The effect of *Moringa oleifera* leaf meal supplementation on tibia strength, morphology and inorganic content of broiler chickens

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Abstract

This study was conducted to assess the effects of dietary *Moringa oleifera* leaf meal (MOLM) supplementation as a possible alternative to antibiotic growth promoters on bone breaking strength (BBS), tibia bone morphology and inorganic ash content in broiler chickens. A total of 2400 one-day-old Cobb-500 broiler chicks of mixed sex were randomly allocated to five dietary treatments in six replications of 80 birds per pen. Dietary treatments were as follows: T1, positive control, 668 g salinomycin and 500 g zinc bacitracin per kg of feed; T2, T3 and T4, graded levels of MOLM, that is (starter (1, 3 and 5 g), grower (3, 9 and 15 g) and finisher (5, 15 and 25 g)) per kg of feed; and T5, a negative control (without supplementation). Birds were provided with feed and water *ad libitum*. Bodyweights (BW) and feed intake (FI) of broilers were measured weekly, and feed conversion ratio (FCR) and average daily gains were calculated accordingly. At 35 days (d) of age, 12 birds per treatment, two from each replicate pen, were randomly selected, stunned electrically at 70 volts, and slaughtered by cervical dislocation. Six left and right tibiae were randomly chosen from each treatment, without being de-fleshed, and were sealed individually in plastic bags to minimize moisture loss. The tibiae were stored at −18 ºC for BBS and tibia ash determination, as well as analyses of their calcium (Ca) and phosphorus (P) composition. Of birds fed diets with higher MOLM levels, T4 had the highest BW, while T1 had the lowest. On days 7 and 21, birds on T4 had the highest average daily gain (ADG) and T1 the lowest, respectively. There were no significant dietary effects in FI on days 7, 21 and 35; while on days 21 and 35, FCR (feed : gain) was highest in MOLM-supplemented birds and lowest in T1 birds, respectively. There were no treatment effects on tibia length (TL), tibia weight (TW) and/or dried tibia weight (DW). A positive correlation (r = 0.667) was observed between TW and DW. There were no treatment effects on BBS and ash percentage, but tibia ash weight was highest in T4 and lowest in T2. Calcium percentage was highest in T1, and lowest in T2 and T5. Phosphorus percentage was lowest in T1 and highest in T5. The highest Ca : P ratio was obtained in T4. The positive correlation observed between BW, TW and TL, and the high P, Ca and ash levels in tibiae from MOLM-supplemented birds depicts the genetic relationship that exists among these parameters. Furthermore, it reveals the effectiveness of MOLM supplementation to enhance nutrient utilization efficiency, increase mineral bioavailability and support bone strength.

Introduction

Bone characteristics are of paramount importance in broiler production, in addition to digestibility and efficiency of feed utilization and gut health (Applegate & Lilburn, 2002). The bone is a living tissue, a composite material of 30% organic matrix and 70% hydroxyapatite, composed mainly of calcium (Ca) and phosphorus (P) in the weight ratio of 2.15 : 1 (Whitehead, 2007). Bone ash content and bone breaking strength (BBS) are the most commonly used parameters for assessing the bioavailability of calcium and phosphorus (EMFEMA, 2002; Shaw *et al.*, 2010). Long bones, particularly the tibia, experience cellular sensitivity to numerous dietary deficiencies (Leach & Lilburn, 1992). The weight of tibia ash has been found to be a sensitive indicator of the amount of available Ca and P in the diet (Hall *et al.*, 2003). Moreover, traditionally, estimates of P needs for growing broilers have been estimated primarily on tibia ash values

(NRC, 1994). In modern meat-type chickens, bone strength and development are closely linked to the expression of genetic potential for maximum lean muscle deposition (Williams *et al.*, 2004). Thus, the increase in dry bone weight and mineral content per unit bone length responds to mechanical forces placed on the bone (McDevitt *et al.*, 2006; Whitehead, 2007; Talaty *et al.*, 2009a). Skeletal integrity of broiler chickens has become a welfare and economic concern (Williams *et al.*, 2004; Almeida Paz & Bruno, 2006; Fleming, 2008). Weak bones are an undesirable trait in growing broiler chickens, which has been linked to 1.1% mortality of birds, and an additional 2.1% condemnation or downgrading of carcasses during processing (Onyango *et al.*, 2006; Talaty *et al.*, 2009b).

