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Effect of Moringa Oleifera Leaf Powder Supplementation on Pectoral Muscle Quality and Morphometric Characteristics of Tibia Bone in Broiler Chickens

■Author(s)

Rehman HF[I] https://orcid.org/0000-0003-0022-7929
Zaneb H[I] https://orcid.org/0000-0002-4664-4990
Masood S[I] https://orcid.org/0000-0002-1104-424x
Yousaf MS[II] https://orcid.org/0000-0002-1106-6289
Ashraf S[I] https://orcid.org/0000-0003-1306-7311
Khan I[I] https://orcid.org/0000-0002-0252-0451
Shah M[I] https://orcid.org/0000-0001-5422-0146
Khilji MS[II] https://orcid.org/0000-0001-6230-9327
Rehman H[II] https://orcid.org/0000-0002-8485-7962

[I] Department of Anatomy and Histology, University of Veterinary and Animal Sciences, Outfall Road, Lahore 54000, Pakistan.
[II] Department of Physiology, University of Veterinary and Animal Sciences, Outfall Road, Lahore 54000, Pakistan.

■Mail Address

Corresponding author e-mail address
Hafsa Zaneb
Department of Anatomy and Histology, University of Veterinary and Animal Sciences, Outfall Road, Lahore 54000, Pakistan.
Phone: +92 42 9921 1449 (Ext 238)
Email: hafsa.zaneb@uvas.edu.pk

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ABSTRACT

Although in-feed antibiotics work for broiler chicken´s growth, they are a source of public health hazard. Therefore, there is a need for alternates which can act as growth promoter without deleterious effects on the health of meat consumers. *Moringa oleifera* is one such phytobiotic which is reported to possess antimicrobial and immuno-modulatory properties. This study investigated the effect of *Moringa oleifera* leaf powder (MOLP) supplementation on meat quality and bone morphometry of broiler. One-day-old chicks (n=100), divided into five groups (four replicates with n=5), were fed a basal diet (control group) or same diet supplemented with 6, 9, 12 or 15 g/kg MOLP. On d-35, two birds per replicate were euthanized to collect samples of breast muscle, blood and tibia bone. The MOLP supplementation significantly increased pH of breast muscle and ash percentage of tibia bone. The diameter of breast muscle fibres and also weight and weight length index of tibia bone significantly increased with 12 and 15 g/kg MOLP. The water holding capacity (WHC) of breast muscle was significantly higher with 9 and 15 g/kg MOLP; whereas robusticity index of tibia bone significantly decreased with 12 and 15 g/kg MOLP inclusion compared to the control group. In conclusion, dietary supplementation of *Moringa oleifera* leaf powder (12g/kg) increased pH, water holding capacity, and muscle fibre diameter of breast muscle and also weight, ash percentage and density indices of tibia bone in broiler chickens.

INTRODUCTION

Broiler meat is not only an affordable source of protein with low levels of collagen, essential vitamins (mainly thiamin, vitamin B6 and pantothenic acid) and minerals (iron, zinc, and copper), but has lower fat content which makes it a healthier choice for consumers (Naji *et al.*, 2013). These nutritional advantages made broiler meat a major part of our diets (Arshad *et al.,* 2016) and therefore increased the pressure on broiler industry for higher meat yield globally. Additionally, consumer preference changed from the whole bird to their processed parts, which further increased the importance of meat yield and its quality characteristics like tenderness, colour, water holding capacity and post-mortem pH changes.

Higher meat yield in broiler is a desirable trait but has negative influence on the development of its bones (Hafeez *et al.*, 2014). Rapid growth rate and higher muscle mass results in metabolic disorders, leading to leg problems or weakness, and difficulties in locomotion (Sgavioli *et al.*, 2016). Bone length, weight and ash percentage are good indicators of the bone health (Ziaie *et al.*, 2011), which in turn depend on its mineral content especially calcium (Hafeez *et al.*, 2014).

To improve the meat yield (Zulkifli *et al.*, 2000) and bone health (Ziaie *et al.*, 2011) in-feed antibiotics have routinely been used in poultry.

