Article

High Dietary Marula (*Sclerocarya birrea subsp. caffra*) Seed (nut) Cake Induces Detrimental Effects on Performance, Carcass Characteristics and Immuno-Physiology of Broiler Chickens

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Abstract: In a completely randomized design (CRD), 400 day-old Ross 308 broilers were randomly allotted to 5 diets with 0, 5, 10, 15 and 20% MSC, each with 8 replicates of 10. Weekly feed intake (FI), body weight gain (BWG) and feed conversion efficiency (FCE) were calculated whilst haemato-biochemistry was measured at d42. Overall, FI was linearly ($P < 0.05$) and quadratically ($P < 0.01$) decreased by MSC, of which the optimum inclusion was 15%, as BWG was linearly ($P < 0.001$) and quadratically ($P < 0.05$) decreased and FCE linearly decreased ($P < 0.01$) by MSC, of which the optimum dietary inclusion was 10%. Also, MSC linearly and quadratically decreased slaughter weight ($P < 0.001$ and $P < 0.05$, respectively), hot carcass weight ($P < 0.001$ and $P < 0.05$, respectively) and cold carcass weight ($P < 0.001$ and $P < 0.05$, respectively). Similarly, it linearly decreased white blood cells ($P < 0.01$) and lymphocytes ($P < 0.05$) as it linearly ($P < 0.001$) and quadratically ($P < 0.01$) decreased symmetric dimethylarginine (SDMA) and linearly ($P < 0.001$) increased serum cholesterol. In conclusion, up to 10% MSC can be incorporated into broiler diets in replacement of SBM without adverse effects.

Keywords: broiler; marula seed cake; oleic acid; blood indices; broiler health

1. Introduction

Expected to reach 9.4 to 10.1 billion people by 2050 with additional 0 to 2.7 billion by 2100 [1], the rapidly growing world human population, together with concomitant improvements in living standards and incomes, has increased the global demand for food (proteins) especially in low- and middle-income (developing) countries particularly in Africa [2]. To meet this demand, global food production needs to increase by 70% by 2050 [3]. Whilst most consumed protein is derived from plants, global trends indicate animal-derived proteins to gradually become predominant sources of food in these countries, similarly to food consumption trends in the developed world [2, 4]. Indeed, white meat consumption is on an upward trajectory in all regions of the world including Africa [4],

 doi:10.20944/preprints202303.0423.v1

with broiler chickens topping global annual production forecasts at 105.26 million metric tonnes in 2023 and a predicted high growth rate of 1.73% in 2019 – 2023 [5]. Consequently, there has been increased need for feed for production of broiler birds [6]. Among other ingredients, high quality (amino acid-rich) protein source(s) is (are) important to incorporate in the diets of these modern birds to ensure efficient production [7]. Intensive genetic selection for rapid growth and massive meat yields over the past decades have heightened the requirements for dietary amino acids in the birds [8, 9].

Due to its high CP content (400 – 480 g/kg DM) and well-balanced profile of highly digestible amino acids, especially the essential ones, in comparison with other oilseed grains [10], SBM is the preferred protein source for broiler diets in sub-Saharan Africa (SSA) and elsewhere [11]. Notwithstanding, the use of SBM in animal feeding is both economically and environmentally unsustainable. Large-scale soya bean cultivation incurs high variable costs and results to deforestation and biodiversity loss [12]. It also increases its carbon footprint (climate change) and pollution arising from high dependence on usage of chemical fertilizers, fuel, machinery and pesticides [13-15]. Indeed, large-scale soya bean cultivation majorly contributes to greenhouse gas emissions emanating from mineralization of organic matter and emission of nitrogen oxide from nitrogen fertilizers [16]. Further, its production contributes significantly to coastal and riverine eutrophication and acidification [17]. Furthermore, use of SBM as animal feed creates concerns about the sustainability of feeding animals with diets based mostly on imported feed proteins [18]. In addition, dual use of SBM as animal feed and human food worsens the feed/food competition thus impacting food security [19].

Against this background, there is urgent need for investigation of protein-rich alternative feed resources that are not only easily accessible and abundantly available particularly to local resource-limited smallholder farmers but also the production of which is non-destructive to the environment such as MSC. Marula (*Sclerocarya birrea*, A. Rich Hochst. subsp. *caffra* (Sond.), Anacardiaceae family) seed cake (MSC) is an industrial by-product (residue) that remains after oil extraction from the seed kernels [20] of fruits fallen from marula trees that are indigenous and abundantly available throughout most of SSA from Niger to South Africa [21-23]. In light of predicted future increases in climate change-associated frequency and severity of droughts in SSA [24, 25], MSC is an ideal alternative dietary protein source for broiler and other animal diets instead of SBM as it is produced from marula tree plants that are moderately resistant to drought and wide-ranging environmental temperatures (27 to 37 °C), that promote marula seed germination [26, 27], as well as rainfall (400 to 1 000 mm per annum) [28, 29]. This feed resource has recently aroused great research interest mainly in Southern Africa due to its high CP (470 g/kg DM) [20, 30] and essential amino acid content similarly to SBM, except for lysine, and residual oil rich in the *n*-9 monounsaturated fatty acid (MUFA) oleic acid (72% to 85%) [31, 32]. Previously, it has been successfully employed as alternative protein source in the diets of cattle [beef: [20]; dairy: [30]], goats [33], Japanese quails [34], and pigs [35, 36]. Whilst MSC has been used also in broiler diets [32, 37], no studies, however, have investigated its dietary effects on the full repertoire of haemato-biochemical parameters including immuno-physiological biomarkers of the birds. Also, no previous studies involved feeding of MSC-containing diets over the full production cycle (day 1 to 42) of broiler chickens. Therefore, the objective of this study was to investigate effects of dietary incremental levels (0 to 20%) of MSC as replacement for SBM on growth performance, internal organs, and carcass characteristics, as well as haemato-biochemical parameters of broiler chickens during the starter (d1 to d14), grower (d15 to d28) and finisher (d29 to d42) phases.

2. Materials and Methods

2.1. The Site description and ethical approval

The rearing and slaughter of birds used in this study was approved by the North-West University (NWU) Animal Production Sciences Research Ethics Committee (Ethical

 doi:10.20944/preprints202303.0423.v1

clearance number: NWU-00806 -22-A5). The study was conducted at NWU Experimental Farm (Molelwane) during the summer season (October – November 2022). The farm is located (coordinates: 25°40.459'S, 26°10.563'E) outside Mahikeng City in the Mahikeng Local Municipality in Ngaka Modiri Molema District, North-West Province of South Africa.

