.17
Lymphoid follicle area (mm ²)	0.17 ± 0.011 ^a	0.11 ± 0.012 ^b	0.14 ± 0.010 ^{ab}	0.14 ± 0.015 ^{ab}	0.16 ± 0.010 ^a	0.14 ± 0.010 ^{ab}	0.17 ± 0.013 ^a	0.01

^{a-b} within the same row, means with different superscripts are significantly different (p < 0.05).

Values represent means ± standard error mean of eight replicates (16 broilers/per group).

NSD: Normal stocking density (10 birds/m²), HSD: High stocking density (16 birds/m²), Se: Selenium, SeNP: Selenium nanoparticles, MOS: Mannan oligosaccharide.

Table 5. Effect of selenium nanoparticles-MOS on morphometric characteristics of tibia bone in 42-day-old broiler chickens reared under higher stocking density.

Parameters	Control (NSD)	Control (HSD)	Se- 0.15 + HSD	MOS + HSD	Se- 0.15 - MOS + HSD	SeNP + HSD	SeNP- MOS + HSD	p-value
Weight (g)	4.36 ± 0.07 ^a	3.75 ± 0.07 ^c	4.00 ± 0.08 ^{bc}	3.89 ± 0.06 ^{bc}	4.17 ± 0.06 ^{ab}	4.09 ± 0.07 ^{ab}	4.14 ± 0.06 ^{ab}	<0.001
Length (mm)	84.75 ± 0.28	83.74 ± 0.23	83.98 ± 0.23	83.95 ± 0.22	83.98 ± 0.24	84.43 ± 0.24	84.50 ± 0.31	0.077
Diaphysial Diameter (mm)	8.73 ± 0.112 ^a	7.58 ± 0.070 ^b	7.61 ± 0.070 ^b	7.47 ± 0.071 ^b	7.69 ± 0.072 ^b	7.69 ± 0.094 ^b	7.72 ± 0.080 ^b	<0.001
MC Diameter (mm)	4.67 ± 0.027 ^b	5.06 ± 0.045 ^a	4.80 ± 0.061 ^b	4.85 ± 0.062 ^{ab}	4.79 ± 0.041 ^b	4.80 ± 0.047 ^b	4.67 ± 0.035 ^b	<0.001
Tibio-tarsal index	46.39 ± 0.76 ^a	33.23 ± 0.70 ^d	36.96 ± 0.43 ^{bc}	34.93 ± 1.20 ^{cd}	37.72 ± 0.75 ^{bc}	37.46 ± 0.99 ^{bc}	39.38 ± 0.66 ^b	<0.001
Robusticity index	5.19 ± 0.024 ^c	5.40 ± 0.041 ^a	5.30 ± 0.036 ^{abc}	5.34 ± 0.030 ^{ab}	5.22 ± 0.034 ^{bc}	5.29 ± 0.034 ^{abc}	5.26 ± 0.021 ^{abc}	<0.001
Weight/length index	51.41 ± 0.77 ^a	44.75 ± 0.92 ^c	47.57 ± 0.95 ^{bc}	46.38 ± 0.77 ^{bc}	49.69 ± 0.83 ^{ab}	48.45 ± 0.88 ^{ab}	49.03 ± 0.67 ^{ab}	<0.001
Ash%	45.51 ± 0.10 ^a	44.57 ± 0.09 ^c	44.81 ± 0.08 ^c	44.58 ± 0.09 ^c	44.95 ± 0.10 ^{bc}	45.22 ± 0.08 ^{ab}	45.30 ± 0.09 ^{ab}	<0.001

^{a-d} within the same row, means with different superscripts are significantly different (p < 0.05).

Values represent means ± standard error mean of eight replicates (16 broilers/per group).

NSD: Normal stocking density (10 birds/m²), HSD: High stocking density (16 birds/m²), Se: Selenium, SeNP: Selenium nanoparticles, MOS: Mannan oligosaccharide, MC: Medullar canal.

weight/length index, and ash% in the SeNP-HSD and SeNP-MOS-HSD groups compared to the HSD group, and this improvement was comparable to the NSD group.

