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# Effects of *in ovo* Injection of Soursop (*Annona muricata*) Leaf Extract on Blood Profile, Immune Organs, and Intestinal Morphology of Noiler Chicks

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## ABSTRACT

Plant extracts in poultry production are widely recognized for their significant benefits in improving productive performance. Specifically, the *in ovo* administration of soursop leaf extract (SLE) shows promise in improving the health and productivity of Noiler chickens. This study aimed to provide valuable insights into the health of the chicks and evaluate the effectiveness and safety of *in ovo* SLE in poultry production by examining the blood profile, internal organs, and intestinal morphology of Noiler chickens. For this experiment, 640 hatching eggs were incubated and randomly divided into four experimental groups, including 0.25 µg SLE, 0.5 µg SLE, 0.75 µg SLE, and a non-injected control group. Three treatment groups received a direct injection of 0.2 ml of the respective SLE concentrations into the air cells of the eggs on the 18th day of incubation. After hatching, chickens from each group were divided into five replicates of 15 chicks each and raised using a completely randomized design. At ten days of age, blood samples were collected from two chicks per replicate for hematology and serum analysis. Two chicks per replicate were sacrificed on day 10 to assess the internal organs and intestinal morphology. The results showed no significant changes in hematological parameters, serum biochemistry, and internal organs in all groups. The soursop leaf extract groups had markedly longer villi, deeper crypts, and thicker muscular walls compared to the control group. In conclusion, the *in ovo* injection of soursop leaf extract at 0.75 µg improved intestinal health by enhancing the intestinal surface structures in Noiler chickens.

**Keywords:** Hematology, Internal organ, *In ovo*, Intestinal morphology, Noiler chicken, Soursop Leaf Extract

## INTRODUCTION

The success of a poultry enterprise is often quickly judged by the growth performance forgetting the health status of the flock which plays such an important role that with time even the growth will be affected if no attention is given. Despite genetic improvement leading to several fast-growing breeds of chicken (Mancinelli et al., 2023), none of them can reach their full genetic potential without proper health management. For this reason, antibiotic growth promoters became an important component of poultry production for an average farmer (Mehdi et al., 2018). However, the restriction on antibiotic growth promoters in poultry production has led to exploring various alternatives, with a preference for natural substances with little to no residual risks. (Bean-Hodgins and Kiarie, 2021).

Among the alternatives to antibiotics growth promoters, phytogenics or botanicals have experienced a

wider exploration due to their tract record in human health management and broad-spectrum scope (Kikusato, 2021; Ndomou and Mube, 2023). Different application methods of phytobiotics have been reported in poultry research with varying results on performance parameters. *In ovo* administration of bioactive substances influences the developing embryos by improving the development and function of the intestine, immune system, and hatching parameters (Ayalew et al., 2023; Kpodo and Proszkowiec-Weglarz, 2023). Substances like carbohydrates, amino acids, vitamins, minerals, prebiotics, probiotics, synbiotics, and phytobiotics have been experimented with and reported to have influenced embryo and chick physiology (Babazadeh and Asasi, 2021; Kpodo and Proszkowiec-Weglarz, 2023). *In ovo* injection of some plant extracts (Moringa and soursop) have also been reported to influence the hatching process by reducing the incubation period which is of economic advantage to the hatchery managers (Bilalissi et al., 2019; Kuka et al., 2024)

Although numerous benefits of phytobiotics have been reported in poultry production, different plants have different chemical compositions which may negatively impact the health of animals. Additionally, the dosage, mode of administration, and duration of usage can lead to toxic effects, leading to inconsistent results (Oladokun et al., 2023). Therefore, the use of every plant material must be carefully evaluated in all possible ways to ascertain the safety of the material used.

Soursop leaf extract has been reported to contain bio-active components such as flavonoids, alkaloids, tannins, saponins, glycosides, ascorbic acid, beta-carotene, and minerals that exhibit antibacterial, antioxidant, anti-inflammatory, and immune-boosting activities leading to improved health and performance of poultry (Oluwayinka et al., 2017; Kuka et al., 2022). *In ovo* injection of soursop leaf extract enhanced chick weight, overall chick quality, survivability, and growth performance of chicks (Kuka et al., 2023).

