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High dietary inclusion of marula seed cake induces detrimental effects on performance, visceromorphometry, and immuno-physiology of broiler chickens

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Abstract

This study investigated the effects of incremental dietary levels of marula seed cake (MSC) in partial replacement of soyabean meal (SBM) and maize on growth performance, viscera macromorphometry, carcass traits, and haemato-biochemistry of broiler chickens during the starter, grower, and finisher phases. In a completely randomized design, 400 day-old Ross 308 broilers were randomly allocated to five diets with 0, 5, 10, 15, and 20% MSC, each with eight replicates of 10 (five of each sex). Weekly feed intake (FI), body weight gain (BWG), and feed conversion efficiency (FCE) were calculated; haemato-biochemistry was measured at day 42. FI was quadratically decreased by dietary MSC, of which the optimum inclusion was 150 g/kg, as BWG and FCE were linearly decreased by the marula by-product. MSC linearly decreased bird slaughter weight and hot and cold carcass weight. White blood cells, lymphocytes, and symmetric dimethylarginine decreased linearly but serum cholesterol concentrations increased linearly. Dietary inclusion of MSC at levels >150 g/kg induced detrimental effects on productive performance, visceral organs and development, carcass traits, and haemato-biochemistry of broiler chickens. There is therefore a need for strategies to resolve antinutritional effects of high dietary MSC to optimize its inclusion in broiler diets.

Keywords: poultry, marula seed cake, nutritional status, oleic acid, health
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Introduction

Soyabean meal (SBM) is the most preferred protein source for broiler diets in sub-Saharan Africa (SSA) and elsewhere, consequent to its high crude protein (CP) content (400–550 g/kg DM) and well-balanced profile of highly digestible amino acids, in comparison with other oilseed grains (Kidd *et al*., 2004). Nevertheless, the use of SBM in animal feeding is both economically and environmentally unsustainable. Large-scale soyabean cultivation incurs high variable costs and results in deforestation, biodiversity loss, climate change, pollution, coastal and riverine eutrophication, and acidification and worsens feed–food competition (Fearnside, 2001). Maize has predominantly been used as a source of energy for broiler diets, but this has increased the feed–food competition and feed costs (Okarter & Liu,

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2010; Odo & Nnadi, 2013). Against this background, there is an urgent need for the investigation of alternative protein and energy-rich feed resources that are not only easily accessible and abundantly available, particularly to resource-limited smallholder farmers, but the production of which should be non-destructive to the environment. An example of such a feed is marula seed cake (MSC).

Marula (*Sclerocarya birrea* subsp. *caffra*) seed cake (MSC) is an industrial by-product that remains after oil extraction from the seed kernels of fruits fallen from marula trees that are indigenous and abundantly available throughout most of SSA from Niger to South Africa (Hall *et al*., 2002; Chirwa & Akinnifesi, 2008; Mlambo *et al*., 2011a; Leakey *et al*., 2022). Considering predicted future increases in climate change-associated frequency and severity of droughts in SSA (Schulze *et al*., 2007; Jiménez *et al*., 2020), MSC is an ideal, alternative dietary protein and energy source for broiler and livestock diets instead of SBM, maize, and other protein and energy ingredients, as it is produced from marula trees that are moderately resistant to drought (Hamidou *et al*., 2014). This feed resource has recently aroused great research interest, mainly in southern Africa, due to its high CP (470 g/kg DM) (Mdziniso *et al*., 2016). The essential and non-essential amino acid content is similar to SBM (except for lysine) and there is a high content of residual oil rich in the *n*-9 monounsaturated fatty acid (MUFA), oleic acid (72–85%) (Mthiyane & Mhlanga, 2017; Malebana *et al*., 2018). The high residual oil content (289.6 to 343.5 g/kg DM) (Mdziniso *et al*., 2016; Mthiyane & Mhlanga, 2017) render it an important energy source and potential replacer of maize and other energy-supplying ingredients in broiler diets. Previously, MSC was successfully used as an alternative protein source in the diet of beef cattle (Mlambo *et al*., 2011a), dairy cattle (Mdziniso *et al*., 2016), goats (Mlambo *et al*., 2011b), Japanese quails (Mazizi *et al*., 2020), and pigs (Mabena *et al*., 2022; Thabethe *et al*., 2022).

To the best of our knowledge, only a few studies have attempted to investigate dietary effects of MSC in broiler chickens and these have investigated the utility of this novel by-product only at the grower and finisher phases (Mthiyane & Mhlanga, 2017; Manyeula *et al*., 2022). No studies have considered responses of the modern bird to dietary MSC during the starter phase when their digestive systems would be envisaged to be the most sensitive to a novel, plant-derived feedstuff. Whilst Manyeula *et al*. (2022) studied this protein-rich by-product in broiler chickens, they measured only limited haematological and serum biochemical responses of the birds. As far as we are aware, there are currently no studies that have investigated dietary effects of MSC on the full repertoire of haematological and serum biochemical parameters, including immuno-physiological biomarkers of the meat-producing birds. Therefore, this study tested the hypothesis that partial dietary replacement of SBM and maize with MSC as a protein and energy source would maintain similar growth performance, visceral organs, carcass traits, haematology, and serum biochemistry responses of broiler chickens over the full production cycle (1–42 d). The objective was therefore to investigate the effects of incremental dietary levels of MSC on growth performance, internal organs, carcass traits, and haemato-biochemical parameters of broiler chickens for the whole production cycle (1–42 d).

