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Performance, hormonal assay, folliculogenesis and reproductive tract morphometry of ISA Brown hens fed diets supplemented with Garlic-tropical leaf meals composite mix

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ABSTRACT

The study investigates the effects of composites of selected tropical leaf meals and garlic on laying hens using performance, hormonal, reproductive tract morphometry, and folliculogenesis as response criteria. Fresh (bitter, moringa, scent, and neem) leaves were harvested, air-dried, and mixed with garlic powder in a ratio of 4:3:1:1:1, respectively to produce the garlic-composite leaf mix (G-CLM). A basal diet was prepared, divided into five equal portions and G-CLM was added at 0gkg-1, 5gkg-1, 10gkg-1, 15gkg-1, and 20gkg-1. Two hundred and seventy, 18 weeks old ISA Brown breed were randomly allotted to the five experimental diets and the trial lasted for 4 months. The results showed that hen-day production was highest at 5gkg-1 supplementation. All reproductive hormones measured were also significantly ($p<0.05$) influenced by the dietary treatments with the exception of follicle-stimulating hormone (FSH). The result showed that significantly ($p<0.05$) larger Preovu one (2.79 cm) and previtellogenic count (23.33) were observed in birds fed 5 gkg-1. In addition, the heaviest tract weight (46.67 g) and ovary weight (33.00 g) were observed in birds fed 20 gkg-1. It was concluded that the usage of G-CLM at 5 gkg-1 could be encouraged in layer nutrition with the aim to improve the clutch size.

Keywords: Garlic-composite leaf mix, hormonal assay, reproductive tract morphometry, folliculogenesis, ISA Brown

INTRODUCTION

Poultry meat and eggs form important components of the diets of humans but their commercial production is constrained by several factors among which the cost of feed is ranked highest. This has resulted into inadequate supply of feedstuffs and finished feeds for poultry farmers. The world population is increasing rapidly (Roser, 2013), which would lead to the growing demand for principal protein sources especially poultry products. To meet this demand, many poultry farmers have resulted into the use of commercial antibiotics to prevent diseases that may reduce productivity. The residual effect in the products of animals managed with antibiotics has been reported (Li *et al.*, 2013). The summation of this was what led to the ban in the use of commercial antibiotics as feed additives within the European Union (EU) in 1999 (Cervantes and ACPV, 2005) and a total ban enforced in 2006. However, this ban has also involuntarily extended to countries out of the EU obviously because of the speculated risk of generating antibiotic resistance in pathogenic microbiota (Windisch *et al.*, 2008). Arising from this is the search for alternative antibiotics in phytogenic additives.

Phytogenic feed additives are of plant origin and are used in animal feeding to promote the performance and health of livestock. It has gained increasing interest in recent times, especially for use in poultry. The pharmaceutical properties of tropical leaf meals, including anti-inflammatory, anticancer, and antibacterial activities have been earlier described (Gong *et al.,* 2016; Ying *et al.,* 2017). It is evident that leaves may possess potent phytogenic activity and may be successfully applied in the management of layers to improve their performance and health.

There have been several research reports on scent leaf extract (Ekwe *et al.*, 2017), neem leaf meal (Olowu *et al.,* 2013), bitter leaf meal (Oleforuh-Okoleh *et al.*, 2015), moringa leaf meal (Swain *et al.*, 2017), supplemented singly in poultry diets, especially in broiler chickens, and the use of garlic meal in layers (Mahmoud *et al.*, 2010). However, nutritional studies on their use as composite leaf mix in layers diet are scanty. Also, data on folliculogenesis and reproductive tract morphometry in laying hens are rare. The objective of this study was therefore to investigate the effects of cured composites of these selected tropical leaf meals and garlic mix on laying hen using hormonal, reproductive, and folliculogenesis as response criteria.

MATERIALS AND METHODS

Experimental site

The feeding trial and the analysis were carried out at the poultry unit of the Teaching and Research Farm (Livestock Section), and the Nutrition Laboratory of the Department of Animal Production and Health of the Federal University of Technology, Akure (FUTA) respectively. Akure is situated on Latitude 7° 15'N and Longitude 5° 12' E in the humid rainforest zone of western Nigeria. It is characterized by two rainfall peaks and high humidity during the rainy season (Agbelade and Akindele, 2013). The mean annual rainfall is about 1500mm and the rains last for about nine months usually from March to November of every year. The mean annual relative humidity is over 75% while the mean annual temperature is about 27°C (Worlddata, 2023).