2.2. Sourcing of marula seed cake and chemical analysis

The MSC was obtained from The Marula Company in Phalaborwa, Limpopo Province, South Africa. On arrival, 100g of MSC was milled using a laboratory mill (screen size: 1 mm) and stored in sealed labelled polyethylene bags at room temperature for chemical analysis. It was then analyzed for dry matter (DM) (method 930.15), ash (method 942.05), EE (method 920.39) and CP (method 954.01) following the guidelines of the Association of Official Analytical Chemists [38] whilst NDF, ADF and ADL were analyzed following procedures of Van Soest et al. [39]. The DM was determined by putting 1 g of sample into pre-weighed crucibles and oven-drying it at 105 °C for 12 h after which it was placed in a desiccator for cooling and then weighed. The DM was then calculated as the difference between the initial sample weight and moisture weight. The ash content was determined by calcinating a dry sample (1 g) in the muffle furnace (Nabertherm GmbH, Germany) for 6 h at 550 °C. Then the OM content was determined by subtracting the % ash from 100%. The EE was determined by extracting crude fat with ether using an automated Soxhlet Fat analyzer ANKOMXT15 extractor following operator's manual (ANKOM Technology, Macedon, NY, USA). The CP content was determined following the Kjeldahl method by analyzing the nitrogen content of each sample and multiplying it by a factor of 6.25. The NDF and ADF were analyzed using an automated ANKOMDELTA fiber analyzer (ANKOM Technology, Macedon, NY, USA). The residue bags of ADF were then immersed in 72% (wt/wt) H_2SO_4 for 3 hours to determine the ADL content. The condensed tannins (CTs) were analyzed according to the method described by Makkar [40]. Briefly, CTs were extracted from 200 mg of plant material using 10 mL of aqueous acetone (70%). Then 0.5 mL of the tannin extract was mixed with 3 mL of butanol-HCl and 0.1 mL of ferric reagent in a test tube. The test tubes were vortexed and subjected to heating on a block at 100 °C for 60 min and allowed to cool at room temperature. The absorbance was then recorded at 550 nm and CTs in % DM as leucocyanidin equivalents were calculated using the following equation:

$$\text{Condensed tannins (\% DM)} = (\text{Absorbance} \times 78.26)/(\text{\% dry matter}) \quad (1)$$

2.3. Diets formulation, experimental design, and management

Five iso-caloric and iso-nitrogenous diets were formulated such that SBM products were replaced with incremental levels of MSC at 0, 5, 10, 15, and 20% to meet the nutritional requirements of broiler chickens at starter (day 1 – 14), grower (day 15 – 28), and finisher (day 29 – 42) phases (Table 1). In a CRD, 400 day-old Ross 308 broiler chicks with average initial weight of 45.61 ± 0.674 g were randomly allocated to the dietary treatments, each with 8 replicate pens of 10 birds (pen: 1.8m high x 1.5m long x 1.5m wide). Each pen had 5 males and 5 females. The experiment was carried out in a deep litter system in a broiler house where temperature and ventilation were manually controlled by opening the curtains during the day (8h00 to 17h00). Each pen had 1 feeder and 1 drinker. On placement, chicks were offered stress pack which provides vitamins and electrolytes for the first 48 hours. Fresh feed and water were offered *ad libitum* throughout the feeding trial.

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Table 1. Ingredient and nutrient composition (g/kg on as-fed basis, unless otherwise stated) of experimental starter (day 1 – 14), grower (day 15 – 28), and finisher (29 – 42) diets.

Ingredients (g/kg)	Starter					Grower					Finisher				
	0	**5**	**10**	**15**	**20**	**0**	**5**	**10**	**15**	**20**	**0**	**5**	**10**	**15**	**20**
Yellow maize	587.7	604.4	545.0	472.4	409.2	594.8	646.7	605.4	528.0	480.3	604.5	642.4	661.0	595.4	532.5
Marula seed cake	0.0	50.0	100.0	150.0	200.0	0.0	50.0	100.0	150.0	200.0	0.0	50.0	100.0	150.0	200.0
Soya bean full fat	124.9	0.0	0.0	0.0	0.0	211.8	21.7	0.0	0.0	0.0	150.0	128.6	0.0	0.0	0.0
Soya bean meal 46.5%	255.0	252.7	125.0	100.6	100.9	165.8	251.9	104.8	117.8	104.6	190.4	149.9	147.4	65.6	66.1
Sunflower oilcake 34%	0.0	59.8	150.0	76.1	0.0	0.0	0.0	148.3	15.0	0.0	0.0	0.0	60.4	76.6	0.0
Wheat bran	0.0	0.0	44.6	163.8	217.7	0.0	0.0	08.6	155.1	104.7	0.0	0.0	0.0	78.7	125.8
Crude soya bean oil	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	27.0	0.0	0.0	0.0	0.0
Silica	0.0	0.0	0.0	0.0	35.1	0.0	0.0	0.0	0.0	76.3	0.0	0.0	0.0	0.0	40.2
L-Lysine	0.0	1.0	3.4	4.0	4.2	0.0	1.1	4.1	4.1	4.6	0.0	0.6	02.5	4.1	4.4
DL-Methionine	1.1	0.6	0.3	0.5	0.0	0.6	0.9	0.5	0.8	0.0	0.8	0.6	0.3	0.3	0.4
L-Threonine	0.0	0.0	0.0	0.4	0.0	0.0	0.0	0.3	0.7	0.0	0.0	0.0	0.0	0.3	0.7
L-Tryptophan	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.1	0.0	0.0	0.0	0.0	0.1	0.3
Limestone, fine	13.9	13.7	13.4	13.8	13.9	12.7	12.7	12.3	12.8	12.5	13.1	13.1	12.9	13.0	13.0
Mono-dicalcium phosphate	9.9	10.4	10.6	10.7	11.4	7.4	8.0	8.3	8.4	9.9	7.7	8.4	9.0	9.2	9.9
Fine salt	2.8	0.3	2.3	2.1	2.1	2.8	2.8	2.1	2.1	2.1	2.8	2.8	2.7	2.1	2.1
Sodium bicarbonate	1.0	1.0	1.7	2.0	2.0	1.0	1.0	2.0	2.0	2.0	1.0	1.0	1.2	2.0	2.0
Choline chloride	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3
Betaine	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3
Quantum blue 10000G	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
BSGF premix	3.0	3.0	3.0	3.0	3.0	2.5	2.5	2.5	2.5	2.5	2.0	2.0	2.0	2.0	2.0
Total	1000	1000	1000	1000	1000	1000	1000	1000	1000	1000	1000	1000	1000	1000	1000
Chemical composition															
Dry matter	879.6	881.3	885.4	887.6	894.2	879.9	880.1	884.6	886.0	898.6	881.9	881.2	883.0	886.3	893.6
Crude protein	210.0	210.0	210.0	210.0	210.0	200.0	200.0	200.0	200.0	200.0	190.0	190.0	190.0	190.0	190.0
Ether extract	49.9	44.1	60.3	76.9	92.2	65.9	48.8	60.9	77.8	91.1	81.0	68.0	61.7	78.1	93.0
Crude fibre	28.7	35.2	52.9	52.9	46.7	30.5	26.8	49.2	42.9	36.3	28.0	28.9	35.4	45.1	38.1
Ash	42.2	43.4	45.6	46.7	46.8	38.5	38.6	41.0	41.7	41.0	37.4	37.7	38.7	40.4	40.3
ME (MJ/Kg)	11.5	11.5	11.5	11.5	11.5	12.0	12.0	12.0	12.0	12.0	12.5	12.5	12.5	12.5	12.5
Calcium	10.0	10.0	10.0	10.0	10.0	9.0	9.0	9.0	9.0	9.0	9.0	9.0	9.0	9.0	9.0
Total phosphorus	5.9	6.5	7.5	8.1	8.4	5.2	5.6	6.6	7.2	7.2	5.1	5.5	6.2	7.0	7.3
Potassium	9.8	8.6	7.4	7.0	6.3	9.3	8.2	6.7	6.6	5.3	8.8	7.7	6.4	5.6	4.8
Sodium	1.6	1.6	1.6	1.6	1.6	1.6	1.6	1.6	1.6	1.6	1.6	1.6	1.6	1.6	1.6
Chloride	2.2	2.4	2.6	2.6	2.6	2.7	2.4	2.6	2.6	2.6	2.2	2.3	2.6	2.6	2.0