The effect of SeNP-MOS supplementation on Se and Cu level in breast muscle, serum, and tibia bone of broilers reared under high stocking density is presented in Table 6. Se and Cu concentrations were low (p < 0.05) in all three tissues (breast muscle, serum, and tibia bone) of the HSD group compared to the NSD group. Se concentration improved (p < 0.05) in all three tissues of the Se-MOS-HSD, SeNP-HSD, and SeNP-MOS-HSD groups compared to the HSD group. Cu concentration improved (p < 0.05) in

the breast muscle and tibia bone of the Se-MOS-HSD and SeNP-OS-HSD groups compared to the HSD group. This aforementioned improvement in Se and Cu concentrations was comparable to the NSD group.

4. Discussion

Demand for broiler breast meat has increased over time; therefore, it is considered the main muscle in the production of boneless meat products [20]. The yield of breast muscle is directly related to hypertrophy of muscle fibres evident through an enlarged diameter of individual muscle cells [21] due to higher protein deposition. Stressors

Table 6. Effect of selenium nanoparticles-MOS on mineral (Se & Cu) concentration in muscle, bone, and serum in 42-day-old broiler chickens reared under higher stocking density.

Parameters	Control (LSD)	Control (HSD)	Se- 0.15 + HSD	MOS + HSD	Se- 0.15 – MOS + HSD	SeNP + HSD	SeNP-MOS + HSD	p-value
Breast muscle								
Se (mg/kg)	0.51 ± 0.019 ^a	0.39 ± 0.013 ^c	0.43 ± 0.017 ^{bc}	0.42 ± 0.010 ^{bc}	0.47 ± 0.010 ^{ab}	0.47 ± 0.016 ^{ab}	0.48 ± 0.013 ^{ab}	<0.001
Cu (mg/kg)	0.698 ± 0.033 ^a	0.556 ± 0.017 ^c	0.576 ± 0.017 ^c	0.621 ± 0.012 ^{abc}	0.665 ± 0.013 ^{ab}	0.606 ± 0.013 ^{bc}	0.682 ± 0.013 ^{ab}	<0.001
Tibia bone								
Se (mg/kg)	0.587 ± 0.041 ^a	0.352 ± 0.020 ^c	0.405 ± 0.026 ^{bc}	0.403 ± 0.016 ^{bc}	0.520 ± 0.028 ^{ab}	0.517 ± 0.026 ^{ab}	0.514 ± 0.030 ^{ab}	<0.001
Cu (mg/kg)	7.41 ± 0.29 ^a	4.66 ± 0.27 ^d	4.88 ± 0.32 ^{cd}	5.07 ± 0.38 ^{bcd}	6.39 ± 0.38 ^{ab}	4.94 ± 0.19 ^{cd}	6.23 ± 0.40 ^{abc}	<0.001
Serum								
Se (mg/L)	0.061 ± 0.001 ^a	0.037 ± 0.001 ^d	0.044 ± 0.003 ^{bcd}	0.041 ± 0.002 ^{cd}	0.050 ± 0.003 ^{abc}	0.052 ± 0.002 ^{abc}	0.053 ± 0.003 ^{ab}	<0.001
Cu (mg/L)	0.219 ± 0.009 ^a	0.123 ± 0.013 ^b	0.158 ± 0.013 ^{ab}	0.176 ± 0.021 ^{ab}	0.166 ± 0.014 ^{ab}	0.167 ± 0.014 ^{ab}	0.179 ± 0.011 ^{ab}	0.002

^{a-c} within the same row, means with different superscripts are significantly different ($p < 0.05$).

Values represent means ± standard error mean of eight replicates (16 broilers/per group).

