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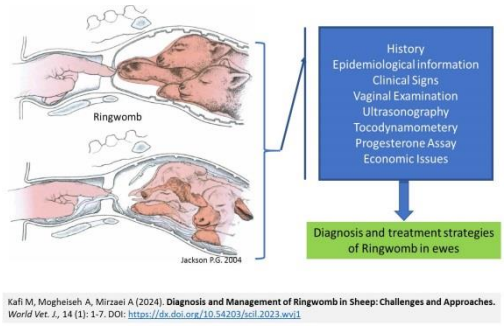
Volume 14 (1); March 25, 2024

Review

Diagnosis and Management of Ringwomb in Sheep: Challenges and Approaches

Kafi M, Mogheiseh A, Mirzaei A.

World Vet. J. 14(1): 1-7, 2024; pii:S232245682400001-14
DOI: https://dx.doi.org/10.54203/scil.2024.wvj1



Kafi M, Mogheiseh A, Mirzaei A (2024). **Diagnosis and Management of Ringwomb in Sheep: Challenges and Approaches.** *World Vet. J.*, 14 (1): 1-7. DOI: https://dx.doi.org/10.54203/scil.2023.wvj1

ABSTRACT: Small ruminants, including sheep and goats, constitute a major part of the livestock population in different countries of the world. About 15-32% of sheep dystocia are due to incomplete dilation of the cervix which is often called ringwomb. This disorder typically occurs during the lambing process, prompting farmers to possibly seek veterinary assistance due to delayed labor. Different causes, such as calcium and phosphorus deficiency, uterine inertia, and fetal inability to enter the cervical canal, could all lead to incomplete dilation of the cervix. In the meantime, many cases of ringwomb occur as idiopathic. Some factors, such as genetics, nutrition, and imbalance of estrogen to progesterone concentration may also contribute to incomplete dilation of the cervix. In practice, it is important to differentiate the ringwomb with similar conditions such as false dilation of the cervix, early dilation syndrome, and vaginal prolapse associated with ringwomb. In this review, the definition of ringwomb in sheep, an exploration of the structure of the sheep's cervix, the normal process of cervix dilation during lambing, etiology and symptoms of ringwomb, differential diagnosis, and different treatments are discussed.
Keywords: Cervix, Collagen, Dystocia, Sheep, Ringwomb

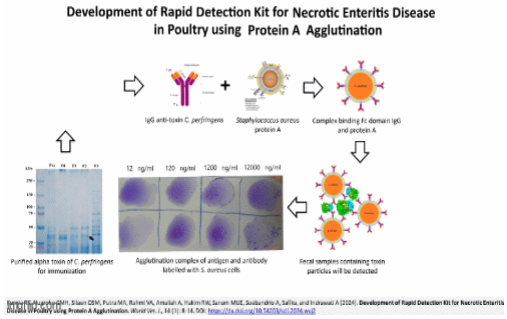
[Full text-PDF] [Crossref Metadata]

Research Paper

Development of Rapid Detection Kit for Necrotic Enteritis Disease in Poultry using Protein A Agglutination

Kurnia RS, Nugroho CMH, Silaen OSM, Putra MA, Rahmi VA, Amaliah A, Hakim RW, Sanam MUE, Soebandrio A, Safika, and Indrawati A.

World Vet. J. 14(1): 8-14, 2024; pii:S232245682400002-14
DOI: https://dx.doi.org/10.54203/scil.2024.wvj2



ABSTRACT: Necrotic enteritis causes significant losses in the global poultry industry, necessitating accurate diagnosis for effective intervention. This study aimed to develop a diagnostic tool for detecting necrotic enteritis in poultry based on the presence of *Clostridium perfringens* (*C. perfringens)* Alpha-toxin in poultry feces. The reagent of the detection kit was developed by conjugation of IgG against *C. perfringens* toxin and *Staphylococcus* cells containing protein A. The IgG antibody was derived from an 8-month-old thin-tailed male sheep immunized with purified 2 ml of *C. perfringens* Alpha-toxin. Sensitivity assays were carried out to determine the detection limit, while *Escherichia coli* (*E. coli*) and *Salmonella enteritidis* (*S. enteritidis*) were used to identify specificity. A purified Alpha-toxin with a protein concentration of 2.8 mg/ml and a specific molecular weight of 43 kDa was successfully obtained. A strong reaction of the hyperimmune antibody (IgG) was also detected in the thin-tailed male sheep serum. The developed rapid detection kit in this study indicated *C. perfringens* Alpha-toxin with a lower concentration (12 ng/ml). Agglutination reactions could differentiate positive control from negative without significant cross-reactivity towards other bacteria (*S. enteritidis* and *E. coli*).
Keywords: Agglutination, *Clostridium perfringens*, Detection, Necrotic Enteritis, Toxin

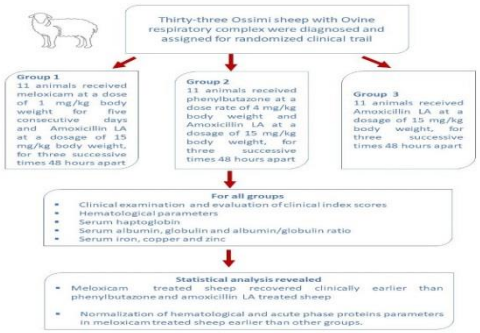
[Full text-PDF] [Crossref Metadata]

Research Paper

Comparative Effects of Meloxicam and Phenylbutazone on Clinical Outcomes and Acute Phase Response in Sheep with Acute Respiratory Disease

Alwayel A., Marzok M, Gioushy M, Kandeel M, Almubarak A, Hamad Y, Shousha S, El-khodery S.

World Vet. J. 14(1): 15-25, 2024; pii:S232245682400003-14
DOI: https://dx.doi.org/10.54203/scil.2024.wvj3



ABSTRACT: The ovine respiratory complex (ORC) is one of the most common respiratory diseases observed in sheep. The objective of the present investigation was to evaluate the comparative therapeutic efficacy of two non-steroidal anti-inflammatory drugs (meloxicam and phenylbutazone) for the treatment of the ORC. For this purpose, 33 Ossimi sheep were randomly assigned into three treatment groups (11 each). Group 1 was treated with amoxicillin long-acting (LA) and meloxicam, group 2 received amoxicillin LA and phenylbutazone, and group 3 was treated with amoxicillin LA alone. Sheep were examined clinically and clinical index scores were recorded before and after treatment. Additionally, blood samples were collected from each sheep. After 14 days of treatment, sheep of group 1 indicated significant improvements in their clinical index scores and a reduction in total leukocyte count. However, there was a significant increase in red blood cell count, hemoglobin, and MCHC%. There was a significant decrease in the serum globulin, copper, and haptoglobin in group 1, compared to sheep of groups 2 and 3. Based on the results of this study, administering meloxicam to sheep with an ovine respiratory complex resulted in significant improvements in clinical outcomes and significant corrections in above mentioned hematological and biochemical parameters. Although phenylbutazone proved to be less effective, it still demonstrated some degree of efficacy in treating this condition. This study suggests that meloxicam may be a more effective treatment option for ORC with phenylbutazone.
Keywords: Acute phase protein, Amoxicillin, Anti-inflammatory, Ovine respiratory complex

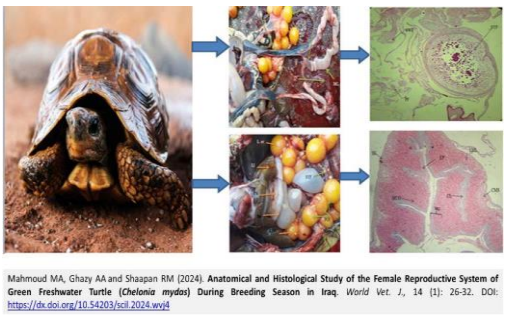
[Full text-PDF] [Crossref Metadata]

Research Paper

Anatomical and Histological Study of the Female Reproductive System of Green Freshwater Turtle (*Chelonia mydas*) During Breeding Season in Iraq

Simawy MSH, AL-Khakani SSA, Fadhil M, Jawad AI, Al-Janabi SM, Al-Rubaie DM, and Hamad RS.

World Vet. J. 14(1): 26-32, 2024; pii:S232245682400004-14
DOI: https://dx.doi.org/10.54203/scil.2024.wvj4



ABSTRACT: Turtles are found in large numbers in Iraqi rivers, due to the availability of a suitable environment for reproduction and food. The breeding season begins from May to the end of October. The current study aimed to evaluate the anatomical and histological characteristics of the green freshwater turtle (*Chelonia mydas*) during the breeding season in Iraq. The samples included eight adult turtles within the age range of 11-14 years that were collected from Shatt Al-Hilla (Iraq) at month June. To investigate the reproductive system histological techniques and hematoxylin and eosin staining were used and before that the animals were anesthetized with chloroform. The results indicated two active ovaries and oviducts which fill the whole abdominal cavity. The mean weights of left and right ovaries and left and right oviducts in the turtles with average weights of 698 ± 0.05 g were 19.5 ± 0.01 g, 22 ± 0.022 g, 3.3 ± 0.05 g, and 4 ± 0.05 g respectively. The mean lengths of carapace, left ovary, right ovary, left oviduct, and right oviduct were 24 ± 0.08 cm, 15.9 ± 0.01 cm, 17± 0.04 cm, 13 ± 0.022 cm, 14 ± 0.056 cm. Anatomically the oviducts include the infundibulum, magnum, isthmus, uterus, and vagina. The infundibulum indicated a funnel-shaped membrane while the magnum was the muscular coiled long tube. The isthmus was shorter and less coiled than the magnum, the uterus appeared as the widest, thickest, and less coiled dark tube and swollen into the posterior to form a cyst-like part, and the vagina was muscular in structure. Histologically, the magnum and uterus were formed from mucosa, muscularis, and serosa. In both parts of the magnum and uterus, were branched crypt-like depressions that appeared devoid of sperm. The widespread distribution of this species in Iraqi rivers could be due to the activity of the ovaries and oviducts during the breeding season, which extends for 6 months.
Keywords: *Chelonia mydas*, Ovary, Oviduct, Turtle

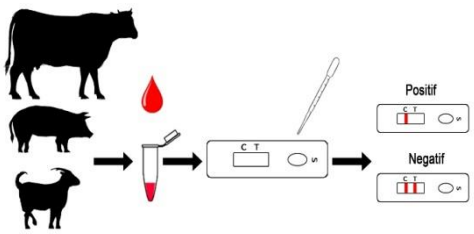
[Full text-PDF] [Crossref Metadata]

Research Paper

Comparative Analysis of Lateral Flow Assay with Indirect ELISA for Detection of Anti-NSP Antibodies of Foot and Mouth Disease

Jauhari A, Munawaroh S, Arnafia W, Sibit D, Rahmahani J, and Suwarno S.

World Vet. J. 14(1): 33-37, 2024; pii:S232245682400005-14
DOI: https://dx.doi.org/10.54203/scil.2024.wvj5



ABSTRACT: Foot-and-mouth disease (FMD) was an exceedingly infectious disease that spread to Indonesia in May 2022. A reliable diagnostic serologic test that can distinguish between infected and vaccinated animals was an important part of FMD (serotype O) control programs in affected areas in Indonesia. For this reason, a non-structural protein (NSP) serological test based on 3ABC proteins has been used. The indirect ELISA serological test requires time, skill, and specialized equipment. An alternative method that can be employed is the lateral flow assay (LFA), which offers the advantages of simplicity and portability, enabling rapid acquisition of results. The objective of this study was to validate the efficacy of a user-friendly anti-NSP antibody LFA for rapid diagnostic purposes. This was done by assessing its sensitivity and specificity in stored samples that had previously been tested using indirect ELISA. There were 32 preserved biological materials from dairy and beef cattle in three provinces in Indonesia that were examined with developed LFA. The results of each sample on LFA were compared to the ELISA result for its sensitivity and specificity according to positive and negative values on both tests. The test had a sensitivity of 95.2% and a specificity of 100%, compared to the indirect ELISA. The measured kappa value is also very good at 0.93, so LFA can be optionally used when examining anti-NSP FMD antibodies. Therefore, the LFA anti-NSP for detecting FMD is considered reliable because of its simplicity and the accuracy of the test results.
Keywords: Antibody, Bovine serum, Foot and mouth disease, Indirect ELISA, Lateral flow assay

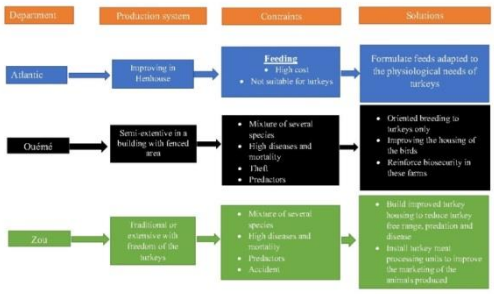
[Full text-PDF] [Crossref Metadata]

Research Paper

Constraints to the Development of Turkey Farming in Southern Benin

Dotché IO, Agbokounou A, Baba L, Adebo N, Okambawa L, Koffi M, And Youssao Abdou Karim I.

World Vet. J. 14(1): 38-52, 2024; pii:S232245682400006-14
DOI: https://dx.doi.org/10.54203/scil.2024.wvj6



ABSTRACT: The turkeys are reared throughout the national territory of Benin, but their breeding is less developed than other poultry species, such as chickens and ducks. The current study aimed to characterize turkey farming in Southern Benin to identify the constraints associated with the farming practice that limit its development. A survey was performed in 104 turkey farms in the Atlantic, Ouémé, and Zou departments. The frequencies of qualitative variables and average quantitative variables were calculated and compared across departments. The investigated variables included turkey housing, feeding practices, reproduction management, health management, difficulties encountered, marketing of animals, and farm products. It was found that the housing, feeding, health monitoring, and constraints varied from one department to another. The turkeys were raised in fence-run buildings in the Ouémé (76.7%), modern poultry houses in the Atlantic (75%), and traditional habitats (42.9%) with a free range in the Zou. The free range prevented farmers from separating the turkeys from other poultry species. The poultry species present with turkeys on studied farms were chickens, ducks, and guinea fowl. The turkeys were fed more with commercial feed in the Atlantic (100%) and Ouémé (92.7%) regions and with cereals and agricultural by-products in Zou (82.1%). The prophylaxis consisted of deworming the birds, vaccinating them against Newcastle disease, controlling bacterial infections with antibiotics, and giving them vitamins in drinking water. The farmers vaccinated more turkeys in Zou than in Atlantic and Ouémé. The vaccination and administration of antibiotics do not prevent the introduction of disease into farms due to poor farm biosecurity, resulting in animal deaths. In conclusion, this study identified the obstacles that limit the development of turkey farming by region in Southern Benin. These barriers are primarily related to housing, feeding, mating, and marketing. Scientific research could potentially solve some of these issues, notably those concerning feeding and mating success. However, housing and marketing concerns would necessitate support from the authorities.
Keywords: Constraint, Feeding, Prophylaxis, Turkey

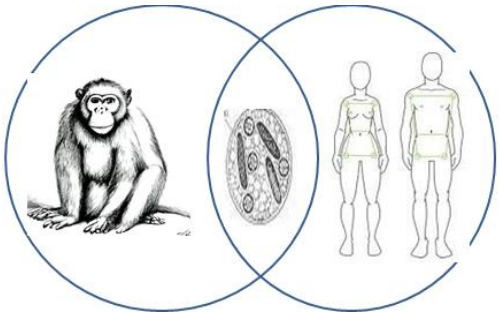
[Full text-PDF] [Crossref Metadata]

Research Paper

Molecular Detection of *Entamoeba* spp. in Monkeys (*Macaca* spp.) in Babylon Province, Iraq

Abdul Abbas ZT and Aziz Anah S.

World Vet. J. 14(1): 53-57, 2024; pii:S232245682400007-14
DOI: https://dx.doi.org/10.54203/scil.2024.wvj7



ABSTRACT: Amoebiasis is a widespread parasitic disease caused by *Entamoeba histolytica* (*E. histolytica*), affecting various hosts, such as humans, birds, and pigs. This study aimed to investigate *Entamoeba* spp. in monkeys (*Macaca* spp.) diagnose them using molecular methods. A total of 33 fecal samples were collected from monkeys (*Macaca* spp.) aged 3-5 years in Babylon province to investigate a common and zoonotic parasitic disease. Initially, microscopic examination was conducted on all samples, and those yielding positive results were preserved for molecular study. The DNA was extracted, and conventional PCR was carried out with a pair of primers to detect the 857 bp fragment of *E. histolytica* SSU rRNA gene. PCR results for 19 fecal samples, previously identified as positive by the direct smear method, from monkeys in the reserves of Babylon province indicated that the presence of the SSUrRNA gene with an 857 bp was 45% in only 15 samples. Sequencing of the SSUrRNA gene revealed 98-100% similarity with *E.histolytica* sequences deposited in International GenBank, which have the sequence numbers OP522013, OP522014, OP522015, OP522016, OP522017, Op626161, Op626162, Op626163, Op626164, and Op626165.
Keywords: *Entamoeba histolytica*, Gene, Monkey, SSU rRNA

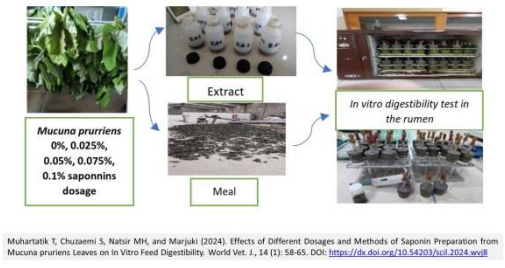
[Full text-PDF] [Crossref Metadata]

Research Paper

Effects of Different Dosages and Methods of Saponin Preparation from *Mucuna pruriens* Leaves on In Vitro Feed Digestibility

Muhartatik T, Chuzaemi S, Natsir MH, and Marjuki.