Bone health problems in chickens are reduced by activity and genetic selection, but nutrition is important as a mitigation strategy to ensure that dietary Ca, vitamin D and P are adequate and are correctly balanced (Fleming, 2008). Absorption and homeostasis of these minerals depend on their dietary ratio, with maximum absorption obtained at 2Ca : 1P often met by supplying NRC (1994) recommendations of 10 g Ca and 4.5 g available P/kg in the broiler starter diet. Broilers are more sensitive to imbalances in mineral supply in their diets (McDevitt *et al.*, 2006). Major ingredients in poultry diets are of plant origin and have approximately 60% - 80% of phosphorus in phytic acid form and/or its salt phytate, rendering it bio-unavailable (Rao *et al.*, 1999; Foidl *et al.*, 2001). A high molar Ca : phytate ratio in the diet may lead to the formation of extremely insoluble Ca-phytate complexes under intestinal conditions, making the phytate molecule inaccessible to the phytase enzyme (Kornegay, 1999). Inadequate dietary sources of calcium result in blood hypocalcaemia, leading to reduced bone strength and mineralization (Talaty *et al.*, 2009a). Typically, supplementation of the phytase enzyme in grain-based poultry diets has become common practice to increase phosphorus bioavailability by 25% - 50%, reduce phosphorus excretion by 15% - 40%, and ensure proper growth and bone development (Harter-Dennis, 1999; Onyango *et al.*, 2006; Coon & Manangi, 2008).

Until recently, the prophylactic use of antibiotic growth promoters in poultry diets improved feed conversion efficiency and further enhanced the digestibility of Ca, P and Mg in the diet. However, after a ban was imposed in the European Union (EU), research efforts to replace them with natural feed additives such as *Moringa oleifera* leaves have increased. *Moringa oleifera* leaves are reported to have potential prebiotic effects and potentially antioxidant phytochemicals, such as chlorogenic acid and caffeic acid (Mbikay, 2012). Caffeic acid has been shown to have powerful inhibitory effects on osteoclastogenesis. Nutritional characterization of dried *M. oleifera* leaves revealed much lower levels of calcium, phosphorus and magnesium contents of 3.65%, 0.3% and 0.5%, respectively (Moyo *et al.*, 2012). Fresh *M. oleifera* leaves have Ca and P contents of 440 mg/100 g and 70 mg/100 g, respectively (Joshi & Mehta, 2010), and up to 21 g/kg phytate (Makkar & Becker, 1997). The underlying effects of the bioactive compounds in *M. oleifera* leaves are not clear. However, they are believed to induce prebiotic effects resulting in increased solubility of minerals because of increased bacterial production of short-chain fatty acids, degradation of mineral complexing phytic acid, and release of bone-modulating factors such as phyto-steroids from the diet (Scholtz *et al.*, 2007). Similar effects have been observed in the presence of antibiotic growth promoters. The current study was therefore designed to assess the potential of using *M. oleifera* leaf meal as an alternative antibiotic growth promoter on BBS, tibia morphology and tibia ash weight when supplemented in broiler diets.

Materials and Methods

The fresh, green and undamaged mature *M. oleifera* leaves used in this study were collected during the month of June from a number of trees from the same village to avoid variations in soil micronutrient content. The leaves were air-dried during the day with no direct sunlight exposure. During drying, the leaves were turned over constantly to avert fungal growth. After 5 days of drying, the leaves were ground to a fine powder to pass through a 0.15 mm sieve. The leaf meal was tightly packaged in polythene plastic bags, sealed, and kept at room temperature until required. The nutrient content of the experimental diets and MOLM samples (Table 1) was determined on the basis of moisture, crude protein, ash and ether extract; as well as the mineral composition according to methods of the Association of Official Analytical Chemists (AOAC, 2000). The techniques described by Van Soest *et al.* (1991) were used to determine neutral detergent fibre (NDF) and acid detergent fibre (ADF) concentrations. The metabolizable energy (ME) content of the diets was calculated based on determined nutrients in the proximate analyses as follows:

[(3.7 × protein) + (8.84 × total fat) + (3.75 × carbohydrates) = 138 kcal/100 g feed]
(× 4.184; to convert kcal to kJ).

The feeding programme consisted of starter (0 to 21 d), grower (22 to 28 d), and finisher (29 to 35 d) basal diets (Table 3), which were formulated to meet the birds' dietary nutrient requirements (NRC, 1994). Each basal feed was split into five treatment (T) groups: T1, positive control, basal diet supplemented with 668 g salinomycin/ton and 500 g albac/ton; T2, T3 and T4, basal diet supplemented with graded levels of MOLM (Table 2), and T5, a negative control, basal diet with no supplementation. Inclusion levels of

M. oleifera leaf meal were increased with change in phase feeding: 1, 3 and 5 g (starter); 3, 9 and 15 g (grower) and 5, 15 and 25 g (finisher) per kg of feed. All diets from starter to finisher were pelleted.