ME = metabolizable energy.

 doi:10.20944/preprints202303.0423.v1

2.4. Feed intake and growth performance measurements

Birds were weekly weighed between 08h00 and 10h00 throughout the feeding trial. Weekly body weights, and daily amounts of feed offered and leftovers were recorded. Initial live weights were measured at arrival and subsequently weekly by weighing all the birds in each pen until week 6. Then BWG (g/bird/week) was calculated by subtracting the initial live weight (g) from the new weight (g) divided by the number of birds per pen. Daily FI (g/day) was calculated by subtracting the weight (g) of the leftover feed from the weight (g) of feed offered divided by the number of birds per pen. The daily FIs were then converted into weekly averages of FI (g/bird/week) by combining pen averages over 7 days whilst FCE was calculated by dividing the weekly BWG by the weekly FI.

2.5. Haemato-biochemistry analysis

A day before slaughter (day 42), blood samples for haematology and serum biochemistry analysis were collected from 16 birds per treatment (2 birds per pen) in the morning under veterinary supervision. Blood was collected from the wing vein with a 21-gauge needle and placed into purple-top EDTA-coated vacutainer tubes for haematological analysis. Red blood cells, hematocrit, hemoglobin, mean corpuscular volume, mean corpuscular hemoglobin, red cell distribution width, reticulocytes, white blood cells, neutrophils, lymphocytes, monocytes, eosinophils, basophils, platelets, and platelet distribution width were analyzed using an automated IDEXX LaserCyte Hematology Analyzer (IDEXX Laboratories (Pty) Ltd., Johannesburg, South Africa). For serum biochemistry analysis, blood samples were collected into red top Vacuette® Serum Clot Activator tubes without EDTA (Greiner Bio-One, GmbH, Frickenhausen, Germany). Serum biochemical parameters including glucose, SDMA, urea, phosphate, calcium, total protein, albumin, globulin, albumin/globulin, alanine transaminase, alkaline phosphatase, gamma-glutamyl transferase, total bilirubin, cholesterol, amylase, and lipase were analyzed using an automated IDEXX Vet Test Chemistry Analyzer (IDEXX Laboratories (Pty) Ltd., Johannesburg, South Africa).

2.6. Slaughter procedure and carcass characteristics measurements

In the evening of day 42, all birds were fasted for 12h (18h00 to 06h00) whilst provided with clean drinking water ad libitum. After group-weighing per pen to obtain the final live weights, birds were transported to Rooigrond abattoir, located ~30 km from NWU experimental site. An hour upon arrival at the abattoir, the birds were sacrificed humanely by cervical dislocation after electrical stunning (70 volts). The jugular vein was cut with a sharp knife at the base of the throat and allowed to bleed for 5 mins. Following thorough bleeding, they were plucked and washed. The heads, necks, and feet were removed. Visceral organs [liver, spleen, heart, gizzard, and intestine (duodenum, jejunum, ileum, colon, and caecum)] were removed by hand through an opening from the vent to the sternum and weighed individually. Hot carcass weight (HCW) was recorded immediately after slaughter at the abattoir and the cold carcass weight (CCW) was recorded 24 h post chilling (4 °C). Subsequently, the chilling loss was calculated and expressed as percentage using the following formula:

$$\text{Chilling loss (\%)} = [\text{HCW (g)} - \text{CCW (g)} / (\text{HCW (g)}] \times 100\% \quad (2)$$

The carcass cuts (breast, wing, thigh, and drumstick) were then removed by cutting from the joints of the carcass and through the shoulder area to remove the backbone from the breast. All cuts were then weighed, and the yields were calculated and expressed as percentages of live bodyweight.

2.7. Statistical analysis

Weekly FI, BWG and FCE data were analyzed using the repeated measures option in the General Linear Model (GLM) procedure whilst overall FI, BWG and FCE as well as haemato-biochemistry, internal organs and carcass characteristics data were analyzed using the PROC GLM procedure of SAS [41]. The least square means (LSMEANS) were

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compared using the probability of difference (PDIFF) option in the LSMEANS statement [41] and differences among them were deemed significant at $P \leq 0.05$. Thereafter, polynomial contrasts (PROC RSREG) were used to assess data for linear and quadratic effects. Response surface regression analysis [41] was performed to estimate the optimum inclusion level of MSC according to the quadratic model: $y = ax^2 + bx + c$, where y = response variable; a and b = coefficients of the quadratic equation; c = intercept; x = MSC level (%); and $-b/2a$ = x value for optimal response.