World Vet. J. 14(1): 58-65, 2024; pii:S232245682400008-14
DOI: https://dx.doi.org/10.54203/scil.2024.wvj8



ABSTRACT: The *Mucuna pruriens* is commonly used in traditional medicine for anti-inflammatory, antibacterial, neuroprotector, antidiabetic, and anti-cancer purposes. The bioactive compounds, such as flavonoid, tannin, and saponin, could improve feed digestion efficiency in ruminants' rumen. The current study aimed to evaluate the effects of different dosages and the two methods of saponin preparation from *Mucuna pruriens* leaves on *in vitro* feed digestibility parameters. A randomized block design with nested arrangements (2×5×3) was used in this study. Two methods of obtaining saponins from *Mucuna pruriens* leaves, including meal (MPLM) and extract (MPLE) of *Mucuna pruriens* leaves, were compared. The nested treatments of the preparation methods were the dosages of the saponin as feed additives in feed samples, involving 0%, 0.025%, 0.050%, 0.075%, and 0.10%. There were 15 samples in each group (five-level dosage and three repetitions). The feed contained 40% forage and 60% concentrate. The obtained results indicated that saponin preparation from *Mucuna pruriens* leaves (MPLM and MPLE) significantly affected dry matter, organic matter, and crude fiber rumen degradability (r-DMD, r-OMD, r-CFD, respectively), as well as NH_3, volatile fatty acid, propionate, butyrate concentrations, acetate-to-propionate (A/P) ratio, acetate, and propionate percentage. However, there was no significant impact on protozoa population, acetate concentration, butyrate percentage, *in vitro* dry matter digestibility (IVDMD), and *in vitro* organic matter digestibility (IVOMD). The MPLM saponin revealed significantly higher values on digestibility parameters except for protozoa, A/P ratio, and acetate percentage. The MPLM saponin dosage of 0.05% showed the highest values for r-DMD (56.48%), r-OMD (56.51%), and r-CFD (54.64%), total Volatile fatty acid (77.71 mM), propionate (21.57 mM), propionate percentage (27.76%), IVDMD (65.95%), and IVOMD (65.86%), but lowest in A/P ratio (2.04). In conclusion, the findings of the present study suggest that the MPLM saponin at a dosage of 0.05% holds promising potential for enhancing the fermentation profile in ruminants.
Keywords: *In vitro*, *Mucuna pruriens*, Nutrient digestibility, Rumen fermentation, Saponin

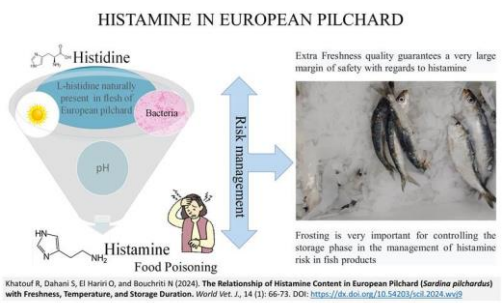
[Full text-PDF] [Crossref Metadata]

Research Paper

The Relationship of Histamine Content in European Pilchard (*Sardina pilchardus*) with Freshness, Temperature, and Storage Duration

Khatouf R, Dahani S, El Hariri O, and Bouchriti N.

World Vet. J. 14(1): 66-73, 2024; pii:S232245682400009-14
DOI: https://dx.doi.org/10.54203/scil.2024.wvj9



ABSTRACT: Histamine food poisoning, stemming from the consumption of certain histamine-rich fish species, such as tuna, mackerel, European pilchards, and herring, is one of the major public health issues worldwide. The present study aimed to evaluate the histamine content in fresh European pilchard (*Sardina pilchardus* Walbaum, 1792) of Mehdia, a coastal city in the north of Morocco. Three randomly selected batches of fresh European pilchards, each weighing 20kg, were obtained from different boats upon landing. The evolution of histamine production was monitored every 8 hours for 6 days, with one batch stored at 0°C and the other at 10°C. The organoleptic characteristics were examined considering sensory evaluation according to the rating system of European Council Regulation No. 2406/96 as common marketing standards for certain fishery products and the quality index method (QIM). The histamine content in European pilchard flesh was determined using the fluorometric method. The results indicated that the average histamine content did not exceed 5 ppm during storage at 0°C. The freshness ratings were highest during the first 3 days, corresponding to QIM values of 0 to 10 at 0°C. On days 4 and 5, the freshness ratings were on quality A, corresponding to QIM values of 11 and 12, and on the last day, they were on quality B, corresponding to a QIM value of 15 with preservation of the organoleptic quality. Statistically, a significant correlation was found between the European pilchard's freshness and the storage duration. In contrast, this correlation between the histamine content and the storage duration was insignificant. At 10°C, the average histamine content exceeded the regulatory limit in force (100 ppm) after 32 hours of storage, and spoilage occurred on day 3. Statistical analysis revealed a strong correlation between the histamine content, storage temperature, the degree of freshness, and the duration of storage. The extra freshness quality index of European pilchard guarantees a very large margin of safety regarding histamine and can be consumed without risk.
Keywords: Degree freshness, European pilchard, Histamine, Sardine, Storage, Temperature

[Full text-PDF] [Crossref Metadata]

Research Paper

The Influence of Mannan Oligosaccharides and Beta Glucan Supplementation on Growth Performance, Blood Constituents, and Cecal Parameters of Broiler Chickens

Osman MA, Motawe HFA, Shoukry MM, El-Komy EM, Khattab MS, Radwan AS, and El-Sayed Hamouda R.

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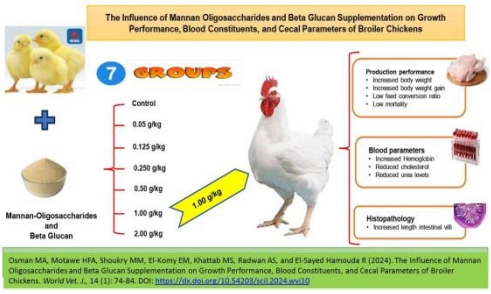
DOI: https://dx.doi.org/10.54203/scil.2024.wvj10



ABSTRACT: Growth promoters in poultry feed have been under severe attention since antibiotics were banned for use in animal diets by the European Union. Thus, it has been important for poultry researchers to find alternatives to antibiotic growth promoters (AGPs) to boost the health and production performance of poultry. This research was conducted to evaluate the effects of adding ALTIMOS® (cell wall of *Saccharomyces cerevisiae*; mannan oligosaccharides [MOS] + beta-glucan [BG]) to broiler diets on productive performance, blood parameters, intestine histopathology, and cecum microbiota of broiler chicken. A total of 252 one-day-old Ross chicks were randomly selected and divided into seven treatments, with six replicates of each treatment. The treatments were the control group (0% feed additives), and groups that received 0.05, 0.125, 0.250, 0.500, 1.0, and 2.0 g MOS+BG /kg basal diet for 35 days feeding trial. The results showed that during most trial periods, the group fed the basal diet supplemented with 1.0 g MOS+BG/kg had the highest body weight and weight gain, as well as the lowest feed consumption and best feed conversion ratio, compared to other treated groups. Moreover, this group had the best productive performance in the accumulative period. The inclusion of MOS+BG at 1.0 g/kg diet showed no significant effect on carcass percent compared to the control group. In addition, the inclusion of MOS+BG at 1.0 g/kg diet resulted in the lowest count of *Escherichia coli* and *Enterococcus* in the cecum, the highest *Lactobacillus* bacteria count among all experimental treatments, and a higher yeast count compared to the control group. The group fed 1.0 g MOS+BG/kg ration had the lowest blood cholesterol, whereas there were no significant differences among all experimental groups in the measured liver functions. Notably, the Hemoglobin percentage in the

group fed MOS+BG at 1.0 g/kg feed was the highest. In the group fed 1.0 and 2.0 g MOS+BG/kg, the intestinal villi length was longer, and the histopathology revealed mild alteration. Overall, the supplementation of 1.0 g MOS+BG/kg diet improved growth performance, blood constituents, and cecum's beneficial bacteria counts of broilers.
Keywords: Beta-glucan, Blood constitute, Broiler chicken, Cecal parameter, Growth Performance, Mannan Oligosaccharide

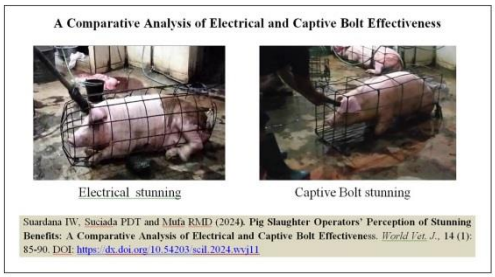
[Full text-PDF] [Crossref Metadata]

Research Paper

Pig Slaughter Operators' Perception of Stunning Benefits: A Comparative Analysis of Electrical and Captive Bolt Effectiveness

Suardana IW, Suciada PDT and Mufa RMD.

World Vet. J. 14(1): 85-90, 2024; pii:S232245682400011-14
DOI: https://dx.doi.org/10.54203/scil.2024.wvj11



ABSTRACT: The pre-slaughter phase, which includes stunning, aims to reduce animal stress, ensuring a more compassionate and efficient process in the meat industry. Various methods are often used in slaughtering pigs, with electrical, mechanical, and chemical stunning being the most common techniques. Several studies have shown that selecting the appropriate method requires operators to comprehensively understand the slaughter process. Therefore, this study focused on evaluating the comprehension of pig slaughter operators regarding the benefits and effectiveness of electrical and captive bolt stunning methods. A total of 17 pigs slaughtered from seven slaughterhouses were selected as samples. Data collection was carried out using questionnaires, interviews, and direct observation. The results showed that operators clearly understood the benefits of stunning in terms of speed and ease. However, their comprehension regarding pig stress reduction before death remained limited. Although captive bolt stunning was known to have various benefits, such as shortening the duration of leg movements after slaughter, it required longer operation time, compared to the electrical method. Therefore, it can be concluded that there is no ideal stunning method as both methods of electrical and captive bolt stunning have their respective advantages and disadvantages.
Keywords: Captive bolt stunning, Effectiveness, Electrical stunning, Comprehension of operator

[Full text-PDF] [Crossref Metadata]

Research Paper

Production Performance and Some Biochemical Traits of Layer Hens Fed on Date Palm Kernel Supplementation

Al-Zuhairi ZA, Mhyson AS, and Mohammed BJ.

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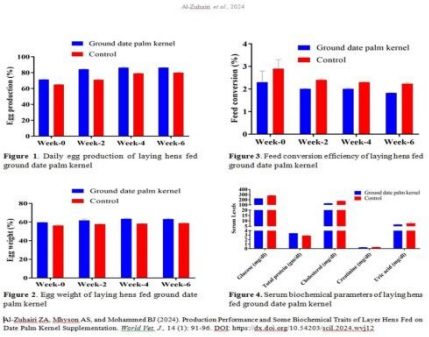
DOI: https://dx.doi.org/10.54203/scil.2024.wvj12



ABSTRACT: To enhance the well-being and productivity of poultry, researchers have conducted investigations into various botanical sources, including date palm kernel, and their bioactive components. The present investigation was conducted to assess the productive performance and certain biochemical characteristics of layer hens that were administered date palm kernel supplementation in their diet. To this end, 40 ISA Brown laying hens (48 weeks old) were used in the current study. The adaptation phase for the chickens lasted for 10 days before the initiation of the experiment. The study lasted 8 weeks. The chickens were then randomly assigned into two groups of 20, with 4 replications (5 chickens per replication). Chickens in the treatment group received 5% of dry matter ground date palm kernel (GDPK), as an additive to the basal diet, and the control group (CL) chickens were fed the basal diet. The eggs were collected daily during the study. At the end of weeks 1, 2, 4, and 6 of the study, egg production percentage, egg weight, and the feed conversion ratio were measured. At the end of the experiment, blood samples were collected to measure the serum levels of glucose, total protein, cholesterol, creatinine, and uric acid. The study findings revealed significant increases in the daily egg production percentage and egg weight during the experiment in the GDPK group, compared to the CL group. The feed conversion ratio recorded significant decreases in the GDPK group when compared to the CL group. Furthermore, the findings indicated significant increases in the serum total protein and significant decreases in the glucose, cholesterol, creatinine, and uric acid concentrations in the GDPK group, compared to the CL group. In conclusion, the results indicated the positive effects of adding ground date palm kernel to the diet of layers on production performance, such as egg weight, conversion ratio, and some biochemical traits, including total protein, glucose, cholesterol, creatinine, and uric acid.

Keywords: Date palm, Egg production, Feed additive, Kernel, Laying hen

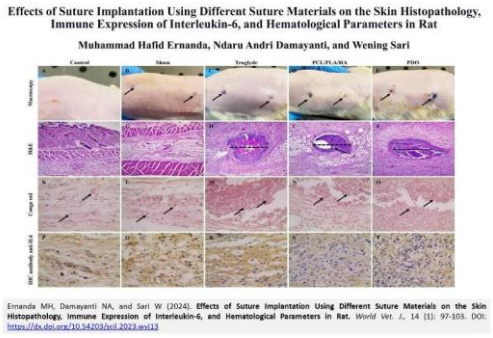
[Full text-PDF] [Crossref Metadata]

Research Paper

Effects of Suture Implantation Using Different Suture Materials on the Skin Histopathology, Immune Expression of Interleukin-6, and Hematological Parameters in Rat

Ernanda MH, Damayanti NA, and Sari W.

World Vet. J. 14(1): 97-103, 2024; pii:S232245682400013-14
DOI: https://dx.doi.org/10.54203/scil.2023.wvj13



ABSTRACT: Suture implantation is a procedure to promote rearrangement of the extracellular matrix. Various cellular responses of post-suture implantation affect the outcome of this procedure. The current study aimed to analyze the effects of suture implantation using polycaprolactone/polylactic acid/hyaluronic acid (PCL/PLA/HA) on skin histopathology, expression of IL-6, and hematological parameters in rat models. To conduct the study, 25 male Sprague Dawley rats, three months old were randomly divided into five groups, including G1 (control), G2 (sham, group injected using skin cannula), and G3-G5 (suture implanted groups). For the suture-implanted groups, a cannula was used using suture materials. Specifically, G3 received truglyde implants, G4 received PCL/PLA/HA implants, and G5 received polydioxanone (PDO) implants. The back skin and blood samples were collected on day 3. Histopathological analysis was conducted on the samples using H and E, Congo red, immunohistochemistry against IL-6, and hematology. The analysis of the data revealed that the group with suture implantation using PCL/PLA/HA had the smallest wound area, compared to the other implanted groups. Further, the PCL/PLA/HA group showed a significant decrease in eosinophils infiltration and IL-6 level on the skin samples after suture implantation. Moreover, there were no significant differences across the groups in most of the hematological parameters after suture implantation, including total erythrocytes, hemoglobin, eosinophil, basophil, and monocyte levels. The total neutrophils increased after suture implantation in all groups, while the total lymphocytes decreased. It can be concluded that the best material according to parameters evaluated in the current study for suture implantation was PCL/PLA/HA.
Keywords: Eosinophil, Histopathology, Hyaluronic Acid, Interleukin-6, Polycaprolactone, Polylactic Acid, Suture implantation

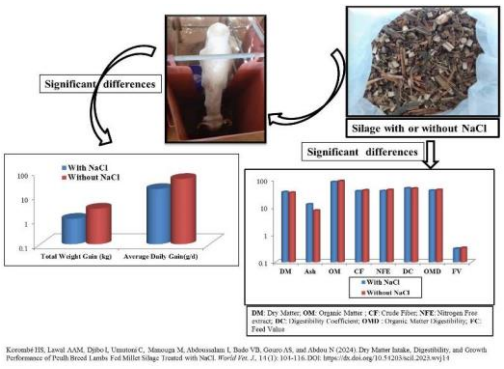
[Full text-PDF] [Crossref Metadata]

Research Paper

Dry Matter Intake, Digestibility, and Growth Performance of Peulh Breed Lambs Fed Millet Silage Treated with NaCl

Korombé HS, Lawal AAM, Djibo I, Umutoni C, Manouga M, Abdoussalam I, Bado VB, Gouro AS, and Abdou N.

World Vet. J. 14(1): 104-116, 2024; pii:S232245682400014-14
DOI: https://dx.doi.org/10.54203/scil.2023.wvj14



ABSTRACT: Livestock feeding is a major challenge in Niger. The aim of this study conducted at the International Crops Research Institute for the Semi-Arid Tropics (ICRISAT) experimental station in Sadoré, Niger, was to assess the effects of adding 1% NaCl to millet stover silage on the dry matter intake, digestibility, and weight performance of Peulh-bred lambs. Four treatments were tested, consisting of millet stover silages of two cultivars (Siaka Millet and Local Sadoré) with or without adding NaCl. The biological material included 32 lambs of Peulh breed Niger aged around 15 months with an average weight of 28.64 kg. They were divided into four blocks of homogeneous average weight and for each block, there were eight lambs. Each treatment was randomly assigned to a block. The trial lasted 75 days, including 15 days of adaptation and 60 days of data collection. Weight evaluation of animals was recorded, and bromatological analyses were carried out. Results indicated that there were significant differences between silages, according to NaCl addition and treatment, for some parameters of chemical composition, feed value, and zootechnical parameters of lambs. Depending on the treatment, moderately high significant differences were recorded for ash, organic matter, and crude

fiber while low significant differences were recorded for digestibility coefficient and organic matter digestibility. Regarding NaCl addition, highly elevated significant differences were recorded for ash and organic matter. These differences were moderately significant for crude fiber and organic matter digestibility. Low significant differences were recorded for dry matter, nitrogen-free extract, digestibility coefficient, feed value, total weight gain, and average daily gain. It is concluded that the addition of 1% NaCl negatively affects the weight development of lambs although it improves the quality of silage parameters such as dry matter, ash, and digestibility coefficient.
Keywords: Millet residue, Salt, Sheep, Silage, Ruminant feed, Zootechnical performance.

[Full text-PDF] [Crossref Metadata]

Research Paper

Effects of Different Methods of Ovulation Induction on Sex Hormones in Serum, and Meat of Rabbit Does

Tverdokhlib Y, Naumenko S, Koshevoy V, Miroshnikova O, Syniahovska K, Kovalova L, and Hryshchuk H.