However, there is currently a lack of research on the impact of *in ovo* soursop leaf extract on the health of Noiler chicks. It is crucial, therefore, to evaluate the potentially toxic effects of bioactive substances on the blood, intestine, and internal organs of Noiler chickens because any harmful effects of a substance consumed by chickens may not be immediately apparent in their growth but can be detected earlier in their tissues.

Hence, the present study aimed to evaluate the effect of *in ovo* administration of soursop leaf extract on the blood profile, intestinal morphology, and internal organs of Noiler chicks.

## MATERIALS AND METHODS

### Ethical approval

The experiment was conducted at the experimental unit of the Regional Center of Excellence in Poultry Science (CERSA) following the CCAC (2009) guidelines and the ethical approval (008/2021/BC-BPA/FDS-UL) from the University of Lomé. All experimental animals were handled with care to minimize any potential suffering

### Soursop leaf extract preparation

Fresh soursop leaves were harvested within Lome, Togo, air-dried at 20$^{o}$C then milled to powder. The extraction followed the procedure of Kuka et al. (2023). The soursop leaf powder was soaked in ethanol (80%) in a ratio of 1:1, and the mixture was set on a shaking machine for 72 hours of regular shaking. The content was filtered twice, first through cotton wool fitted in a filtration funnel and finally through a coffee filter bag (# 6) in a filtration funnel. The filtrate was concentrated at 40°C in a rotary evaporator equipped with a chiller and vacuum pump to produce the leaf extract that was then refrigerated for later use. In preparation for injection, a stock solution (1000 µg) was created by reconstituting ten (10) milligrams of the extract with ten milliliters of saline solution (0.9% NaCl). Another 10µg working solution was created by reconstituting one milliliter of the stock solution with 100 milliliters of saline solution. To obtain the 0.25, 0.5, and 0.75 µg concentrations needed for the injection, 0.25 ml, 0.5 ml, and 0.75 ml of the working solution were finally reconstituted with 10 ml of saline solution (Kuka et al., 2023).

### Experimental design and *in ovo* injection

A completely randomized design was used for the experiment, 756 Noiler chicken eggs were purchased from a breeder farm in Lome, Togo. These eggs were weighed and assigned unique codes for easy identification and monitoring during the experiment. Subsequently, the eggs were incubated at 37.7$^{o}$C and a relative humidity of 60% using a Royal Pas Reform (SmartPro TM) combi incubator from the Netherlands. Throughout the incubation period, which lasted until day 18, the eggs were regularly turned at a 45$^{o}$ angle, once every hour. On day 18, the eggs were examined under intense light (candling) to identify and remove any infertile ones. The remaining

eggs, which displayed evidence of developing embryos, were then randomly divided into four groups, each containing 160 eggs.

The broad ends of the eggs were perforated using 21 g needles, and the extract was injected into the air cell through the holes using an automatic syringe with 22 gauge (13mm), after that, the holes were sealed with adhesive tape. The control group comprised normal (non-injected) eggs; groups one, two, and three were injected with 0.25 µg/ml, 0.5 µg/ml, and 0.75 µg/ml of soursop leaf extract, respectively at day 18 of incubation. All the eggs were then transferred into the hatching baskets in three replicates and allowed to hatch at 37.7$^{o}$C and 70% relative humidity.

Since there was no discernible difference between the saline-injected and non-injected eggs in earlier works (Karamik and Kop-Bozbay, 2020; Atan and Kop-Bozbay, 2021), no group of eggs received saline injection in this experiment. A volume of 0.2 milliliters was administered to each egg within the air cell. Hatched chicks were raised for ten days in five replicates of fifteen (15) each, based on the groupings. The chicks had 24 hours of light and *ad libitum* feeding. The chick mash contained 20% crude protein and 2800 Kcal/kg ME (Table 1) as recommended by Amo Sieberer Hatchery LTD. According to the manufacturer's instructions, the chicks received multivitamins (Introvit A + Oral, Netherlands) in their water and were vaccinated following the schedule for Noiler chickens.