Materials and Methods

The rearing and slaughter of broiler chickens used in this study was approved by the North-West University (NWU) Animal Production Sciences Research Ethics Committee (Approval #: NWU-00806-22-A5). The study was conducted at NWU Molelwane Experimental Farm during the summer season (October–November 2022). The farm is located (GPS 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.

Yellow maize, SBM, and all other dietary ingredients (except MSC) were purchased from Simplegrow Agric Services (Pty) Ltd (Centurion, South Africa) whereas MSC was obtained from The Marula Company in Phalaborwa, Limpopo Province, South Africa. Upon arrival, 100 g of the by-product and experimental diets (Table 1) were milled using a laboratory mill (screen size: 1 mm) and stored in sealed, labelled polyethylene bags at room temperature (20–25 °C) for chemical analysis. Samples were then analysed for dry matter (DM) (method 930.15), ash (method 942.05), ether extract (EE) (method 920.39), CP (method 954.01), minerals (calcium and phosphorus) (method 991.25), and amino acids (lysine, methionine, and threonine) (method 982.30), following the guidelines of the AOAC (2005). Neutral detergent fibre (NDF), acid detergent fibre (ADF), and acid detergent lignin (ADL) were analysed following the procedures of van Soest *et al*. (1991). Condensed tannins (as leucocyanidin equivalents) were analysed according to the method described by Makkar (2000).

Five iso-caloric-nitrogenous diets were formulated such that they contained incremental levels (0, 5, 10, 15, and 20%) of MSC to partially replace SBM and maize to meet the nutritional requirements of broiler chickens at starter (d1–14), grower (d15–28), and finisher (d29–42) phases (Table 1) (NRC,

1994). In a completely randomised design, 400 day-old Ross 308 broiler chicks with an average initial weight of 45.61 ± 0.67 g were randomly allocated to the dietary treatments, each with eight replicate pens (size = 1.8m high × 1.5m long × 1.5m wide) of 10 birds. Each pen had five males and five females. The experiment was carried out in a deep litter system in an environmentally-controlled broiler house where the temperature was maintained within 18 and 21 °C. The broiler house had semi-automatic curtains that were opened during the day (08:00 to 17:00) to allow natural light and closed in the evenings/night until the next morning (17:00 to 08:00). However, in week 1, artificial light was provided 24 h a day using electric lights in addition to infra-red lamps (one per pen) for brooding. Thereafter, infra-red lamps were removed, and electric lighting was only provided for 12 h (18:00 to 06:00) a day until the end of the experiment. Each pen had one feeder and one drinker. On placement, chicks were offered StressPack, which provided vitamins and electrolytes for the first 48 h. Fresh feed and water were offered *ad libitum* throughout the feeding trial. All pens were checked daily for mortalities, and dead birds were removed and recorded.

Following measurement of initial live weights upon arrival, broiler chickens were weighed weekly between 08:00 and 10:00 throughout the feeding trial by weighing all the birds in each pen until week 6. The weekly body weights, daily amounts of feed offered, and leftovers were recorded. Then BWG (g/bird/week) was calculated by subtracting the previous weekly body weight (g) from the current body weight (g), divided by the number of broilers per pen. Daily FI (g/bird/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 FI values were then converted into weekly averages of FI (g/bird/week) by combining pen averages over 7 d; FCE was calculated by dividing the weekly BWG by the weekly FI.

A day before slaughter (day 42), blood samples for haematology and serum biochemistry analysis were collected from 16 birds per treatment (two 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 using an automated IDEXX LaserCyte Haematology Analyzer (IDEXX Laboratories (Pty) Ltd, Johannesburg, South Africa). The heterophil-to-lymphocyte ratio was calculated as heterophil divided by the lymphocyte values. 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 were analysed using an automated IDEXX Vet Test Chemistry Analyzer (IDEXX Laboratories (Pty) Ltd, Johannesburg, South Africa).

On the evening of day 42, six birds per pen (three males: three females) were fasted for 12 h (18:00 to 06:00) but provided with clean drinking water *ad libitum*. Next morning after group-weighing per pen to obtain the final live (slaughter) weights, birds were transported to Rooigrond poultry abattoir, ~30 km from the NWU experimental site. An hour after arrival at the abattoir, they 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 min. Following thorough bleeding, feathers were un-plucked, and the carcasses washed. The heads, necks, and feet were removed. Visceral organs were also 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, whereas cold carcass weight (CCW) was recorded 24 h post chilling (4 °C). Subsequently, chilling loss was calculated and expressed as a percentage using the formula:

$$Chilling\ loss\ (\%) = \frac{(HCW - CCW)}{(HCW)} \times 100\% \quad (1)$$

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. The internal organs and carcass cuts were then weighed and expressed as percentages of the HCW.