Lately, imposition of ban on in-feed antibiotics usage by the European Union (European commission regulation, 2003) started a new era of research to explore their alternatives (Demir *et al*., 2003). Probiotics, prebiotics, synbiotics, acidifiers and phytobiotics offer some of the alternative approaches to the in-feed antibiotics (Griggs & Jacob, 2005). Phytobiotics are plant derived feed additives (Hashemi & Davoodi, 2010) with biologically active substances and antimicrobial properties (Demir *et al*., 2003).

Moring oleifera leaf powder (MOLP) is a phytobiotic derived from the *Moringa oleifera* plant, well known for its medicinal usage in humans (Nkukwana *et al*., 2014a). It has bioactive compounds which possess immuno-modulatory and anti-microbial (Bukar *et al*., 2010) properties when supplemented in animal or broiler feed. The MOLP also contains significant amount of natural anti-oxidants (vitamin E and selenium), minerals (calcium, phosphorus and magnesium,) and phytochemicals such as caffeic acid (Moyo *et al*., *2012*). Caffeic acid inhibits the development of osteoclasts, and natural anti-oxidants decrease lipid oxidation in meat. *Moringa oleifera* leaves are also believed to possess potential prebiotic effects (Nkukwana *et al*., 2014b). This study, therefore, aimed to document the effect of *Moringa oleifera* leaf powder (MOLP) supplementation on meat quality and morphometric characteristics of tibia bone in broiler chicken.

MATERIALS & METHODS

All experimental protocols were approved by Ethical Review Committee for the use of lab animals, University of Veterinary and Animal Sciences via letter no. DR/328 dated: 27-05-2016.

The details regarding formulation and supplementation of MOLP, management of birds, and experimental design have been described earlier (Khan *et al.,* 2017). Briefly, a total of 100-day-old Hubbard chicks (male and female) were randomly divided into five groups with four replicates containing five birds in each replicate. The chicks were reared on wood shaving litter in an environmentally controlled house in the Department of Physiology, in the University of Veterinary and Animal Sciences Lahore, Pakistan. The temperature and relative humidity (RH) of the experimental house was maintained at 35 ± 1 °C and 70 ± 5%, respectively during the first week. Later on, the temperature was decreased by 3 °C per week until it reached 26 ± 1°C with RH 65 ± 5% on day 21 and it was maintained till the end of the trial, that is day 35. Birds in the control group were fed a corn based basal diet (BD) free of antibiotic feed additives or coccidiostats. The birds in the experimental groups were fed the same BD supplemented with *Moringa oleifera* leaf powder at 6 g/kg (6 g/kg MOLP group), 9 g/kg (9 g/kg MOLP group), 12 g/kg (12 g/kg MOLP group) and 15 g/kg (15 g/kg MOLP group) (Nkukwana *et al.,* 2014c). For the preparation of MOLP, undamaged fresh green *Moringa oleifera* leaves were harvested during the month of February. To avoid leaching, the leaves were dried without direct sun light exposure. During the drying period the leaves were regularly turned to avoid fungal growth. Afterwards, the dried leaves were ground to extracta fine powder. Fresh water and feed were offered *ad libitum*. The basal diet (Table 1) was formulated to meet the requirements of poultry by the NRC (National Research Council, 1994).

Table 1 – Ingredient and nutritive value of the basal diet(g/kg).

Ingredients	Ingredients g/kg
Corn	585.0
Soybean meal 44%	250.0
Sunflower meal	35.0
Canola meal	80.0
Vegetable oil	15.0
Dicalcium phosphate	9.0
Limestone	15.1
Common salt	5.0
DL-Methionine	2.1
L-Lysine HCl	1.2
Vitamin premix[1]	1.3
Micro mineral premix[2]	1.3
Nutrient contents	
Crude protein	207.2
Metabolisable energy (MJ)	12.2
Calcium	9.1
Phosphorus	6.1

[1]Provided vitamins per kg of the feed: vitamin A (retinol), 3.3 mg; vitamin B12 (cyanocobalamin), 0.0132 mg; vitamin D3 (cholecalciferol), 0.055 mg; vitamin E (alpha--tocopherol), 14.74 mg; choline chloride, 440mg; riboflavin, 8.8mg; pantothenic acid, 22mg; ethoxyquin, 250mg; menadione, 2.2mg; pyridoxine, 4.4mg; folic acid, 1.1mg; biotin, 0.22; thiamin, 4.4mg.