Table 1 Analysed *Moringa oleifera* leaf meal (MOLM) composition

Analysed nutrient composition in g/kg unless where stated	
Metabolizable energy (MJ/kg)	11.4
Crude protein	267.6
Crude fibre	157.2
Ether extract	56.3
Moisture	78.3
Ash	108.1
Acid detergent fibre	137.9
Neutral detergent fibre	200.8
Analysed mineral composition in mg/kg unless where stated	
Phosphorus (g/kg)	3.2
Potassium (g/kg)	24.3
Calcium (g/kg)	28.1
Magnesium (g/kg)	10.1
Sodium (g/kg)	8.0
Iron	192.0
Copper	5.7
Zinc	23.8
Manganese	86.8
Boron	33.1
Aluminium	160.0

Table 2 Dietary inclusion levels of *Moringa oleifera* leaf meal (MOLM)

Phase feeding regimen	Dietary treatments (MOLM g/kg feed)		
	T2	T3	T4
Starter (0 to 21 d)	1	3	5
Grower (22 to 27 d)	3	9	15
Finisher (28 to 35 d)	5	15	25

A total of 2400 day-old Cobb-500 broiler chicks of mixed sex were purchased from a commercial hatchery, weighed and randomly allocated to 30 floor pens containing fresh wood shavings to the depth of 10 cm in an environmentally controlled house. The experiment was a completely randomized design divided into five dietary treatments with six replicate groups of 80 birds per pen (5 diets × 6 replicates). House temperature was set and maintained at 34 ºC during the first week, then reduced by 3 ºC per week until 22 ºC was reached, and was maintained at this level until the end of the experiment. Birds were vaccinated for Marek's disease and infectious bursal disease at hatchery, but no vaccines were administered during rearing. Chicks were inspected daily, and dead birds were removed after recording mortality (pen, date and BW). Feed and fresh water were offered *ad libitum* throughout the 35-day rearing period. Care and management of birds were in accordance with principles of animal care in experimentation (NRC, 1985). The

experiment was subjected to an assessment for its ethical acceptability and approved by the Ethics Committees of the University of Fort Hare (Animal Ethics No.: NKU01-1SWAP01).

At 35 days of age, 12 birds were randomly selected per treatment, two per replicate pen (pen means served as the experimental unit for statistical analysis), and were individually weighed to ascertain whether they represented the average pen weight. Birds were then fasted for 8 hours with water offered *ad libitum*, and were reweighed before they were slaughtered by cervical dislocation following electrical stunning at 70 volts. After bleeding, scalding, plucking and washing, the feet, head and neck were removed. The left and right tibiae from six birds per treatment were randomly chosen; without being de-fleshed, and were individually sealed in plastic bags to minimize moisture loss. Tibiae were stored at −18 ºC for BBS and tibia ash determination, as well as the analyses of their calcium (Ca) and phosphorus (P) composition.

Table 3 Composition of basal starter, grower, and finisher diets on as fed basis

Item	Phase feeding regimen		
	Starter (0 to 21 d)	Grower (22 to 27 d)	Finisher (28 to 35 d)
Feed ingredients (g/kg)			
Fine maize	610.3	664.4	660.4
Soya oilcake	311.5	249.1	253.5
Fishmeal	25.4	33.3	40.0
Sunflower oil	20.0	25.0	16.9
Limestone	14.8	14.0	13.6
Monocalcium phosphate	8.6	7.1	7.6
Methionine	3.1	1.7	2.4
Salt	3.3	2.9	3.1
*Vitamin + mineral premix	1.2	1.2	1.2
Sodium Bicarbonate	0.9		
Choline Chloride	0.8	0.5	0.2
Lysine	0.2	0.6	0.8
Treonine	0.1	0.2	0.3
Analysed composition (g/kg)			
Crude protein	246.2	206.6	200.6
Crude fibre	30.9	14.1	32.8
Ether extract	50.0	54.7	45.2
Calcium	11.2	10.1	9.8
Available phosphorus	7.7	7.8	6.8
Calculated composition			
Metabolizable energy (MJ/kg)	14.08	14.42	13.88

*Supplied per kg of feed: 60 mg iron; 40 mg manganese; 4 mg copper; 70 mg zinc; 0.8 mg iodine; 0.3 mg selenium; 9000 IU vitamin A; 2000 IU vitamin D; 15 IU vitamin E; 2 mg vitamin K; 1 mg vitamin B_{12}; 0.30 mg biotin; 250 mg choline chloride; 0.75 mg folic acid; 20 mg niacin; 5.3 mg pantothenic acid; 7.5 mg pyridoxine; 7.5 mg riboflavin; 2.1 mg thiamine.

When ready for use, tibiae were defrosted overnight at 4 ºC. After thawing, they were de-fleshed and cleaned of all tissue, including cartilage caps (periosteum) by hand, and weighed. The length (cm) of each bone was determined with a ruler (the cross-section area was not calculated). The breaking strength of each tibia was determined by the three-point bending test using an Instron 4502 (Instron Ltd., High Wycombe, UK) material testing machine with default specimen dimensions setup as circular geometry, 10 mm diameter, anvil height of 30 mm and crosshead speed of 50 mm/min. Vertical hydraulic force was applied at the

midpoint of the bone shaft to minimize splintering. The load, defined as force (Newton (N)) of cross-sectional area, represented bone strength, while the modulus measured rigidity, as related to stress and strain.