Weekly FI, BWG, and FCE data were analysed as repeated measures in SAS (2012) using the statistical model:

$$y_{ijk} = \mu + \alpha_i + \beta_j + (\alpha\beta)_{ij} + \varepsilon_{ijk} \quad (2)$$

where y_{ijk} = response variable, μ = overall mean, α_i = effect of diet, β_j = effect of week, $(\alpha\beta)_{ij}$ = diet × week interaction effect, and ε_{ijk} = random error.

Overall FI, BWG, and FCE as well as data on haemato-biochemistry, internal organs, and carcass characteristics were analysed following the general linear model (GLM) procedure using the statistical model:

$$y_{ij} = \mu + \alpha_i + \varepsilon_{ij} \quad (3)$$

where y_{ij} = response variable, μ = overall mean, α_i = effect of diet, and $+\varepsilon_{ij}$ = random error. The least square means (LSMeans) were compared using the probability of difference (PDIFF) option in the LSMeans statement and differences among them were deemed significant at $P \leq 0.05$.

Thereafter, the response surface regression (PROC RSREG) analysis was performed to estimate the optimum inclusion level of MSC according to the quadratic model:

$$y = ax^2 + bx + c \quad (4)$$

where y = response variable; a and b = coefficients of the quadratic equation; c = intercept; x = MSC level (g/kg); and $-b/2a$ = x value for optimal response.

Results and Discussion

The proximate composition of MSC is shown in Table 2. MSC contains high CP (471.8 g/kg DM) and OM contents, with low levels of EE, ash, fibre (NDF, ADF, and ADL) and CTs. In keeping with previous studies (Mdziniso *et al*., 2016; Mthiyane & Mhlanga, 2017), our results found MSC to have a similar CP content as SBM. Considering an amino acid composition similar to that of SBM, except for lysine (Malebana *et al*., 2018; Mthiyane & Mhlanga, 2017), MSC offers potential to replace SBM in poultry diets in southern Africa and elsewhere. Local marula oil-extracting factories have improved their efficiency of oil extraction from marula kernels, as evidenced by the relatively low residual oil content of MSC used in this study compared to previous MSC products (289.6–343.5 g/kg DM) (Mdziniso *et al*., 2016; Mthiyane & Mhlanga, 2017). With more improvements in oil extraction efficiency, iso-energetic MSC-containing broiler and livestock diets can henceforth be formulated with greater ease and the feed product is expected to have less problems with fungal and hence mycotoxin infestation, as observed previously (Mthiyane & Mhlanga, 2017).

Of interest also is the relatively low fibre content in MSC used in this study in comparison to values observed in previous studies (Hlongwana *et al*., 2021; Mabena *et al*., 2022). The observed low fibre content of MSC renders this by-product even more ideal for use in diets of broiler chickens and other non-ruminants that are unable to utilize high fibre-containing diets (Zijlstra *et al*., 2012; Jiménez-Moreno & Mateos, 2013). The current study showed MSC to be richer in ash, an indicator of the mineral content, compared to previous MSC by-products (48.5–54.3 g/kg DM) (Mthiyane & Mhlanga, 2017; Hlongwana *et al*., 2021; Mabena *et al*., 2022). The concentration of condensed tannins in MSC used in this study is higher than that reported by Malebana *et al*. (2018) yet within the normal range considered safe for the by-product to be used in broiler diets without induction of adverse effects on bird growth (Hidayat *et al*., 2021). Previous studies have shown that inclusion of up to 3% tannins in broiler diets improves gut health and digestive performance (Tandiang *et al*., 2014; Huang *et al*., 2018).

Table 1 Ingredient and nutrient composition (g/kg on as-fed basis, unless otherwise stated) of experimental starter (d1–14), grower (d15–28), and finisher (d29–42) diets

	MSC Inclusion Level (%)														
	Starter					Grower					Finisher				
Ingredient	**0**	**50**	**100**	**150**	**200**	**0**	**50**	**100**	**150**	**200**	**0**	**50**	**100**	**150**	**200**
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
SBM (CP: 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
MSC	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
Soyabean 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
Sunflower meal (CP: 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 soyabean 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
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
*Mineral–vitamin 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
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
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-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
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
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
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
Total	1000	1000	1000	1000	1000	1000	1000	1000	1000	1000	1000	1000	1000	1000	1000
Analysed 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	210.4	210.1	210.7	210.5	200.3	200.3	200.1	200.0	200.7	190.2	190.0	190.8	190.3	190.4
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
Metabolizable energy (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
Lysine	1.15	1.01	1.04	1.03	10.1	1.09	1.05	1.03	1.02	1.00	1.02	0.95	0.93	0.91	0.89
Methionine	0.39	0.37	0.37	0.37	0.30	0.33	0.37	0.37	0.37	0.30	0.34	0.32	0.32	0.32	0.32
Threonine	0.66	0.62	0.57	0.57	0.50	0.62	0.59	0.57	0.57	0.48	0.59	0.56	0.52	0.50	0.50