**Table 1.** The starter diet of Noiler chicken administered *in ovo* soursop leaf extract

| Ingredients | Amount (kg) |
|---|---|
| Maize | 53.00 |
| Soya bean meal (46%) | 33.00 |
| Wheat bran | 7.00 |
| Concentrate (5% JLC)* | 5.00 |
| Oyster shells | 2.00 |
| Total | 100.00 |
| **Nutrients composition** | |
| Crude protein (%) | 20.00 |
| Metabolizable Energy (kcal) | 2800.00 |
| Crude fibre (%) | 4.01 |
| Calcium (%) | 1.04 |
| Phosphorus (%) | 0.45 |
| Methionine (%) | 0.50 |
| Lysine (%) | 1.00 |

*Jubaili layer concentrate: Crude protein, 30%; Crude fats, 2%, Calcium, 4%; Phosphorus, 4.1%; Lysine, 2.0%; Methionine, 3.0%; Methionine + cystine, 3.5%; Sodium, 2.0%, Met. Energy, 2280 Kcal/kg; Vitamins; Minerals, enzymes. A diet formulated according to Amo Farm Sieberer Hatchery Ltd.

**Sample collection**

Two chicks per replicate were sampled for blood on day 10 in the morning, and one ml of blood was collected from the jugular vein with the aid of a needle and syringe into anticoagulant (Ethylenediaminetetracetic acid) bottles for haematological analysis. Another 2 ml of blood was collected into plane tubes and allowed to clot. Blood samples were transported to the Regional Center of Excellence in Poultry Science (CERSA) laboratory at the University of Lome, Togo, using ice box to maintain cold conditions. An automatic hematology analyzer (DH36, Dymind Biotechnology Co. Ltd Shenzhen, China) was used to analyze the following parameters: hematocrit (HCT %), red blood cell (RBC $10^{12}$/L) count, hemoglobin (Hb g/L) level, white blood cell (WBC $10^{9}$/L) count, lymphocytes, granulocytes, mean corpuscular volume (MCV fL), mean corpuscular hemoglobin (MCH pg), mean corpuscular hemoglobin concentration (MCHC g/L), and platelet count (PLT $10^{9}$/L). The analyzer uses impedance for WBC, RBC, and PLT analysis, and cyanide-free colorimetry for the Hb test (Dymind Technologies). Coagulated samples were centrifuged at 3000 rpm for 15 minutes to extract serum for further analysis. Levels of total protein, albumin, globulin (g/dL), alanine aminotransferase (ALT U/L), aspartate aminotransferase (AST U/L), and triglycerides (g/dL) in the serum were determined using specific reagents (Cypress diagnostic, Belgium) and the Gen5™ Microplate Reader and Imager Software (BioTek Instruments USA), following the manufacturer's instructions.

The heart, liver, spleen, thymus, and bursa of Fabricius from two chicks per replicate were weighed with a sensitive balance, and the weights were expressed to the percentage of the body weight using the formula below to obtain the organ's relative weight.

$$Relative\ weight = \frac{Organ\ weight\ (g)}{Chick\ weight\ (g)} \times 100$$

(Formula 1)

Two chicks from each replicate were sacrificed by cervical dislocation and dissected on day 10. One-inch mid-section of the ileum was cut, flushed with distilled water, and then fixed in a 10% formalin solution. The samples were processed using standard histological procedures, including dehydration, clearing, and embedding in paraffin wax. Six (6 mm) thick sections were sliced from each sample, placed on glass slides, and stained with Hematoxylin-Eosin (H & E) stain. Microscopic observation and examination were conducted using a light microscope (Olympus, Japan). Villus height,

crypt depth, thickness of muscularis, and mucosae were determined**.** Photomicrography of the H & E slides was done at X40 magnification with a Digital Microscope camera, Amscope® 319CU CMOS Camera. The morphometric analyses were performed using TS View CX Image® Software, version 6.2.4.3, and Motic Images Plus 2.0 ML (China).

**Statistical analysis**

Data collected was computed using one-way analysis of variance (ANOVA) in SPSS Software (Version 23, 2018). Differences between treatment means were compared using Duncan's multiple range test and significant differences were considered at $p \leq 0.05$.

