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ORIGINAL ARTICLE

LIGHT MICROSCOPIC STUDIES OF THE UPPER DIGESTIVE TRACT OF ADULT SPUR-WINGED GEESE (*PLECTROPTERUS GAMBENSIS*)

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Ethical considerations: When reporting experiments on animals Observation of the ARRIVE guidelines 2.0: Updated guidelines for reporting animal research, published on July 14, 2020 (DOI: 10.1371/journal.pbio.3000410), is applied. The authors ensure that all procedures were performed in compliance with the guidelines for animal care of their institutions or with national/international guidelines.

ABSTRACT

The present study elucidated the histoarchitecture of the upper digestive tract (UDT) of six Spur-winged geese (SWG), a wildfowl species of the Sudano-Sahelian wetlands of northern Nigeria. Tissue samples collected from different parts of the UDT were processed by the routine paraffin technique of light microscopy. The beak was lined by stratified squamous keratinized epithelium. The underlying dermis consisted of dense areolar tissue, blood vessels, and encapsulated nerve endings of Herbst corpuscles. The apical part of the tongue was lined by stratified squamous epithelium, which was heavily cornified on the ventral surface. The lingual submucosa comprised a mass of compound tubular mucous glands that exhibited moderate reactivity to Alcian blue. The esophagus was lined by stratified squamous non-keratinized epithelium, while the subepithelial area showed a few lymphatic nodules and polymorphic alveolar mucous glands. The mucosal glands were modified at the esophageal-proventricular junction into crypt-like folds. The proventricular wall comprised mainly proventricular glands, consisting of straight alveolar tubules lined by oxyntico-peptic cells, especially towards the basal portion. The proventricular plicae at the isthmus were modified into organized gizzard glands. The gizzard mucosa was lined by a hard cuticle layer. The gizzard-duodenal junction presented mucosal folds of variable heights, lymphatic nodules, and duodenal villi.

Key words: histology; spur-winged geese; upper digestive tract

INTRODUCTION

The spur-winged geese (SWG) of the family *Anatidae* are closely related to geese and shelducks but are distinct in a number of anatomical features, and thus are classed under the subfamily *Plectropterinae* [1]. The International Union for Conservation of Nature (IUCN) has classified these birds as a "least concern" species. They are herbivorous waterfowl, which usually eat seeds and vegetative parts of aquatic and terrestrial plants [2, 3]. The digestive system of birds, when compared to mammals, exhibits less interspecific variation [4] but evolves through a multitude of evolutionary changes to become a unique anatomo-physiological entity [5], presenting a number of significant structural differences between species and breeds [6].

There is a paucity of information on the detailed anatomic description of the digestive apparatus of the SWG. Knowledge of the functional anatomy of the digestive tract is essential for an improved understanding of the adaptive feeding habits in this species and for avian comparative anatomy. Thus, the present study was aimed at providing a detailed description of the upper digestive tract and some associated glands of the SWG, using histology and histochemical techniques.

MATERIALS AND METHODS

Sample collection

A total of six apparently healthy adult SWG, comprising three males and three females each, were used for this study. The birds were purchased from local sellers at the Moromoro local market and the *Zabarmari* ward of the Jere Local Government Area (LGA), Borno State (11.8024° N, 13.1931° E.), Nigeria. The birds were euthanized by a combination of diazepam (4 mg/kg) and ketamine HCl (60 mg/kg) intramuscularly as described by Onuk et al. [7]. The procedure was conducted as per the ARRIVE guideline and conformed to the guidelines of the Animal Use and Ethics Committee (AUEC) of the Faculty of Veterinary Medicine, University of Maiduguri, Nigeria. (Ethical approval number: AUP-R004/2023).

Histoarchitectural studies

Tissue samples (approximately 5 mm^3) from the upper and lower beaks, tongue (apex, body, and root segments), esophagus (proximal and middle segments), crop, proventriculus (esophageal-proventricular junction, middle, and proventricular-gizzard junction segments) and ventriculus (middle and ventricular-duodenal junction segments) were collected and fixed in 10% neutral buffered formalin for approximately 48 hours. The hard tissue (beaks and bony palate) was decalcified using the formic acid-sodium citrate method [8].

The tissues were further processed by a routine paraffin technique, and serial sections of 5-6 µm were cut and stained with Harris's hematoxylin and eosin (H&E) for general tissue architecture, Mallory's trichome stain for collagen fibers, and Alcian Blue (AB) (pH 2.5) for mucosubstances. All stained sections were examined, and photomicrographs were captured at different magnifications (x40, x100, and x400) using the I-SCOPE DN1177D LED microscope.

RESULTS

Upper beak

The upper beak was covered by an epidermis and the underlying dermis (Fig. 1a). The epidermis comprised a stratified squamous epithelium consisting of stratum basale, stratum spinosum, and stratum corneum (Fig. 1b). The stratum basale comprised 2-3 layers of basal cells, having a basophilic nucleus. The stratum spinosum comprised a layer of fusiform-shaped cells with flattened nuclei. The stratum corneum presented cells having pyknotic nuclei and few areas of vacuolations. These strata were covered by a very hard, dense cornified layer, and at some places, the free surface of the cornified layer presented a discontinuous layer of flattened cells (Fig. 1a).

The dermis consists of connective tissue with blood vessels and encapsulated nerve endings, the Herbst corpuscles. The Herbst corpuscle was ovoid-shaped, surrounded by a distinct cellular capsule. It presented a central axial nerve fiber surrounded by concentric lamellae (Fig. 1b).

The lamina epithelialis of the palatine mucosa comprised the stratum basale, a distinct stratum lucidum, and the stratum corneum (Fig. 2a). The lamina propria consisted of loose connective tissue, and the subepithelial area of the propria presented papillae of loose connective tissue, while the deeper layer was dense connective tissue and presented the palatine gland (Fig. 2b).

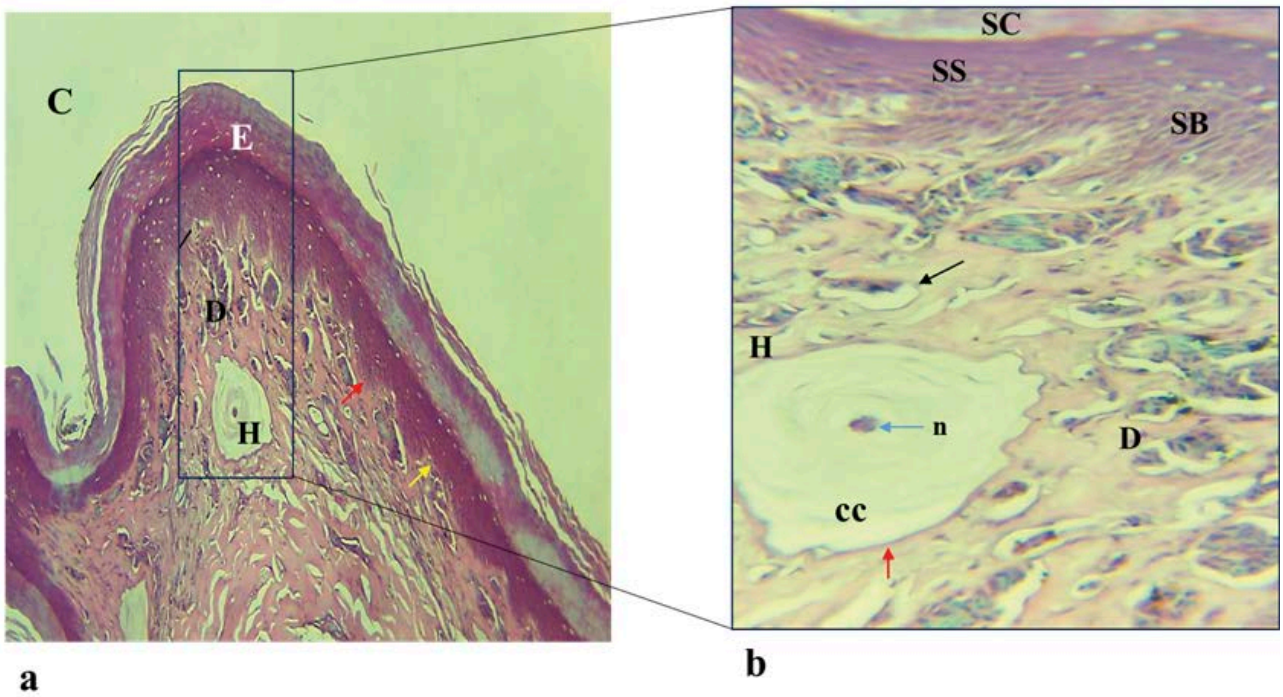

Fig. 1. Photomicrograph of upper beak showing: a. C. oral cavity, E. epidermis, D. dermis, epidermal peg (red arrow), dermal papilla (yellow arrow), H. Herbst corpuscle (H&E, ×100), b. higher magnification (H&E, ×400) of the epidermis showing: SB. stratum basale, SS. stratum spinosum, SC. stratum corneum, D. dermis with blood vessel (black arrow), cc. concentric core, and n. nerve of the Herbst corpuscle.

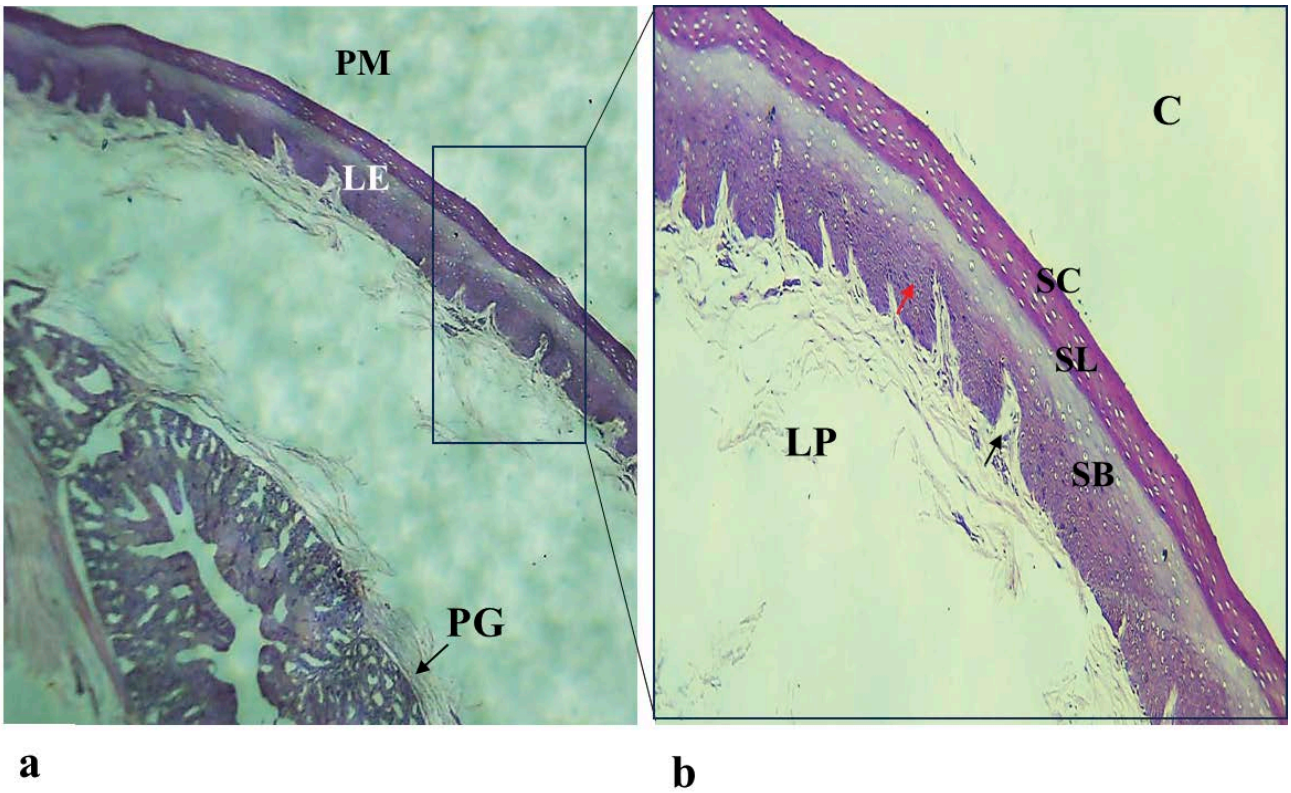

Fig. 2. Photomicrograph of cross section of the palate showing: a. PM. palatine mucosa, includes the LE. lamina epithelialis, PG. palatine gland deeper propria (H&E, ×40), b. higher magnification (H&E, ×400) of the lamina epithelialis showing: SB. stratum basale, SL. stratum lucidum, SC. stratum corneum, epidermal peg (red arrow), dermal papilla (black arrow).

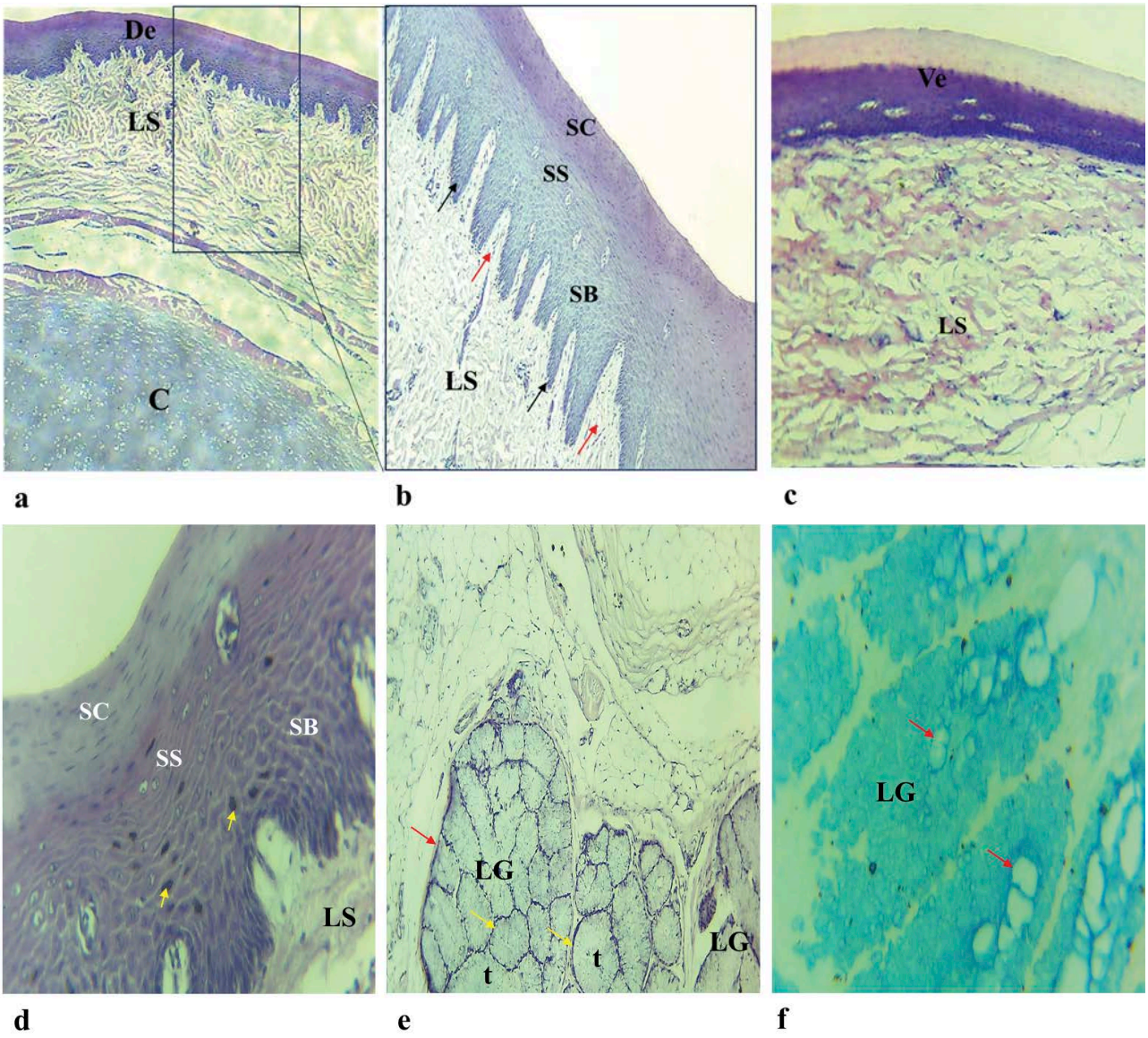

Fig 3. Photomicrograph of transverse section of the tongue (apex) showing: a. De. dorsal epithelium, LS. lingual submucosa, C. cartilage (H&E, ×40), b. higher magnification (H&E, ×100) of the dorsal epithelial surface showing: SB. stratum basale, SS. stratum spinosum, SC. stratum corneum, LS. lingual submucosa, epidermal pegs (black arrows), dermal papilla (red arrows), c. longitudinal section of the tongue (apex) showing: Ve. ventral epithelium, LS. lingual submucosa (H&E, ×100), d. transverse section of the tongue (body) showing: SB. stratum basale, SS. stratum spinosum, SC. stratum corneum, LS. lingual submucosa, melanocytes (yellow arrow) (H&E, ×400), e. transverse section of the tongue (root) showing; LG. lingual salivary glands, surrounded by connective tissue capsule (red arrow), t. glandular tubules, separated by connective tissue septa (yellow arrows) (H&E, ×100), f. transverse section of the tongue (root) showing: weakly sulfated lingual salivary glands (red arrows), LG. lingual gland. (AB ×100).

Tongue

The tongue was lined by a thick stratified squamous epithelium that was well developed dorsally (Fig. 3a). This epithelium is comprised of stratum basale, stratum spinosum, and stratum corneum. The free surface of the dorsal epithelium was straight, while the deeper layer presented a well-developed epidermal peg, alternating between papillae of the lingual submucosa (Fig. 3b). The entoglossum, forming the rostral end of the hyoid apparatus, was cartilaginous and surrounded by a perichondrium (Fig. 3a).

The ventral epithelium at the tongue apex was covered by a thick cornified layer of keratin (Fig. 3c). It was devoid of epidermal pegs but presented strata similar to the dorsal epithelium. The histoarchitecture of the body and root of the tongue was similar to that of the lingual apex. However, melanocytes were observed irregularly distributed within the cells of the stratum basale and stratum spinosum, especially at the tongue body (Fig. 3d). In addition, striated lingual muscle and lingual glands were observed towards the deeper surface of the lingual submucosa, adjacent to a

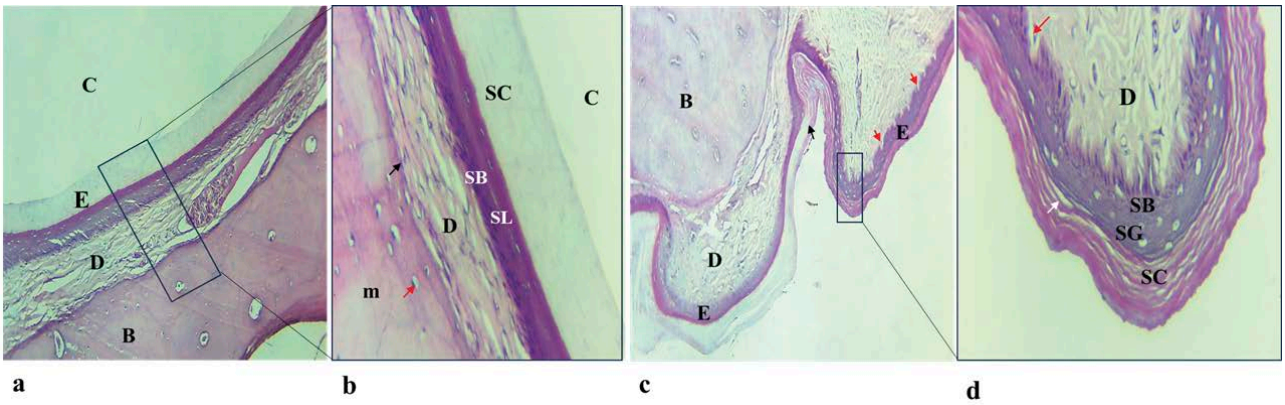

Fig. 4. Photomicrograph of lower beak showing; a. C. oral cavity, E. epidermis, D. dermis, B. dentary bone, (H&E, ×100), b. higher magnification (H&E, ×400) of the dorsal epithelial surface showing: SB. stratum basale, SL. stratum lucidum, SC. stratum corneum, D. dermis, periosteum (black arrow), m. bone matrix, osteocyte within lacunae (red arrow) (H&E, ×400), c. lateral lower beak showing: B. bone, E. epidermis, D. dermal papillae (red arrows), the hard cornified layer of the epidermis transitioning into softer form arranged in stacks (black arrow) (H&E, ×100), d. higher magnification (H&E, ×400) of lateral skin fold of lower beak showing: SB. stratum basale, SG. stratum granulosum, SC. stratum corneum, D. dermal papilla (red arrow).

cluster of adipose tissue (Fig. 3e). The lingual gland consisted of a compound tubular gland that exhibited low to moderate reaction to weakly sulfated muco-substance and sialo-mucin (Fig. 3f).

Lower beak

The free surface of the epidermis of the lower beak presented a very thick layer of relatively soft keratin that was translucent in appearance (Fig. 4a). The epidermis consists of the stratum basale, spinosum, and corneum (Fig. 4b). The bony core of the lower beak was composed of dentine bone; the bony matrix housed osteocytes with eccentric nuclei (Fig. 4b).

At the lateral commissure of the upper and lower beak, the hard cornified layer of the epidermis becomes soft and is arranged in irregular stacks (Fig. 4c). At the adjacent skin fold of the lower beak, the epidermis showed stratum basale and stratum corneum, whereas the dermis consisted of relatively loose areolar connective tissue and presented numerous dermal papillae (Fig. 4d).

Esophagus

The mucosal wall of the esophagus consists of 4 layers (Fig. 5a). The tunica mucosa is comprised of lamina epithelialis, lamina propria, and lamina muscularis mucosae (Fig. 5a-b). In certain regions, lymphoid follicles were observed in the sub-epithelial area, with infiltration of lymphoid cells toward the basal portion of the epithelium (Fig. 5a-b). The lamina propria was comprised of loose connective tissue and contained sub-epithelial glands that were compound alveolar glands. In some areas, a lymphoid nodule was seen adjacent to the mucosal gland (Fig. 5b-c).

The submucosa was poorly developed, comprising an indistinct layer of fine connective tissue. The lamina muscularis mucosae was situated immediately beneath the alveolar glands, and at some places, the fibers of the muscularis mucosae extended between the mucosal glands and sub-epithelial area of the mucosa. The tunica muscularis consisted of thick inner circular and thin outer longitudinal smooth muscles, separated by fine connective tissue fibers (Fig. 5c).

Pseudo-crop

The diverticulum of the pseudo-crop was lined by stratified squamous non-keratinized epithelium, and in some places lympho-epithelium was observed (Fig. 6a). A few mucosal glands were observed in the sub-epithelial area of the lamina propria (Fig. 6a). The body of the pseudo-crop presented a similar epithelial lining as the diverticulum, and the propria consisted of loose connective tissue, while mucosal glands were absent. The lamina muscularis mucosae was prominent, presenting a thick band of smooth muscle layer (Fig. 6b).

The esophageal-proventricular junction was a short transitory zone, where the esophageal mucous glands were modified into crypt-like folds of the proventriculus (Fig. 6c). A strand of muscle fibers passed inward over the anterior surface of the glandular lobule of the proventriculus and connects the main mass of the muscularis mucosa with the scant tissue of the submucosa (Fig. 6d). Heavy infiltration of lymphatic cells forming large foci in the lamina propria was observed.

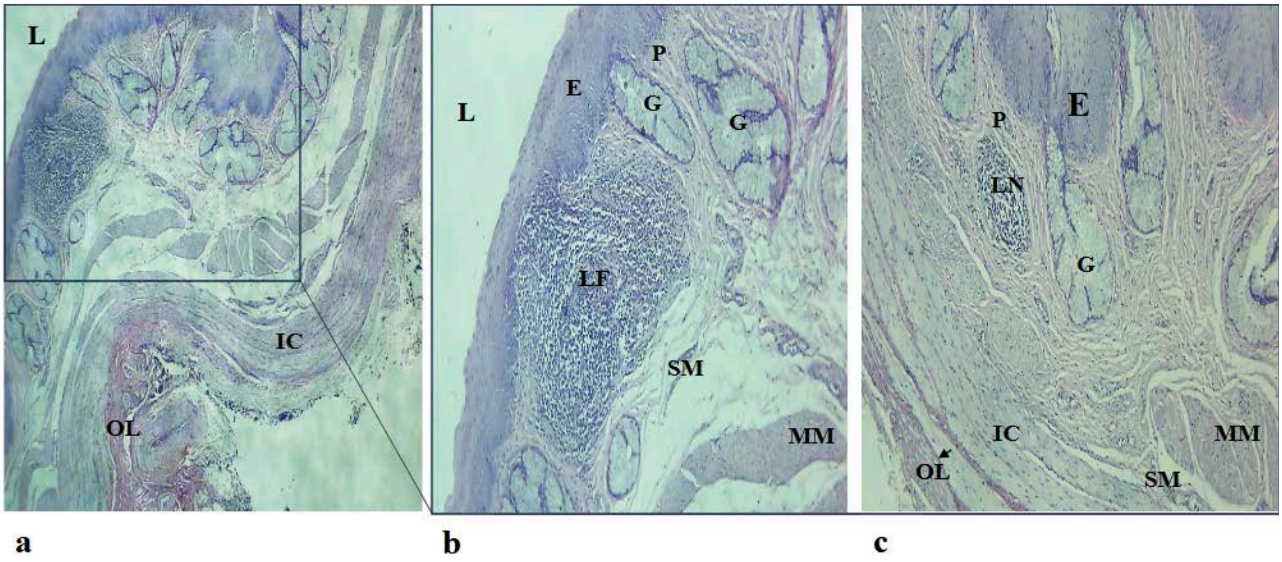

Figure 5. Photomicrograph of transverse section of esophagus showing: a. L. lumen, IC. inner circular and OL. outer longitudinal muscle layer of tunica muscularis, (H&E, ×40), b. higher magnification (H&E, ×100) of a transverse section of esophagus showing: L. lumen, E. stratified squamous epithelium, LF. lymphoid foci, LP. lamina propria, G. mucosal glands, MM. muscularis mucosae, SM. submucosa, c. transverse section of esophagus showing: E. stratified squamous epithelium, LP. lamina propria, LN. lymphoid nodule, G. mucosal gland, MM. muscularis mucosae, SM. submucosa, IC. inner circular and OL. outer longitudinal muscle layer of tunica muscularis (H&E, ×100).

Proventriculus

The mucosal lining of the proventriculus was thrown into folds of varying height. The greater part of the proventricular wall was composed of proventricular glands, presenting a number of rounds to polymorphic lobules. These lobules were surrounded by a connective tissue capsule, which separated the muscularis mucosa into a thin inner and a thick outer part (Fig. 7a-b).

The wall of each lobule was composed of numerous straight alveoli radiating from a central cavity of the lumen (Fig. 7b). The tunica muscularis consisted of a prominent inner circular and outer longitudinal muscle layer (Fig. 7b). The apical portion of the proventricular gland was lined by microscopic folds (plicae) of varying height. The plicae were lined with simple columnar epithelium (Fig. 7c). At the basal area of the proventricular gland, the inner and outer parts of the muscularis mucosae were prominent (Fig. 7d).

The straight alveoli joined to form a short tertiary duct and were lined by simple cuboidal epithelium (Fig. 7d). The central cavity of the lobule was lined by simple columnar ciliated epithelium with a few goblet cells. A sec-

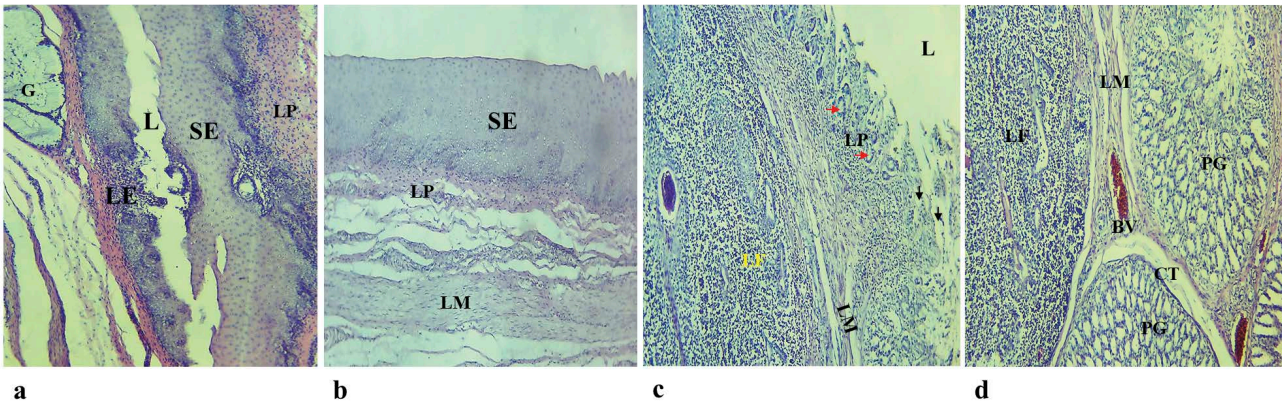

Fig. 6. Photomicrograph of transverse section of pseudo-crop (diverticulum) showing: a. SE. stratified squamous keratinized epithelium, L. lumen, LP. lamina propria, LE. Lymphoepithelium, G. gland (H&E, ×100), b. transverse section of pseudo-crop (body) showing: SE. stratified squamous keratinized epithelium, LP. lamina propria, LM. Lamina muscularis mucosa (H&E, × 100), c. transverse section of apical part of esophageal-proventricular junction showing: L. lumen, LP. lamina propria, LM. lamina muscularis mucosae, lf. lymphoid foci, esophageal mucosal glands (red arrows), crypt-like folds of proventriculus (black arrows), (H&E, ×100), d. transverse section mid-portion of esophageal-proventricular junction showing: lf. lymphoid foci, LM. lamina muscularis mucosae, BV. blood vessel, CT. connective tissue, Pg. proventricular gland (H&E, ×100).

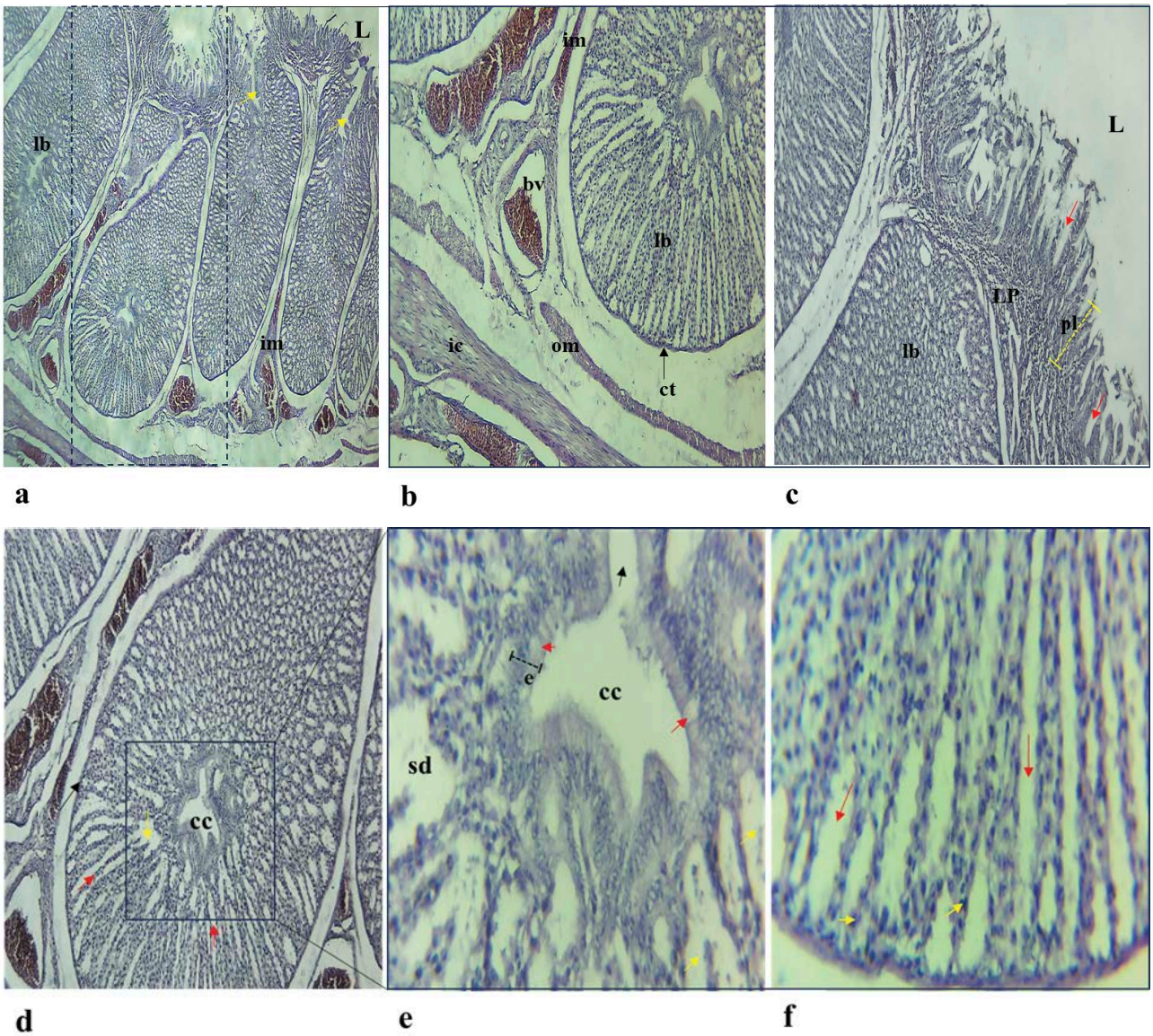

Fig. 7. Photomicrograph of transverse section of proventricular wall showing: a. L. lumen, lb. lobules of proventricular glands, primary ducts (yellow arrow), im. inner part of muscularis mucosae (H&E, ×100), b. higher magnification of transverse section of proventricular wall (basal part) showing: im. thin inner and om. thick outer part of muscularis mucosae, bv. blood vessel, lb. lobule of proventricular gland, ct. connective tissue capsule (black arrow), ic. inner circular muscle of muscularis externa (H&E, ×100), c. transverse section of proventricular wall (apical part) showing: L. lumen, pl. microscopic folds or plicae having sulci (red arrows), LP. lamina propria, lb. lobule of proventricular gland (H&E, ×100), d. proventricular gland lobule showing: cc. central cavity, glandular tubules (red arrows) joining to from a short tertiary duct (yellow arrow), each lobule is surrounded by connective tissue capsule (black arrow). (H&E, ×100), e. higher magnification (H&E, ×400) of the cc. central cavity, lined by columnar ciliated epithelium with Goblet cells (red arrows), sd. secondary duct, tubulus (yellow arrow), f. proventricular gland showing: straight tubules (red arrows) lined by low cuboidal epithelium, oxyntico-peptic cells (yellow arrows). (H&E, ×100).

ondary duct extended from the central cavity and was lined by the epithelium of the central cavity (Fig. 7e). The duct of the straight alveolar secretory units was lined by low cuboidal epithelium, and towards the basal portion of these alveoli, there were oxyntic peptic cells having a darkly basophilic oval nucleus (Fig. 7f).

The *isthmus* was a short transitory zone, in which the proventricular gland terminated abruptly. The proventricular plicae were gradually modified into the gizzard gland. The fine muscle fibers of the internal portion of the muscularis mucosa extended over the posterior edge of the last proventricular lobule to join the main mass of the muscularis mucosa (Fig. 8a). The outer portion of the muscularis mucosa merged with the circular layer of muscularis externa at the terminal portion of the proventriculus to form the main mass of the muscularis externa (Fig. 8b).

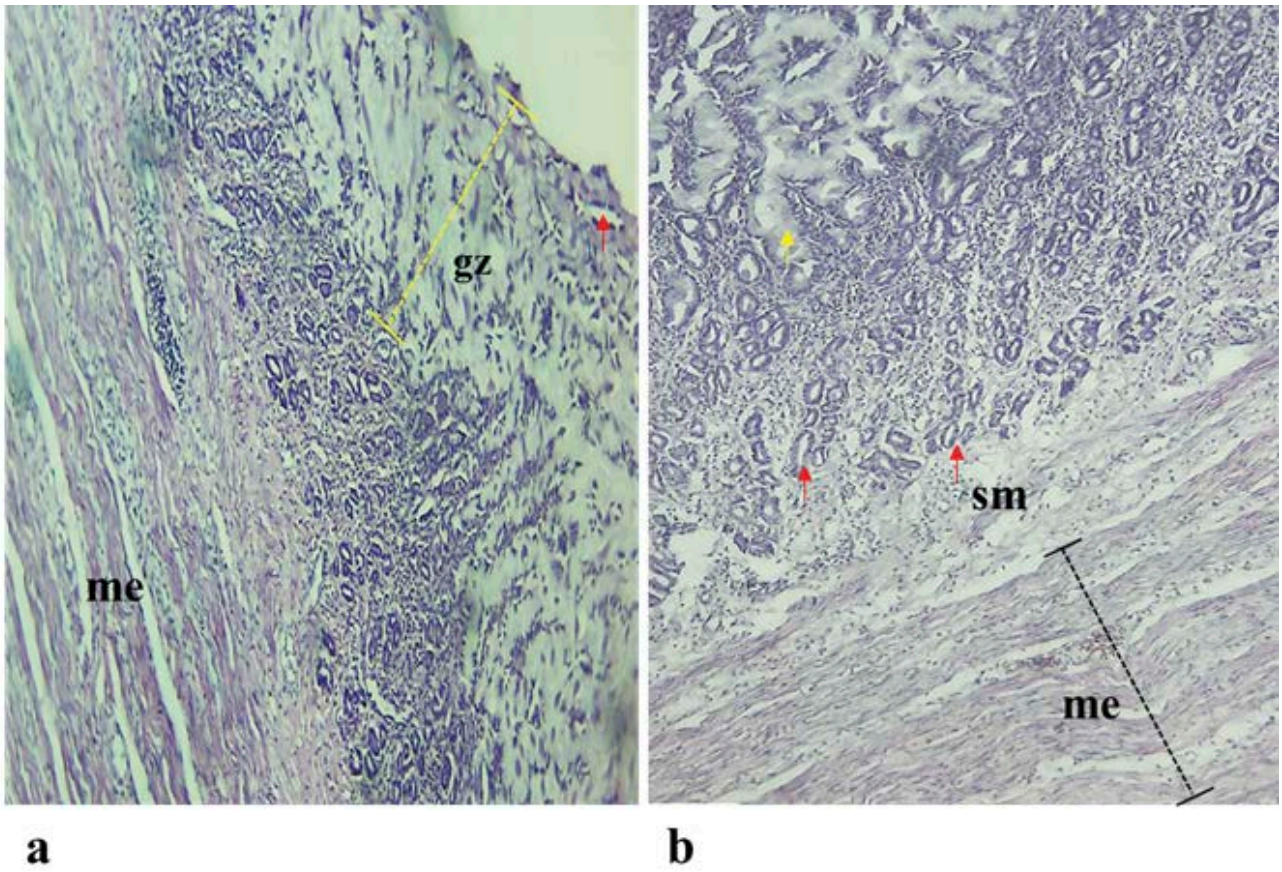

Fig. 8. Photomicrograph of proventricular-gizzard junction (isthmus) showing: a. gz. Gizzard lining covering the mucosa of the isthmus, proventricular plicae transitioning into the gizzard gland (red arrow), me. thick circular muscle layer, (H&E, ×100), b. mucoid secretory product (yellow arrow) lining the cuticle, gastric glands (red arrows), sm. submucosa, me. thick circular muscle layer (H&E, ×100).

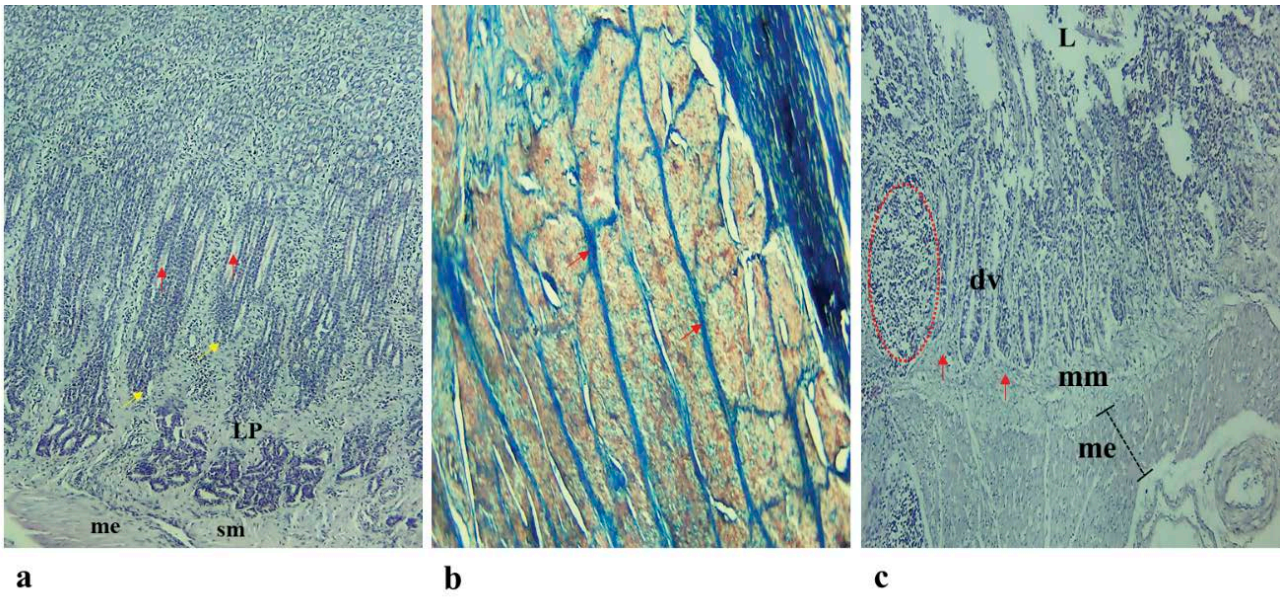

Fig. 9. Photomicrograph of gizzard showing: a. gastric pits (red arrows), gizzard glands (yellow arrows), LP. lamina propria, sm. submucosa, me. muscularis externa, (H&E, ×100), b. muscle fasciculi surrounded by connective tissue perimysium positive for collagen (red arrow), (MT ×100), c. gizzard-duodenal junction showing: L. lumen, dv. duodenal villi, lymphoid nodule (red dotted circle), mm. muscularis mucosae, having folds (red arrow), me. muscularis externa (H&E, ×100).

Ventriculus

The lining of the gizzard mucosa was formed by secretions of the gizzard gland oriented both parallel and perpendicular to the surface of the gizzard mucosa (Fig. 9a). The tunica propria was overlain by gizzard glandular tubules, while the submucosa was covered by a layer of dense connective tissue (Fig. 9a). The muscularis externa of the gizzard was well developed, comprising bundles of muscle fascicles surrounded by fine collagen connective tissue fibers (Fig. 9b).

The gizzard-duodenal junction was also a short transitory zone, where the mucosa was characterized by folds of variable sizes. There was also a lymphatic nodule that was both diffuse and nodular. The first few duodenal villi became discernible at this junction, and ventral to these villi, the duodenal mucosa formed a prominent fold of varying heights. The muscularis externa was prominent at this junction (Fig. 9c).

DISCUSSION

The true adult geese are essentially vegetarian [9]; thus, the anatomical adaptations of their digestive apparatus are a reflection of their feeding habit. For instance, the Herbst corpuscle observed in the upper beak of SWG was described as a sensory receptor used for selection and assessment of prehended foodstuffs, particularly in dabbling waterbirds [10]. Although SWG are surface feeders, they dabble on the surface of shallow waters for small invertebrates [9]. Furthermore, these Herbst corpuscles function as pressure-sensitive mechanoreceptors, detecting pressure and vibration from the surrounding environment [11].

The thick and keratinized epithelial lining of the SWG tongue corresponds to recent findings of Abdelhakeem et al. [12] in adult Egyptian geese. On the contrary, their studies reported the presence of lingual nail as thickened para-keratinized epithelium of the ventral surface of the tongue apex. In the SWG, the lingual nail existed as a gross cornified horny plate at the rostral tip of the premaxillary and the lower beak but was not a structure of the tongue. According to Jackowiak and Ludwig [13], a strongly keratinized epithelium was seen mainly in herbivorous and granivorous birds, while a lesser degree of keratinization was seen primarily in aquatic birds [14]. Furthermore, several lamellated sensory corpuscles and nerve endings were observed in the lingual nail area in the Egyptian geese [12]. In the SWG, sensory corpuscles were observed only in the dermis of the beak.