Table 4 Analysed nutrient composition of the experimental diets and MOLM on dry matter basis in g/kg

Dietary treatment	ME (MJ/kg)	CP (g/kg)	CF (g/kg)	EE (g/kg)	ADF (g/kg)	NDF (g/kg)	P (g/kg)	Ca (g/kg)
				Starter (1 to 21 days)				
T1	13.9	224	37	44	46	110	10	10
T2	13.9	235	40	47	46	105	10	10
T3	13.8	241	46	49	52	102	11	10
T4	14.1	239	37	57	41	111	10	13
T5	14.0	246	37	50	35	109	11	11
				Grower (22 to 27 days)				
T1	14.0	196	43	51	54	134	7.0	10
T2	13.9	196	42	55	46	134	7.0	10
T3	13.9	204	41	54	43	140	8.0	11
T4	13.8	205	40	47	50	122	8.0	11
T5	14.0	207	44	55	55	109	8.0	10
				Finisher (28 to 35 days)				
T1	13.7	217	43	49	59	110	7.0	11
T2	13.9	210	40	50	47	133	7.0	10
T3	13.7	222	36	46	44	119	7.0	11
T4	13.6	218	42	47	56	124	7.0	11
T5	13.8	201	40	45	50	123	7.0	10

T_1, positive control, 668 mg salinomycin, 500 mg zinc bacitracin; T_2, T_3 and T_4, graded levels of MOLM;
T_5: a negative control diet with no supplementation.
CP = crude protein; ME = metabolizable energy; CF = crude fibre; EE = ether extract ADF = acid detergent fibre;
NDF = neutral detergent fibre.

      Sheared tibiae pieces were collected and dried at 100 ºC for 24 hours, cooled in a desiccator, and defatted by soaking in 200 mL anhydrous ether for 24 h to remove moisture, as well as polar and non-polar lipids. The dried fat-free bones were weighed, then ashed in a muffle furnace overnight at 600 ºC. The ash was weighed. Tibiae were analysed for Ca and P composition. Percentage of tibia ash was calculated as (tibia ash weight ÷ tibia dry weight) × 100; and tibia ash index was calculated as tibia ash percentage ÷ tibia length.
      The morphological, physical and chemical data were subjected to analysis of variance (ANOVA) using SPSS (2011) to determine the effect of dietary treatments. Duncan's multiple range test was used to compare treatment means. Data were presented as the least square means with standard error. The bodyweights (BW), BBS, tibia weight (TW), dried tibia weight (DW) and tibia ash mineral content (Ca and P) were regressed against tibia ash percentage. Pearson correlation coefficients between BW, tibia characteristics, tibia ash and mineral content (Ca and P) were calculated using SPSS (2011). Differences were considered significant at $P < 0.05$. The model was:

$$Y_{ij} = \mu + \alpha_i + e_{ij}$$

where Y_{ij} = response variable, μ = the common mean, α_i = the effect of dietary treatment (T1, T2, T3, T4 and T5) and e_{ij} = the random error.

Results and Discussion
      Body weight, average daily gain (ADG), feed intake (FI) and feed : gain ratio (FCR) values at days 7, 21 and 35 are presented in Table 5. There were no differences in BW at placement and at d 35. However, on

d 7 and 21, birds on T4 had the highest BW (P <0.05), while T1 had the lowest, respectively. Similarly, on d 7 and 21, birds on T4 had the highest (P <0.05) ADGs, and were lowest in T1, respectively. The results of the current study indicated that MOLM exerted a feed intake regulating effect in broiler chickens at dietary inclusion levels ranging between 1 and 25 g/kg in starter, grower and finisher diets, respectively. There were no significant dietary effects (P >0.05) in FI on d 7, 21 and 35, while on d 21 and d 35, FCR was significantly higher in birds that were fed diets supplemented with MOLM and was lower in T1 birds (P <0.05).

The present findings seem to agree with previous reports that plant extracts have an influence on FI and FCR. Although in a number of cases FI and FCR were not changed, the extracts had positive effects on BW, ADG and energy utilization (Wallace *et al.*, 2010). Phenolic acids used as additives have beneficial effects on nutrient utilization by stimulating the production of digestive enzymes such as trypsin and amylase (Windisch *et al.*, 2008). Similar findings on growth performance and digestive organ size were observed by Nkukwana *et al.* (2014). Other authors have shown that cinnamic acid, a phenolic acid present in *M. oleifera* leaves, can cause increased glycolysis and increased utilization of glucose for energy production (Kasetti *et al.*, 2012; Mbikay, 2012). This may justify the increased BW and FCR in birds that were supplemented with MOLM in their diets, a response that is often observed with in-feed antibiotic inclusion. On the other hand, dietary cholecalciferol or P was reported to improve BW gain, FI, feed efficiency and percentage tibia ash of broiler chicks (Onyango *et al.*, 2006).