*Premix contained 0.12 g biotin; 0.7 mg folic acid; 30 mg niacin; 10 mg pantothenic acid; 11,000 IU vitamin A; 2.5 mg vitamin B1; 4.5 mg vitamin B2; 1 mg vitamin B6; 2500 IU vitamin D3; 25 IU vitamin E; 2.0 mg vitamin K3; 8.0 mg copper sulphate; 80 mg ferrous sulphate; 100 mg magnesium sulphate; 0.25 mg sodium selenite; 0.34 mg potassium iodine; 79 mg zinc sulphate. MSC = marula seed cake, SBM = soyabean meal

Table 2 Nutritional composition of marula seed cake (g/kg DM, unless otherwise stated)

Nutrient	Composition (g/kg DM)
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 (%)	0.11

Table 3 shows the effects of incremental levels of MSC on weekly and overall FI, BWG, and FCE, as well as mortality of birds. Repeated measures analysis revealed a significant diet × week interaction on weekly BWG (P <0.05) but not FI (P >0.05) and FCE (P >0.05). Regarding FI, dietary MSC induced a linear decrease (P <0.01) in this parameter in week 1, a quadratic decrease in weeks 2 (P <0.05), 3 (P <0.01), and 4 (P = 0.001), as well as a linear decrease in week 5 (P <0.01). There was no effect (P >0.05) of dietary MSC inclusion on FI in week 6. Overall, FI was quadratically decreased (P <0.01) by dietary inclusion of incremental levels of MSC. In terms of FI, the optimum dietary inclusion level of the marula by-product was 150 g/kg, beyond which there was a sharp decrease in this parameter.

Dietary inclusion of MSC induced a linear decrease in BWG in weeks 1 (P = 0.001) and 3 (P <0.01), a quadratic decrease in week 4 (P = 0.001), and a linear decrease in week 5 (P <0.01). However, there was no effect (P >0.05) of dietary MSC on BWG in weeks 2 and 6. Overall, BWG was linearly decreased (P = 0.001) by dietary inclusion of increasing levels of MSC, with the optimum inclusion level, in terms of this parameter, of 100 g/kg, beyond which BWG decreased.

Inclusion of dietary incremental levels of MSC linearly decreased FCE in broilers in weeks 1 (P <0.01), 3 (P <0.01), and 5 (P <0.01), whereas FCE decreased quadratically from week 4 (P <0.01). Similar to BWG, there was no effect (P >0.05) of dietary MSC on FCE in weeks 2 and 6. Overall, FCE decreased linearly (P <0.01) with increasing dietary levels of MSC in broiler diets, with the optimum inclusion level of the marula by-product, in terms of this parameter, found to be 100 g/kg, beyond which FCE decreased. The results also indicated no effect (P >0.05) of dietary MSC on all performance parameters (FI, BWG, and FI) in broiler chickens during week 6 of the study. Similarly, mortality was not affected (P >0.05) by dietary MSC (Table 3).

As part of the investigation of the nutritive value of MSC for broiler chickens, this study tested effects of incremental dietary inclusion of the marula oil extraction by-product on broiler growth performance and mortality during the whole production cycle from d1 to 42. The observed increase in FI from 0 to 150 g/kg, as well as BWG and FCE from 0 to 100 g/kg of dietary MSC inclusion level beyond which they decreased, corroborates previous observations in pig studies by Thabethe *et al*. (2020), Hlongwana *et al*. (2021), and Mabena *et al*. (2022) but contradicts those of Mazizi *et al*. (2019) 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 (Mthiyane & Mhlanga, 2017); 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 (150 to 200 g/kg) dietary MSC inclusion levels may also be related to the high oleic acid content in the residual oil-rich MSC. Dietary oleic acid was previously shown to decrease food intake through induction of satiety in mice (Igarashi *et al*., 2022) and to decrease food energy intakes in humans (Mennella *et al*., 2015) by eliciting the production of oleoylethanolamide (Schwartz *et al*., 2008). Oleoylethanolamide is known to decrease food intake (Rodriguez de Fonseca *et al*., 2001; Fu *et al*., 2003; Piomelli, 2013) through a mechanism involving the histaminergic system (Provensi *et al*., 2014). Oleic acid has the capacity to regulate body weight in animals, as was demonstrated through a decrease in this parameter in rats fed a diet supplemented with 100 g/kg olive oil (Nogoy *et al*., 2020), a rich source of oleic acid

(70–80%). Mechanistically, this might occur via high MUFA diets exhibiting greater rates of oxidation, leading to decreased body weight (Liu *et al*., 2016). Another putative mechanism might involve oleic acid inducement of a laxative (cathartic) effect, as has been demonstrated in rats (Beubler & Juan, 1979). These effects of oleic acid may therefore explain the decreased FI, BWG, and FCE in broiler chickens fed high dietary levels of MSC in the current study. These mechanisms may also explain the observed decrease in slaughter weight, HCW, and CCW in these broilers when fed high (150–200 g/kg) dietary inclusion levels of MSC.