3. Results

3.1. Proximate composition of MSC

The proximate composition of MSC is shown in Table 2. The results indicated MSC with a notably high CP (471.8 g/kg DM) and OM contents, with low levels of EE, ash, fiber (NDF, ADF and ADL) and CTs.

Table 2: Chemical composition of MSC (g/kg DM).

Nutrient	Composition
Dry matter	938.7
Crude protein	471.8
Ether extract	168.2
Ash	75.1
Organic matter	924.9
Neutral detergent fibre	122.1
Acid detergent fibre	62.3
Acid detergent lignin	27.4
Condensed tannins	1.076

3.2 Effect of MSC on growth performance

Table 3 shows the effect of incremental levels of MSC on weekly and overall FI, BWG and FCE. Regarding FI, dietary MSC inclusion induced a linear [$y = -0.009(\pm0.047)x + 139.690(\pm4.112)$, $R^2 = 0.174$] decrease ($P < 0.01$) in this parameter in week 1, a quadratic [$y = -0.243(\pm0.094)x^2 + 4.073(\pm1.967)x + 225.874(\pm8.304)$, $R^2 = 0.146$] decrease ($P < 0.05$) in week 2, a linear [$y = -0.558(\pm0.169)x + 395.262(\pm14.926)$, $R^2 = 0.094$] and quadratic [$y = -0.558(\pm0.169)x^2 + 8.921(\pm3.536)x + 395.262(\pm14.926$, $R^2 = 0.205$] decrease ($P < 0.05$ and $P < 0.01$, respectively) in week 3, a quadratic [$y = -0.862(\pm0.248)x^2 + 16.392(\pm5.179)x + 589.079(\pm21.861)$, $R^2 = 0.244$] decrease ($P < 0.01$) in week 4 and both a linear [$y = -0.804(\pm0.269)x + 806.622(\pm23.703)$, $R^2 = 0.175$] and quadratic [$y = -0.804(\pm0.269)x^2 + 11.116(\pm5.616)x + 806.622(\pm23.703)$, $R^2 = 0.160$] decrease ($P < 0.01$, for both contrasts) in week 5. On the other hand, there was no effect ($P > 0.05$) of dietary MSC inclusion on FI in week 6. However, analysis of overall FI showed this parameter to have been linearly [$y = -2.738\ (\pm0.950)x + 3157.385(\pm83.652)$, $R^2 = 0.1051$] and quadratically [$y = -2.738(\pm0.950)x^2 + 41.795(\pm19.819)x + 3157.385(\pm83.652)$, $R^2 = 0.1640$] decreased ($P < 0.05$ and $P < 0.01$, respectively) by dietary inclusion of incremental levels of MSC. In terms of FI, the optimum dietary inclusion level of MSC was 15% beyond which there was a sharp decrease in this parameter.

Concerning BWG, dietary inclusion of MSC induced a linear [$y = -0.047(\pm0.027)x + 91.881(\pm2.406)$, $R^2 = 0.5116$] decrease ($P < 0.001$) in this parameter in week 1, a linear [$y = -0.393(\pm0.159)x + 284.192(\pm13.989)$, $R^2 = 0.212$] and quadratic [$y = -0.393(\pm0.159)x^2 + 4.651(\pm3.314)x + 284.192(\pm13.989)$, $R^2 = 0.112$] decrease ($P < 0.01$ and $P < 0.05$, respectively) in week 3, a quadratic [$y = -0.789(\pm0.169)x^2 + 16.167(\pm3.518)x + 322.166(\pm14.848)$, $R^2 = 0.370$] decrease ($P < 0.001$) in week 4, and a linear [$y = -0.288(\pm0.228)x + 504.403(\pm20.086)$, $R^2 = 0.315$] decrease ($P < 0.001$) in week 5. However, there was no effect ($P > 0.05$) of dietary MSC inclusion on BWG in weeks 2 and 6. Notwithstanding, analysis of overall BWG showed this parameter to have been linearly [$y = -1.355(\pm0.629)x + 2066.411(\pm55.388)$, $R^2 =$

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0.328] and quadratically [y = -1.355(±0.629)x^2 + 10.295(±13.122)x + 2066.411(±55.388), R^2 = 0.0748] decreased ($P < 0.001$ and $P < 0.05$, respectively) by dietary inclusion of increasing levels of MSC. Regarding BWG, the optimum dietary inclusion level of MSC was found to be 10% beyond which there was a decrease in this parameter (Table 3).

As far as FCE is concerned, inclusion of dietary incremental levels of MSC linearly decreased this parameter in broilers in week 1 [y = -0.0003(±0.0002)x + 0.662(±0.016), R^2 = 0.279; $P < 0.001$], week 3 [y = -0.00006(±0.0002)x + 0.712(±0.020), R^2 = 0.195; $P < 0.01$], and week 5 [y = 0.0002(±0.0002)x + 0.625(±0.016), R^2 = 0.2170; $P < 0.01$]. Similarly, MSC inclusion quadratically decreased FCE in broilers in week 4 [y = -0.0005(±0.0002)x^2 +0.011(±0.003)x + 0.546(±0.014), R^2 = 0.163; $P < 0.01$]. Otherwise, similarly to BWG, there was no effect ($P > 0.05$) of dietary MSC on FCE in weeks 2 and 6. Notwithstanding, analysis of overall performance data showed a linear [y = -0.005(0.003)x + 0.656(0.013), R^2 = 0.2198] decrease ($P < 0.01$) in FCE with the inclusion of increasing levels of MSC in broiler diets. Overall, our FCE data showed the optimum dietary inclusion level of MSC to be 10% beyond which there was a decrease in the efficiency of broilers to convert feed into body weight (Table 3). Interestingly, results also showed no effects ($P > 0.05$) of dietary MSC at all inclusion levels including at 20% on all performance parameters (FI, BWG and FI) in broilers in week 6 of the study (Table 3).

Table 3. Effect of dietary inclusion of MSC on weekly and overall feed intake, body weight gain and feed conversion efficiency of broiler chickens.