The compound tubular lingual salivary glands of SWG were similar to findings reported in Egyptian geese [12]. The lingual glands found in the Muscovy ducks and African pied crows are of the simple tubular type [15], while in the laughing dove and quail, they were tubulo-alveolar, having a strong affinity to AB stain [16, 17]. The lingual salivary glands function by secreting mucinous saliva, acting as a lubricant to aid swallowing of ingested food as a protective cover for the mucous membrane of the upper digestive tract [18].

The esophageal mucosal wall of SWG comprised four histological layers, as observed in many avian species [19], and the crop was indistinct, present in the form of a simple fusiform dilation, as reported in the common moorhen [20]. Generally, birds with a fully developed crop temporarily store ingesta, soften it, and predigest poorly digestible food particle [10].

The histo-structure of the crop mucosal wall showed species-specific characteristics. In the SWG, the mucosal lining was stratified squamous non-keratinized, as reported in pigeons, ducks, Japanese quail, and cattle egrets [21]. Similarly, compound alveolar mucosal glands were seen in the subepithelial area of the propria in SWG. In the cattle egret, the gland was of the simple branched alveolar type and compound tubulo-alveolar in domestic ducks [22]. Mucus produced by the mucosal glands of the esophagus and crop helps lubricate the lumen of the esophagus and crop. In addition, it provides a protective coat to the epithelium against friction [23].

The stomach of grain- and plant-eating birds, including the chicken, pigeon, geese, and ducks, is distinctly divided into the glandular proventriculus and the muscular ventriculus [10]. The gizzard in avians functions in grinding and macerating tough ingesta through powerful muscular development [24].

The wall of the proventriculus in SWG was mainly comprised of proventricular glands, as reported in domestic duck [25]. In the moorhen, these were divided into superficial and deep proventricular glands [20]. The gizzard tunica mucosa of the SWG was lined by the secretory cuticle of the gizzard gland, as reported in broilers [24], and had a yellowish layer of cuticle in domestic duck [21].

Dyce et al. [4] reported that cuticles are species-specific. Abumandour [26] reported that only a small area of the mucous membrane of the ventriculus of the eurasian hobby bird was covered by a cuticle layer, while the remaining luminal surface lacked this structure. The thickness of the cuticle was strongly correlated with the type of food: it was thin in frugivorous and nectarivorous birds, and thick in granivorous birds [27].

The gizzard tubular glands of SWG are mostly lined by chief cells, which stained basophilic. These cells secrete proteolytic enzymes called pepsin [28]. The muscularis layer of turkey gizzard was thick and consisted of bundles of parallel smooth muscle fibers, as observed in our findings.

CONCLUSION

The present study provided detailed microscopic features of the upper digestive tract of SWG, which revealed many similarities with the domestic duck. Noticeable features observed were compared with those of other avian species for a better understanding of the functional morphology of the digestive tract as it relates to the adaptive feeding habits in the SWG.

Data Availability Statement

The raw data that supports the findings of this study will be made available by the authors without undue reservation.

Ethical Statement

This study follows ethical guidelines and approval from the Animal Use and Ethics Committee (AUEC) of the Faculty Veterinary Medicine, University of Maiduguri (Approval no: AUP-R004/2023).

Conflict of Interest

The authors have no conflict of interest to declare.

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Generative AI Statement

The manuscript was prepared without the use of generative AI or AI-assisted technologies.

Authors' Contributions

IAG – Concept and results interpretation; SB and MMK – Sample collection and processing; YBM and MZ – Literature search/Resources; IAG and BGG – Manuscript writing and critical review.

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ORIGINAL ARTICLE

PREVALENCE OF GASTROINTESTINAL PARASITES ON VEGETABLES IN SOME SELECTED MARKETS AND FARMS IN ZARIA METROPOLIS, KADUNA STATE, NIGERIA

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Ethical considerations: When reporting experiments on animals Observation of the ARRIVE guidelines 2.0: Updated guidelines for reporting animal research, published on July 14, 2020 (DOI: 10.1371/journal.pbio.3000410), is applied. The authors ensure that all procedures were performed in compliance with the guidelines for animal care of their institutions or with national/international guidelines.

ABSTRACT

Parasitic infections pose significant threats to human and animal health, particularly in developing countries, where they contribute to socio-economic challenges. This study investigated the prevalence of parasites on vegetables cultivated and sold in farms and markets in Zaria metropolis, Kaduna State, Nigeria. A total of 105 vegetable samples were analyzed using simple sedimentation and saturated salt flotation techniques with a zinc sulfate sucrose medium (specific gravity: 1.21). Overall, 22.9% of the samples were contaminated with parasites. The detected parasites included nematode larvae (8.6%), *Strongyloides* spp. (1.0%), Strongyle eggs (5.7%), *Spirocerca lupi* (1.9%), *Heterakis* spp. (1.0%), *Aspicularis tetraptera* (1.9%), *Entamoeba* spp. (1.0%), Coccidia spp. (2.9%), *Balantidium* spp. (1.0%), and *Fasciola* spp. (1.0%). Contamination rates were highest in cucumber (*Cucumis sativus*, 28.6%) and carrot (*Daucus carota*, 28.6%) samples, followed by cabbage (*Brassica oleracea*, 21.2%), onion (*Allium cepa*, 20.0%), and lettuce (*Lactuca sativa*, 17.6%). A significant association was observed between parasite contamination and the two local government areas in Zaria ($\chi^2 = 5.379$, $p = 0.020$). Public health concerns were underscored by the presence of nematode larvae suggestive of *Strongyloides stercoralis*, a helminth capable of reproducing in the environment and secreting larvae in feces. These findings emphasize the need for improved agricultural practices, such as composting human and animal waste before use as manure. Thorough washing of vegetables is strongly recommended to reduce the risk of parasitic infections.

Keywords: farm; gastrointestinal parasite; market; vegetable; Zaria metropolis

INTRODUCTION

Parasitic infections remain a significant global health challenge, particularly in developing countries where poor sanitation exacerbates their socioeconomic impact [1]. Parasites, broadly classified into ectoparasites and endoparasites, include helminths such as roundworms (*Nematodes*), tapeworms (*Cestodes*), flukes (*Trematodes*), and thorny-headed worms (*Acanthocephalans*), as well as protozoans. These organisms often require both definitive and intermediate hosts for their life cycle [2]. Vegetables, an essential component of human diets, are frequently implicated in the transmission of enteric parasites due to contamination during cultivation, harvest, transport, and storage. Common contaminants include *Ascaris lumbricoides*, *Cryptosporidium* spp., *Entamoeba histolytica*, *Giardia intestinalis*, and hookworm species [3, 4]. Studies from various countries, including Ethiopia [4], Ghana [3], and Nigeria [5, 6], highlight a high prevalence of parasitic contamination on vegetables. Recent evidence further confirms that this problem remains widespread. A 2024 systematic review and meta-analysis reported pooled contamination rates of approximately 44–47% for fruits and vegetables globally, with helminths such as *Ascaris* spp. and protozoans like *Giardia* spp. being the most frequently detected parasites, highlighting the persistent public health concern associated with fresh produce consumption [7].

In Nigeria, parasitic contamination is under-researched, with limited studies reporting contamination rates in markets and farms. Nevertheless, recent findings from Ado-Ekiti Metropolis revealed that about 65% of vegetables sampled from local markets were contaminated with medically important parasites, including *Strongyloides stercoralis*, *Giardia lamblia*, and *Ascaris lumbricoides*, indicating a substantial risk to consumers [8]. Earlier studies have similarly identified *Ascaris* spp., hookworms, *Trichuris* spp., and *Strongyloides* spp. on vegetables sold in Maiduguri [6], Jos [5], and Ilorin [9]. Contributing factors include the use of untreated manure, wastewater irrigation, and poor post-harvest practices [10]. Environmental factors such as climate, rainfall, and soil type further influence parasite prevalence. The increasing trend of consuming raw or lightly cooked vegetables to preserve nutrients may heighten the risk of parasitic infections [11]. Practices such as using untreated sewage as fertilizer are prevalent in some regions, facilitating the transfer of parasites from soil to crops and humans [12]. Supporting this concern, a 2024 community-based study in Ethiopia reported a contamination rate of approximately 46.1% among vegetables and fruits sold in peri-urban markets, reinforcing the role of fresh produce as a major route for intestinal parasite transmission in low-resource settings [13]. Addressing this issue requires raising awareness among farmers and consumers, improving agricultural practices, and implementing strict hygiene measures. Research on the prevalence of gastrointestinal parasites in vegetables is vital for informing public health interventions, particularly in regions like Zaria metropolis, Nigeria, where parasitic diseases are endemic. This study aims to determine the prevalence of gastrointestinal parasites on vegetables from selected farms and markets in Zaria.

MATERIALS AND METHODS

Study Area

This study was carried out within the Zaria metropolitan authority, which comprises two local government areas, namely, Zaria and Sabon Gari. Zaria is located in the Northern Guinea Savannah zone within latitudes 10º 59′ 18″ N and longitudes 7º 41′ 46″ E at an altitude of 550-700 meters above sea level and a total land area of 300 km^2. Sabon Gari LGA is located at 11º 9′ 50″ N, 7º 41′ 49″ E, with a land mass of 263 km^2.

Study Design

A cross-sectional study was conducted using a purposive sampling technique to collect samples of five different types of vegetables from four farms and four markets. At the market, vegetables were displayed on tables, while at the farms, they were placed on the ground.

Sample Collection

The vegetables include lettuce (*Lactuca sativa*), cabbage (*Brassica oleracea*), cucumber (*Cucumis sativus*), onion (*Allium cepa*), and carrots (*Daucus carota*). A total of 105 samples were randomly collected and labeled from the above-mentioned farms and markets. The vegetables were placed in clean polythene bags and transported to the laboratory. The samples were stored at 4°C until processed.

Laboratory Procedures

A modification of the method by Abougrain et al. [14] was used as follows: Two hundred and fifty grams of each fresh, unwashed vegetable sample from both markets and farms were weighed into a sterile plastic bowl and washed with 250 ml of physiological saline solution (0.95% NaCl). The washing water was left for 10 hours to allow sedimentation, after which the top layer was discarded. The remaining washing water was centrifuged at 1500 g for 5 minutes. The supernatant was discarded, and the sediment was collected into 30 ml centrifuge tubes. Each of the 105 samples was processed independently.

1. Simple Sedimentation

The sediment was allowed to stand for 30 minutes, and the supernatant was discarded. A thin smear of each sediment was made using a Pasteur pipette on a clean glass slide. The slides were observed under a light microscope using 10x and 40x objective lenses.

2. Saturated Salt Flotation

The supernatant was discarded, and the residue was gently agitated in a sucrose flotation medium with a specific gravity of 1.21, then sieved through gauze placed on a funnel into a test tube. Each test tube was filled to the brim with the flotation medium to form a convex meniscus. A cover slip was placed on top of the test tube for 30 minutes, then carefully removed and placed on a clean glass slide. The slide was examined under a microscope using 40x and 10x objective lenses for the presence of parasite eggs and oocysts. The morphological characteristics of the eggs, as described by Soulsby [15], were used for identification.

Data Analysis

The Statistical Package for the Social Sciences (SPSS), version 21, was used to analyze the data. Chi-square tests were performed to assess the association between parasite eggs and the factors studied. P-values ≤ 0.05 were considered significant. The number of positive samples was divided by the total number of samples to calculate the estimated prevalence.

RESULTS

The overall prevalence of parasitic contamination was 22.9% (24/105). Specifically, the prevalence in Sabon Gari and Zaria Local Government Areas (LGAs) was 31.6% (18/57) and 12.5% (6/48), respectively. Statistical analysis revealed a significant association between the prevalence of parasite eggs and the LGAs sampled ($p = 0.02$) (Table 1). When comparing sources, the prevalence of parasite eggs was slightly higher in vegetables sampled from markets (28.0%) compared to farms (18.2%), although this difference was not statistically significant ($p = 0.231$) (Table 1). The prevalence of parasite eggs was highest in cucumbers and onions (28.6%) compared to cabbage (21.2%), carrots (20.0%), and lettuce (17.6%). However, the differences were not statistically significant ($p = 0.903$) (Table 1).

Among market samples, the prevalence of parasite eggs varied across the four markets studied: Sabon Gari Market (33.0%), Samaru Market (41.7%), Tudun Wada Market (21.4%), and Galma Market (11.1%) (Table 2). For farm samples, prevalence rates were 12.5% in Shika Farm,

Table 1. Prevalence of gastrointestinal parasite eggs/oocysts on vegetables in Zaria metropolis, Kaduna State, Nigeria

Variable	Category	Number examined	Number positive	Prevalence (%)	Chi-square	P-value
LGA	Sabon Gari	57	18	31.6	5.379	0.020
	Zaria	48	6	12.5		
Source	Market	50	14	28.0	1.432	0.231
	Farm	55	10	18.2		
Type of vegetable	Cabbage	33	7	21.2	1.053	0.903
	Carrot	20	4	20.0		
	Lettuce	17	3	17.6		
	Cucumber	14	4	28.6		
	Onion	21	6	28.6		
	Total	105	24	22.9		

Table 2. Distribution of gastrointestinal parasite eggs/oocysts on vegetables across selected markets and farms in Zaria metropolis, Kaduna State, Nigeria

Source	Category	Total No. Examined	Number Positive	Prevalence (%)	Chi-Square	P-value
Market	Sabon Gari	15	5	33.0	4.744	0.315
	Samaru	12	5	41.7		
	Tudun Wada	14	3	21.4		
	Galma	9	1	11.1		
	Total	50	14	28.0		
Farm	Shika	16	2	12.5	8.085	0.89
	Bomo	13	5	38.5		
	Tudun Wada	11	3	27.3		
	Gyallesu	15	0	0.0		
	Total	55	10	18.2		

Table 3. Prevalence of nematode, trematode and protozoan eggs/oocysts on vegetables in Zaria metropolis, Kaduna State, Nigeria

LGA	No. examined	Prevalence Nematode (%)	Prevalence Trematode (%)	Prevalence Protozoan (%)	Fisher Exact	P-value
Sabon Gari	57	16 (28.1)	2 (3.5)	1(1.8)	11.407	0.010
Zaria	48	3 (6.2)	0 (0.0)	3(6.2)		
Total	105	19 (21.1)	2 (1.9)	4 (3.8)		

38.5% in Bomo Farm, 27.3% in Tudun Wada Farm, and no parasite was observed in Gyallesu Farm (Table 2).

The types of parasites observed included nematodes, trematodes, and protozoans (Fig. 1). In Sabon Gari LGA, the prevalence of these categories was 28.1%, 3.5%, and 1.8%, respectively. In Zaria LGA, the prevalence rates were both 6.2% for nematodes and protozoans. A significant association was also observed between the prevalence of parasite categories and the LGAs sampled ($p = 0.01$) (Table 3).

DISCUSSION

The prevalence of helminth and protozoan eggs on freshly cultivated and marketed vegetables from the Zaria and Sabon Gari local government areas of Kaduna State is of public health significance, but it is lower than reports from Jos Plateau (36.0%) [5], Ilorin (40%) [16], Abeokuta (73.5%) [17], and Port Harcourt (29.5%) [18]. Conversely, it is higher than findings from Ibadan (11.6%) [19] and Maiduguri (3.5%) [6]. Variations in prevalence across these studies may be attributed to differences in sample types, collection methods, seasonal factors, farming practices, and water or soil quality. The vegetables examined in this study included cabbage, lettuce, carrots, cucumber, and onions. Detected parasites included *Strongyloides stercoralis*, nematode larvae, strongyle eggs, *Spirocerca*, *Heterakis*, *Aspicularis*, *Entamoeba histolytica*, coccidia, *Balantidium*, and *Fasciola*. Cucumbers and onions exhibited the highest contamination levels, differing from previous studies [20, 21] which did not sample cucumbers. Amaechi et al. [16] found lettuce to be the most contaminated, while Tchounga et al. [1] reported cabbage as the least contaminated.

Nematode larvae, likely *Strongyloides stercoralis*, were the most prevalent parasite, indicating fecal contamination. This poses significant public health risks due to the parasite's ability to proliferate independently of a host. Strongyle eggs were also common, possibly linked to manure types and treatments used in vegetable cultivation. Vegetables from Bomo farms had the highest contamination, likely due to irrigation with water from River Bomo, which is contaminated by open defecation, refuse dumping, and runoff from pit latrines and sludge, consistent with observations by Dauda et al. [22]. High contamination in Samaru and Sabon Gari markets may stem from their sourcing of vegetables from Bomo farms, poor handling practices, lack of adequate water for washing, and poor storage conditions. The detection of *Aspicularis tetraptera*

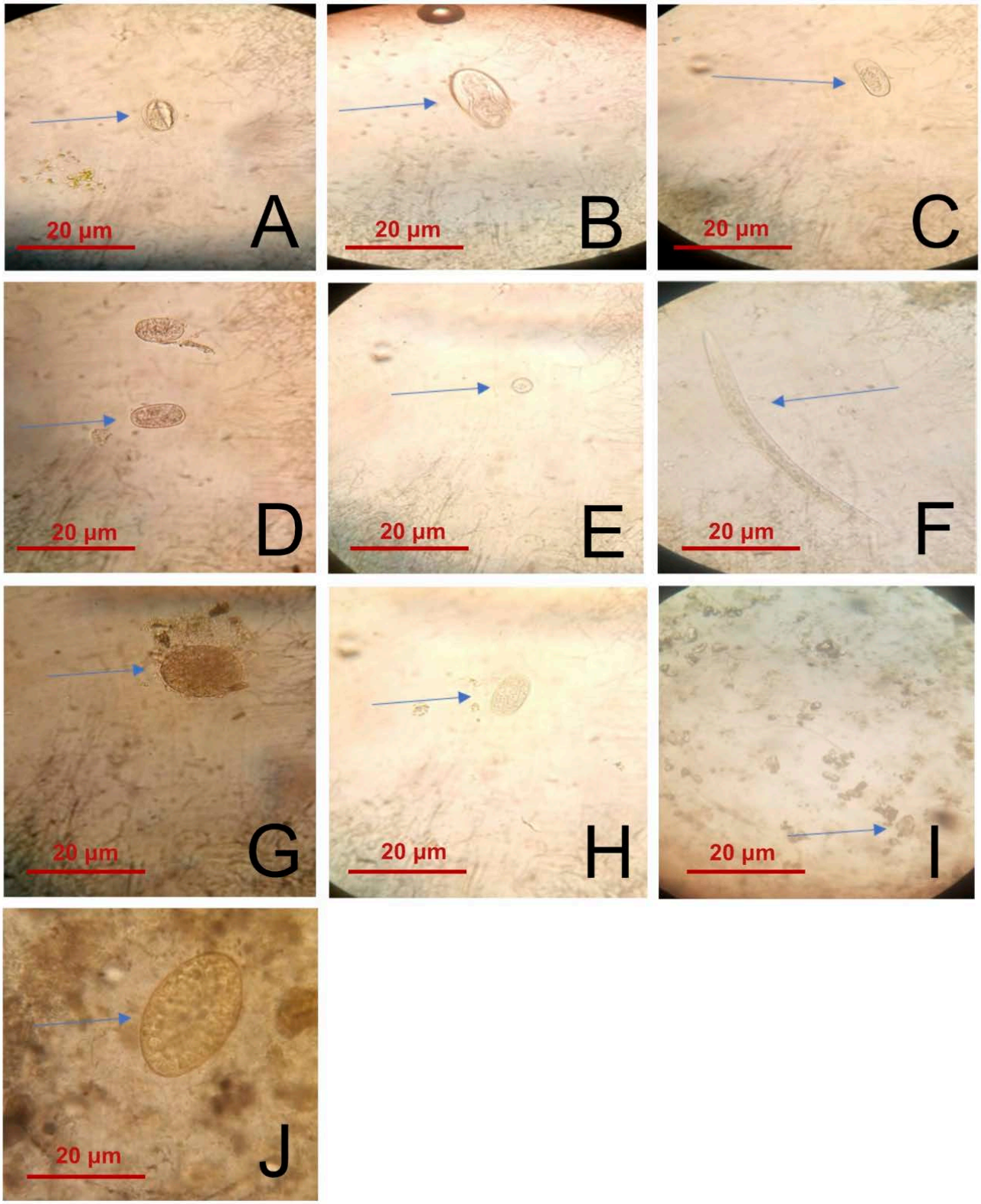

Fig. 1. Gastrointestinal parasite eggs and oocysts on vegetables sampled; A: *Strongyloides* egg, B: *Aspicularis tetraptera* egg, C: *Spirocerca lup*i egg, D: *Strongyle* egg, E: Coccidia oocysts, F: Nematode larvae, G: *Balantidium* cysts, H: *Heterakis* egg, I: *Entamoeba* cysts, J: *Fasciola* egg (using a light microscope at x40 magnification as captured with a digital camera)

eggs in market samples suggests rodent contamination during storage.

A higher prevalence of contamination was observed in market-sourced vegetables compared to farm-sourced ones. This likely reflects additional contamination during handling, washing with contaminated water, or inadequate storage facilities. These findings underscore the need for improved farming, handling, and hygiene practices to mitigate health risks associated with vegetable consumption in the region.

CONCLUSIONS

This study confirms a 22.9% prevalence of helminth and protozoan contamination in vegetable samples from farms and markets in the Zaria metropolis. Nematode larvae, likely *Strongyloides*, and strongyle eggs were the most common parasites, underscoring the risk of consuming contaminated vegetables. These findings highlight the importance of monitoring and improving hygiene practices in vegetable cultivation and marketing to reduce the risk of parasitic contamination and protect public health.

Health education on personal hygiene and safe eating practices should be prioritized in the study area. Markets must be equipped with modern sanitary amenities such as toilets, running water, and proper drainage systems to enhance hygiene. Preventing contamination requires integrated strategies, including proper wastewater treatment, crop restrictions, and improved wastewater application techniques.

Data Availability Statement

The raw data of this article will be made available by the authors without undue reservation.

Ethical Statement

Ethical approval was not necessary for this work.

Conflict of Interest

No conflict of interest.

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Authors' contributions

AAI, EDO, and CVO designed the research; AAI and CVO collected the samples; EDO and AAI analyzed and interpreted the data. AAI, EDO, and CVO contributed to writing the manuscript. All authors read and approved the final manuscript.

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REVIEW ARTICLE

BACTERIAL CAUSES OF NEONATAL MORTALITY IN SHEEP: A REVIEW

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ABSTRACT

Sheep farming plays a significant role in global livestock production, contributing to the output of meat, wool, and milk, and providing income for millions of livestock farmers, especially in developing countries. In this review, the role of bacteria in the aetiology and pathology of neonatal lamb mortality, including management and prevention strategies, is discussed. Several bacteria are known to cause neonatal mortality in sheep, with *Escherichia coli* consistently identified as the most common pathogen, responsible for 15–20% of neonatal deaths worldwide. Other important bacterial pathogens include *Salmonella* spp., *Pasteurella multocida*, and *Mannheimia haemolytica*. Additionally, *Brucella melitensis* and *Campylobacter fetus* subsp. *fetus*, among others, have also been recognised as bacterial causes of neonatal mortality in lambs. This review demonstrates that bacterial infections cause lamb losses in sheep during the first two weeks of life. Effective management and preventative strategies—such as prompt vaccination, adequate colostrum intake, good sanitation, responsible antibiotic use, and supportive care—can significantly reduce bacterial infections in newborn lambs. This reduction will lower neonatal mortality rates, support sustainable sheep production, and contribute to global food security.

Keywords: bacteria; neonate; mortality; sheep production

INTRODUCTION

Sheep farming plays a significant, albeit variable, role in global livestock production, contributing to the production of meat, wool, and milk, and providing income for many farmers, especially in developing countries [1].

Early mortality in lambs is a significant constraint to sustainable sheep production, with multifactorial factors

attributed to both infectious and non-infectious causes [2]. Lamb mortality before weaning reduces sheep fertility and profitability for sheep farmers. In certain regions, this condition can also develop into significant income losses [3].

Neonatal mortality in sheep is defined as lamb deaths within the first week of life, with the highest risk in the initial 48 hours [4]. Lamb mortality rates vary, ranging from 10 to 25% between pregnancy diagnosis and sale, depending on the production system used [4], with early neonatal mortality (within 48 hours) accounting for 62.1% of neonatal deaths in some studies [4]. Neonatal lambs are most susceptible to infectious diseases on the day they are born, with up to 50% of all pre-weaning mortality occurring during this period [3].

Predisposing factors include insufficient colostrum intake, inadequate monitoring during lambing, hypothermia, and poor feeding practices [5]. Low birth weight, young or primiparous ewes, and low dam weight at lambing also increase mortality risk [6]. Respiratory disorders, diarrhoea, starvation, and trauma caused by bacterial infections are considered major causes of neonatal mortality in lambs [6, 7, 8, 9, 10].

The sheep placenta is epitheliochorial and does not allow the humoral transfer of immune components from the ewe to the lamb. Newborn lambs are thus born markedly hypogammaglobulinaemic and depend entirely on passive transfer of colostral immunoglobulins, acquired by suckling, for immunological protection after lambing.

Passive immunity must be acquired within the first 24 hours in newborn lambs, as gut closure to immunoglobulin absorption occurs between 24 and 36 hours after birth [11]. When adequate passive transfer of immunity does not happen, lambs are said to suffer from ‘failure of transfer of passive immunity’, which puts neonatal lambs at increased risk of morbidity and mortality [11].

Lamb mortality is associated with low immunoglobulin status 24 hours after birth [11], demonstrating the importance of ensuring good colostrum intake in newborn lambs. Adequate colostrum intake relies on two complementary factors: the ability of the lamb to ingest sufficient amounts (through vigorous sucking behaviour) and the concentration and quantities of colostral immunoglobulins available from the ewe [11].

Bacterial infections are a major cause of neonatal death, accounting for 36% of early neonatal deaths in one study [12]. *Escherichia coli* is consistently identified as the most prevalent pathogen, responsible for 14–30.8% of neonatal deaths [12]. Other common bacterial causes include *Pasteurella multocida*, *Clostridium perfringens*, and *Staphylococcus aureus* [4]. *Listeria monocytogenes* and *Campylobacter fetus* have also been implicated [13].

Besides infections, traumatic lesions and congenital malformations also contribute to neonatal mortality [12]. *Escherichia coli* is frequently isolated from lambs that die in the first two weeks of life and is often associated with diarrhoea and septicaemia [12, 13]. *Pasteurella* spp. are commonly found in respiratory infections and are significant contributors to neonatal mortality [13]. *Salmonella* spp. can cause enteritis and septicaemia in lambs [13].

Most bacterial-related deaths occur within the first few days of life. For example, in one study, the highest mortality rate was observed in lambs under 15 days old [12]. Septicaemia and pneumonia are frequently identified as causes of death, with *E. coli* being a common agent in septicaemic cases [12].

This paper aims to review the role of bacteria in the aetiology and pathology of neonatal lamb mortality and also suggest management and prevention strategies.

Economic Impact of Bacterial-Caused Neonatal Mortality on Sheep Farming

Bacterial-caused neonatal mortality in sheep has significant economic implications, directly affecting farmers’ income and sustainable livestock production. Lamb mortality rates, often reaching 15–20% globally, represent a significant loss of potential income from meat, wool, and breeding stock [14].

Each lamb lost equates to a direct financial loss, with studies estimating the value of a single lamb at approximately $28.5 to $90, depending on the production system and market conditions [14].

Diarrhoea and pneumonia are the major clinical signs associated with infectious diseases leading to pre-weaning mortality [15, 16]. Diarrhoea is the most devastating clinical sign in newborn lambs under 21 days of age, which is accompanied by enteritis, abdominal pain, and an increase in faecal fluidity [17].

Indirect costs further compound the economic burden [15, 16]. These include expenses related to veterinary services, medications (e.g., antibiotics for bacterial infections like *E. coli*), and labour for boarding sick lambs [15]. Additionally, farms with high neonatal mortality experience

reduced productivity due to fewer surviving lambs reaching marketable age or affecting replacement stock [17, 18].

Preventable bacterial infections also lead to increased reliance on antibiotics, raising concerns about global antimicrobial resistance and regulatory pressures [19]. The cost-effectiveness of preventive measures, such as vaccination and improved hygiene practices, is evident in reducing mortality rates and associated losses [15].

Beyond finances, neonatal mortality imposes emotional stress on farmers, leading to frustration and emotional trauma over preventable deaths [16]. This mental toll can affect decision-making and farm management practices [16].

Common Bacterial Pathogens in Neonatal Mortality in Sheep

- ***Escherichia coli* (*E. coli*):** This bacterium is frequently identified as a major cause of neonatal lamb mortality [11, 12]. It is often associated with septicaemia and gastrointestinal infections and is isolated in a significant proportion of cases, such as 65% of septicaemic cases and 30.8% of bacterial infections overall [11, 12].
- ***Pasteurella* spp.:** These bacteria are commonly linked to respiratory infections and are significant contributors to neonatal mortality in sheep. *Pasteurella multocida* is specifically noted in some studies [12].
- ***Salmonella* spp.:** These pathogens can cause enteritis and septicaemia in lambs, contributing to neonatal mortality [18].

Common Infections and Conditions

- **Septicaemia:** Often caused by *E. coli*, this condition is a leading cause of neonatal lamb mortality [11].
- **Pneumonia:** Respiratory infections, including those caused by *Pasteurella* spp., are common in neonatal lambs [11, 12].
- **Gastrointestinal Infections:** Diarrhoea and enteritis are frequently associated with bacterial pathogens like *E. coli* and *Salmonella* spp. [12].

Escherichia coli

Pathogenesis of *Escherichia coli* in Neonatal Lamb Mortality

Escherichia coli contributes to neonatal lamb mortality primarily through septicaemia and endotoxaemia, often following intestinal colonisation and is reported to be the most harmful organism in the first two weeks of birth. Since the immune system of the lambs has not been fully developed. [11, 20]. Lambs typically acquire pathogenic *E. coli* via faecal-oral transmission in contaminated environments or vertical exposure during parturition [11]. After ingestion, non-enterotoxigenic strains colonise the gut mucosa, triggering catarrhal gastroenteritis and compromising intestinal barrier integrity [20]. This allows bacterial translocation into the bloodstream, leading to systemic infection – a process implicated in 65% of septicaemia neonatal lamb deaths [11].

Key pathogenic mechanisms include:

- **Endotoxin release:** Lipopolysaccharides (LPS) from *E. coli* cell walls induce systemic inflammation, causing elevated serum endotoxin levels linked to poor prognosis [20, 21].
- **Organ invasion:** Post-bacteraemia, the bacteria cause interstitial pneumonia, hepatitis, and nephritis through hematogenous spread [11, 20].
- **Microbiome dysbiosis:** Antibiotic treatment exacerbates endotoxaemia by altering gut microbiota, enabling secondary pathogens like *Proteus mirabilis* to proliferate [19].

Pathological findings show distinct patterns: untreated lambs develop generalised inflammatory lesions (meningoencephalitis, interstitial nephritis), while antibiotic-treated cases exhibit enterotoxemic damage with intestinal ischemia [19]. Virulent strains like O78: NM demonstrate nasal mucosal tropism, suggesting alternative entry routes beyond the gastrointestinal tract [20].

Susceptibility is influenced by delayed colostrum intake, impairing passive immunity, high bacterial load exposure in contaminated lambing areas, and gut microbiota composition, where resistant lambs maintain lower jejunal bacterial counts [11, 20, 22]. Approximately 14% of neonatal deaths are directly attributed to *E. coli* infections, with mortality peaking within 48 hours post-parturition [11, 20].

Clinical Manifestations of *Escherichia coli* in Neonatal Lamb Mortality

Bacterial pathogens are responsible for high rates of mortality in lambs, with *E. coli* being considered an important cause of diarrhoea in lambs [10].

The clinical manifestations of *Escherichia coli* infections in neonatal lambs include signs of septicaemia, enteritis, and respiratory distress [11, 13]. Lambs affected by septicaemia *E. coli* often exhibit weakness, lethargy, loss of appetite, and rapid progression to recumbency or sudden death [13]. Neurological symptoms such as ataxia, seizures, or muscle spasticity may also occur before death [11].

Enteric infections caused by *E. coli* lead to diarrhoea, dehydration, and electrolyte imbalances, which can further compromise the lamb's condition [13, 19]. Respiratory involvement may manifest as laboured breathing or nasal discharge, especially in cases of mixed bacterial infections [13].

Post-mortem findings frequently reveal systemic inflammation, including interstitial pneumonia, gastrointestinal lesions, and evidence of bacteraemia [11]. Infected lambs may also show pulmonary oedema and pericardial effusion [11].

Mode of Transmission of *Escherichia coli* in Neonatal Lamb Mortality

Escherichia coli transmission in neonatal lamb mortality primarily occurs through vertical transmission during parturition and environmental exposure [23, 24]. Lambs can ingest the bacteria from contaminated vaginal secretions during parturition, particularly if the ewe is colonised [24]. Post-parturition, faecal-oral transmission is common in unsanitary environments, with poor hygiene in lambing areas facilitating bacterial spread [24, 25]. Contaminated colostrum or milk, especially when bottle-fed under unhygienic conditions, also introduces pathogens [23, 24]. In severe cases, *E. coli* enters the bloodstream, causing septicaemia linked to 14% of neonatal deaths [11]. Risk factors include delayed colostrum intake, overcrowding, and unhygienic obstetric manipulations during dystocia and wet conditions that promote bacterial survival [23, 24]. Early-life infections often arise within 24–48 hours, emphasising the critical perinatal exposure window [11, 25].

***Streptococcus* spp.**

Pathogenesis of *Streptococcus* spp. in Neonatal Mortality in Sheep

Streptococcus agalactiae (Group B *Streptococcus*, GBS) is a leading cause of neonatal infections, including sepsis, meningitis, and pneumonia [26, 27]. Group B *Streptococcus* colonises the maternal genital tract and can be transmitted to lambs during parturition [28]. The pathogenesis involves several virulence factors that enable bacterial adherence, invasion, and immune evasion. Key virulence factors include the haemolytic pigment, hyaluronidase, pili, adhesins, and the sialic acid-rich capsular polysaccharide [26, 27]. These factors facilitate colonisation of epithelial surfaces, invasion of host barriers, and evasion of immune clearance [28]. The completion of GBS genome sequencing has provided insights into the organism's metabolism and gene regulation, offering potential targets for novel preventive strategies [29]. Current prevention methods include maternal screening and antibiotic prophylaxis, while vaccine development remains an active area of research [27].

Respiratory syncytial virus (RSV) and *Streptococcus pneumoniae* co-infections can lead to severe respiratory disease, particularly in neonates. In a lamb model, RSV–*S. pneumoniae* co-infection resulted in increased lung pathology, neutrophil infiltration, and cytokine dysregulation, despite lower bacterial loads [30]. Respiratory syncytial virus infection enhances *S. pneumoniae* virulence through direct binding of RSV G protein to pneumococcal penicillin-binding protein 1a, leading to increased bacterial adherence and upregulation of virulence genes [31]. In preterm lambs, RSV infection induced a heightened pro-inflammatory response, characterised by increased cytokine production and neutrophil recruitment, but with reduced neutrophil activation [32]. Respiratory syncytial virus infection in neonatal lambs also caused lung inflammation, architectural remodelling of lung-draining lymph nodes, and activation of caspase-mediated cell death pathways [32]. These findings highlight the complex interactions between RSV and *S. pneumoniae* in exacerbating respiratory disease severity.

Clinical Manifestation of *Streptococcus* spp. in Neonatal Mortality in Sheep

Group B *Streptococcus* (GBS) is a significant cause of neonatal infections, presenting as early-onset disease (EOD) within the first week of life or late-onset disease (LOD) between 7–89 days [33]. EOD typically manifests within 24 hours of parturition, with symptoms including sepsis, pneumonia, and meningitis [34, 35]. Common clinical presentations include fever, poor activity, respiratory distress, and irritability [35]. EOD has a higher incidence

of pneumonia and mortality compared to LOD, while meningitis is more prevalent in LOD cases [35].

Group B *Streptococcus* infections can lead to severe complications, including neurodevelopmental injuries and long-term disabilities [33]. Globally, GBS infections affect over 300,000 neonates annually, resulting in 90,000 deaths [33]. Prevention strategies, such as intrapartum antibiotic prophylaxis, are crucial in reducing the incidence of GBS infections in neonates [36].

Mode of Transmission of *Streptococcus* spp. in Neonatal Mortality in Sheep

The transmission of *Streptococcus spp.* in neonatal lamb mortality occurs through vertical, horizontal, and environmental routes. **Vertical transmission** primarily involves exposure to maternal vaginal or faecal secretions during parturition, where *Streptococcus agalactiae* (Group B *Streptococcus*) colonises mucosal surfaces of lambs [37]. **Horizontal transmission** arises postnatally via contaminated environments, such as bedding, equipment, or milk, particularly in settings with poor hygiene [37, 38]. The **navel** serves as a critical entry point for pathogens like *Streptococcus dysgalactiae*, especially if not properly disinfected after parturition [38]. Additionally, wounds from management practices (e.g., tail docking, castration) create portals for bacterial invasion [38]. Co-infections with viruses like respiratory syncytial virus (RSV) may exacerbate respiratory transmission of *Streptococcus pneumoniae* through aerosolised droplets [30]. Maternal colonisation and environmental persistence of *Streptococcus* spp. in lambing areas further amplify infection risks, particularly in overcrowded or unsanitary conditions [38]. Prophylactic measures, including navel disinfection and improved biosecurity, are essential to mitigate transmission [38].

Clostridium perfringens

Pathogenesis of *Clostridium perfringens* in Neonatal Mortality in Sheep

In lambs, the infection often begins with the ingestion of *C. perfringens* spores or vegetative cells from contaminated environments or maternal faeces. Once in the intestine, the bacteria multiply rapidly and colonise epithelial cells, releasing toxins. This toxin disrupts cell membranes by hydrolysing phospholipids, leading to necrosis, vascular damage, and systemic absorption of toxins [39]. The systemic effects include haemolysis, jaundice (icterus), and haemoglobinuria due to the destruction of red blood cells [39].

Severe cases result in sudden death due to shock and multi-organ failure. Post-mortem findings include centrilobular hepatic necrosis, acute tubular injury in the kidneys, splenic congestion, and pulmonary oedema [39]. Factors such as inadequate colostrum intake and stress increase susceptibility. Diagnosis is challenging due to the widespread presence of *C. perfringens* in healthy animals; quantitative toxin detection methods are needed for confirmation [39]. Preventive measures include vaccination against clostridial toxins and improved hygiene during lambing.

Clinical Manifestation of *Clostridium perfringens* in Neonatal Mortality in Sheep

Clostridium perfringens causes severe enteric diseases in sheep, particularly affecting neonatal lambs. Type B and C infections typically result in lamb diarrhoea, characterised by sudden death or acute symptoms like cessation of nursing, listlessness, and bloody diarrhoea [40]. Type D enterotoxaemia often presents with neurological signs and sudden death in well-conditioned lambs [41]. Diagnosis relies on clinical signs, post-mortem findings, and laboratory confirmation, with toxin detection in intestinal contents being crucial [40]. Histopathological examination of the brain is particularly useful for type D diagnosis [40]. *C. perfringens* infections can cause rapid, severe illness with high mortality rates, as observed in neonatal foals [42]. The first reported case of *C. perfringens* type B in New Zealand highlights the potential for geographical spread and emphasises the importance of vaccination in prevention [43].

Gross findings include generalised icterus, red urine in the bladder, and organ congestion [39]. Post-mortem findings often reveal haemorrhagic enteritis with mucosal ulceration and systemic lesions such as pulmonary oedema and splenic congestion [39].

Mode of Transmission of *Clostridium perfringens* in Neonatal Mortality in Sheep

Clostridium perfringens is a common pathogen in livestock, causing enteritis and mortality, particularly in neonates [44]. The bacteria are ubiquitous in environments

such as soil, water, and animal intestines [45]. Transmission occurs through ingestion of spores or vegetative cells from contaminated sources, including colostrum and feed [45]. Predisposing factors include environmental contamination, intestinal pH, imbalance of gut microflora, and nutrition [45]. In lambs, *C. perfringens* types A, B, and D are prevalent, with higher infection rates observed in cold periods [44]. Neonatal lambs are particularly susceptible due to underdeveloped immune defences [42].

Listeria monocytogenes

Pathogenesis of *Listeria monocytogenes* in Neonatal Mortality in Sheep

Listeria monocytogenes is a Gram-positive, facultative intracellular pathogen that causes severe infections in humans and animals [46].

Lambs that develop septicemic disease may acquire infection from contamination on the ewe's teat or from the ingestion of milk containing the organism from ewes with subclinical bacteremia, through the navel from the environment, and also as a congenital infection [47, 48].

The bacterium invades host cells using proteins like internalin (InlA/InlB), which interact with cellular receptors to trigger internalization [48]. It also uses Act-A, a protein that is important in intracellular movement by acting on polymerisation and is also thought to play a role in cell tropism (adhesion) and invasion.

Once inside, *L. monocytogenes* escapes the phagosome using listeriolysin O and replicates in the cytoplasm [49]. The pathogen then spreads cell-to-cell by exploiting host actin polymerisation machinery through its ActA protein [50]. This intracellular lifecycle allows *L. monocytogenes* to evade host immune responses and establish systemic infections [47]. The bacteria can cross the intestinal barrier, leading to infection of the liver, brain, and placenta [47]. Despite antibiotic treatment, listeriosis has a high mortality rate of 25–30%, primarily due to severe meningoencephalitis [47].

Clinical Manifestation of *Listeria monocytogenes* in Neonatal Mortality in Sheep

Listeria monocytogenes is a pathogenic bacterium that can cause severe infections, particularly in neonates. Clinical manifestations primarily include septicaemia and meningoencephalitis [51, 52]. Neonatal listeriosis often presents with nonspecific symptoms such as fever, depression, and respiratory distress [53, 54]. The infection can be transmitted from dam to foetus, with maternal symptoms including fever and diarrhoea [54]. Preterm neonates may exhibit more severe symptoms compared to full-term infants [54]. Diagnosis is confirmed through positive blood cultures, and treatment typically involves intravenous administration of ampicillin or penicillin for 1–2 weeks [54]. Cephalosporins are ineffective against *L. monocytogenes* [54]. Despite treatment, mortality rates remain high due to the combination of immunocompromised hosts and often delayed diagnosis [52].

Mode of Transmission of *Listeria monocytogenes* in Neonatal Mortality in Sheep

Listeria monocytogenes can cause severe neonatal infections through both vertical and horizontal transmission. Vertical transmission occurs transplacentally or during parturition, leading to early-onset listeriosis [52, 55]. Horizontal transmission can happen postnatally through ingestion or contact with contaminated sources [56]. Neonates are particularly susceptible due to their immature immune systems and gut microbiota [56]. Nosocomial infections have been reported, with cross-contamination occurring through shared equipment or personnel [57]. The placenta plays a crucial role in protecting the foetus, but *L. monocytogenes* can evade this barrier using virulence factors like internalin proteins [55]. Various animal models have been used to study *L. monocytogenes* transmission to investigate their pathogenesis [55]. Proper disinfection of medical equipment is essential to prevent nosocomial spread [57].

Pasteurella multocida

Pathogenesis of *Pasteurella multocida* in Neonatal Mortality in Sheep

Pasteurella multocida is a significant bacterial pathogen causing respiratory diseases in various animals, including neonatal lambs. The bacterium typically resides in the upper respiratory tract of healthy animals but can become pathogenic under stress conditions [58]. Key virulence factors include the capsule, lipopolysaccharide, and *Pasteurella multocida* toxin, which aid in evading host immunity and promote bacterial survival [58, 59]. *P. multocida* often acts as a secondary pathogen following prima-

ry viral infections that compromise respiratory defences [60]. Stressful conditions, such as transport, heat stress, and overcrowding, impair the animal's immune response, allowing *P. multocida* to proliferate and invade lung tissue, leading to bronchopneumonia [59, 60]. The disease progresses rapidly, with clinical signs ranging from mild respiratory distress to acute death, characterised by severe fibrinous or fibrinopurulent bronchopneumonia and fibrinous pleurisy [60].

Clinical Manifestation of *Pasteurella multocida* in Neonatal Mortality in Sheep

Pasteurella multocida infections in neonatal lambs can cause severe respiratory distress and systemic illness, often leading to acute death without prior clinical signs [11].

Clinical manifestations include depression, lethargy, inappetence, coughing, and nasal discharge [61]. Post-mortem findings typically reveal lung consolidation and pleuropneumonia [62]. *P. multocida* is a significant cause of neonatal mortality in sheep, with infections often resulting in septicaemia or pneumonia [11]. The prevalence of lung lesions can be high in lambs, with severe lesions significantly reducing growth performance [62]. Risk factors for neonatal pasteurellosis include animal exposure, vertical transmission, low birth weight, and early onset of symptoms [63]. Prevention and control strategies should focus on appropriate antimicrobial selection, reliable diagnosis, and development of vaccines targeting relevant serotypes [61].