World Vet. J. 14(1): 117-128, 2024; pii:S232245682400015-14
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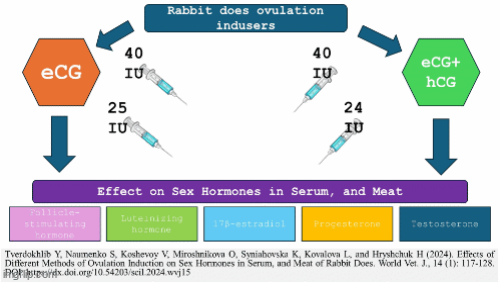


ABSTRACT: High indicators of reproductive function in rabbits can be achieved using hormonal inducers of ovulation, in particular analogs of gonadotropin-releasing hormone, serum, and chorionic gonadotropins. Therefore, the aim of this study was to evaluate the dynamics of sex hormones in the blood serum and meat of rabbit does during ovulation stimulation over 5 consecutive pregnancies. For this purpose, 60 Hyla rabbit does were randomly divided into five groups of 12, ensuring four animals per group with three replicates. Animals of the first and second experimental groups, animals received intramuscular injections of serum gonadotropin, 40 IU and 25 IU respectively, three days prior to artificial insemination. Females of the third and fourth groups were administered combined doses of serum and chorionic gonadotropins (40 IU and 24 IU, respectively) during the same period. Rabbits of the control group were stimulated to ovulate by subcutaneous injection of 0.2 ml analog of gonadotropin-releasing hormone after artificial insemination. Long-term administration of gonadotropins revealed dose-dependent effects. Hyperprogesteronemia was detected in rabbit does (40 IU), while hyperandrogenia was noted in females (24 IU) during the combined administration of gonadotropins. The use of serum gonadotropin at a dose of 25 IU contributed to an increase in the level of follicle-stimulating, luteinizing hormone and progesterone while decreasing 17β-estradiol. A high dose (40 IU) in rabbit does did not cause significant fluctuations of hormones in blood serum, but decreased luteinizing hormone and progesterone. Long-term use of a gonadotropin-releasing hormone analog was accompanied by a pronounced decrease in the level of luteinizing hormone, as well as 17β-estradiol. However, the meat of all experimental animals did not increase the content of steroid hormones (testosterone and 17β-estradiol). It can be concluded that ovulation stimulation in rabbit does using a serum gonadotropin dose of 25 IU and the recommended dose of a gonadotropin-releasing hormone analog does not negatively impact the hormonal balance.
Keywords: Analogue of gonadotropin-releasing hormone, Artificial insemination, Equine chorionic gonadotropin, Human chorionic gonadotropin, Rabbit does

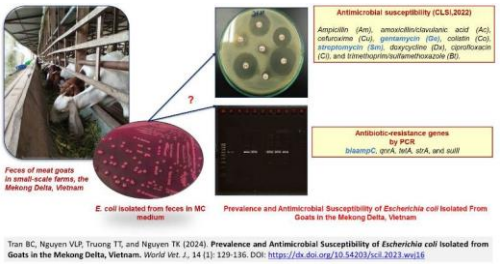
[Full text-PDF] [Crossref Metadata]

Research Paper

Prevalence and Antimicrobial Susceptibility of Escherichia coli Isolated from Goats in the Mekong Delta, Vietnam

Tran BC, Nguyen VLP, Truong TT, and Nguyen TK.

World Vet. J. 14(1): 129-136, 2024; pii:S232245682400016-14
DOI: https://dx.doi.org/10.54203/scil.2023.wvj16



ABSTRACT: Escherichia coli is one of the severe pathogens causing severe diarrhea and resistance to antibiotics in domestic animals, including goats. From April to June 2023, 122 fresh feces of hybrid Boer goats of different ages and genders were collected randomly in the small-scale farms in the Mekong Delta, Vietnam, to clarify the prevalence and antibiotic resistance of E. coli isolated from those feces. By the traditional culture method, of 122 samples, 87 fecal samples were positive for E. coli (71.31%). There were no statistically significant differences in the prevalence of E. coli among male or female goats and ages (< 6 months and ≥ 6 months). E. coli was detected in goats over 6 months and under 6 months at 76.56% and 65.52%, respectively, while

88.20% and 85.42% in male and female goats. The antimicrobial susceptibility of E. coli strains to 7 examined antibiotics was conducted using the Kirby-Bauer disk diffusion method. The results indicated that E. coli was sensitive 100% to colistin (10 µg), amoxicillin/clavulanic acid (20/10 µg), cefuroxime (30 µg), doxycycline (30 µg), ciprofloxacin (5 µg), and 87.50% to ampicillin (10 µg) and bactrim (trimethoprim/sulfamethoxazole, 1.25/23.75 µg), respectively. However, those E. coli strains were highly resistant to streptomycin (93.75%), and 93.67% of E. coli strains were resistant to one to three antibiotics. Among them, the resistant pattern of Ge+Sm (gentamycin + streptomycin) was the most frequent detection (43.75%). The prevalence rate of antibiotic resistance genes (blaampC, tetA, qnrA, strA, and sulII) in E. coli strains isolated from goat feces was detected by PCR. Among them, gene blaampC was the most predominant (96.88%), followed by qnrA (68.75%). Furthermore, 81.25% of E. coli strains harbored two to five antibiotic-resistance genes, and the gene pattern of blaampC + tetA + qnrA was the most popular (21.88 %). The antibiotic resistance and harbored antibiotic resistance genes in E. coli strains isolated from goat feces increase animal and public health concerns.
Keywords: Antibiotic resistance gene, Antimicrobial susceptibility, E. coli, Goat, Small-scale farm

[Full text-PDF] [Crossref Metadata]

Research Paper

The Role of Neutrophils and NETosis in Local Immunity of Feline Inflammatory Aural Polyps

Zhelavskyi M, Maryniuk M, Drobot M.

World Vet. J. 14(1): 137-144, 2024; pii:S232245682400017-14
DOI: https://dx.doi.org/10.54203/scil.2024.wvj17

Zhelavskyi M, Maryniuk M, Drobot M (2024). **The Role of Neutrophils and NETosis in Local Immunity of Feline Inflammatory Aural Polyps.** *World Vet. J.*, 14 (1): 137-144. DOI: https://dx.doi.org/10.54203/scil.2024.wvj17

ABSTRACT: Feline inflammatory aural polyps are abnormal growths that can occur in the ear canals of cats, particularly in the middle ear. These polyps are frequently linked to persistent inflammation and can result in a range of ear-related complicated pathologies. The etiology is multifactorial. The purpose of the research was to study the cytology of an inflammatory polyp in a cat and to study the role of neutrophils and their mechanisms on the formation of extracellular protective traps by neutrophils (NETs). A 4-year-old, female spayed, Scottish fold cat, weighing 3.5 kg sent to a veterinary clinic (Mirra-Vet, Kyiv, Ukraine). Clinical, otoscopic methods, and laboratory methods of cytological diagnostics were used for the research. At the onset of the clinical investigation, exudate discharge from the ear and a painful response were observed. Upon detailed otoscopy, a polyp in the ear canal was diagnosed. An increase in the number of leukocytes (23.2×109/L), their absolute content, and an increase in the percentage of neutrophils (48.2 %) in the leukogram. Assessing the capacity of neutrophils to generate NETs (Neutrophil Extracellular Traps) was determined after samples were collected using a cytologic brush. Cytological analysis of samples from the inter-tragic incisure area highlighted a significant presence of neutrophils, forming extracellular protective traps. The results revealed free NETs in separate areas of the slides. The findings indicated the formation of cooperative groups among neutrophils, other phagocytes, and epithelial cells, along with slender nuclear streaks. During the treatment (Otoflox, 2 drops per ear), the inflammatory reaction disappears, polyp size decreases, exudative reactions decrease, and neutrophil activity decreases. After 3 days of treatment, the animal's condition improved. The ear was clean without sulfur and lesions. The complete treatment course spanned 7 days. During the treatment, the inflammatory reaction disappeared, polyp size decreased, exudative reactions decreased, and neutrophil activity decreased. Experimental studies have shown that during the inflammatory reaction in the ear, protective mechanisms of local immune defense are activated. Activated neutrophils perform their function through phagocytosis and the formation of NETs. These studies contribute to supplementing the data on the immunopathological mechanisms of feline inflammatory polyps.
Keywords: Ear polyps, Feline inflammatory, Local immunity, Neutrophils

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Research Paper

The Effects of Different Concentrations of Vitamin D3 on Immunological Parameters of Immunosuppressed Rats Induced

Kmosh SM, and Al-Naely AJ.

World Vet. J. 14(1): 145-150, 2024;
pii:S232245682400018-14

DOI: https://dx.doi.org/10.54203/scil.2024.wvj18

ABSTRACT: Vitamin D3 receptor is expressed in several types of immune cells suggesting that Vitamin D3 could have immune regulatory roles. The current study was conducted to

investigate the role of Vitamin D3 in reducing the toxicity of the cisplatin on some Immunological parameters in the rat model. The current experiment was conducted on 80 adult white male rats within the age range of 9-12 weeks. The animals were divided into eight groups (10 animals in each group). The control group was dosed with the physiological solution until the end of experiment (C). Rats in the second treatment were injected with cisplatin (2 mg/kg, T1). Rats in the third (T2), fourth (T3), and fifth (T4) groups were injected with cisplatin at a concentration (2 mg/kg) and received Vitamin D3 at levels of 5000 IU, 10,000 IU, and 15,000 IU, respectively. The rats in the sixth (T5), seventh (T6), and eighth (T7) groups were subjected to Vitamin D3 at concentrations of 5000 IU,10,000 IU, and 15,000 IU, respectively. At the end of the experiment, which lasted 21 days, the animals were anesthetized, their weights were recorded, and blood samples were collected. The findings revealed a significant elevation in the levels of interleukin-12, tumor necrosis factor-alpha, C-reactive protein, lymphocyte percentage, monocyte percentage, and eosinophil percentage within group T1 compared to the control and other treatment groups that received Vitamin D3. The average percentage of white blood cells and neutrophils in group T1 was significantly lesser than other groups. It can be concluded that supplementation of different Vitamin D3 levels (5000-10,000 IU) have positive influences on the immunological parameters of immunosuppressed rats.

Keywords: Cisplatin, Immunosuppressant, Interleukin, Vitamin D3

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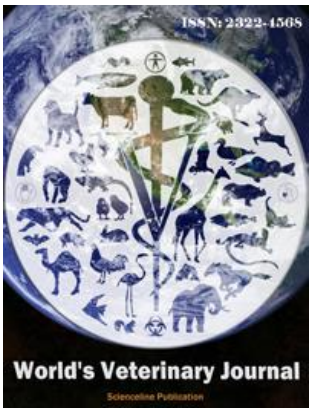

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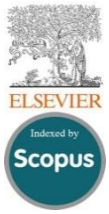

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Diagnosis and Management of Ringwomb in Sheep: Challenges and Approaches

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REVIEW ARTICLE
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ABSTRACT

Small ruminants, including sheep and goats, constitute a major part of the livestock population in different countries of the world. About 15-32% of sheep dystocia are due to incomplete dilation of the cervix which is often called ringwomb. This disorder typically occurs during the lambing process, prompting farmers to possibly seek veterinary assistance due to delayed labor. Different causes, such as calcium and phosphorus deficiency, uterine inertia, and fetal inability to enter the cervical canal, could all lead to incomplete dilation of the cervix. In the meantime, many cases of ringwomb occur as idiopathic. Some factors, such as genetics, nutrition, and imbalance of estrogen to progesterone concentration may also contribute to incomplete dilation of the cervix. In practice, it is important to differentiate the ringwomb with similar conditions such as false dilation of the cervix, early dilation syndrome, and vaginal prolapse associated with ringwomb. In this review, the definition of ringwomb in sheep, an exploration of the structure of the sheep's cervix, the normal process of cervix dilation during lambing, etiology and symptoms of ringwomb, differential diagnosis, and different treatments are discussed.

Keywords: Cervix, Collagen, Dystocia, Sheep, Ringwomb

INTRODUCTION

Incomplete dilation of the cervix is one of the most common causes of dystocia in sheep and goats. This abnormal condition is specifically called ringwomb in sheep and goats (Voigt et al., 2021). This condition usually occurs in ewes older than three years (multiparous) and sometimes with polytocous pregnancies (Parkinson et al., 2019; Cowley et al., 2023). The prevalence of ringwomb varies in different seasons and between sheep breeding units (Mavrogianni, 2017). Breed and body condition scores do not have a strong relationship with the incidence of ringwomb. Most literature considers 15-32% of sheep dystocia due to ringwomb; however, some researchers report a much higher prevalence (Kerr, 1999; Parkinson et al., 2019). The first scientific report describing ringwomb dates back to the 1930s (Kerr, 1999). Later, in the 1950s, a more detailed description of the condition was published (Mackinnon and Bayliss, 1952). Ringwomb occurs without a specific cause. Multiple and different causes can lead to this situation. Although the precise cause of ringwomb in the ewes is not clear, there may be a significant percentage of this condition along with vaginal prolapse (Mostefai et al., 2018). In the present review, the authors aim to discuss different approaches and challenges for the diagnosis and treatment of ringwomb in sheep. The content of this review is based on the available literature and the authors' clinical experiences and observations on ringwomb in sheep at the Department of Clinical Sciences School of Veterinary Medicine, Shiraz University.

Uterine cervix anatomy

The cervical structure varies among different breeds of ewes (Kershaw et al., 2005; Naqvi et al., 2005). In Iran, a study in the Zel breed, different shapes of protrusion of the external os of the cervix into the vaginal fornix, such as duckbill, papilla, flap, slit, and rose were reported (Marzban Abbasabadi et al., 2017). Further, the papilla shape was found to be the most common among Afshari and Kurdish breeds, while the flap shape was the most commonly reported shape of the external os of the cervix among the Makuei breed (Soufyeh et al., 2014). In Iranian native breeds, the average cervical length in the luteal phase is not much different, compared to the follicular phase (6.3 cm versus 6.1 cm) (Marzban Abbasabadi et al., 2017). The average cervical length in the Afshari, Kurdish, and Makuei breeds is 4.11, 4.02, and 4.05 cm, respectively (Soufyeh et al., 2014). Additionally, in Sanjabi breed the average cervical length is 5.5 cm (Habibizad et al., 2015). The average cervical diameter in Afshari, Kurdish, and Makuei breeds is 0.95, 0.95, and 0.96 cm, respectively (Soufyeh et al., 2014). However, the average cervical diameter in the Merinos (Australian brees), Costelasta, and Assaf (south-west Asia) breeds stands at 1.03 cm (Kaabi et al., 2006). In Afshari, Kurdish, and Makuei breeds of Iran, the average number of folds is 7.4, ranging from 5 to 10 folds in each cervix. The size and distance between the folds, from the outer part of the cervix (the vaginal side) to the inner part (the uterus body side), decrease.

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The distance between the first and second folds is greater than the intervals between the other folds. The interior space of the cervix is a funnel-like shape. The number of cervical folds in Afshari, Kurdish, and Makuei breeds has been reported as 5.65, 5.62, and 5.5 on average, respectively (Soufyeh et al., 2014).

Normal cervical dilation

The cervix is closed after the establishment of pregnancy, and the external os is gradually covered with secretions from the cervical mucosa. This process plays an important role in maintaining pregnancy and keeping the uterus nearly sterile. Over time, these secretions become more viscous and harder, causing complete obstruction of the cervical canal (Menzies, 2007). As the delivery approaches, however, the mucosal substance softens and gradually exits through the cervical external os. Following the hormonal changes that occur before and during parturition, the cervix and pelvic ligaments as well as the perineal area are relaxed. The alterations in the cervix are a consequence of various mechanical, hormonal, neural, and biochemical mechanisms (Jackson, 2004). Reducing progesterone and increasing estrogen, relaxin, and prostaglandins have a significant role in softening and dilating the cervix. Biochemical changes, such as a decrease in the concentration of collagen, proteoglycan, and hyaluronidase, and a small but significant increase in tissue hydration, also occur in the cervical tissues following these hormonal changes. These changes serve to soften and relax the cervical tissue. The activity of leukocyte-specific enzyme, myeloperoxidase, and changes in leukocyte concentration in cervical tissue could participate in the reorganization of cervical connective tissue (Menzies, 2007). Although much of the cervical tissue is composed of connective tissue, about 20-30% of it consists of smooth muscle, which plays an important role in opening the cervix during delivery (Marzban Abbasabadi et al., 2017). Disruption of hormonal changes near delivery can cause incomplete dilation of the cervix. The exact processes involved in the dilation of the cervix are not yet well understood in sheep. For example, the effect of relaxin on the cervix has been proven in all species, as it softens the cervix before delivery; however, in sheep, cervical relaxation may not be dependent on the blood relaxin changes (Taverne and Noakes, 2019).

Lambing stages

Natural lambing occurs in three stages, including preparation of the birth canal (relaxation and opening), preparation of the fetus for exit, and expulsion of the fetus and placenta. During the preparatory stage, regular contractions of the longitudinal and circular muscles of the uterus and the dilation and opening of the cervix occur. This stage is completed in 2-6 hours. In the second stage of labor, the amniotic membrane, head, and forelegs or hind legs enter the birth canal (Taverne and Noakes, 2019). Uterine contractions intensify as a result of the activation of the Ferguson reflex. This occurs when the fetal membranes and fluids apply pressure to the internal os of the cervix, stimulating sensory neurons. As a result, more oxytocin is released from the posterior pituitary gland, increasing the intrauterine pressure and gradually pushing the fetus into the cervix. Additionally, voluntary abdominal contractions commence due to the activation of the pelvic reflex. This occurs when the fetal membranes and fetus are present in the pelvic cavity. This stage typically lasts 30-120 minutes. The third stage is the expulsion of the placenta, which takes between 5-8 hours (Menzies, 2007).