Parameter	Week	Dietary inclusion of MSC (%)					SEM	*P*-value	
		0	5	10	15	20		Linear	Quadratic
FI (g/bird)	1	137.95^{a}	140.52^{a}	131.27^{a}	126.92^{ab}	125.50^{b}	4.406	0.0084	0.8510
	2	225.98^{ab}	238.29^{a}	247.26^{a}	227.50^{ab}	211.63^{b}	8.988	0.1653	0.0141
	3	394.61^{ab}	425.07^{a}	435.11^{a}	395.71^{ab}	353.27^{b}	16.210	0.0317	0.0022
	4	591.32^{b}	636.09^{ab}	693.48^{a}	618.69^{b}	578.63^{b}	23.008	0.5639	0.0013
	5	805.49^{a}	845.47^{a}	846.03^{a}	777.47^{ab}	713.24^{b}	25.698	0.0035	0.0050
	6	1005.29	996.22	989.60	983.21	928.31	30.465	0.0843	0.4168
BWG (g/bird)	1	90.60^{a}	92.71^{a}	86.09^{a}	77.13^{b}	72.26^{b}	2.537	<.0001	0.0966
	2	137.89	139.37	154.42	131.34	124.37	7.524	0.1533	0.0610
	3	279.99^{ab}	302.17^{a}	302.99^{a}	245.65^{b}	228.18^{b}	14.678	0.0016	0.0182
	4	326.00^{b}	370.14^{b}	421.40^{a}	378.46^{ab}	331.69^{b}	15.742	0.6953	<.0001
	5	503.77^{a}	493.42^{a}	492.50^{a}	423.24^{b}	396.74^{b}	21.536	0.0002	0.2155
	6	717.97	698.18	601.77	614.61	595.86	47.733	0.3350	0.5299
FCE	1	0.66^{a}	0.66^{a}	0.66^{a}	0.61^{b}	0.58^{b}	0.017	0.0004	0.1125
	2	061	0.58	0.62	0.58	0.59	0.018	0.5260	0.8758
	3	0.70^{a}	0.71^{a}	0.70^{a}	0.62^{b}	0.65^{b}	0.019	0.0049	0.7768
	4	0.55^{b}	0.58^{ab}	0.61^{a}	0.61^{a}	0.58^{ab}	0.016	0.0925	0.0083
	5	0.63^{a}	0.58^{a}	0.58^{a}	0.54^{b}	0.56^{b}	0.018	0.0024	0.2770
	6	0.72	0.71	0.62	0.63	0.64	0.053	0.1517	0.4020
Overall FI (g/bird)		3158.64^{a}	3281.67^{a}	3342.76^{a}	3129.56^{a}	2910.59^{b}	90.822	0.0268	0.0066
Overall BWG (g/bird)		2056.22^{a}	2095.98^{a}	2059.18^{a}	1870.42^{b}	1749.10^{b}	59.720	<.0001	0.0379
Overall FCE		0.65^{a}	0.64^{a}	0.62^{ab}	0.59^{b}	0.60^{b}	0.039	0.0025	0.4808

Means in the same row with different superscripts (^{ab}) are significantly different. BWG = body weight gain, FCE = feed conversion efficiency, FI = feed intake, MSC = marula seed cake, and SEM = standard error of the mean.

3.3 Effect of MSC on internal organs and carcass characteristics

The internal organs and carcass characteristics of broiler chickens fed diets supplemented with varying inclusion levels of MSC are shown in Tables 4 and 5. There were neither linear nor quadratic effects ($P > 0.05$) of MSC on the weights and lengths of all internal organs (Table 4). Similarly, there were neither linear nor quadratic effects ($P >$

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0.05) of MSC on carcass characteristics, except for the slaughter weight, HCW and CCW (Table 5). In this regard, dietary inclusion of MSC linearly and quadratically decreased the slaughter weights ($y = -1.356(\pm0.631)x + 2112.052(\pm55.551)$, $R^2 = 0.328$, $P < 0.001$; and $y = -1.356(\pm0.631)x^2 + 10.315(\pm13.161)x + 2112.052(\pm55.551)$, $R^2 = 0.075$, $P < 0.05$; respectively), HCW [$y = -1.026(\pm0.453)x + 1482.653(\pm39.901)$, $R^2 = 0.301$, $P < 0.001$; and $y = -1.026(\pm0.453)x^2 + 9.082(\pm9.454)x + 1482.653(\pm39.901)$, $R^2 = 0.085$, $P < 0.05$; respectively] and CCW [$y = -1.001(\pm0.451)x + 1451.337(\pm39.703)$, $R^2 = 0.3089$, $P < 0.001$; and $y = -1.001(\pm0.451)x^2 + 8.464(\pm9.406)x + 1451.337(\pm39.703)$, $R^2 = 0.0812$, $P < 0.05$; respectively] of broilers in response to increasing dietary inclusion levels of MSC.

Table 4. Effect of dietary MSC inclusion on weights and lengths of internal organs of broiler chickens.

Parameters	Dietary inclusion of MSC (%)					SEM	*P*-value	
	0	5	10	15	20		Linear	Quadratic
Liver (g)	35.35	39.01	39.53	36.42	33.31	2.700	0.4282	0.0896
Spleen (g)	2.36	2.74	2.58	2.13	2.35	0.295	0.4944	0.5755
Proventriculus (g)	8.70	8.82	8.14	8.66	8.25	0.502	0.5031	0.9381
Gizzard (g)	37.00	40.15	36.50	35.15	39.97	1.951	0.8836	0.4591
Duodenum weight (g)	16.34	17.02	16.45	15.70	14.61	1.458	0.2934	0.4870
Jejunum weight (g)	29.68	30.42	26.03	24.88	26.01	4.284	0.4938	0.5605
Ileum weight (g)	25.99	24.26	24.63	23.07	22.22	1.955	0.1563	0.9819
Caecum weight (g)	10.94	13.18	16.76	10.91	12.27	2.667	0.9635	0.2696
Colon weight (g)	2.23	2.20	2.28	1.96	2.64	0.287	0.5308	0.3513
Duodenum length (cm)	28.81	30.21	30.39	29.78	30.22	1.484	0.6063	0.6206
Jejunum length (cm)	61.49	65.48	65.51	61.87	60.40	2.453	0.4514	0.1142
Ileum length (cm)	66.89	73.16	73.38	68.03	68.48	15.595	0.1911	0.3950
Caecum length (cm)	18.88	18.74	18.60	18.44	17.95	0.616	0.3651	0.6174
Colon length (cm)	4.83	4.79	5.28	4.75	5.11	0.402	0.6790	0.8939

MSC = marula seed cake, SEM = standard error of the mean.