NSD: Normal stocking density (10 birds/m²), HSD: High stocking density (16 birds/m²), Se: Selenium, SeNP: Selenium nanoparticles, MOS: Mannan oligosaccharide, Se: Selenium, Cu: Copper.

influence muscle yield by decreasing muscle fibre diameter through reducing protein deposition and by decreasing the proliferation potential of satellite cells in muscles. The effect of HSD stress was pronounced on muscle fibre diameter, area, and density. The present study is the first to report the effect of HSD and SeNP-MOS supplementation on breast muscle histomorphometry. Stressors related to heat stress are known to decrease muscle fibre diameter and prevent hypertrophy by reducing the expression of mRNA of different growth factors (IGF- 1) and transcription factors (MyoD and MyoG). These factors repress downstream of mTOR which results in decreased protein synthesis and deposition in muscle fibre [22]. We assume that the HSD might have activated the same pathways for reduced expression of these aforementioned factors resulting in decreased muscle fibre diameter and increased density/mm². Se plays an important role in protecting skeletal muscle cells from degradation by increasing selenoprotein W (Sel-W) in muscle fibres and activating protein synthesis. Se is reported to increase protein synthesis by increasing IGF-1 or by mimicking insulin thus activating the insulin signalling cascade which leads to increased protein synthesis and deposition [23]. Se also increases the proliferation of satellite cells and their incorporation into muscle fibre cells [23,24]. The same studies suggested that supplementation of Se increased the expression of myogenic regulatory factors (MRFs) MyoD and MyoG in normal [23] and stressed conditions [24]. The MOS through its role in improving the GIT environment might have helped in higher absorption of nutrients and trace minerals such as Se and Cu, which is evident from their higher concentration in breast muscle in the current study. Selenium (as selenite or SeNP) and MOS

enhanced each other's individual effects; therefore, their combination proved more effective in alleviating harmful effects of HSD on breast muscle fibre diameter and muscle fibre area. This could potentially result in higher muscle yield for consumers.

Meat pH and WHC are considered two of the most important factors influencing the tenderness and juiciness of processed breast meat. The higher ultimate pH (pHu) of meat is directly related to higher WHC in breast meat [20]. The HSD affects meat quality by decreasing WHC and increasing oxidative reactions resulting in lowering of muscle pH [25]. The HSD is also reported to reduce fibrillar protein water holding capacity, which means that the contractions at rigor mortis would have caused the dripping of loosely bound water [26]. Some studies also suggested that higher muscle fibre size results in higher pHu and lower drip loss, which supports our findings that HSD lowered the muscle fibre diameter and pHu and increased the drip loss [27]. The SeNP is reported to delay the metabolic conversion of glucose to lactic acid which helps to maintain muscle pH. [28]. The supplementation of SeNP-MOS did not improve the pH of HSD-stressed birds but there was a numeric increase in pH values. Higher pH value favours water retention and leads to higher WHC. Moreover, higher bioavailability of Se in breast muscle, as observed in the SeNP-MOS supplemented group in our study, might have improved muscle antioxidant status, protected muscle cell membrane integrity, and prevented water loss. The SeNP-MOS also improved the growth performance of HSD-stressed broiler in the current study which could have a direct link with higher pHu and lower drip loss% in breast muscles.

Stressors generally reduce the amount of parenchyma of lymphoid organs by increasing lymphocyte apoptosis in immune organs. The HSD-related stress significantly influences the development of immune organs in broilers and consequently may increase the incidence of diseases. In our study, HSD decreased the lymphoid tissue area in caecal tonsil and bursa of Fabricius. These findings are in agreement with those of Muniz et al. [29], who reported decreased volume of lymphoid follicles of bursa in HSD-stressed broilers. Exposure to HSD causes oxidative stress on cellular level, which could be linked to reduced availability of Se from GIT, as reflected in reduced Se concentration in different tissues in the HSD group. The Se deficiency leads to improper proliferation of lymphocytes linked with decreased serum IL-1 and IL-2 [30]. The MOS could have protected GIT tract resulting in proper absorption and bioavailability of Se in both Se-MOS- and SeNP-MOS-supplemented HSD broilers. The higher Se at tissue levels may have led to increase in the secretion of IL-1 and IL-2, resulting in the proliferation of B and T cells, respectively, as evidenced by higher lymphoid area of caecal tonsil and bursa of Fabricius.