The internal organs and carcass traits 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 5). Similarly, there were neither linear nor quadratic effects (P >0.05) of MSC on all carcass traits, except for the SW, HCW, and CCW (Table 5). In this regard, we observed linear decreases in SW (P = 0.001), HCW (P = 0.001), and CCW (P = 0.001) of broilers in response to increasing dietary inclusion levels of MSC.

Considering the lack of effect of dietary MSC on the weights and lengths of all visceral organs, it would therefore appear that this alternative feed resource does not contain detrimental antinutritional factors and is thus safe to incorporate at 50 to 100 g/kg inclusion levels in broiler chicken diets. This notion is further supported by the lack of effect of dietary MSC on mortality in this study. Generally, the feeding of alternative, plant-derived feedstuffs or their extracts with high levels of antinutritional factors and fibre is associated with increased size of the digestive tract (JøRgensen *et al*., 1996; Egbu *et al*., 2022). Further, the lack of effect of dietary MSC on all performance parameters even at high inclusion levels of MSC (200 g/kg) in week 6 suggests age-dependent attainment of adaptation to consumption of the novel, alternative protein source in broiler chickens. This was reflected in the significant diet × week interaction effect on weekly BWG. 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 the ability to secrete digestive enzymes with concomitant improved digestive ability, as they grow older (Ravindran & Abdollahi, 2021). If MSC contained any antinutritional substances, their adverse impact appears to have decreased as the age of birds advanced, similarly to previous observations (Rao *et al*., 2013; Erdaw *et al*., 2017).

Table 4 Effect of dietary MSC inclusion on weights (% of HCW, unless stated otherwise) and lengths of internal organs of broiler chickens

Parameters	Dietary inclusion of MSC (g/kg)					SEM	*P*-value	
	0	50	100	150	200		Linear	Quadratic
Liver (%)	1.68	1.82	1.88	1.91	1.86	0.119	0.246	0.392
Spleen (%)	0.11	0.13	0.12	0.11	0.13	0.014	0.593	0.942
Proventriculus (%)	0.42	0.41	0.39	0.45	0.46	0.023	0.080	0.167
Gizzard (%)	1.76	1.87	1.74	1.84	2.23	0.082	0.170	0.195
Duodenum (%)	0.78	0.80	0.78	0.81	0.82	0.067	0.615	0.920
Jejunum (%)	1.39	1.42	1.23	1.31	1.46	0.049	0.988	0.147
Ileum (%)	1.25	1.14	1.17	1.22	1.24	0.099	0.827	0.429
Caecum (%)	0.52	0.62	0.78	0.57	0.68	0.118	0.478	0.452
Colon (%)	0.11	0.10	0.11	0.10	0.15	0.015	0.074	0.112
Duodenum length (cm)	28.81	30.21	30.39	29.78	30.22	1.484	0.606	0.621
Jejunum length (cm)	61.49	65.48	65.51	61.87	60.40	2.453	0.451	0.114
Ileum length (cm)	66.89	73.16	73.38	68.03	68.48	15.595	0.191	0.395
Caecum length (cm)	18.88	18.74	18.60	18.44	17.95	0.616	0.365	0.617
Colon length (cm)	4.83	4.79	5.28	4.75	5.11	0.402	0.679	0.894

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

Table 5 Effect of dietary MSC inclusion on carcass traits (% of CCW, unless stated otherwise) of broiler chickens

Parameters	Dietary inclusion of MSC (g/kg)					SEM	*P*-value	
	0	50	100	150	200		Linear	Quadratic
SW (g)	2101.79[a]	2141.79[a]	2104.99[a]	1915.72[b]	1794.64[b]	59.894	0.001	0.038
HCW (g)	1478.57[ab]	1504.47[a]	1489.28[a]	1361.61[b]	1264.29[b]	43.208	0.001	0.030
CCW (g)	1446.62[a]	1472.38[a]	1452.97[a]	1326.67[b]	1230.75[b]	42.999	0.001	0.033
Chilling loss (%)	2.17	2.14	2.46	2.56	2.67	0.189	0.061	0.921
Dressing %	70.47	70.22	70.73	71.03	70.45	0.618	0.691	0.706
Breast (%)	21.65	20.23	20.65	21.55	19.88	0.969	0.342	0.796
Drumstick (%)	7.28	6.75	7.46	7.12	7.21	0.297	0.819	0.871
Thigh (%)	9.15	7.66	8.87	8.39	8.87	0.301	0.882	0.083
Wing (%)	5.82	5.14	6.04	5.63	5.98	0.231	0.313	0.420
Back length (cm)	20.02	18.35	30.56	18.74	18.91	4.607	0.904	0.256

Means in the same row with different superscripts ([ab]) are significantly different (P <0.05). SW = slaughter weight, HCW = hot carcass weight, CCW = cold carcass weight, MSC = marula seed cake, SEM = standard error of the mean

The haematological responses of broiler chickens to graded dietary levels of MSC are shown in Table 6. The results demonstrated no effects (P >0.05) of MSC inclusion on all haematological parameters, except for white blood cells and lymphocytes. In this regard, MSC linearly decreased white blood cells (P <0.01) and lymphocytes (P <0.05) of birds. The lack of effect of dietary MSC inclusion on most haematological parameters of the modern birds in this study is also indicative of reasonable biosafety of the by-product in relation to the health of the birds.