Collection of the candidate leaf meals

The bitter leaves, moringa leaves, scent leaves, and neem leaves were harvested around Akure axis in Ondo State, Nigeria, while the garlic bulbs were purchased from a reputable vendor in Akure. The leaves were identified and authenticated by the curator in the FUTA Herbarium Centre. The leaves were deposited for referral purposes (Table 1).

Table 1. Voucher specimen number of plant materials of use in the formulation of the composite leaf mix fed to laying hens

Plant materials	FUTA Herbarium Number
Bitter (*Vernonia amygdalina*) leaves	0328
Moringa (*Moringa oleifera*) leaves	0324
Scent (*Ocimum gralissimum*) leaves	0323
Neem (*Azadirachta indica*) leaves	0322
Garlic (*Allum sativum*) powder	0330

Thereafter, the leaves were chopped and air-dried to reduce the moisture content and to ensure nutrient retention. The air-dried leaves were milled to produce leaf meals. The leaf meals (Bitter leaves, Moringa leaves, Scent leaves, and Neem leaves) and garlic were mixed in a ratio of 4:3:1:1:1, respectively to produce the Garlic-composite leaves mix (G-CLM) which was stored in polyethylene bags, kept under moisture-free conditions prior to use. Samples of the G-CLM were analyzed (Table 2) for proximate composition using AOAC (1990) methods while the calcium and magnesium were analyzed using atomic absorption spectrophotometry and phosphorus using the vanadomolybdophosphoric method.

Table 2. Proximate composition, energy and mineral content of Garlic-composite leaves mix

Nutrient	Composition
Moisture Content	12.90

Crude Protein (%)	32.83
Metabolizable Energy (kcal/kg)	3096.20
Crude Fat (%)	10.42
Fibre (%)	18.30
Ash (%)	14.36
Nitrogen Free Extract (%)	11.19
Calcium (ppm)	38.00
Magnesium (ppm)	45.70
Phosphorus (ppm)	3.05

Diets Preparation

A basal diet was prepared using the formula in Table 3. The basal diet was divided into 5 equal portions. Portion 1 was the control diet without G-CLM addition while G-CLM was added to Portions 2, 3, 4, and 5 at 5 gkg^{-1}, 10 gkg^{-1}, 15 gkg^{-1}, and 20 gkg^{-1} and designated as Diets 2, 3, 4 and 5 respectively.

Table 3. Gross Composition of the experimental diets (Layers)

Ingredient	**0 gkg^{-1}**	**5 gkg^{-1}**	**10 gkg^{-1}**	**15 gkg^{-1}**	**20 gkg^{-1}**
Maize	55.45	55.45	55.45	55.45	55.45
Soybean meal	9.0	9.0	9.0	9.0	9.0
Groundnut cake	16.5	16.5	16.5	16.5	16.5
Wheat offal	6.5	6.5	6.5	6.5	6.5
Dicalcium phosphate	1.5	1.5	1.5	1.5	1.5
Fish meal	0.5	0.5	0.5	0.5	0.5
Limestone	9.7	9.7	9.7	9.7	9.7
Lysine	0.15	0.15	0.15	0.15	0.15
Methionine	0.15	0.15	0.15	0.15	0.15
Salt	0.3	0.3	0.3	0.3	0.3
Premix	0.25	0.25	0.25	0.25	0.25
G-CLM	-	0.05	0.10	0.15	0.20
Total	100.00	100.05	100.10	100.15	100.20
Analyzed Composition					
Crude protein (%)	16.14	16.14	16.32	16.33	16.52
Crude fibre	11.31	11.51	11.63	11.91	11.95
Calculated Composition					
Metabolizable Energy (kcal/kg)	2873.36	2992.88	3012.34	3107.74	3234.20
Calcium (%)	4.01	4.01	4.01	4.01	4.01
Available phosphorus (%)	0.64	0.64	0.64	0.64	0.64
Methionine	0.41	0.41	0.41	0.41	0.41
Lysine	0.84	0.84	0.84	0.84	0.84

G-CLM = Garlic-Composite Leaf Mix; Metabolizable Energy = 35 (%CP) + 85 (%CF) + 35 (NFE)

Experimental Animal Management

Two hundred and seventy (270) point-of-lay of ISA Brown breed at 18 weeks were purchased from a reputable farm. The birds were weighed and randomly allotted to the experimental treatments on arrival. The birds (layers) were assigned to 5 dietary treatments of six (6) replicates per treatment having 9 birds each in a completely randomized design. The experimental birds were fed their respective diets throughout the duration of the experiment which spanned four (4) months.