Etiology of incomplete dilation of cervix

Although calcium and phosphorus deficiency, uterine inertia, and fetal inability to enter the cervical canal have been reported among the causes of ringwomb in sheep and goats, none of them may be the main cause of this condition. The genetic basis for the occurrence of ringwomb is likely due to the occurrence of this condition in consecutive generations of affected ewes (Jacobson et al., 2020). The profile of estrogen and progesterone secretion in the affected ewes does not provide a clear interpretation of the occurrence of ringwomb. However, a set of studies suggests that the increasing ratio of estrogen to progesterone at parturition may be a contributing factor in the development of ringwomb (Mavrogianni, 2017). Accordingly, the consumption of estrogenic diets by pregnant ewes can disrupt the physiological process of cervical dilation by disrupting the hormonal balance of estrogen and progesterone immediately before and during parturition (Srinivas and Sreenu, 2009). An imbalance in estrogen to progesterone ratio disrupts the synthesis and secretion of prostaglandins E and F in the cervical tissue, consequently causing failure in the depolymerization of cervical collagen tissue. Changes in collagen tissue are necessary for the progress of the first stage of parturition. Although no reports confirm the possibility of experimentally creating ringwomb in ewes, the injection of stilbestrol in the near stages of parturition has created a state similar to incomplete cervical dilatation (Hindson et al., 1967). Additionally, the daily injection of progesterone in the last week of pregnancy has disrupted the dilation of the cervix (Kerr, 1999).

Clinical symptoms

Incomplete dilation of the cervix may be seen during normal or delayed deliveries. On veterinary examination, the most obvious clinical feature is the placenta hanging from the vulva in ewes with incomplete cervical dilation and

without labor straining (Khan and Erdoğan, 2019). However, this definition encompasses a range of cases, from those in which the cervix has not opened at all to those in which a fetal organ has passed through the cervix. Many clinicians accept this description, but cases of an incomplete abortion (where the fetus remains in the uterus, the cervix is partially open, and the fetal membranes are outside the birth canal and hanging from the vulva) must be distinguished from ringwomb (Mavrogianni, 2017). Like ewes in a normal lambing process, affected ewes are not typically separated from the herd. Swelling and softening of the vulva and pelvic structure may not be noticeable, and mammary gland growth and colostrum accumulation may be slower than in normal conditions. The affected ewe may not enter the second stage of lambing after a period of restlessness and abdominal straining (Kerr, 1999). Leaving the animal in such a condition can lead to detachment of the placenta from the uterus, resulting in the death of the fetus. Upon examination of the birth canal, the cervix may be closed or partially open to the extent of one or two fingers. If the membranes of the fetus are intact and inside the uterus, it may be advisable to wait for the next stages of lambing. However, if the cervix is partially open and the membranes of the fetus are torn in the vagina or hanging from the vulva, then this is a case of ringwomb with no doubt (Khan and Erdoğan, 2019). After a two-hour period of examination for the progress of cervical dilation, if there is no significant change, treatment should not be delayed. Experiences have shown that the cervix will not open by simply waiting in cases of ringwomb. Some researchers believe that ringwomb does not usually occur in two consecutive lambing seasons in the same ewe, unless the cervix was damaged during the previous parturition. Ewes with ringwomb may be found recumbent on the ground with both hind legs outstretched and having difficulty breathing (Jacobson et al., 2020).

Differential diagnosis

Incomplete dilation of the cervix with similar clinical signs may occur, which must be carefully investigated to distinguish one from the other during the clinical examination. False dilation of the cervix, early dilation syndrome (EDS), vaginal prolapse associated with ringwomb, and failure to initiate lambing can all lead to incorrect diagnosis and treatment (Mavrogianni, 2017).

False dilation of the cervix

Sometimes the farmer dilates the cervix by digital manipulation before the end of the first stage of natural parturition. Depending on when the intervention takes place, the cervix may open and the lamb may be removed, or it may not be possible to open the cervix (Jacobson et al., 2020). The authors' experiences show that the cervix eventually closes in cases where it is not possible to fully open it. In some cases, due to the abnormal positioning of the fetus inside the uterus, the necessary pressure from the fetus is not applied to the cervix and therefore the cervix remains closed (Kerr, 1999).

Early dilatation syndrome

The clinical symptoms of this syndrome are largely similar to ringwomb, the main difference is that early dilatation syndrome (EDS) of the cervix is usually seen in primiparous ewes. This syndrome occurs in the last two to three weeks of pregnancy leading to natural lambing. In this situation, the mammary gland has not grown much and there is no colostrum, while in some animals, significant changes occur in a very short time, for example, overnight, and the mammary gland grows. Many cases of EDS suggest that an abortion is taking place (Kerr, 1999). On examination, fetal membranes are often hanging from the vulva and the lamb inside the uterus is either dead or crushed and macerated. If the lamb is alive, it is generally weak and premature. In these ewes, the cervix is open by one to two fingers, and in cases of maceration of the lamb in the uterus, the vaginal discharge is usually foul-smelling. Attempting to open the cervix with massage is usually futile and can cause rupture of the cervix, peritonitis, and death of the ewe. Early dilation syndrome of the cervix does not appear to have a genetic basis. If an infectious agent is responsible for the occurrence of early dilation syndrome of the cervix followed by an abortion, 5 to 30% of the herd may be affected (Kerr, 1999).

In order to accurately diagnose cases that have not yet entered the first stage of lambing, and where the ewe may have shown transient symptoms similar to parturition (such as brief straining or the discharge of vaginal-mucosal secretions), ultrasound examination can be used to determine the gestational age based on indices such as the diameter of the fetal kidney, evaluation of uterine contractions using a tocodynamometer, and measurement of blood progesterone concentration (Lye and Freitag, 1990; Mukasa-Mugerwa and Viviani, 1992). Among these methods, ultrasound examination is more convenient and practical, and if progesterone measurement kits are available, levels of progesterone above 1-2 ng/ml in the blood will indicate the absence of labor (Mukasa-Mugerwa and Viviani, 1992; Kalkan et al., 1996). In a hospital or veterinary clinic where it is possible to check the uterine contractions using a tocodynamometer, the uterus is expected to have the lowest uterine contractions in the pregnancy period, if it has not yet entered the first stage of labor.

Diagnosis and treatment of incomplete dilation of cervix

Considering the available facilities, the authors suggest two strategies for the diagnosis and treatment of ringwomb. In the first strategy, epidemiological information, clinical symptoms, and an ultrasound machine (Figure 1) are used for diagnosis and treatment. This solution can be used under farm conditions.

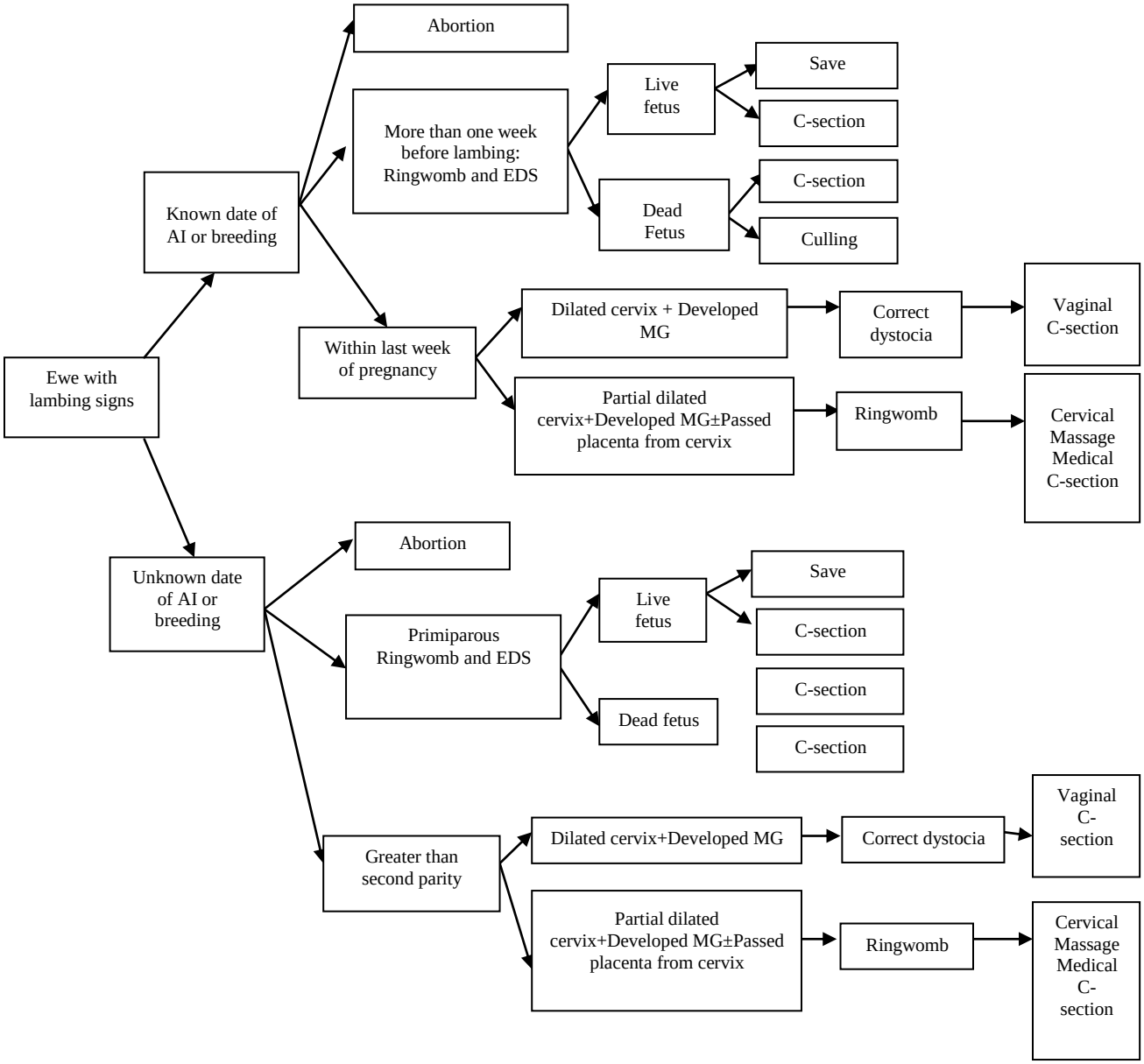

Figure 1. Diagnosis and treatment of ewes affected by ringwomb. General health of ewes needs to be considered in all cases. EDS: Early dilation syndrome; C-section: Cesarean section; AI: Artificial insemination; MG: Mammary gland.

In the second strategy, ultrasonography, tocodynamometery, and blood progesterone measurement can be used in veterinary clinics or hospital conditions (Figure 2). With these tools, along with history and clinical symptoms, it is possible to make a reliable differential diagnosis of incomplete dilation of the cervix. In the first stage, gestational age is determined by measuring the diameter of the fetus's kidney, rib bone, and aorta, and further viability of the fetus is evaluated by assessing the heart rate, fetal movements, fetal fluids, and placenta via the abdominal ultrasound examination. Abortion may be the case if there are signs of labor with a gestational age lower than normal or if the fetus is dead. In these cases, the farmer should be informed of the possibility of abortion and the necessary investigations should be carried out regarding the history and cause of abortion. Additionally, the farmer should be advised on the possibility of culling the affected ewe as it may approach abortion. To maximize the economic profit of the farmer, a valid and reliable prognosis should be given. An intra-vaginal examination can be performed using a hand covered with a glove and lubricated with a water-soluble gel. The vaginal examination provides valuable information about the degree of relaxation and dilation of the cervix and the opening of the birth canal, as well as the contents inside the vagina. Using a tocodynamometer, it is possible to measure the number and intensity of uterine contractions. In the normal course of pregnancy, uterine contractions in the range of 10 mm Hg can be distinguished from the first stage of labor with uterine contractions at regular intervals up to 40-50 mm Hg (Lye and Freitag, 1990). It is important to mention that uterine inertia may cause a wrong diagnosis. In these conditions, by considering the history, clinical symptoms, and the results of clinical and ultrasound examinations, a more correct diagnosis can be made. If it is possible to measure blood progesterone using animal-side kits, the concentration of progesterone can indicate the normal or abnormal pregnancy process. A blood progesterone level of less than 1-2 ng/ml indicates the beginning of lambing or the early stages of abortion (Mukasa-Mugerwa and Viviani, 1992).

Treatment

The following options are suggested for the treatment of ringwomb in farm conditions. Manual dilation of the cervix, injection of muscle relaxants, and cesarean section can be used (Mavrogianni, 2017; Khan and Erdoğan, 2019). From an economic perspective, the farmer may also be advised with the option of culling in specific cases. Some veterinarians have considered the treatment of ringwomb using cervical massage with fingers in some cases. However, many practitioners believe that if the cervix opens with this method, the cause of dystocia may have not been necessarily ringwomb. Cervical massage can be done carefully for 20 to 30 minutes. Care must be taken; the cervix of an ewe with incomplete dilation of the cervix is very vulnerable. It is crucial to exercise caution to avoid any tearing of the cervix of an ewe with incomplete dilation. Based on the above reasons and clinical experiences, it appears that the only approach to address ringwomb is conducting a cesarean section.

The use of muscle relaxants alone in cases of ringwomb has not resulted in high success. Muscle relaxants such as hyoscine do not have the ability to open the cervix selectively. Hormone products such as estradiol, oxytocin, and prostaglandin E2 have been used alone or in combination, but no significant success has been achieved (Padilha-Nakaghi et al., 2020). Hyoscine can be helpful when administered during the first stage of lambing as it relaxes the smooth muscle of the cervix.

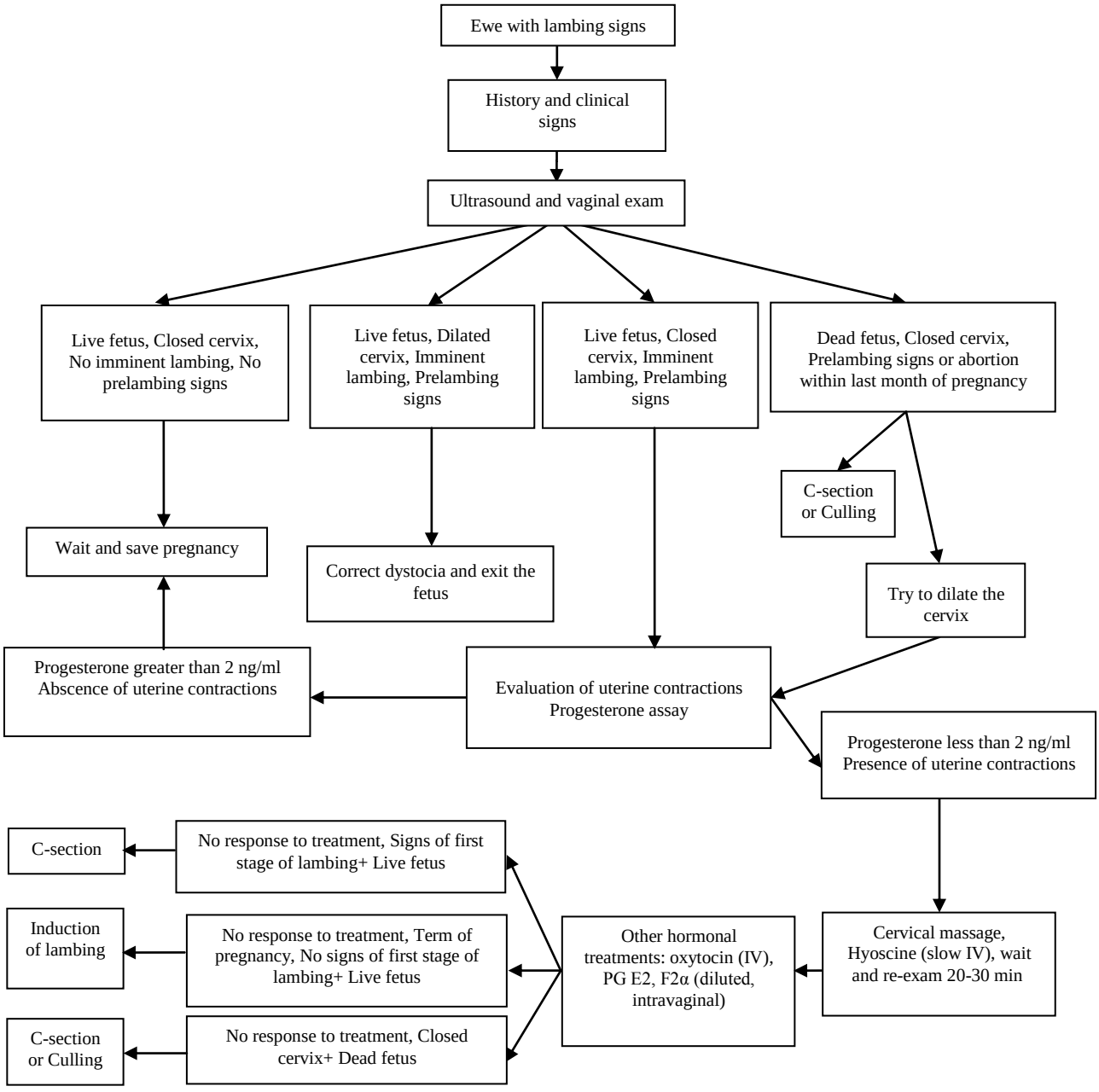

Figure 2. Differential diagnosis, treatment, and management of ewes affected with ringwomb using vaginal examination, ultrasound exam, progesterone measurement, and evaluation of uterine contractions in pregnant ewes with imminent signs of lambing. C-section: Cesarean section; IV: Intravenous; PG: Prostaglandin.

CONCLUSION

To diagnose the causes of incomplete dilation of the cervix in ewes, it is important to employ the history, clinical symptoms, and birth stages, as well as using methods including ultrasonography, tocodynamometery, and on-farm progesterone assay. Meanwhile, to maximize the economic profit of the farmer, a valid and reliable prognosis should be given considering the cost-benefit of treatment and the possibility of culling the affected ewes. Two different approaches for the diagnosis and treatment of ringworm were presented in this review. It is necessary to conduct studies aiming to identify the risk factors associated with the occurrence of ringwomb and genetic predispositions in different breeds.