The kernels of marula fruits are a safe and delicious source of nutrition generally enjoyed by millions of predominantly rural people in numerous countries in Africa without any health perturbations. Generally, they are consumed as a snack (Mashau *et al*., 2022), incorporated into porridge and boiled meat as flavour enhancers (Petje, 2009), or their extracted oil is used for meat preservation (Duke, 1989; Maroyi, 2013). In poultry nutrition, their dietary consumption in the form of MSC has also elicited no deleterious effects in Japanese quails (Mazizi *et al*., 2019). Hence, their detrimental effects on broiler chicken white blood cells and lymphocytes at dietary inclusion levels beyond 100 g/kg as observed in the current study was unexpected. Notwithstanding, some studies have reported inhibitory effects of oleic acid in its neat form (Menendez *et al*., 2005; Carrillo *et al*., 2011; Hidalgo *et al*., 2011) or as dietary olive oil (Verlengia *et al*., 2003; Llado *et al*., 2010) or cashew kernel oil (Yaqoob *et al*., 1995) on lymphocytes and their proliferation in different tissues, including blood.

Consumption of the oleic acid-rich Mediterranean diet decreases the number of leukocytes and platelets in human subjects (Ambring *et al*., 2016). The mechanism underlying these deleterious effects of oleic acid on leukocytes, including lymphocytes, may involve the MUFA-induced cellular oxidative stress, mitochondrial depolarization (Cury-Boaventura et al., 2004, 2005 & 2006; Ambring *et al*., 2016) and apoptosis (Jeffery *et al*., 1996 & 1997). The current study is the first to investigate the full repertoire of haematology in broiler chickens. 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. The molecular mechanisms underlying the observed MSC-induced perturbations in immunological parameters of broilers and other chicken breeds need to be elucidated.

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

Parameter	Week	Dietary inclusion of MSC (g/kg)					SEM	*P*-value				
		0	50	100	150	200		Linear	Quadratic	Diet	Week	Diet × Week
FI (g/bird)	1	137.95^{a}	140.52^{a}	131.27^{a}	126.92^{ab}	125.50^{b}	4.406	0.008	0.851			
	2	225.98^{ab}	238.29^{a}	247.26^{a}	227.50^{ab}	211.63^{b}	8.988	0.165	0.014			
	3	394.61^{ab}	425.07^{a}	435.11^{a}	395.71^{ab}	353.27^{b}	16.210	0.032	0.002			
	4	591.32^{b}	636.09^{ab}	693.48^{a}	618.69^{b}	578.63^{b}	23.008	0.564	0.001			
	5	805.49^{a}	845.47^{a}	846.03^{a}	777.47^{ab}	713.24^{b}	25.698	0.004	0.005			
	6	1005.29	996.22	989.60	983.21	928.31	30.465	0.084	0.417	0.001	0.001	0.207
BWG (g/bird)	1	90.60^{a}	92.71^{a}	86.09^{a}	77.13^{b}	72.26^{b}	2.537	0.001	0.097			
	2	137.89	139.37	154.42	131.34	124.37	7.524	0.153	0.061			
	3	279.99^{ab}	302.17^{a}	302.99^{a}	245.65^{b}	228.18^{b}	14.678	0.002	0.018	0.001	0.001	0.028
	4	326.00^{b}	370.14^{b}	421.40^{a}	378.46^{ab}	331.69^{b}	15.742	0.695	0.001			
	5	503.77^{a}	493.42^{a}	492.50^{a}	423.24^{b}	396.74^{b}	21.536	0.002	0.216			
	6	717.97	698.18	601.77	614.61	595.86	47.733	0.335	0.529			
FCE (gain/feed)	1	0.66^{a}	0.66^{a}	0.66^{a}	0.61^{b}	0.58^{b}	0.017	0.004	0.113			
	2	061	0.58	0.62	0.58	0.59	0.018	0.526	0.876			
	3	0.70^{a}	0.71^{a}	0.70^{a}	0.62^{b}	0.65^{b}	0.019	0.005	0.777	0.004	0.001	0.251
	4	0.55^{b}	0.58^{ab}	0.61^{a}	0.61^{a}	0.58^{ab}	0.016	0.093	0.008			
	5	0.63^{a}	0.58^{a}	0.58^{a}	0.54^{b}	0.56^{b}	0.018	0.002	0.277			
	6	0.72	0.71	0.62	0.63	0.64	0.053	0.152	0.402			
Overall FI (g/bird)		3158.64^{a}	3281.67^{a}	3342.76^{a}	3129.56^{a}	2910.59^{b}	90.822	0.027	0.007			
Overall BWG (g/bird)		2056.22^{a}	2095.98^{a}	2059.18^{a}	1870.42^{b}	1749.10^{b}	59.720	0.001	0.038			
Overall FCE (gain/feed)		0.65^{a}	0.64^{a}	0.62^{ab}	0.59^{b}	0.60^{b}	0.039	0.003	0.481			
Mortality (%)		8.90	5.59	5.59	7.86	6.63	0.280	0.751	0.449			