Table 5 Effect of diets supplemented with or without *Moringa oleifera* leaf meal (MOLM) on bodyweight, average daily gain, feed intake and feed conversion ratio (feed : gain) of broilers chickens

Experimental period (d) and parameter	Dietary Treatments					SEM	*P*-value
	T1	T2	T3	T4	T5		
Bodyweight(g)							
0 d	45	46	45	46	46	0.2	0.818
7 d	165[b]	176[ab]	176[ab]	181[a]	172[ab]	1.5	0.010
21 d	888[b]	928[a]	932[a]	955[a]	919[ab]	5.5	< 0.001
35 d	2119	2147	2236	2174	2177	14.0	0.094
Average daily gain (g)							
7d	23.6[c]	25.2[ab]	25.1[ab]	25.8[a]	24.6[bc]	0.1	0.010
21 d	42.3[b]	44.2[a]	44.4[a]	45.5[a]	43.7[ab]	0.3	< 0.001
35 d	60.6	61.3	63.9	62.1	62.2	0.4	0.094
Feed intake (g)							
7 d	153	157	152	158	149	1.4	0.246
21 d	1087	1042	1101	1084	1043	9.0	0.120
35 d	3230	3160	3219	3147	3109	18.9	0.218
Feed : gain (g:g)							
7 d	1.08	1.12	1.16	1.14	1.17	0.01	0.173
21 d	1.22[b]	1.12[a]	1.18[ab]	1.14[a]	1.14[a]	0.01	0.013
35 d	1.53[b]	1.47[ab]	1.44[a]	1.45[a]	1.43[a]	0.01	0.053

[a–c] Means within the same row that do not share a common superscript are significantly different (P <0.05). n = 6. T_1, positive control, 668 mg salinomycin, 500 mg zinc bacitracin; T_2, T_3 and T_4 contained graded levels of MOLM; T_5: a negative control diet with no supplementation.

Table 6 shows the effects of diets supplemented with or without *M. oleifera* leaf meal on tibia morphology (weight and length) before and after drying and removal of fat, as well as on BBS. The dietary supplementation of MOLM had no significant effect on tibia characteristics. No statistical differences were observed on TW among treatment groups, although T1 tibiae were lighter, even after drying. Tibiae from T2 birds were longer than those in other treatments, while those from T4 birds were shorter. Although no treatment effects were observed in the TW/tibia length (TL) ratio, it was higher in T3, T4 and T5; and lower in T1. The dried defatted tibiae (DW) were heavier in T3 (11.20 ± 0.347) and lighter in T1 (9.44 ± 0.562) birds.

Nonetheless, LW, which is the difference between TW and DW, was lowest in T1 and highest in T3, although it was generally higher in tibiae from birds that were supplemented with MOLM. Previously, a depression in BW and tibia ash has been reported in diets with lowest available P and increased Ca levels without phytase supplementation (Yan *et al.*, 2006).

Table 6 Least square means (± standard errors) of tibia characteristics of broilers (35 d) fed diets supplemented with or without *Moringa oleifera* leaf meal (MOLM)

Variable (n = 6)	Dietary treatments					*P*-value
	T1	T2	T3	T4	T5	
TL (cm)	9.8 ± 0.09	10.0 ± 0.04	9.9 ± 0.01	9.6 ± 0.04	9.8 ± 0.25	0.229
TW (g)	10.0 ± 0.60	11.1 ± 0.46	11.7 ± 0.79	11.4 ± 0.86	11.6 ± 0.35	0.322
DW (g)	9.4 ± 0.56	10.5 ± 0.49	11.2 ± 0.35	10.7 ± 0.62	11.1 ± 0.33	0.116
LW (g)	2.4[b] ± 0.74	5.0[a] ± 0.26	5.8[a] ± 0.22	5.5[a] ± 0.24	5.7[a] ± 0.13	< 0.001
TW/TL (g/cm)	1.0 ± 0.06	1.1 ± 0.05	1.2 ± 0.08	1.2 ± 0.09	1.2 ± 0.04	0.274
Tibia ash index	5.0 ± 0.09	4.9 ± 0.03	4.9 ± 0.05	5.0 ± 0.02	4.9 ± 0.12	0.454
BBS (N)	395.0 ± 38.83	346.9 ± 41.26	371.9 ± 24.75	375.8 ± 34.09	338.3 ± 22.86	0.753

[a,b,c,d,e]Means within a row with no common superscript differ significantly (*P* <0.05).
T₁, positive control, 668 mg salinomycin, 500 mg zinc bacitracin; T₂, T₃ and T₄ contained graded levels of *M. oleifera* leaf meal (MOLM); and T₅: a negative control diet with no supplementation.
TW = tibia weight; TL = tibia length; DW = dried defatted tibia weight; LW = loss in tibia weight;
BBS = bone breaking strength.