## RESULTS AND DISCUSSION

Table 2, presents the effect of *in ovo* injection of soursop leaf extract on the hematology of Noiler chicks. No significant differences ($p > 0.05$) were observed among the parameters analyzed in all groups. The finding indicated that the chickens did not suffer any impairment due to the treatment effect in this study. Furthermore, it is possible that the doses of soursop leaf extract used in the present study were not high enough to induce a significant change in the parameters. In contrast to these findings, Asa et al. (2022) found a significant increase in red blood cell (RBC) and hemoglobin (Hb) levels in broiler chickens that received a combination of carbohydrates and antioxidants through *in ovo* administration. The discrepancy between the present study and other reports may be attributed to differences in the timing of sample collection, as the chickens in the present experiment were sampled at ten days old. Likewise, Oke et al. (2022) observed a significant difference in RBC levels of broiler chicks at hatch but could not find any variation in blood parameters at the finisher stage when black cumin was administered through *in ovo* injection. The higher percentage of granulocytes in the injected groups could imply the mobilization of defense cells in response to infection or abnormal conditions (Ünal et al., 2022).

Serum biochemical parameters revealed no significant differences, except for ALT (Table 3). *In ovo* feeding of soursop leaf extract at 0.25 µg, had a significantly higher level of ALT ($p < 0.037$) compared to the other treatment groups. This result indicated the possibility of a negative effect which that concentration could not amend. However, it was worth noting that the values reported in this study fell within the range of values reported for Noiler chickens (Idowu et al., 2021). No significant differences ($p > 0.05$) were observed in the immune and vital organs of the chicks among the treatment groups (Table 4). This result indicated that the extract did not cause any possible inflammation in the assessed organs. Visual observation also confirmed that the organs were not inflamed. This report corresponds to those of other researchers who used plant extracts, indicating the potential safety of *in ovo* phytobiotics. Saki and Salary (2015) who administered *in ovo* thyme and savory extracts in broiler chicken reported similarities in organ weights. Likewise, Bakyaraj et al. (2011) observed that *in ovo* administration of amino acids, trace minerals, fatty acids and vitamins (Vit. A 200 IU, vit. D3 20 IU, vit. E1 IU, thiamine 72 µg, riboflavin 144 µg, pyridoxine 140 µg, pantothenic acid 400 µg, niacin 140 µg, vit. C 8 mg, linoleic acid 20 mg) did not influence the spleen and bursa weights but influenced the thymus weight of the broiler chicks.

On the contrary, El-Kholy et al. (2021) reported that *in ovo* administration of cinnamon, thyme, and clove extract had a significant difference in bursa weight without a difference in thymus and spleen weights. The size of the thymus, spleen, and bursa of Fabricius are used alongside other indicators to determine the health status of chickens (Lutful Kabir, 2009; Sikandar et al., 2017).

The effect of *in ovo* injection of soursop leaf extract on the intestinal morphology of Noiler chicks was presented in Table 5 and Figure 1. Villus height, crypt depth, muscular, and mucosae thickness were significantly different ($p < 0.05$) among the treatment groups. *In ovo* injection of soursop leaf extract presented higher values of villus height, crypt depth, and muscular thickness than the un-injected group. Ilea villi height was longer in the 0.75 µg group while the crypt was deeper in the 0.25 µg group ($p < 0.05$). The improvement in the intestinal surface structures in the present study could be inferred from the influence of the antibiotic and anti-inflammatory properties of soursop leaf extract. Researchers have established that *in ovo* injection of nutrients improves intestinal development leading to longer intestinal villi (Kadam et al., 2013; Siwek et al., 2018). Furthermore, *in ovo* administration of bioactive compounds such as probiotics and plant extracts can create the right environment for colonizing the embryonic gut by beneficial microbe that promotes intestinal health (Yadav et al., 2016; Shehata et al., 2022).

The outcome of this study agrees with Cheled-Shoval et al. (2011) who reported greater villus height and intestinal muscle thickness in chickens caused by *in ovo* injection of mannan oligosaccharide. Similar results were

obtained by other researchers who injected *in ovo Baccilus subtilis* and raffinose in broiler chickens (Oladokun et al., 2021; Shehata et al., 2022). Furthermore, oral administration of soursop leaf extract in broiler chicken was reported to have improved villus height, crypt depth, and feed conversion ratio (Kuka et al., 2022).