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Availability of data and materials
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Development of Rapid Detection Kit for Necrotic Enteritis Disease in Poultry using Protein A Agglutination

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ABSTRACT

Necrotic enteritis causes significant losses in the global poultry industry, necessitating accurate diagnosis for effective intervention. This study aimed to develop a diagnostic tool for detecting necrotic enteritis in poultry based on the presence of *Clostridium perfringens* (*C. perfringens)* Alpha-toxin in poultry feces. The reagent of the detection kit was developed by conjugation of IgG against *C. perfringens* toxin and *Staphylococcus* cells containing protein A. The IgG antibody was derived from an 8-month-old thin-tailed male sheep immunized with purified 2 ml of *C. perfringens* Alpha-toxin. Sensitivity assays were carried out to determine the detection limit, while *Escherichia coli* (*E. coli*) and *Salmonella enteritidis* (*S. enteritidis*) were used to identify specificity. A purified Alpha-toxin with a protein concentration of 2.8 mg/ml and a specific molecular weight of 43 kDa was successfully obtained. A strong reaction of the hyperimmune antibody (IgG) was also detected in the thin-tailed male sheep serum. The developed rapid detection kit in this study indicated *C. perfringens* Alpha-toxin with a lower concentration (12 ng/ml). Agglutination reactions could differentiate positive control from negative without significant cross-reactivity towards other bacteria (*S. enteritidis* and *E. coli*).

Keywords: Agglutination, *Clostridium perfringens*, Detection, Necrotic Enteritis, Toxin

INTRODUCTION

Necrotic enteritis, among enteric diseases, inflicts substantial losses by reducing production and increasing mortality, leading to an annual financial setback of around $6 billion for the global poultry industry (Wade and Keyburn, 2015). This poultry affliction is characterized by lesions scattered throughout the small intestine, contributing to growth retardation, reduced feed efficiency, and a mortality rate ranging from 10% to 40% within a single flock (Lacey et al., 2018; Zahoor et al., 2018). The subclinical manifestation of the disease results in decreased body weight or impaired body weight gain and adversely affects the feed conversion ratio. Necrotic enteritis is a complex ailment, with coccidia infection standing out as a crucial predisposing factor due to its detrimental impact on intestinal epithelial integrity, thereby promoting the colonization of *C. perfringens* (Shojadoost et al., 2012; Moore, 2016).

Over the years, antibiotic growth promoters (AGPs) and anticoccidial drugs have been widely used to uphold intestinal health and mitigate disease risks, especially in the context of intensive commercial production conditions (Daniel et al., 2011). Nevertheless, the extensive application of antimicrobials in animal feed is now considered imprudent due to concerns about its potential contribution to antibiotic-resistant pathogenic bacteria that could be transmitted to humans (Seal et al., 2013; Broderick et al., 2021). Consequently, it is pivotal to diagnose necrotic enteritis through observations in both field and laboratory animal studies. This diagnosis is crucial for determining disease control and intervention strategies, such as vaccination (Abd El-Hack et al., 2022).

Diagnosis of necrotic enteric diseases poses challenges. A preliminary assessment can be made by considering the flock's history and gross lesions. Necrotic enteritis diagnosis was confirmed by histopathology due to the damage of intestinal epithelium and lesion in the duodenum, jejunum, ileum, and cecum. Acute lesions include friability of the

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small intestine, filled with reddish or dark brown pseudo membranes over the mucosal-associated with multifocal to coalescent ulcers (Santiani et al., 2023). However, this method requires a series of processes, specific tools, specialized laboratories, and professional interpretation.

Accurate identification of clostridial enteric disease is challenging, particularly for lacking familiarity with this specific disease or other common poultry enteric diseases such as coccidiosis. Different interpretations exist regarding the implications and visual characteristics of intestine autolytic changes, particularly at the microscopic level. These changes start shortly after death and are often mistaken for pathological alterations (Smyth, 2016). In the current study, a diagnostic tool kit for necrotic enteritis was developed to detect the presence of *C. perfringens* Alpha-toxin in the feces of poultry suspected of being infected with the disease. Sensitivity and specificity tests were also carried out to determine the potential, benefits, and effectiveness of the kit in the field.

MATERIALS AND METHODS

Ethical approval

All research methods and practices and the use of animals have been approved by the Animal Ethics Committee, School of Veterinary Medicine and Biomedical Sciences, Institut Pertanian Bogor (IPB) University, Bogor, Indonesia, with certificate number 096/KEH/SKE/VIII/2023.

Study time, location, and animal

This research was conducted at various locations from January to August 2023. The production and purification of alpha toxin from *C. perfringens* were carried out at the research and development (RND) unit of PT. Medika Satwa Laboratoris, Indonesia. Antibody production was performed using 8-month-old thin-tailed male sheep in the animal testing facility of PT. Medika Satwa Laboratoris, Indonesia. The manufacturing and testing of the detection kit were conducted at the Laboratory for Development and Production of Biological Materials at IPB University, Indonesia.

Clostridium perfringens alpha-toxin production and purification

The production of alpha-toxins from *C. perfringens* was conducted in accordance with the previous study (Kurnia et al., 2022) to obtain crude toxin. Subsequently, the potential toxin activity was determined based on hemolytic activity with readings on spectrophotometry (UV-Vis Spectrophotometry Genesys, Thermo Scientific, USA) at a wavelength of 540 nm. Furthermore, the crude toxin was purified through several stages, starting with ammonium sulfate precipitation to obtain a protein toxin concentrate. This was followed by ion exchange, where the toxin protein fractions were determined based on hemolytic activity, concentration, and molecular weight (Ochi et al., 2004; Duong-Ly and Gabelli, 2014).

Antibodies production

The purified toxin protein was injected subcutaneously into 8-month-old thin-tailed male sheep twice, at a 28-day interval, to obtain serum containing IgG of anti-alpha-toxin. Antibodies were collected from sheep that had previously hyperimmunized. Five native sheep with an average weight of 55 ± 1.5 were injected with 2 ml of toxoid. After 2 weeks of the second injection, the immunity level of each animal was determined from each serum by antigen-antibody reaction using an agar gel immunodiffusion assay. The purification of IgG was performed using the addition of rivanol (2-ethyoxy-6,9-diaminoacridine lactate) followed by ammonium sulphate precipitation (Vargas et al., 2012). Subsequently, the concentration and purity of IgG from serum were measured using UV-Vis Spectrophotometry Genesys, Thermo Scientific, USA at 260/280 nm wavelength.

Preparation and IgG conjugation of Protein A

In the current study, *Staphylococcus aureus* (*S. aureus*) strain Cowan I (ATCC 12598) obtained from IPB University was used to produce large amounts of protein A. The preparation of stabilized staphylococci followed a previously established protocol that employed protein A from *S. aureus* cells to facilitate binding IgG, ensuring that the antigen-antibody reaction manifested as co-agglutination (Arnafia et al., 2017). *S. aureus* Cowan I was grown in blood agar at 37°C for 24 hours. The bacterial cells were collected by centrifugation (10000× g, 20 minutes), followed by three washes with phosphate buffer saline (PBS, pH 7.4). The cells were then suspended in 0.5% formalin-PBS (v/v) solution and incubated for 3 hours at 25°C. Subsequently, the cells were washed with PBS and resuspended in PBS at a concentration of 10% (v/v). The suspension was then heated at 80°C for an hour, washed three times with PBS, and resuspended in PBS at the concentration of 10% (v/v). The cells were stained by resuspending in 10% crystal violet. After incubation for 20 minutes at 25°C, the cells were filtered in sterile cotton, centrifuged at 10.000× g for 20 minutes,

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and resuspended in PBS-sodium azide 0.1% at the concentration of 10% (v/v). Monospecific IgG of anti-alpha-toxin with a concentration of 2.1 mg/ml were mixed with 300 µl stabilized *Staphylococcus* cells and allowed to attach for 3 hours at 37℃. The suspension was centrifuged at 5000× g for 5 minutes and washed twice with 0.02 M phosphate (pH 7.3)-buffered 0.85% saline (PBS). The cells were then suspended in PBS to achieve a final volume of 1 ml. The antibody-conjugated staphylococci were then stored at 4°C until they were used as direct co-agglutination reagents.

Sensitivity and specificity test

In the current study, the sensitivity of a kit prototype to detect alpha-toxin of *C. perfringens* was evaluated using an agglutination reaction (Kurnia et al., 2022). The purified *C. perfringens* alpha toxin was diluted in PBS so that graded concentrations of the toxin could be tested to determine sensitivity (detection limit) in suspension. In the experiment, 15 µL from each concentration of toxin suspension was mixed with 15 µL of kit reagent on a glass slide. The agglutination reaction was examined for 30 seconds. *Salmonella enteritidis* (*S. enteritidis*) ATCC 13076, and *Escherichia coli* (*E. coli*) ATCC 25922 bacteria isolates were used to determine the specificity (cross-reactivity) of the kit prototype. All tested bacteria were cultured on a blood agar medium for 24 hours at 37°C. A single colony from each tested bacteria was suspended in 10 µL of PBS, mixed with 10 µL kit reagent on a glass slide, and observed for 10 seconds. Specific gene detection by PCR was compared with the result of agglutination to determine the specificity of the kit prototype. Fresh overnight bacterial cultures in the brain heart infusion (BHI) were used for nucleic acid extraction using the boiling method. All the DNA extracted from the bacteria was examined using PCR with a specific primer. The primer sequence of specific uspA gene *E. coli* was 5′-CCGATACGCCTGCCAATCAGT-3′ and 5′-ACGCAGACCGTAGGCCAGAT-3′ (Rubio et al., 2019). The primer sequence of *S. enteritidis*-specific gene was 5′-ATATCGTCGTTGCTGCTTCC-3′ and 5′- CATTGTTCCACCGTCACTTTG-3′ (Hardiati et al., 2021).

Limit of detection of the development kit in fecal sample

Simulated fecal samples were prepared by adding the *C. perfringens* alpha toxin serial dilutions to negative control poultry feces. The toxin was added to the fecal sample with various concentrations (12000 ng/ml, 1200 ng/ml, 120 ng/ml, 12 ng/ml). Subsequently, 250 µl of the fecal sample was transferred into a microtube and added with 250 µl of 0.2 M AMP (2-Amino-2-methyl-1-propanol) buffer. Then, 500 µl of suspension was centrifuged at 10000× g for 5 minutes. Using a kit prototype, the supernatant was used as a sample for the co-agglutination procedure.

RESULTS

Purification of *Clostridium perfringens* toxins

The findings indicated that ion exchange was effective in purifying the toxin, with the elution step using 0.2 Mol NaCl releasing pure alpha toxin protein fraction based on a higher specific activity of 5.67 U/mg with a protein concentration of 2.8 mg/ml (Figure 1). The specific molecular weight of that protein fraction is also known to be 43 kDa based on observations using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

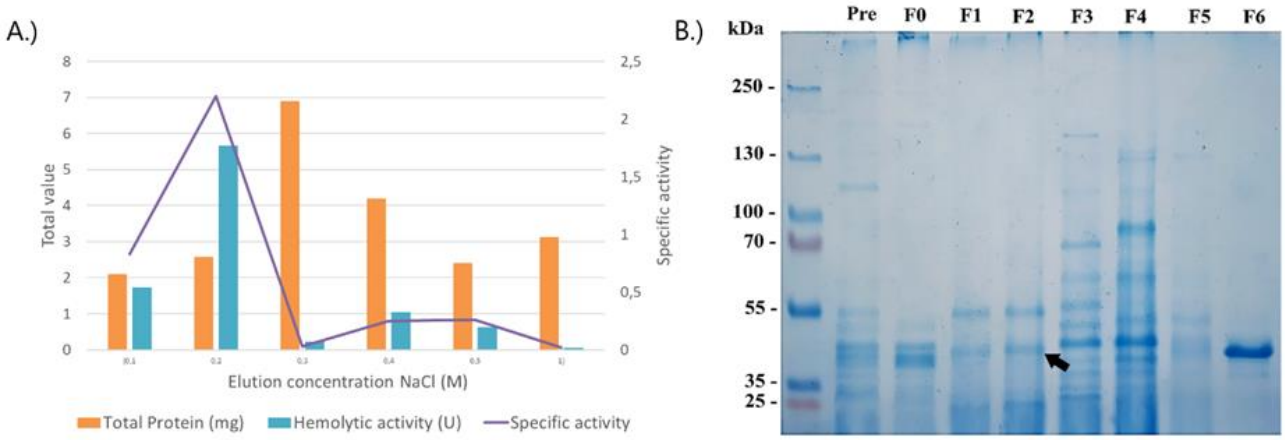

Figure 1. The protein fraction of *Clostridium perfringens* alpha-toxin. **A:** Total value (mg/U) analysis of protein fraction by ion exchange purification at gradual NaCl elution. A higher specific activity value of alpha toxin appears at 0.2 M NaCl concentration. **B:** SDS-PAGE analysis shows that protein fraction 2 (F2) contains specific molecular weight at 43 kDa and 56 kDa. Pre: Crude protein before purification; F0: Dilution of 0 M NaCl; F1: Dilution of 0.1 M NaCl; F2: Dilution of 0.2 M NaCl; F3: Dilution of 0.4 M NaCl; F4: Dilution of 0.6 M NaCl; F5: Dilution of 0.8 M NaCl; F6: Dilution of 1 M NaCl

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Production of antibodies anti-*Clostridium perfringens* alpha-toxin

The antibody obtained from sheep's serum immunized by *C. perfringens* toxin showed a response by an appearance of thin precipitin lines in the second week post-induction (Ab1) at Agar Gel Immunodiffusion Assay. Meanwhile, the precipitin lines appear more distinct in the fourth week and second week post-booster (Ab2 and Ab3). The purification of IgG from the serum indicated a thick single precipitin line (Figure 2). This finding revealed a strong reaction between the toxin antigen and the IgG antibodies obtained.

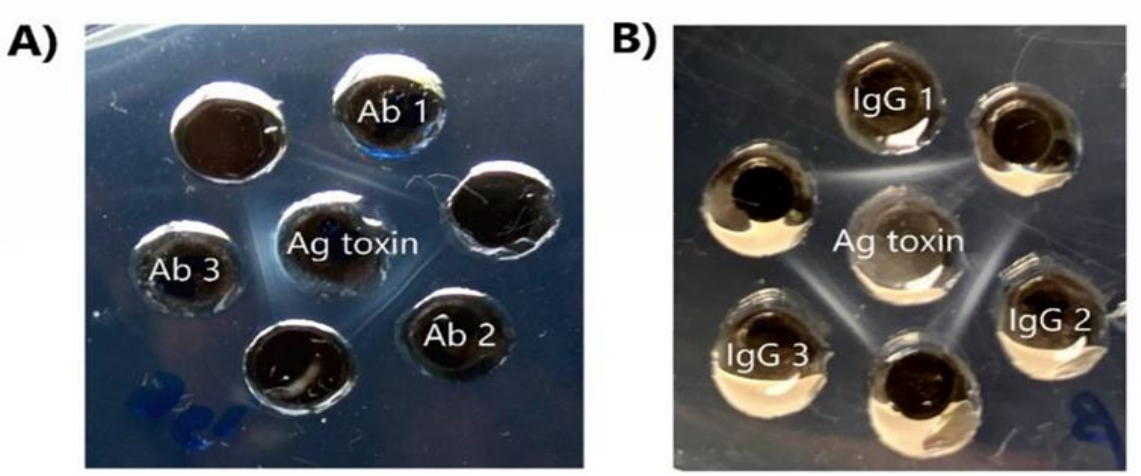

Figure 2. Agar gel immunodiffusion assay reaction of *C. perfringens* alpha toxin and specific antibody. **A:** Antigen and serum in the second-week post-induction (Ab1), reaction in the fourth-week post-induction (Ab2), and second-week post-booster (Ab3). **B:** Antigen and purified IgG in the second-week post-induction (IgG1), the reaction in the fourth-week post-induction (IgG2), and second-week post-booster (IgG3).

Sensitivity and specificity of agglutination kit reagent

Conjugation between IgG antibodies against *C. perfringens* toxin and *Staphylococcus* cell wall was observed through an agglutination reaction when the *C. perfringens* Alpha-toxin was added to the reagent as a control. To assess sensitivity, decreasing concentrations were tested. Consequently, the agglutination reaction became increasingly faint as the concentration of the control toxin decreased. At this stage, it can be determined that the agglutination reaction can detect the presence of the toxin with a lower concentration based on serial dilutions of the *C. perfringens* alpha toxin at approximately 12 ng/ml (Figure 3A).

Table 1. The comparison of co-agglutination reaction results for the detection of *Clostridium perfringens* alpha-toxin with PCR results to confirm the bacterial identity

Bacterial antigen	Agglutination reaction	PCR with specific primer
Clostridium perfringens alpha-toxin	+	-
Salmonella enteritidis ATCC	-	+
Escherichia coli ATCC	-	+

ATCC: American Type Culture Collection

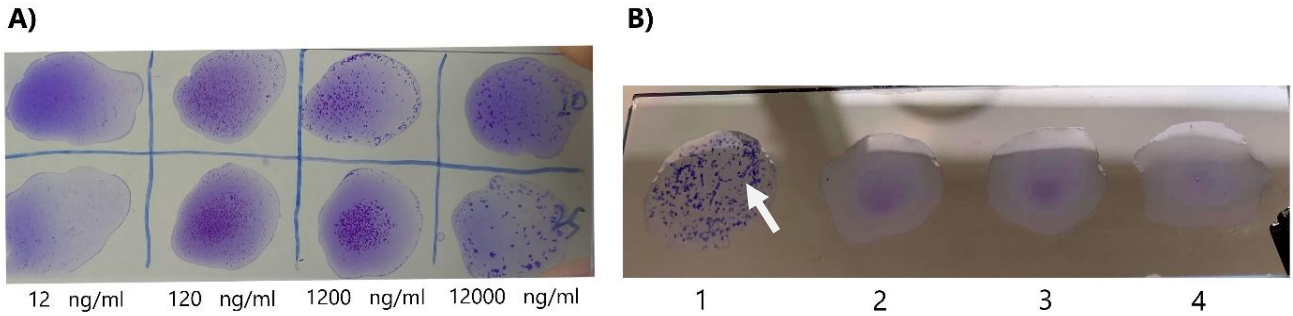

Figure 3. Agglutination reaction of the developed reagent. **A:** Sensitivity of the reagent by decreased toxin (12 µg/ml – 12 ng/ml) shows that the agglutination reaction becomes increasingly faint. **B:** Determination of specificity (cross-reactivity) of kit prototype, agglutination reaction of *Clostridium perfringens* alpha with reagent (1); reaction of *Salmonella enteritidis* with reagent (2); reaction of *Escherichia coli* with reagent (3); negative control PBS (4).