Data Collection

The hen day production (%HDP) was calculated as follows:

$$\% \text{ HDP} = \frac{Egg\ Laid}{Number\ of\ Hen\ in\ Stock} \times 100$$

At the close of the feeding trial (112 days), sixty (60) birds (12 per treatment) were humanely slaughtered and blood samples were collected into heparin bottles and non-heparin bottles. From non-heparin bottles, sera were separated following centrifugation at 3000rpm for about 10 minutes. The sera samples were then taken to the Chemical Pathology Laboratory of Bridge Science Laboratory, Ilorin, Nigeria, where an enzyme-based immunoassay procedure was employed to determine progesterone, testosterone, luteinizing hormone, follicle-stimulating hormone, and estrogen in the serum samples using commercial kits.

The selected birds were dissected and the various parts of the reproductive tract which included the ovary, infundibulum, magnum, isthmus, uterus, vagina, and the entire reproductive tract were weighed and recorded accordingly. The ovary and the entire reproductive tract were weighed using an electronic sensitive scale, while the length of the infundibulum, magnum, isthmus, uterus, and vagina were measured using a ruler graduated to the nearest 1.0 mm.

The number of follicles in each of the birds slaughtered was taken. The follicles were divided into matured/Graafian follicles (follicles whose dimension is 2.5 cm or 25 mm and above); stage 3, pre-ovulatory or maturing follicles (20-24mm); stage 2, pre-ovulatory follicles (15-19mm); stage 1, pre-ovulatory follicles (10-14mm) and previtellogenic follicles (Myres et al., 2004). The dimension of the follicles in each of the birds was also taken and recorded. The dimension of the follicles was taken using a divider and a measuring tape graduated to the nearest 0.01 mm.

Data Analysis

All data collected were subjected to analysis of variance using the statistical software package SPSS (Version 25) and the Duncan Multiple Range Test of the software was used for mean separation. The results were depicted as Mean ± Standard Error of Mean.

Model Specification:

$Yij = \mu + Ti + \varepsilon_{ij}$

Where;

Yij = Individual outcome

μ = Grand mean

Ti = Treatment effect

ε_{ij} = Error variability

RESULTS

Figure 1 shows the effect of G-CLM supplementation on the egg laying pattern of ISA Brown Hens. Hens fed diet supplemented with 5 gkg^{-1} G-CLM recorded the highest (80.6%) hen day production (%HDP). However, birds placed on 10 – 20 G-CLM supplemented diets recorded lower HDP than the control (79.71%).