Means in the same row with different superscripts (^{ab}) are significantly different (P <0.05). BWG = body weight gain, FCE = feed conversion efficiency, FI = feed intake, MSC = marula seed cake, SEM = standard error of the mean

Table 6 Effect of dietary inclusion of MSC on haematological parameters of broiler chickens

Parameter	Dietary inclusion of MSC (g/kg)					SEM	*P*-value	
	0	50	100	150	200		Linear	Quadratic
Red blood cells ($\times10^{12}$/L)	1.39	1.39	1.08	1.18	1.29	0.153	0.385	0.282
Haematocrit (L/L)	8.11	8.35	6.57	7.29	7.69	0.486	0.073	0.599
Haemoglobin (g/dL)	9.03	10.34	7.39	7.54	13.13	0.808	0.069	0.698
MCV (fL)	42.500	55.35	42.09	46.13	49.66	2.896	0.259	0.918
MCH (pg)	35.91	46.04	36.68	38.76	43.75	2.817	0.355	0.970
White blood cells ($\times10^{9}$/L)	9.15^{ab}	14.85^{a}	11.58^{a}	8.23^{b}	8.57^{b}	2.053	0.009	0.160
Heterophils ($\times10^{9}$/L)	3.15	5.83	5.29	4.06	4.07	0.911	0.193	0.086
Lymphocytes ($\times10^{9}$/L)	2.73^{b}	5.89^{a}	2.84^{b}	1.93^{b}	2.12^{b}	0.997	0.034	0.253
Heterophil/Lymphocyte ratio	1.72	2.47	2.33	4.38	2.31	1.176	0.410	0.437
Monocytes ($\times10^{9}$/L)	1.55	2.43	2.81	1.78	1.85	0.698	0.316	0.334
Eosinophils ($\times10^{9}$/L)	0.56	0.59	0.55	0.38	0.47	0.144	0.152	0.406
Basophils ($\times10^{9}$/L)	0.06	0.08	0.09	0.09	0.05	0.018	0.158	0.201
Platelet (K/µL)	150.19	28.13	90.19	76.94	177.50	24.505	0.712	0.119
PDW (%)	18.61	14.30	12.78	11.99	13.16	1.412	0.077	0.851

Means in the same row with different superscripts (^{ab}) are significantly different (P <0.05). MCH = mean corpuscular hemoglobin, MCV = mean corpuscular volume, PDW = platelet distribution width, MSC = marula seed cake, 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 MSC on all serum biochemistry parameters, except for symmetric dimethylarginine (SDMA) and cholesterol. In this respect, dietary MSC linearly decreased (P <0.01) serum SDMA concentrations as the MSC level increased from 0 to 150 g/kg, beyond which it increased with 150 to 200 g/kg MSC. In contrast, serum cholesterol showed a linear increase (P = 0.001) to increasing dietary inclusion levels of MSC.

The safety of MSC as broiler chicken feed at low (50–150 g/kg) and its apparent toxicity at high (200 g/kg) dietary inclusion levels was clearly represented in the SDMA responses. Strongly correlated with renal function, SDMA is a biomarker of acute kidney injury (Kielstein *et al*., 2006) and has previously been measured in broiler and quail studies of alternative protein sources and phytogenic feed additives (Marareni & Mnisi, 2020; Matshogo *et al*., 2021). Considering the linearly decreasing serum SDMA concentrations in broilers fed diets with 0–150 g/kg MSC, it is evident that MSC is safe to use at these relatively low dietary inclusion levels but induced kidney injury in the chickens when it was included at a higher (200 g/kg) content, mirroring the observed decremental responses in performance and immunological parameters of birds fed diets with such high dietary inclusion levels of the marula oil extraction by-product.