[2]Supplied minerals per kg of the feed: Cu (CuSO4), 20mg; Zn (ZnO), 200mg; Mn (MnSO4), 240mg; Fe (FeSO4), 120mg; I (KI), 0.92mg; Ca, 150 to 180mg.

On d-35, eight birds from each group (1 male and 1 female per replicate) were randomly selected and decapitated. Skin and viscera of the birds were removed manually. From each bird, three samples of the breast muscle (*Pectoralis major*) were cut perpendicular to the longitudinal axis of the muscle fibres. One of the three samples were fixed in 10% neutral buffered formalin solution and processed for light microscopy through paraffin embedding technique followed by haematoxylin and eosin staining technique (Bancroft *et al*., 2013). The remaining two samples were utilized

for measuring drip loss, an indicator of water holding capacity (WHC), and pH of the muscle, respectively.

For muscle fibre per unit area count, pictures were captured at 4x objective lens magnification from three different areas of the slide. A circle of 0.5mm diameter was made on captured pictures with the help of Prog Res®2.1.1 Capture Prog Camera Control Software. The circle was divided in two equal halves and all muscle fibres located inside the circle along with muscle fibre touching the boundary line on the right half of the circle were counted. Muscle fibres touching the boundary line of the left half of the circle were excluded from the count. Muscle fibre/ mm^2 was measured by following a mathematical formula,

Muscle fibre/ $mm^2 = (1/\pi r^2)$* muscle fibre counted in 0.5mm radius circle

For muscle fibre diameter, pictures of slides were captured on 10x objective lens from five different areas on slide. On each picture, a grid of 0.6mm length and 0.26 width was made with the help of Prog Res®2.1.1 Capture Prog Camera Control Software. The grid consisted of 5 columns and 2 rows (a total of 10 boxes in one grid). Muscle fibres were selected from the alternate boxes of the grid. Their vertical and horizontal internal diameters were measured, and their average was reported.

Water holding capacity (drip loss) of meat was measured by Honikel's gravimetric method as described by Honikel (1998) for raw whole meat. Breast muscle (*Pectoralis major*) pH was measured by putting the piercing knob of the digital pH meter (Cyberscan 510pH, Eutech, Singapore) in the thickest part of the muscle sample (Guardia *et al*., 2014) at an interval of 0 hours, 12 hours and 24 hours post slaughtering. Calibration buffers, Citrate-Phosphate Buffer pH 4, 7 and Carbonate-Bicarbonate Buffer pH 10, were used to calibrate the pH meter. In-between pH measurements, meat samples were refrigerated.

The right tibia of each bird was separated at the drumstick with intact flesh. Each bone was labelled and immersed in boiling water (100 °C) for ten minutes. Thereafter, tibiae were cooled at room temperature, de-fleshed by hand and patellae were removed. Bones were air dried for 24 hours at room temperature. The tibia weight, length, bone outer diameter and medullary canal diameter were measured based on methods described by Mutuş *et al*. (2006). Briefly, tibiotarsal bone weight was measured using a digital weighing balance (BL 220H, Shimadzu, Tokyo, Japan). The length of the bone was measured using digital vernier callipers. Each bone was marked at its mid length and this marked point was used to measure bone outer diameter. The bone was broken at marked mid-point and thickness of lateral and medial walls were measured with digital vernier callipers. For medullary canal diameter calculation, the combined thickness of medial and lateral walls was subtracted from outer bone diameter. The tibio-tarsal index was measured by the following formula: tibiotarsal index= (outer bone diameter - medullary canal diameter/ outer bone diameter) x 100. The robusticity index was determined by the following formula: robusticity index = bone length (mm) / cube root of bone weight (mg) as described by Kocabagli (2001). The bone weight/length index was measured by dividing the tibia weight (mg) by its length (mm) as mentioned by Seedor*et al*. (2005). For bone ash determination, bone fragments were dried in a hot air oven at 105°C for 24 hours, burnt in a muffle furnace at 600 °C for 6 hours. The percentage bone ash was measured relative to dry tibia weight (Mutuş *et al*., 2006). At d-35, blood was collected at the time of de-capitation in sterile vacutainers and centrifuged at 3000 rpm for 5 minutes to harvest serum and was stored at -20°C until further analysis. For measuring serum alkaline phosphatase (ALP) activity, an alkaline phosphatase kit (ALP kit Fortress Diagnostics, United Kingdom) was used and its absorbance was measured at 405 nm wavelength in spectrophotometer (UV-2800, Biotechnology Medical Services, USA) as mentioned in the kit (Jawad *et al*., 2014).