Table 5. Effect of dietary MSC inclusion on carcass characteristics of broiler chickens.

Parameters	Dietary inclusion of MSC (%)					SEM	*P*-value	
	0	5	10	15	20		Linear	Quadratic
SW (g/bird)	2101.79^{a}	2141.79^{a}	2104.99^{a}	1915.72^{b}	1794.64^{b}	59.894	<.0001	0.0382
HCW (g)	1478.57^{ab}	1504.47^{a}	1489.28^{a}	1361.61^{b}	1264.29^{b}	43.208	0.0001	0.0296
CCW (g)	1446.62^{a}	1472.38^{a}	1452.97^{a}	1326.67^{b}	1230.75^{b}	42.999	0.0001	0.0327
Chilling loss (%)	2.17	2.14	2.46	2.56	2.67	0.189	0.0607	0.9205
Dressing %	70.47	70.22	70.73	71.03	70.45	0.618	0.6907	0.7064
Breast (%)	12.85	10.47	12.86	12.14	12.89	0.667	0.3412	0.7603
Drumstick (%)	5.03	4.64	5.15	4.93	4.94	0.211	0.8706	0.9064
Thigh (%)	6.30	5.26	6.11	5.80	6.08	0.199	0.8816	0.0906
Wing (%)	4.01	3.53	4.17	3.89	4.10	0.155	0.3181	0.4674
Back length (cm)	20.02	18.35	30.56	18.74	18.91	4.607	0.9035	0.2559

Means in the same row with different superscripts (^{abc}) are significantly different. SW = slaughter weight, HCW = hot carcass weight, CCW = cold carcass weight, MSC = marula seed cake, SEM = standard error of the mean.

3.4 Effect of MSC on hemato-biochemistry

The hematological responses of broiler chickens to dietary graded levels of MSC are shown in Table 6. The results demonstrated no effects ($P > 0.05$) dietary MSC inclusion on all hematological parameters, except for white blood cells and lymphocytes. In this regard, MSC inclusion in broiler diets linearly decreased white blood cells [$y = -0.034(\pm0.024)x + 13.230(\pm2.021)$, $R^2 = 0.219$, $P < 0.01$] and lymphocytes [$y = -0.016(\pm0.014)x + 4.635(\pm1.192)$,

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R^2 = 0.154, P < 0.05]. The optimum inclusion level of MSC showed to be 10% for white blood cells and 5% for lymphocytes.

Table 6. Effect of dietary inclusion of MSC on hematological parameters of broiler chickens.

Parameter	Dietary inclusion of MSC (%)					SEM	*P*-value	
	0	5	10	15	20		Linear	Quadratic
Red blood cells (×10^{12}/L)	1.39	1.39	1.08	1.18	1.29	0.153	0.3853	0.2817
Hematocrit (L/L)	8.11	8.35	6.57	7.29	7.69	0.486	0.0728	0.5995
Hemoglobin (g/dL)	9.03	10.34	7.39	7.54	13.13	0.808	0.0692	0.6981
MCV (fL)	42.500	55.35	42.09	46.13	49.66	2.896	0.2591	0.9182
MCH (pg)	35.91	46.04	36.68	38.76	43.75	2.817	0.3553	0.9697
White blood cells (×10^{9}/L)	9.15^{ab}	14.85^{a}	11.58^{a}	8.23^{b}	8.57^{b}	2.053	0.0093	0.1595
Neutrophils (×10^{9}/L)	3.15	5.83	5.29	4.06	4.07	0.911	0.1932	0.0855
Lymphocytes (×10^{9}/L)	2.73^{b}	5.89^{a}	2.84^{b}	1.93^{b}	2.12^{b}	0.997	0.0343	0.2527
Monocytes (×10^{9}/L)	1.55	2.43	2.81	1.78	1.85	0.698	0.3159	0.3335
Eosinophils (×10^{9}/L)	0.56	0.59	0.55	0.38	0.47	0.144	0.1522	0.4057
Basophils (×10^{9}/L)	0.06	0.08	0.09	0.09	0.05	0.018	0.1582	0.2006
Platelet (K/µL)	150.19	28.13	90.19	76.94	177.50	24.505	0.7122	0.1187
PDW (%)	18.61	14.30	12.78	11.99	13.16	1.412	0.0769	0.8512

Means in the same row with different superscripts (ab) are significantly different. MCH = mean corpuscular hemoglobin, MCV = mean corpuscular volume, PDW = platelet distribution width, MSC = marula seed cake, and SEM = standard error of the mean.

The effects of dietary incorporation of graded levels of MSC on serum biochemical parameters of broiler chickens are shown in Table 7. Results showed no effect (P > 0.05) of dietary MSC on all serum biochemical parameters, except for SDMA and cholesterol. In this connection, dietary MSC linearly [y = 0.043(±0.014)x + 26.089(±1.322), R^2 = 0.263, P < 0.001] and quadratically [y = 0.043(±0.014)x^2 -1.213(±0.307)x + 26.089(±1.322), R^2 =0.1478, P < 0.01] decreased the serum SDMA concentrations as the marula by-product level increased from 0 to 15% beyond which it increased from 15 to 20%. In contrast, serum cholesterol concentrations showed a linearly [y = 0.0002(±0.0012)x +2.281(±0.107), R^2 = 0.418, P < 0.001] increasing response to increasing dietary inclusion levels of MSC.

Table 7. Effect of dietary MSC inclusion on serum biochemistry of broiler chickens.