Determination of agglutination kit reagent specificity was conducted based on a comparison with another

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diagnostic test using PCR (Figure 4). It appeared that no agglutination reaction occurred by adding *E. coli* and *Salmonella* antigens with the reagent, indicating that there is no cross-reactivity with other antigen bacteria (Figure 3B). Negative control by PBS was also carried out to make sure no self-agglutination formation particles occurred in the reaction.

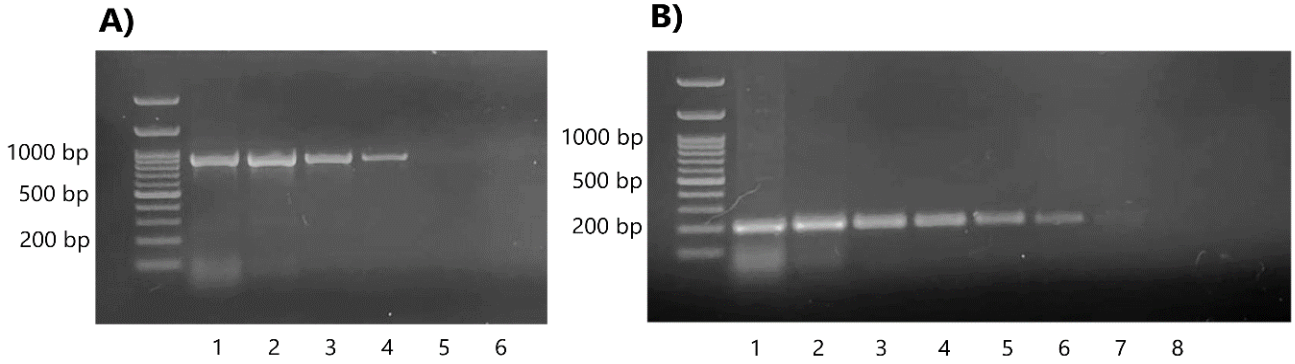

Figure 4. PCR confirmation identity of *Escherichia coli* and *Salmonella enteritidis* by specific primer. **A:** An approximate 880 bp band size represents uspA gene that specifically identifies *Escherichia coli* isolate. Lane 6 is non-template control (NTC), while 1-5 was bacterial genomic DNA template at amounts from 210 ng to 21 pg. **B:** An approximate 206 bp band size represents specific gene of *Salmonella enteritidis*. Lane 8 is NTC, while 1-7 was bacterial genomic DNA template from 193 ng to 19 pg.

DISCUSSION

The rapid detection kit developed in this study utilized a co-agglutination reaction with two primary elements. These components included *S. aureus* cells with protein A (*S. aureus* Cowan I) that act as a foundational matrix, while IgG of anti-alpha-toxin of *C. perfringens* type A serves as the agent to detect antigens within the sample. Protein A of *S. aureus is* known to have a high affinity for the Fc region of IgG (O'Seaghdha et al., 2006). The protein is abundantly present on the bacterial cell wall and composed of a signal sequence. Each of its repeating Ig-binding domains adopts a three-helical structure that can bind to the Fc region of IgG via helices I and II (Cruz et al., 2021). While the Fc region attaches to the protein A structure of *Staphylococcus aureus*, Fab region of IgG detects explicitly the antigen in the fecal sample. The co-agglutination reaction co-occurs with *C. perfringens* type A toxin, resulting from the binding between antigens and antibodies already bound to *Staphylococcus* cell wall.

Digestive tract infections represent a significant concern within the poultry industry and have resulted in substantial economic losses (Salem and Attia, 2021). Necrotic enteritis (NE), whether in clinical or subclinical form, is a major gastrointestinal ailment in poultry, severely impacting profitability in the poultry sector (Bansal et al., 2021; Salem et al., 2021). Necrotic enteritis continues to pose challenges, particularly in regions with poorly managed poultry operations. In the diagnostic process for NE, errors often occur, particularly in the context of bacterial infections. This leads to an upsurge in the use of antibiotics on farms as a response to NE (Fathima et al., 2022). To counteract the increasing consumption of antibiotics, which could potentially contribute to antibiotic resistance, a diagnostic kit for NE has been developed. Recently, various methods, such as ELISA, have been explored and created to rapidly detect NE. However, ELISA comes with certain limitations, such as the need for expensive, highly specialized laboratory equipment and the requirement for well-trained personnel (Nnachi et al., 2022).

The co-agglutination technique has evolved to detect certain bacteria, including toxins from *C. perfringens*. The co-agglutination technique offers a range of advantages, including sensitivity, specificity, speed, simplicity, cost-effectiveness, and reliability (Dong et al., 2019). The construction of a reagent can be optimized to distinguish between positive and negative controls clearly. Sensitivity observation revealed that alpha-toxin from *C. perfringens* can be detected by reagent developed at a lower concentration of 12 ng/ml in the current study. Based on a previous study, the concentration of *C. perfringens* toxin ranged from 0.19 to 17.6 ng per gram of wet feces was found in poultry with confirmed NE lesions, so native toxin was detected in the digestive or fecal droppings (Lee et al., 2020; 2021). This finding supports the idea that the reagent developed in this research will be able to detect *C. perfringens* toxin within the appropriate range concentrations in the field.

Reagents developed in the current study were also assessed for their reaction with other bacteria related to digestive disease. These bacteria, including *E. coli* and *S. enteritidis*, had no cross-reaction based on agglutination observation. A single colony used for current detection indicated the absence of an agglutination. Bacterial identification was further validated by PCR to detect specific genes that identify certain bacteria based on DNA material genetics in gradually

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decreasing concentrations. The diagnosis of diseases caused by bacteria or viruses, with the analysis at the molecular level, poses unique challenges when detecting protein structures like toxins (Pawaiya et al., 2020). In line with the findings of the current study, Serroni et al. (2022) have found that the kit development has the potential to detect toxins in digestive diseases with a rapid reaction, considering toxin protein structures were time-dependent proteolytic degradation itself in fecal samples.

CONCLUSION

The results of the study indicated that the kit developed in this study could detect a reagent for Necrotic enteritis. The conjugation of IgG of anti-alpha toxin of *C. perfringens* type A, with *Staphylococcus* protein A produced an agglutination reaction when samples containing *C. perfringens* toxin were added. The sensitivity of the kit also indicated that the toxin concentration could be detected within the appropriate range in a field case of Necrotic Enteritis, with well-established specificity. However, further studies regarding the stability, with several quality control observations of this diagnostic kit, need to be conducted to determine optimal results.

DECLARATIONS

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Authors' contributions

Ryan Septa Kurnia, Christian Marco Hadi Nugroho, Otto Sahat Marua Silaen, Muhammad Ade Putra, Vivin Aulia Rahmi, Alya Amaliah, Safika, and Agustin Indrawati conceived, designed, collected, and analyzed data and wrote the manuscript. Rani Wardani Hakim, Maxs U.E Sanam, Amin Soebandrio, Safika, Agustin Indrawati designed, supervised the study, and reviewed the manuscript. All authors read and approved the final draft of the manuscript for publication.

Competing interests

The authors declared that there are no competing interests.

Ethical considerations

The authors declare that this manuscript is original and is not being considered elsewhere for publication. Other ethical issues, including consent to publish, misconduct, fabrication of data, and redundancy, have been checked by the authors.

Availability of data and materials

All data of the current study are available upon reasonable requests from the authors.

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Comparative Effects of Meloxicam and Phenylbutazone on Clinical Outcomes and Acute Phase Response in Sheep with Acute Respiratory Disease

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ABSTRACT

The ovine respiratory complex (ORC) is one of the most common respiratory diseases observed in sheep. The objective of the present investigation was to evaluate the comparative therapeutic efficacy of two non-steroidal anti-inflammatory drugs (meloxicam and phenylbutazone) for the treatment of the ORC. For this purpose, 33 Ossimi sheep were randomly assigned into three treatment groups (11 each). Group 1 was treated with amoxicillin long-acting (LA) and meloxicam, group 2 received amoxicillin LA and phenylbutazone, and group 3 was treated with amoxicillin LA alone. Sheep were examined clinically and clinical index scores were recorded before and after treatment. Additionally, blood samples were collected from each sheep. After 14 days of treatment, sheep of group 1 indicated significant improvements in their clinical index scores and a reduction in total leukocyte count. However, there was a significant increase in red blood cell count, hemoglobin, and MCHC%. There was a significant decrease in the serum globulin, copper, and haptoglobin in group 1, compared to sheep of groups 2 and 3. Based on the results of this study, administering meloxicam to sheep with an ovine respiratory complex resulted in significant improvements in clinical outcomes and significant corrections in above mentioned hematological and biochemical parameters. Although phenylbutazone proved to be less effective, it still demonstrated some degree of efficacy in treating this condition. This study suggests that meloxicam may be a more effective treatment option for ORC with phenylbutazone.

Keywords: Acute phase protein, Amoxicillin, Anti-inflammatory, Ovine respiratory complex

INTRODUCTION

Ovine respiratory complex (ORC) is one of the main diseases affecting small ruminants in many countries (Tibbo et al., 2001; Navarro et al., 2019). It commonly affects individuals or groups of sheep and frequently requires a combination of infectious agents as well as managemental risk factors (Hindson and Winter, 2008). The ovine respiratory complex is a feedlot production health concern in sheep-rearing countries (González et al., 2016; Lacasta et al., 2019)**.** The outcomes of ORC include increased mortality, reduced growth rate, waste in the slaughterhouse, medicine expenditures, and labor expenses related to treatment are the outcomes of ORC (Baghezza et al., 2021).

Sheep are more vulnerable to respiratory infections owing to the interactions between several viral and bacterial pathogens, impaired pulmonary defense mechanisms, and husbandry-related factors (Brogden et al., 1998). The bacteria most implicated in ORC are *Mannheimia haemolytica*, *Mycoplasma ovipneumoniae*, *Pasteurella multocida*, *Bibersteinia trehalosi*, *Trueperella pyogenes*, and *Escherichia coli* (Saura Armelles, 2017). A compromised immune system can lead to the invasion and growth of pathogenic bacteria in lung tissue, with subsequent inflammation and severe clinical signs (Mosier, 2014; Pahal et al., 2018; Franco et al., 2019).

Clinically, ORC can be acute or chronic (Kumar et al., 2014), where acute ORC in sheep is characterized by fever, depression, weight loss, mucopurulent discharge, increased respiratory rate, and crackles in the anterior thorax (Gilmour et al., 1983). In contrast, sheep affected by chronic ORC experience chronic soft cough and mucopurulent nasal discharge (Scott, 2015).

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Nonsteroidal anti-inflammatory drugs (NSAIDs) have been used as a supplementary treatment alongside antimicrobial therapy to address ORC because they possess analgesic, antipyretic, and anti-inflammatory properties (Politis et al., 2019). Meloxicam is an oxicam-class NSAID with powerful anti-inflammatory, analgesic, and antipyretic effects (Xu et al., 2014). It operates by inhibiting cyclooxygenase-2 enzymes, which lessens the production of tumor necrosis factor, an inflammatory cytokine that is produced during respiratory tract infection (Bednarek et al., 2003; Hirsch et al., 2003; Curry et al., 2005). In cattle, meloxicam has been clinically evaluated widely as an adjunctive therapy for the treatment of respiratory tract diseases (Salamon et al., 2000; Schmidt et al., 2000). Similarly, Phenylbutazone is an effective NSAID with antipyretic and analgesic properties and has been used in veterinary medicine for more than five decades to treat musculoskeletal system inflammation, soft tissue inflammation, and laminitis (Flood and Stewart, 2022). While Phenylbutazone has been proven to be very effective in treating respiratory disease in calves owing to its potent anti-inflammatory effects, there is a paucity of information on its effectiveness as a supplementary therapy for treating ORC in sheep (El-Deeb et al., 2021).

The effect of anti-inflammatory drugs on acute phase proteins in calves with respiratory diseases has been presented (El-Deeb et al., 2021), and in sheep with pneumonic pasteurellosis (El-Deeb and Elmoslemany, 2016). However, there is little information about the effect of meloxicam on clinical and acute phase proteins (APPs) in sheep with ORC.

Therefore, the aim of the present study was to compare the therapeutic efficacy of meloxicam and phenylbutazone on the clinical outcomes, hematological parameters, and selected APPs in Ossimi sheep affected by ORC.

MATERIAL AND METHODS

Ethical approval

Ethical approval for this experiment was given by the committee of King Faisal University No. KFU-REC-2023-NoV-ETHICS-1371.

Animals and clinical examination

In this study, a total of 33 Ossimi sheep with ORC were examined. To diagnose ORC, a systemic clinical examination was conducted with signs of anorexia, depression, coughing, nasal discharge, and high body temperature (≥ 40°C; Lacasta et al., 2019). On the first examination and before treatment, clinical findings for each sheep were identified and scored, and the sum of such clinical index scores was recorded (Table 1). Sheep were randomly selected from different herds in Dakahlia and Qalubia governorates. The age of the sheep ranged from three months to two years (Mean 12 ± 55 months).

The body weight of examined sheep ranged from 4 to 40 kg (Mean 19 ±8.4 kg). Sheep were raised in smallholder flocks and kept indoors. Sheep were fed on green fooders and 250 gm concentrates daily The animals were randomly allocated to three equally sized treatment groups, each containing 11 sheep. The sheep were diagnosed to have ORC based on clinical examination (Lacasta et al., 2019).

Briefly, the presence of cough, nasal discharge, and abnormal lung sound on auscultation are the major findings. In addition, evidence of systemic signs was confirmatory. Systemic signs were fever (≥ 40°C), anorexia, and depression. The present clinical trial was conducted according to the CONSORT statement guidelines (2010).

Table 1. Description and scores of clinical signs in sheep with acute respiratory disease

	Clinical variable	Level and description
1	Cough	Absent: 0 Dry cough: 1 Moist cough: 2
2	Nasal discharge	Absent: 0 Serous discharge: 1 Mucoid discharge: 2 Mucopurulent: 3 Purulent: 4
3	Ocular discharge	Absent: 0 Serous discharge: 1 Mucoid discharge: 2 Purulent: 3
4	Heart rate (beat/min)	70-90: 0 90-100: 1 100-120: 2 >120: 3
5	Respiratory rate (cycle/min)	20-40: 0 40-50: 1 50-60: 2 >60 : 3
6	Temperature	39-40^{0}C: 0 >40-41^{0}C: 1 >41-42^{0}C : 2 >42^{0}C : 3
7	Appetite	Inappetance: 1 Anorexia:2
8	Alertness	Alert: 0 Mild depression: 1 Severe depression: 2
9	Dyspnea	Absent: 0 Mild dyspnea: 1 Severe dyspnea: 2
10	Lung sound	Normal sound: 0 exaggerated vesicular sound: 1 wheezes: 2 crackles: 3
11	Conjunctivitis	Absent: 0 Mild conjunctivitis: 1 Severe conjunctivitis: 2

Treatment protocol

Group 1 received subcutaneous injections of Amoxicillin LA at a dosage of 15 mg/kg body weight, administered three times successively with a 48-hour interval between each injection (El-Deeb et al., 2021), and intramuscular administration of meloxicam **(**METACAM, Boehringer Ingelheim Animal Health, USA Inc.) at a dosage of 1 mg/kg body weight for five consecutive days (Metacam, 2016). Group 2 received treatment that involved subcutaneous administration of Amoxicillin LA (Amoxypen LA, MSD Animal Health) at a dosage of 15 mg/kg body weight, for three successive times 48 hours apart, and intravenous administration of phenylbutazone (Buta-Fenil, AM Trading, Lab. Tornel, Mexico) at a dose of 4 mg/kg body weight for five consecutive days (El-Deeb et al., 2021). Group 3 was treated only with Amoxicillin LA, at the same dose and protocol as group 1 and group 2.

Clinical follow-up

To assess the health of the sheep, clinical examination and clinical index scores were recorded for each animal on the first day of the visit (T0), day 7 (T1), and day 14 (T2) post-treatment.

Blood samples

Two blood samples were collected from each sheep under investigation through jugular vein puncture. While sampling, no sedative drugs have been used. Sampling was performed at the time of the first visit and on days 7 and 14 post-treatment. One of the blood samples (5 ml) was collected on anticoagulant (5 mg sodium ethylene diamine tetra acetic acid) for the evaluation of total and differential leukocytic counts. The second five mL of blood sample was collected without anti-coagulant for analysis of biochemical parameters including iron, zinc, copper, haptoglobin, albumin, total protein, and globulin concentrations.

Hematological analysis

The red blood cell (RBCs, 10^6/µl), hemoglobin (Hb, gm/dl), PCV%, total leukocytic, and differential leukocyte (WBCs10^3/µl) counts were determined using a hematology analyzer (MS9-5, Melet Schloesing Laboratories, France) according to (Feldman et al., 2000).

Biochemical examination

Iron was measured colorimetrically using commercial test kits (Fortess Diagnostics Limited Co., United Kingdom). Zinc and copper levels were estimated colorimetrically using commercial test kits (Biodiagnostic Co., Egypt). Albumin and total protein levels were measured colorimetrically using commercial test kits (Stanbio, Boerne). Finally, haptoglobin was measured by the nephelometric method using commercial test kits (Turbox, Orion diagnostica Oy, Finland). Nephelometry was applied as a simple, accurate, and reliable technique for the determination of haptoglobin (Shih et al., 2014). The haptoglobin was measured at a wavelength of 600 nm following the instruction of the manufacturer of the used test kits.

Statistical analysis

A commercial software program (GraphPad version 5.0, USA) was used for statistical analyses. First, the groups were assessed for homogeneity using the Kruskal–Walli's test. As the data were found to be homogenous, the mean and standard deviation for variables were presented. For the clinical index score, analysis of variance test (ANOVA) and Bonferroni multiple comparison tests as post hoc was performed to find the significant changes. A general linear model with repeated measures ANOVA was used to evaluate the effect of anti-inflammatory drugs on biochemical variables. Wilks' lambda test was used as an indicator of significant changes. Where such a test was found to be significant, analysis of variance test (ANOVA) and Tukey's comparison test as post hoc was performed. For all data, the outcomes were considered significant when $p < 0.05$.