Intestinal structures and their well-being were of utmost significance because they provide the surfaces for nutrient absorption. Changes in these structures affect the optimum functioning of the intestine thereby affecting the growth and general performance of the animal. Increased absorptive surface of the intestine and nutrient absorption were associated with longer villi of the intestine, the outcome was an improved feed conversion ratio and growth performance (Prakatur et al., 2019**)**. The improved intestinal morphology in the present study relates to the improved feed conversion ratio and growth performance of Noiler chicks reported by Kuka et al. (2023). The present study and other studies have shown that soursop leaf extract has the potential to promote intestinal health and development in chickens (Kuka et al., 2022; Artawiguna et al., 2023).

**Table 2.** Effect of *in ovo* injection of soursop leaf extract on hematology of Noiler chickens at 10 days of age

| Parameters | Treatment (Soursop leaf extract) | | | | p-value |
|---|---|---|---|---|---|
| | Control | 0.25 (µg/ml) | 0.50 (µg/ml) | 0.75 (µg/ml) | |
| HCT (%) | 25.30 ± 0.09 | 29.40 ± 0.04 | 28.40 ± 0.09 | 31.20 ± 0.12 | 0.656 |
| RBC ($10^{12}$/L) | 1.91 ± 0.65 | 2.14 ± 0.30 | 2.12 ± 0.58 | 2.33 ± 0.85 | 0.666 |
| Hgb (g/L) | 107.43 ± 37.45 | 119.00 ± 15.98 | 116.86 ± 36.61 | 127.00 ± 47.47 | 0.793 |
| MCV (fL) | 131.39 ± 7.01 | 137.10 ± 5.43 | 133.20 ± 5.76 | 133.24 ± 4.52 | 0.322 |
| MCH (pg) | 55.97 ± 2.59 | 55.53 ± 2.38 | 54.81 ± 2.20 | 54.33 ± 1.29 | 0.510 |
| MCHC (g/L) | 416.29 ± 15.70 | 405.00 ± 11.46 | 411.71 ± 5.68 | 407.71 ± 7.95 | 0.062 |
| PLT ($10^9$/L) | 1.71 ± 1.38 | 1.29 ± 0.48 | 1.71 ± 2.05 | 1.00 ± 1.29 | 0.736 |
| WBC ($10^9$/L) | 51.82 ± 21.29 | 61.03 ± 5.36 | 61.67 ± 8.45 | 59.39 ± 19.34 | 0.608 |
| Lymph (%) | 0.88 ± 0.04 | 0.89 ± 0.02 | 0.89 ± 0.03 | 0.91 ± 0.02 | 0.182 |
| Gran (%) | 0.04 ± 0.02$^a$ | 0.02 ± 0.01$^b$ | 0.03 ± 0.01$^a$ | 0.02 ± 0.01$^b$ | 0.050 |

HCT: Hematocrit, RBC: Red blood cell, Hgb: Hemoglobin, MCV: Mean corpuscular volume, Lymp: Lymphocytes, MCH: Mean corpuscular hemoglobin, MCHC: Mean corpuscular hemoglobin concentration, PLT: Platelets, Gran: Granulocytes. $^{a,b}$: means within a row with different superscripts are significantly different

**Table 3.** Effect of *in ovo* injection of soursop leaf extract on serum biochemistry of Noiler chickens at 10 days of age

| Parameters | Treatment (Soursop leaf extract) | | | | p-value |
|---|---|---|---|---|---|
| | Control | 0.25 (µg/ml) | 0.50 (µg/ml) | 0.75 (µg/ml) | |
| Total Protein (g/dl) | 4.71 ± 0.49 | 4.25 ± 0.47 | 4.12 ± 0.49 | 4.14 ± 0.44 | 0.061 |
| Albumin (g/dl) | 2.98 ± 0.21 | 2.91 ± 0.15 | 2.66 ± 0.42 | 2.63 ± 0.49 | 0.140 |
| Globulin (g/dl) | 1.83 ± 0.43 | 1.34 ± 0.53 | 1.46 ± 0.65 | 1.50 ± 0.75 | 0.419 |
| ALT (U/L) | 20.18 ± 5.11$^a$ | 15.23 ± 3.66$^b$ | 15.10 ± 2.87$^b$ | 14.59 ± 4.51$^b$ | 0.037 |
| AST (U/L) | 32.64 ± 16.77 | 21.83 ± 9.89 | 23.65 ± 10.07 | 25.64 ± 13.11 | 0.367 |
| Triglycerides (g/dl) | 197.68 ± 46.07 | 172.69 ± 27.85 | 165.42 ± 30.23 | 167.82 ± 25.88 | 0.218 |

ALT: Alanine aminotransferase, AST: Aspartate aminotransferase. $^{a,b}$ Means with different superscripts within a row are significantly different.