Bone weight, length, and ash contents are important indicators of bone health and metabolism [31] influenced by different stressors. Higher stocking density has been associated with compromised musculo-skeletal development and is directly linked with reduced activity in birds raised under HSD [32]. Our results are consistent with previous studies which reported that HSD negatively affected bone strength and walking ability in broilers during 4th to 6th week of age [32, 33]. Similarly, Hall [34] concluded that stocking density and bone quality are inversely related. Effects of HSD on bone can be explained in multiple ways such as lowered activity of birds [32], reduced expression of both parathyroid hormone-related protein and insulin-like growth factor-1 [35], which are required for normal bone growth. Stressors generally inhibit osteoblastic differentiation by reduced expression of selenoproteins such as glutathione peroxidases in bone microarchitecture [36]. The HSD through its generalized effect could also lead to decreased nutrient availability for bone development due to compromised digestive and absorptive function of digestive tract [37]. Selenium nano-particle supplementation could have played a more potent role in mitigating HSD-led oxidative stress in bone microarchitectural environment. There are at least nine selenoproteins expressed in developing osteoblast protecting it from oxidative stress and helping them in normal functioning [36]. The MOS is considered beneficial for balancing gut microbial ecology; therefore, it could have played an indirect role in increasing the mineral absorption [38]. Prebiotics are also considered to improve mineral absorption through caeca by attracting large quantity of water, thereby raising fluidity and

dissolving more minerals for higher absorption [39]. As observed in current study, Selenium as selenite or as SeNP and MOS enhanced each other's individual effects, so their combination proved more potent in mitigating harmful effects of HSD on bone weight, medullary canal diameter, bone density indices, and bone ash content.

Microminerals such as copper (Cu) and Se are required in many metabolic pathways and are considered essential for normal growth and development of broilers. Cu is required for proper functioning of enzymes required for iron metabolism and cytochrome oxidase system [40]. Se is an important component of selenoenzymes performing antioxidant activity. The HSD decreased the level of both Cu and Se in bone, serum and breast muscle. The HSD has been reported to negatively influence intestinal microarchitecture [41] leading to decrease in intestinal absorptive function, which could be a reason for decreased mineral concentrations observed in the investigated tissues. Lower bioavailability of Se leads to imbalanced oxidant/antioxidant status and favours oxidative free radical thus leading to tissue damage. Moreover, lower Cu levels in serum decreased the bone mineral density and increased the serum cholesterol levels [42]. This could be related to our findings for muscle and bone tissue, where HSD negatively influenced their morphometric characteristics. Supplementation of Se-MOS and SeNP-MOS improved the Cu and Se concentrations in serum, bone, and muscle. MOS through its localized effect on intestinal development and Se through generalized protection against oxidative damage might have augmented each other's effects thus leading to higher Se and Cu concentrations. There is no positive or negative interaction of Se supplementation with Cu metabolism [43], so increase in Cu concentration could be related to overall positive impact of Se-MOS and SeNP-MOS in HSD stress.

5. Conclusion

Based on these results, we conclude that higher stocking density (16 bird/m²) is a stressful situation for birds in tropical environment which can negatively influence meat yield and quality, immune organ development, and tibia bone health. Moreover, SeNP-MOS proved to be the most superior combination from all the investigated supplementations in partially alleviating the deleterious effects of HSD in broilers. Further studies are required to exactly document the molecular mechanisms through which SeNP-MOS stimulates the muscle, immune organ, and tibia bone development.