RESULTS

The clinical disease index score indicated that significant improvement in sheep treated with a combination of meloxicam and amoxicillin LA in group 1, compared to those treated with a combination of amoxicillin LA and phenylbutazone in group 2 and those treated with amoxicillin LA alone in group 3. This improvement was significant on 7 ($p < 0.01$) and 14 ($p < 0.01$) days post-treatment. Furthermore, complete recovery was achieved in the sheep of group 1 with a clinical index score of 1, whereas the sheep of groups 2 and 3 had clinical index scores of six and seven, respectively, 14 days post-treatment. The respiratory rate and heart rate returned to normal levels in sheep of group 1 and group 2 more rapidly than those of group 3.

The red blood cell indicated Hb concentration, RBC count, and MCHC% significantly increased in sheep of group 1, compared to those of groups 2 and 3 at 14 days post-treatment ($p < 0.01$; Figure 1). However, the total leukocyte count significantly decreased in the sheep of group 1, compared to those of group 3 on 14 days post-treatment ($p < 0.01$). The neutrophil percentage significantly decreased in sheep of group 1, compared to those of group 2 and group 3 on 7 days and 14 days post-treatment ($p < 0.05$). In contrast, lymphocyte percentage was significantly higher in sheep of group 1 compared to those of group 2 and group 3 on 7 days and 14 days post-treatment ($p < 0.05$; Figure 2). Serum

haptoglobin showed a significant decrease in sheep in group 1, compared to those of group 2 and group 3 on 7 days and 14 days post-treatment ($p < 0.01$; Figure 3). Similarly, serum globulin revealed a significant decrease in sheep of group 1, compared to those of group 3 on 14 days post-treatment ($p < 0.05$). Both serum albumin and albumin/globulin (A/G) ratio indicated a significant increase in sheep of group 1 compared with those of group 3 on 14 days post-treatment ($p < 0.05$; Figure 4). Serum iron levels rose significantly in sheep of group 1 compared with those of group 3 on 14 days post-treatment ($p < 0.05$; Figure 5). However, the serum copper levels in sheep of group 1 demonstrated a significant decrease, compared to those of group 3 on 7 and 14 days post-treatment ($p < 0.05$; Figure 6). Serum zinc levels significantly increased in sheep of group 1, compared to those of group 3 on 14 days post-treatment ($p < 0.05$), and significantlyincreased compared to those of groups 2 and 3 on 14 days post-treatment ($p < 0.05$; Figure 7).

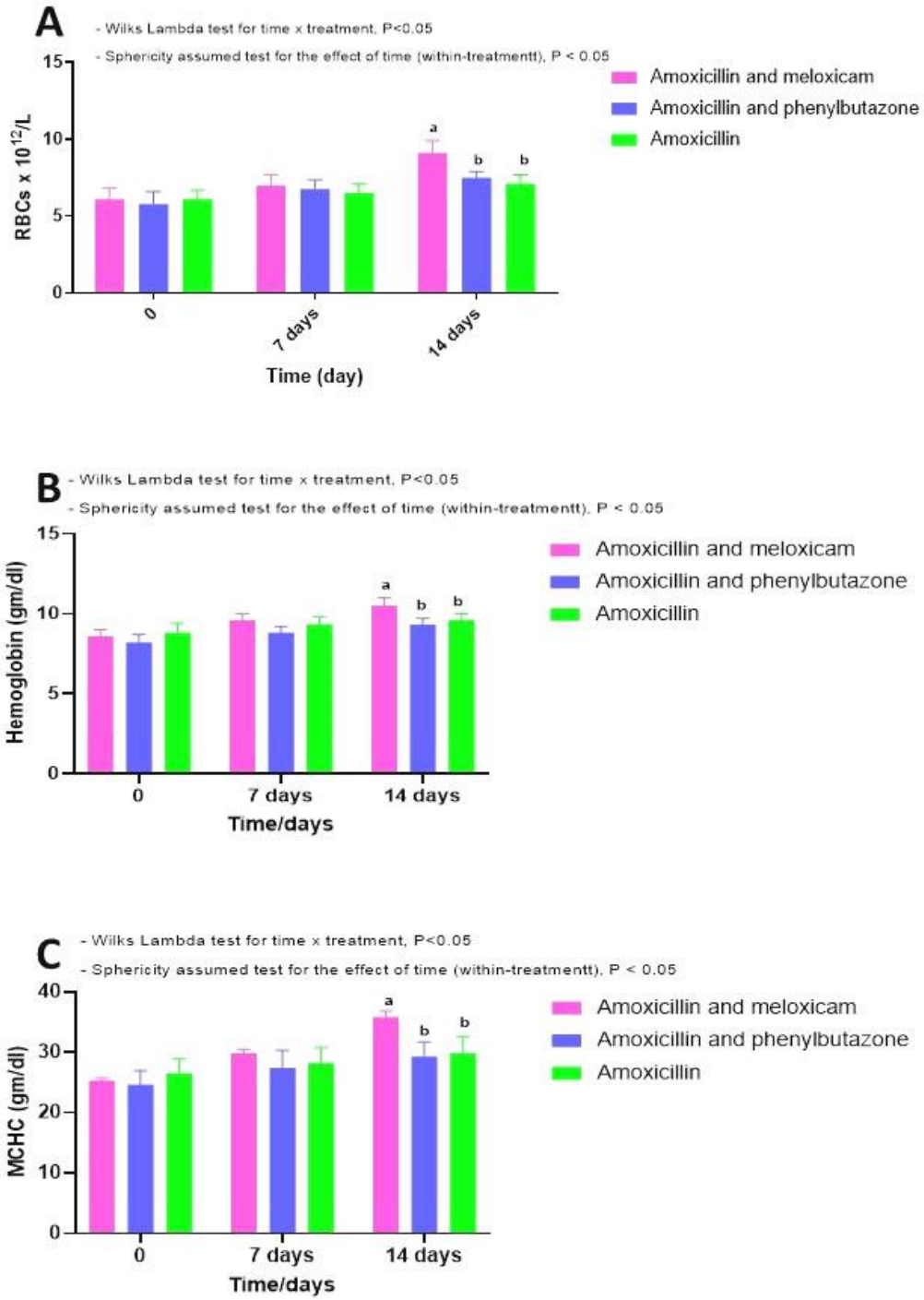

Figure 1. Mean values of hematological indices in sheep with ovine respiratory disease before and after treatment with amoxicillin LA and meloxicam, amoxicillin LA and phenylbutazone and with amoxicillin LA alone. [a,b:] Means with different superscript letters are significantly different at $p < 0.05$.

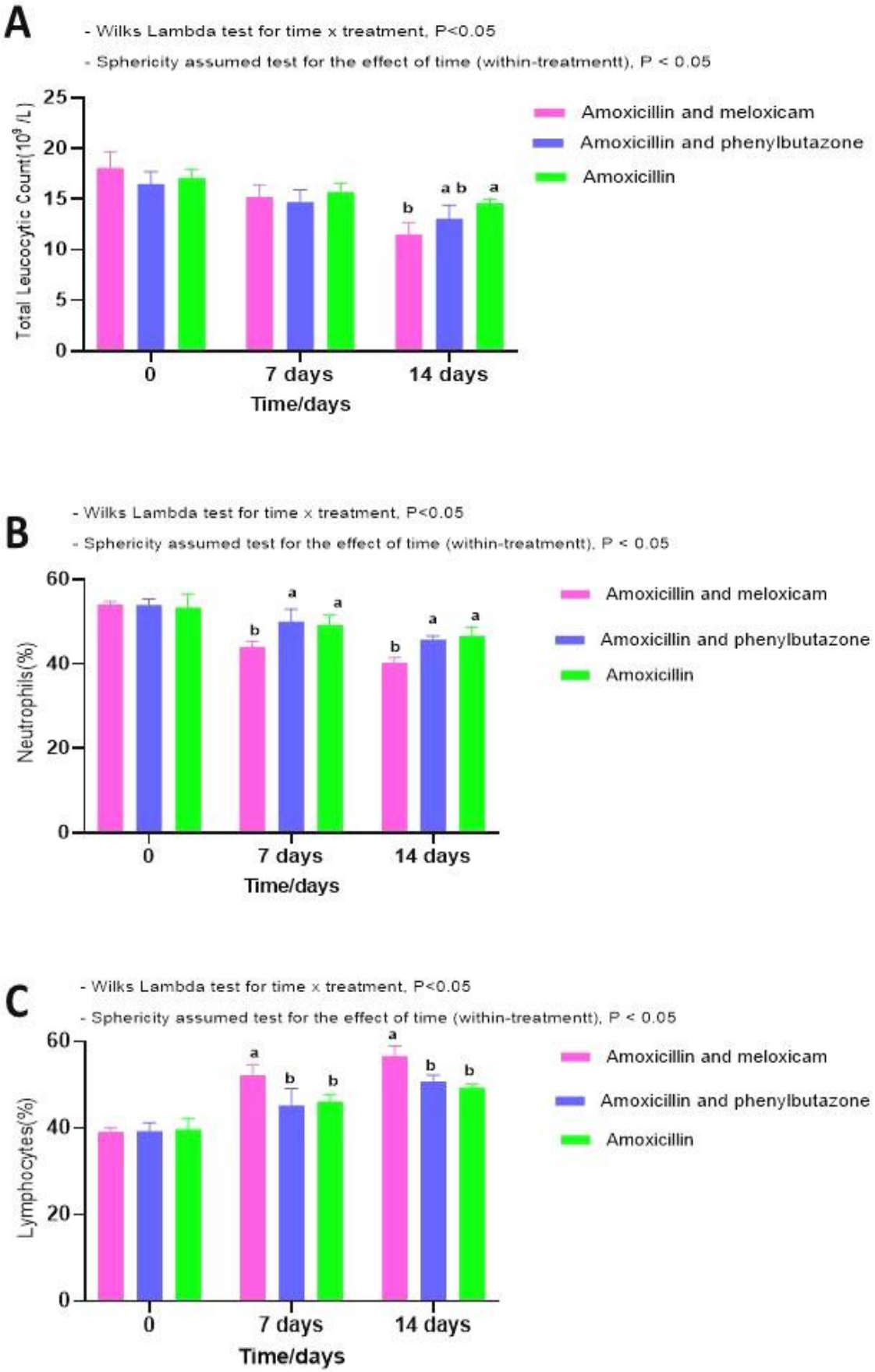

Figure 2. Mean values of total and differential leukocytic count in sheep with ovine respiratory disease before and after treatment with amoxicillin LA and meloxicam, amoxicillin LA and phenylbutazone and with amoxicillin LA alone. [a,b:] Means with different superscript letters are significantly different at $p < 0.05$.

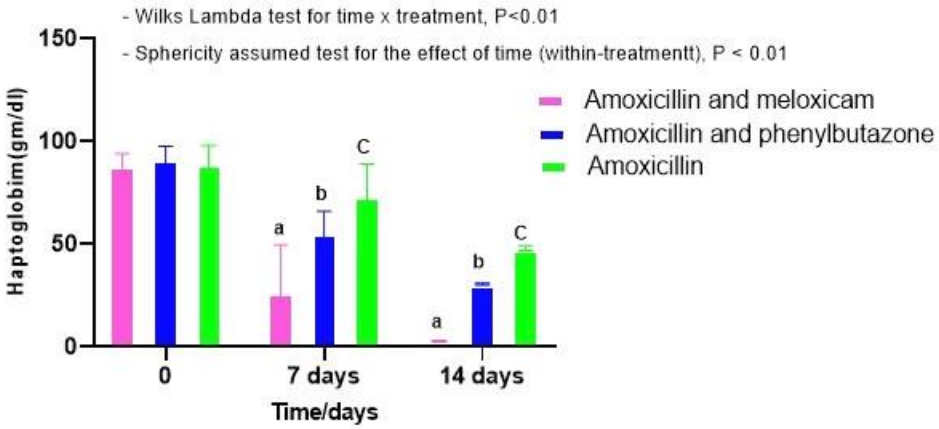

Figure 3. Mean values of haptoglobin in sheep with ovine respiratory disease before and after treatment with amoxicillin LA and meloxicam, amoxicillin LA and phenylbutazone and with amoxicillin LA alone. [a,b,c:] Means with different superscript letters are significantly different at $p < 0.05$.

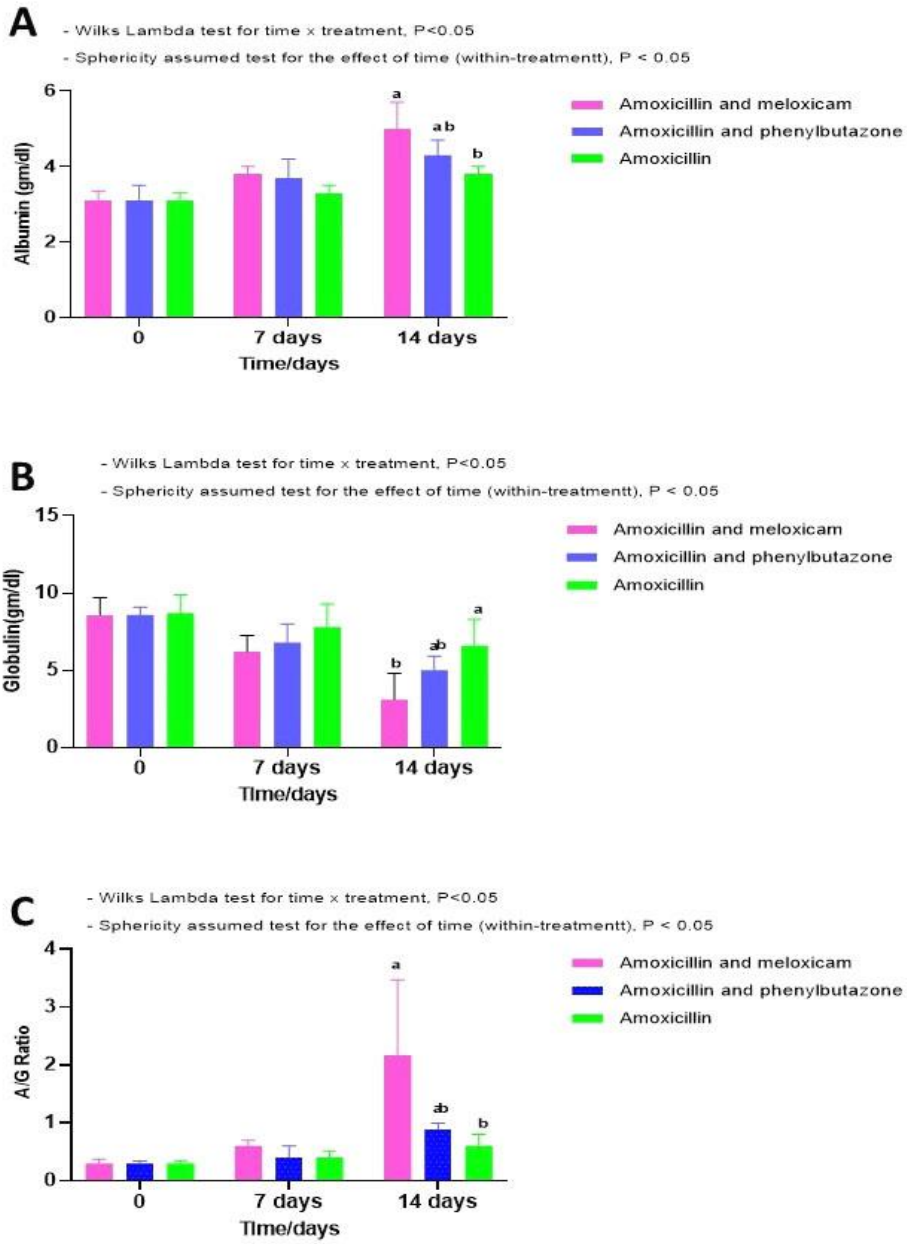

Figure 4. Mean values of serum protein in sheep with ovine respiratory disease before and after treatment with amoxicillin LA and meloxicam, amoxicillin LA and phenylbutazone and with amoxicillin LA alone. [a,b:] Means with different superscript letters are significantly different at $p < 0.05$.

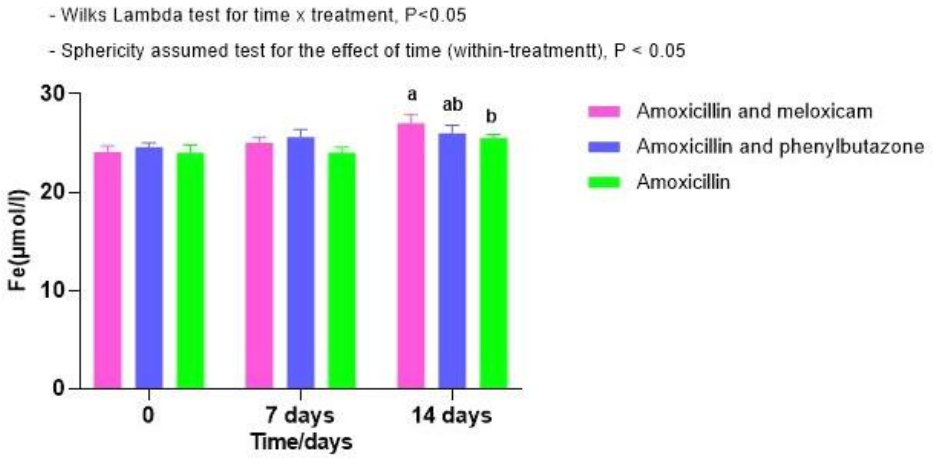

Figure 5. Mean values of serum iron in sheep with ovine respiratory disease before and after treatment with amoxicillin LA and meloxicam, amoxicillin LA and phenylbutazone and with amoxicillin LA alone. [a,b:] Means with different superscript letters are significantly different at $p < 0.05$.