Usually the greatest need for nonphytate P is for improvement in tibia ash, followed by BW and feed conversion efficiency (Yan *et al.*, 2001), which was partly the case in the current study. Other authors reported an increase in total gain and improvement in tibia breaking strength at four weeks of age when the dietary concentration of nonphytate P was 0.5% in comparison with a diet containing only 0.35% (Shaw *et al.*, 2010). As a result, in instances where phytase is supplemented, the higher BW gain may be attributable to an increase in P availability and possibly feed intake (Rao *et al.*, 1999). In the current study, basal diets in all three phases were formulated to contain 0.5% P, and there were no variations in P concentration between treatments, thus making it difficult to explain the observed performance. There was good agreement between calculated and analysed levels of Ca, but surprisingly the total P concentrations in the starter phase for all experimental diets were 0.3% higher than the formulated specification. This could have contributed to bone strength of birds from an early age; which might explain why there were no differences in tibia characteristics. Generally, broilers have been found to be sensitive to imbalances in mineral supply in their diets (McDevitt *et al.*, 2006).

Despite this, the lack of effect on BBS from dietary MOLM supplementation is difficult to explain, because although calculated and analysed P and Ca levels were not different between treatments (Table 2), the extra Ca and P in Moringa leaves were expected to become systemically available to the birds. Bone ash content and BBS are the most commonly used parameters for assessing bioavailability for Ca and P (EMFEMA, 2002; Shaw *et al.*, 2010). The ability of the bone architecture of an immature growing skeleton to withstand loads was reported to be genetically related, being further influenced by whether feed was restricted or offered *ad libitum* to the birds (Williams *et al.*, 2004). Generally, the P requirement of the chick decreases with age. However, owing to poor utilization, a few studies have focused on P requirements after three or four weeks of age to correspond with increases in feed intake by the broiler when faecal excretion is higher (Yan *et al.*, 2001). Onyango *et al.* (2006) reported an increase in ileal digestibility of dry matter, energy, nitrogen and P, but an accompanying decrease in the digestibility of Ca when the dietary level of P was increased. A similar observation was reported by McDevitt *et al.* (2006), who notes that dry bone weight and mineral content per unit length of modern broiler strains increase in direct proportion to the increase in BW. In contrast, inadequate dietary sources of Ca result in blood hypocalcaemia, leading to reduced bone strength and mineralization (Talaty *et al.*, 2009a).

As with other tibia characteristics, there were no statistical differences in tibia ash percentage (Table 7). Although tibia ash weight was not significantly different between T4 and T1, it was highest in T4 and

lowest in T2 (*P* <0.05). This implies that the increase in dry bone weight and mineral content per unit bone length responds to mechanical forces placed on the bone (McDevitt *et al.*, 2006; Whitehead, 2007; Talaty *et al.*, 2009b). The weight of tibia ash has been found to be a more sensitive indicator of the amount of Ca and P in the diet (Hall *et al.*, 2003). The normal content of starter diets is about 10 g Ca and 4.5 g available P/kg diet (NRC, 1994; Fleming, 2008). In view of the Ca and P levels in T1 and T4, including their Ca : P ratios, it is unlikely that low systemic mineral concentrations are the reason for the observed lower bone ash content in T4 and T5 (Williams *et al.*, 2004).

Thus, the lack of difference in tibia ash percentage and BBS seen in this study suggests limited mineral availability and utilization efficiency at tissue level of birds fed the MOLM-supplemented diets. As a result it may be speculated that the bioavailability of Ca and P was limited in these birds or that birds had a problem in utilizing the minerals quickly enough because of their rapid growth rate (Williams *et al.*, 2000). Furthermore, bioactive compounds in *M. oleifera* leaves have been reported to have a regulatory effect on birds' gastrointestinal tract, resulting in improved nutrient utilization efficiency (Mbikay, 2012), hence an increase in tibia ash percentage of these birds. Because birds in the negative control performed as well as birds in T1 and MOLM-supplemented diets, this agrees with the notion that well-nourished healthy chicks do not respond to antibiotic supplements when they are housed under clean and disinfected conditions (Hernandez *et al.*, 2004). Although the percentage of tibia ash is commonly and routinely used as a measure of bone mineralization, the weight of the tibia ash proved to be a more sensitive indicator of bone mineralization (Hall *et al.*, 2003). Moreover, bone ash can be a misleading measure of bone response because increases in total bone growth are possible without altering the percentage of bone ash (Cheng *et al.*, 2004).