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References

- Li W, Wei F, Xu B, Sun Q, Deng W et al. Effect of stocking density and alpha-lipoic acid on the growth performance, physiological and oxidative stress and immune response of broilers. *Asian-Australasian Journal of Animal Sciences* 2019; 32 (12): 1914-1922. doi:10.5713/ajas.18.0939
- Yanai T, Abo-Samaha MI, El-Kazaz SE, Tohamy HG. Effect of stocking density on productive performance, behaviour, and histopathology of the lymphoid organs in broiler chickens. *European Poultry Science* 2018; 82: 1-15. doi: 10.1399/eps.2018.247
- Wu Y, Wang Y, Wu W, Yin D, Sun X et al. Effects of nicotinamide and sodium butyrate on meat quality and muscle ubiquitination degradation genes in broilers reared at a high stocking density. *Poultry Science* 2020; 99 (3): 1462-1470. doi: 10.1016/j.psj.2019.10.070
- Ma H, Xu B, Li W, Wei F, Kim WK et al. Effects of alpha-lipoic acid on the behavior, serum indicators, and bone quality of broilers under stocking density stress. *Poultry Science* 2020; 99 (10): 4653-4661. doi: 10.1016/j.psj.2020.05.007
- Surai PF, Kochish II. Nutritional modulation of the antioxidant capacities in poultry: the case of selenium. *Poultry science* 2019; 98 (10): 4231-4239. doi: 10.3382/ps/pey406
- Visha P, Selvaraj P, Jayachandran S. Selenoprotein gene (Sel W) expression in the bursa of broiler chickens as influenced by dietary supplementation of nanoselenium. *Journal of Entomology and Zoology Studies* 2020; 8 (03): 392-396.
- Tang JY, He Z, Liu YG, Jia G, Liu GM et al. Effect of supplementing hydroxy selenomethionine on meat quality of yellow feather broiler. *Poultry Science* 2021; 100 (10): 1-13. doi: 10.1016/j.psj.2021.101389
- Khan AZ, Khan IU, Afzal S, Khan S, Khan MA et al. Effects of Selenium-enriched probiotics on lameness and growth improvement in broiler chickens under heat stress condition. *Acta Veterinaria Eurasia* 2021; 47 (1): 19-28. doi: 10.5152/actavet.2020.20044
- Sevim Ö, Ahsan U, Tatlı O, Kuter E, Khamseh EK et al. Effect of nano-selenium and high stocking density on performance, carcass yield, meat quality and feathering score of broilers. *Ankara Üniversitesi Veteriner Fakültesi Dergisi* 2022. 1-24. doi: 10.33988/auvfd.83812
- Abdel-Moneim AM, Shehata AM, Mohamed NG, Elbaz AM, Ibrahim NS. Synergistic effect of *Spirulina platensis* and selenium nanoparticles on growth performance, serum metabolites, immune responses, and antioxidant capacity of heat-stressed broiler chickens. *Biological Trace Element Research* 2022; 200 (2): 768-779. doi: 10.1007/s12011-021-02662-w
- Kridtayopas C, Rakangtong C, Bunchasak C, Loongyai W. 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* 2019; 98 (10): 4595-4605. doi: org/10.3382/ps/pez152