Mode of Transmission of *Pasteurella multocida* in Neonatal Mortality in Sheep

Pasteurella multocida is a significant cause of neonatal infections, with transmission occurring through various routes. While animal exposure, particularly to cats and dogs, is a major risk factor [63], vertical transmission from ewe to lamb during parturition has been documented [64]. Horizontal human-to-animal transmission, though rare, has also been reported [65]. Neonatal pasteurellosis often presents as bacteraemia with or without meningitis, with onset after 72 hours of age significantly associated with meningitis [63]. Treatment typically involves β-lactam antibiotics, sometimes combined with aminoglycosides or chloramphenicol [63]. In a broader context of neonatal infections in lambs, *P. multocida* is one of several bacterial species implicated in both early (hebdomadal) and later (post-hebdomadal) neonatal deaths, with infections often resulting from umbilical contamination at parturition or ingestion [66].

Other Bacterial Infections

Staphylococcus aureus

Staphylococcus aureus is a commensal bacterium that can become an opportunistic pathogen, causing various infections, including skin lesions, pneumonia, and bloodstream infections [67, 68]. The bacterium employs several virulence factors, notably Panton-Valentine leukocidin (PVL) and alpha-toxin (Hla), which are associated with necrotic skin infections and severe pneumonia [69]. During the transition from colonisation to invasion, *S. aureus* upregulates genes involved in adhesion, toxin production, and nutrient acquisition [67]. In bloodstream infections, *S. aureus* secretes coagulases and displays agglutinins, leading to abscess formation [68]. The pathogen also recruits and destroys immune cells, facilitating its spread to new sites or hosts [68].

Methicillin-resistant *Staphylococcus aureus* (MRSA) poses significant challenges in livestock settings due to its resistance to multiple antibiotics [70]. MRSA can cause various infections, ranging from skin and soft tissue infections to severe systemic conditions [70]. In livestock, MRSA is associated with suppurative skin conditions, wound infections, and mastitis [71]. The emergence of livestock-associated MRSA, particularly ST-398, has raised concerns about zoonotic transmission to farmers, veterinarians, and abattoir workers [71]. MRSA has been detected in retail meat and raw milk, indicating potential foodborne transmission risks [72]. Preventive measures include maintaining hygiene during animal handling and processing, reducing antibiotic use in livestock, and screening animals and their products for MRSA [71]. Combining herbal extracts with antibiotics shows promise in combating MRSA and reducing antibiotic resistance [73].

***Salmonella* spp.**

Salmonella enterica is a significant zoonotic pathogen affecting both humans and animals, capable of causing gastroenteritis and systemic infections [74]. In neonatal

lambs, *S. enterica* subsp. *diarizonae* can lead to diarrhoea and histopathological changes in the abomasum and small intestine [75]. The pathogen's ability to invade the intestinal epithelium is crucial for infection, with invasion occurring as early as 15 minutes post-infection in calves [76]. However, the magnitude of intestinal invasion does not necessarily correlate with host specificity in sheep [77]. The host response to *Salmonella* infection involves upregulation of chemokines and cytokines, leading to neutrophilic inflammation [76]. While some *Salmonella* serovars have broad host ranges, others are host-adapted, influencing their ability to persist in systemic sites and cause disease [74, 77].

***Mycoplasma* spp.**

Mycoplasma ovipneumoniae plays a significant role in respiratory diseases of sheep, particularly in lambs. It is commonly isolated from pneumonic lungs and associated with various pathological findings, including suppurative bronchopneumonia and interstitial pneumonia [78]. *M. ovipneumoniae* contributes to lamb mortality, often in conjunction with other pathogens like *Mannheimia* spp. and *Pasteurella multocida* [79]. The bacteria colonise the respiratory tract, causing ciliostasis and eliciting an exudate that allows secondary bacterial infections [80]. Chronic non-progressive pneumonia (CNP) in lambs is frequently associated with *M. ovipneumoniae*, with multiple strains often present in affected lungs [80]. Transmission occurs through direct contact or inhalation of aerosols, with ewes potentially serving as persistent carriers [79]. While *M. ovipneumoniae* is a primary agent in ovine respiratory diseases, other mycoplasmas like *M. arginini* appear to play a less significant role [78].

Some Less Frequently Isolated Bacteria

Corynebacterium pseudotuberculosis and *Mannheimia haemolytica* [12].

Pathophysiology of Bacterial Infections in Neonatal Lambs

Neonatal lambs have an immature immune system that makes them susceptible to most bacterial infections. This immunological immaturity is characterised by low leukocyte counts, reduced neutrophil phagocytic activity, and diminished cytokine responses to pathogen-associated molecular patterns [81]. Neonatal lambs exhibit a distinct innate immune system biased towards TH2-/TH17-polarizing and anti-inflammatory cytokine production, with impaired TH1-polarising cytokine production [82]. This leaves them particularly susceptible to intracellular pathogens. They also have deficient expression of complement and antimicrobial proteins and peptides, contributing to their vulnerability to pyogenic bacteria [82]. The immature immune system, combined with the need for invasive veterinary procedures, makes neonatal lambs highly susceptible to common pathogens [83]. This vulnerability is exacerbated in compromised newborn lambs, such as those requiring intensive care [84]. This immunologic immaturity creates a vulnerable window during the first postnatal week, allowing pathogens like *Escherichia coli*, *Streptococcus* spp., and *Clostridium perfringens* to invade and colonise mucosal surfaces [81].

Infectious Routes and Colonisation

The gastrointestinal tract of newborn lambs is rapidly colonised by microbes after parturition, with the initial microbiome significantly influenced by early neonatal events and feeding modes [85]. Suckling lambs acquire microbes primarily from the dam's teats and ambient air, while artificially bottle-fed lambs receive bacteria mainly from the dam's vagina, air, and pen floor [86]. Bottle feeding can increase potential microbes like *Escherichia*/*Shigella* and delay anaerobic microbe establishment [86].

Bacterial Virulence and Toxins

Bacterial pathogens produce various toxins and virulence factors that play crucial roles in disease progression. *Escherichia coli* strains possess multiple virulence factors, including adhesion proteins, secretion systems, and toxins such as heat-labile enterotoxins and Shiga toxins [87]. *Clostridium perfringens* produces enzymes like alpha toxin (phospholipase C) and kappa toxin (collagenase), which disrupt membrane integrity, as well as toxins affecting vascular endothelium and haemolysins [88]. *Staphylococcus aureus* synthesises alpha-toxin and toxic shock syndrome toxin, while other bacterial toxins include botulinum and tetanus neurotoxins [89]. These toxins employ diverse mechanisms to affect eukaryotic cells, contributing to pathogenicity. These toxins exacerbate systemic inflammation, triggering cytokine storms and multi-organ failure.

Immune Evasion and Outcomes

Streptococcus species employ various mechanisms to evade host immune responses. *Streptococcus pyogenes* utilises streptolysin O to induce rapid macrophage apoptosis, hindering immune clearance and enhancing virulence [90]. Both *S. pyogenes* and *S. pneumoniae* secrete complement evasion factors that interfere with opsonisation and terminal pathway lysis [91]. *S. pneumoniae*'s polysaccharide capsule protects against complement-mediated opsonophagocytic killing, while its surface-associated glycosidases modulate host defence proteins by removing glycans, affecting their stability and function [92]. These glycosidases also contribute to biofilm formation, enhancing bacterial resilience against antimicrobials and immune attacks. Additionally, they facilitate bacterial adherence and nutrient acquisition by hydrolysing host glycans [92].

Clostridium perfringens, particularly types B and D, poses significant threats to neonatal lambs, causing sudden death through enterotoxaemia and systemic vascular damage [43]. *C. perfringens* type B, newly reported in New Zealand, produces both beta and epsilon toxins, leading to haemorrhagic enteritis and vascular damage [43]. The epsilon toxin of type D causes cerebral endothelial damage, resulting in vasogenic oedema and focal necrosis [43].

Clostridial infections are part of a broader spectrum of neonatal infections in lambs, which account for approximately 7.5% of lamb mortalities [66]. These infections often result from umbilical contamination or ingestion [66]. Fortunately, effective control of clostridial diseases can be achieved through toxoid vaccines, with protection transferred to neonatal lambs via colostrum [93].

Escherichia coli infections pose a significant threat to neonatal lambs, causing high mortality rates and economic losses in sheep farms [4, 94]. *E. coli* was identified as the primary bacterial cause of neonatal mortality in lambs, representing 63.4% of isolates [4]. Vaccination of pregnant ewes with *E. coli* surface proteins or formalin-inactivated bacterins can provide passive immunity to newborn lambs, significantly reducing morbidity and mortality rates [95, 96]. Lambs born to vaccinated ewes demonstrate increased resistance to both homologous and heterologous *E. coli* challenge exposures [95]. Risk factors associated with neonatal mortality include lack of vaccination, not separating neonates from adults, and starvation-mothering exposure [4]. While antibiotics were used to treat infections, probiotics were found to be more effective in controlling new infection rates [94].

Management and Prevention Strategies

The management and prevention of bacterial infections in neonatal lambs are critical to reducing neonatal mortality and ensuring the overall health of lambs, particularly during their first few days of life. Effective strategies include vaccination, promotion of colostrum intake, maintaining sanitation and biosecurity, implementing antibiotic therapy, and providing supportive care.

Vaccination programs play a significant role in the prevention of infections such as *E. coli* and Clostridia, which are common causes of neonatal distress. Studies show the importance of vaccines in providing immunity against specific pathogens, which can significantly reduce the incidence of disease outbreaks in lambing seasons. Furthermore, the timing of vaccine administration aligns with lamb rearing practices to ensure optimal protection, especially against diseases like pneumonia triggered by pathogens such as *Mannheimia haemolytica* [11].

The intake of colostrum, the first milk produced by ewes, is vital to lamb survival and health. Research indicates that an adequate amount of colostrum can substantially lower the chances of infections, as it provides essential antibodies and promotes the development of the gut microbiota, enhancing the neonate's immune response [97]. For instance, studies show that high immunoglobulin G levels in lambs correlate with better outcomes post-colostrum intake, thus supporting their physiological capabilities against bacterial infections [98]. This passive immunity can diminish the necessity for prophylactic antibiotics and decrease mortality rates associated with bacterial diseases like watery mouth caused by *E. coli* [97].

Sanitation and biosecurity are foundational aspects of managing bacterial infections. Good farm management practices, such as daily cleaning of lambing pens, can mitigate the build-up of pathogenic microorganisms in the environment, lowering flock mortality rates [11]. Regular disinfection of the pen of pregnant ewes waiting for parturition, cleaning of teats and equipment, feeding sufficient colostrum, and timely change of bedding are among the measures that can reduce infection in neonatal lambs.

Specifically, the improper handling of lambs during critical neonatal procedures like castration and tail docking without adequate sanitation has been associated with the prevalence of infections such as joint ill [38]. Instituting rigorous biosecurity protocols can minimise the risk of contagious infections, supporting both individual and herd health.

Antibiotic therapy is another key strategy, particularly in treating established infections. However, judicious use remains essential to avert antimicrobial resistance. For instance, while antibiotics like gamithromycin have shown effectiveness against *Mycoplasma* infections, caution is recommended due to the increasing resistance seen in various pathogens [99]. In managing infections, supportive care, which encompasses hydration and nutritional support, remains crucial alongside antibiotic treatment to bolster the recovery of affected lambs [30].

CONCLUSION

This review discussed the role of bacteria in the aetiology and pathology of neonatal lamb mortality, including management and prevention strategies. Several bacteria were reported to cause neonatal mortality in sheep, most especially in the first two weeks of life, with *Escherichia coli* consistently identified as the most prevalent pathogen globally. Other significant bacterial pathogens include *Salmonella* spp., *Pasteurella multocida*, and *Mannheimia haemolytica*. Additionally, *Brucella melitensis* and *Campylobacter fetus* subsp. *fetus,* among others, were also identified as bacterial causes of neonatal mortality in lambs. Overall, the integration of vaccination strategies, robust sanitation practices, promotion of colostrum intake, appropriate use of antibiotics, and provision of supportive care forms a comprehensive approach to managing bacterial infections in neonatal lambs. This multi-faceted strategy will not only reduce neonatal mortality but also foster sustainable livestock productivity, especially in developing countries.

Generative AI Statement

The authors declare that no generative AI and AI- assisted technologies were used in writing this manuscript.

Authors' Contributions

All the Authors contributed to the design, writing, editing and proof reading of this manuscript. All the authors read and approved the final manuscript.

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ORIGINAL ARTICLE

PROTECTIVE EFFECTS OF PIROXICAM AGAINST ANTHRACENE-INDUCED HAEMATOLOGICAL AND BIOCHEMICAL ALTERATION IN FEMALE RATS

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Ethical considerations: When reporting experiments on animals Observation of the ARRIVE guidelines 2.0: Updated guidelines for reporting animal research, published on July 14, 2020 (DOI: 10.1371/journal.pbio.3000410), is applied. The authors ensure that all procedures were performed in compliance with the guidelines for animal care of their institutions or with national/international guidelines.

ABSTRACT

The study aimed to evaluate the protective effects of piroxicam and carboplatin against anthracene-induced haematological and biochemical alterations in female rats using randomized controlled trial. Thirty-six female rats of seven weeks old that weighed 166.25 ± 26.79 g divided into six groups of six each per group were used for the study. Each of the rats of group 1, 3, 4, 5 and 6 was administered 100 mg/kg of oral anthracene, for three weeks, except group 2, which was administered 2.5 mg/kg of water. Thereafter, each rat in groups 1 and 2 was administered water, group 3 (piroxicam low dose), group 4 (carboplatin/piroxicam), group 5 (piroxicam high dose) and group 6 (carboplatin) for a period of three weeks. Blood sample (2 ml) was collected from each rat for haematology and serum biochemistry. The rats were euthanised using 100 mg/kg of sodium pentobarbitone. Body weight, packed cell volume, erythrocyte, leucocyte and neutrophil counts were significantly decreased ($p < 0.05$), causing emaciation, anaemia, and immunodepression. Lymphocytes, monocytes, basophils, and eosinophils were significantly increased ($p < 0.05$) causing immunostimulation. Urea, creatinine, aspartate aminotransferase, alanine aminotransferase and alkaline phosphatase were significantly decreased ($p < 0.05$), indicating malnutrition, reduced kidney and liver functions.

Keywords: amelioration; anaemia; anthracene; carboplatin; hepatitis; nephritis; piroxicam; rat

INTRODUCTION

Anthracene is a lung cancer-promoting polycyclic aromatic hydrocarbon (PAH) that consists of three benzene rings [1]. Exposure to anthracene in humans happens mainly through tobacco smoke [2]. It is susceptible to degradation in the presence of light [3]. Prolonged exposure causes a variety of adverse reactions [4], such as acute dermatitis, photophobia, nausea, adynamia, loss of appetite, gastroenteritis, and chronic bronchitis [5]. Anthracene has a toxic effect on the kidney and liver [6]. The toxicity effect of anthracene is dose-specific (quantitative) and exposure-specific (qualitative) [7]. It causes binucleated micronuclei, nuclear buds, and fragmented apoptotic cells [8]. However, therapeutic and toxic agents could have positive and negative effects on animals' erythrocytes [9]. Piroxicam reduces pain, inflammation, and mastitis [10], and it is excreted in urine and faeces [11]. The safe therapeutic dose of piroxicam is low, but a higher dose is toxic [12]. Therefore, there is a need for a protective agent for haematological and biochemical changes. Hence, the protective potential of piroxicam and anthracene was investigated in female rats.

MATERIALS AND METHODS

Animals and housing

Seven-week-old female rats weighing 166.25 ± 26.7 g were housed in metal cages and kept in the Departmental Laboratory of Veterinary Physiology and Biochemistry, College of Veterinary Medicine, Joseph Sarwuan Tarka University, Makurdi, Benue State, Nigeria. Water and food were provided *ad libitum*. Care was provided for the animals as recommended by the Ethical Committee, College of Veterinary Medicine, Joseph Sarwuan Tarka University, Makurdi, Nigeria, given the permit number (JOSTUM/CVM/ETHICS/2025/07).

Chemicals and drugs

Drugs

Piroxicam [Shanxi Federal Pharmaceutical Co. Ltd., China; Batch no 121103; NAFDAC no 04-6809] and carboplatin [Batch no. L01XAO2; NAFDAC no. A4-100071, German Pharmacy Company Ltd., Germany] were purchased from a pharmacy in Makurdi, Nigeria and used for the experiment.

Preparation of anthracene solution

Approximately 20 g of anthracene powder was dissolved in 80 ml of distilled water and stirred to make a homogenous 20% (200 mg/ml) solution that gave a homogenous stable solution of pH 6.

Preparation of piroxicam solution

Approximately 20 g of piroxicam powder from capsules was dissolved in 80 ml of distilled water and stirred to make a homogenous 20% (200 mg/ml) solution that gave a homogenous stable solution of pH 6.

Preparation of carboplatin solution

Approximately 20 g of carboplatin powder was dissolved in 80 ml of distilled water and stirred to make a homogenous 20% (200 mg/ml) solution that gave a homogenous stable solution of pH 6.

Selection of piroxicam, anthracene, and carboplatin therapeutic doses

The therapeutic dose selection of piroxicam was based on the LD50 of the animals determined according to the method reported by Saganuwan and Orinya [9]. An induction dose of 100 mg/kg body weight of oral anthracene administered to the experimental animals was selected based on the reported LD_{50} of >5000 mg/kg body weight, whereas the experimental rats were treated according to the reported method [9–12]. Therapeutic doses of carboplatin were calculated based on the data reported by Thermo Fisher Scientific [13].

Experimental design

A repeated measures randomized controlled trial was adopted for the study. A total of thirty-six female rats divided into six groups of six each were used for the study. Groups 1, 3, 4, 5 and 6 were administered 100 mg/kg of anthracene orally for three weeks, whereas group 2 was administered 2.5 ml of water orally.

Group	Drugs used
1	Tumor control group (induced + water)
2	Normal control group (positive control)
3	Piroxicam low-dose group (2.5 mg/kg) (anthracene, piroxicam)
4	Combination therapy group (piroxicam 2.5 mg/kg + carboplatin 2.5 mg/kg) (anthracene, piroxicam, carboplatin)
5	Piroxicam high-dose group (5.0 mg/kg) (anthracene, piroxicam)
6	Carboplatin group (2.5 mg/kg) (anthracene, carboplatin)

Sample collection and analysis

A pretreatment blood sample (2ml) was collected from the tail vein of each rat. After collection of blood samples on days 0, 7, and 21, groups 3, 4, 5, and 6 were treated with piroxicam (2.5 mg/kg), a combination of carboplatin (2.5 mg/kg) co-administered with piroxicam (2.5mg/kg), piroxicam (5 mg/kg) and carboplatin (2.5 mg/kg), respectively. Haematological and biochemical parameters were induced but not treated in group 1. Groups 1 and 2 were treated with 2.5 mg/kg of water [11, 14]. Animals were euthanised on day 42 using intraperitoneal injection of 100 mg/kg body weight of pentobarbital sodium [15, 16].

Determination of hematological parameters

Total blood volume, plasma volume, and erythrocyte volumes were determined mathematically [17, 18]. The formula is presented as follows:

$$Total\ blood\ volume\ (TBV) = Plasma\ volume\ (PV) \times \frac{100}{100 - Haematocrit}$$

Total blood volume was determined using the method of Saganuwan and Onyeyili [18]. The red blood cells and white blood cells were counted using the method of Brown [19] and Cheasebrough [20].

Determination of renal parameters

Serum creatinine and urea were determined using the established methods [21, 22]. Plasma creatinine was determined according to the method reported by Saganuwan [23]. Plasma creatinine was determined mathematically, using the formula given below:

$$Serum\ creatinine = \frac{Plasma\ creatinine\ (Pcr)}{1440} \times 1000$$

Determination of hepatic enzyme parameters

Liver enzymes such as serum glutamic oxaloacetic transaminase (SGOT), serum glutamic pyruvic transaminase (SGPT), and alkaline phosphatase (ALP) were determined using the established methods [24, 25, 26]. The ratio of aspartate to alanine aminotransferase was determined according to the method of Williams and Hoofnagle.

A ratio greater than 2.0 denotes alcohol liver disease, and less than 1.0 denotes chronic hepatitis and chronic cholestatic syndrome [27].

Statistical analyses

All the data generated were presented as mean ± standard error of mean (SEM). Two-way repeated measures analysis of variance was used to analyze the data, and a post-hoc test was used to determine significant differences among six group means [28]. Neutrophil-lymphocyte ratio was calculated according to the method reported by Zaherec [29]. The SGOT/SGPT ratio was calculated using the method reported by Cohen and Kaplan [30].

RESULTS

Body weights

The effects of anthracene and piroxicam on the body weight of female rats are presented in Table 1. The weights of the animals significantly increased on day 7 and decreased on days 21, 35, and 42. However, there was no significant increase ($p > 0.05$) in the group induced with anthracene and treated with carboplatin (2.5 mg/kg), the groups treated with carboplatin (2.5 mg/kg)/piroxicam (2.5 mg/kg), the group treated with piroxicam (5 mg/kg) on the 35th and 42nd days, respectively (Table1).

Erythrocyte parameters

The effects of anthracene and piroxicam on the packed cell volume of female rats are presented in Table 2. There was significant decrease ($p < 0.05$) in packed cell volume (PCV) throughout the period of experimentation except for groups 4 and 5, which increased significantly on day 42. However, there was a significant increase ($p < 0.05$) in PCV in the group induced but not treated on day 21, 35, and 42, as well as groups (1, 3, and 5) induced but not treated with carboplatin (2.5mg/kg), respectively (Table 2). Erythrocyte counts decreased significantly ($p < 0.05$) throughout the period of experimentation. There was no significant increase ($p > 0.05$) in the erythrocyte counts on days 35 and 42 of the group induced with anthracene but not treated with piroxicam. However, significant increase in the erythrocyte counts ($p > 0.05$) was observed on day zero in the group neither induced nor treated with either piroxicam or carboplatin (Table 2).

Total blood volume, plasma volume, and red blood cells volumes were significantly decreased in all the groups ($p < 0.05$) except the group administered carboplatin and piroxicam (Table 2).

Table 1. Effects of anthracene, piroxicam and carboplatin on body weight (g) of female rats

Experimental groups	Treatments	Days of treatment				
		0	7	21	35	42
1.	Induced not treated Water (2.5ml) (Negative control)	148.00±8.09^{bd}	149.67±7.91^{bd}	166.16±7.43^{ad}	147.00±13.49^{bd}	149.67±12.91^{bd}
2.	Neither induced nor treated Water (2.5ml) (Positive control)	143.83±8.92^{bd}	147.33±9.07^{ad}	144.00±10.04^{bd}	130.17±5.75^{bd}	134.33±4.84^{bd}
3.	Induced and treated Piroxicam (2.5 mg/kg)	145.50±4.35^{bd}	149.00±3.79^{cd}	145.17±9.79^{bd}	130.60±13.68^{bd}	131.40±14.15^{bd}
4.	Induced and treated Carboplatin (2.5 mg/kg) Piroxicam (2.5 mg/kg)	146.17±4.08^{bd}	149.83±4.09^{bd}	134.50±15.92^{bd}	155.60±24.35^{bc}	166.25±26.79^{ad}
5.	Induced and treated Piroxicam (5 mg/kg)	151.67±7.97^{bc}	154.50±7.85^{bc}	190.67±18.97^{ac}	146.80±11.33^{bd}	146.60±11.89^{bd}
6.	Induced and treated Carboplatin (2.5 mg/kg)	151.17±3.60^{bd}	153.50±3.72^{ad}	139.17±6.47^{bd}	132.40±8.12^{bd}	137.75±9.79^{bd}

Keys: a = significantly higher along the row ($p < 0.05$); b = significantly lower along the row ($p < 0.05$); c = significantly higher along the column ($p < 0.05$); d = significantly lower along the column ($p < 0.05$)

Leucocyte parameters

Leucocyte counts were significantly decreased ($p < 0.05$) in all the piroxicam and anthracene-treated groups except for group 4 on days 21, 35 and 42 (Table 3). The monocyte counts were significantly ($p < 0.05$) increased in groups 1 and 2 on days 7, 21, 35, and 42 and decreased significantly in groups 3 and 6 on day 42, respectively (Table 3). Lymphocyte counts decreased significantly ($p < 0.05$) on days 7, 21, and 35 and increased significantly ($p < 0.05$) on day 42 in groups 1, 2, 3, 4, and 6, except in group 5 (Table 3). Neutrophil counts were significantly increased ($p < 0.05$) on days 7, 21, and 35, except in group 1. Meanwhile, the neutrophil counts of groups 1, 3, 4, and 6 were significantly decreased ($p < 0.05$) on day 42 except for groups 2 and 5, respectively. Neutrophil-lymphocyte ratios of the rats are presented in Table 3. The neutrophil-lymphocyte ratio was significantly increased in groups 2, and 3 (days 7, 21, 35), groups 4 and 5 (days 7, 21), and 6 (days 0, 7, 21 and 35), respectively. However, there was a significant decrease ($p < 0.05$) in neutrophil-lymphocyte ratio in all the groups except group 2 (positive control). Basophil counts were significantly increased ($p < 0.05$) on day 35 and decreased significantly ($p < 0.05$) in groups 2 and 4, they increased significantly in groups 3 and 5, respectively (Table 3). The counts of eosinophils were significantly increased in all the groups on days 7, 21, 35, and 42, except for groups 4 and 6, which decreased significantly ($p < 0.05$) (Table 3).

Biochemical parameters

Urea was significantly increased ($p < 0.05$) on day 21 and eventually decreased on day 42 in all the groups, respectively (Table 4). Serum creatinine was significantly increased on day 21 in the group neither induced nor treated (G 2), induced and treated with piroxicam (2.5 mg/kg), and induced and treated with carboplatin (G 6), but decreased in the group treated with piroxicam (5 mg/kg) (G 5), respectively (Table 4). Plasma creatinine was significantly decreased ($p < 0.05$) on day 42 ($p < 0.05$) in all the groups, except the group administered anthracene and water (Table 4).

Aspartate aminotransferase (AST) was significantly decreased ($p < 0.05$) in groups 2, 3, 4, and 5 and increased in groups 1 and 6 on day 21, and on day 42 (Table 5). Alanine aminotransferase (ALT) was significantly decreased ($p < 0.05$) in groups 1, 2, 3 and 5, but increased in groups 4 and 6 on day 21. However, on day 0 and 42, the values of ALT were not significantly different ($p > 0.05$). Alkaline phosphate values were significantly higher on day 21 but relatively the same on days 0 and 42 (Table 5). The significant increase ($p < 0.05$) in SGOT/SGPT ratio was observed in the groups 2 (3.95±0.69), 3 (3.41±0.62), 4 (2.30±0.27), 5 (2.85±0.23), and 6 (3.07±0.10) as compared to group 1 (1.64±0.41), respectively (Table 5).

Table 2. Effects of anthracene, piroxicam and carboplatin on erythrocyte parameters of female rats

Experimental groups	Treatments	Packed cell volume (%) Days of treatment				
		0	7	21	35	42
1.	Induced not treated Water (2.5ml) (Negative control)	45.83±0.5^{bc}	47.17±1.14^{ac}	45.83±1.16^{bc}	46.67±1.15^{bd}	45.17±1.60^{bc}
2.	Neither induced nor treated Water (2.5ml) (Positive control)	41.33±2.8^{bd}	45.83±1.74^{ad}	40.00±2.76^{bd}	40.17±3.30^{bd}	39.67±1.9^{bd}
3.	Induced and treated Piroxicam (2.5 mg/kg)	42.33±2.1^{ad}	38.17±1.62^{bd}	36.17±2.88^{bd}	41.80±2.65^{bd}	40.00±1.6^{bd}
4.	Induced and treated Carboplatin (2.5 mg/kg) Piroxicam (2.5 mg/kg)	38.16±1.8^{bd}	39.33±3.17^{bd}	35.83±3.44^{bd}	41.40±1.40^{bd}	44.00±1.6^{ad}
5.	Induced and treated Piroxicam (5 mg/kg)	41.00±2.9^{bd}	36.50±3.36^{bd}	32.17±3.55^{bd}	41.00±1.30^{bd}	43.00±1.1^{ad}
6.	Induced and treated Carboplatin (2.5 mg/kg)	41.50±1.8^{bd}	41.17±2.64^{bd}	34.17±2.77^{bd}	47.00±1.47^{ac}	42.50±1.94^{bd}
		Erythrocyte counts (x10^{12}/L)				
1.	Induced not treated Water (2.5ml) (Negative control)	9.43±0.51^{bd}	7.28±0.23^{bd}	7.33±0.40^{bd}	8.98±0.29^{bc}	9.57±0.39^{ac}
2.	Neither induced nor treated Water (2.5ml) (Positive control)	10.04±0.45^{ac}	6.92±0.21^{bd}	6.75±0.48^{bd}	7.84±0.53^{bd}	9.03±0.31^{bd}
3.	Induced and treated Piroxicam (2.5 mg/kg)	9.49±0.40^{ad}	6.95±0.35^{bd}	7.44±0.71^{bc}	8.17±0.66^{bd}	8.69±0.43^{bd}
4.	Induced and treated Carboplatin (2.5 mg/kg) Piroxicam (2.5 mg/kg)	9.03±0.31^{ad}	7.43±0.59^{bd}	6.66±0.22^{bd}	8.20±0.66^{bd}	8.77±0.56^{bd}
5.	Induced and treated Piroxicam (5 mg/kg)	9.42±0.50^{ad}	7.17±0.66^{bd}	6.76±0.59^{bd}	8.00±0.49^{bd}	8.61±0.54^{bd}
6.	Induced and treated Carboplatin (2.5 mg/kg)	9.49±0.53^{ad}	7.64±0.65^{bc}	6.98±0.43^{bd}	7.45±0.97^{bd}	8.20±0.75^{bd}
		Blood volume (mL)				
1.	Induced not treated Water (2.5ml) (Negative control)	11.84±0.64^{bd}	11.97±0.63^{bd}	13.29±0.59^{ad}	11.76±1.08^{bd}	11.97±1.03^{bd}
2.	Neither induced nor treated Water (2.5ml) (Positive control)	11.51±0.71^{bd}	11.79±0.73^{ad}	11.52±0.80^{bd}	10.41±0.46^{bd}	10.75±0.39^{bd}
3.	Induced and treated Piroxicam (2.5 mg/kg)	11.64±0.35^{bd}	11.92±0.30^{ad}	11.61±0.78^{bd}	10.44±1.09^{bd}	10.51±1.13^{bd}
4.	Induced and treated Carboplatin (2.5 mg/kg) Piroxicam (2.5 mg/kg)	11.69±0.33^{bd}	11.99±0.33^{bd}	10.76±1.33^{bd}	12.45±1.95^{bc}	13.30±2.14^{ac}
5.	Induced and treated Piroxicam (5 mg/kg)	12.13±0.64^{bc}	12.36±0.63^{bc}	15.25±1.52^{ac}	11.74±0.91^{bd}	11.73±0.95^{bd}
6.	Induced and treated Carboplatin (2.5 mg/kg)	12.09±0.29^{bd}	12.28±0.29^{ad}	11.13±0.52^{bd}	10.59±0.65^{bd}	11.02±0.78^{bd}
		Plasma volume (mL)				
1.	Induced not treated Water (2.5ml) (Negative control)	6.41±0.64^{bd}	6.33±1.13^{bd}	7.20±1.15^{ad}	6.27±1.07^{bd}	6.57±1.01^{bd}
2.	Neither induced nor treated Water (2.5ml) (Positive control)	6.75±0.69^{bd}	6.39±0.72^{bd}	6.91±0.78^{cd}	6.23±0.44^{bd}	6.49±0.38^{bd}
3.	Induced and treated Piroxicam (2.5 mg/kg)	6.71±0.34^{bd}	7.37±0.30^{bd}	7.41±0.76^{ad}	6.08±1.06^{bd}	6.31±0.71^{bd}
4.	Induced and treated Carboplatin (2.5 mg/kg) Piroxicam (2.5 mg/kg)	7.23±0.32^{bc}	7.28±0.32^{bd}	6.91±1.29^{bd}	7.30±1.92^{bc}	7.45±2.11^{ac}
5.	Induced and treated Piroxicam (5 mg/kg)	7.16±0.62^{bc}	7.85±0.61^{bc}	10.35±1.47^{ac}	6.93±0.99^{bd}	6.69±0.94^{bd}
6.	Induced and treated Carboplatin (2.5 mg/kg)	7.07±0.78^{bd}	7.23±0.29^{bd}	7.33±0.51^{ad}	5.62±0.64^{bd}	6.34±0.77^{bd}
		Erythrocyte volume (x10^{12}/L)				
1.	Induced not treated Water (2.5ml) (Negative control)	5.43±0.00^{bc}	5.64±0.50^{bc}	6.09±0.56^{ac}	5.49±0.01^{bc}	5.40±0.02^{bd}
2.	Neither induced nor treated Water (2.5ml) (Positive control)	4.76±0.02^{bd}	5.40±0.01^{ad}	4.61±0.02^{bd}	4.18±0.02^{bd}	4.26±0.01^{bd}
3.	Induced and treated Piroxicam (2.5 mg/kg)	4.93±0.01^{ad}	4.55±0.00^{bd}	4.20±0.02^{bd}	4.36±0.03^{bd}	4.20±0.42^{bc}
4.	Induced and treated Carboplatin (2.5 mg/kg) Piroxicam (2.5 mg/kg)	4.46±0.01^{bd}	4.71±0.01^{bd}	3.85±0.04^{bd}	5.15±0.03^{bd}	5.85±0.03^{ac}
5.	Induced and treated Piroxicam (5 mg/kg)	4.97±0.02^{bd}	4.51±0.02^{bd}	4.90±0.05^{bd}	4.81±0.08^{bd}	5.04±0.11^{ad}
6.	Induced and treated Carboplatin (2.5 mg/kg)	5.02±0.01^{bd}	5.05±0.01^{ad}	3.80±0.01^{bd}	4.97±0.01^{bc}	4.68±0.01^{bd}

Keys: a = significantly higher along the row ($p < 0.05$); b = significantly lower along the row($p < 0.05$); c = significantly higher along the column($p < 0.05$); d = significantly lower along the column($p < 0.05$)

Table 3. Effects of anthracene, piroxicam and carboplatin on differential leucocyte counts

Experimental groups	Treatments	Leucocyte counts ($x10^9$/L) Days of treatment				
		0	7	21	35	42
1.	Induced not treated Water (2.5ml) (Negative control)	4.28±0.28^{bd}	4.35±0.26^{ac}	4.03±0.09^{bd}	3.85±0.18^{bd}	4.20±0.19^{bc}
2.	Neither induced nor treated Water (2.5ml) (Positive control)	4.35±0.31^{ad}	3.75±0.23^{bd}	4.02±0.08^{bd}	4.17±0.24^{bd}	3.98±0.20^{bd}
3.	Induced and treated Piroxicam (2.5 mg/kg)	4.67±0.25^{ac}	3.97±0.08^{bd}	3.67±0.25^{bd}	4.02±0.16^{bd}	4.04±0.28^{bd}
4.	Induced and treated Carboplatin (2.5 mg/kg) Piroxicam (2.5 mg/kg)	3.68±0.21^{bd}	4.15±0.32^{bd}	4.13±0.35^{bc}	4.38±0.22^{ac}	4.05±0.34^{bd}
5.	Induced and treated Piroxicam (5 mg/kg)	3.95±0.29^{bd}	4.20±0.34^{ad}	3.55±0.19^{bd}	3.82±0.24^{bd}	3.88±0.24^{bd}
6.	Induced and treated Carboplatin (2.5 mg/kg)	3.88±0.27^{bd}	3.92±0.32^{ad}	3.80±0.08^{bd}	3.77±0.31^{bd}	3.60±0.25^{bd}
		Monocytes (%)				
1.	Induced not treated Water (2.5ml) (Negative control)	2.00±0.41^{bd}	2.00±0.26^{bd}	2.33±0.33^{bd}	2.33±0.33^{ad}	2.33±0.33^{ad}
2.	Neither induced nor treated Water (2.5ml) (Positive control)	2.00±0.45^{bd}	3.00±0.57^{bc}	2.50±0.43^{bd}	2.00±0.36^{bd}	3.83±0.65^{ac}
3.	Induced and treated Piroxicam (2.5 mg/kg)	3.67±0.33^{ac}	2.67±0.56^{bd}	2.17±0.31^{bd}	2.20±0.37^{bd}	2.60±0.40^{bd}
4.	Induced and treated Carboplatin (2.5 mg/kg) Piroxicam (2.5 mg/kg)	3.17±0.60^{ad}	2.50±0.43^{bd}	2.50±0.43^{bd}	2.20±0.58^{bd}	3.00±0.41^{bd}
5.	Induced and treated Piroxicam (5 mg/kg)	2.17±0.60^{bd}	2.83±0.40^{bd}	1.67±0.49^{bd}	3.40±0.51^{ac}	3.00±0.52^{bd}
6.	Induced and treated Carboplatin (2.5 mg/kg)	3.17±0.60^{ad}	3.00±0.57^{bc}	3.00±0.36^{bc}	2.75±0.25^{bd}	2.00±0.40^{bd}
		Lymphocyte counts (%)				
1.	Induced not treated Water (2.5ml) (Negative control)	59.33±2.94^{bd}	60.83±1.30^{bc}	57.00±2.78^{bc}	66.00±0.96^{ac}	65.33±1.94^{bd}
2.	Neither induced nor treated Water (2.5ml) (Positive control)	59.83±2.57^{ad}	47.00±5.57^{bd}	40.50±3.86^{bd}	54.17±2.79^{bd}	58.17±1.07^{bd}
3.	Induced and treated Piroxicam (2.5 mg/kg)	59.83±2.87^{bd}	40.00±2.82^{bd}	36.67±2.70^{bd}	52.60±1.83^{bd}	64.40±2.02^{ad}
4.	Induced and treated Carboplatin (2.5 mg/kg) Piroxicam (2.5 mg/kg)	65.17±2.2^{bc}	41.83±0.95^{bd}	37.67±1.08^{bd}	55.40±2.27^{bd}	68.75±2.17^{ac}
5.	Induced and treated Piroxicam (5 mg/kg)	64.83±1.9^{ad}	37.33±3.18^{bd}	43.00±4.53^{bd}	54.80±1.96^{bd}	62.00±3.11^{bd}
6.	Induced and treated Carboplatin (2.5 mg/kg)	33.67±4.0^{bd}	36.17±1.35^{bd}	45.17±7.17^{bd}	52.25±2.09^{bd}	68.50±1.32^{ad}
		Neutrophil counts (%)				
1.	Induced not treated Water (2.5ml) (Negative control)	37.00±3.04^{bc}	34.67±1.28^{bd}	37.33±2.36^{ad}	29.33±1.14^{bd}	30.33±1.76^{bd}
2.	Neither induced nor treated Water (2.5ml) (Positive control)	34.67±3.12^{bd}	48.17±5.72^{bd}	54.83±3.92^{ad}	41.17±2.33^{bd}	36.17±0.91^{bc}
3.	Induced and treated Piroxicam (2.5 mg/kg)	36.00±3.02^{bd}	55.50±2.94^{bd}	58.33±2.55^{ac}	42.60±2.38^{bc}	30.80±2.01^{bd}
4.	Induced and treated Carboplatin (2.5 mg/kg) Piroxicam (2.5 mg/kg)	30.67±1.65^{bd}	52.67±1.17^{bd}	57.33±1.63^{ad}	39.80±2.52^{bd}	27.00±1.96^{bd}
5.	Induced and treated Piroxicam (5 mg/kg)	31.35±2.01^{bd}	56.67±2.68^{ad}	53.00±4.38^{bd}	39.80±1.56^{bd}	32.20±3.08^{bd}
6.	Induced and treated Carboplatin (2.5 mg/kg)	33.67±4.08^{bd}	58.50±1.34^{ac}	49.67±7.37^{bd}	42.25±1.65^{bd}	28.50±1.55^{bd}
		Neutrophil-lymphocyte ratio				
1.	Induced not treated Water (2.5ml) (Negative control)	0.62±0.42^{ad}	0.57±0.40^{bd}	0.65±0.35^{ad}	0.44±0.20^{bc}	0.46±0.23^{bd}

2.	Neither induced nor treated Water (2.5ml) (Positive control)	0.58±0.20^{bd}	1.02±0.42^{ac}	1.35±0.41^{ac}	0.76±0.34^{bc}	0.62±0.35^{bc}
3.	Induced and treated Piroxicam (2.5 mg/kg)	0.60±0.18^{bd}	1.39±0.43^{ac}	1.59±0.39^{ac}	0.81±0.22^{bc}	0.48±0.17^{bd}
4.	Induced and treated Carboplatin (2.5 mg/kg) Piroxicam (2.5 mg/kg)	0.47±0.13^{bd}	1.26±0.42^{ac}	1.52±0.25^{ac}	0.72±0.19^{bd}	0.39±0.15^{bd}
5.	Induced and treated Piroxicam (5 mg/kg)	0.48±0.18^{bd}	1.52±0.34^{ac}	1.23±0.39^{ac}	0.73±0.32^{bd}	0.52±0.17^{bd}
6.	Induced and treated Carboplatin (2.5 mg/kg)	1.00±0.17^{ac}	1.62±0.41^{ac}	1.10±0.17^{ac}	0.81±0.32^{bc}	0.42±0.20^{bd}
				Basophils (%)		
1.	Induced not treated Water (2.5ml) (Negative control)	1.17±0.40^{bc}	1.50±0.43^{bd}	1.83±0.31^{ac}	1.17±0.31^{bd}	1.17±0.40^{bd}
2.	Neither induced nor treated Water (2.5ml) (Positive control)	1.17±0.16^{bc}	1.33±0.33^{bd}	1.33±0.33^{bd}	1.67±0.49^{ad}	1.00±0.36^{bd}
3.	Induced and treated Piroxicam (2.5 mg/kg)	0.16±0.16^{bd}	1.17±0.31^{bd}	1.83±0.31^{ac}	1.40±0.40^{bd}	1.20±0.37^{bd}
4.	Induced and treated Carboplatin (2.5 mg/kg) Piroxicam (2.5 mg/kg)	0.50±0.22^{bd}	1.67±0.21^{bc}	1.83±0.49^{ac}	1.60±0.24^{bd}	0.75±0.47^{bd}
5.	Induced and treated Piroxicam (5 mg/kg)	1.00±0.36^{bd}	1.33±0.42^{bd}	1.50±0.43^{bd}	1.20±0.20^{bd}	1.80±0.37^{ac}
6.	Induced and treated Carboplatin (2.5 mg/kg)	0.66±0.49^{bd}	1.33±0.33^{bd}	1.33±0.21^{bd}	1.75±0.85^{ac}	0.75±0.25^{bd}
				Eosinophil counts (%)		
1.	Induced not treated Water (2.5ml) (Negative control)	0.50±0.22^{bd}	1.00±0.25^{bd}	1.50±0.34^{ac}	1.17±0.31^{bd}	0.83±0.31^{bd}
2.	Neither induced nor treated Water (2.5ml) (Positive control)	0.66±0.21^{bc}	0.83±0.31^{bd}	1.00±0.36^{ad}	1.00±0.45^{ad}	0.83±0.16^{bd}
3.	Induced and treated Piroxicam (2.5 mg/kg)	0.33±0.21^{bd}	0.66±0.21^{bd}	1.00±0.36^{bd}	1.20±0.37^{ac}	1.00±0.32^{bc}
4.	Induced and treated Carboplatin (2.5 mg/kg) Piroxicam (2.5 mg/kg)	0.50±0.34^{bd}	1.16±0.31^{bc}	1.17±0.63^{ad}	1.00±0.32^{bd}	0.50±0.28^{bd}
5.	Induced and treated Piroxicam (5 mg/kg)	0.66±0.21^{bc}	0.83±0.40^{bd}	0.83±0.40^{bd}	0.80±0.37^{bd}	1.00±0.45^{ac}
6.	Induced and treated Carboplatin (2.5 mg/kg)	0.33±0.21^{bd}	1.00±0.26^{ad}	0.83±0.30^{bd}	1.00±0.41^{ad}	0.25±0.25^{bd}

Keys: a = significantly higher along the row ($p < 0.05$); b = significantly lower along the row ($p < 0.05$); c = significantly higher along the column ($p < 0.05$); d = significantly lower along the column ($p < 0.05$); neutrophil-lymphocyte ratio = 1–2 (normal); >3.0–0.7 (pathological); 2.3–3.0 (cancer atherosclerosis, infection, inflammation, stress, psychiatric disorder); 2.5–5.0 (solid tumor)