**Table 4.** Effect of *in ovo* injection of soursop leaf extract on relative organs' weight of Noiler chickens at 10 days of age

| Organs | Treatment (Soursop leaf extract) | | | | p-value |
|---|---|---|---|---|---|
| | Control | 0.25 (µg/ml) | 0.50 (µg/ml) | 0.75 (µg/ml) | |
| Liver (%) | 3.68 ± 1.10 | 3.52 ± 0.64 | 3.24 ± 0.32 | 3.57 ± 0.38 | 0.609 |
| Heart (%) | 0.74 ± 0.17 | 0.75 ± 0.12 | 0.75 ± 0.08 | 0.84 ± 0.09 | 0.282 |
| Spleen (%) | 0.14 ± 0.05 | 0.19 ± 0.27 | 0.09 ± 0.02 | 0.10 ± 0.05 | 0.450 |
| Bursa (%) | 0.29 ± 0.08 | 0.26 ± 0.05 | 0.27 ± 0.19 | 0.24 ± 0.08 | 0.801 |
| Thymus (%) | 0.16 ± 0.05 | 0.15 ± 0.04 | 0.22 ±0.07 | 0.24 ± 0.12 | 0.118 |

N = 10

**Table 5.** Effect of *in ovo* injection of soursop leaf extract on intestinal morphology of Noiler chickens at 10 days of age

| Parameters | Treatment (Soursop leaf extract) | | | | p-value |
|---|---|---|---|---|---|
| | Control | 0.25 (µg) | 0.50 (µg) | 0.75 (µg) | |
| Villus height (µm) | 307.42 ± 91.26[b] | 343.50 ± 77.51[ab] | 315.66 ± 82.99[b] | 394.52 ± 94.45[a] | 0.009 |
| Crypt depth (µm) | 133.92 ± 34.06[b] | 167.48 ± 41.43[a] | 140.87 ± 31.51[b] | 159.71 ± 50.29[ab] | 0.032 |
| Villus crypt ratio | 2.44 ± 0.99[ab] | 2.14 ± 0.55[b] | 2.33 ± 0.17[ab] | 2.76 ± 1.16[a] | 0.014 |
| Muscular thickness (µm) | 374.61 ± 56.47[c] | 496.82 ± 99.33[ab] | 453.11 ± 89.75[b] | 540.99 ± 65.71[a] | < 0.001 |
| Mucosae thickness (µm) | 140.38 ± 50.15[a] | 112.26 ± 33.42[ab] | 103.99 ± 23.67[b] | 136.11 ± 48.08[ab] | 0.012 |

[a,b] Means with different superscripts within a row are significantly different. n = 40