- Zhang SY, Zhang J, Wang HY, Chen HY. Synthesis of selenium nanoparticles in the presence of polysaccharides. *Materials Letters* 2004; 58 (21): 2590-2594. doi: 10.1016/j.matlet.2004.03.031
- Honikel KO. Reference methods for the assessment of physical characteristics of meat. *Meat Science* 1998; 49 (4): 447-457. doi: 10.1016/S0309-1740(98)00034-5
- Bancroft JD, Layton C. The hematoxylin and eosin. In: Suvarna SK, Layton C, Bancroft JD (editors). *Bancroft's theory and practice of histological techniques*. 8th ed. London, United Kingdom: Churchill Livingstone, Elsevier health sciences; 2013. pp. 126-135.
- Rehman HF, Zaneb H, Masood S, Yousaf MS, Ashraf S et al. Effect of Moringa oleifera leaf powder supplementation on breast muscle quality and morphometric characteristics of tibia bone in broiler chickens. *Brazilian Journal of Poultry Science* 2018; 20 (04): 817-824. doi: 10.1590/1806-9061-2017-0609
- Mutuş R, Kocabağlı N, Alp M, Acar NÜ, Eren MU et al. The effect of dietary probiotic supplementation on tibial bone characteristics and strength in broilers. *Poultry Science* 2006; 85 (9): 1621-1625. doi: 10.1093/ps/85.9.1621
- Kocabağlı N. The effect of dietary phytase supplementation at different levels on tibial bone characteristics and strength in broilers. *Turkish Journal of Veterinary and Animal Sciences* 2001; 25 (5): 797-802.
- Saeed AA, Sandhu MA, Khilji MS, Yousaf MS, Rehman HU et al. Effects of dietary chromium supplementation on muscle and bone mineral interaction in broiler chicken. *Journal of Trace Elements in Medicine and Biology* 2017; 42: 25-29. doi: 10.1016/j.jtemb.2017.03.007
- Norman GR, Streiner DL. *Biostatistics: The bare essentials*. 3rd ed. Hamilton, Ontario, Canada: BC Decker Inc; 2008. Pp. 77-81.
- Pekel AY, Tatlı O, Sevim Ö, Kuter E, Ahsan U et al. Effects of reducing dietary amino acid density and stocking density on growth performance, carcass characteristics, meat quality, and occurrence of white striping in broiler chickens. *Poultry Science* 202; 99 (12): 7178-7191. doi: 10.1016/j.psj.2020.08.077
- Soglia F, Petracchi M, Davoli R, Zappaterra M. A critical review of the mechanisms involved in the occurrence of growth-related abnormalities affecting broiler chicken breast muscles. *Poultry Science* 2021; 100 (6): 1-20. doi: 10.1016/j.psj.2021.101180
- Ma B, He X, Lu Z, Zhang L, Li J et al. Chronic heat stress affects muscle hypertrophy, muscle protein synthesis and uptake of amino acid in broilers via insulin like growth factor-mammalian target of rapamycin signal pathway. *Poultry Science* 2018; 97 (12): 4150-4158. doi: 10.3382/ps/pey291
- Gao J, Nie W, Wang F, Guo Y. Maternal selenium supplementation enhanced skeletal muscle development through increasing protein synthesis and SelW mRNA levels of their offspring. *Biological Trace Element Research* 2018; 86 (1): 238-248. doi: 10.1007/s12011-018-1288-z