Table 4. Effects of anthracene, piroxicam and carboplatin on kidney function parameters of female rats

Experimental groups	Treatments	Urea (mmol/L) Days of treatment 0	21	42
1.	Induced not treated Water (2.5ml) (Negative control)	6.19±0.32^{bc}	12.45±0.15^{ad}	6.19±0.32^{bc}
2.	Neither induced nor treated Water (2.5ml) (Positive control)	3.12±1.46^{bd}	12.60±0.40^{ad}	3.22±1.46^{bd}
3.	Induced and treated Piroxicam (2.5 mg/kg)	3.23±0.48^{bd}	11.50±1.40^{ad}	3.23±0.48^{bd}
4.	Induced and treated Carboplatin (2.5 mg/kg) Piroxicam (2.5 mg/kg)	5.59±0.02^{bd}	11.15±0.45^{ad}	5.59±0.01^{bd}
5.	Induced and treated Piroxicam (5 mg/kg)	5.87±0.81^{bc}	14.10±0.10^{ac}	5.87±0.18^{bd}
6.	Induced and treated Carboplatin (2.5 mg/kg)	4.19±0.89^{bd}	10.03±0.47^{ad}	4.19±0.89^{bd}
		Serum creatinine (µmol/L)		
1.	Induced not treated Water (2.5ml) (Negative control)	99.50±9.00^{ac}	66.85±19.05^{bd}	99.45±9.05^{ac}
2.	Neither induced nor treated Water (2.5ml) (Positive control)	43.25±3.98^{bd}	77.60±0.50^{ad}	58.25±2.49^{bd}
3.	Induced and treated Piroxicam (2.5 mg/kg)	50.65±2.35^{bd}	86.70±3.90^{ad}	50.65±2.35^{bd}
4.	Induced and treated Carboplatin (2.5 mg/kg) Piroxicam (2.5 mg/kg)	77.50±1.50^{bd}	81.15±6.15^{ad}	77.50±1.50^{bd}
5.	Induced and treated Piroxicam (5 mg/kg)	91.95±16.85^{ad}	88.50±1.31^{bc}	91.95±16.85^{ad}
6.	Induced and treated Carboplatin (2.5 mg/kg)	57.65±8.35^{bd}	73.25±9.35^{ad}	57.65±8.35^{bd}
		Plasma creatinine (µmol/L)		
1.	Induced not treated Water (2.5ml) (Negative control)	143.28±12.96^{ac}	96.25±27.43^{bd}	143.23±13.03^{ac}
2.	Neither induced nor treated Water (2.5ml) (Positive control)	62.28±5.73^{bd}	111.74±0.72^{ad}	83.88±3.59^{bd}
3.	Induced and treated Piroxicam (2.5 mg/kg)	72.94±3.38^{bd}	124.85±5.62^{ad}	72.94±3.38^{bd}
4.	Induced and treated Carboplatin (2.5 mg/kg) Piroxicam (2.5 mg/kg)	111.60±2.16^{bd}	116.86±8.86^{ad}	111.60±2.16^{bd}
5.	Induced and treated Piroxicam (5 mg/kg)	132.41±24.26^{ad}	127.44±1.89^{bc}	132.41±24.26^{ad}
6.	Induced and treated Carboplatin (2.5 mg/kg)	83.02±12.02^{bd}	105.48±13.64^{ad}	83.02±12.02^{bd}

Keys: a = significantly higher along the row ($p < 0.05$); b = significantly lower along the row ($p < 0.05$); c = significantly higher along the column ($p < 0.05$); d = significantly lower along the column ($p < 0.05$)

Table 5. Effects of anthracene, piroxicam and carboplatin on liver function parameters of female rats

Experimental groups	Treatments	Aspartate aminotransferase U/L (SGOT)		
		Days of treatment		
		0	21	42
1.	Induced not treated Water (2.5ml) (Negative control)	164.10±0.60^{bd}	183.80±2.00^{ad}	164.10±0.60^{bd}
2.	Neither induced nor treated Water (2.5ml) (Positive control)	190.10±5.50^{ad}	42.60±1.49^{bd}	190.10±5.50^{ad}
3.	Induced and treated Piroxicam (2.5 mg/kg)	293.65±2.99^{ad}	114.70±1.81^{bd}	293.65±2.99^{ad}
4.	Induced and treated Carboplatin (2.5 mg/kg) Piroxicam (2.5 mg/kg)	230.25±2.16^{ad}	176.55±2.44^{bd}	230.25±2.16^{ad}
5.	Induced and treated Piroxicam (5 mg/kg)	318.40±3.26^{ac}	104.85±7.28^{bd}	318.40±3.26^{ac}
6.	Induced and treated Carboplatin (2.5 mg/kg)	252.75±4.37^{bd}	266.75±9.95^{ac}	252.75±4.37^{bd}
		Alanine aminotransferase U/L (SGPT)		
1.	Induced not treated Water (2.5ml) (Negative control)	100.30±0.60^{ad}	77.45±1.05^{bd}	100.30±0.60^{ad}
2.	Neither induced nor treated Water (2.5ml) (Positive control)	190.10±5.50^{ac}	28.40±2.38^{bd}	48.15±28.05^{bd}
3.	Induced and treated Piroxicam (2.5 mg/kg)	86.20±1.96^{ad}	64.62±1.26^{bd}	86.20±1.96^{ad}
4.	Induced and treated Carboplatin (2.5 mg/kg) Piroxicam (2.5 mg/kg)	100.20±3.27^{bd}	103.05±4.35^{ad}	100.20±3.27^{bd}
5.	Induced and treated Piroxicam (5 mg/kg)	111.80±5.80^{ad}	43.20±4.60^{bd}	111.80±5.80^{ac}
6.	Induced and treated Carboplatin (2.5 mg/kg)	82.25±17.15^{bd}	121.90±5.80^{ac}	82.25±17.15^{bd}
		SGOT-SGPT ratio		
1.	Induced not treated Water (2.5ml) (Negative control)	1.64±0.41^{bd}	2.37±0.78^{ac}	1.64±0.41^{bd}
2.	Neither induced nor treated Water (2.5ml) (Positive control)	1.90±0.41^{bd}	1.50±0.26^{bd}	3.95±0.69^{ac}
3.	Induced and treated Piroxicam (2.5 mg/kg)	3.41±0.62^{ac}	1.77±0.59^{bd}	3.41±0.62^{ad}
4.	Induced and treated Carboplatin (2.5 mg/kg) Piroxicam (2.5 mg/kg)	2.30±0.27^{ad}	1.71±0.23^{bd}	2.30±0.27^{ad}
5.	Induced and treated Piroxicam (5 mg/kg)	2.85±0.23^{ad}	2.43±0.65^{bc}	2.85±0.23^{ac}
6.	Induced and treated Carboplatin (2.5 mg/kg)	3.07±0.10^{ad}	2.19±0.29^{bd}	3.07±0.10^{ad}
		Alkaline phosphatase U/L (ALP)		
1.	Induced not treated Water (2.5ml) (Negative control)	9.25±0.45^{ac}	25.85±8.15^{bc}	9.25±0.45^{ac}
2.	Neither induced nor treated Water (2.5ml) (Positive control)	6.90±4.60^{bd}	17.55±0.15^{ac}	6.90±4.60^{bd}
3.	Induced and treated Piroxicam (2.5 mg/kg)	6.00±0.50^{bd}	9.45±8.25^{ad}	6.00±0.50^{bd}
4.	Induced and treated Carboplatin (2.5 mg/kg) Piroxicam (2.5 mg/kg)	7.40±1.10^{bd}	9.75±4.55^{ad}	7.40±1.10^{bd}
5.	Induced and treated Piroxicam (5 mg/kg)	5.50±0.30^{bd}	15.35±0.05^{ad}	5.50±0.30^{bd}
6.	Induced and treated Carboplatin (2.5 mg/kg)	7.05±1.25^{bd}	12.95±0.45^{ad}	7.05±1.25^{bd}

Keys: a = significantly higher along the row ($p < 0.05$); b = significantly lower along the row ($p < 0.05$); c = significantly higher along the column ($p < 0.05$); d = significantly lower along the column ($p < 0.05$); SGOT/SGPT ratio = >1.0 (chronic liver inflammation; <1.0 hepatitis, non-alcoholic fatty disease, bile duct problem); 1 (drug induced liver inflammatory)

DISCUSSION

Effects of anthracene, piroxicam, and carboplatin on weight gain

The increased weight gain observed on day 35, and day 21 in group 4 (2.5 mg/kg carboplatin/2.5 mg/kg piroxicam) and the weight loss observed in group 5 (5 mg/kg piroxicam) show that a combination of carboplatin/piroxicam has weight-increasing potential and piroxicam alone has weight reducing potential. However, increased weight gain observed in group 4 shows that carboplatin could improve weight when co-administered with piroxicam for a period of 3 weeks [31, 32, 33].

Effects of anthracene, carboplatin, and piroxicam on haematological parameters

The decreased PCV caused by anthracene and restored by carboplatin and piroxicam shows that both can be used to ameliorate anthracene-induced haemolysis in rats [31, 34]. However, the decreased erythrocyte counts in all the anthracene- and piroxicam-treated groups show that erythrocyte counts are independent of the packed cell volume. Hence, piroxicam and anthracene can cause anaemia [35] that could be exacerbated by some anti-cancer drugs [36], as observed in the group administered carboplatin. Also, it was reported that decreased red blood cell width distributions (RWDs) as observed in the present study is a strong index of survival in cancer patients [37]. The leucopenia observed in the present study is in disagreement with the findings that leucocytosis is a prognostic marker of lung cancer [37–39]. Also, the leucocyte count is independent of physical activity in lung cancer patients [40]. This further suggested that leukocytosis is an important biomarker for increased risk of lung damage [41]. However, tumour-related leukocytosis is related importantly to non-small cell carcinoma, and it is an ominous prognostic marker [39]. Leucocytosis could be attributed to monocytosis and lymphocytosis observed in the present study. The observed neutrophilia and neutropenia in the treated group agree with the report indicating that neutrophils could be used as a biomarker of cancer detection. Tumour-associated neutrophilias (TANs) could be controlled by tumour microenvironment and aid in tumour progression. Hence, TANs could be harmful and beneficial [42]. Certainly, neutrophilia is a negative prognostic factor in lung cancer patients, but other white blood cells do not affect patient's survival [43]. Basophilia and eosinophilia observed in the present study show that they could be used as prognostic biomarkers for piroxicam treatment. Whereas basopenia and eosinopenia could be prognostic biomarkers for carboplatin treatment [44, 45].

Effects of anthracene, carboplatin, and piroxicam on biochemical parameters

The administration of piroxicam may cause a significant increase in the levels of ALT and AST [31, 46], is characterized by a remarkable cellular infiltration of hepatic tissue by certain chemicals [47, 48]. The decreased alanine aminotransferase and alkaline phosphatase observed in the present study show that anthracene and carboplatin could inhibit the hepatic enzymes. However, 5 mg/kg of piroxicam restored the enzymes to their normal values. Piroxicam could modulate humoral immune system, induce liver enzymes, enhance renal function, and reduce the allometric parameter of the liver [49]. Meanwhile, delayed absorption and distribution of piroxicam could provide the desired therapeutic effect with less toxicity [50], suggesting a strong correlation between the two enzymes in liver problems [51]. The aspartate alanine amino transferase level greater than 2.0 in the present study indicates hepatic inflammation. This effect may be due to keto-enol tautomerism of piroxicam. The enol functional group could have been responsible for the rise in the ratio [52]. Some tautomers cause damage to tissues [53], indicating the importance of toxicity studies of drugs in animals for identification of potential hazards [54]. Hydroxyl and oxygen functional groups are responsible for tissue damage as revealed by liver enzyme markers [55], suggesting that the knowledge of therapeutics could be highly beneficial in anthracene poisoning [56]. The restoration of urea and creatinine to normal after sharp increases shows that a carboplatin dose could be administered based on normal renal function [57]. Hence, body surface area, body weight, creatinine clearance, glomerular filtration rate, creatinine half-life, and age are necessary parameters for assessing renotoxicity that may be caused by carboplatin and piroxicam [23]. Lower neutrocyte-lymphocyte ratio (NLR) is associated with reduced systemic inflammation and longer survival [58]. The increased SGOT/SGPT ratio observed in the present study shows that the livers of the group 2–6 rats were inflamed, indicating that the SGOT/SGPT ratio is a marker of liver damage [30].

Mechanisms of action of anthracene, carboplatin, and piroxicam

The metabolite of anthracene (2-methly-1-benzofurann-3-carbaldelyde) increases oxygen affinity of haemoglobin to prevent polymerization of haemoglobin thereby inhibiting red blood cell sickling, leading to metabolic instability caused by the aldehyde functional group [59, 60]. Anthracene could be converted to anthraquinone [61]. Presence of aldehyde functional and ketone functional groups in the anthracene metabolites suggest that anthracene could be used to induce cell damage [62]. Hence, it could be an inducer and an inhibitor of biological activities [63]. Carboplatin, a derivative of cisplatin, causes DNA damage by forming adducts with platinum, leading to inhibition of replication, transcription, and cell death [64]. Piroxicam inhibits tissue cyclooxygenases 1 and 2 (COX-1 and -2) resulting in reduced synthesis of pro-inflammatory prostaglandins that mediate inflammation and pain [65]. Piroxicam also inhibits N-methyl-D-aspartate receptor [66], suggesting that piroxicam could inhibit aspartate aminotransferase enzyme. Aspartate, glutamate, D-alanine, asparagine and L-alanine are derived from an intermediate of citric acid cycle [67].

Study limitations and recommendations for future research

The species of animals, sex, age, and the research environment are major limitations, connoting that general laboratory practice (GLP) and standard operating procedure (SOP) could be responsible for the variations in the results. Methods of the haematological and biochemical analyses of the parameters, and the data generated, could contribute greatly to the differences in the reported results. Meanwhile, we recommend biochemical, molecular, cellular, and physiological elucidation of the derangements in the parameters. Hence, further studies are necessary aimed at elucidating pathophysiology, biochemical pharmacology, and biochemical toxicology of anthracene, carboplation, and piroxicam and their mechanistic interplay. Relationship between obesity, anaemia, polycythemia, immune stimulation, and transminitis should be studied. Hyperuremia, hypecreatinaemia and hyperphosphataemia associated with impaired kidneys, particularly, chronic kidney disease, need to be studied in relation to anthracene-carboplatin-piroxicam interplay.

CONCLUSION

Anaemia, leucopenia, monocytopenia, lymphopenia, neutrophilia, basophilia, eosinopenia, increased urea, creatinine, aspartate aminotransferase, alanine aminotransferase, alkaline phosphate were observed, suggesting imunomodulatory potentials of anthracene, carboplatin, and piroxicam.

Ethical Statement

All the rats used for the study were handled according to the guide principles of the Animal Ethics Committee of Joseph Sarwuan Tarka University Makurdi given the permit number (JOSTUM/CVM/ETHICS/2025/07).

Conflict of Interest

The authors have no conflict of interest to disclose.

Data Availability Statement

The raw data of this article will be made available by the authors, without undue reservation.

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Authors' Contributions

SAS designed the study and analyzed the data. AMU carried out the study and collected the data. SAS, AMU, AMV, and TA wrote, proofread and approved the manuscript.

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REVIEW ARTICLE

GASTRIC LESIONS IN DOMESTIC PIGS AND WILD BOARS: ETIOLOGY, PATHOLOGY AND PREDISPOSING FACTORS

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Ethical considerations: When reporting experiments on animals Observation of the ARRIVE guidelines 2.0: Updated guidelines for reporting animal research, published on July 14, 2020 (DOI: 10.1371/journal.pbio.3000410), is applied. The authors ensure that all procedures were performed in compliance with the guidelines for animal care of their institutions or with national/international guidelines.

ABSTRACT

Gastric lesions represent a significant health concern in domestic pigs and, to a lesser extent, in wild boars. Their development reflects a multifactorial interaction of dietary characteristics, stress exposure, microbial balance, and infection with *Helicobacter* spp. Domestic pigs show a high prevalence and severity of lesions, primarily in the *pars oesophagea*, driven by finely ground low-fibre feed, intensive housing, and dysbiosis. In contrast, wild boars typically display mild, often asymptomatic lesions associated with their fibre-rich diet, natural social structure, and stable microbiota. This review compares etiological factors and pathological features across both populations and highlights implications for prevention. Special attention is given to the ecology and zoonotic relevance of *Helicobacter suis*. Understanding these mechanisms is essential for optimizing nutritional strategies, minimizing stress, and mitigating risks of interspecies transmission.

Key words: domestic pigs; gastric ulcers; *Helicobacter suis*; microbiota; pathology; prevention; stress; wild boars

INTRODUCTION

Gastric lesions in pigs constitute an important welfare and productivity issue, with consequences ranging from reduced growth rates to sudden death [1, 2]. Domestic pigs are particularly vulnerable due to the combined effects of intensive management, low-structural diets, and repeated exposure to stress [3, 4]. Anatomically, the non-glandular *pars oesophagea*, lined by stratified squamous epithelium and lacking mucous protection, represents the primary site affected by parakeratosis, erosions, and ulceration [5, 6].

Recent research has highlighted the value of comparing domestic pigs with wild boars, which share the same species lineage but differ markedly in diet, environment, and microbiota [7, 8]. Wild boars consume diverse, fibre-rich natural diets and experience lower chronic stress, factors that influence gastric physiology and markedly reduce ulcer severity [9, 10]. They also serve as reservoirs for sev-

eral *Helicobacter* species, including *H. suis*, *H. apri*, and *H. pylori*, with implications for animal and human health [11, 12].

This review synthesizes etiological, pathological, microbial, and zoonotic aspects of gastric lesions in domestic pigs and wild boars to identify key mechanisms and inform prevention strategies.

LITERATURE SEARCH STRATEGY

Relevant literature was identified through PubMed, Scopus, Web of Science, and Google Scholar using combinations of the terms: gastric ulcers, *pars oesophagea*, *Helicobacter suis*, wild boar, domestic pig, gastric microbiota, and porcine stomach pathology. Publications from 1995 to 2025 were considered, with emphasis on peer-reviewed articles. Additional sources were obtained from the bibliographies of selected papers. Studies describing etiology, risk factors, microbiology, diet, pathology, or comparative aspects between domestic pigs and wild boars were included.

ETIOLOGICAL FACTORS

Feed structure and dietary composition

Feed particle size is one of the strongest determinants of gastric lesion development. Finely ground or pelleted diets reduce gastric content stratification, allowing acidic digesta to reach the *pars oesophagea* and initiate epithelial damage [13, 14]. Low-fibre diets further enhance acidity and reduce mucosal resilience, whereas coarse or fibre-rich feeds promote buffering and protective layering [15, 16, 17]. High-fibre feeding enhances digesta layering, reduces acid backflow, and supports beneficial microbiota [7, 15].

Although the importance of feed structure in gastric ulcer development is well established, the role of dietary composition remains less clearly defined. Several studies indicate that cereal type, fibre level, feeding regime, and the inclusion of organic acids or selected micronutrients may modulate ulcer risk; however, the reported effects remain inconsistent and highly context-dependent [3, 14, 16, 18].

Wild boars consume structurally rich natural feeds: roots, fruits, vegetation, and insects, supporting digesta separation and reducing acid contact [7, 19]. Seasonal dietary changes alter gastric pH and microbiota but rarely lead to severe lesions [8]. These mechanisms clarify why domestic pigs, exposed to intense production pressures, consistently develop more severe lesions compared to wild boars. Environmental and nutritional constraints, rather than inherent anatomical susceptibility, account for the contrasting pathological outcomes [3, 20].

Stress and environmental conditions

Chronic stress in domestic pigs results from high stocking density, regrouping, transport, and competition for feed and space. These factors activate endocrine pathways that increase gastric acidity, reduce mucosal perfusion, and impair tissue repair [21, 22]. Stress also disrupts microbial balance and enhances susceptibility to ulceration. Stress-induced hyperacidity decreases epithelial repair capacity, further compromising gastric mucosal integrity [13, 14, 18, 23].

Wild boars, in contrast, live in stable social groups, display natural behaviour, and maintain continuous movement, all of which support gastric motility and mucosal integrity [9, 10].

Gastric microbiota and microbial dysbiosis

A stable microbiota plays a crucial role in maintaining gastric homeostasis. Domestic pigs frequently exhibit reduced *Lactobacillus* spp. and increased pro-inflammatory genera such as *Fusobacterium* [23, 24]. These shifts facilitate inflammation, compromise the mucus barrier, and predispose mucosa to erosion. Dysbiosis weakens mucosal protection and interacts synergistically with dietary factors and stress to increase ulcer risk [13, 18].

Wild boars generally maintain a stable microbiota, which supports gastric resilience and contributes to the typically mild or asymptomatic lesions observed in this population [7, 10].

Helicobacter suis is widely recognized as a significant pathogen in pigs, inducing chronic gastritis, epithelial alteration, and enhanced sensitivity to concurrent stressors [25, 26]. Experimental infection confirms its capacity to elicit mucosal inflammation and impair growth [27]. Wild boars often harbour *H. suis*, *H. apri*, and occasionally *H. pylori* with minimal pathology, acting as natural reservoirs [11, 28].

These observations highlight the significance of *Helicobacter* spp. in both domestic pigs and wild boars and

set the stage for considering their potential zoonotic relevance, as discussed below.

Helicobacter spp. and zoonotic aspects

Helicobacter suis is the primary *Helicobacter* species in pigs but also infects humans, where it is linked to chronic gastritis and more severe gastric disorders [29, 30]. Genetic analyses reveal similarities between strains recovered from pigs, wild boars, and human patients, supporting zoonotic transmission potential [11, 31].

Wild boars play a key ecological role by maintaining a reservoir of diverse *Helicobacter* species [28, 32], increasing the relevance of wildlife-livestock interfaces for disease control. Biosecurity and monitoring are therefore critical in mixed-use landscapes.

Key differences in etiological factors, pathogenic mechanisms, and resulting gastric pathology between domestic pigs and wild boars are summarized in Tables 1 and 2.

COMPARATIVE PATHOLOGY OF DOMESTIC PIGS AND WILD BOARS

Domestic pigs

Lesions in domestic pigs predominantly involve the *pars oesophagea*, with pathology ranging from subclinical hyperkeratosis to deep, hemorrhagic ulceration [15, 33, 34]. Subacute lesions present as thickened, roughened epithelium due to parakeratosis [5]. Erosion progresses to crateriform ulcers penetrating submucosa, often accompanied by necrotic debris and clotted blood [35]. Sudden death may occur when ulcers erode sub-mucosal arteries [36] (Fig. 1).

Microscopically, lesions reveal epithelial necrosis, inflammatory infiltrates of lymphocytes and neutrophils, vascular dilation, and mucin depletion [28, 33]. *Helicobacter suis* infection induces lymphoplasmacytic gastritis, epithelial atrophy, and mucus barrier disruption, often in combination with *Fusobacterium gastrosuis* [37].

Clinical signs include pale mucosae, reduced feed intake, lethargy, decreased weight gain, and, in chronic cases, iron-deficiency anaemia.

Wild boars

Wild boars generally exhibit mild gastric lesions characterized by superficial hyperkeratosis or limited erosions [12, 38] (Fig. 2, Fig. 3). Deep ulceration and hemorrhage are rare, and histological inflammation is minimal [39]. Their high-fibre diet and stable microbiota promote mucosal resilience [10], while natural activity patterns support gastric motility and perfusion.

Despite their carrier status for multiple *Helicobacter* spp., wild boars seldom show extensive glandular pathology, underscoring the importance of environmental moderation of disease expression [11].

Table 1. Comparison of the etiology and pathogenesis of gastric lesions in domestic pigs and wild boars (authors' own table)

Factor	Domestic pigs	Wild boars
Primary type of feed	Finely ground/pelleted diet, low fibre content	Diverse natural diet, high fibre content
Effect of feed on *pars oesophagea*	Increased digesta mixing → acidic content contacts epithelium → parakeratosis, erosions	Thicker digesta layer → reduced acid backflow
Aggressive factors	Acid, pepsin, mechanical abrasion, stress-induced hyperacidity	Mainly seasonal changes in diet and energy intake
Protective factors	Weaker → low fibre, stress, rapid growth, intensive production	Stronger → fibre, stable microbiota, lower stress
***Helicobacter* spp.**	*H. suis* → major pathogen; synergizes with *Fusobacterium gastrosuis*	*H. suis, H. apri, H. pylori* → mostly asymptomatic
Microbiota	Affected by dysbiosis with finely ground feed → reduced *Lactobacillus*	Stable, diverse, adapted to high fibre
Key trigger of ulceration	Fine particle size + stress + *H. suis*	Seasonal dietary changes, rarely pathogenic bacteria
Ulcer prevalence	High, mainly in finishing pigs	Low, mostly mild lesions
Clinical course	Often peracute, silent bleeding, sudden deaths	Mostly latent, clinically silent
Severity of pathology	Moderate to high	Low

Table 2. Comparison of gastric pathology, risk factors, and predisposing conditions in domestic pigs and wild boars (authors' own table)

Parameter	Domestic pigs (*Sus scrofa domesticus*)	Wild boars (*Sus scrofa*)
Lesion prevalence	High: 30–70%, mainly in *pars oesophagea*	Low: 5–10%, mostly mild erosions or hyperkeratosis
Most common location	*Pars oesophagea* – deep erosions and ulcers	*Pars oesophagea* – mainly superficial changes
Typical pathologies	Parakeratosis, deep ulcerations, bleeding, scarring	Mild parakeratosis, superficial erosions, rarely ulcerations
Diet	Finely ground or pelleted feed, low fibre content, monodiet	Diverse natural diet: roots, fruits, fibre, insects, vegetation
Nutritional effects	High risk: low structural fibre → higher acidity in the cardia	Protective: fibre → more stable digestion → less acid–epithelium contact
Microbiota	Lower diversity, frequent dysbiosis; reduced *Lactobacillus*, increased *Fusobacterium*	Higher diversity, more stable microbiota; lower dysbiosis risk
***Helicobacter* spp.**	*H. suis* commonly pathogenic → chronic gastritis, ulcerations, dysbiosis	Frequent carriers of *H. suis, H. apri, H. pylori* – mostly asymptomatic
Zoonotic potential	Infected populations may transmit *H. suis* to humans	Reservoir of *Helicobacter* spp., high zoonotic relevance
Stress factors	High: stocking density, transport, handling, hierarchy, low enrichment	Low to moderate: natural environment, free movement
Effect of stress on stomach	Increased cortisol → hyperacidity → deep ulcers → higher risk	Lower stress → more stable mucosal barrier
Role of genetics	Selected for high growth → may be more susceptible	Less selected, more robust digestion
Clinical signs	Often hidden → sudden death possible due to bleeding; anemia, melena, weakness	Mostly asymptomatic, rare clinical signs
Economic impact	Significant: reduced growth, poor feed conversion, mortality	Minimal (mainly in wild populations)
Environmental factors	Intensive housing → limited movement → higher stress	Forest environment → natural behaviour, seasonal dietary shifts
Protective factors	Coarse fibre, probiotics, good stress management	Natural diet, low stress, microbial diversity

IMPLICATIONS FOR PREVENTION AND MANAGEMENT

Preventive strategies must consider the interplay of diet, stress, microbiota, and infection pressure. Increasing structural fibre, reducing excessive feed grinding, maintaining consistent feeding routines, and ensuring gradual dietary transitions significantly lower ulcer risk [40, 41, 42]. Stress reduction through stable group management, minimized transport, adequate space, bedding, and enrichment improves mucosal protection [22, 43].

Microbiota-supporting interventions, including probiotics, fermented feeds, and organic acids, can reinforce gastric stability [44]. Monitoring and controlling *H. suis* infections remain essential, particularly in herds with recurrent ulcer problems [24].

Economically, gastric lesions contribute to decreased growth, poorer feed conversion, and sudden mortality in domestic pigs, supporting the integration of nutritional and welfare-based prevention within herd health planning [3, 45].

Preventive approaches targeting nutrition, stress reduction, microbiota stabilization, and infection control in domestic pigs and wild boars are summarized in Table 3.

CONCLUSIONS

Gastric lesions in domestic pigs and wild boars arise from the interaction of nutritional, microbial, environmental, and host-related factors. Domestic pigs are predisposed to severe *pars oesophagea* lesions due to finely ground low-fibre diets, high stress exposure, and microbiota instability, often compounded by *H. suis* infection. Wild boars, by contrast, typically develop mild lesions associated with their natural diet, low stress levels, and stable gastric microbiota.

Comparative evaluation highlights that management and dietary structure, more than species-related anatomy,

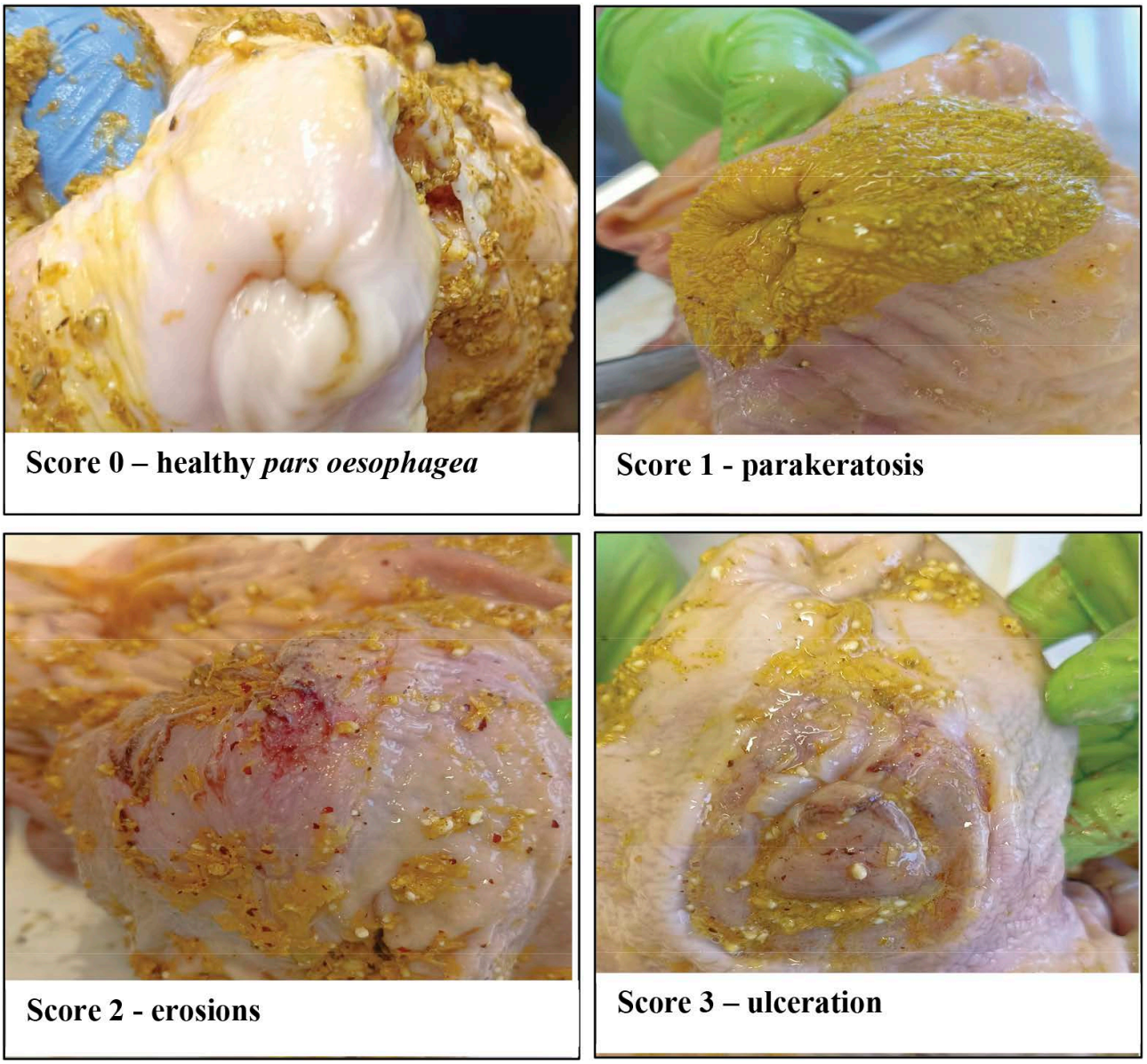

Fig. 1. Lesion score of the gastric mucosa in a finishing pig from 0 to 3
Source: (authors' own photographs)

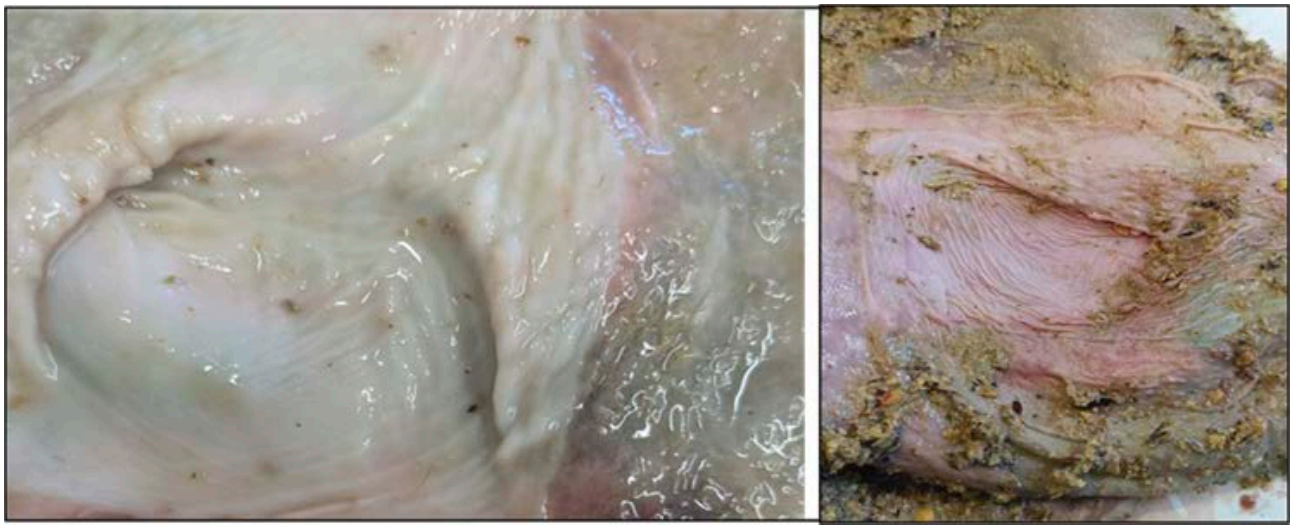

Fig. 2. Stomach of a wild sow and yearling with healthy mucosa – score 0
Source: (authors' own photographs)

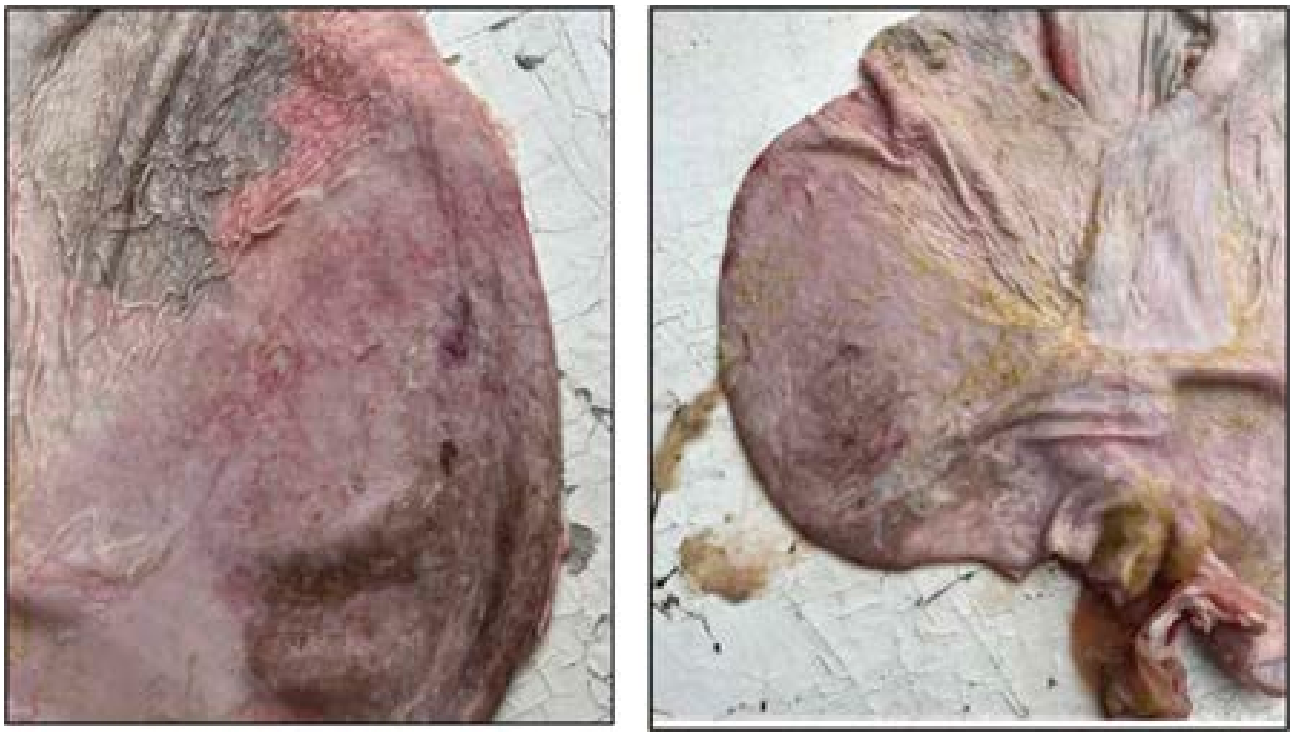

Fig. 3. Stomach of a wild boar (yearling) – glandular (fundic) region with erosions present
Source: (authors' own photographs)

Table 3. Preventive strategies – comparison of domestic pigs and wild boars (authors' own table)

Prevention area	Domestic pigs	Wild boars
Nutrition and feeding	- Increase fibre content (wheat bran, beet pulp) - Use coarser particle size - Avoid extremely fine grinding - Consistent feeding and gradual feed transitions	No intervention – natural diet provides sufficient structure and fibre
Management	- Minimize stress (transport, regrouping) - Optimize stocking density - Improve bedding and environmental enrichment	Not applicable (natural conditions)
Microbiota	- Probiotics (*Lactobacillus spp.)* - Fermented feed - Support SCFA production - Monitoring	Stable microbiota → minimal need for intervention
Control of *H. suis* infection	- Farm hygiene - Age group separation	Natural reservoir; interventions only feasible in farmed wild boars
Seasonal risks	Low	High → fluctuations in diet may alter pH and microbiota

determine gastric outcomes. Future research should focus on:

(1) characterizing gastric microbiota composition and seasonal variability in wild boars;

(2) clarifying host–pathogen interactions involving *Helicobacter* spp.;

(3) developing targeted feeding and welfare practices to reduce ulcer prevalence; and

(4) assessing zoonotic transmission risks at wildlife–livestock interfaces.

Comprehensive integration of nutritional strategies, stress mitigation, microbial monitoring, and biosecurity offers the most effective pathway to reducing ulcer incidence while improving welfare and production efficiency in domestic pigs.

Ethical Statement

No Ethical Approval was necessary for this study.

Conflict of Interest

The authors declare no conflicts of interest.

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Generative AI statement

The authors declare that no generative AI or AI-assisted technologies were used in the writing of this manuscript.

Authors' Contributions

ZK – Conceptualization, Methodology, Literature review, Writing – original draft, Writing – review and editing, Supervision.

JN – Conceptualization, Methodology, Investigation, Writing – original draft, Writing – review and editing, Funding acquisition, Supervision.

KB – Literature review, Writing – original draft, Visualization.

JČ – Contribution to wildlife ecology section, Writing – review and editing.

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ORIGINAL ARTICLE

TOXICOLOGICAL ASSESSMENT OF *LAGENARIA BREVIFLORA* WHOLE FRUIT EXTRACT IN JUVENILE AFRICAN CATFISH (*CLARIAS GARIEPINUS*)

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Ethical considerations: When reporting experiments on animals Observation of the ARRIVE guidelines 2.0: Updated guidelines for reporting animal research, published on July 14, 2020 (DOI: 10.1371/journal.pbio.3000410), is applied. The authors ensure that all procedures were performed in compliance with the guidelines for animal care of their institutions or with national/international guidelines.

ABSTRACT

Plant extracts have been used in farmed fish to control fish bacterial infection, fish fry predators, to replace chemical pesticides and piscicides. This study was carried out to assess the toxicological effect of methanol fruit extract of *L. breviflora* in juvenile African catfish (*Clarias gariepinus*). Forty-five (45) juvenile catfish were randomly and equally divided into 9 groups. The first five groups were administered varying concentrations of *L. breviflora* fruit extracts (25 mg/L, 50 mg/L, 75 mg/L, 100 mg/L, 125 mg/L) and LC_{50} was calculated to be 63.9 mg/L. The remaining four groups were labelled A to D. The fish were exposed daily to graded concentrations of *L. breviflora*: Group A (Control), Group B (6.25 mg/L), Group C (12.5 mg/L) and Group D (25 mg/L) for 14 days. Blood samples of exposed fish showed significant decreases in the red blood cell count, packed cell volume, haemoglobin concentration, mean corpuscular volume, and mean corpuscular haemoglobin. The histology results show erosion of the epidermis and hyperplastic alarm cells in the skin, areas of diffuse vacuolation, periportal degeneration and necrosis in the liver, mild degeneration of the lamella core, congestion of the submucosa and expanded lamella core in the skin. The plant extract can therefore be recommended for use at concentrations < 10 mg/L.

Keywords: aquaculture; *Clarias gariepinus; Lagenaria breviflora*; toxicology

INTRODUCTION

Aquatic food in the form of fish is increasingly appreciated for nutritional value addition through fish consumption, which is driven by growing demand for wholesome and healthy fish [1]. Production of fish species like African catfish is important for the high demand of fish in Nigeria, and the reason for the species is due to its characteristics,

such as fast-growing, productive, efficient, and adaptive nature to the environment. In addition, aquaculture attributes of *C. gariepinus* include rapid growth, good feed conversion, excellent flesh quality, and high plasticity in its feeding habits, as well as good market potential [2].

The increased demand for African catfish is high, and many low-income earners depend on catfish as a major protein source [3]. Nigeria is making a substantial and prominent contribution to aquaculture production in Africa [4]. Nigeria is the largest aquaculture producer in Sub-Saharan Africa and ranks second in Africa. Nigeria is a distinguished second top producer of catfish, and this accounts for 64% of total fish production annually [4]. Despite being the top producer of catfish, Nigeria faces significant obstacles in bridging the demand-supply gap in its fishery market. Meanwhile, the growth of aquaculture is harshly endangered by the challenges and difficulties, such as extreme temperature fluctuation and diseases [5].

Moreover, diseases outbreaks due to bacterial infection pose serious economic devastation to the rapid growth of aquaculture in Nigeria [6]. The morbidity and mortality caused by bacterial infection are usually high due to environmental and biological factors. The causes of bacterial infection in farms include lack of biosecurity measures, stocking density, poor water quality, contaminated feed, and lack of prompt diagnosis and treatment of infected fish [7].

Over the last several decades, plant extracts have been used in farmed fish to control fish bacterial infection and fish fry predators, to replace chemical pesticides and piscicides due to extensive and indiscriminate use of these non-biodegradable synthetic chemicals, which have had harmful effects on aquatic environments and pose a high risk to non-targeted organisms [8, 9]. Plant extracts are considered promising agents because of their eco-friendliness, ease of availability, high efficiency, rapid biodegradability, and reduced toxicity [10]. The roles of plant extracts include disease management, water quality improvement, antibacterial effects (as an alternative to antibiotics), growth promotion, immunity improvement, and anti-stress effects [11].

Recently, the acceptance of traditional medicine as an alternative form of healthcare and the development of antimicrobial resistance to the available antibiotics have led to the increased isolation and identification of secondary metabolites produced by plants and their use as active principles in medicinal preparations. One such plant that has been used is *Lagenaria breviflora* (*L. breviflora*). *L. breviflora* was used in herbal treatment of inflammatory diseases such as colitis, skin diseases, jaundice, ulcers, and congestive heart failure [12]. Previous reports indicated that *L. breviflora* possessed antibacterial, antioxidant, anti-nociceptive, and anti-inflammatory attributes [12, 13]. The effects of dietary *Lagenaria breviflora* leaf extract (LBLE) on the growth performance, feed utilisation, and haematological parameters of juvenile African catfish were assessed by Paray et al. [14]. The usage of *L. Breviflora* in livestock production cannot be exaggerated, and rural rearers extensively utilized the fruit as curative and preventive options. *L. breviflora* was reported to contain phytochemicals, bioactive molecules, and vitamins such as flavonoids, carotenoids, alkaloids, phenols, 1,2-benzenedicarboxylic acid, mono[2-ethylhexyl], and vitamins C, B, and E, which can act as anti-inflammatories, antioxidants, and antibacterial agents, meanwhile, observed toxic effect of the extract may be due to the presence of alkaloids and other constituents [15]. This study was carried out to assess the toxicological effect of the methanol fruit extract of *L. breviflora* in juvenile African catfish (*Clarias gariepinus*).