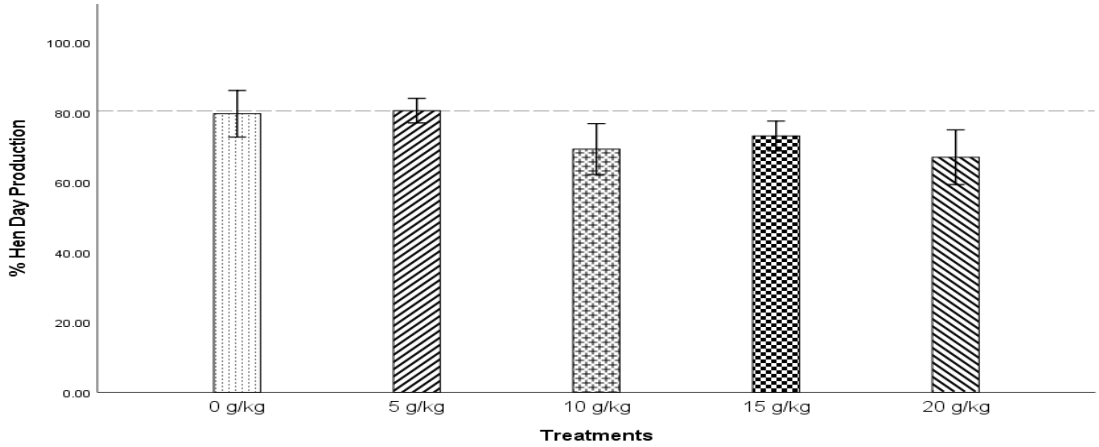

Figure 1. Effects of G-CLM on egg laying pattern of ISA Brown Hens

Table 4 shows the effects of garlic-composite leaves mix (G-CLM) supplementation on the reproductive hormones of laying birds. The results showed that all reproductive hormones measured were significantly ($p<0.05$) influenced by the dietary treatments with the exception of follicle-stimulating hormone (FSH). It was revealed that the gonadotropin-releasing hormone (GnRH) of birds fed diets containing 5 gkg^{-1} G-CLM was the highest but compared with those fed 10 gkg^{-1}, 15 $gkg^{-1}$and 20 gkg^{-1} G-CLM-based diets. Furthermore, birds fed diets containing G-CLM had comparable increased luteinizing hormone (LH) levels with the control (0.13 mIU/ml) except in birds fed diets containing 5 gkg^{-1} (0.11 mIU/ml). However, the LH levels of

birds fed diets containing 5 gkg^{-1} and 20 gkg^{-1} were similar ($p > 0.05$). Also, the estradiol of birds fed diets containing 15 gkg^{-1} and 20 gkg^{-1} G-CLM was similar ($p > 0.05$), they were however significantly ($p < 0.05$) lower than those reported for birds fed on diets containing 5 gkg^{-1} G-CLM but higher than those fed diet containing 0 gkg^{-1} and 10 gkg^{-1} G-CLM. In addition, birds fed diets containing 20 gkg^{-1} G-CLM had the highest progesterone levels which compared with those observed for birds fed diets containing 0 gkg^{-1} and 5 gkg^{-1} G-CLM but significantly ($p < 0.05$) higher than those fed diets containing 10 gkg^{-1} and 15 gkg^{-1} G-CLM.

Table 4. Effects of composite mix supplementation on reproductive hormone of laying birds

Treatment	Control	5 gkg^{-1}	10 gkg^{-1}	15 gkg^{-1}	20 gkg^{-1}	P-Value
GnRH (pg/ml)	1179.56 ± 92.94^{b}	2742.44 ± 376.00^{a}	2392.60 ± 753.56^{ab}	1480.12 ± 89.03^{ab}	1503.80 ± 150.37^{ab}	0.050*
FSH (mIU/ml)	0.21 ± 0.03	0.17 ± 0.01	0.22 ± 0.01	0.22 ± 0.04	0.20 ± 0.01	0.673
LH (mIU/ml)	0.13 ± 0.01^{a}	0.11 ± 0.01^{b}	0.15 ± 0.01^{a}	0.14 ± 0.01^{a}	0.12 ± 0.01^{ab}	0.024*
Estradiol (pg/ml)	42.66 ± 17.32^{c}	530.10 ± 38.63^{a}	101.01 ± 0.77^{c}	388.79 ± 50.33^{b}	294.94 ± 46.48^{b}	<0.001*
Progesterone (ng/ml)	36.51 ± 2.04^{ab}	34.01 ± 3.44^{ab}	30.26 ± 2.67^{b}	28.38 ± 0.53^{b}	43.60 ± 5.28^{a}	0.050*

* = Significant; ^{a,b} means with different superscript across rows are significantly ($p < 0.05$) different; FSH = Follicle Stimulating Hormone; **LH** = Luteinizing Hormone; GnRH = Gonadotropin-Releasing

The effects of garlic-leaf composite mix supplementation on laying hens on the folliculogenesis of laying hens are as presented in Table 5. The previtellogenic value was highest (23.33 ± 3.93) in birds fed diets containing 5 gkg^{-1} G-CLM but compared ($p > 0.05$) with those fed diets containing 10 gkg^{-1} – 20 gkg^{-1} G-CLM and higher ($p<0.05$) than those fed diets containing 0 gkg^{-1} G-CLM. However, birds fed the control and those fed diets supplemented with G-CLM at 10, 15 gkg^{-1}, and 20 gkg^{-1} are similar ($p > 0.05$). Also, the length of preovulatory one was highest (2.79 ± 0.19) in birds fed diet containing 5 gkg^{-1} G-CLM and this compared with values observed for birds on 10 gkg^{-1} and 15 gkg^{-1} G-CLM-based diets but higher than those fed 0 gkg^{-1} and 20 gkg^{-1} G-CLM-based diets.