Parameters	Dietary inclusion of MSC (%)					SEM	*P*-value	
	0	5	10	15	20		Linear	Quadratic
Glucose (mmol/L)	6.77	6.89	7.11	7.58	6.31	0.618	0.7945	0.3096
SDMA (µg/dL)	24.81^{a}	23^{a}	17.13^{b}	14.94^{b}	19.38^{c}	1.288	0.0004	0.0055
Urea (mmol/L)	0.60	0.60	0.94	0.64	1.21	0.302	0.1949	0.6993
Phosphate (mmol/L)	4.01	3.92	3.96	3.84	3.95	0.207	0.8650	0.9039
Calcium (mmol/L)	2.38	2.38	2.38	2.38	2.41	0.028	0.5402	0.3899
Total protein (g/L)	36.56	33.88	36.56	31.69	35.63	1.846	0.7380	0.9870
Albumin (g/L)	14.44	13.63	14.38	13.38	14.63	0.630	0.3311	0.7323
Globulin (g/L)	22.19	20.13	22.00	18.38	21.25	1.243	0.9587	0.8870
Albumin/globulin	0.66	0.69	0.66	0.74	0.69	0.017	0.2273	0.7167
Alanine transaminase (U/L)	27.81	25.56	24.25	29.00	28.75	3.047	0.2092	0.7226
Alkaline phosphatase (U/L)	680.25	693.06	743.88	937.50	686.38	90.729	0.5163	0.3411
Total bilirubin (µmol/L)	16.63	9.19	16.63	11.19	10.38	3.563	0.9456	0.4044
Cholesterol (mmol/L)	2.39^{b}	2.44^{b}	2.67^{ab}	2.65^{b}	3.01^{a}	0.121	<.0001	0.8563
Amylase (U/L)	400.25	477.13	389.06	536.06	304.06	75.174	0.7409	0.1356
Lipase (U/L)	208.44	208.88	187.44	405.44	232.69	83.141	0.3949	0.7378

Means in the same row with different superscripts (^{abc}) are significantly different. MSC = marula seed cake, SDMA = symmetric dimethylarginine, SEM = standard error of the mean.

 doi:10.20944/preprints202303.0423.v1

4. Discussion

This study was undertaken to investigate MSC as an alternative to the economically and environmentally unsustainable conventional SBM protein source for broiler chicken diets. In keeping with previous studies [30, 32], our results found MSC to have a similar CP content as SBM. Considering its amino acid composition also mimicking that of SBM, except for lysine [31, 32], MSC offers great promise to replace SBM in poultry diets in Southern Africa and elsewhere. Interestingly also, local marula oil-extracting factories have improved their efficiency of oil extraction from marula kernels as evidenced by relatively low residual oil content in MSC used in this study compared to previous MSC products (289.6 – 343.5 g/kg DM: [30, 32]. With more improvements in oil extraction efficiency, iso-energetic MSC-containing broiler and other animal diets can now be formulated with greater ease and the product is expected to have less problems with fungal and hence mycotoxin infestation as observed previously [32]. Of interest also is the relatively low fibre content in MSC used in this study in comparison to values observed in previous studies (244.0 & 357.3, 223.2 & 245.6, and 98.1 & 114.3 g/kg DM for NDF, ADF and ADL, respectively: [35, 42]). The observed low fibre content of MSC render the product even more ideal for use in diets of broiler chickens and other non-ruminants that are unable to utilize high fibre-containing diets[43, 44]. Further, our study showed MSC to be richer in ash, an indicator of the mineral content, compared to observations in previous studies (48.5 – 54.3 g/kg DM: [32, 35, 42]). Furthermore, the concentration of CTs observed in MSC used in this study is higher than that reported by Malebana et al. [31] yet within the normal range that is considered safe for the feed product to be used in broiler diets without induction of adverse effects on bird growth performance [45]. Indeed, previous studies have shown that inclusion of up to 3% tannins in broiler diets improves gut health and digestive performance [46, 47].

As part of the investigation of the nutritive value of MSC for broiler chickens, this study tested effects of incremental dietary inclusion levels of the marula by-product in replacement of soya bean products on bird growth performance during the whole production cycle from 1 to 42 days (starter, grower, and finisher phases). The observed increase in FI from 0 to 15%, as well as BWG and FCE from 0 to 10%, of dietary MSC inclusion level beyond which they decreased corroborates observations in pig studies by Hlongwana et al. [42], Mabena et al. [35] and Thabethe et al. [36] but contradicts those of Mazizi et al. [48] in Japanese quails. In a previous study, the decrease in performance parameters with increasing inclusion levels of MSC was suspected to be induced by extensive lipid peroxidation and mycotoxin infestation of MSC [32]. Indeed, these researchers found high lipid peroxidation and low concentrations of mycotoxins deoxynivalenol (DON) and T-2 toxin in MSC. Notwithstanding, there is a possibility that the observed decrease in performance at high (15 to 20%) dietary MSC inclusion levels may also be related to the high oleic acid content in the residual oil-rich MSC. Indeed, oleic acid was previously shown to decrease food energy intakes in humans [49] through its eliciting of production of oleoylethanolamide [50], which is known to decrease food intake [51-53] through a mechanism involving the histaminergic system [54]. Oleic acid also has capacity to regulate body weight in animals as was demonstrated through a decrease in this parameter in rats fed a diet supplemented with 10% olive oil [55], a rich (70 - 80%) source of oleic acid. Mechanistically, this might occur through high MUFA diets exhibiting greater rates of oxidation leading to decreased body weight [56]. Hence, such detrimental effects of oleic acid on food intake and body weight may explain the decreased FI, BWG and FCE in broiler chickens fed high dietary levels of MSC in this study. These mechanisms may also explain the observed significant decrease in slaughter weight, hot carcass weight and cold carcass weight in broilers fed high (15 to 20%) dietary inclusion levels of MSC. Otherwise, considering the lack of effect of dietary MSC on the weights and lengths of all internal organs, it would therefore appear that the marula by-product does not contain detrimental antinutritional factors and is thus safe to incorporate at 5 to 10% inclusion levels in broiler diets. Generally, feeding of alternative plant-derived feedstuffs or their

 doi:10.20944/preprints202303.0423.v1

extracts with high levels of antinutritional factors and fibre is associated with increased size and length of the digestive system [57, 58]. Further, the observation of lack of effects of dietary MSC on all performance parameters even at as high inclusion levels of the marula by-product as 20% in week 6 suggests age-dependent attainment of adaptation to consumption of the novel alternative protein source by birds. Indeed, the digestive system of broiler chickens undergoes major anatomical and physiological changes as the birds grow with increases in size and length as well as ability to secrete digestive enzymes, alongside improved digestive ability, as they grow older [59]. If the MSC contained any antinutritional substances including oleic acid, their adverse impacts appear to have decreased as the age of birds advanced, similarly to previous observations [60, 61].