MATERIALS AND METHODS

Preparation of plant extract

Fresh fruits of *L. breviflora* were purchased from a commercial seller in the market. The fruits were air-dried for 6 weeks at room temperature. The dried blended *L. breviflora* whole fruit (2.3 kg) was extracted in a glass container using 96% methanol (7 L). The mixture was constantly stirred and allowed to extract for 72 hrs. The filtrate was filtered using Whatman filter paper (1 mm). A repeat extraction was done and filtered. The combined filtrate was then concentrated with a rotary evaporator (Heidolph Laborota 400 efficient, made in Germany, model 517-01002-002) set at 40°C, after which the concentrate was further concentrated using a vacuum oven set at 40°C with a pressure of 700 mmHg. The weight of the crude extract was 99 g and the percentage yield was 4.3%.

Experimental animals and acclimatization

Forty-five juveniles of *Clarias gariepinus* (n = 45) with an average weight of 25.28 ± 0.81 g, length of 15.30 ± 0.6

cm were procured from a commercial fish farm in Ibadan. The fish were stored in 100 L plastic tanks and acclimatized for 2 weeks. The fish were earlier disinfected in 250 ppm formalin for 1 hour and then randomly examined and observed to be free from ectoparasites, lesions, or clinical signs of any disease. Following acclimatization, the fish were randomly and equally divided into 9 groups in 2 L of water, and each group was labelled.

Acute toxicity

An acute toxicity test of *L. breviflora* fruit crude extracts (methanol) was carried out to determine the lethal concentrations (LC_{50}) to *C. gariepinus*. The *C. gariepinus* juveniles were subjected to *L. breviflora* fruit extract (methanol) toxicity test for 96 hours to determine the toxic concentration range and the related timing. The test was carried out using 2L plastic tanks, and the fish were distributed equally into 5 different groups; 20 fish were divided into five groups (n = 4). The groups were A, B, C, D, and E. Varying concentrations of *L. breviflora* fruits extracts (methanol) were added to the water at 25 mg/L, 50 mg/L, 75 mg/L, 100 mg/L, and 125 mg/L respectively. Every 12 hours, mortality rates were monitored and recorded. The LC_{50} was calculated from the graph plotted.

Sub-chronic toxicity

Sub-chronic toxicity test of *L. breviflora* fruit methanol extracts was carried out to determine the toxic effect of the plant. The *C. gariepinus* juveniles were subjected to *L. breviflora* fruit crude extract (methanol) toxicity test for 14 days, followed by collection of a blood sample and harvesting of organs on day 15. The test was carried out using 2L plastic tanks, and the fish were distributed equally into 4 different groups. Twenty-four fish were divided into four groups (n = 6). The groups were A, B, C, and D. The fish were exposed daily to graded concentrations of *L. breviflora:* Group A (Control, 0 mg/L), Group B (6.25 mg/L), Group C (12.5 mg/L) and Group D (25 mg/L). The fish were exposed to fresh extract-treated water daily.

Haematology

Blood was collected into lithium heparinized tubes from the *C. gariepinus* juvenile on day 15 post extract exposure using the caudal vein for determination of haematological parameters. Heparinised capillary tubes were filled with blood to about two-thirds of the tube and sealed with plasticine to determine the packed cell volume. The tubes were placed in the haematocrit centrifuge (Perkin Elmer, USA) for 5–6 min at 3000 rpm and the packed cell volume (PCV) was read in the haematocrit reader. Haematology was carried out according to the method described by Adeshina et al. [16]. The following parameters were measured: packed cell volume (PCV); haemoglobin (Hb); red blood cell (RBC); white blood cell (WBC); counts, mean cell haemoglobin (MCH), mean cell haemoglobin concentration (MCHC), and mean cell volume (MCV).

Histology

Four fishes per group were sacrificed by stunning, and tissue samples of the gills, and other lymphoid organs (liver, spleen and kidney) were harvested and fixed in 10% formalin. Tissue specimens were obtained from skin, liver, kidney, and gills for histological examination. Four fish each (n = 4) were sampled from each exposed and control group. Tissue specimens from skin, liver, kidney, and gill were fixed with 10% neutral buffered formalin, dehydrated, infiltrated, embedded in paraffin, and stained with haematoxylin and eosin according to Roberts [17].

Statistical analysis

Statistical software SPSS version 23 was employed for statistical analysis of data. Haematology parameters were subjected to one-way ANOVA. Differences were considered significant at $p \leq 0.05$ for all the datasets.

Table 1. Number of dead and mortality rate of *Clarias gariepinus* juveniles following acute exposure to graded concentrations of the *Lagenaria breviflora* whole fruit methanol extract

Concentration	24 Hrs	48 Hrs	72 Hrs	96 Hrs
25 mg/L	0 (0%)	1 (25%)	0 (0%)	0 (0%)
50 mg/L	0 (0%)	0 (0%)	1 (25%)	0 (0%)
75 mg/L	1 (25%)	1 (25%)	0 (0%)	0 (0%)
100 mg/L	4 (100%)	0 (0%)	0 (0%)	0 (0%)
125 mg/L	4 (100%)	0 (0%)	0 (0%)	0 (0%)

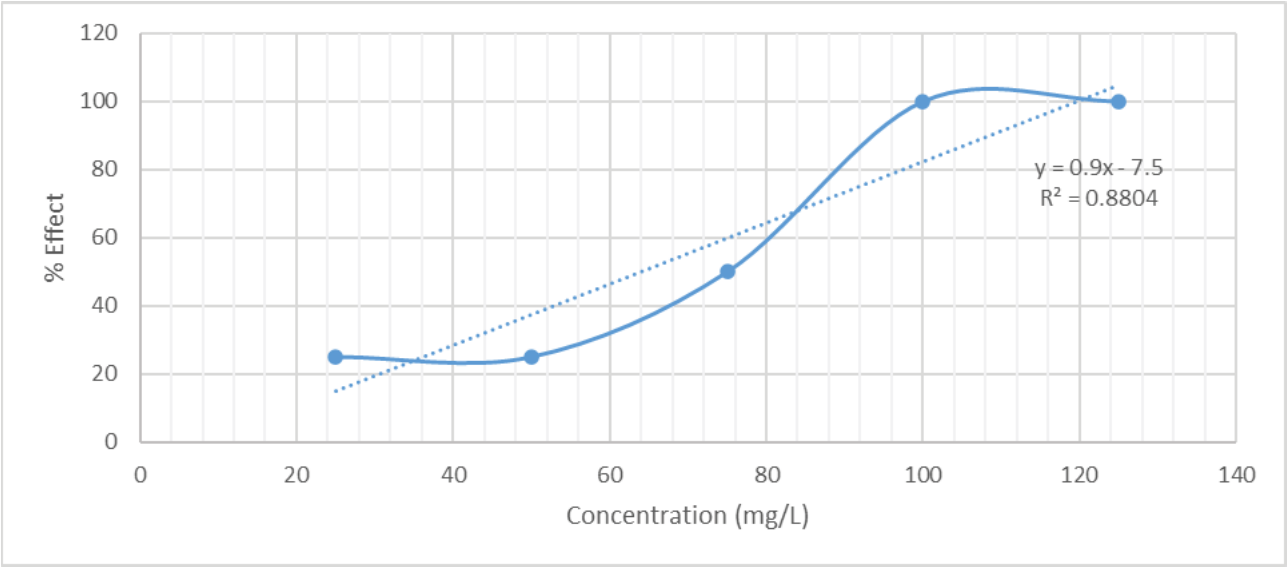

Fig. 1. LC_{50} concentration of *Clarias gariepinus* juveniles following acute exposure to graded concentrations of the *Lagenaria breviflora* whole fruit methanol extract; LC_{50} = 63.9 mg/L

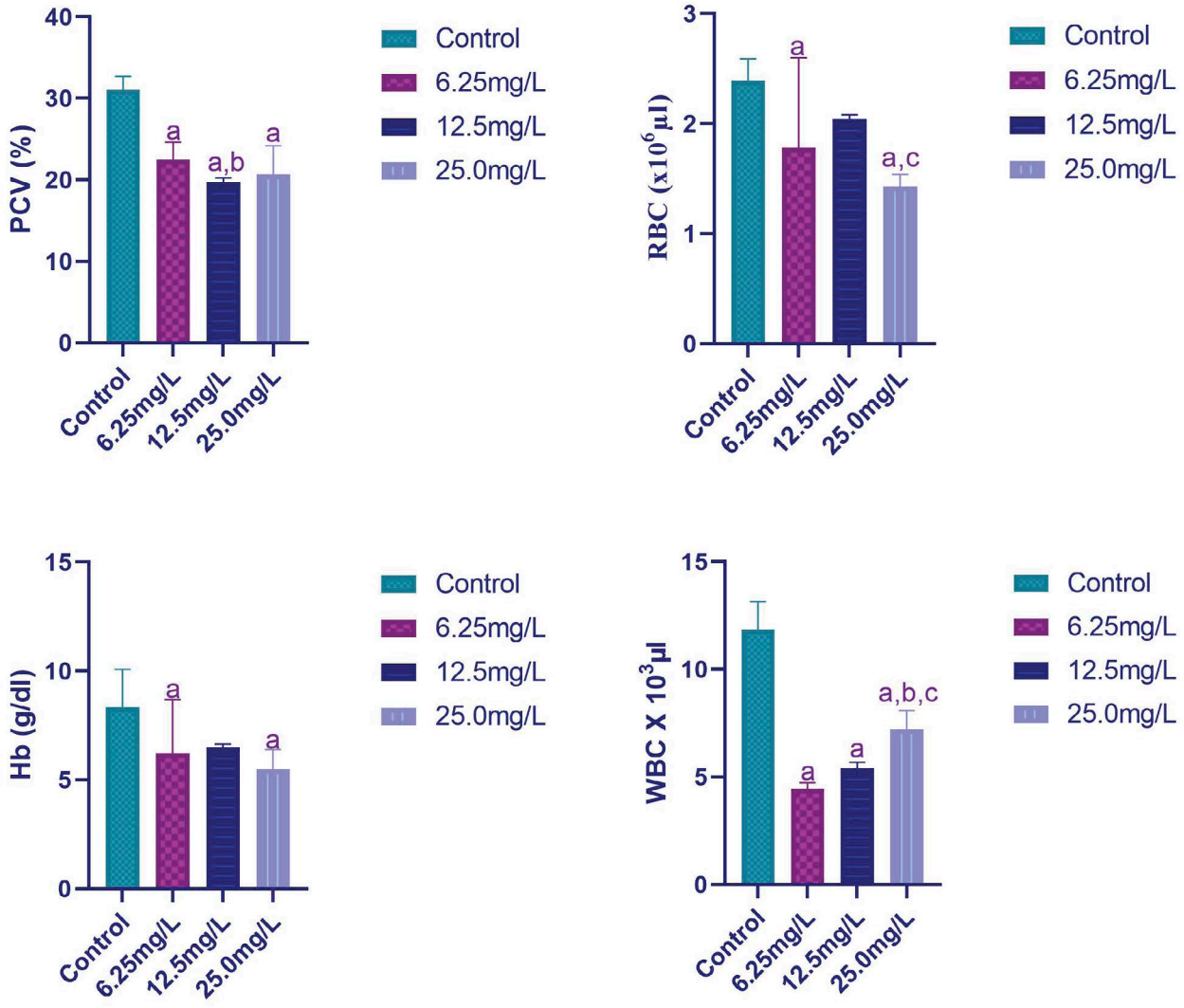

Fig. 2. Haematology of *Clarias gariepinus* juveniles following sub-chronic exposure to graded concentrations of the *Lagenaria breviflora* whole fruit methanol extract (values with different superscripts indicate statistical significance at $p < 0.05$)

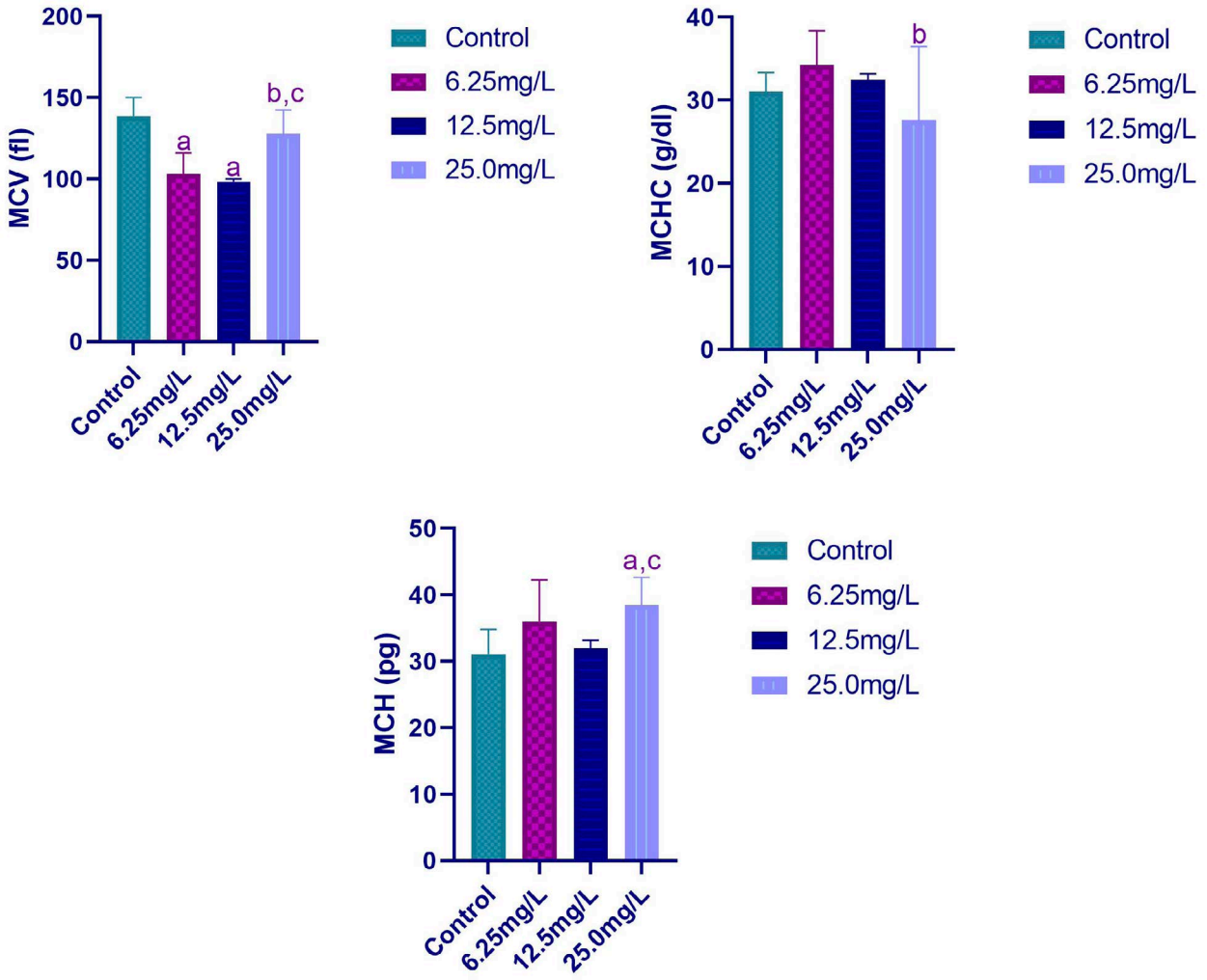

Fig. 3. Red cell indices of *Clarias gariepinus* juveniles following sub-chronic exposure to graded concentrations of the *Lagenaria breviflora* whole fruit methanol extract (values with different superscripts indicate statistical significance at $p < 0.05$)

RESULTS

The fish in all the groups showed no obvious clinical signs on observation for 24 hours post-exposure to graded concentrations of *L. breviflora* methanol extract during acute toxicity. Mortality rate is shown in Table 1. LC_{50} was calculated to be 63.9 mg/L as shown in Figure 1. There was a significant decrease in the PCV ($p < 0.05$) of fish exposed to 6.25 mg/L, 12.5 mg/L and 25 mg/L compared with the control. A significant decrease ($p < 0.05$) was also observed in fish exposed to 12.5 mg/L of the extract compared to 6.25 mg/L. A significant decrease was also seen in the RBC ($p < 0.05$) of fish exposed to 6.25 mg/L and 25 mg/L compared with the control. In fish exposed to 25 mg/L of the extract compared to 12.5 mg/L, a significant decrease $p < 0.05$ was also observed. There is a significant decrease in Hb ($p < 0.05$) of fish exposed to 6.25 mg/L and 25 mg/L of the extract compared to the control (Fig. 2). A significant decrease was detected in the MCV ($p < 0.05$) of fish exposed to 6.25 mg/L and 12.5 mg/L compared with the control. There was also a significant increase ($p < 0.05$) observed in fish exposed to 25 mg/L of the extract compared to 6.25 mg/L and 12.5 mg/L. There was a significant increase ($p < 0.05$) in the MCH of fish exposed to 25 mg/L compared with the control and 12.5 mg/L of the extract.

A significant decrease in the MCHC ($p < 0.05$) of fish exposed to 25 mg/L compared to 6.25 mg/L of the extract (Fig. 3). There was a significant decrease in the WBC ($p < 0.05$) of fish exposed to 6.25 mg/L, 12.5 mg/L, and 25 mg/L compared with the control. A significant increase ($p < 0.05$) was also observed in fish exposed to 25 mg/L of the extract compared to 6.25 mg/L and 12.5 mg/L (Fig. 2).

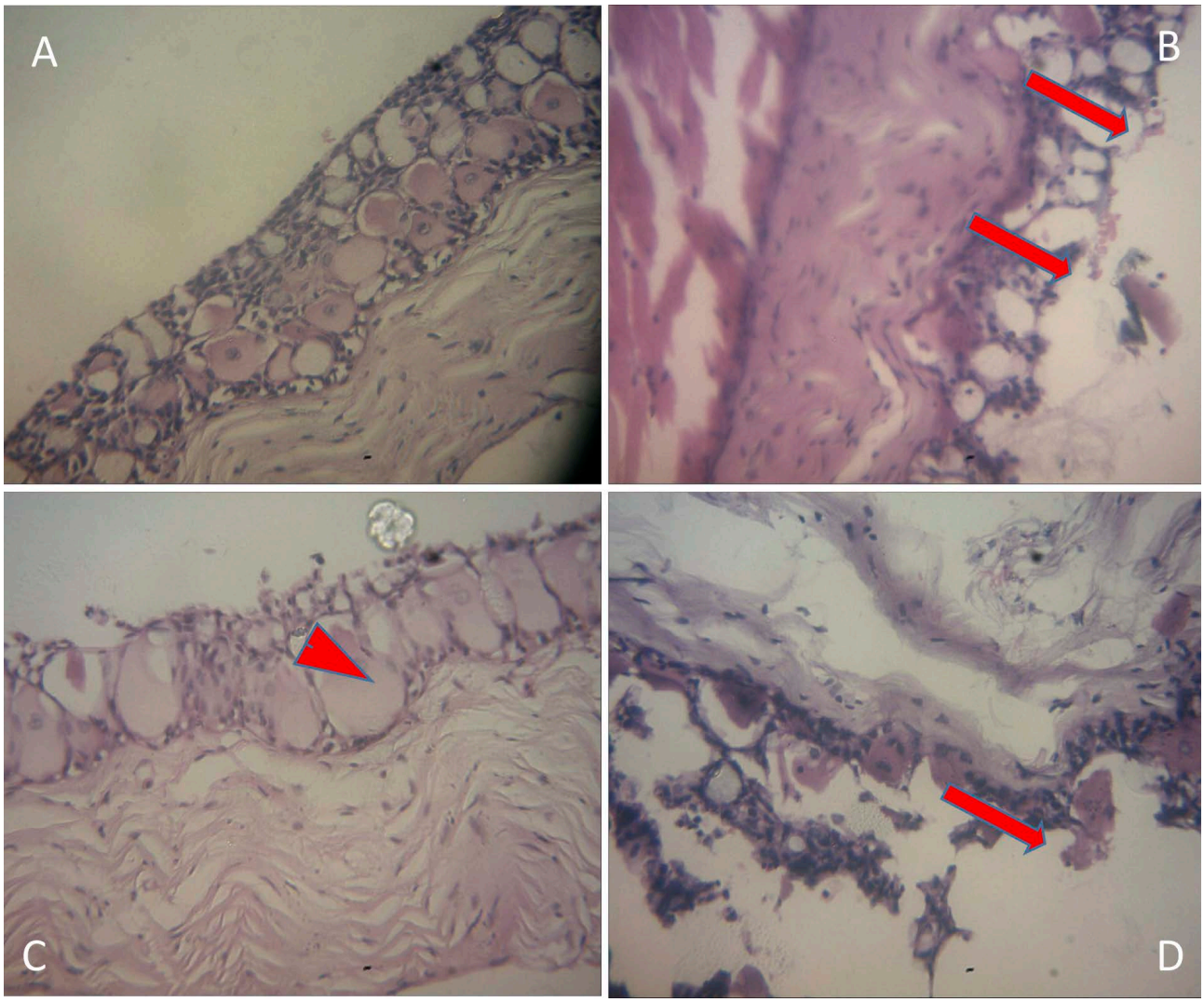

Fig. 4. Histology of the skin of *Clarias gariepinus* juveniles following sub-chronic exposure to graded concentrations of the *Lagenaria breviflora* whole fruit methanol extract; H&Ex400; A (Control) – Normal, B (6.25 mg/L) – Erosion of the epidermis (arrow), C (12.5 mg/L) – Hyperplastic alarm cells (red arrow head), D (25 mg/L) – Erosion of the epidermis (red arrow)

The histology showed erosion of the epidermis in the skin of fish exposed to 6.25 mg/L, hyperplastic alarm cell in 12.5 mg/L, and erosion of the epidermis in 25 mg/L (Fig. 4). The histology of the liver showed periportal degeneration and necrosis in the fish exposed to 6.25 mg/L, periportal degeneration and necrosis with vacuolation in 12.5 mg/L, and periportal degeneration and necrosis in 25 mg/L (Fig. 5). The histology of the kidney showed moderate congestion of the interstitium in the fish exposed to 25 mg/L, meanwhile, there was abnormality observed in fish exposed to 6.25 mg/L and 12.5 mg/L, respectively (Fig. 6). The histology of the gill showed mild degeneration of the lamella core and oedema in the fish exposed to 6.25 mg/L, moderate congestion of the submucosa and expanded lamella connective tissue in 12.5 mg/L, and moderate congestion of the submucosa in 25 mg/L (Fig. 7).

DISCUSSION

In this study, there was a significant reduction in the PCV, RBC, HB, and WBC at the different doses of the extract when compared with the control. Increases in mean MCHC at dose 6.25 mg/L and 12.5 mg/L were not significant when compared with the control but a significant decrease was noticed at a concentration of 25.0 mg/L. The MCH results show a significant increase when the extract at dose 25.0 mg/L is compared with the control and 12.5 mg/L. There is a significant decrease in MCV when the extract at doses of 6.25 mg/L and 12.5 mg/L is compared with the control.

The low PCV, MCV, and a significant decrease in the highest concentration of MCHC are indicative of microcytic hypochromic anaemia [18]. This type of anaemia

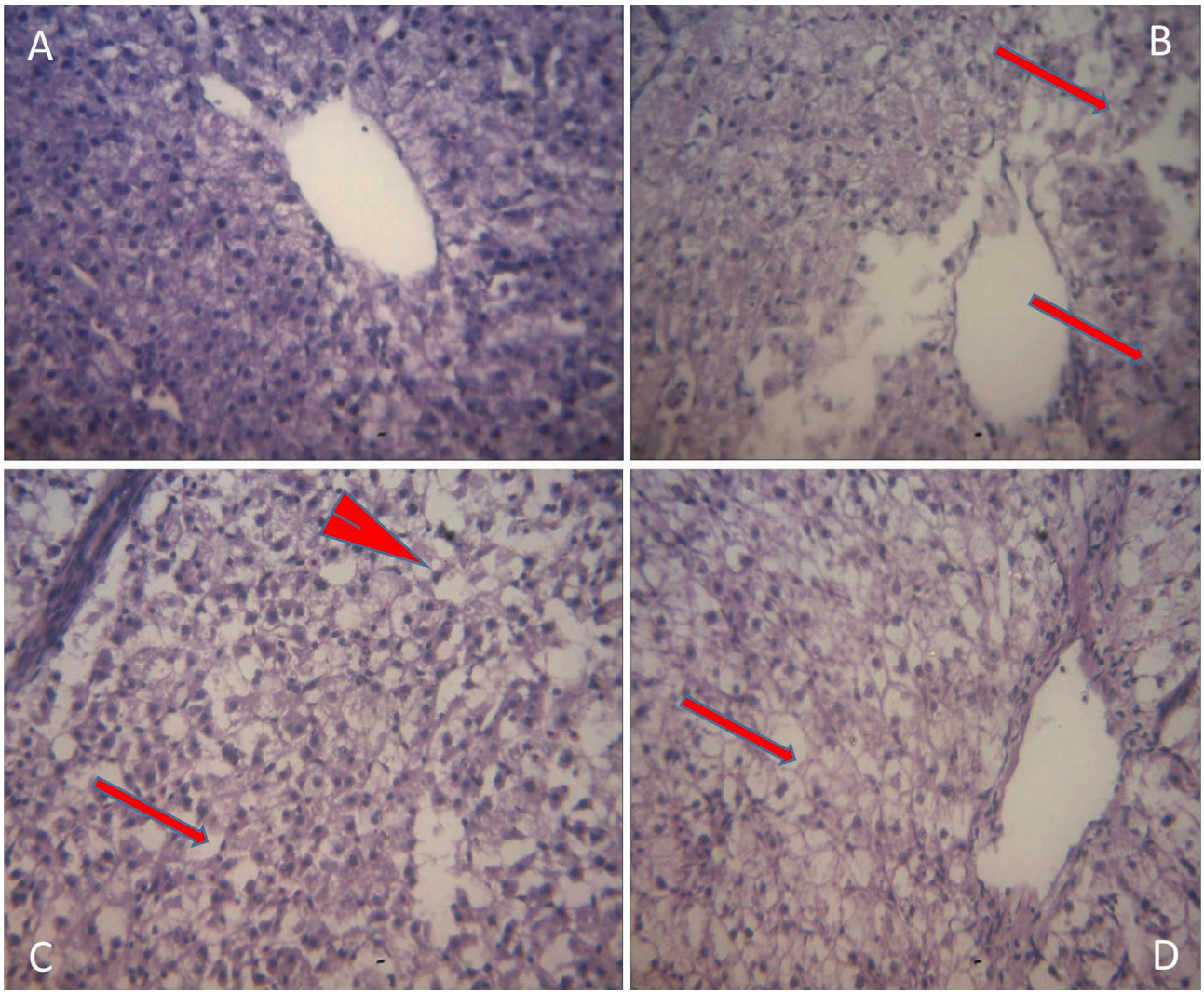

Fig. 5. Histopathology of the liver of *Clarias gariepinus* juveniles following sub-chronic exposure to graded concentrations of the *Lagenaria breviflora* whole fruit methanol extract; H&Ex40; A (Control) – Normal, B (6.25 mg/L) – Periportal degeneration and necrosis (arrow), C (12.5 mg/L) – Periportal degeneration and necrosis (arrow) with vacuolation (red arrow head), D (25 mg/L) – Periportal degeneration and necrosis (red arrow)

has also been reported in healthy fish subjected to nonylphenol and octylphenol [19]. The low WBC count may be due to prolonged stress in this situation, induced by the sub-chronic exposure to the extract [20]. Short-term stress sometimes results in an increase in WBC, but chronic and/or strong stress usually causes leukopenia [21].

The gills, skin, and liver of fish exposed to all concentrations of the extract showed signs of toxicity compared to the unexposed fish. However, the kidneys of fish exposed to the extract at concentrations of 6.25 mg/L and 12.5 mg/L were normal compared to the control, while 25 mg/L showed signs of toxicity following exposure to the extract. A significant pathology in the kidney was mainly the moderate congestion of the interstitium at 25 mg/L. This study indicates that the primary organs exposed to the extract (skin and gills) may be involved with detoxification of the extract, as evident by the direct changes in the organs.

Periportal degeneration and necrosis, vacuolation, an increase in sinusoidal gaps, and poor hepatic cord structure were observed following sub-chronic exposure to *L. breviflora*. Vacuolation is due to the metabolic changes that occurred in the cytoplasm of the hepatocytes. According to Ezhilarasan et al. [22], oxidative stress is the main cause of liver necrosis. The changes in the liver may also explain the reduction of the haematological parameters in the fish. The primary organs responsible for erythropoiesis in fish are the kidneys. The main role of RBCs is to carry oxygen in the blood by the hemoglobin molecule. Therefore, erythropoiesis needs to be tightly regulated to maintain

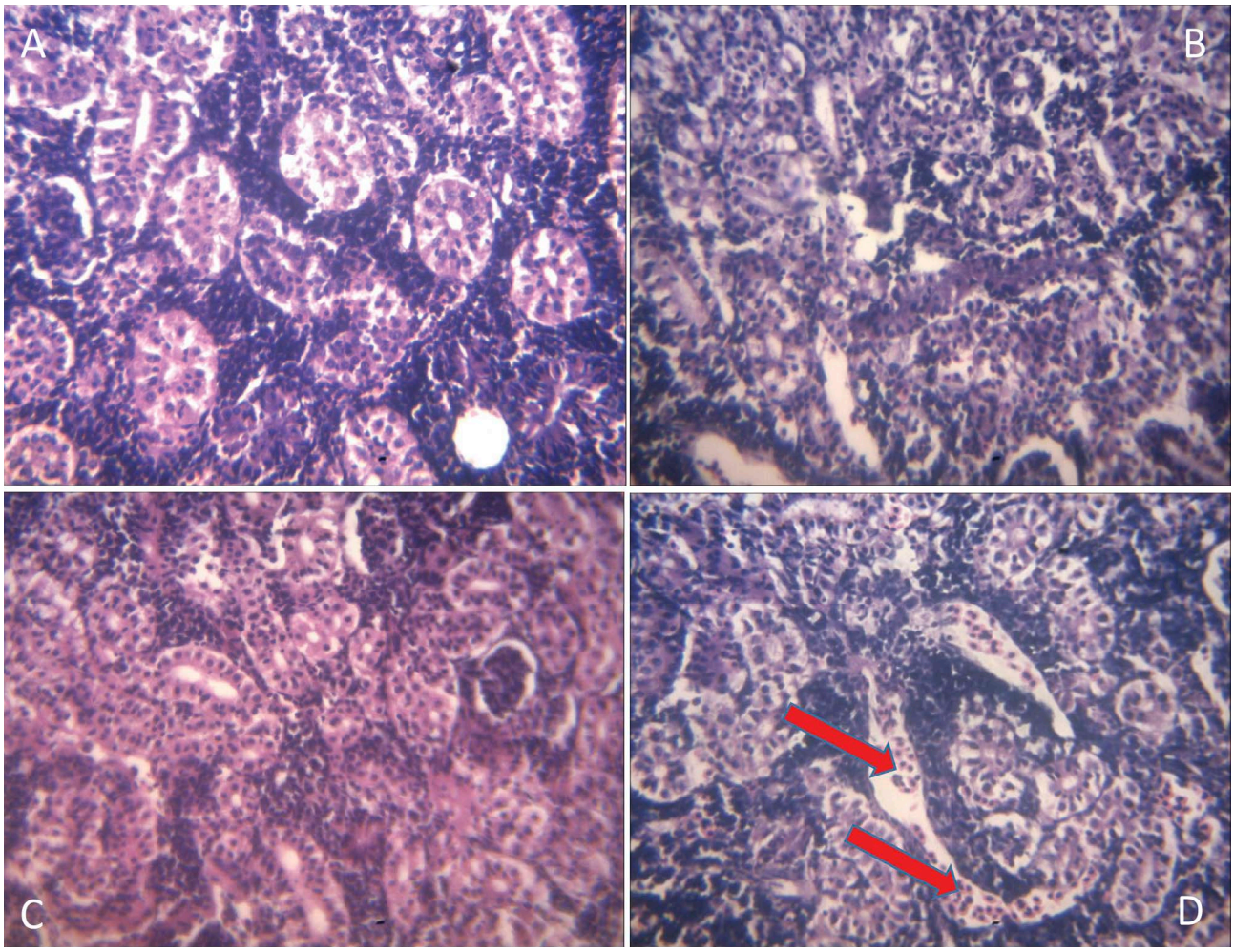

Fig. 6. Histopathology of the kidney of *Clarias gariepinus* juveniles following sub-chronic exposure to graded concentrations of the *Lagenaria breviflora* whole fruit methanol extract; H&Ex40; A (Control) – Normal, B (6.25 mg/L) - Normal, C (12.5 mg/L) - Normal, D (25 mg/L) – Moderate congestion of the interstitium (red arrow)

homeostasis and to meet changes in oxygen supply and demand.

The study's findings of epithelial detachment and oedematous changes in the gill lamellae of the exposed fish suggest that the observed changes may have been caused by increased capillary permeability of the blood vessels of the afflicted gills. Similar oedematous gills in *C. gariepinus* treated with sub-lethal concentrations of methanol extract of *Raphia hookeri* were described by Adeogun et al. [23]. Despite the fact that mucous secretion helps to stop toxicants from reaching the gill epithelium, these effects may disrupt the gas exchange process and decrease respiration [24].

There was erosion of the epidermis, which may be a sequel to the loss of the protective functions carried out by the mucous cells. Prolonged exposure to the plant extract may have predisposed the skin to extensive loss and rapid exhaustion of the mucous cells. According to Chandel et al. [25], *C. batrachus* skin exposed to the air experienced similar tear and wear that led to sloughing off of skin surfaces and haemorrhage.

CONCLUSION

The detected changes in the blood and histological abnormalities in the gills, liver, and skin of exposed fish, as well as the reported mortality in some of the exposed fish at acute toxicity, show that the ethanol extract of *L. breviflora* is toxic at the doses of exposure, particularly at concentrations >12.5 mg/L. The plant extract can therefore be recommended for use at concentrations <10 mg/L.

In conclusion, this work reporte d the safety evaluation of *L. breviflora* extract administered to *C. gariepinus*. It also contributed to basic research into alternative medicinal therapies in aquaculture.

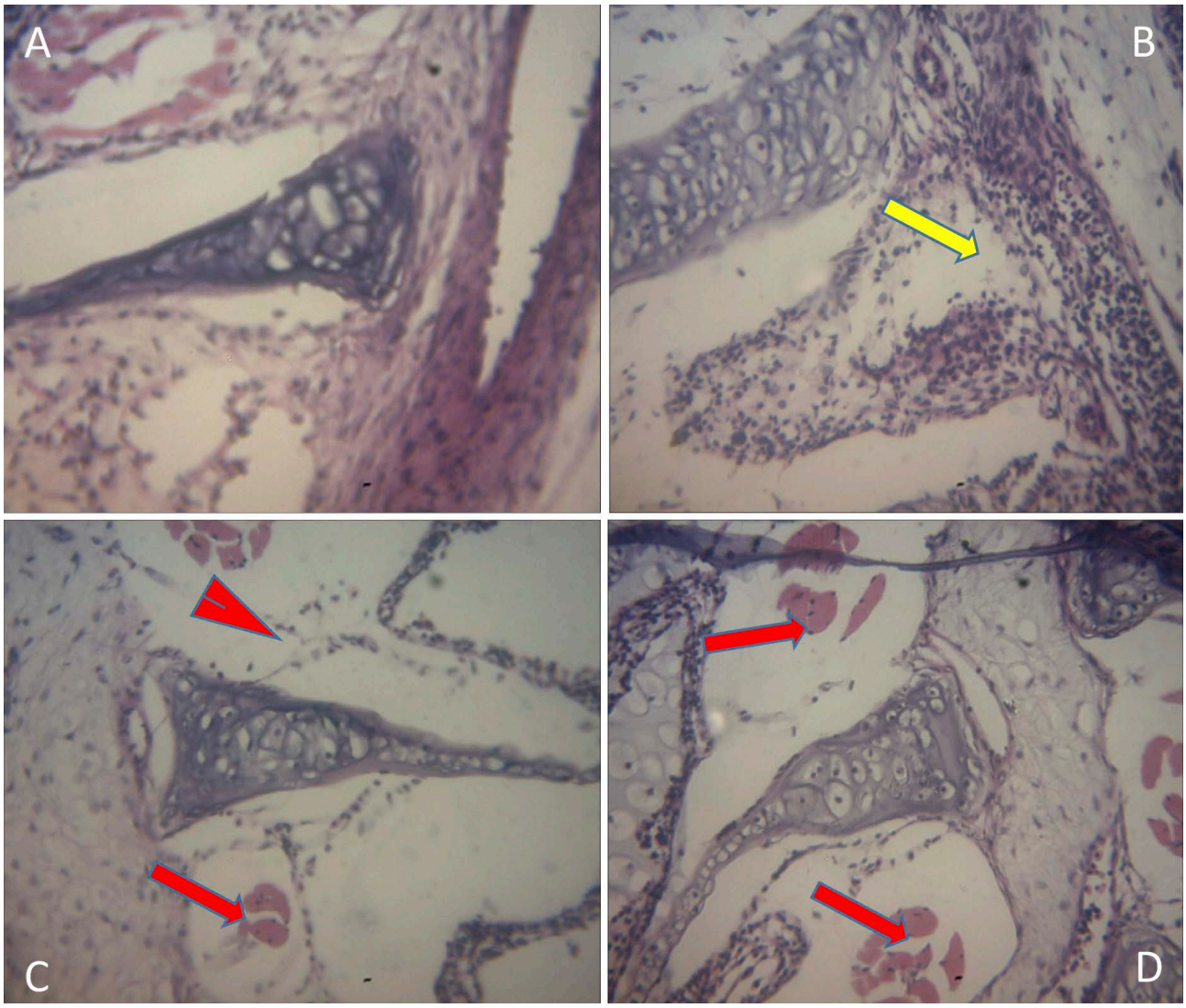

Fig. 7. Histopathology of the gills of *Clarias gariepinus* juveniles following sub-chronic exposure to graded concentrations of the *Lagenaria breviflora* whole fruit methanol extract; H&Ex400; A (Control) – (Normal), B (6.25mg/L) – Mild degeneration of the lamella core and oedema (yellow arrow), C (12.5mg/L) – Moderate congestion of the submucosa (red arrow) and expanded lamella connective tissue (arrow head), D (25mg/L) – Moderate congestion of the submucosa (red arrow)

Ethical Approval

The study adhered to the ethical guidelines for animal research and was approved by the University of Ibadan's Animal Care, Use, and Research Ethics Committee (Approval No.: NHREC/ UIACUREC/08/12/2024).

Conflict of Interest

There is no competing interest to declare concerning this research work.

Availability of Data and Materials

The data that support the findings of this study are available on request from the corresponding author.

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Generative AI Statement

This manuscript does not involve any generative AI.

Authors' Contributions

Motivation / Concept: OA
Design: OA, OR
Control/Supervision: OA, OR, GA
Data Collection and /or Processing: SA
Analysis and /or Interpretation: OA, OR, SA, TA, BO
Literature Review: SA, TA, BO, GA
Writing the Article: SA, TA, BO, GA
Critical Review: OR, OA

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ORIGINAL ARTICLE

BONE INDICES AND MINERAL INTERACTION IN ORGANS AND BONES OF BROILER CHICKENS FED DIET SUPPLEMENTED DIFFERENT ZINC TYPES

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Ethical considerations: When reporting experiments on animals Observation of the ARRIVE guidelines 2.0: Updated guidelines for reporting animal research, published on July 14, 2020 (DOI: 10.1371/journal.pbio.3000410), is applied. The authors ensure that all procedures were performed in compliance with the guidelines for animal care of their institutions or with national/international guidelines.

ABSTRACT

A 42-day trial was carried out to assess the influence of dietary supplementation of three zinc types and their combinations on bone indices and interaction with other minerals in the tissues and bones of broiler chickens. Two hundred and sixteen (216) Arbor Acres day-old broiler chicks were divided into six groups to consist of 36 birds and assigned to six treatments, which include zinc-methionine (Zn-met), zinc-oxide (ZnO), nano zinc (NZn), NZn + Zn-met, NZn + ZnO and control (antibiotics). Each treatment was replicated thrice with 12 birds. Data collected were subjected to one-way analysis of variance. Phosphorus was the only mineral that varied in the liver. Heart and meat mineral content were not significantly (P > 0.05) affected except for zinc and phosphorus respectively. Supplementation of nano-zinc alone increased bone weight, and bone robusticity was higher generally in birds fed diets fortified with zinc. Zn retention was markedly reduced in birds supplemented with Zn-met and NZn + ZnO. Sole supplementation of nano-zinc significantly elevated phosphorus (37.96 mg/dl) in bones, while Cu content was higher in all zinc-supplemented groups except the ZnO group. This study concludes that nano-zinc supplementation increased tibia bone length and weight, while bone ash and calcium were not affected by different zinc types and their combinations.

Keywords: antibiotics; bone morphometry; mineral retention; zinc types; zinc supplementation

INTRODUCTION

Broiler health is a major concern arising from its impact on their growth, welfare, and productivity. Skeletal issue is another common problem in broilers stemming from rapid growth rates exceeding skeletal development, nutritional imbalance, or infectious agents. Birds with deformed limbs have walking problems, and their sitting on the bedding also affects the quality of meat and the profitability of poultry production [1]. In addition to this situation, broiler chickens with weak legs have worse performance because they have reduced feed intake [2]. This often leads to problems of bone breakage during handling and transportation and during processing at slaughterhouse [3].

To address some of these problems, there has been increased research into the adoption of various alternatives to synthetic drugs, such as antibiotics, to minimize the risk associated with their use on both broilers and consumers. The use of minerals such as zinc, calcium, copper, selenium, etc., has been used for the improvement of broiler health and productivity.

Zinc, a nutritionally indispensable trace element, plays three major biological roles in the body: as a catalyst, and as a structural and regulatory ion [4]. It has a catalytic, coactive, or structural role in a wide variety of enzymes that regulate many physiological processes, including metabolism and growth [5]. Burrell et al. [6] opined that the National Research Council (NRC) recommendation of 40 mg/kg of zinc in broiler diets may have only considered growth performance as a criterion. However, higher levels of Zn may need to be supplemented during the entire period of production but not exceeding 70 mg/kg [7]. It is well known that a wide range of metals, including zinc, possess antimicrobial activity [8].

Different forms of zinc supplementation, including inorganic (e.g., zinc sulfate, zinc oxide), organic (e.g., zinc amino acid complexes), and nano-sized zinc, are used in broiler chicken diets to enhance mineral retention, bone development, and meat quality. Minerals from inorganic sources interact between mineral cations and other dietary components when fed to animals [9], leading to the formation of insoluble complexes (phytates) with much lower availability to animals. Organic or chelated forms of microminerals may have higher bioavailability than inorganic sources, mainly because of their lower interaction with other dietary constituents. Recently, ZnO nanoparticles (ZnNPs with sizes within 1–100 nm) are getting more attention for use in the nutrition of livestock to address dietary requirements and to promote animal growth due to the larger surface area, higher surface activity, catalytic efficiency, and stronger adsorbing quality compared with the conventional use of Zn [10].

Swain et al. [10] reported that minerals are transported directly to target organs and are easily absorbed and distributed into organs and tissues of broiler chickens. Also, bones function as a reserve of most of the trace minerals in broilers, including Zn; thus bone characteristics such as tibia bone index, bone strength, bone mineral concentration [11], and bone ash have often been used as sensitive indicators of bone status and response to both macro- and trace-mineral supplementation [12].

Overall, the strategic supplementation of different zinc forms and their combinations in broiler diets is crucial for optimizing mineral retention in organs, enhancing bone morphometry and mineralization, and improving meat quality, thus supporting productive performance and health in poultry production systems. Hence, this study was conducted to investigate the possible influence of the three aforementioned zinc types and their proportionate combinations in the diet on bone morphometry and mineral retention in some selected organs and bones of broiler chickens.

MATERIALS AND METHODS

Experimental site

The experiment was carried out at the Directorate of University Farms of the Federal University of Agriculture, Abeokuta, Ogun State, Nigeria. The farm area lies within latitude 7°13’49’’N, longitude 3°26’11.98’’E, and altitude 76 m above the sea level. The vegetation is located within the derived savannah zone of southwestern Nigeria. The area is characterised by a humid climate, a mean annual rainfall of 1037 mm and a temperature of about 30.4°C [13].