Table 7 Least square means (± standard errors) of tibia ash and mineral composition of broilers (35 d) fed diets supplemented with or without *Moringa oleifera* leaf meal (MOLM)

Variable (n = 6)	Dietary treatments					*P*-value
	T1	T2	T3	T4	T5	
Ca (%)	31.4[b] ± 2.17	29.7[b] ± 0.95	32.2[b] ± 1.82	39.0[a] ± 1.73	30.6[b] ± 0.56	<0.001
P (%)	15.7[c] ± 0.17	16.7[bc] ± 0.19	17.0[b] ± 0.30	17.5[ab] ± 0.23	18.1[a] ± 0.27	<0.001
Ca: P (%)	2.0[ab] ± 0.07	1.8[b] ± 0.06	1.9[ab] ± 0.13	2.2[a] ± 0.10	1.7[b] ± 0.02	0.002
Ash (%)	49.5 ± 0.64	48.7 ± 0.17	48.3 ± 0.53	48.2 ± 0.30	48.0 ± 0.39	0.149
Ash weight (g)	52.0[a] ± 1.49	41.1[b] ± 2.89	50.3[ab] ± 2.12	54.3[a] ± 0.44	49.7[ab] ± 3.43	0.006

[a,b,c] Means within a row with no common superscript differ significantly (*P* <0.05).
T$_1$, positive control, 668 mg salinomycin, 500 mg zinc bacitracin; T$_2$, T$_3$ and T$_4$ contained graded levels of *M. oleifera* leaf meal (MOLM); T$_5$: a negative control diet with no supplementation.
Ca = calcium; P = phosphorus.

The highest tibia Ca content (*P* <0.05) was noted in T4, with T2 having the lowest (39.0 ± 1.73 vs. 29.7 ± 0.95; Table 7) amount. Similarly, T4 had the highest (*P* <0.05) P concentration, and lowest in T1 (17.5 ± 0.23 vs. 15.7 ± 0.17). The highest (*P* <0.05) Ca : P ratio was obtained in T4 (2.24 ± 0.104) and was at its lowest in T5 (1.69 ± 0.017). According to Williams *et al.* (2000), the variations in bone ash Ca and P among treatments bear no resemblance to the composition of these minerals in the diets. Major ingredients in poultry diets are of plant origin, with 60% - 80% of P being in the form of phytic acid, rendering it biologically unavailable, implying that it cannot be utilized. Instead, it is excreted to the environment (Kornegay, 1999; Foidl *et al.*, 2001). Generally, Ca has a relatively high availability from most feedstuffs, but the availability of phosphorus varies largely depending on the source (Waldenstedt, 2006). As a result, in P deficiency, it is unlikely that birds would respond to phytase supplementation if Ca was also limiting (Yan *et al.*, 2006).

Critically, although phytase was not added in the current study, a high molar Ca : phytate ratio in the diet has been reported to result in the formation of extremely insoluble Ca-phytate complexes under intestinal conditions, making the phytate molecule inaccessible to the phytase enzyme (Kornegay, 1999), particularly in weak acid or neutral pH conditions of the gastrointestinal tract of chickens (Pallauf & Rimbach, 1997). Nonetheless, absorption and homeostasis of dietary Ca and P are believed to be maximized at a dietary ratio of 2Ca : 1P ratio (NRC, 1994; Whitehead, 2007). The mature bone mineral, however, is generally accepted to have a structure close to that of the calcium phosphate, hydroxyapatite, which has a

molar Ca : P ratio of 1·67 : 1 (Williams *et al.*, 2000). Surprisingly, the current study shows a Ca : P ratio of 1.69 : 1, which resulted in the lowest tibia ash and breaking strength. On the contrary, while the transient high Ca: P ratio is influenced by dietary available P, it seems unlikely to have any effect on bone strength (Williams *et al.*, 2000).

Tibia Ca was positively correlated with tibia ash percentage, and TW before and after drying, but not with BW, BBS, P content or tibia length (Table 9). The BBS was positively correlated to tibia ash percentage, TW, tibia length and bone phosphorus content, but no significant differences (P <0.05) were observed between these variables. In modern meat-type chickens, bone strength and development are closely linked to the expression of genetic potential for maximum lean muscle deposition (Williams *et al.*, 2004). Overall, the results of the current study suggest that bone mineral content and bone mineral density may be more sensitive indicators of dietary Ca and P concentrations in broilers than tibia breaking strength (Venäläinen *et al.*, 2006).

Furthermore, other factors such as management, nutrition, hygiene, and concurrent diseases may influence leg integrity, both under field and experimental conditions, even when diet contents appear to be adequate (Waldenstedt, 2006). Although Lewis *et al.* (2009) found little probability that photoperiod may contribute to tibial integrity in practical terms, photoperiod does influence bone development during early growth through vitamin D metabolism. Broiler skin plays an important role in vitamin D metabolism, because it contains vitamin D precursors, which can be converted to vitamin D_3 when birds are exposed to ultraviolet light (Onyango *et al.*, 2006). It is common knowledge that in the presence of vitamin D, the systemic availability and utilization of Ca and P are increased. Therefore, under continuous lighting, as in the present study, the observed ash weight, Ca/P ratio, as well as Ca and P in tibia ash could be expected. Nutritionally, another study showed an increase in bone density when broilers were fed diets containing *Solanum glaucophyllum*, a plant containing a glycoside of 1,25-dihydroxyvitamin D when compared with the nonphytate P basal diet group (Cheng *et al.*, 2004).