24. Liu Y, He A, Tang J, Shah AM, Jia G et al. Selenium alleviates the negative effect of heat stress on myogenic differentiation of C2C12 cells with the response of selenogenome. *Journal of Thermal Biology* 2021; 97: 102874-102882. doi: 10.1016/j.jtherbio.2021.102874
25. Chen X, Zhang L, Li J, Gao F, Zhou G. Hydrogen peroxide-induced change in meat quality of the breast muscle of broilers is mediated by ROS generation, apoptosis, and autophagy in the NF- κ B signal pathway. *Journal of Agricultural and Food Chemistry* 2017; 65 (19): 3986-3994. doi:10.1021/acs.jafc.7b01267
26. Nasr MA, Alkhedaide AQ, Ramadan AA, Abd-El Salam EH, Hussein MA. Potential impact of stocking density on growth, carcass traits, indicators of biochemical and oxidative stress and meat quality of different broiler breeds. *Poultry Science* 2021; 100 (11): 1-12. doi: 10.1016/j.psj.2021.101442
27. Berri C, Le Bihan-Duval E, Debut M, Sante-Lhoutellier V, Baéza E, G et al. Consequence of muscle hypertrophy on characteristics of Pectoralis major muscle and breast meat quality of broiler chickens. *Journal of Animal Science* 2007; 85 (8): 2005-2011. doi: 10.2527/jas.2006-398
28. Khan I, Zaneb H, Masood S, Ashraf S, Rehman HF et al. Supplemental selenium nanoparticles-loaded to chitosan improves meat quality, breast muscle histology, Tibia bone morphometry and tissue mineral retention in broilers. *Pakistan Veterinary Journal* 2022; 42 (2): 236-240. doi: 10.29261/pakvetj/2022.007
29. Muniz EC, Fascina VB, Pires PP, Carrijo AS, Guimarães EB. Histomorphology of bursa of Fabricius: effects of stock densities on commercial broilers. *Brazilian Journal of Poultry Science* 2006; 8 (4): 217-220. doi: 10.1590/S1516-635X2006000400003
30. Zhang ZW, Wang QH, Zhang JL, Li S, Wang XL et al. Effects of oxidative stress on immunosuppression induced by selenium deficiency in chickens. *Biological Trace Element Research* 2012; 49 (3): 352-361. doi: 10.1007/s12011-012-9439-0
31. Ma H, Xu B, Li W, Wei F, Kim WK et al. Effects of alpha-lipoic acid on the behavior, serum indicators, and bone quality of broilers under stocking density stress. *Poultry Science* 2020; 99 (10): 4653-4661. doi: 10.1016/j.psj.2020.05.007
32. Buijs S, Van Poucke E, Van Dongen S, Lens L, Baert J et al. The influence of stocking density on broiler chicken bone quality and fluctuating asymmetry. *Poultry Science* 2012; 91 (8): 1759-1767. doi: 10.3382/ps.2011-01859
33. Sørensen P, Su G, Kestin SC. Effects of age and stocking density on leg weakness in broiler chickens. *Poultry Science* 2000; 79 (6): 864-870. doi: 10.1093/ps/79.6.864
34. Hall AL. The effect of stocking density on the welfare and behaviour of broiler chickens reared commercially. *Animal Welfare* 2001; 10 (1): 23-40.
35. Li XM, Zhang MH, Liu SM, Feng JH, Ma DD et al. Effects of stocking density on growth performance, growth regulatory factors, and endocrine hormones in broilers under appropriate environments. *Poultry Science* 2008; 98 (12): 6611-6617. doi: 10.3382/ps/pez505
36. Zeng H, Cao JJ, Combs GF. Selenium in bone health: roles in antioxidant protection and cell proliferation. *Nutrients* 2013; 5 (1): 97-110. doi:10.3390/nu5010097
37. Sugiharto S. Dietary strategies to alleviate high-stocking-density-induced stress in broiler chickens—a comprehensive review. *Archives Animal Breeding* 2022; 65 (1): 21-36. doi: 10.5194/aab-65-21-2022
38. Sohail MU, Rahman ZU, Ijaz A, Yousaf MS, Ashraf K et al. Single or combined effects of mannan-oligosaccharides and probiotic supplements on the total oxidants, total antioxidants, enzymatic antioxidants, liver enzymes, and serum trace minerals in cyclic heat-stressed broilers. *Poultry Science* 2011; 90 (11): 2573-2577. doi: 10.3382/ps.2011-01502
39. Leblebici ÖD, Aydoğan İ. The effects of Mannan oligosaccharide and Chitosan oligosaccharide on performance and blood parameters of broilers. *Journal of Poultry Research* 2018; 15 (1): 18-22.
40. Samanta B, Biswas A, Ghosh PR. Effects of dietary copper supplementation on production performance and plasma biochemical parameters in broiler chickens. *British Poultry Science* 2011; 52 (5): 573-577. doi: 10.1080/00071668.2011.608649
41. Sun ZW, Fan QH, Wang XX, Guo YM, Wang HJ et al. High stocking density alters bone-related calcium and phosphorus metabolism by changing intestinal absorption in broiler chickens. *Poultry science* 2018; 97 (1): 219-226. doi: 10.3382/ps/pex294
42. Kaya A, Altın A, ÖZpınar A. Effect of copper deficiency on blood lipid profile and haematological parameters in broilers. *Journal of Veterinary Medicine Series A* 2006; 53 (8): 399-404.
43. Buckley WT, Huckin SN, Fisher LJ, Eigendorf GK. Effect of selenium supplementation on copper metabolism in dairy cows. *Canadian Journal of Animal Science* 1986; 66 (4): 1009-1018.