Source of test ingredients

The different zinc types (organic, inorganic, and nano-zinc) were acquired from a reputable pharmacy in Abeokuta, Ogun State. Feed-grade inorganic zinc oxide (95% purity and Zn content not less than 76.3%) was manufactured

by Guangdong Guanghua Sci-Tech Co., Ltd.; nano-zinc (100 nm) was produced by TATA Chemicals, India; and the zinc-methionine (contains 12% zinc and 27.3% methionine) was manufactured by Zinpro Corporation, USA.

Experimental design and management of birds

A total of 216 Arbor Acres day-old broiler chicks were acquired from a reliable hatchery. The pen was cleared and disinfected prior to the arrival of the birds. Birds were raised under intensive management using a deep litter system over a period of 42 days. The day-old chicks were allocated into 6 treatments, and each treatment group was subdivided into three (3) replicates with 12 birds each. The treatments include supplementation of 60 mg of nano-zinc (NZn), zinc oxide (ZnO), zinc methionine (Zn-met), NZn + ZnO (30 mg each), NZn + Zn-met (30 mg each) in the diets and the control. Brooding was carried out for 2 weeks with extended daylight (about 5 hours) provided for chicks to feed well and a heat source (charcoal pot) to maintain the environmental temperature suitable for chicks' growth. The birds were fed commercial diets at both starter (22% crude protein, 3000 Kcal/kg metabolisable energy, 5.0 g/kg crude fat, 4.3% crude fibre, 1.2% calcium,, 0.45% phoshorus, 0.56% methionine and 1.2% lysine) and finisher (18% crude protein, 3150 Kcal/kg metabolizable energy, 5.5 g/kg crude fat, 3.0% crude fibre, 1.2% calcium, 0.44% phoshorus, 0.5% methionine and 1.2% lysine). Medications (vitamins and coccidiostat) and vaccines (Gumboro and Lasota) were given as at when due during the study. Antibiotics (enrofloxacin) was applied in water for the control treatment only according to the manufacturer's recommendation, while various zinc types served as alternatives to antibiotics in the respective treatment groups.

Data collection

Mineral retention in blood, muscle, and organs

On the 42nd day, one bird was selected from each replicate. Blood samples (2 ml) were collected from each bird via jugular vein into plain bottles, after which the birds were slaughtered. A small piece of the breast muscle (5 g) and organs such as the liver, heart, and spleen were removed from each bird and stored in airtight plastic containers to determine mineral content [14]. The concentrations of Zn, Cu, P, and Ca were determined using Inductively Coupled Plasma-Mass Spectrometry (ICP-MS).

Bone morphometry

The left tibia bones of the slaughtered birds were collected, labeled, and soaked in boiling water (100°C) for 10 mins to facilitate removal of adhering muscles and connective tissues. The width of the bone was measured with vernier calipers, which was expressed in centimeters (cm). The tibia weight (g), tibia length (cm), and tibia robusticity index were obtained as described by Mutus et al. [15]. The bone index was obtained as described by Seedor et al. [16].

Tibia index=(Tibia weight)/(Tibia length)

Robusticity index=(Bone length)/((Bone weight)1/3

Bone mineral analysis

The left tibia bones were air-dried and taken to the laboratory for analysis of the ash content and some minerals (Ca, P, Cu, and Zn). Tibia samples were subjected to oven-drying at 105°C for 24 h, cooled in a desiccator, and weighed. Thereafter, bone samples were ashed in a muffle furnace at 600°C for 24 h in crucibles according to the method recommended by AOAC [17]. Ash content of the tibia and femur was expressed as a percentage of dry bone weight. For mineral content analysis, ash samples were subjected to digestion using a mixture of nitric (65% HNO_3) and perchloric ($HClO_4$) acid at 2:1 v/v [18]. Following digestion, samples were allowed to cool at room temperature. The content of calcium (Ca), phosphorus (P), copper (Cu), and zinc (Zn) in digested samples was determined as described by Skoog et al. [19] using atomic absorption spectrophotometry.

Data analysis

Data collected were subjected to one-way analysis of variance in a Completely Randomized Design using SPSS (version 23.0). Significant differences among means were separated using Duncan's multiple range test as contained in the software at a 5% level of significance ($P < 0.05$).

RESULTS

Effect of dietary supplementation of different zinc sources and their combinations on mineral retention in some organs and meat of broiler chickens

The effect of dietary supplementation of different zinc sources and combinations on mineral retention in some organs and meat of broiler chickens is shown in Table 1. Re-

sults showed there was no significant (P > 0.05) difference in mineral composition of the spleen. All mineral content of liver determined was not significantly (P > 0.05) affected except (P < 0.05) phosphorus. Phosphorus was least in the organic (2.77 mg/dl) zinc group and highest in birds fed a diet supplemented with 30 mg/kg each of nano + inorganic (4.23 mg/dl). Heart and meat mineral content were not significantly (P > 0.05) affected except for zinc (Zn) and phosphorus (P), respectively. The highest values for Zn were observed in birds fed a diet supplemented with organic (90.67 µg/dl) zinc, inorganic (87.00 µg/dl) zinc, and the control birds (84.27 µg/dl), while the least value was recorded in the nano + inorganic (52.47 µg/dl) group for heart. Phosphorus content for meat was highest in birds fed a diet supplemented with inorganic (3.80 mg/dl) zinc and organic zinc (3.53 mg/dl), while the least value was recorded at 60 mg/kg nano-zinc (2.53 mg/dl) group.

Effect of dietary supplementation of different zinc sources and their combinations on bone morphometry of broiler chickens

The effect of dietary supplementation of different zinc sources and combinations on bone morphometry of broiler chickens is shown in Table 2. The result revealed that different zinc supplementation in the diet of broiler chickens had a significant (P < 0.05) effect on the bone length, weight, and robusticity index. Highest values for bone length were recorded in nano and organic groups (10.09 cm and 10.01 cm, respectively), while the least value was recorded in inorganic (9.29 cm) group. The highest value for weight was observed in birds fed a diet supplemented with nano-zinc (7.47 g), and the least value was observed in birds fed a diet supplemented with inorganic (5.50 g) zinc. The robusticity index was highest in birds fed a diet supplemented with nano + inorganic (5.42 cm/g) zinc and the least recorded in control (5.01 cm/g) birds.

Table 1. Mineral composition in some organs and meat of broiler chickens fed diets supplemented with different zinc sources and their combinations

	Zinc types and combination supplementations							
Parameters	**NZn**	**Zn–met**	**ZnO**	**NZn+ Zn-met**	**NZn + ZnO**	**Control**	**SEM**	**P-value**
Spleen								
Ca (mg/dl)	12.63	12.97	11.70	12.20	12.20	12.40	0.21	0.67
Zn (µg/dl)	63.03	84.37	71.17	71.07	85.20	76.17	3.02	0.24
Cu (µg/dl)	238.73	258.33	214.10	235.40	307.90	272.77	15.12	0.61
P (mg/dl)	4.13	3.93	3.50	3.53	3.13	3.70	0.16	0.63
Liver								
Ca (mg/dl)	11.20	10.33	10.93	12.13	17.77	11.70	1.01	0.32
Zn (µg/dl)	79.13	92.83	64.93	86.90	66.63	79.63	4.86	0.57
Cu (µg/dl)	161.80	187.57	187.57	212.17	244.23	200.57	13.34	0.67
P (mg/dl)	3.40^{ab}	2.77^{b}	3.67^{ab}	3.93^{ab}	4.23^{a}	3.37^{ab}	0.17	0.03
Heart								
Ca (mg/dl)	11.50	10.80	10.60	12.17	11.7	11.77	0.22	0.32
Zn (µg/dl)	78.93^{ab}	90.67^{a}	87.00^{a}	74.43^{ab}	52.47^{b}	84.27^{a}	4.04	0.04
Cu (µg/dl)	210.50	187.57	187.57	281.60	193.93	207.73	12.69	0.25
P (mg/dl)	3.23	3.67	4.07	4.10	4.27	3.40	0.15	0.26
Meat								
Ca (mg/dl)	10.60	11.07	10.40	10.70	10.70	10.63	0.18	0.97
Zn (µg/dl)	79.57	88.47	85.97	64.30	76.77	74.60	4.44	0.73
Cu (µg/dl)	181.20	182.83	185.90	203.87	210.00	172.33	7.34	0.73
P (mg/dl)	2.53^{c}	3.53^{a}	3.80^{a}	3.40^{ab}	3.47^{ab}	2.77^{bc}	0.13	0.02

^{abc}Means within a row with different superscripts differ significantly (P < 0.05)

Effect of dietary supplementation of different zinc sources and their combinations on bone minerals of broiler chickens

The effect of dietary supplementation of different zinc sources and combinations on bone minerals of broiler chickens is shown in Table 3. All parameters measured were significantly ($P < 0.05$) affected except ($P > 0.05$) calcium and ash. Zinc (Zn) and phosphorus (P) content were least in the organic (28.25 and 0.86 mg/100mg) group, respectively. The highest P content was observed in nano (37.96 mg/100mg) group, and the highest Zn content recorded in inorganic (1.62 mg/100mg) group. The highest Cu was recorded in birds fed a diet supplemented with 30 mg/kg each of nano + organic (0.63 mg/100mg) and birds in the organic (0.62 mg/100mg) group. The least Cu was observed with birds in inorganic and control (0.47 and 0.49 mg/100mg, respectively) groups.

DISCUSSION

Zn concentration in organs and tissues is often used as an indicator of the Zn status and storage in the body of animals as well as the level of Zn consumed by birds, because low levels are considered an early symptom of zinc deficiency [20]. Swain et al. [10] reported that minerals are transported directly to target organs and are easily absorbed and distributed into organs and tissues of broiler chickens. Mineral retention, particularly of calcium and copper was not significantly influenced in all organs and meat assessed in this study. This suggests that zinc types and supplementation did not interfere with calcium and copper availability, absorption, and retention in broiler chickens. In a related study, Ayoola et al. [21] reported similar Ca retention in organs of broiler chickens fed diets supplemented with different levels of nano-zinc.

Zinc retention only varied in the heart, and the variation did not give a clear picture of the influence of zinc supplementation because the control with no zinc supplementation had similar retention as some of those groups supplemented. In a similar study, Zhang et al. [22] recorded no significance in zinc retention in the liver of broiler chickens when zinc supplementation was varied between 0 and 120 g/kg. They added that the variation recorded in the breast muscle was not consistent with the dosages of supplementation at both 21 and 42 days of age.

Birds provided a diet with Zn-met had the least retention, whereas birds fed a mixture of nano-zinc and ZnO showed better phosphorus retention in the liver. Furthermore, compared to the use of nano-zinc, phosphorus re-

Table 2. Bone morphometry of broiler chickens fed diets supplemented with different zinc sources and their combinations

	Zinc types and combination supplementations							
Parameters	**NZn**	**Zn–met**	**ZnO**	**NZn+ Zn-met**	**NZn+ ZnO**	**Control**	**SEM**	**P-value**
Bone length (cm)	10.09^{a}	10.01^{a}	9.29^{b}	9.84^{ab}	9.87^{ab}	9.68^{ab}	0.10	0.02
Width (cm)	0.78	0.71	0.70	0.70	0.66	0.79	0.02	0.20
Weight (g)	7.47^{a}	7.13^{ab}	5.50^{b}	6.47^{ab}	6.07^{ab}	7.20^{ab}	0.25	0.02
Total bone index (g/cm)	0.74	0.71	0.59	0.66	0.61	0.74	0.02	0.16
Robusticity index (cm/g)	5.17^{ab}	5.21^{ab}	5.25^{ab}	5.29^{ab}	5.42^{a}	5.01^{b}	0.15	0.02

^{ab}Means within a row with different superscripts differ significantly($P<0.05$)

Table 3. Bone minerals (Ca, P, Cu and Zn) of broiler chickens fed diet supplemented with different zinc sources and their combinations

	Zinc types and combination supplementations							
Parameters	**NZn**	**Zn–met**	**ZnO**	**NZn+ Zn-met**	**NZn+ ZnO**	**Control**	**SEM**	**P-value**
Calcium (mg/100mg)	114.67	111.64	118.98	103.37	118.01	121.35	2.35	0.28
Phosphorus(mg/100mg)	37.96^{a}	28.25^{b}	31.55^{ab}	31.29^{ab}	28.25^{b}	30.67^{ab}	1.17	0.04
Zinc (mg/100mg)	1.17^{ab}	1.01^{b}	1.62^{a}	1.20^{ab}	0.86^{b}	1.17^{ab}	0.08	0.02
Copper (mg/100mg)	0.55^{ab}	0.62^{a}	0.47^{b}	0.63^{a}	0.51^{ab}	0.49^{b}	0.02	0.03
Ash (g/100g)	28.3	27.67	27.6	27.47	26.1	27.79	0.78	0.99

^{ab}Means within a row with different superscripts differ significantly ($P < 0.05$)

tention in meat showed better retention when conventional sources of zinc (ZnO and Zn-met) were utilized alone or in conjunction with Zn nanoparticles. According to this study, zinc has a crucial role in enhancing phosphorus absorption, distribution, and retention in tissues and organs. The noteworthy zinc result from the heart indicates a reduction in or lack of the antagonistic action between divalent metals, allowing zinc to have a fuller synergetic, beneficial influence on mineral absorption and meeting the zinc needs of broilers [23].

Bone characteristics like tibia bone index, bone strength, bone mineral concentration, and bone ash have frequently been used as sensitive indicators of bone status and response to both macro- and trace-mineral supplementation. Bones function as a reserve of most of the trace minerals in broilers, including Zn [12]. Additionally, a prominent sign of the adequate intake of minerals in a chicken diet is the quality of the bone [24].

In this study, dietary zinc supplementation resulted in an increase in tibia weight, length, and robusticity index, which is indicative of a moderate tibia bone width and density. According to Seedor et al. [16], a larger ratio of bone weight to bone length denotes greater bone density and enhanced bone strength. This ratio depicts the principle of bone mineralization [25]. The zinc supplementation in the form of nano-zinc-alone demonstrates its ability to increase tibia bone index due to improved bone length and weight, even without any significant differences in tibia bone density.

In comparison to birds given inorganic zinc supplements, the bioavailability of nano-zinc particles, organic zinc, and their mixtures was shown to be higher. This may be explained by the fact that different forms of zinc and their combinations were easily taken up by the digestive system and used to improve the bone length and weight of birds. Bone appears to be a functional store of zinc that could be quickly mobilized for any urgent need, as evidenced by the positive relationship between the robusticity index and weight and length. This suggests that different forms of zinc and their combinations supplemented in diet favour the formation of stronger bone and that Zn can be utilized when deficiency occurs [26].

The results of this study are consistent with the report of Cufadar et al. [27], which indicates that different sources of zinc exhibited similar results at various inclusion levels (20, 40, 60, 80, and 100 mg/kg) in diets, although tibia Ca and P were not negatively affected. The findings of this study contradict those of Idowu et al. [28], which reported a significant increase in Ca concentration in the tibia of birds supplemented with conventional (organic and inorganic) zinc sources. According to Ao et al. [23], increased Zn binding affinity for Ca would limit its retention in the tibia. This claim contradicts this study's findings because the tibia's calcium retention was not. Zinc sulphate and zinc methionine chelate-supplemented birds showed notably greater zinc reserves, which is compatible with the much higher zinc deposition in the tibia seen in these birds [29].

The bone ash found in this investigation is consistent with the findings of Kumar et al. [30], who found no significant difference in the percentage of tibia ash between various zinc sources (ZnS, ZnM, and HME 100) given at the same amount. Additionally, Sahraei et al. [24] found that when conventional (organic and inorganic) sources were added to broiler chickens' diet at three dosage levels (100, 150, and 200 mg/kg), the zinc concentration in the tibia ash did not change. This is explained by the fact that zinc (Zn) is a necessary component of the enzyme system that produces vitamin D3, which controls the retention of tibia ash and the absorption of minerals (P, Cu, and Ca) in broiler chickens [31].

CONCLUSIONS

This study concludes that different zinc types and their combinations did not influence zinc retention in organs except the heart, with no consistency. Phosphorus abundance, however, varied significantly in liver and meat. Bone length and weight were increased by nano-zinc supplementation, while bone ash and calcium were not affected by different zinc types and their combinations. Hence, any of the zinc types studied can be safely incorporated at the considered dosage into the diet of broiler chickens without any negative implication on mineral retention in organs. Also, nano zinc supplementation has a superior tendency to improve bone length and weight, which could possibly enhance better bone strength and minimize the chances of skeletal problems in broiler chickens.

Data Availability Statement

The raw data of this article will be made available by the authors, without undue reservation.

Ethical Statement

The research was approved by the Animal Ethics and Welfare Committee of the College of Animal Science and Livestock Production, Federal University of Agriculture, Abeokuta. The Approval Reference number is FUNAAB/COLANIM/AEWC/2025/002.

Conflict of Interest

There is no conflict of interest whatsoever as regards this study among the authors.

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Generative AI Statement

There was no use of generative AI in the writing of this manuscript.

Authors' Contributions

The idea of this research was originally conceived and mapped out by Ayoola Akeem A. and developed by all authors. Ajayi Oluwatosin D. and Oke Oluwaseun C. collected the field data. Ekunseitan Deji A. and Sanda Adeyinka J. assisted with analysis of data and interpretation of results. Ayoola Akeem A. and Oluwatubosun Olawale B. were involved in the preparation of the manuscript for publication.

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ORIGINAL ARTICLE

HUSBANDRY PRACTICES, BIOSECURITY MEASURES OF PIG FARMERS AND THEIR AWARENESS OF TRANSMISSIBLE GASTROENTERITIS IN KWARA AND OYO STATES, NIGERIA

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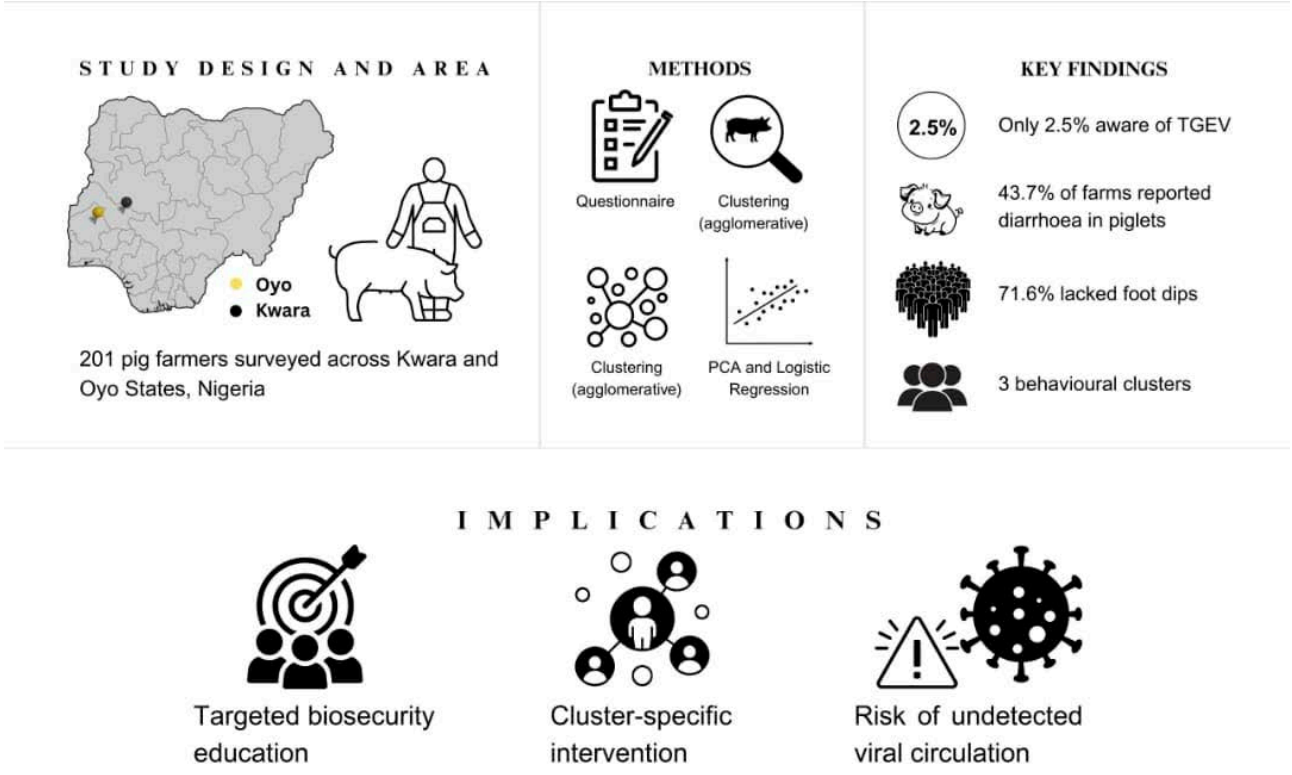
Ethical considerations: When reporting experiments on animals Observation of the ARRIVE guidelines 2.0: Updated guidelines for reporting animal research, published on July 14, 2020 (DOI: 10.1371/journal.pbio.3000410), is applied. The authors ensure that all procedures were performed in compliance with the guidelines for animal care of their institutions or with national/international guidelines.

ABSTRACT

Transmissible gastroenteritis (TGE) is a highly contagious enteric viral disease of swine with serious implications for pig health and productivity. However, data on its occurrence and impact in Nigeria remain scarce. This study assessed pig farmers' awareness of TGE, alongside husbandry and biosecurity practices, across 201 farms in Kwara and Oyo States. Structured questionnaires and on-site observations were analyzed using descriptive statistics, agglomerative hierarchical clustering, principal component analysis (PCA), and logistic regression. Awareness was strikingly low—only 2.5% of respondents had heard of TGE, and 12.9% understood biosecurity. Essential infrastructure was lacking: 71.6% of farms had no foot dips, and 73.1% lacked staff access control. Although just 5.5% of pigs reportedly showed diarrhoea alone, 57.7% presented diarrhoea with other symptoms, and 43.7% of farmers observed signs in piglets aged 0–3 months, suggesting underreporting and possible silent circulation. Cluster analysis defined three farm profiles: Cluster 1 (13.9%) with strong compliance, Cluster 0 (73.1%) with basic hygiene but no personnel control, and Cluster 2 (12.9%) with minimal adherence. PCA reinforced these divisions, highlighting foot dips, staff access, and segregation as key drivers. Findings expose critical gaps in biosecurity and awareness, emphasizing the need for targeted education, veterinary outreach, and surveillance to strengthen Nigeria's pig sector.

Keywords: clustering analysis; husbandry practices; Nigeria; PCA; pig farming; targeted biosecurity; TGE awareness; transmissible gastroenteritis (TGE)

GRAPHICAL ABSTRACT



INTRODUCTION

Transmissible gastroenteritis (TGE) is an acute, rapidly spreading viral disease of pigs of all ages characterised by profuse watery diarrhoea, vomiting, dehydration, and high mortality in piglets less than two weeks old. It is caused by the TGE virus (TGEV), which is a member of the family *Coronaviridae*, genus *Alphacoronavirus,* and species *Alphacoronavirus-1*, and constitutes a threat to the pork industry worldwide [1–3]. In addition to pigs, infections have been documented in foxes, dogs, and cats [4]. As an enteric pathogen, TGEV predominantly targets and affects the villous epithelial cells of the small intestine, disrupting normal absorption mechanisms and causing severe diarrhoea and dehydration in infected pigs [5]. Infection has been reported to occur through faecal-oral, respiratory (aerosol), and vertical transmission via milk from infected sows, with these three routes of spread for TGEV [2, 6]. Apart from direct contact with infected pigs, TGEV is also spread via contaminated fomites such as feeding equipment, clothing, or vehicles, and mechanical vectors, including farm personnel, rodents, and birds [7].

Due to the multiple transmission pathways, biosecurity remains the fundamental element in the prevention and control of Transmissible Gastroenteritis Virus (TGEV). The implementation of rigorous biosecurity measures, such as restricting access to the farm, disinfecting equipment, isolating new or ill animals, and upholding high standards of sanitation, effectively reduces the introduction and dissemination of viruses, thereby protecting both animal and public health [8–10]. Additionally, robust farm biosecurity fortifies the resilience of livestock systems against emerging and re-emerging diseases and promotes sustainable pig production [11]. In Africa, the epidemiology and impacts of TGE remain poorly defined and less well understood [8]. Apart from reports of its identification based on clinical history, post-mortem pathology, virus isolation and/or serology (neutralization test, enzyme-linked immunosorbent assay) in Kenya [9, 10], Uganda [11], South Africa [12], Central African Republic, Rwanda and Togo [13], and Nigeria [14], there is limited information on its occurrence in Africa. In regions with intensive pig production, particularly in Western and Southern Africa [15], the potential for TGE outbreaks is higher due to increased close contact among pigs on farms.

Prior to the report of Aiki-Raji et al. [15], there was no information on the prevalence and impact of TGE in Nigeria. In a study conducted on pigs sampled from livestock

farms and abattoirs in Ogun and Oyo States, southwest Nigeria, these researchers found a higher TGE seroprevalence in abattoir pigs (33.3%) than in farm pigs (27.7%). Since pigs are not vaccinated against TGEV in Nigeria, the detection of TGEV antibodies in pig sera could be attributed to natural exposure to the virus. This study was therefore carried out to evaluate the husbandry practices and biosecurity measures of pig farmers, as well as their knowledge of TGE in Kwara and Oyo States of Nigeria. It is anticipated that the findings will offer valuable insights into the current understanding and preventative practices regarding TGE in these two states and will serve as a foundation for developing strategies for effective disease control.

MATERIAL AND METHODS

Study design

A cross-sectional study was conducted between April 22 and June 9, 2024, in Kwara and Oyo States, two of the largest pig-producing states in Nigeria [15]. These states were purposively selected due to their significant pig farming activity, contrasting agro-ecological zones, high pig population density, and strategic economic importance in the swine value chain.

Participant selection and sampling criteria

Participants were adult pig farmers (≥ 20 years) engaged in continuous commercial pig farming. Eligible farms were purposively selected based on the following criteria:

(i) a minimum herd size of 50 pigs,
(ii) ongoing commercial operation,
(iii) willingness to grant access for field assessment, and
(iv) logistical accessibility.

Although being in pig farming for a minimum of 5 years was originally intended as a criterion, actual respondent profiles varied, and this was retained during analysis to reflect real-world situations.

Survey instrument, reliability assessment, and questionnaire design

A semi-structured questionnaire was administered via both Google Forms and in-person interviews. It included four sections:

(i) Demographics: location, gender, age, years of experience in pig farming, level of education, herd size, staff employed, and management type, amongst others.
(ii) Husbandry practices: age and source of pigs and type of feed used.
(iii) Biosecurity measures.
(iv) Knowledge of TGE.

The questionnaire was reviewed by three independent experts (a veterinarian, a research scientist, and an extension officer) to ensure content validity. A pilot test with 20 farmers (excluded from the main study) refined question clarity. Cronbach's alpha ($\alpha = 0.80$) confirmed acceptable internal reliability for biosecurity and TGE knowledge items, which depict the awareness scale items primarily based on binary and Likert-type responses. This indicates an adequate level of internal reliability and is considered sufficient for exploratory public health research, particularly in field-based cross-sectional studies involving behavioural and awareness constructs [16]. The final version of the questionnaire is available as a supplementary file and through the following link:

https://docs.google.com/forms/d/e/1FAIpQLSeYuWyx8GMzo92gH-AVZ7x0VltotxVj7-Ibgn740xHthbO9Hg/viewform

Data analysis

Descriptive statistics (frequencies, percentages, central tendencies) were used to summarise demographic and practice-related data using Microsoft Excel and the IBM® SPSS® v17 software package for statistical analysis (Pennsylvania, USA). This descriptive methodology was adopted, as previously reported [17], to assess the knowledge of TGE, farmers' perceptions of the disease, their husbandry and management practices, understanding of routine biosecurity measures, and common TGE symptoms. To identify latent patterns in farm behaviour, unsupervised machine learning (agglomerative clustering with $k = 3$) was applied to biosecurity behaviour patterns among respondents. The optimal cluster count was determined using the silhouette score compared with K-means. Clusters were profiled to reflect varying levels of awareness and implementation. Chi-square testing was conducted to assess associations between cluster membership and geographic location. Finally, logistic regression models were built using Python (statsmodels v0.14.4) to assess predictors of (a) absence of foot dips (proxy for poor biosecurity) and (b) TGE aware-

ness. Results were reported as odds ratios (ORs), 95% confidence intervals (CIs), and p-values < 0.05.

RESULTS

Socio-demography

A total of 201 pig farmers (Kwara, n = 100; Oyo, n = 101) out of 120 contacted in each state participated, yielding an approximately 83% response rate per state. Most (71.6%, n = 144) were males aged 50–60 years (31.8%). Most (58.7%, n = 118) had secondary education, while 20.4% (n = 41) had no formal education. Despite a criterion of ≥ 5 years' experience, 74.1% had less than 5 years in pig farming (Table 1).

Husbandry practices

Most farms (68.2%) used intensive management, and 81.6% categorised pigs by age. Herd sizes varied; 34.2% had fewer than 20 pigs, while 25.4% had over 80. Nearly half (49.3%) used self-compounded feeds; others relied on local industry feeds like brewery wastes and kitchen scraps. Although 65.7% kept records, these were mostly basic, noting animal purchases or treatments (Table 2).

Biosecurity awareness and practices

Only 12.9% (n = 26) knew the term "biosecurity," but many practised cleaning (100%) and disinfection (84.3%). Structural biosecurity was weak: 71.6% lacked foot dips, 73.1% had no staff entry protocols, and 84.4% allowed visitors without strict controls. Only 14% enforced full protocols, and 34.8% routinely segregated animals (Table 3).

Clustering of biosecurity behaviours

Agglomerative hierarchical clustering was applied to seven standardized binary biosecurity indicators: foot dip, staff and visitor protocols, segregation, cleaning, disinfection, and hygiene measures. A three-cluster solution (k = 3) was selected based on silhouette scores (agglomerative = 0.53; K-means = 0.42), showing better cohesion and separation with the agglomerative method. The resulting clusters represented distinct profiles of biosecurity awareness and implementation:

Cluster 1 (13.9%): High-compliance farms implementing comprehensive protocols, including foot dips (92.9%), visitor control (85.7%), and staff routines (89.3%).

Cluster 0 (73.1%): Practised core hygiene (100% cleaning; 84.3% disinfection) but lacked staff (11.6%) and visitor controls (15.6%).

Table 1. Demographics of respondents

Variable	Category	Frequency (n)	Percentage (%)
Age group (years)	20–30	45	22.4
	31–40	23	11.4
	41–50	45	22.4
	51–60	64	31.8
	≥61	24	11.9
Gender	Male	144	71.6
	Female	57	28.4
Location	Oyo	101	50.2
	Kwara	100	49.8
Academic status (level of education)	No formal education	41	20.4
	Primary/Secondary education	118	58.7
	Tertiary education	42	20.9
Years of experience as a pig farmer (years)	1–3	76	37.8
	4–6	73	36.3
	7–9	23	11.4
	10–12	3	1.5
	13–15	11	5.5
	>15	15	7.5

Table 2. Husbandry practices associated with pig farming in Kwara and Oyo States

Variable	Category	Frequency (n)	Percentage (%)
Flock size (n = 196)	1–20	67	34.2
	21–40	22	11.2
	41–60	24	12.2
	61–80	33	16.8
	81–100	31	15.8
	101–120	10	5.1
	121–140	3	1.5
	141–160	0	0.0
	161–180	4	2.0
	181–200	2	1.0
Sectioning of pigs based on groups (n = 201)	Yes	164	81.6
	No	37	18.6
Record keeping (n = 201)	Yes	132	65.7
	No	69	34.3
Management practice (n = 201)	Intensive	137	68.2
	Semi-intensive	14	7.0
	Extensive	50	24.9
Source of pigs (n = 201)	Nigeria	198	98.5
	Imported	3	1.5
Age range (months) (n = 200)	Above 12 only	8	4.0
	6–12 only	34	17.0
	0–6, Above 12	12	6.0
	0–6, 6–12, Above 12	103	51.5
	0–6, 6–12	12	6.0
	0–6 only	31	15.5
Type of feed (n = 201)	Self-compounded, Local, Kitchen waste	31	15.4
	Self-compounded, Local	1	0.5
	Self-compounded only	99	49.3
	Others	1	0.5
	Local only	63	31.3
	Kitchen waste only	3	1.5
	Imported only	3	1.5

Cluster 2 (12.9%): Minimal adoption of recommended practices with poor foot dip use (3.8%) and complete absence of staff protocols.

A Principal Component Analysis (PCA) was performed on the standardised indicators to verify the cluster structure. The first two components (PC1 and PC2) explained 58.4% of the total variance. Clusters were distinguished in the PCA scatterplot (Fig. 1). A PCA loading heatmap (Fig. 2) indicated that foot dip, staff and visitor protocols, and segregation were the main contributors to PC1. A summary heatmap (Fig. 3) illustrates the proportion of farmers in each cluster who have adopted key biosecurity practices, while Table 4 presents the distribution of farmers across clusters by state.

Awareness of TGE and clinical observations

Awareness of TGE among surveyed pig farmers was remarkably low, with only 5 respondents (2.5%) recognizing clinical signs of the disease and correctly identifying it as a cause of diarrhoea in piglets. Despite this, clinical signs consistent with TGE were often reported. Specifically, 43.7% (n = 86) of farmers observed diarrhoea in piglets aged 0–3 months, the most vulnerable age group for severe disease, while 57.7% reported diarrhoea along with other

Table 3. Biosecurity practices associated with pig farming in Kwara and Oyo States

Variable	Category	Frequency (n)	Percentage (%)
Biosecurity awareness (n = 201)	Yes	26	12.9
	No	175	87.1
Presence of foot dip on farms (n = 201)	Yes	57	28.4
	No	144	71.6
Awareness of biosecurity protocols (n = 201)	Cleaning only	94	46.8
	Cleaning and disinfection only	16	8.0
	Disinfection only	57	28.4
	Segregation only	1	0.5
	Segregation, cleaning, disinfection	33	16.4
Biosecurity protocols practiced (n = 201)	Cleaning only	82	40.8
	Cleaning and disinfection only	39	19.4
	Disinfection only	48	23.9
	None	1	0.5
	Segregation cleaning only	1	0.5
	Segregation, cleaning, disinfection	30	14.9
Protocol for staff (n = 201)	Yes	54	26.9
	No	147	73.1

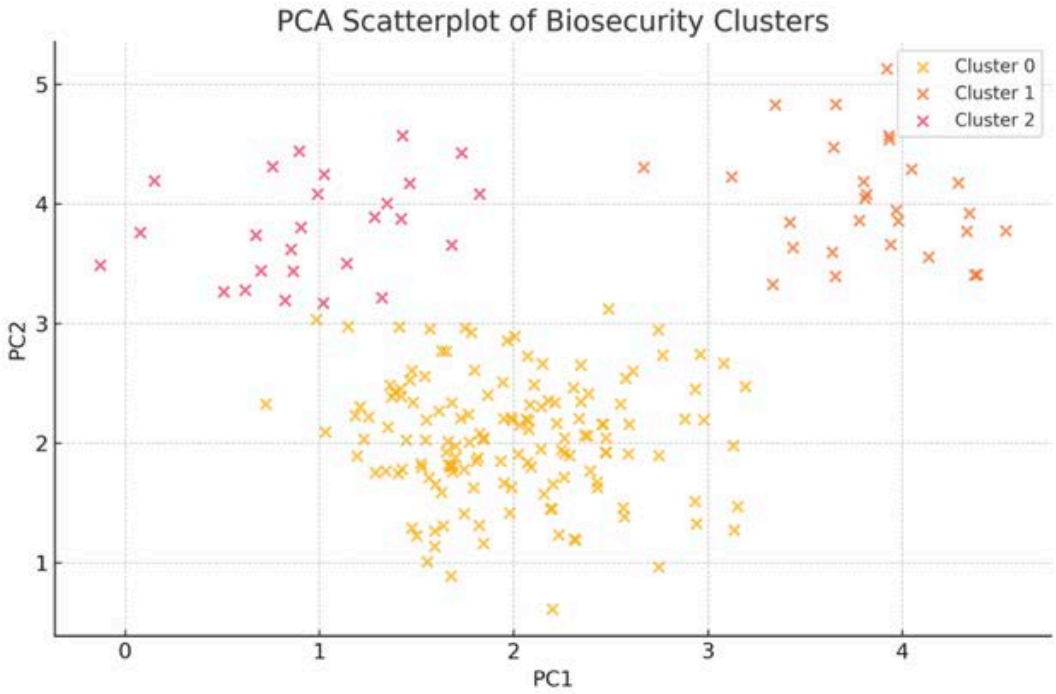


Fig. 1. Principal Component Analysis (PCA) scatterplot showing separation of farmers (n = 201) based on standardized biosecurity indicators. Each point represents a farmer and is colored by cluster membership (k = 3), illustrating clear cluster separation along the first two principal components (PC1 and PC2).

symptoms, and only 5.5% observed diarrhoea as an isolated sign. Logistic regression analysis showed that TGE awareness did not vary significantly based on age, gender, education level, location, or years of farming experience ($p > 0.3$ for all variables). Although awareness was marginally higher among farmers with primary or secondary education compared to those with no formal or tertiary education, this association was not statistically significant (OR: 1.43; 95% CI: 0.13–72.55). Similarly, male respondents had higher odds of being aware of TGE than females

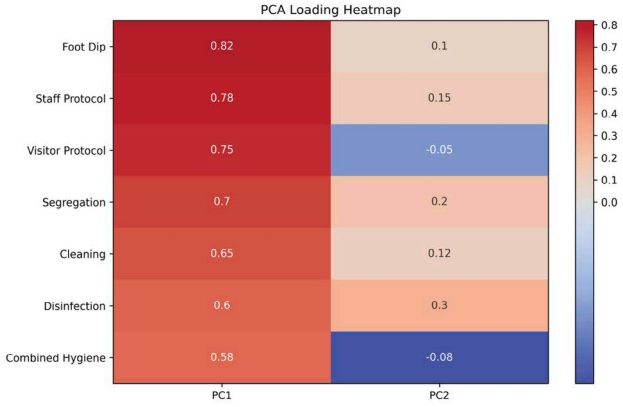


Fig. 2. PCA loading heatmap displaying the contribution of each biosecurity indicator to the first two principal components. Darker shades indicate higher loading values; foot dip, staff and visitor protocol, and segregation contributing most strongly to PC1.

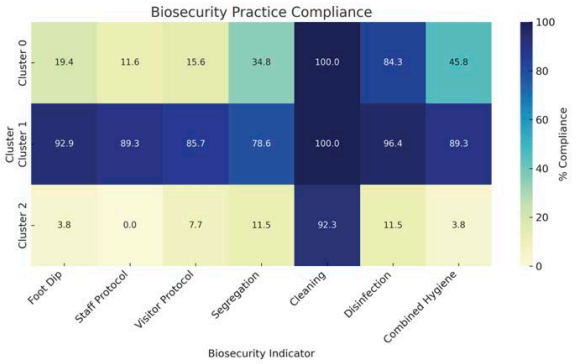


Fig. 3. Heatmap illustrating the proportion (%) of pig farmers within each biosecurity cluster demonstrating positive implementation of key biosecurity practices. Cluster 1 shows the highest compliance across all indicators; Cluster 2 exhibits minimal adherence.

Table 4. Distribution of pig farmers by biosecurity cluster and states

Cluster	Kwara State (n, %)	Oyo State (n, %)	Total (n, %)
0	77 (52.4)	70 (47.6)	147 (73.1)
1	11 (39.3)	17 (60.7)	28 (13.9)
2	11 (42.3)	15 (57.7)	26 (12.9)
Total	99 (49.3)	102 (50.7)	201 (100.0)

(OR: 1.60; 95% CI: 0.15–80.18), but this difference was also not significant (Table 5).

Regression analysis

Logistic regression identified predictors of poor biosecurity, defined as no foot dips. Significant factors were farm location, education, and farming experience. Farms in Kwara State had higher odds of lacking foot dips than those in Oyo (OR = 98.9; $p < 0.001$). Farmers with secondary education showed better compliance than those with less or more education. Longer farming experience (≥ 10 years) was linked to poor biosecurity, with such farmers being 7.53 times more likely to lack foot dips than those with ≤ 6years (OR = 7.53; $p = 0.026$) (Table 6).

DISCUSSION

In the present study, we assessed the husbandry practices and biosecurity measures of pig farmers as well as their knowledge of TGE in Kwara and Oyo States of Nigeria. The results provided several important insights into the current state of pig production in these states, highlighting significant gaps in husbandry and biosecurity practices, as well as knowledge of the disease.

The study population primarily consisted of male farmers (71.6%) aged 51–60 years, with most (58.7%) having only secondary education, while just 20.9% attended tertiary institutions. This demographic profile aligns with findings from other studies on small-scale pig farming in developing countries, which often show a prevalence of middle-aged to older male farmers with limited formal education [18, 19]. Our findings regarding these demographic characteristics (age group, gender, and years of experience and expertise) support those of other studies in Nigeria [20, 21] and Zambia [22]. The relatively low education level of these farmers may impact their ability to adopt modern animal husbandry practices and disease control measures, highlighting the importance of continuous education and training, as experience alone does not ensure compliance with evolving biosecurity standards.

Most farmers in this study had limited experience, with the majority having only 1–3 years of pig farming experience. This indicates potential recent growth in the pig farming sector in these regions, possibly driven by factors such as increased demand for pork, low start-up costs for pig farming, and government initiatives to promote livestock production. However, the limited experience of many farmers also points to a need for targeted training and support programs to enhance their farming practices and disease management skills. The study further shows that the majority (68.2%) of farmers practised the intensive production system, aligning with global trends [23]. While intensification can boost productivity and improve disease control, it presents various challenges in developing countries, especially regarding biosecurity and disease management, due to higher animal densities, frequent an-

Table 5. Awareness of TGE

Variable	Category	Frequency (n)	Percentage (%)
Awareness of TGEV (n = 201)	Yes	5	2.5
	No	196	97.5
Age range of diarrheal observed	0–3 months	86	43.7
	0–3 months, 4–6 months	20	10.2
	0–3 months, 4–6 months, 6 months and above	12	6.1
	0–3 months, 6 months and above	2	1.0
	4–6 months	50	25.4
	4–6 months, 6 months and above	11	5.6
	6 months and above	13	6.6
	Unknown	3	1.5
Awareness of TGEV as agent of diarrhoea	Yes	5	2.5
	No	196	97.5
Other common pig diseases noticed	Anthrax	1	2.9
	ASF only	12	35.3
	ASF and Foot Rot	11	32.4
	Cough	1	2.9
	Foot Rot only	3	8.8
	Undisclosed	6	17.6
Carcass disposal method	Burning	3	1.5
	Burying	117	58.2
	Burning, Burying	1	0.5
	Burying, Open dumping	4	2.0
	Incineration	22	10.9
	Open dumping	47	23.4
	Undisclosed	7	3.5

ASF = African Swine Fever virus

Table 6. Binary logistic regression for predictors of poor biosecurity (foot dip absence)

Predictor	Comparison	Odds Ratio (95% CI)	p-value
State	Kwara vs Oyo	98.9 (8.7–1126.0)	< 0.001**
Gender	Male vs. Female	1.28 (0.44–3.71)	0.655
Age group (years)	30–40 vs ≤ 30	1.83 (0.45–7.48)	0.400
	40–50 vs ≤ 30	1.34 (0.31–5.79)	0.695
	50–60 vs ≤ 30	2.52 (0.35–18.03)	0.357
	≥ 60 vs ≤ 30	1.76 (0.10–31.15)	0.701
Education level	Secondary education vs. No formal	0.22 (0.06–0.73)	0.013*
	Secondary education vs. Tertiary	0.24 (0.06–0.90)	0.035*
Farming experience (years)	7–9 vs ≤ 6	2.55 (0.54–11.99)	0.235
	≥ 10 vs ≤ 6	7.53 (1.28–44.36)	0.026*

*Significant at $p < 0.05$; **Highly significant at $p < 0.01$.

imal and human movement, and reliance on shared equipment [24, 25]. Our findings, which show that over 68% of farms were intensive but 73.1% lacked staff control protocols, highlight this contradiction — intensification is not accompanied by adequate biosecurity.