Table 5. Effects of composite mix supplementation on laying hens on the folliculogenesis of laying hens

Parameters	Control	5 gkg^{-1}	10 gkg^{-1}	15 gkg^{-1}	20 gkg^{-1}	P-Value

Matured follicle	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00	1.000
Preovu three	2.33 ± 0.67	2.00 ± 0.00	1.00 ± 0.00	1.67 ± 0.33	4.00 ± 1.73	0.212
Preovu two	1.67 ± 0.33	1.67 ± 0.33	1.00 ± 0.00	1.67 ± 0.33	2.00 ± 0.58	0.452
Preovu one	1.67 ± 0.33	1.67 ± 0.33	1.00 ± 0.00	1.33 ± 0.33	1.50 ± 0.29	0.483
Previtellogenic	12.33 ± 1.86^{b}	23.33 ± 3.93^{a}	17.50 ± 0.29^{ab}	19.33 ± 1.86^{ab}	16.50 ± 0.29^{ab}	0.046*
Gfollicle (cm)	0.46 ± 0.23	0.47 ± 0.05	0.82 ± 0.09	0.66 ± 0.08	0.46 ± 0.20	0.361
Preovu three (cm)	3.18 ± 1.81	1.51 ± 0.21	1.68 ± 0.17	1.16 ± 0.17	0.97 ± 0.42	0.418
Preovu two (cm)	1.73 ± 0.21	2.40 ± 0.14	1.99 ± 0.05	1.46 ± 0.73	1.46 ± 0.36	0.424
Preovu one (cm)	1.98 ± 0.22^{bc}	2.79 ± 0.19^{a}	2.51 ± 0.01^{abc}	2.66 ± 0.10^{ab}	1.81 ± 0.42^{c}	0.054*

* = Significant at 5%; ^{a,b} means with different superscript across rows are significantly ($p < 0.05$) different

Table 6 shows that none of the reproductive tract morphometry parameters of the laying hens measured was significantly ($p > 0.05$) influenced by the dietary treatment.

Table 6. Effects of composite mix supplementation on the reproductive tract morphometry of laying birds

Parameters	**Control**	**5 gkg^{-1}**	**10 gkg^{-1}**	**15 gkg^{-1}**	**20 gkg^{-1}**	**P-Value**
Weight of entire tract (g)	23.33 ± 4.18	43.33 ± 2.73	32.00 ± 12.12	33.67 ± 6.44	46.67 ± 3.18	0.178
Weight of ovary (g)	19.67 ± 3.84	30.33 ± 3.84	20.67 ± 7.97	31.00 ± 4.00	33.00 ± 1.15	0.210
Infundibulum (cm)	12.50 ± 0.58	10.33 ± 0.93	10.67 ± 1.17	16.00 ± 3.51	10.67 ± 0.33	0.145
Magnum (cm)	33.33 ± 2.67	29.43 ± 3.84	25.50 ± 4.01	30.33 ± 4.17	36.00 ± 3.18	0.364
Isthmus (cm)	9.17 ± 0.73	12.17 ± 1.48	10.00 ± 0.29	12.17 ± 1.67	10.83 ± 0.73	0.292
Uterus (cm)	9.00 ± 1.15	7.67 ± 0.44	8.50 ± 1.61	7.83 ± 0.44	7.67 ± 0.60	0.819
Vagina (cm)	7.00 ± 2.52	3.50 ± 0.76	4.00 ± 1.76	3.83 ± 1.09	4.83 ± 0.17	0.506

DISCUSSION

The laying performance of ISA Brown hens used in this study declined by the supplementation of G-CLM at 10 gkg^{-1} – 20 gkg^{-1} compared with the control. Although, *Allium sativa* has been reported to have egg production improvement properties in layers (Azeke and Ekpo, 2009), *Moringa oleifera, Vernonia amygdalina,* and *Ocinun gratissimum* used singly has also been reported by Adegbenro (2015) to facilitate egg production by stimulating health and appetite but this was not the case in the present study. The plausible reason for the current finding could be attributed to the possible interactions among the various phytogens concentration in the leaves and garlic used which might have led to the decline in egg production performance of the hens on consumption. Thus, the combination of these leaves meal and garlic in the ratio used in this study beyond 5 gkg^{-1} may not have beneficial effects on the laying performance.