The lack of significant effects of dietary MSC inclusion on most haemato-biochemical parameters of broiler chickens in this study is indicative of reasonable biosafety of the marula by-product in relation to the health of the birds. Indeed, the kernels of marula fruits are a safe and delicious source of nutrition generally indulged upon by millions of mainly rural people in numerous countries in Africa without any health perturbations [62, 63]. Generally, they are consumed as a snack [64], incorporated into porridge and boiled meat as flavor enhancers [65] or their extracted oil used for meat preservation [66-68]. In poultry nutrition, their dietary consumption in the form of MSC has also elicited no deleterious effects in Japanese quails [48]. Hence, their deleterious effects on broiler chicken white blood cells including lymphocytes at dietary inclusion levels beyond 10% and 5%, respectively, as observed in this study, was unexpected. Notwithstanding, many studies have reported inhibitory effects of oleic acid in its neat form [69-71] or as dietary olive oil [72, 73] or cashew kernel oil [74] on lymphocytes and their proliferation in different tissues including blood [74]. Also, consumption of an oleic acid-rich Mediterranean diet decreased the number of leukocytes and platelets in human subjects [75]. The mechanisms underlying these deleterious effects of oleic acid on leukocytes including lymphocytes seem to involve the MUFA-induced cellular oxidative stress, mitochondrial depolarization [75-78] and apoptosis [79, 80]. This was the first study to investigate the full repertoire of haemato-biochemical parameters including immuno-physiological biomarkers in broilers. Hence, it remains to be seen in future studies whether diets supplemented with high levels of MSC would induce similar deleterious effects on leukocytes in other breeds of chicken. Also, there is need for elucidation of molecular mechanisms underlying the observed MSC-induced perturbations in immunological parameters of broilers and other breeds of chicken.

The safety of MSC as broiler chicken feed at low (0 to 15%) and its apparent toxicity at high (20%) dietary inclusion levels was clearly embodied in SDMA responses. Correlated well with renal function, SDMA is a biomarker of acute kidney injury [81] and has previously been measured in broiler and quail studies of alternative protein sources and phytogenic feed additives [82, 83]. Considering the linearly decreasing serum SDMA concentrations in broilers fed diets with 0 to 15% MSC, it is evident that MSC was safe to use at these relatively low dietary inclusion levels but induced kidney injury in the birds when it was included at a higher (20%) level, mirroring the observed decremental responses in performance and immunological parameters of birds fed diets with high dietary inclusion levels of the marula by-product. As mentioned above, it is argued that the high oleic acid content of MSC particularly at 20% inclusion level of the marula by-product induced cellular oxidative stress [76-78] and possibly apoptosis as well [79, 80] in the chickens. Lending support to this contention are previous observations of elevated SDMA levels in disease conditions involving oxidative stress including diabetes mellitus[84], atherosclerosis [85] inflammation [86, 87], apoptosis [88], and compromised immune function [89]. In fact, some studies have postulated that SDMA itself may be an inducer of oxidative stress by elevating reactive oxygen species in monocytes [90] whilst enhancing NADPH-oxidase through endothelial Toll-like receptor-2 activation [91]. Further, literature evidence reporting abrogative effects of administration of antioxidants including epigallocatechin-3-gallate [92], melatonin [93], N-acetylcysteine [94], and vitamin E [95] on kidney injury, measured as asymmetric dimethylarginine (ADMA), a structural isomer of SDMA [96],

 doi:10.20944/preprints202303.0423.v1

further reinforces the contention of oleic acid having induced oxidative stress in broilers at high dietary MSC inclusion levels. Unfortunately, our data could not be compared with literature values as this was the first study to investigate SDMA responses to dietary MSC supplementation in broilers. In future studies, there is need for investigation of biomarkers of and mechanisms underlying oxidative stress in birds fed high MSC-containing diets.

Another intriguing finding in the current study was the linear increase in bird serum cholesterol responses to incremental dietary inclusion levels of MSC. Whilst our serum cholesterol values were about 1.8 times lower than plasma cholesterol ones previously observed in quails [97], there are currently no comparable literature serum cholesterol responses to dietary MSC supplementation in broilers. However, it is evident that the observed increase in the chicken serum cholesterol concentrations with increasing dietary MSC inclusion levels in the current study is again associated with oleic acid. Indeed, a previous study showed consumption of oleic acid-rich olive oil to increase blood plasma and adipose tissue concentrations of high-density lipoprotein cholesterol (HDL-C) [55]. Apparently, the MUFA has unique ability to selectively increase the levels of the health-beneficial blood HDL-C whilst decreasing those of its cardiovascular disease (CVD)-associated low-density lipoprotein cholesterol (LDL-C) counterpart [55, 98, 99] resulting to its attenuation of CVD risk in hypercholesterolemic patients [100, 101]. Hence, it will be necessary to measure concentrations of both HDL-C and LDL-C in MSC-fed broilers in future studies in order to discern which exactly between the two cholesterol species is responsible for the observed elevation in serum cholesterol levels of birds fed incremental marula by-product-containing diets. Also, the observed dietary MSC-associated increase in bird serum cholesterol suggests a need for investigation of underlying molecular mechanisms in terms of cholesterol biosynthesis in future studies.

5. Conclusions

In conclusion, up to 10% MSC can be incorporated into broiler diets in replacement of SBM without adverse effects on growth performance, carcass yield and immuno-physiology of birds. Notwithstanding, there is need for strategies to resolve the antinutritional effects of oleic acid, or any other antinutritional substance that might occur in the marula by-product, in order to optimize its inclusion in broiler diets.

Author Contributions: Conceptualization, M.S.M. and D.M.N.M.; methodology, M.S.M. and D.M.N.M.; validation, M.S.M.; D.M.N.M.; D.C.O.; and M.M.; formal analysis, M.S.M.; and D.M.N.M.; investigation, M.S.M.; and D.M.N.M.; resources, D.M.N.M.; data curation, M.S.M.; writing—original draft preparation, M.S.M.; writing—review and editing, D.M.N.M.; supervision, D.M.N.M.; D.C.O.; and M.M.; project administration, D.M.N.M.; funding acquisition, D.M.N.M. All authors have read and agreed to the published version of the manuscript.

Funding: The first author acknowledges the PhD Scholarship from the National Research Foundation (NRF) (Grant no: 141668) and North-West University bursary. Also, financial support from the NWU School of Agricultural Sciences and Department of Animal Science is greatly acknowledged.

Institutional Review Board Statement: The animal study protocol was approved by the North-West University (NWU) Animal Production Sciences Research Ethics Committee (Ethical clearance number: NWU-00806 -22-A5).

Data Availability Statement: The data used in supporting the conclusions of this article will be made available by the corresponding author, without undue reservation.

Conflicts of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript; or in the decision to publish the results.

 doi:10.20944/preprints202303.0423.v1

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