Table 8 Pearson correlation coefficients (r) among tibia characteristics of broilers (35 d) fed diets supplemented with or without *Moringa oleifera* leaf meal (MOLM)

Variable	BW	TW	DW	LW	TL	BBS	Ca	P	TW/TL
Ash	−0.256	−0.524**	−0.434*	−0.327	0.103	0.042	0.124	−0.499*	−0.532**
BW		−0.070[NS]	0.246[NS]	0.315	0.121	−0.079	−0.050	0.205[NS]	−0.090[NS]
TW			0.667**	0.563***	−0.055	0.216	0.213	−0.098[NS]	0.977***
DW				0.700***	0.038	−0.172	0.357	−0.016[NS]	0.632***
LW					−0.034	−0.059	0.195	0.539**	0.551**
TL						0.227	−0.250	−0.151[NS]	−0.265[NS]
BBS							−0.059	0.029[NS]	0.164[NS]
Ca								0.078[NS]	0.241[NS]
P									−0.044[NS]

NS = non-significant; *P ≤0.05; **P ≤0.01; ***P ≤0.001; Ca = calcium; P = phosphorus; BW = bodyweight; BBS = bone breaking strength; TW = tibia weight; DW = Dried defatted tibia weight; LW = loss in tibia weight; TL = tibia length.

Phosphorus, on the other hand, was positively correlated with LW, TW, DW and TL, while it was negatively correlated with and significantly different from tibia ash percentage (r = −0.499; P <0.01). However, although no correlation or linear relationships were observed between BW at 35 days of age and TW, DW or TL (Tables 8 and 9), a positive correlation (r = 0.667) and a highly significant difference (P <0.01) were observed between TW and DW. Perhaps, the positive correlation observed between BW, dry bone weight and bone length depicts the genetic relationship that exists among these parameters. Tibiae from birds in the positive control, T1, had the lowest (P <0.05) loss in TW following drying and fat removal from these bones, compared with birds that were supplemented with MOLM. Tibia weight loss owing to removal of moisture, polar and non-polar lipids was positively correlated and significantly different to TW (r = 0.563; P <0.01) and DW (r = 0.700; P <0.01). The positive effects of using plant extracts as additives has been attributed to increased solubility of minerals owing to increased bacterial production of short-chain fatty

acids, enlargement of the absorption surface by promoting proliferation of enterocytes and increased expression of calcium binding proteins (Scholz-Ahrens *et al.*, 2007).

Table 9 Linear relationship between tibia ash, bodyweight and tibia characteristics of broilers (35 d) fed diets supplemented with or without *Moringa oleifera* leaf meal (MOLM)

Variable	Equation	R^2	Coefficient		SE		Sig. level
			Intercept	x-variable	Intercept	x-variable	
BW	y = 0.0024x + 2.049	0.87	3.012	−0.017	0.600	0.012	NS
BBS	y = 2.588x + 236.16	0.94	225.664	2.884	631.961	13.024	NS
Ca	y = 0.141x + 25.50	0.86	8.805	0.490	35.849	0.739	NS
P	y = 0.033x +15.33	0.98	37.924	−0.431	6.875	0.142	**
TW	y = 0.054x + 8.479	0.97	47.014	−0.739	11.011	0.227	**
DW	y = 0.042x + 8.488	0.94	34.150	−4.486	9.243	0.190	*
TL	y = 0.010x + 9.134	0.90	8.460	0.028	2.453	0.051	NS

NS: non-significant; *P <0.05; **P <0.01; Ca = calcium; P = phosphorus; BW = bodyweight; BBS = bone breaking strength; TW = tibia weight; DW = dried defatted tibia weight; TL = tibia length.

Conclusion

Commercial meat-type chickens have been developed for improved growth and meat production. However, the skeleton remains a potential weak link relative to providing physical support to heavier BW at a very young age. Bone morphology and mineral density are important factors in measuring bone quality in relation to their support for muscle growth and development. The increased tibia Ca and P contents, Ca/P ratio and ash weight of birds that were supplemented with MOLM indicate that *Moringa oleifera* leaves has potential to be used as an alternative additive to AGPs. The improved BW, ADG and FCR could be a reflection of the attributes induced by bioactive compounds in MOLM to influence intestinal nutrient utilization, thereby improving tibia integrity and inorganic composition of tibia bones. Nonetheless, in future studies utilization efficiency of P and Ca should be measured when MOLM is supplemented in broiler diets. The use of phytase may also be recommended. This would give a clear understanding of the amount of these minerals that are utilized by the bird, and at what rate. It would also be necessary to quantify the excreted levels.

Acknowledgements

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