Studies have shown that performance in terms of egg laying is dependent on hormonal factors which are mainly involved in the hypothalamic-pituitary-gonadal axis (HPGa) in birds (Du *et al.,* 2020). This is because the reproductive organ development, follicular development, maturation, and ovulation in chickens are mainly regulated by the HPGa (Liu *et al.,* 2020). The result of this study showed that G-CLM supplementation enhanced gonadotropin-releasing hormone (GnRH) production from the pituitary glands in treated ISA Brown laying hens. The GnRH is responsible for stimulating the secretion of the luteinizing hormone (LH) and follicle-stimulating hormone (FSH) which play a particularly important role in the course of follicular development and ovulation. The reduction in FSH in birds fed 5 gkg^{-1} and 20 gkg^{-1} (though not statistically significant ($p > 0.05$)) may result in poor follicular growth. This observation may have been a result of a high concentration of prolactin to a level that exceeds the levels required in the egg production process. Prastiya *et al.* (2022) reported that high concentration of prolactin inhibits GnRH secretion which then results in the inhibition of egg production in the birds. However, such is not the case in this study because GnRH concentration in this study was highest in 5 gkg^{-1} which recorded the lowest FSH concentration. Therefore, the lower FSH levels observed in this study may be attributed to other physiological changes which include acid-to-base imbalance, immunosuppression, poor feed efficiency, and body weight.

The result obtained in this study further showed that G-CLM supplementation at 5 gkg^{-1} reduced LH secretion. This suggests that the supplementation of G-CLM in laying birds might be effective at higher levels of 10 gkg^{-1}, 15 gkg^{-1}, and 20 gkg^{-1} supplementations. The improvement in LH may be attributed to the garlic composition in the G-CLM phytogenic additive, which has been reported to have improved LH concentration as reported by Ekeleme-Egedigwe *et al.* (2019) and El-Akabawy and El-Sherif (2016) in rats, Modaresi and Mohajer (2015) in mice, and Ismael and Ameen (2012) in quail. FSH and LH stimulate the conversion of cholesterol to 17α-

hydroxypregnenolone which is then converted to estradiol hormone. The phytogenic mixture of G-CLM in this study significantly improved estradiol hormone concentration which is the most potent form of estrogen in birds. Estrogen has a feedback effect on the hypothalamus and pituitary gland that promote follicular development (Tarumi *et al.,* 2014). However, this finding contradicts that of Ismael and Ameen (2022) who reported that the inclusion of garlic powder at 2% and 4% reduced estradiol concentration. Furthermore, only G-CLM supplementation at 20 gkg^{-1} significantly increased progesterone concentration more than the control. The progesterone hormone helps in follicle release by weakening the stigma tissue during ovulation (Rawding, 2013).

In this study, the previtellogenic count was significantly higher in the treated birds than in the control. However, the influence of G-CLM supplementation did not follow a particular trend. This could have an influence on estrogen secretion in the birds, as avian estrogen is produced primarily in the small follicles (Baba *et al.,* 2017). A previous report showed that estrogen contributes to follicle-stimulating hormone mRNA expression in a granulosa cell culture (Baba *et al.,* 2017). The increase in previtellogenic count in the treated birds is expected to result in higher FSH levels, such was not the case in this study. Follicle development and activation in chicks are also promoted by the estrogen hormone (Zhao *et al.,* 2017). In addition, the secretion of yolk precursors from the liver and incorporation of yolk precursors into the developing yolky follicles is stimulated by estrogen (Stephens *et al.*, 2016).

In the current study, the dimension of preovulatory one was significantly increased in birds fed diets containing 5 – 15 gkg^{-1} G-CLM which could be as a result of the higher level of GnRH. It has been reported that as follicles grow, their dependence on gonadotrophins for development and survival is increased (Dunlop and Anderson, 2014). The result of folliculogenesis of ISA Brown as observed in this study did not correlate with the hormonal assay. Therefore, the discrepancies in the interaction of folliculogenesis and hormonal assay observed in this study may be attributed to dietary treatments amongst other factors because reproduction is a multifarious and regimented process, that is influenced by hormonal and transcription regulators, nutrition, breed, and physiological status differences (Shen *et al.,* 2022).

The reproductive tract morphometry was not statistically affected by the treatment effect in this study, suggesting that identical reproductive tract morphometry was triggered by the dietary supplementation of G-CLM in the laying hens. This thus contradicts the findings of Olateju *et al.* (2022) who reported a significant increase in reproductive tract morphometry in ISA Brown pullets fed diets containing phytogenic addictive (Clove leaf meal).

CONCLUSION

The results of the present study revealed that dietary G-CLM improved HDP at 5 gkg-1. Although there was no particular trend, some reproductive hormone concentration and previtellogenic follicle dimension were improved by G-CLM addition.

Based on the findings of this study, the usage of G-CLM at 5 gkg-1 should be encouraged in layer nutrition.

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