As mentioned above, it is argued that the high oleic acid content of MSC, particularly at 200 g/kg inclusion of the alternative feed resource, induced cellular oxidative stress (Cury-Boaventura *et al*., 2004, 2005, 2006) and possibly apoptosis (Jeffery *et al*., 1996 & 1997) in the chickens. Lending support to this contention are previous observations of elevated SDMA levels in disease conditions involving oxidative stress, including diabetes mellitus, atherosclerosis, inflammation, apoptosis, and compromised immune function (Tain & Hsu, 2017). Some studies have postulated that SDMA itself may be an inducer of oxidative stress by elevating reactive oxygen species in monocytes (Schepers *et al*., 2009) whilst enhancing NADPH-oxidase through endothelial Toll-like receptor-2 activation (Speer *et al*., 2013). Evidence reporting abrogative effects of antioxidants, including epigal-locatechin-3-gallate, melatonin, N-acetylcysteine, vitamin E on kidney injury, measured as asymmetric dimethylarginine (ADMA), a structural isomer of SDMA (Tain & Hsu, 2017) further reinforces the contention that oleic acid is pro-oxidative in broiler chickens at high dietary MSC inclusion levels. The current study is the first to investigate SDMA responses to dietary MSC supplementation in broiler chickens and thus there are currently no comparable values in the scientific literature. In future studies, there is a need to investigate biomarkers of and mechanisms underlying oxidative stress in birds fed high MSC-containing diets.

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

Parameters	Dietary inclusion of MSC (g/kg)					SEM	*P*-value	
	0	50	100	150	200		Linear	Quadratic
Glucose (mmol/L)	6.77	6.89	7.11	7.58	6.31	0.618	0.795	0.310
SDMA (µg/dL)	24.81[a]	23.00[a]	17.13[b]	14.94[b]	19.38[c]	1.288	0.004	0.006
Urea (mmol/L)	0.60	0.60	0.94	0.64	1.21	0.302	0.195	0.699
Phosphate (mmol/L)	4.01	3.92	3.96	3.84	3.95	0.207	0.865	0.904
Calcium (mmol/L)	2.38	2.38	2.38	2.38	2.41	0.028	0.540	0.390
Total protein (g/L)	36.56	33.88	36.56	31.69	35.63	1.846	0.738	0.987
Albumin (g/L)	14.44	13.63	14.38	13.38	14.63	0.630	0.331	0.732
Globulin (g/L)	22.19	20.13	22.00	18.38	21.25	1.243	0.959	0.887
Albumin/globulin	0.66	0.69	0.66	0.74	0.69	0.017	0.227	0.717
ALT (U/L)	27.81	25.56	24.25	29.00	28.75	3.047	0.209	0.723
ALP (U/L)	680.25	693.06	743.88	937.50	686.38	90.729	0.516	0.341
Total bilirubin (µmol/L)	16.63	9.19	16.63	11.19	10.38	3.563	0.946	0.404
Cholesterol (mmol/L)	2.39[b]	2.44[b]	2.67[ab]	2.65[b]	3.01[a]	0.121	0.001	0.856
Amylase (U/L)	400.25	477.13	389.06	536.06	304.06	75.174	0.741	0.136
Lipase (U/L)	208.44	208.88	187.44	405.44	232.69	83.141	0.395	0.738

Means in the same row with different superscripts ([abc]) are significantly different (P <0.05). ALT = alanine transaminase, ALP = alkaline phosphatase, MSC = marula seed cake, SDMA = symmetric dimethylarginine, SEM = standard error of the mean

The linear increase in broiler serum cholesterol responses to incremental dietary inclusion levels of MSC was interesting. Whilst our serum cholesterol values were ~1.8 times lower than plasma cholesterol values previously observed in quails (Mazizi *et al*., 2022), 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 associated with oleic acid. A previous study reported that the consumption of oleic acid-rich olive oil increased blood plasma and adipose tissue concentrations of high-density lipoprotein cholesterol (HDL-C) (Nogoy *et al*., 2020). Apparently, the MUFA has the unique ability to selectively increase the content of the health-beneficial blood HDL-C, whilst decreasing that of its cardiovascular disease (CVD)-associated, low-density lipoprotein cholesterol (LDL-C) counterpart (Rudel *et al*., 1995; Kwon & Choi, 2015; Nogoy *et al*., 2020), resulting in the attenuation of CVD risk in patients with hypercholesterolaemia (Zambón *et al*., 2000; Bemelmans *et al*., 2002). Hence, it will be necessary to measure concentrations of both HDL-C and LDL-C in MSC-fed broilers in future studies to discern which of the two cholesterol moieties is responsible for the observed elevation in serum cholesterol levels of birds fed incremental marula by-product-containing diets. The observed dietary MSC-associated increase in broiler serum cholesterol suggests a need for investigation of underlying molecular mechanisms in terms of cholesterol biosynthesis in future studies.

Conclusion

Up to 150 g/kg of MSC can be incorporated in broiler chicken diets to substitute SBM without adverse effects on growth performance, visceral organ sizes, carcass yield, and immuno-physiology of birds. There is a need for strategies to resolve antinutritional effects of high dietary levels of MSC to optimize its inclusion in broiler diets so as to completely replace SBM.

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Author Contributions

MSM conceptualised the study, collected the data, conducted the statistical analyses, interpreted the results, and wrote the initial draft of this manuscript; DMNM conceptualised the study, developed the original hypotheses, was involved in supervision, collaborated in the interpretation of results, and finalised the manuscript; DCO was involved in supervision, collaborated in the interpretation of results, and finalised the manuscript; MM was also involved in supervision, collaborated in the interpretation of results, and finalised the manuscript.

Conflict 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.

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