Data were analysed using one-way analysis of variance and presented as mean ± SEM (SPSS V. 13.3 Chicago IL, USA). The group differences were compared with the Duncan's Multiple Range test. Differences were considered significant at $p<0.05$. Additionally, regression (linear, cubic and quadratic) models were run to study dose-dependent responses.

RESULTS

The supplementation of *Moringa oleifera* leaf powder (MOLP) in diet increased water holding capacity of breast muscle. The water holding capacity (WHC) is expressed through drip loss, the higher the drip loss the lower the WHC. Drip loss was high ($p<0.05$) in control group (3.32) compared to9 g/kg MOLP, 15 g/kg MOLP groups (2.2 and 2.39 respectively) (Table 2). For all the MOLP supplemented groups, the pH values of breast muscle at 0 hours, 12 hours and 24 hours post-mortem was higher ($p<0.05$) than the control group (Table 2).

Table 2 – Effect of *Moringa oleifera* leaf powder supplementation on drip loss, pH, muscle fibre density and muscle fibre diameter of breast muscle.

Parameter	Control	6 g/kg MOLP	9 g/kg MOLP	12 g/kg MOLP	15 g/kg MOLP	P-Value (ANOVA)	*p*-values of regression model		
							Linear	Quadratic	Cubic
Drip loss (%)	3.32±0.42[a]	2.601±0.20[ab]	2.2±0.10[b]	2.70±0.20[ab]	2.39±0.21[b]	0.032	0.041	0.028	0.029
pH (0H)	5.81±0.05[c]	6.20±0.11[b]	6.39±0.11[b]	6.48±0.05[a]	6.51±0.10[a]	0.005	<0.001	<0.001	<0.001
pH (12H)	5.72±0.04[b]	6.02±0.06[a]	6.09±0.07[a]	6.01±0.12[a]	6.23±0.03[a]	0.005	<0.001	0.001	<0.001
pH (24H)	5.60±0.04[b]	5.81±0.05[a]	5.85±0.04[a]	5.85±0.10[a]	5.91±0.06[a]	0.007	0.003	0.006	0.010
Muscle fibre density[§]	642.44±14.93	632.27±9.91	624.48±14.10	627.48±12.36	627.31±9.63	0.859	0.367	0.533	0.736
Muscle fibre diameter[†]	37.98±0.59[b]	38.54±0.47[b]	39.09±0.42[ab]	40.14±0.46[a]	40.06±0.56[a]	0.010	<0.001	0.002	0.004

Eight (8) birds were evaluated from each group

[a–b] within the same row, means with different superscripts are significantly different ($p<0.05$).

Values represent the Mean ± SEM of four replicates.

MOLP = *Moringa oleifera* leaf powder

[§]Muscle fibre density: Total number of muscle fibre/mm^2 of muscle area

[†]Muscle fibre diameter values: (µm)

When studying the effects of MOLP supplementation on breast muscle histomorphometry, it was found that muscle fibre density of all MOLP supplementation groups was numerically lower at all the investigated level but did not differ statistically ($p>0.05$) compared to control group (Table 2). The muscle fibre diameter of breast muscle was higher ($p<0.05$) in 12 g/kg MOLP and 15 g/kg MOLP groups (40.14 and 40.06 µm respectively) than the control group (37.98 µm) (Table 2).