One of the most striking findings from this study is the poor knowledge of biosecurity among pig farmers, with only 12.9% (n = 26) being familiar with the term. These gaps were further explored through unsupervised clustering, which revealed three distinct farmer profiles based on

biosecurity awareness and implementation. Cluster 1 represented farmers with strong biosecurity knowledge and consistent practice, reflecting high compliance with recommended protocols. In contrast, Cluster 2 included those with minimal awareness and weak implementation, highlighting the highest-risk group. Cluster 0 encompassed farmers who engaged in moderate practices, such as cleaning and disinfection, but lacked comprehensive protocols, including visitor and staff controls. The absence of a clear regional pattern across clusters suggests that these biosecurity gaps are widespread rather than state-specific. Importantly, the presence of farmers who engaged in good practices without formal awareness highlights a disconnect between conceptual understanding and routine behaviour. This emphasises the need for training interventions that prioritise practical application over terminology, given the pivotal role of biosecurity in swine disease prevention and control [26, 27]. The lack of foot dips and protocols for staff in 71.6% and 73.1% of farms, respectively, underscores the significant gaps in biosecurity practices on those farms. These results are consistent with those of other workers who reported suboptimal biosecurity measures on small-scale pig farms in developing countries [25, 28].

Perhaps the most important finding of this study is the extremely low level (2.5%) of knowledge of TGE among the respondents. Our findings show that the majority of the sampled population (57.7%) observed diarrhoea and other related symptoms of TGE on their farms. However, the extremely low level of knowledge of TGE among the respondents, the presence of clinical signs consistent with the disease, and the low level of biosecurity suggest that the disease may be present but remains unrecognised; these patterns indicate that TGEV, or other enteric pathogens with similar presentations, may be circulating undetected on many farms. This aligns with studies in Kenya and Uganda, where a similar phenomenon was reported for enteric viral infections [19]. This low awareness level is worrisome, as TGE causes significant morbidity, mortality, and economic losses, particularly in piglets [29]. Further, it is consistent with the findings of other studies conducted in Nigeria and elsewhere [30–32] and highlights the need for increased education and awareness creation regarding TGE. The disease is endemic in the tropics, and biosecurity practices are the best way to ensure its spread is controlled, while unvaccinated piglets are protected from infection with the virus. With 87.1% of respondents being unaware of the concept of biosecurity, the likelihood of TGEV spreading in southwest Nigeria is very high. The persistence of diarrhoea across multiple age groups, especially in young pigs, is a warning sign that needs further investigation. Although various pathogens can cause diarrhoea, the age distribution and clinical signs observed in this study are consistent with TGEV infection [33, 34].

LIMITATIONS

Since this study was conducted in only two states of Nigeria, the results may not be a true reflection of the situation in other parts of the country. However, considering that these are two of the largest pig-producing states in Nigeria [15], further research involving other states is recommended to provide a more comprehensive picture of TGE knowledge and pig farming practices across the country. Furthermore, laboratory confirmation of TGE occurrence in farms reporting clinical symptoms suggestive of the disease would provide valuable epidemiological data that can influence the development of effective prevention and control strategies against the disease.

RECOMMENDATIONS AND CONCLUSIONS

The results of this study highlight the urgent need for education and awareness programmes that focus on TGE among pig farmers in Kwara and Oyo States of Nigeria. The observed gap between the low-level awareness of TGE and the reported clinical symptoms associated with the disease suggests that TGE may be underdiagnosed/misdiagnosed and possibly endemic in these states. Furthermore, there is a clear need to improve biosecurity practices on the studied pig farms. While some farmers had introduced basic biosecurity measures, comprehensive and standardized protocols remain lacking.

Recommended biosecurity measures include:

1. Installation and regular use of foot dips/baths at entrances to pig pens.
2. Provision of aprons or protective gear for farm visitors.
3. Implementation of compulsory showers before entry into animal production facilities.
4. Establishment of formalized, consistent biosecurity protocols across farms.

5. Educational initiatives should emphasize not only best practices but also how to translate them into routine, enforceable procedures.
6. Tailored biosecurity programmes should be developed based on the farmer profiles identified via the clustering analysis:

- Cluster 1 (Informed and Compliant Farmers): Sustain momentum through periodic refresher trainings and updated biosecurity guidelines. These farmers can be engaged as peer educators or local biosecurity champions. Innovative tools such as mobile-based disease reporting systems and standard operating procedure (SOP) checklists should be piloted within this group to strengthen monitoring and compliance.
- Cluster 0 (Cautious but Under-informed Farmers): Implement targeted awareness campaigns that build on existing practices, such as routine cleaning. Practical training sessions and simplified SOP templates should be introduced to help formalize and expand current hygiene behaviors into full biosecurity protocols.
- Cluster 2 (High-Risk and Unaware Farmers): Prioritize basic biosecurity education using grassroots strategies, including local language radio messages, religious institutions, and farmer cooperatives. Provide starter hygiene kits (e.g., foot dips, protective wear), integrate training with feed and input delivery systems, and assign trained extension officers for direct field engagement.

7. The use of machine learning techniques in this study shows promise for targeted extension interventions and data-driven swine health planning.
8. The dominance of local pig sourcing highlights the importance of establishing robust national disease surveillance and control programmes.
9. Collaboration between pig farmers, state veterinary services, and the Federal Ministry of Agriculture and Food Security will be crucial for the successful implementation of any surveillance and control initiatives.
10. Effective TGE prevention and control will require the development and enforcement of standard protocols aimed at preventing the introduction and spread of the disease at the farm level.
11. Given that TGEV is an RNA virus prone to genetic recombination, resulting in both high- and low-pathogenicity field strains [35–37], active surveillance will be essential to determine the disease's endemicity and the nature of circulating strains.
12. This surveillance will inform the establishment of effective biosecurity guidelines, especially among breeders and high-risk farms.
13. As supported by MacLachlan & Dubovi [38], TGE control protocols should include depopulation of affected herds during severe outbreaks, isolation of diseased animals to prevent transmission, routine cleaning and disinfection of facilities and equipment, strict enforcement of biosecurity measures across all pig farming operations, and vaccination, which remains the most effective preventive strategy for TGE [39]. The availability and usage of vaccination should be prioritized within national control frameworks.

Data Availability Statement

Repository name: Mendeley Data
Direct URL to data: doi: 10.17632/t8fv4bgcxn.1

Ethical Statement

Ethical clearance was obtained from the Kwara State Ministry of Agriculture and Rural Development (Approval number: ERC/MARD/2024/09/001) and the Oyo State Ministry of Agriculture (Approval number: VD 922/29). Informed consent was secured from all participants in accordance with the World Medical Association Declaration of Helsinki Ethical Principles (WMA, 2015) and the National Health Research Ethics Committee, Nigeria (NHREC, 2014).

Conflict of Interest

The authors have no conflict of interest to declare.

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Authors' Contributions

Conceptualization: BIO, HOT, JOA, DOO; Methodology: BIO, JOA, AOA; Field data and Sampling: BIO, IDO, OBT, TJA, AJT; Data analysis: BIO, IDO, AH; Resources

and Supervision: BIO, JOA, DOO; Writing-original draft preparation: BIO, AOA, IDO, AH, OJI, FAA, MAA, OBT; Writing-review and Editing: BIO, AOA, AH, HKA; Final Edit: BIO, HKA, DOO.

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Appendix

Supplementary materials.

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ORIGINAL ARTICLE

CIRCANNUAL CYCLE OF THE SERTOLI CELL NUCLEOLUS IN ROE DEER, *CAPREOLUS CAPREOLUS*, AND SEASONAL CHANGES OF ITS LEYDIG CELLS: AN ULTRASTRUCTURAL STUDY

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ABSTRACT

Ruminant Sertoli cells, instead of a nucleolus with nucleolonema, have a vesicular nucleolus, first described as a multivesicular nuclear body (MNB). It consists of membrane vesicles and tubules covered with ribosome-like granules. We studied the testes of several domestic and wild ruminants using transmission electron microscopy. In domestic ruminants with continuous spermatogenesis, the MNB occurs throughout the year. In roe deer, the seasonal breeder, the most developed MNB is during the rut. During testicular rest, Sertoli cells have a nucleolus without vesicles. The cycle of Sertoli cells nucleolus is related to the onset and cessation of spermatogenesis and cyclical changes in Leydig cell, which include their regression, apoptosis and autophagy. Before and during spermatogenesis, Leydig cells have all the features of steroid-producing cells. At the end of the rut, the amount of smooth endoplasmic reticulum (SER) decreases rapidly and lipid droplets and glycogen appear in their cytoplasm. In January, small inactive Leydig cells contain lipid droplets, remnants of SER and glycogen. Our observations indicate that the MNB performs a major function of the nucleolus – a key role in ribosome biogenesis. Why membrane vesicles are present in the MNB, and what their role is in ruminant Sertoli cells, remains a mystery more than 50 years after its discovery.

Key words: Leydig cell; roe deer; Sertoli cell nucleolus cycle; ultrastructure

INTRODUCTION

Sertoli cell, also termed sustentacular, supporting, or "nurse" cell, *Sustentocytus testis,* also *Epitheliocytus sustentans,* is a unique cell. It has more functions than any other among over 200 cell types of the body does, making it a unique cell necessary for spermatogenesis [1, 2, 3, 4, 5, 6]. Apart from support, protection and nutrition of

developing germ cells via various signal pathways [3, 7, 8], Sertoli cells have other functions crucial for spermatogenesis [5, 9, 10, 11]. They form a blood-testis barrier – BTB [12, 13, 14, 15, 16, 17, 18], and transport nutrients and many regulatory factors through it to developing germ cells and hereby create a specific environment necessary for spermatogenesis [2, 3, 19, 20]. The BTB physically divides the seminiferous epithelium into basal and apical (or adluminal) compartments [13, 15, 16, 17] and is pivotal to spermatogenesis [2, 3, 20, 21]. Sertoli cells communicate with each other via gap junctions [22, 23, 24, 25]. Sertoli cells are phagocytic [26], they remove residual bodies [27, 28], phagocytose degenerated [29, 30] and apoptotic germ cells [31, 32]. Phagocytosis is morphologically the most noticeable function of Sertoli cells [33], and is an essential event for spermatogenesis [10]. Sertoli cells also play a role in the transport of germ cells across seminiferous epithelium [34, 35] and in spermiation – the release of sperms from seminiferous epithelium [3, 36, 37, 38, 39, 40, 41]. Sertoli cells also secrete isosmotic fluid, which carries immotile testicular sperms from their site of origin to the rete testis and excurrent ducts [9, 42, 43, 44]. Specific Sertoli cells in the tubuli recti form a plug-like structure, serving a valve preventing the reflux of fluid from the rete testis into the seminiferous tubules [45, 46, 47, 48]. Sertoli cells are polarized; showing bidirectional both endocrine and exocrine [49] as well as paracrine [2, 6, 50, 51, 52, 53] and autocrine activity [2, 11, 54, 55, 56]. They produce anti-Müllerian hormone – AMH [57, 58, 59], inhibin [60, 61] and activin [56] and androgen-binding protein – ABP [62, 63, 64, 65]. Sertoli cells also secrete many regulatory growth factors, which play a key role in local regulation of spermatogenesis [2, 8, 52, 54] and Leydig cells [53, 66,]. The secretory products of Sertoli cells are pivotal in fostering germ cell development and directing the appropriate maturation of sperm [11]. Creation of BTB [5, 13, 18] and secretion of numerous factors necessary for normal development of germ cells [8, 52, 54, 55] are the two basic functions of Sertoli cell, which are essential for spermatogenesis [2, 4, 19, 21]. Through them, Sertoli cells create and control the special environment within the seminiferous tubules necessary for the progression of germ cells to spermatozoa [2, 3, 16]. In addition to their essential role for germ cell development, Sertoli cells also play a role in regulating androgen production in the testes [53, 66]; all of this makes Sertoli cells "key drivers of testicular function" [5]. Sertoli cells also form unique tubulobulbar complexes around spermatids [67, 68, 69, and others], which are cytoskeleton-related structures indispensable for spermiation [34, 41].

The wide range of functions of Sertoli cell is reflected in its diverse structure and vice versa. There is no other cell in the body with such a diverse structure as the Sertoli cell. The ultrastructure of mammalian Sertoli cells has been reviewed by many authors [1, 27, 70, 71, 72, 73, 74, 75, 76]. Unusual characteristics of Sertoli cells include a nucleolus containing membrane vesicles, which is found only in ruminant Sertoli cells. First, Nicander et al. [77] described the ontogenetic development of vacuolated nucleolus in "indifferent cells" in seminiferous tubules of three-and-a-half to five-and-a-half-month old bull calves. Then Nicander [78] was first to observe "vacuoles within and around the nucleolus" of the bull and ram Sertoli cells. Zibrin [79] published the first electron micrographs of the Sertoli cell´s unique nucleolus of normal adult bulls and was the first to describe in detail this structure consisting of membrane vesicles covered with ribosome-like granules as a multivesicular nuclear body (MNB) with nucleolar activity. This was confirmed by all who later studied the Sertoli cells of the bull under an electron microscope [46, 47, 70, 72, 74, 80, 81, 82, 83, 84, 85, 86, 87]. The MNB, later also called vesicular nucleolus, was found in Sertoli cell in other domestic and wild ruminants such as ram [46, 70, 85, 88], gerenuk [70], chamois [86], Cape buffalo [70], roe deer [88], water buffalo [90, 91, 92], lesser mouse deer [93], West African dwarf goats [94, 95, 96], Shiba goat [97, 98] and Egyptian Nubian goat [99]. The only known exception so far is the fallow deer, *Dama dama;* its Sertoli cells have a reticular nucleolus with nucleolonema [74, 89, 100] instead of the MNB - vesicular nucleolus.

In this retrospective paper, we summarize our 25-year previous study of the Sertoli nucleolus in various ruminants, focusing on the structure of the roe deer Sertoli cell nucleolus throughout the year as well as the ultrastructure of its Leydig cells. In addition to the results of our electron microscopic observation, we present a minireview of Sertoli cell structure and function. We also include the short history of the discovery of the MNB, later also called the vesicular nucleolus, because some reviews of Sertoli cells did not respect it or just simply ignored it [72, 73, 76]. More than half a century has passed since its discovery [78, 79] and it is still not known what causes its develop-

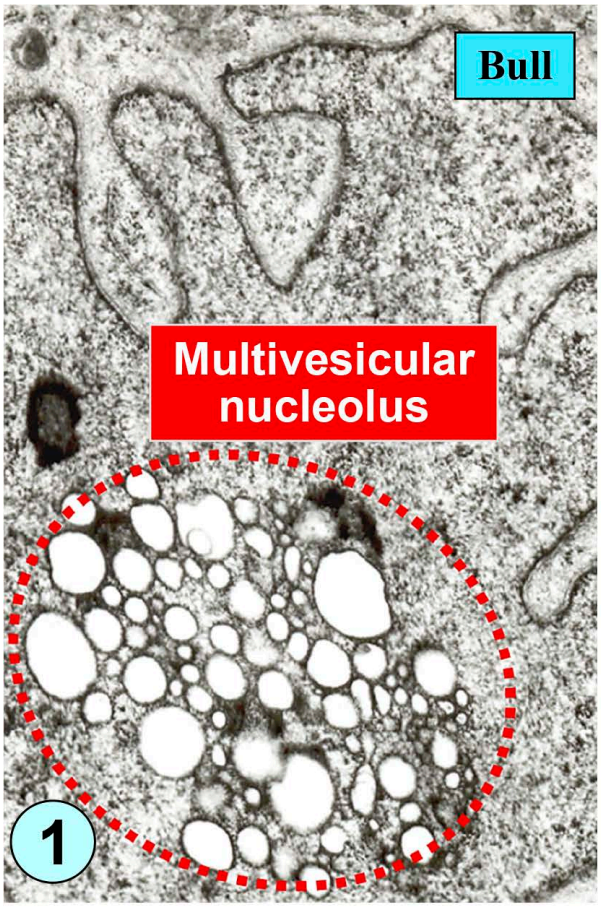
ment, what its function is, why MNB is found in Sertoli cells and why only in ruminants. Therefore, one more goal of this paper is to draw attention to further study of MNB in Sertoli cells of ruminants.

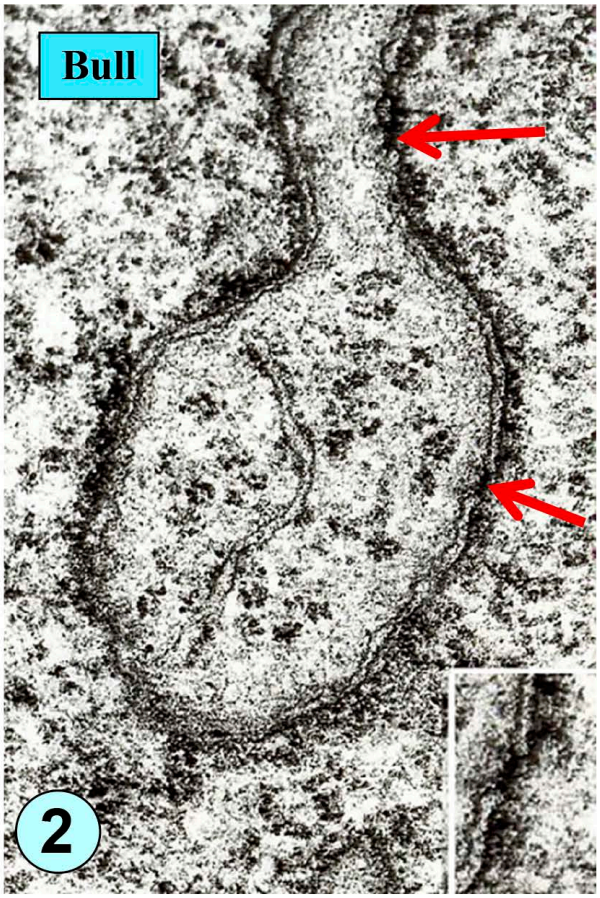
MATERIAL AND METHODS

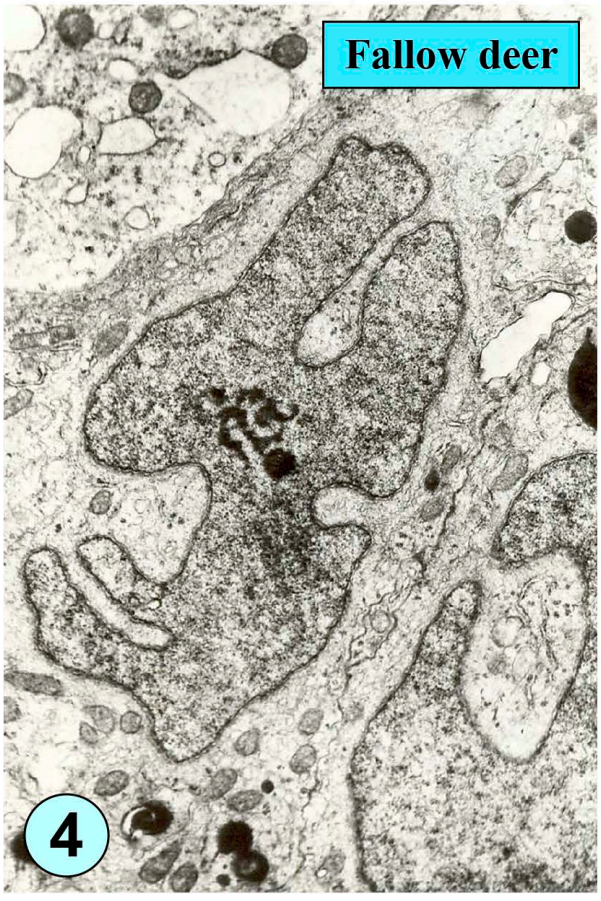
We studied testes of roe deer *(Capreolus capreolus),* fallow deer *(Dama dama),* bull*,* ram and goat and several other domestic mammals such as the stallion, boar, and dog and rodents mouse and rat by conventional transmission electron microscopy (TEM). We obtained the testes of seven roe deer 2- to 7-years-old shot on January 1, April 15, mid-June, mid-July and the end of August, testes of a 9-month-old fallow deer and of four 2- to 7-year-old fallow deer shot in heat in October - November. Eight bull testes samples we collected from 2-year-old healthy adult bulls slaughtered at a local slaughterhouse. The testes of two adult rams and a goat we collected from animals used for anatomy dissections in our department. Small, 1-3 mm^3 pieces of tissue were immediately fixed in 3% glutaraldehyde (Sigma-Aldrich, Bratislava, Slovak Republic) in 0.15 M cacodylate buffer pH 7.2 for 3 hours, or in a mixture of 2.5% paraformaldehyde and 2% glutaraldehyde, both in 0.15 M phosphate buffer solution pH 7.2–7.4 and followed by 1% OsO_4 (Fluka Chemie AG, Buchs, Switzerland) in the same buffer for 2 hours. Rinsed samples, rapidly dehydrated in acetone, we further processed routinely and embedded in Durcupan ACM (Sigma-Aldrich Chemie GmbH, Germany). Since the first observations of bull Sertoli cell and its nucleolus of the first author date back to the late 60s of the last century, he fixed his first bull testis samples in 3% glutaraldehyde in 0.1 M phosphate buffer with 5% sucrose pH 7.4–7.6 for 2 hours each. Fixed samples were rapidly dehydrated in alcohol followed by propylene oxide and routinely embedded in Araldite or Durcupan ACM. We cut ultrathin sections on the TESLA BS 490 ultramicrotome using glass knives, double stained with uranyl acetate and lead citrate (Merck, Bratislava, Slovak Republic) and photographed in electron microscopes Tesla BS 500, JEM 1200 EX and JEM-100 CX (JEOL, Japan). From all testes, immediately after sampling for TEM, we took samples for histological examination under a light microscope (LM). We fixed slices of testis 3 to 5 mm thick in 4% neutral formaldehyde in 0.1 M phosphate buffer pH 7.2–7.4 and processed routinely. Paraffin sections 3-5 μm thick were stained with hematoxylin and eosin (H&E).

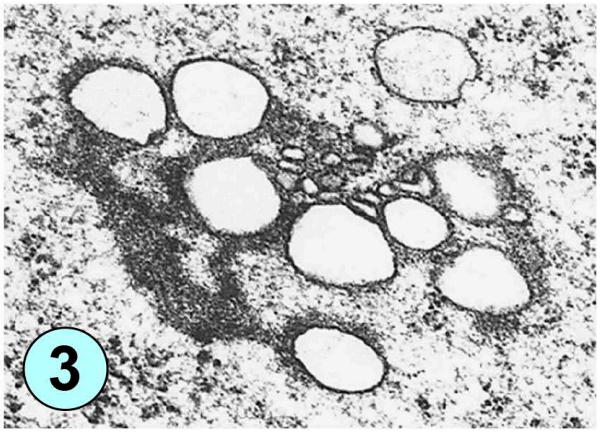
RESULTS

The largest and best-developed MNB among the Sertoli cells of ruminants is in the bull. The typical MNB also known as the vesicular nucleolus of bull Sertoli cells as in Fig. 1 consists of membrane vesicles of various sizes and a few tubules, both covered with ribosome-like granules and a small amount of fibrillar material. In all non-ruminants, we observed a reticular nucleolus with nucleolonema, and no membrane vesicles. We did not observe any seasonal changes in the nucleus and nucleolus in the bull Sertoli cell; the MNB – vesicular nucleolus is found throughout the year. The Sertoli cell's nucleus in all ruminant species has a folded surface, its nuclear envelope forms numerous deep invaginations towards the MNB. Invaginations contain ribosomes and occasionally one or two cisternae of rough endoplasmic reticulum (Fig. 2). The structure of the Sertoli cell of the roe deer and its nucleolus during spermatogenesis and in rut (Fig. 3) looks like that of other ruminants, including the MNB of the bull and other ruminants except the fallow deer, *Dama dama* (Figs. 4, 5). Sertoli cells of fallow deer also have a euchromatic nucleus with a folded surface and deep invaginations of nuclear envelope that are full of ribosomes (Fig. 4), but instead of a vesicular nucleolus, they have a reticular nucleolus with a nucleolonema (Fig. 5). We found such a more or less similar nucleolus with a nucleolonema in Sertoli cells of dog, cat, fox, stallion, boar, mouse, and rat. Rat and all rodent Sertoli cells have a tripartite nucleolus with a prominent reticular nucleolus flanked by two round, electron-dense heterochromatic bodies. The roe deer is a seasonal breeder. Its spermatogenesis begins in March - April. Sertoli cells are activated at about the same time, or a little earlier. Many mostly small vesicles appear in the nucleolonema of fibrillar material and 1-2 large vesicles are found on the edge of the reticular nucleolus (Fig. 6). The largest, best-developed MNB of roe deer Sertoli cell with the most numerous large round vesicles occurs during the rut from mid-July to mid-August. The nucleolus mainly consists of large and smaller vesicles (Fig. 7) and is quite similar to the MNB of bull Sertoli cells. Unlike the bull, a web of fine filaments covers most of the vesicles. In July, some of the


Bull
Multivesicular nucleolus
1


Bull
2


Fallow deer
4


3

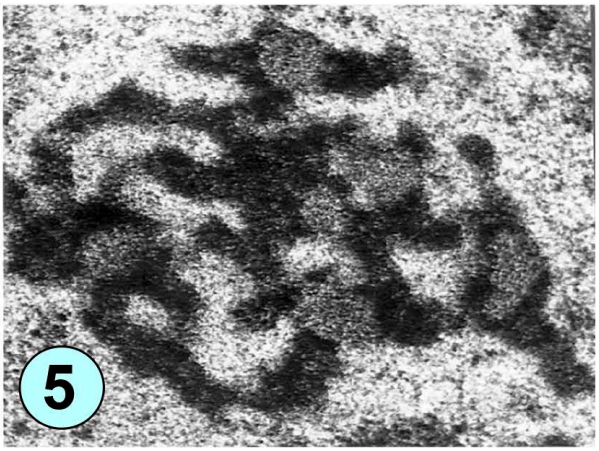

5

Fig. 1. Multivesicular nucleolus – a typical multivesicular nuclear body (MNB) of a bull Sertoli cell. The nuclear envelope forms numerous folds towards the MNB. 9,500 x.
Fig. 2. Close-up view of invagination of the nuclear envelope of a bull Sertoli cell with many free polyribosomes and a few membrane-bound to RER cisterna. Nuclear pores (arrows) and in an inset, and perinuclear vimentin filaments are also clearly visible. 60,000 x; inset 90,000 x.
Fig. 3. Vesicular nucleolus with a rudimentary nucleolonema of Sertoli cell of the roe deer in rut when the MNB in this seasonal breeder is the largest. The roe deer shot in July. 30,000 x.
Fig. 4. Sertoli cell of the fallow deer with an elaborated nucleus and a reticular nucleolus. Large invaginations of nuclear membrane contain free ribosomes and a few profiles of RER cisternae. The fallow deer was shot during rut in October. 8,000 x.
Fig. 5. Reticular nucleolus with nucleolonema in Sertoli cell of the fallow deer in rut. The fallow deer is the only so far known exception among ruminants, in which no large, round vesicles are found in the nucleolus of its Sertoli cells in active testis or during the rut. 33,500 x.

vesicles, mostly large ones, were partially covered with ribosome-like granules. The nucleolonema is barely visible. In August, when the rut ends, spermatogenesis also ends. Residual bodies, which are remnants of phagocytosed dead or apoptotic spermatogenic elements and sperms, we found in the Sertoli cells. In January, the testis is at its most dormant. The outline of the nucleus of Sertoli cells smoothed, there are almost no invaginations of the nucleus envelope. Sertoli cells have a reticular nucleolus with a dense distinct nucleolonema (Fig. 9). Vesicles in the Sertoli cells' nucleoli have almost completely disappeared (Fig. 10), we observed 1-2 small vesicles in the nucleolonema of about every fourth nucleolus of Sertoli cell. Spermatogenesis does not take place. The seminiferous epithelium consists of Sertoli cells (Fig. 11) and spermatogonia, but in some seminiferous tubules there were few spermatocytes in addition to spermatogonia. No advanced stages of spermatogenesis such as round or elongated spermatids were present (Fig. 11).

We also observed seasonal changes in the ultrastructure of Leydig cells that corresponded to their varying steroidogenic activity during the year. In January, at the maximum rest in the testis, Leydig cells were small, had an irregular shape and contained lipid droplets (Figs. 12, 14, 15), membranous whorls of SER residues (Figs. 13–15), various dense bodies – the end products of lysosomal degradation (Fig. 13) and a small amount of glycogen (Figs. 13, 14). We also found autophagosomes with membrane whorls of residual SER, lipid droplets, and mitochondria in many inactive Leydig cells in January (Figs. 12, 14). Some autophagosomes contained remnants of glycogen, most of the glycogen was free in aggregates in the cytoplasm (Fig. 14). These were residues of glycogen, which we first observed as round glycogen aggregates together with fat droplets and rapid decrease of SER volume in Leydig cells in August (Fig. 16), which causes the well-known sudden drop of testosterone levels. In August and January, we also observed chromatin margination of Leydig cells and nuclear fragmentation in some cells, both typical of apoptosis. It is noteworthy that in mid-August, at the end of heat, the vesicular nucleus of Sertoli cells is still well developed (Fig. 17), but in some places already there is some nucleolonema with tiny vesicles or tubules (Fig. 17, arrows), typical of the reticular nucleolus at the beginning of the cycle after the testicular quiescence in January. A detailed description of seasonal changes throughout the year in the structure of roe deer Leydig cells will be the subject of a separate paper.

DISCUSSION

The nucleolus, the most prominent structure in a cell nucleus, is a membrane-less intranuclear organelle involved in ribosome biogenesis and protein synthesis. Its primary functions are ribosomal RNAs synthesis, processing, and assembly of ribosome subunits in ribosomes biogenesis [101, 102, 103, 104, 105, 106 and others]. As a result, the nucleolus is a necessary part of the proteosynthetic apparatus of the cell [107, 108, 109 and many others]. Early studies of Sertoli cells identified that they synthesize and secrete over 60 proteins, a very diverse range that includes the hormones inhibin B, anti-Müllerian hormone, activin, transport proteins like androgen-binding protein (ABP), transferrin, ceruloplasmin and testibumin, several growth factors e.g., clusterin, proteins with enzymatic activities such as plasminogen, cytokines e.g., stem cell factor (SCF), and others [8, 54, 55, 56, 110, 111, 112]. More recent proteomic and genomic analyses identified that Sertoli cells synthesize hundreds of different proteins and polypeptides, which are essential for supporting spermatogenesis and regulating testicular functions [113, 114]. This requires that Sertoli cells must have an efficient proteosynthetic system first decoding a large part of a cell genome and including an active nucleolus producing ribosomes. Indeed, the synthesis of a large number of differ-

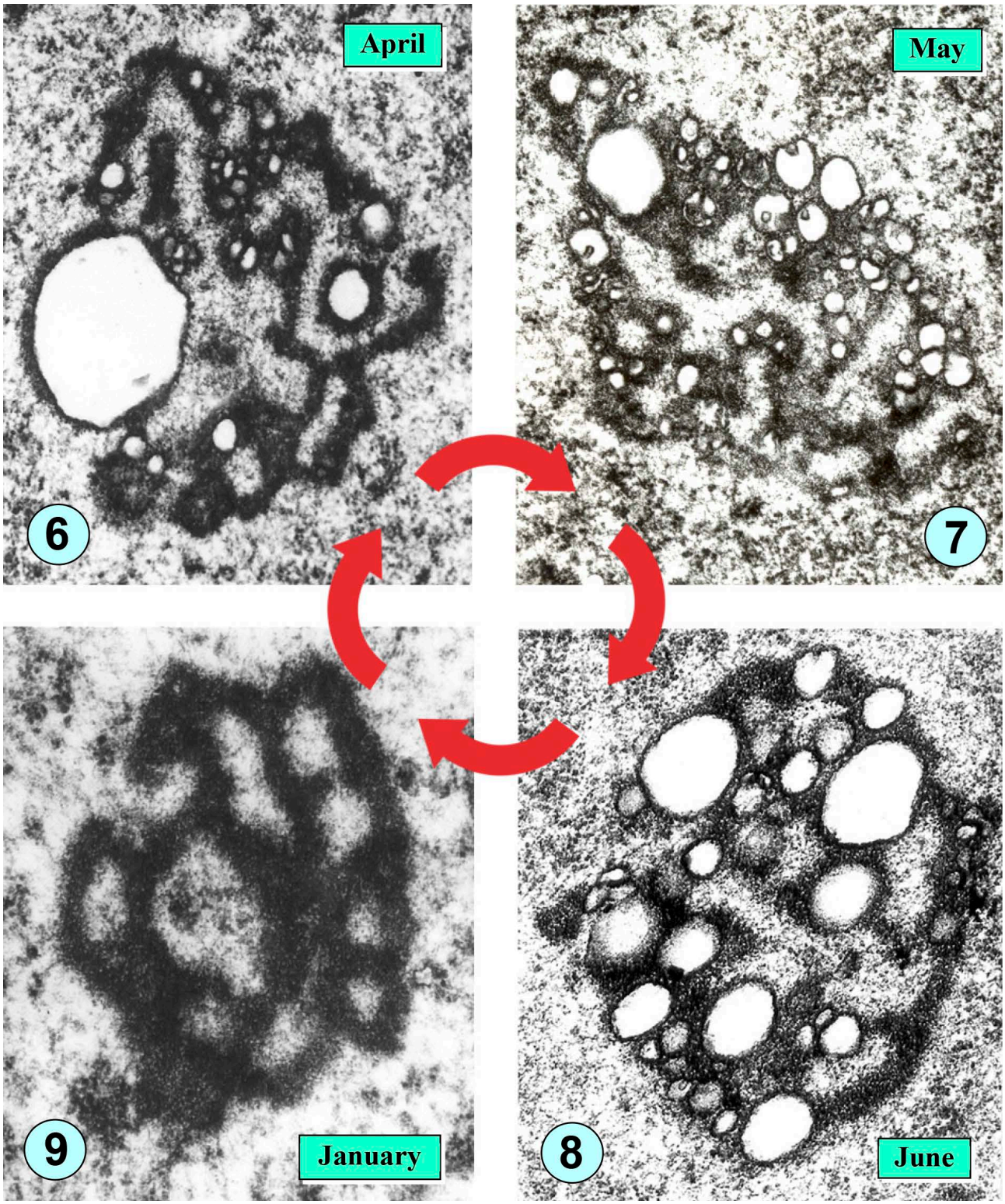


Fig. 6. Vesicular nucleolus of a Sertoli cell at onset of spermatogenesis. April. 28,000 x.
Fig. 9. Reticular nucleolus of a Sertoli cell of roe deer in January. 30,000 x.

Fig. 7. Vesicular nucleolus of a Sertoli cell of roe deer before the rut in May. 18,500 x.
Fig. 8. Part of vesicular nucleolus of Sertoli cell in June; roe deer shot in rut. 32,000 x.

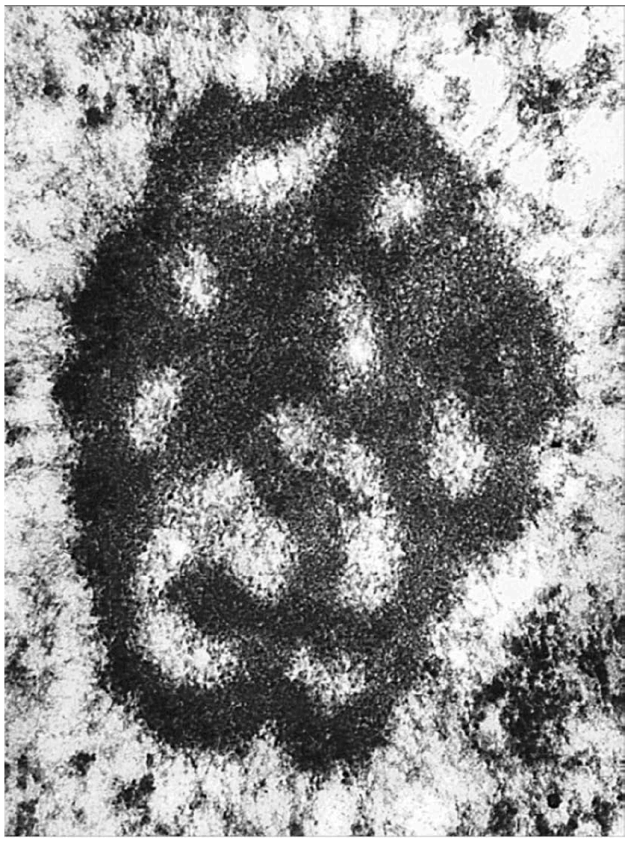


Fig. 10. Reticular nucleolus with a distinct nucleolonema of Sertoli cell of roe deer in January. It consists mainly of fibrillar part, granular compartment is small. 32,000 x.

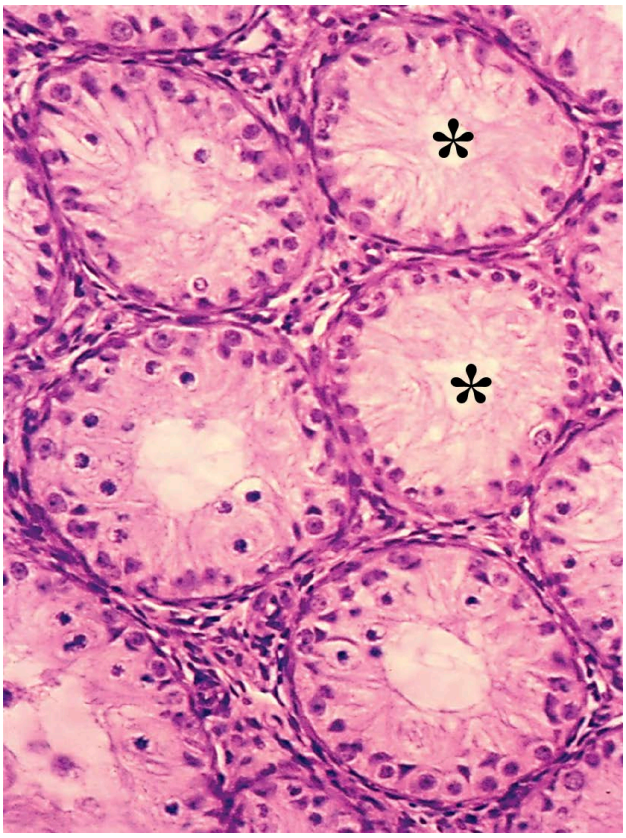


Fig. 11. Histology of the quiescent testis of a roe deer in January. No developing germ cells are in seminiferous tubules marked by an asterisk, not many are in others. LM 400 x.

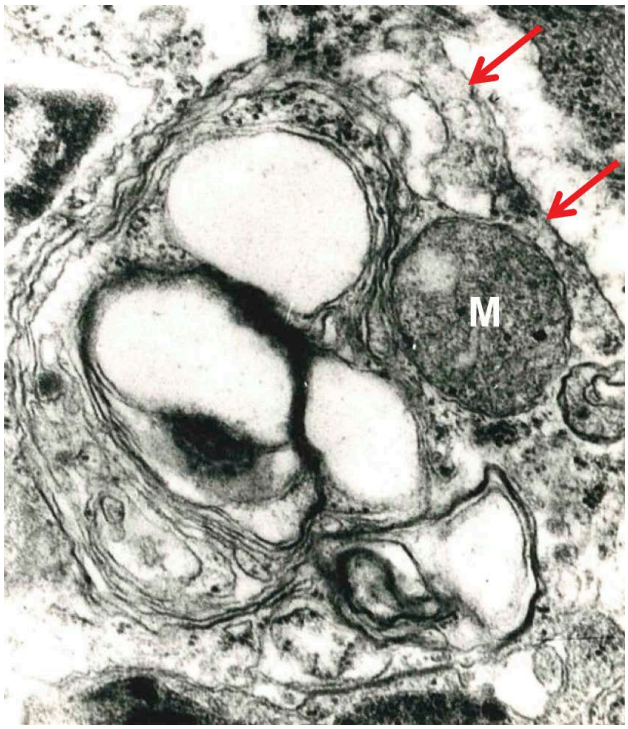


Fig. 12. Autophagosome in inactive Leydig cell with the membranous whorls of residual SER and mitochondrion (M). 29,600 x.

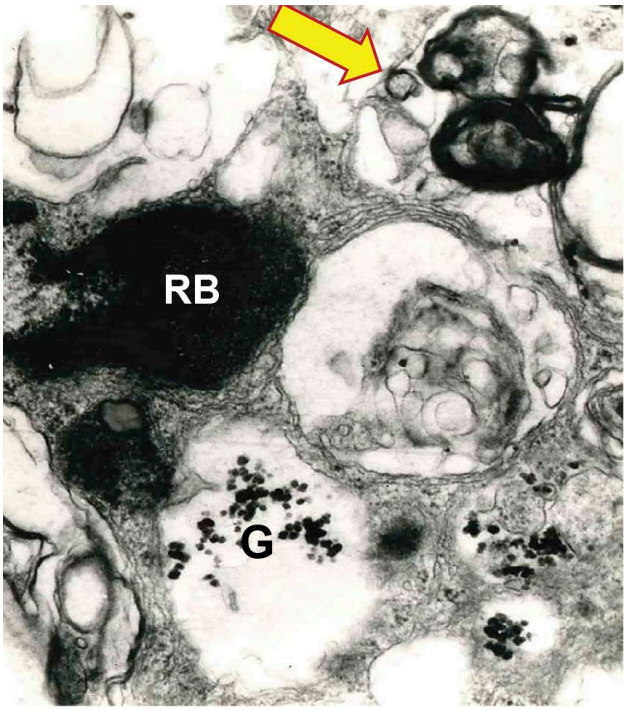


Fig. 13. Detail of an inactive Leydig cell in January. Note the residual SER (arrowhead), glycogen (G), residual body (RB). 29,600 x.

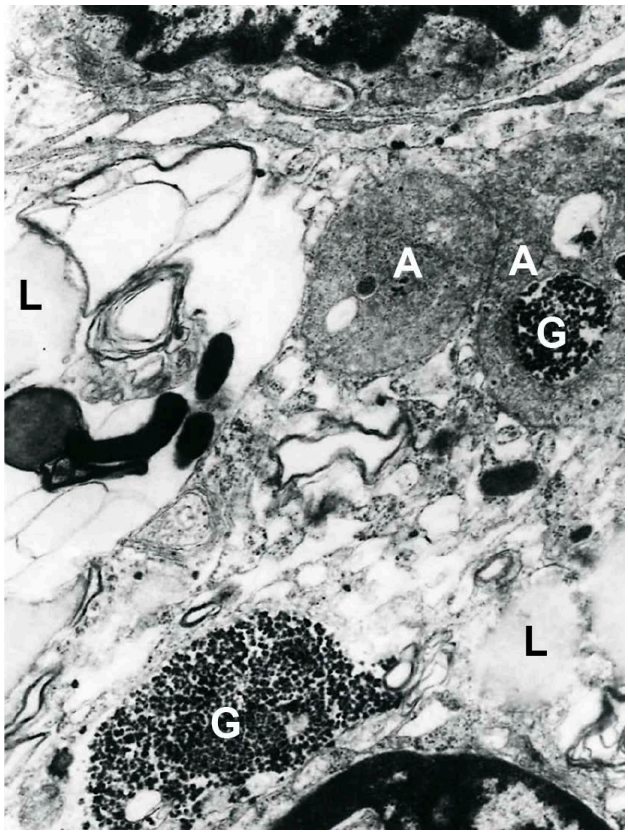


Fig. 14. Autophagic vacuoles (A), one with glycogen (G), lipid droplets (L), SER and glycogen in non-active Leydig cell. 16,600 x.

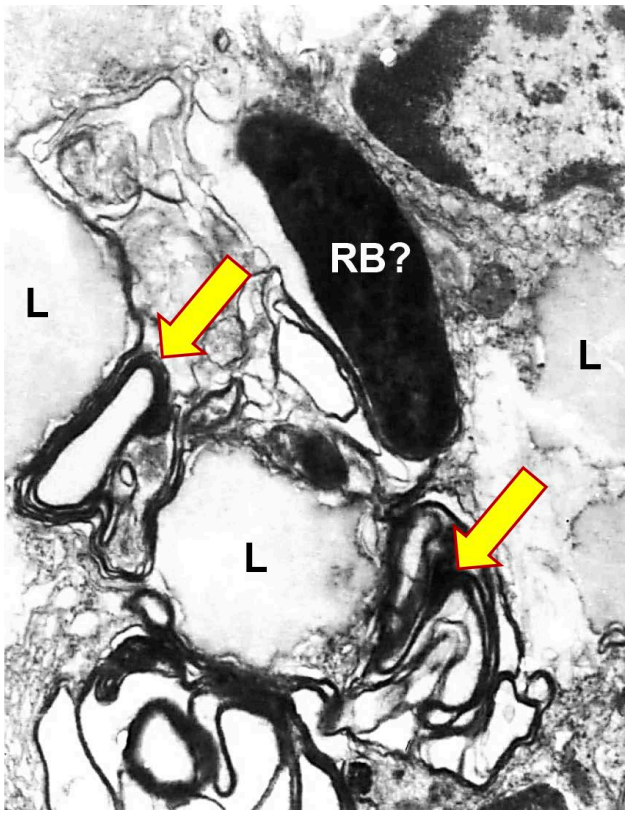


Fig. 15. Detail of non-active Leydig cell. The myelin-like whorls of residual SER (arrows), lipid droplets (L), residual body (RB). 20,200 x.