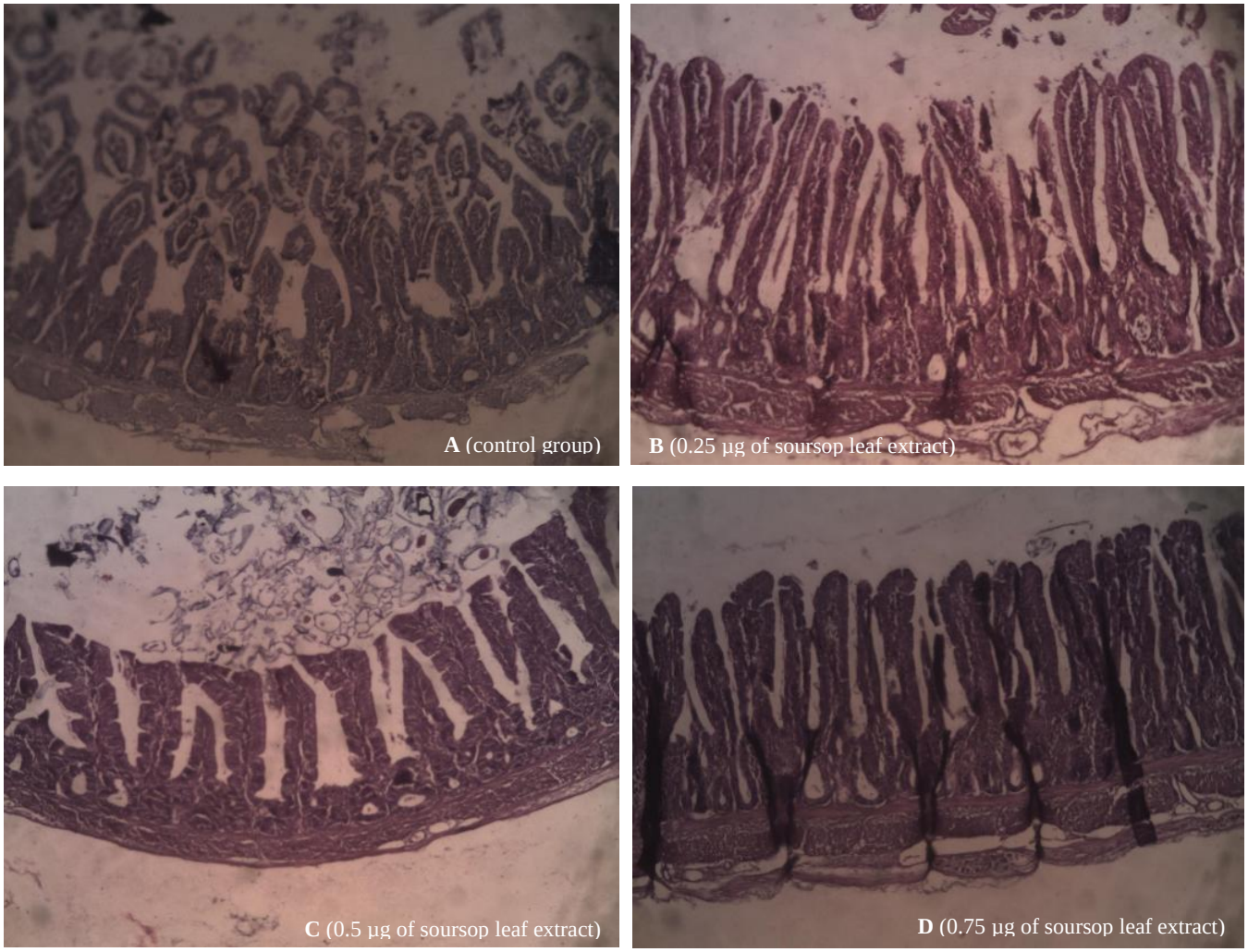


**Figure 1.** Effects of *in ovo* injection of sour sop leaf extract on intestinal structures of Noiler chickens at 10 days of of age. Viewed at X40 magnification. 2024.

## CONCLUSION

*In ovo* administration of soursop leaf extract in Noiler chickens positively influenced intestinal villi, crypt depth, and muscular wall thickness. These effects can improve nutrient absorption leading to enhanced growth of the chickens. Furthermore, *in ovo* injection of soursop leaf extract did not cause a significant change in the blood profile and internal organs of the chickens which showed their normal state of health. We recommend that higher concentrations of soursop leaf extract should be experimented with to ascertain the benefits and safety of chicken production. Also, an expanded immune response

of chicken administered *in ovo* soursop leaf extract should be studied.

## DECLARATIONS

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### Authors' contributions

Kokou Tona, Simplice Karou, and Batomayena Bakoma validated the protocol, provided access to facilities, and supervised the experiment, Timothy Kuka conducted the experiment and wrote the manuscript; Oumbortime N'nanle assisted with validation and laboratory analysis. All authors contributed to the experiment and the write-up of the manuscript and approved the last edition of the article before submission to the journal.

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### Competing interests

The authors declare that there is no personal or professional conflict of interest with the present study.

### Ethical considerations

The authors have avoided plagiarism, misconduct, data falsification, and double submission/publication and have given consent to publish this article.

### Availability of data and materials

The original contributions presented in the study are included in the article/supplementary material. For inquiries, please contact the corresponding author.

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