For tibia bone morphometric results, the supplementation of MOLP did not affect ($p>0.05$) tibia bone length, bone and medullary canal diameters (Table 3). The weight of tibia was higher ($p<0.05$) in 12 g/kg MOLP and 15 g/kg MOLP groups (4.08 and 4.01g respectively) compared to 6 g/kg MOLP, 9 g/kg MOLP and control group (3.66, 3.66 and 3.51 g respectively) (Table 3). The ash percentage of tibia bone for all the MOLP supplemented groups (48.61, 48.87, 49.37 and 49.11 % respectively) was higher ($p<0.05$) than the control group (47.31%) (Table 3). The supplementation level of 12 g/kg and 15 g/kg MOLP ($p<0.05$) increased the weight/length index (50.33 and 49.37 respectively) of the tibia when compared to 6 g/kg MOLP, 9 g/kg MOLP and control groups (44.99, 45.19and 43.27 respectively) (Table 3). The MOLP supplementation did not affect ($p>0.05$) tibio-tarsal index in any of the experimental group. Robusticity Index of tibia bone was lower ($p<0.05$) in 12 g/kg and 15 g/kg MOLP supplemented group (5.07 and 5.1 respectively) as compared to the control group (5.35) (Table 3). The serum alkaline phosphatase (ALP) level (U/l; Means ± SEM) did not vary ($p>0.05$) among the experimental groups.

Table 3 – Effect of *Moringa oleifera* leaf powder supplementation on Morphometric parameters and density indices of tibia bone.

Parameters	Control	6 g/kg MOLP	9 g/kg MOLP	12 g/kg MOLP	15 g/kg MOLP	*p*-Value (ANOVA)	*p*-values of regression model		
							Linear	Quadratic	Cubic
Tibia bone length[†]	81.28±0.76	81.64±1.06	81.18±0.63	81.14±0.67	81.16±0.46	0.98	0.744	0.944	0.968
Tibia bone weight[§]	3.51±0.10[b]	3.66±0.14[b]	3.66±0.09[b]	4.08±0.07[a]	4.01±0.06[a]	0.001	<0.001	<0.001	0.001
Tibia bone diameter[†]	7.07±0.05	7.05±0.08	7.13±0.08	7.21±0.06	7.16±0.06	0.553	0.142	0.335	0.380
Medullary canal diameter[†]	4.14±0.09	4.01±0.12	4.19±0.15	4.17±0.12	4.06±0.06	0.773	1.000	0.934	0.722
Tibia bone ash percentage	47.31±0.22[b]	48.61±0.17[a]	48.87±0.42[a]	49.37±0.25[a]	49.11±0.54[a]	0.002	<0.001	<0.001	0.001
W/L index	43.27±1.51[b]	44.99±1.85[b]	45.19±1.25[b]	50.33±0.82[a]	49.37±0.52[a]	0.001	<0.001	0.001	0.001
Tibiotarsal index	41.38±1.20	43.35±1.196	41.25±1.833	42.27±1.65	43.27±0.93	0.730	0.538	0.809	0.738
Robusticity index	5.35±0.08[a]	5.30±0.09[ab]	5.26±0.06[ab]	5.07±0.04[c]	5.10±0.01[bc]	0.022	0.002	0.007	0.013
Alkaline phosphatase†	1167.09±60.20	1273.65±48.89	1203.62±83.41	1289.77±123.51	1101.91±70.6	0.222	0.680	0.381	0.567

Eight (8) birds were evaluated from each group

[a-c] within the same row, means with different superscripts are significantly different ($p<0.05$)

Values represent the Mean ± SEM of four replicates.