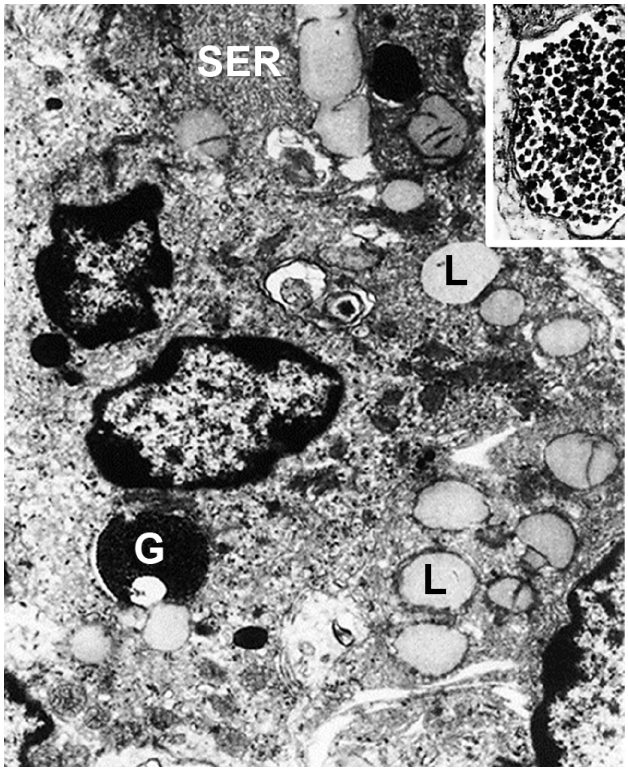


Fig. 16. Leydig cell at the end of rut. Some SER still present, several lipid droplets (L), glycogen (G), marginated chromatin. Mid-August. 9,000 x. Inset Glycogen at 20,200 x.

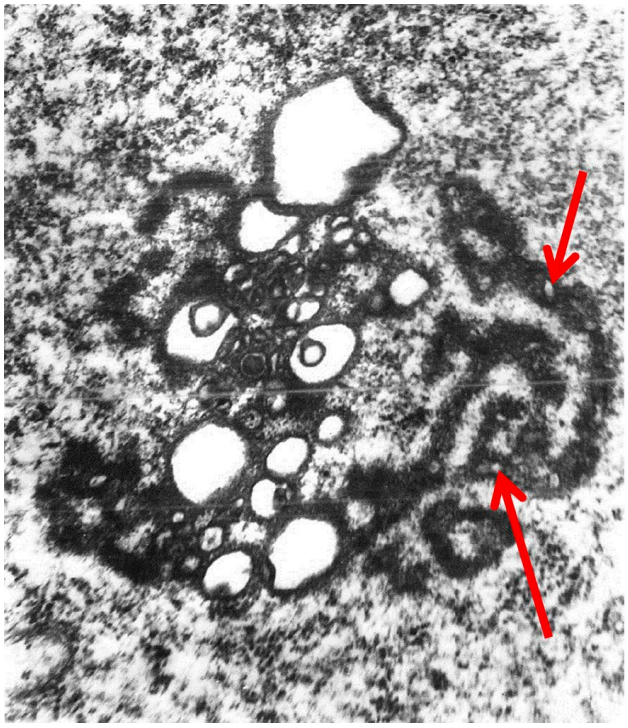


Fig. 17. Large vesicular nucleolus of Sertoli cell with a small nucleolonema and very small vesicles and tubules in it (arrows) at the end of rut. Roe deer shot in Mid-August. 19,500 x.

ent proteins and polypeptides in Sertoli cells corresponds to their structure/ultrastructure. And indeed, Sertoli cells have a very euchromatic nucleus with a prominent species-specific nucleolus. In most species, the nucleolus is reticular with a distinct nucleolonema, which in rodents is flanked by two round, dense heterochromatic satellite karyosomes. While the euchromatic nuclei of the other three most euchromatic cells in the body: nerve cells, hepatocytes and blastomeres are round, the nuclear surface of the adult Sertoli cell is folded, and their nuclear envelope numerous forms numerous deep invaginations towards nucleolus, and towards the MNB in ruminants. The nuclear envelope in invaginations has numerous nuclear pores; invaginations expand and contain many ribosomes.

One of the main characteristics of nucleolus is that they do not have membranes. The nucleolus of ruminant Sertoli cells consisting of membranous vesicles is unique; there is no other analogue of it in the animal kingdom. The only other exception is the nucleolar channel system (NCS) in epithelial cells of human endometrium [115, 116, 117, 118, 119, 120, 121 and many others]. NCS is composed of several rows of coiled tubules embedded in an amorphous matrix, the outer surface of the tubules are numerous electron-dense particles resembling ribosomes [117, 118, 119, 120, 121, 122]. The progesterone dependence of NCS in the human endometrial epithelial cells was found shortly after its discovery [115, 116, 117] by Kohorn et al. [123]. Currently NCS is a well-established ultrastructural hallmark of the post-ovulation mid-secretory human endometrium, and a potential marker of uterine receptivity [121, 122, 124, 125, 126]. Its transient presence has been associated with fertility [122, 126, 127, 128, 129], but its presence or absence cannot serve as a predictor of infertility [126]. On the other hand, a delay or absence of NCS formation in endometrial cells has been found in some infertile women with unexplained sterility [130, 131, 132]. Dockery et al. [132] observed its impaired development in women with unexplained infertility with a relative excess of luteal estrogen. Guffanti et al. [124] found that NCSs contain proteins of the nuclear pore complexes, inner nuclear membrane, nuclear lamina and endoplasmic reticulum. They used monoclonal antibodies, phase contrast and double fluorescence microscopy, which allows detection of NCS by light microscopy, a great technique to recognize NCS in endometrium and exploit for reproduction without electron microscopy. This will allow applying theoretical knowledge about NCS in practice even despite that the composition and function of the membrane system of NCS have not yet been fully clarified [121, 128, 129]. However, less than a decade after the discovery of NCS [115, 116, 117], Kohorn et al. [123] already demonstrated in vitro that NCS formation is caused by progesterone and subsequent research has confirmed that NCS formation depends on a specific progesterone threshold [127, 128, 133 and others]. On the other hand, more than half a century has passed since the discovery of the Sertoli cell MNB of the bull [79], and we still do not know what causes its development and why. It is still unknown whether its production depends on follicle-stimulating hormone (FSH) or another hormone and why MNB only occurs in ruminants. It is worth noting that MNB – vesicular nucleolus of Sertoli cells in ruminants has been included in veterinary histology textbooks [134, 135]. In contrast, NCS in the women´s endometrium is a clinically significant marker of endometrial receptivity and the "window of implantation" [122, 123, 124, 128, 129] relevant to reproduction, but despite this, no human histology textbook mentions it yet.

Andriana et al. [98], in their thorough study of the postnatal development of the MNB in the Sertoli cell of Shiba goats, found that it arises as narrow channels from the inner membrane of the nuclear envelope, gradually enlarges and moves from the periphery to the central region. We did not find such successive stages of MNB development as Andriana et al. [98] because its seasonal development in adult roe deer is probably faster. Nevertheless, it is very likely that it develops in the same way in roe deer and all ruminants except fallow deer, which does not have MNBs. A reticular nucleolus is present in Sertoli cells of fallow deer throughout the year, even in adults [74, 89, 100]. MNB is stunted or less developed in some breeds of goat [95, 96] and sheep [85]. Due to the large number of vesicles in Sertoli cells in adult bulls and most other ruminants, we consider multivesicular nucleolus – MVN to be a more appropriate name instead of vesicular nucleolus.

All authors who studied Sertoli and Leydig cells in seasonal breeders [136, 137, 138, 139 and others] found significant changes in their structure throughout the year. Both cells in the testes undergo significant ultrastructural changes throughout the annual reproductive cycle. Spermatogenic arrest results in the accumulation of lipids and primary and secondary lysosomes in Sertoli cells [99, 137, 138, 139]. Also worth noting is the change in nuclear shape in prepubertal versus adult and in inactive versus active

Sertoli cells. The nuclei of prepubertal Sertoli cells and inactive Sertoli cells of seasonal breeders of the quiescent testis are round or oval with a smooth surface; and if they have few invaginations, these are wide and shallow. Cameron et al. [140], in their model experiments on rats, found that FSH causes invaginations of the nuclear envelope of Sertoli cells containing a large number of polyribosomes. The nuclei of adult Sertoli cells in domestic ruminants and Sertoli cells of seasonal breeders before and during spermatogenesis also have several deep invaginations towards the MVN. Near the MVN, the invaginations widen and contain many ribosomes. All the above suggest that the MVN, like the reticular nucleus with nucleolonema, has a key role in ribosome biogenesis.

Simultaneously with changing roe deer Sertoli cell structure and nucleolar cycle, we observed circannual seasonal changes in the structure of Leydig cells. Their main function is secretion of testosterone, necessary for spermatogenesis and hence fertility [66]. The best structural measure of the endocrine activity of Leydig cells and testosterone production is the volume of their smooth endoplasmic reticulum (SER) [28, 141, 142, 143, 144]. We found substantial changes in SER volume of roe deer Leydig cells throughout the year, which responded to changing testosterone blood/serum levels found by [145, 146, 147, 148]. Testosterone production in roe deer shows a biphasic pattern. There is mild increase in April; the highest testosterone levels occur during the peak of the rutting season in mid-July in mid-August, then it drops rapidly to very low concentrations [146, 147, 148]. In January, we found almost no SER in the Leydig cells of deer, which corresponds to the lowest level of testosterone in the blood, close to zero. Leydig cells (at least in rodents) have a tremendous regenerative capacity, which has been found by many authors after selective destruction of Leydig cells with ethylene dimethanesulfonate (EDS) [149, 150, 151, 152, 153]. We assume that in roe deer, the inactive Leydig cells full of lipid droplets, remnants of SER and glycogen clusters either regenerate or are removed by macrophages and a new generation of Leydig cells is formed in the spring. Since the duration of apoptosis can range from a few hours to several days [154, 155, 156], it is unusual that we found apoptotic Leydig cells with chromatin margination not only in August but also in January, several months after the rut. Autophagy also occurs at a high rate in Leydig cells [157, 158, 159] and plays a significant role in regulating testosterone production by influencing uptake of cholesterol [158, 160]. In Leydig cells, autophagosomes preferentially sequester organelles important for steroid production such as mitochondria and SER [159, 161, 162]. In the autophagosomes of roe deer Leydig cells, we also found almost exclusively mitochondria and residual SER. Apoptosis plays a vital role in tissue homeostasis and is a vital part of various cellular processes, including normal cell turnover and atrophy [155, 156, 163, 164], and is also involved in seasonal regression of testis spermatogenic activity [165]. In recent years, other cellular mechanisms were found to be involved in the decrease of the activity of the seminiferous epithelium during the non-reproductive period, such as changes in Sertoli cell morphology and function, and autophagy. Autophagy maintains intracellular homeostasis by the process, in which cells degrade and recycle their own proteins and organelles [160, 164, 166, 167, 168, 169]. Both apoptosis and autophagy can occur simultaneously in the same cell [169, 170, 171, 172]; they often occur sequentially with autophagy preceding apoptosis [171]. We also observed both autophagy and apoptosis in the same roe deer Leydig cells. Sertoli and Leydig cells in adults (at least in rodents) are stable cell populations throughout the year that do not normally proliferate [144, 173, 174, 175], even in roe deer, a seasonal breeder [145, 176, 177], though they can regenerate if experimentally eliminated. This could explain that we never observed macrophages in the interstitium of the roe deer testis in the non-breeding season, which remove dead Leydig cells e.g., after EDS [149, 150, 151, 152, 153]. Dead Leydig cells are cleared rapidly, as already "4 and 14 days after EDS, macrophages were the dominant cell in the interstitial space" and 14 days after EDS administration, inclusions from dead Leydig cells in macrophages almost disappeared [150].

The seasonal breeding rodents are used as a model to study structure-function relationships in the testis [e.g., 136, 137, 139, 178, 179]. Klonisch et al. [177] state the roe deer "as an excellent non-rodent model for studying seasonal regulation of testis function with naturally changing photoperiod". We also recommend a roe deer, a ruminant and seasonal breeder, similar to economically important cattle with continuous spermiogenesis, as the best natural model for studying the regulation of spermatogenesis, already proposed by Klonisch et al. [177] without knowing about seasonal circannual changes of nucleolus in their Sertoli cells. Since the nucleolus participates "in the cell proliferation, resting state, differentiation, maturation, ag-

ing and death" [108], the MVN in ruminant Sertoli cells and its annual structural cycle in roe deer is not a cytological rarity or curiosity, but can serve as a simple morphological marker of ruminant Sertoli cell activity. Since membrane vesicles appear in the nucleolus of Sertoli cell precursors of bull calves [77], and MVN in Sertoli cells of bull [82, 89], goat [94, 97, 98], water buffalo [90] and roe deer [73, 88] develops before the onset of spermatogenesis (regardless of whether during puberty or before rut in adult seasonal breeder), we suppose MVN is related to the regulation of spermatogenesis. It has been 140 years since Enrico Sertoli first identified and described cells in the seminiferous tubules of the testes in 1865 that now bear his name. Based on the current PubMed and Scopus databases, approximately 24,000 to 26,000 studies on Sertoli cells have been conducted to date and there are still many reasons and challenges for further research on them. Among other, because of obstacles to the transfer of basic research findings into practical use in reproduction and clinical therapy. More than one-half century has passed since the discovery of MVN, later also known as the vesicular nucleolus, in the bull Sertoli cells and its function remains unknown as well as why it only occurs in ruminants. Further study of ruminant Sertoli cells is also needed to clarify the role of MVN in Sertoli cells and its function for spermatogenesis.

CONCLUSION

The nucleolus as a vital cell structure, which plays a crucial role in protein synthesis by producing ribosome subunits. Without the nucleolus, cells would not be able to produce the ribosomes necessary for making proteins. Sertoli cells synthesize hundreds of proteins and polypeptides therefore have a very euchromatic nucleus and species-specific prominent nucleolus. The ruminant Sertoli cells' nucleolus consists of membrane vesicles covered with ribosome-like small granules, first described as a multivesicular nuclear body – MNB, later also known as a vesicular nucleolus. Given the large number of round membrane vesicles in it, we suggest a more appropriate name multivesicular nucleolus – MVN. Unlike domestic ruminants, which have continuous spermatogenesis and reproduce continuously throughout the year, and MVN also occurs throughout the year, in the roe deer, a seasonal breeder with seasonal spermatogenesis, the fully developed MVN occurs only before the onset and during spermatogenesis. In January, in regressed testis, the nuclei of Sertoli cells of roe deer lack large membrane vesicles and have a reticular nucleolus. The seasonal MVN development in Sertoli cells of adult roe deer resembles its ontogenetic development in continuously breeding ruminant. The formation, disappearance and lack of membrane vesicles in the nucleolus of adult deer Sertoli cells repeats itself every year, creating a circannual seasonal cycle of the Sertoli nucleolus that precedes the circannual cycle of Leydig cells. The latter mainly consist of the loss of the smooth endoplasmic reticulum after rut, and accumulation and storage of fat droplets and glycogen in inactive Leydig cells. The function of MVB is still unclear. Its structure and deep folds of the Sertoli cell nuclear envelope towards the MVN containing numerous ribosomes suggest its role in ribosome production. Since membranes in the nucleolus of ruminant Sertoli cells appear before spermatogenesis, we believe that MVN play a role in its regulation, at least at its onset. What the exact purpose of the membrane vesicles in Sertoli cells is, and why the MVN is found only in ruminants, still remains a mystery.

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Conflict of Interest

None of the authors has any conflicts of interest.

Data Availability Statement

The raw data of this article will be made available by the authors, without undue reservation.

Ethical Statement

This study did not require any ethical approval, as it is not required if the study material comes from animals slaughtered in an abattoir, from hunted game, or obtained by castration. During the game protection season, animals were shot with exceptions granted by the Ministry of Agriculture and Forestry of the Slovak Republic and the Directorate of East Slovakian Forests.

Authors' Contributions

M. Z.: Conception and design of the study, specimen collection, electron microscopy, drafting the manuscript, final approval and accountability. K. H.: methodology, processing of specimens, electron microscopy, bibliography search, manuscript revision. J. P.: high-resolution electron microscope photography, manuscript revision. All authors read and approved the published version of the manuscript.

Generative AI Statement

The authors declare that they did not use any AI generation in the creation of this manuscript.

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The paper is dedicated to the memory of two outstanding researchers in reproduction and in particular Sertoli cells. Lonnie D. Russel (December 11, 1944 – July 11, 2001) was an exceptionally successful researcher with 16,395 citations, passionate about the Sertoli cell. Lennart Nicander (November 4, 1923 – May 8, 1991) was the first to observe membrane vacuoles in the nucleolus of a bull Sertoli cell.

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ORIGINAL ARTICLE

SEASONAL VARIATION IN MILK COMPOSITION AND PHYSIOLOGICAL INDICATORS IN DAIRY COWS: A ONE-YEAR STUDY FROM EASTERN SLOVAKIA

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Ethical considerations: When reporting experiments on animals Observation of the ARRIVE guidelines 2.0: Updated guidelines for reporting animal research, published on July 14, 2020 (DOI: 10.1371/journal.pbio.3000410), is applied. The authors ensure that all procedures were performed in compliance with the guidelines for animal care of their institutions or with national/international guidelines.

ABSTRACT

This study aimed to evaluate seasonal variations in the main components of raw cow's milk and to assess the relationships between milk composition and selected physiological indicators, including milk urea nitrogen (MUN), lactoferrin (LF), and somatic cell count (SCC). A total of 3000 milk samples from 250 clinically healthy dairy cows were collected across four seasons on a commercial farm in eastern Slovakia. Milk fat and protein exhibited significant seasonal differences ($p < 0.001$), with the lowest values in summer and the highest in autumn. Lactose concentrations remained relatively stable throughout the year, showing no significant seasonal variation. MUN showed notable seasonal variation, with significantly lower levels in winter compared with other seasons, reflecting changes in feeding regime and nitrogen metabolism. Lactoferrin levels were lowest in summer and highest in autumn, likely reflecting physiological changes associated with the lactation stage. Seasonal changes in SCC were not statistically significant, although a trend toward higher values in summer was observed. These findings highlight the influence of nutrition, physiological state, and environmental conditions on milk composition and udder health. Understanding seasonal dynamics is essential for optimizing dairy herd management, mastitis monitoring, and technological quality of raw milk.

Key words: lactoferrin; milk; milk composition; milk fat; season; somatic cell count

INTRODUCTION

The composition of bovine milk is a complex biological matrix determined by a wide range of genetic, physiological, environmental, and technological factors, including its fat, protein, lactose, mineral, and bioactive component profile. Among the most influential variables are season, nutritional status, udder health, stage and number of lactations, and overall herd management conditions. Numerous studies in Europe and beyond have confirmed that seasonal factors are among the key determinants of milk composition, particularly affecting fat, protein, lactose, urea nitrogen, and somatic cell count [1, 2, 3, 4].

Seasonal variation in milk composition has been observed not only in Europe but also in other continents with different climatic and production conditions. In Western Europe, long-term monitoring in the Netherlands confirmed significant seasonal changes in milk fat and protein, with the lowest values during summer and the highest in winter [5]. Similar trends were reported in Mediterranean systems, where high summer temperatures strongly reduced milk solids [2].

In Asia, studies from China demonstrated pronounced seasonal fluctuations in milk urea nitrogen and protein content related to feeding regime and climate [6], while Indian research highlighted the role of heat stress and metabolic load in shaping seasonal milk composition and SCC [7]. Outside Europe, seasonal changes in milk composition have also been described in intensive and semi-intensive systems in North America and other temperate regions, where summer heat stress and pasture quality play a major role [1].

These international findings indicate that although the direction of seasonal changes in milk composition is largely consistent worldwide, the magnitude of these changes depends strongly on regional climate, feeding strategies, and management systems. Therefore, region-specific data, such as those from eastern Slovakia, are essential for understanding local production conditions and for designing appropriate nutritional and health-management strategies.

Milk fat and protein levels typically decline during the summer due to elevated temperatures, heat stress, and seasonal changes in feeding, particularly the increased proportion of succulent feeds (green forage, pasture herbage) in the ration, whereas during the winter these parameters tend to be more stable and reach higher values [5, 8]. Lactose, the most stable component of milk, exhibits minimal seasonal variation; however, its concentration may be affected by metabolic stress, udder infections, and the energy balance of dairy cows [7]. The urea nitrogen content of milk (MUN) is an essential indicator of nitrogen balance and dietary protein utilization efficiency. It is well known that MUN rises primarily during the grazing season when the intake of rapidly degradable nitrogen is higher [9, 6], and excessive levels may negatively affect fertility and the metabolic load of dairy cows.

Somatic cell count (SCC) is a key indicator of udder health, with elevated values closely linked to subclinical mastitis, alterations in milk composition, and deteriorated technological properties, particularly in cheese production [10, 7]. An increased SCC is associated with reduced casein content, proteolytic degradation of milk proteins, and lower cheese yield and quality. Seasonal fluctuations in SCC have been reported, with higher levels in summer, mainly due to heat stress and increased environmental bacterial load [8].

While many international studies have explored seasonal variations in milk quality, there is still limited information on conditions in Slovakia, particularly on large-scale farms in mountainous areas. Therefore, this study aims to analyse seasonal changes in the physicochemical composition of milk, urea nitrogen concentration, and SCC under intensive dairy production conditions in eastern Slovakia over a 1-year period. The study also includes an assessment of relationships among individual parameters, which may contribute to a better understanding of the interactions among nutrition, udder health, and milk technological quality.

Unlike most previous studies that focus mainly on traditional milk quality indicators (fat, protein, lactose, urea nitrogen, SCC), this work also integrates lactoferrin as an additional functional and immunological marker. The combined evaluation of lactoferrin with classical technological and physiological indicators represents a novel approach under Slovak production conditions and allows a more complex assessment of mammary gland status and milk quality across seasons.

MATERIALS AND METHODS

Farm Characteristics and Housing Conditions

The study was conducted at an agricultural cooperative located in the eastern Slovak region, situated on the

northern foothills of the Levoča Mountains (49.2761° N, 20.6834° E) at an altitude of approximately 550–570 m above sea level. The area lies within a mountainous production zone characterised by a continental climate with warm summers and cold winters typical of central Europe (Slovakia has a primarily humid continental climate with warm summers and cold winters).

The annual average temperature in Slovakia generally ranges from about 9°C to 11°C, with January averages below 0°C and July averages around 18–21°C. Annual precipitation in mountainous and highland regions of Slovakia is relatively high, often about 700–800 mm or more per year, with monthly maxima during summer months and lower totals in winter [11, 12, 13].

The herd comprised approximately 700 livestock units, including 250 Slovak Spotted dairy cows. The cows were kept in a free-stall system with solid bedding.

The animals were fed a total mixed ration (TMR) prepared directly on the farm, with its composition adjusted according to season. During winter, the ration was based mainly on maize silage, grass silage, hay, concentrate mixture, and mineral–vitamin supplements. In spring and summer, fresh green forage or pasture herbage formed an important part of the ration, supplemented with silage and concentrates to ensure adequate energy and protein supply. In autumn, the diet gradually shifted back to a higher proportion of conserved forages.

Cows had continuous ad libitum access to fresh drinking water from automatic water troughs throughout the year. Milking was carried out twice daily in a parallel milking parlour (Agromont, Slovakia) equipped with twelve fixed stalls arranged in two opposing rows. The average annual milk yield of the dairy cows exceeded 7,000 L per cow.

Animal Group

A total of n = 250 clinically healthy dairy cows in different parities (1st to 8th lactation; average parity 2.67) were included in the study. The cows were at various stages of lactation, with days in milk ranging from 2 to 625 (mean 119). Including the full physiological spectrum of lactation stages enabled an objective assessment of seasonal effects without selection bias.

Milk Sampling

Individual milk samples were collected once per month from each lactating cow over a 1-year period (March 2024 – February 2025). Cows in the dry period were not sampled. Because not all cows were in milk throughout the entire year, the total number of samples was slightly lower than the theoretical maximum (12 × 250 = 3000). In total, 3000 raw milk samples were obtained from approximately 250 cows, corresponding to about 2500–3000 samples depending on lactation stage and dry periods.

Sampling was always performed during the morning milking to minimise technological variability and ensure comparability between samples.

Before sampling, a clinical examination of the udder and a milk assessment using the California Mastitis Test (CMT) were performed. Only CMT-negative composite samples (mixed milk from all four quarters of the udder) were collected, thereby eliminating intra-udder quarter variability. In total, 3000 CMT-negative composite milk samples were included in the final analysis.

Sample Transport and Processing

Samples were collected into sterile containers and immediately cooled to 5°C. Within 6 hours of collection, they were transported to the Laboratory of the Department of

Hygiene, Technology and Food Safety at the University of Veterinary Medicine and Pharmacy in Košice (Slovakia) for analysis.

Laboratory Analyses

The analysis included the determination of the following basic physicochemical parameters:

- fat (%)
- protein (%)
- lactose (%)
- milk urea nitrogen (MUN; mg/dL)
- somatic cell count (SCC; $\times 10^3$ cells/mL)

In the laboratory, samples were analysed using a LactoScan MCCV analyser from Milkotronic Ltd., Bulgaria. All analyses were conducted in accordance with the relevant international standards ISO 21543 and STN EN ISO 13366-2 [14, 15].

MUN concentration was assessed in relation to the dynamics of milk composition and milk yield. Samples for MUN determination were analysed at the Central Dairy Laboratory, which is accredited according to ISO/IEC 17025, in cooperation with the State Breeding Services of the Slovak Republic, using data from the national herd-recording information system [16].

Because lactoferrin content cannot be routinely determined with LactoScan instruments, samples were processed separately at the Central Dairy Laboratory. Milk was first centrifuged (1,400 × g, 45 min), and the fat layer was removed. Lactoferrin in the skimmed milk fraction was then quantified using an immunochemical method – a quantitative ELISA assay based on antibodies specific to bovine lactoferrin.

Before analysis, milk samples were diluted 1:10,000 following the protocol [17] to ensure that measured values fell within the calibration curve range. The ELISA assay utilised goat anti-bovine lactoferrin antibodies coated onto microtiter plates and a secondary antibody conjugated with peroxidase for detection. After the enzymatic reaction and absorbance measurement, lactoferrin concentrations were calculated from the calibration curve and converted back to the original (undiluted) sample.

Statistical Analysis

Data were divided according to four seasons: spring (March–May), summer (June–August), autumn (September–November), and winter (December–February). For each season, mean values and standard deviations (SD) were calculated for all monitored parameters (fat, protein, lactose, urea nitrogen, lactoferrin, SCC). Differences between seasons were evaluated using one-way analysis of variance (ANOVA) followed by Tukey's post hoc test at a significance level of $\alpha = 0.05$. Statistically significant differences between means are reported together with the corresponding p-values.

In addition, relationships between variables were assessed using Pearson's correlation and linear regression across the entire dataset and within each season. Particular attention was given to correlations between the basic milk components (fat, protein, lactose) and urea nitrogen, lactoferrin, and SCC, as these relationships can reveal interactions between nutrition, udder health, and milk composition. Statistical analyses were performed using IBM SPSS Statistics version 20.

RESULTS

The results from monthly monitoring over 1 year indicate marked seasonal variations in the biochemical composition of raw cow's milk, as well as in selected physiological indicators reflecting nutritional status and udder health. Data are shown as mean, median, standard deviation, minimum, and maximum for each season.

Seasonal Changes in the Basic Milk Components

Fat

Milk fat content showed a clear seasonal pattern. The lowest average values were recorded during the summer (3.83%), whereas the highest values were observed in the autumn (4.20%). The difference between summer and autumn was highly significant ($p < 0.001$). Fat content in autumn was also significantly higher compared with spring and winter (by approximately 0.2 percentage points, $p < 0.01$). In contrast, no significant difference was found between winter (3.98%) and spring (3.95%) (Table 1).

Protein

Protein content exhibited a seasonal trend similar to that of fat. The lowest mean value was observed in summer (3.49%), while the highest was recorded in autumn (3.68%). Autumn and winter values were significantly

Table 1. Seasonal average values of basic milk components (Mean ± SD)

Season	Autumn	Winter	Spring	Summer
Fat (%)	4.20 ± 0.58[a]	3.98 ± 0.55[a,b]	3.95 ± 0.54[a,b]	3.83 ± 0.57[b]
Protein (%)	3.68 ± 0.28[a]	3.60 ± 0.27[a,b]	3.52 ± 0.26[a,b]	3.49 ± 0.29[b]
Lactose (%)	4.94 ± 0.28	4.87 ± 0.33	4.87 ± 0.29	4.93 ± 0.28
SCC (10^3 cells/mL)	274.99 ± 58.44	366.60 ± 10.70	364.79 ± 76.64	423.44 ± 86.72
MUN (mg/dL)	35.29 ± 8.49[b]	26.57 ± 5.58[a]	35.81 ± 7.74[b]	34.39 ± 6.84[b]
Lactoferrin (mg/dL)	171.96 ± 40.05[b]	157.96 ± 36.46[b]	158.20 ± 37.73[b]	153.79 ± 52.40[a]

Values in rows labeled with different letters (a,b) exhibit statistical significance ($P < 0.001$).

higher than those in spring and summer ($p < 0.001$). The difference between autumn and summer (approximately 0.19 percentage points) was highly significant ($p < 0.001$). However, no significant difference was observed between autumn and winter, suggesting that both seasons represent periods with peak milk protein concentration. The lower protein content in summer is linked to an imbalance in the energy-to-protein ratio during grazing. In contrast, the higher intake of concentrated feed in winter supports increased milk protein synthesis. Seasonal minimum and maximum values of all monitored parameters are summarized in Table 2.

Table 2. Seasonal minimum and maximum of the monitored parameters

Parameter	Lowest Value (Season)	Highest Value (Season)
Fat (%)	Summer (3.83)	Autumn (4.20)
Protein (%)	Summer (3.49)	Autumn (3.68)
Lactose (%)	Spring (4.87)	Autumn (4.94)
Milk urea nitrogen (mg/dL)	Winter (26.57)	Spring (35.81)
Lactoferrin (mg/L)	Summer (153.79)	Autumn (171.96)

Lactose

Lactose was the most stable of all monitored milk components. Its values fluctuated only minimally, within a narrow range of approximately 4.87–4.94%. The lowest concentrations were recorded during winter and spring, whereas summer and autumn showed slightly elevated levels. No significant seasonal differences were observed.

Seasonal Differences in Milk Urea Nitrogen

Milk urea nitrogen (MUN) exhibited one of the most pronounced seasonal dynamics among the evaluated parameters. The lowest concentrations were observed in winter, with an average value of approximately 27 mg/dL, which was significantly lower than those in all other seasons ($p < 0.001$). In spring, summer, and autumn, MUN concentrations were markedly higher, ranging from 34 to 36 mg/dl, with the highest levels recorded in spring. No significant differences were found among spring, summer, and autumn, indicating that these periods shared similarly elevated urea nitrogen concentrations (Figure 1).

The increased MUN levels in spring and summer likely reflect a higher intake of rapidly degradable nitrogen during the grazing period, along with a less balanced dietary energy-to-protein ratio. The spring peak may be associated with the lush, nitrogen-rich pasture at the onset of the vegetation season, while summer levels reflect the continued presence of fresh forage. Conversely, the winter minimum suggests a more balanced ratio with a lower proportion of rapidly degradable protein.

Values exceeding 35 mg/dL are considered borderline in terms of reproductive performance, as chronically elevated MUN has been associated with a 5–10% reduction in conception rates. In our dataset, spring values most closely approached this threshold, suggesting a potential excess of dietary protein relative to energy early in the grazing season. Extremely low MUN values (<15 mg/dL), which would indicate protein deficiency in the ration, were not observed.

Seasonal Differences in Lactoferrin Content

The lowest mean values of lactoferrin were observed in summer (154 ± 52 mg/L) and were significantly lower than in all other seasons ($p < 0.001$). Higher values were recorded in autumn (172 ± 40 mg/L), while winter and spring exhibited intermediate concentrations. No significant differences were detected among spring, winter, and autumn values ($p > 0.05$), indicating that the observed seasonal variation was primarily due to lower lactoferrin concentrations in summer.

The reduction in lactoferrin during summer is likely related to heat stress, which can decrease feed intake, milk production, and mammary protein synthesis. The higher

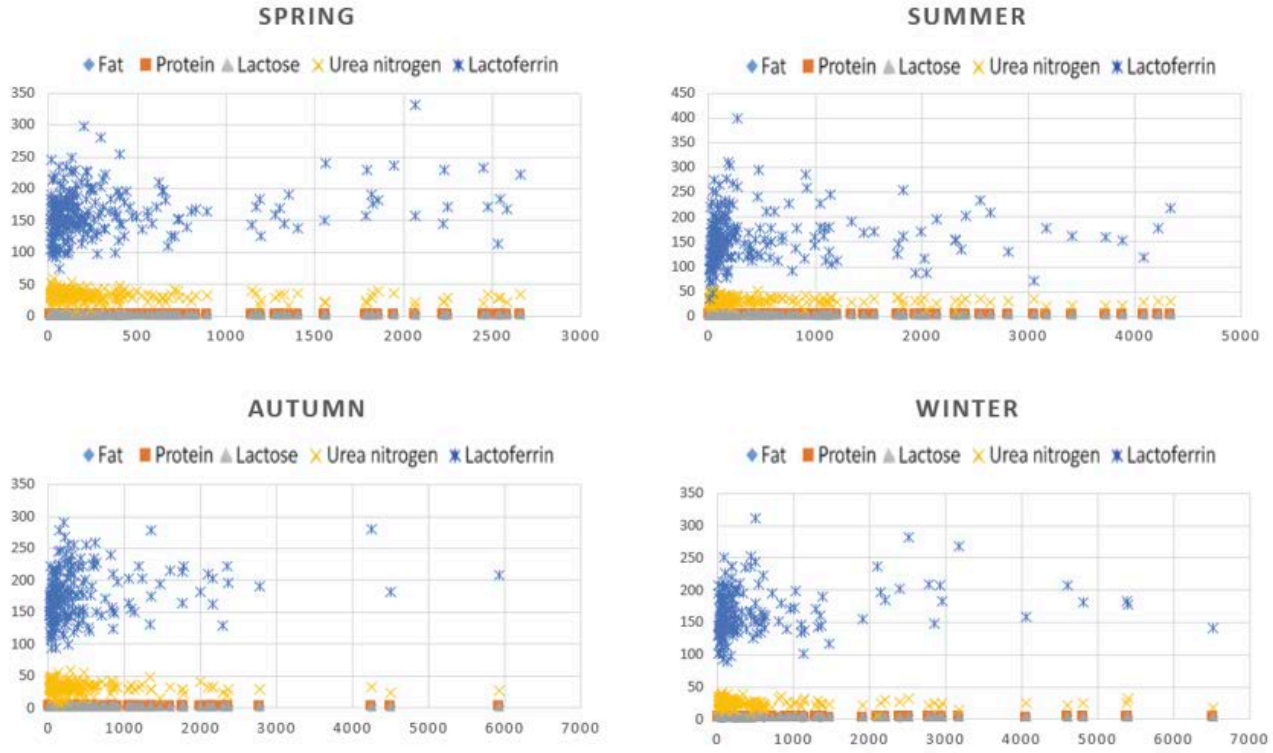

Fig. 1. Seasonal scatter distribution of milk composition parameters (fat, protein, lactose, urea nitrogen, lactoferrin)

concentrations observed in autumn likely reflect physiological changes associated with late lactation and the dry-off period, when lactoferrin naturally increases in milk as part of normal mammary gland function. Although lactoferrin, as an acute-phase protein, can reach considerably higher concentrations during inflammation or in colostrum, the seasonal variation observed in milk from healthy cows (approximately 150–170 mg/L) indicates predominantly physiological rather than pathological influences.

Somatic Cell Count

Somatic cell count (SCC) fluctuated between seasons; however, despite numerical differences, no statistically significant seasonal effect was detected ($p > 0.05$; $p = 0.0653$). The high between-cow variability, reflected in large standard deviations, likely masked more subtle seasonal patterns (Figure 2).

Geometric means showed that the lowest SCC values occurred in autumn (approximately 2.75×10^5 cells/mL). In contrast, higher values were recorded in winter and spring (around 3.6×10^5 cells/mL), with the highest levels observed during the summer months (approximately 4.2×10^5 cells/mL). These trends suggest that summer is the most critical period for milk hygiene, likely due to increased environmental stress and a higher bacterial load associated with warm, humid conditions. Median SCC values likewise confirmed that the cleanest milk was produced in autumn, whereas the highest SCC occurred in summer.

DISCUSSION

The results of this study confirm that season is a significant factor influencing the elemental composition of cow's milk as well as selected physiological indicators. The identified differences correspond with long-established trends described in the literature; however, the data obtained under large-scale farming conditions in eastern

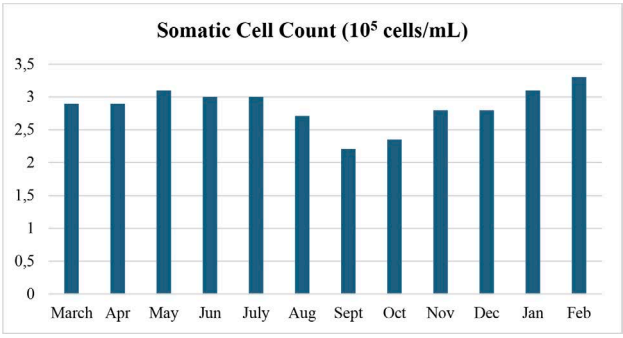

Fig. 2. Monthly and seasonal changes in Somatic Cell Count (×10⁵ cells/mL)

Slovakia provide an important contribution to knowledge derived from other climatic and production systems.

The lowest fat and protein contents were detected during the summer, which agrees with several studies reporting the negative effect of heat stress on milk solids concentration [5, 8]. Elevated temperatures, reduced feed intake, and metabolic adjustments reduce fatty acid synthesis, resulting in lower milk fat concentration. At the same time, altered degradation and utilisation of nitrogenous compounds may contribute to reduced protein content. Conversely, the highest values of these parameters were recorded in autumn, when environmental conditions are more stable, and cows are often in later stages of lactation, which may positively influence milk composition.

Comparable seasonal patterns have also been described in Central and Eastern European production systems. Studies from Poland, the Czech Republic, Hungary, and the Balkans report a similar decline of fat and protein during summer and their increase in autumn and winter, largely attributed to heat stress and changes in feeding strategies [18]. Similar autumn–winter increases in milk fat and protein were documented in Poland under grazing/feeding-season conditions [19], while Hungarian monitoring data likewise confirmed clear within-year seasonal shifts in raw milk fat and protein [20]. Consistent results have also been reported from the Balkan region, where multi-year datasets showed the lowest fat and protein values during summer and the highest during winter [18]. Evidence from Slovakia further supports this regional pattern, with the lowest fat and protein contents repeatedly observed in summer [21]. The magnitude of seasonal differences observed in the present study therefore corresponds well with data from neighbouring countries, indicating that climatic and nutritional drivers of milk composition are highly comparable across this region.

Lactose concentration remained relatively stable throughout the year, reflecting its fundamental biological function and its strong link to milk's osmotic pressure [7]. Lactose is less sensitive to nutritional or environmental fluctuations, and therefore its variability is generally lower than that of fat and protein.

Milk urea nitrogen showed one of the most pronounced seasonal fluctuations. The highest values in spring and summer are typical for the grazing period, when the intake of rapidly degradable nitrogen is increased [9, 6]. Excessive MUN levels may indicate an imbalance between dietary nitrogen and available energy. This can negatively influence not only milk composition but also fertility and metabolic load in dairy cows. The low MUN values observed during winter reflect a more stable ratio based on conserved forage.

Lactoferrin concentrations were lowest in summer and highest in autumn. Lactoferrin is an immunologically active mammary gland protein [10]. The summer decrease likely reflects physiological factors such as reduced protein synthesis during late lactation, whereas the autumn increase is associated with the late stage of lactation and the dry-off period.

However, the magnitude of seasonal variation is strongly influenced by local environmental and production conditions. The studied farm is located in a mountainous area of eastern Slovakia, characterised by cooler winters, relatively mild summers, and a shorter vegetation period compared with lowland regions. These conditions influence forage availability, grazing duration, and the structure of the ration during the year. In such areas, the transition between pasture-based and conserved-forage feeding is more pronounced, which may explain the clear seasonal differences observed in fat, protein, MUN, and lactoferrin. Unlike lowland intensive systems, where feeding regimes are often more uniform throughout the year, mountain production zones show stronger contrasts between winter and summer diets, as well as greater sensitivity to climatic stress. Therefore, although the general effect of season on milk composition is well known, region-specific studies such as this one are necessary to understand how global trends are modified by local climate, altitude, and feeding systems.

Somatic cell count did not exhibit statistically significant seasonality in this study, although a trend toward higher SCC in summer was observed. This trend is consistent with findings from other authors [22], who report elevated SCC at temperatures above 25°C due to increased bacterial load, reduced environmental hygiene, and decreased cow comfort [7]. It is important to note that the average SCC throughout the year was approximately 3.6×10^5 cells/mL, which is below the EU legislative limit for raw milk (400,000 cells/mL), indicating that the cows were clinically healthy throughout the study period. Milk with elevated SCC contains less lactose and casein and higher levels of proteolytic enzymes, which negatively affect cheese making properties. The high individual vari-

ability in SCC likely contributed to the lack of statistical significance, despite the summer trend.

Several biologically relevant relationships between milk composition and physiological indicators were observed. Milk protein content tended to be lower when MUN values were higher, which may reflect metabolic strain caused by an excess of rapidly degradable nitrogen without an adequate energy supply. Similar trends have been reported in other studies, where elevated MUN was associated with reduced milk protein and poorer fertility [9]. No consistent association between lactoferrin and SCC was observed in this study. SCC fluctuated seasonally, with the highest values during summer, likely due to increased environmental stress and higher bacterial load. Lactoferrin concentrations were within the physiological range (approximately 150–170 mg/L), reflecting normal seasonal changes associated with late lactation and the dry-off period rather than pathological processes. While lactoferrin can reach higher levels during subclinical or clinical mastitis, such conditions were not detected in this herd.

The inclusion of lactoferrin alongside traditional milk quality indicators provides a more comprehensive understanding of mammary gland physiology and its seasonal dynamics. In this context, the analysis confirms that nutritional management and udder health monitoring should take into account the seasonal dynamics of the parameters assessed. Regular assessment of indicators such as MUN, lactoferrin, and SCC can help optimize lactation strategy, improve reproductive and production performance, and increase technological milk quality, while also creating a solid foundation for further research into the interactions between nutrition, physiological status, and mammary gland defense mechanisms.

CONCLUSIONS

The results of the year-long monitoring of raw cow's milk quality under intensive dairy production conditions in eastern Slovakia confirmed that the season is a significant factor influencing the elemental composition of milk and selected physiological indicators. The lowest fat and protein contents were recorded during the summer, whereas the highest values of both components occurred in autumn. Lactose remained relatively stable throughout the year, showing the lowest seasonal variability of all monitored constituents.

Milk urea nitrogen displayed the most pronounced seasonal dynamics – low winter values reflected a stable ration, while elevated concentrations in spring and summer indicated an excess of rapidly degradable nitrogen during the vegetation period. Lactoferrin concentrations were lowest in summer and highest in autumn, likely reflecting physiological changes associated with the late stage of lactation and the dry-off period.

Somatic cell count (SCC) did not differ significantly among seasons, although a trend toward higher values was observed during the summer months, possibly reflecting increased mammary gland stress.

Overall, the analysis confirms that nutritional management and udder health monitoring should account for the seasonal dynamics of the parameters under evaluation. Regular assessment of indicators such as MUN, lactoferrin, and SCC can help optimise feeding strategies, improve reproductive and production performance, and enhance the technological quality of milk. These results provide a valuable basis for further research into the interactions between nutrition, physiological status, and the defence mechanisms of the mammary gland.

Data Availability Statement

The raw data of this article will be made available by the authors, without undue reservation.

Ethical Statement

Ethical approval was not necessary for this work.

Conflict of Interest

No conflict of interest.

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Generative AI Statement

Authors disclosed that no form of generative AI or AI-assisted technologies was used in the writing process.

Authors' Contributions

S.O. contributed to the study design, provided methodological supervision, assisted with data interpretation, and critically revised the manuscript, including statistical analysis.

J.Z. conceived and designed the study, performed the laboratory analyses, and prepared the manuscript draft.

F.Z. carried out sampling.

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