MOLP =*Moringa oleifera* leaf powder

[†]Bone length and diameters: mm

[§]Bone weight: g

†Alkaline phosphatase: U/l

DISCUSSION

This study demonstrated the effects of MOLP supplementation on meat quality and morphometric features of tibia bone in broilers. Water holding capacity (WHC) and meat pH are crucial qualitative traits of broiler meat which affect the appearance of the products as well as their juiciness, cooking losses (Karthivashan *et al*., 2015) and meat tenderness, a quality which mainly determines consumer preference (Lomiwes *et al*., 2014). Our results revealed that breast muscles of the birds receiving MOLP supplementation had higher pH values and those receiving 9 and 15g of MOLP were presented with higher WHC of muscles when compared to the non-supplemented group. Additionally, linear, quadratic and cubic relationships were observed for these parameters. These results of pH are in agreement with those of Wapi *et al.* (2014) who observed that the group supplemented with 750g/t MOL meal, had higher (p<0.05) pH compared to all other experimental groups. Berri *et al*. (2007) also reported that higher pre-rigor pH resulted in higher WHC of breast muscle in broilers. The result of the current study also showed that pH values in all experimental groups gradually decreased till the final reading taken at 24 hours post-mortem, an observation in accordance with that of Kadim *et al.* (2009). MOLP is a good source of vitamin E and selenium (Moyo *et al*., 2011). Owing to the presence of these contents, we can hypothesize that the MOLP supplementation might have stabilized the muscular membrane by activating antioxidants and preventing free radicals (Alabi *et al.,* 2017). Moreover, higher initial muscle pH values in MOLP supplemented group might have contributed towards the stabilization of the volume of myofibrils by reducing the protein denaturation, thus leading to conservation of water inside the muscle cells (Honikel, 1998).

The weight of a muscle is the function of the total number of fibres present in a particular muscle along with their cross sectional area (Tumova & Teimouri, 2009). The results showed that 12 g/kg and 15 g/kg MOLP supplementation increase (p<0.05) the muscle fibre diameter. Regarding MOLP supplementation in broiler, the authors could not find any report indicating its influence on muscle histomorphometry. But Cohen-Zinder *et al.* (2017) did report higher crude protein content and sarcomere length in the muscle of *Moringa oleifera* fed lambs. The increased fibre size of skeletal muscles is also associated with higher pH (Berri *et al*., 2007) and lowered drip loss (Duclos *et al*., 2007). It may be assumed that MOLP supplementation increased protein deposition as indicated by the increased muscle fibre diameter and thus translated into higher muscle weight and dressing percentage as previously observed by Alabi *et al.* (2017) in birds fed with *Moringa oleifera* leaf extract. This is substantiated by the fact that body weight of 12 g/kg and 15 g/kg MOLP supplemented groups was also higher in the current experiment (Published Data, continuation of previous study, Khan *et al.,* 2017).

In modern meat-type chicken, higher muscle to bone ratio leads to skeletal abnormalities and fractures of bone (Kwiatkowska *et al.,* 2017). Bone condition is directly related to the_bioavailability of calcium (Ca) and phosphorus (P) at tissue level. The most common parameters used to check the bioavailability of Ca and P are bone breaking strength (BBS) along with ash content of bones (Shaw *et al.*, 2010). The current study demonstrated that weight, ash percentage, W/L and Robusticity index of MOLP supplemented birds tibia bone showed linear, quadratic and cubic pattern of increase in response to dose increment, with peak values obtained with 12 g/kg MOLP supplementation. Our findings are in disagreement with those of Nkukwana *et al. (*2014b) who reported that *Moringa oleifera* leaf meal (MOLM) had no effect on tibia bone characteristics. This variation in results of bone characteristics may be due to phasic increment of MOLM in starter, grower and finisher ration in the afore-mentioned experiment. The increase in bone weight and ash percentage are indictors of good mineralization of bone which might be due to the positive effect of *Moringa oleifera* leave bioactive compounds on gastrointestinal tract leading to improved nutrient and mineral absorption (Mbikay, 2012) and decreased excretion of calcium from body (Parikh *et al*., 2015). Another possible explanation for improved tibia bone parameters is the presence of Phytoestrogens flavonoids in MOLP. Phytoestrogens inhibits activities of osteoclasts; promote mineralization through osteoblast and protein synthesis in bone (Sirotkin & Harrath, 2014).

Alkaline phosphatase (ALP) is a family of glycoproteins that are present in blood. The two most important isoforms of ALP come from liver and bone (Tilgar *et al*., 2008). The ALP level in blood is a good indicator of liver and bone health. Our results showed no difference in ALP levels among all the groups. Similar observations were reported by Melesse *et al*. (2013) and Ewuola *et al*. (2012) for chicken and rabbit, respectively. Therefore, it may be assumed that MOLP supplementation has no toxic effect on bone health and functionality of liver as assessed through serum ALP analysis.

In conclusion, supplementation of *Moringa oleifera* leaf powder at an inclusion rate of 12 g/kg in the diet of broilers improved meat quality indicators as well as weight, ash percentage and density indices of tibia bone.

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