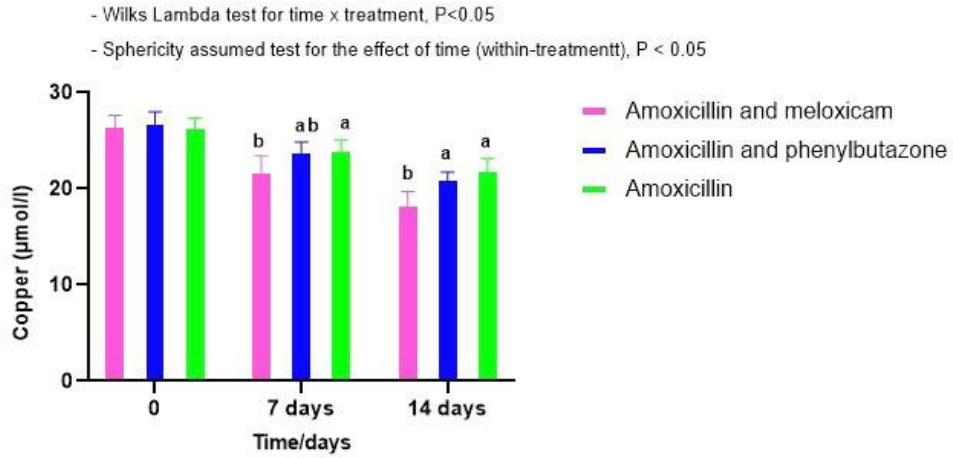

Figure 6. Mean values serum copper in sheep with ovine respiratory disease before and after treatment with amoxicillin LA and meloxicam, amoxicillin LA and phenylbutazone and with amoxicillin LA alone. [a,b,:] Means with different superscript letters are significantly different at $p < 0.05$.

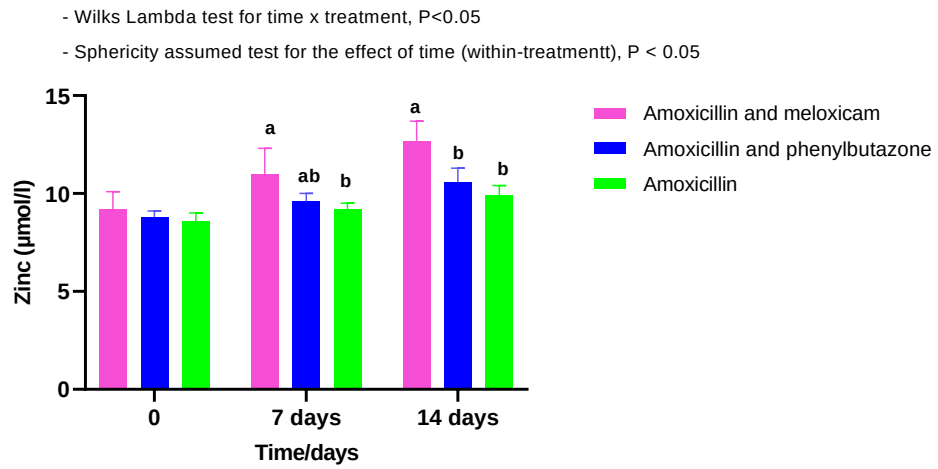

Figure 7. Mean values serum zinc in sheep with ovine respiratory disease before and after treatment with amoxicillin LA and meloxicam, amoxicillin LA and phenylbutazone and with amoxicillin LA alone. [a,b:] Means with different superscript letters are significantly different at $p < 0.05$.

DISCUSSION

In the present study, significant improvement in the clinical index score was recorded in sheep treated with meloxicam compared to those treated with phenylbutazone and those treated with Amoxicillin LA alone. This finding is consistent with that of Bednarek et al. (2003) who stated that administering a combination of oxytetracycline and meloxicam to calves with enzootic bronchopneumonia led to a significantly faster improvement in the clinical illness index score than treatment with a combination of oxytetracycline and flumethasone, or oxytetracycline alone. Additionally, Friton et al. (2005) found that administering a combination of meloxicam and antibiotics to feedlot cattle with bovine respiratory disease resulted in an improvement of clinical signs and a reduction of lung lesions compared to those treated with antibiotics alone. Similarly, Georgoulakis et al. (2006) observed that combining meloxicam with chlortetracycline in the treatment of growing pigs with porcine respiratory disease complex infection resulted in quicker recovery from respiratory inflammation caused by viruses and bacteria compared to pigs treated with chlortetracycline alone.

According to Dudek et al. (2020), an increase in neutrophil levels is typically observed during the onset of pneumonia in calves. Neutrophils play a crucial role in defense mechanisms through phagocytosis and oxidative burst (Jimbo et al., 2017). Therefore, the significant decrease in neutrophils and increase in lymphocyte percentage observed in the group of sheep treated with meloxicam compared to the other treatment groups may be due to the anti-inflammatory effect of meloxicam.

Concerning APPs, meloxicam could significantly decrease serum haptoglobin and globulin but increase serum albumin and albumin/globulin (A/G) ratio compared to phenylbutazone and Amoxicillin LA alone. This finding suggests rapid recovery and improvement in meloxicam treated group than others. Haptoglobin is an acute-positive protein that is a clinically relevant criterion for evaluating the occurrence and severity of inflammatory reactions in cattle, such as pneumonia (Eckersall and Bell, 2010). It is a scavenger protein with antioxidant, antimicrobial, and anti-inflammatory (Gulhar et al., 2018). The anti-inflammatory effect of haptoglobin is due to its ability to bind with CD11b/CD18 proteins which are found on neutrophils (El Ghmati et al., 1996). Additionally, other plasma proteins (albumin and globulin) play an essential role in the inflammation process and are usually used to evaluate the level of malnutrition and the seriousness of an illness (Laky et al., 2007). Albumin is considered the primary negative APP found in all animal species (Cray et al., 2009). According to Aldred and Schreiber (2020) during inflammation, albumin synthesis decreases, and amino acids are utilized for the creation of positive APPs. In addition, Otal et al. (2022) attributed the decreased serum albumin levels during inflammation to an increase in the volume of distributed albumin due to increased capillary permeability, leading to the escape of serum albumin. In contrast, elevated levels of globulin are associated with chronic inflammation and indicate prolonged exposure to various proinflammatory cytokines (Gopal et al., 2010). Furthermore, the significant increase in globulin in bovine respiratory disease-affected calves may be due to immune system activation caused by pathogens (Abd El-Raof and Hassan, 1999). The A/G ratio, a biomarker that combines albumin and globulin, indicates the status of inflammation and nutrition (Yang et al., 2022). Various respiratory disorders have also been associated with A/G abnormalities in patients with respiratory diseases (Qin et al., 2018; Chen et al., 2022).

Regarding copper, zinc, and iron meloxicam-treated sheep recovered their normal level of copper, zinc, and iron earlier than those of phenylbutazone and Amoxicillin LA groups. This finding may be attributed to the potent anti-inflammatory effect of meloxicam. Generally, in farm animals, as a result of infection and toxemia, there is hypozincemia, hypoferremia, and hypercupremia (Constable et al., 2016). Moreover, infection in farm animals is found to be associated with oxidative stress (Lykkesfeldt and Svendsen, 2007). The increased serum level of copper before treatment may be due to an increase in the production of the copper-binding protein, ceruloplasmin. This protein scavenges free radicals and acts as an antioxidant during infection and inflammation (Fox et al., 1995). In calves, Galarza et al. (2021) attributed the decrease in serum zinc levels during infection to the release of interleukin 1β and interleukin 6. In mice, the release of cytokines during infection has been found to facilitate Zinc uptake from the bloodstream (Aydemir et al., 2012). Additionally, decreased hepatic production of albumin (transporting zinc), transferrin, and lactoferrin leads to lower levels of zinc and iron in the blood (Gruys et al., 2005). In a study, it has been demonstrated that meloxicam could ameliorate the oxidative stress caused by extraneous exercise by strengthening the superoxide dismutase activity (Gunes et al., 2011). However, the combination of meloxicam and injectable trace elements did not affect the morbidity and serum levels of trace element on 45 days post-treatment in fattening calves (Hartschuh, 2015).

CONCLUSION

In conclusion, meloxicam-treated sheep had earlier clinical recovery and early normalization of blood parameters than those treated with phenylbutazone or amoxicillin LA alone. Meloxicam is an alternative effective anti-inflammatory drug for sheep in clinical practice. Further studies are needed to investigate the effect of meloxicam on specific infections in sheep with respiratory signs and to evaluate the cytokine response to treatment with meloxicam.

DECLARATIONS

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Authors' contributions

Abdullah Khalid Alwayel did the conceptualization and drafting of the manuscript, Mohamed Marzok supervised the project and manuscript submission, Magdy Gioushy conducted the investigation and drafting of the manuscript; Mahmoud Kandeel and Adel Almubarak did project administration and resources; Yaser Hamad did data analysis; Saad Shousha did interpretation and writing, Sabry El-khodery did editing and reviewing of the manuscript. All authors have read and agreed to the published version of the manuscript.

Competing interests

There are no competing interests to disclose.

Ethical considerations

Ethical issues (including plagiarism, consent to publish, misconduct, data fabrication and/or falsification, double publication and/or submission, and redundancy) have been checked by all the authors.

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Availability of data and materials

All data of this study are available by reasonable requests from authors.

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Anatomical and Histological Study of the Female Reproductive System of Green Freshwater Turtle (*Chelonia mydas*) During Breeding Season in Iraq

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ABSTRACT

Turtles are found in large numbers in Iraqi rivers, due to the availability of a suitable environment for reproduction and food. The breeding season begins from May to the end of October. The current study aimed to evaluate the anatomical and histological characteristics of the green freshwater turtle (*Chelonia mydas*) during the breeding season in Iraq. The samples included eight adult turtles within the age range of 11-14 years that were collected from Shatt Al-Hilla (Iraq) at month June. To investigate the reproductive system histological techniques and hematoxylin and eosin staining were used and before that the animals were anesthetized with chloroform. The results indicated two active ovaries and oviducts which fill the whole abdominal cavity. The mean weights of left and right ovaries and left and right oviducts in the turtles with average weights of 698 ± 0.05 g were 19.5 ± 0.01 g, 22 ± 0.022 g, 3.3 ± 0.05 g, and 4 ± 0.05 g respectively. The mean lengths of carapace, left ovary, right ovary, left oviduct, and right oviduct were 24 ± 0.08 cm, 15.9 ± 0.01 cm, 17± 0.04 cm, 13 ± 0.022 cm, 14 ± 0.056 cm. Anatomically the oviducts include the infundibulum, magnum, isthmus, uterus, and vagina. The infundibulum indicated a funnel-shaped membrane while the magnum was the muscular coiled long tube. The isthmus was shorter and less coiled than the magnum, the uterus appeared as the widest, thickest, and less coiled dark tube and swollen into the posterior to form a cyst-like part, and the vagina was muscular in structure. Histologically, the magnum and uterus were formed from mucosa, muscularis, and serosa. In both parts of the magnum and uterus, were branched crypt-like depressions that appeared devoid of sperm. The widespread distribution of this species in Iraqi rivers could be due to the activity of the ovaries and oviducts during the breeding season, which extends for 6 months.

Keywords: *Chelonia mydas*, Ovary, Oviduct, Turtle

INTRODUCTION

Various studies have extensively explored the morphology of the ovaries and oviducts of turtles, focusing on different aspects of the reproductive system (Cabral et al., 2011; Silva et al., 2011; Chaves et al., 2012; Firmiano et al., 2012). Some other researchers examined the morphology and functions of the female turtle's reproductive organs, offering comprehensive insights into breeding behavior and nesting (Peixoto et al., 2012; Souza et al., 2014).

In female turtles (*Podocnemis lewyana*), the reproductive system entailed two active ovaries (left and right), two oviducts, and suspensory ligaments (Yntema, 1981; Callebaut et al., 1997; Sánchez-Ospina et al., 2014). The ovaries have a long tubular shape with a thickness of 2 mm, width of 2 mm, and length of 20 mm. The reproductive system of marine turtles consists of two ovaries and oviducts with suspensory ligaments (Wyneken, 2001). The oviduct starts near the ovary and is divided into five regions (Girling, 2002). After ovulation, the corpus luteal is formed, and progesterone production occurs as in domestic mammals (Wyneken, 2001; Hafez and Hafez, 2004). A macroscopic study by Faillab et al. (2018) in sea green turtles (*Chelonia mydas*) indicated the medium length of 2.57 cm and width of 7.90 cm for ovaries.

Histologically, the ovaries are created from the cortex and medulla, having various follicles ranging from primordial oocytes to mature ova found in the cortex. The follicles, categorized based on size, undergo different stages of development, known as folliculogenesis, previtellogenesis, and vitellogenesis (small, medium, and large; Nainan et al., 2010; Pérez-Bermudez et al., 2012). In the hawksbill turtle (*Eretmochelys imbricate*), the size of follicles ranges from 19.2 to 24.9 µm (Pérez-Bermudez et al., 2012). The medulla is composed of smooth muscle, blood vessels, fibroblasts, lymphoid tissue, and collagen fibers (Callebaut et al., 1997; Nainan et al., 2010; Pérez-Bermudez et al., 2012). There are differences in the size of ovaries considering the age of turtles and the follicles (Callebaut et al., 1997).

Microscopic analysis of the oviducts reveals distinct layers, as described by Palmer and Guillette (1988). The mucosa is composed of ciliated epithelium and connective tissue containing glands. The muscularis layer consists of

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smooth muscle, providing the necessary contractile function. The outermost layer, known as the serosa, forms the protective outer covering of the oviducts. Therefore, the current study aimed to evaluate the anatomical and histological characteristics of the green freshwater turtle (*Chelonia mydas*) during the breeding season (June) in Iraq.

METHODS AND MATERIALS

Ethical approval

The current study was conducted according to the ethical guidelines of the Department of Anatomy and Histology, college of Veterinary Medicine, Al-Qasim Green University, Babylon, Iraq (No: 2023, 8/9/2023).

Study animals

In the current study, eight female adult turtles (*Chelonia mydas*) were used as the samples. The turtles had an average weight of 698 grams and an age range of 11-14 years and were collected from Shatt Al-Hilla (Iraq) at month June 2023. Data on length straight carapace length (SCL) was measured from the middle of the nuchal notch to the posterior-most tip of the caudal peduncle and weights were systematically recorded for analysis. Anesthesia, using chloroform in a closed chamber, was administered to facilitate the removal of the plastron (MacLean et al., 2008).

Dissection process

The dissection process involved a precise cut through the neck skin, lateral extension, and circumferential cutting around the axillary region near the plastron. The incisions followed the seam created by the marginal and inframarginal cuts. The methodology employed in this study was based on a study by Wyneken (2001).

Macroscopic study histological examination

For the macroscopic study, data on the ovary and oviducts of the turtles, including shape, location, length, and weight, were recorded. Additionally, specific details about parts of the oviduct were recorded. Histological examination focused on the ovary, magnum, and uterus. In this regard, formalin 10% was first used to fix the samples, followed by dehydration, clearing, embedding, sectioning (5-7µm), and mounting. The sections were stained by Haematoxylin and Eosin (Suvarna et al., 2012).

RESULTS

Anatomical results

In breeding season which extends from May to the end of October, the reproductive system of Iraqi green freshwater turtle is composed of two active ovaries and two active oviducts. The ovaries occupied the whole abdominal cavity on both the left and right sides, extending from the liver cranially to the end of the coelomic cavity caudally. The ovaries had the shape of a cluster of grapes. Within the ovaries, follicles of varying sizes representing different stages of growth (Figure 1).

The mean weights of the left and right ovaries were recorded as 19.5 ± 0.01 gm and 22 ± 0.022 gm, respectively. Accordingly, the mean weights of the left and right oviducts were 3.3 ± 0.05 and 4 ± 0.05 gm, respectively. Regarding the length, SCL was measured as 24 ± 0.08 cm. The lengths of the left and right ovaries were 15.9 ± 0.01 cm and 17 ± 0.04 cm, respectively, while the lengths of the left and right oviducts were 13 ± 0.022 cm and 14 ± 0.056 cm, respectively (Tables 1 and 2). Both left and right oviducts exhibited a highly convoluted tube structure, comprising four distinct parts. The infundibulum, characterized by a very transparent and thin funnel membrane, was divided into two segments including a thin, flattened, diaphanous wall known as the funnel and another thick wall termed the tubular part. The magnum, a long and curvy tube with a white appearance, displayed a robust blood vessel supply on its external surface. This section featured a large diameter, a long and coiled portion, and a thick wall (Figure 2). The isthmus, shorter and less coiled than the magnum, was followed by the uterus.

The uterus, the widest and thickest segment, presented as a dark tube, swelling posteriorly to form a cyst-like part. Its thickness was comparable to that of the magnum, and it seamlessly transitioned into the vagina. The vagina, a muscular and short segment, led to the cloaca, completing the reproductive anatomy (Figure 2).

Histological results

In the histological examination, the outer layer of the cortex was observed to be composed of cuboidal cells, forming the epithelium. Within the cortex, both mature and immature follicles were identified. The oocytes within these follicles exhibited a central rounded nucleus with bushy chromatin, surrounded by cytoplasm in a fibrillar morphology. However, visualization of the medulla of the ovaries proved to be challenging (Figure 3).

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Considering the histological structure of the magnum and uterus, both organs were found to be comprised of mucosa, muscularis, and serosa layers, arranged from internal to external. The mucosa in both the magnum and uterus featured multiple longitudinal folds lined by the ciliated secretory epithelium of the pseudostratified columnar type. Notably, the mucosal folds in the uterus appeared narrower and taller, compared to those in the magnum. Some folds in both the magnum and uterus exhibited a leaf-like appearance. There are branched crypt-like depressions lined by cuboidal cells, the crypts see a lot at the caudal part of the oviduct (Figures 4, 5).

Table 1. The mean weight of Iraqi green freshwater turtle (*Chelonia mydas*) ovary, and oviduct

Anatomical parameters	Mean ± SD (gr)
Turtles weight	698±0.05
Left ovary	19.5±0.01
Right ovary	22±0.022
Left oviduct	3.3±0.05
Right oviduct	4±0.05

Table 2. The mean length of Iraqi green freshwater turtle (*Chelonia mydas*) ovary, and oviduct

Anatomical parameters	Mean ± SD (cm)
Turtles (carapace)	24±0.08
Left ovary	15.9±0.01
Right ovary	17±0.04
Left oviduct	13±0.022
Right oviduct	14±0.056

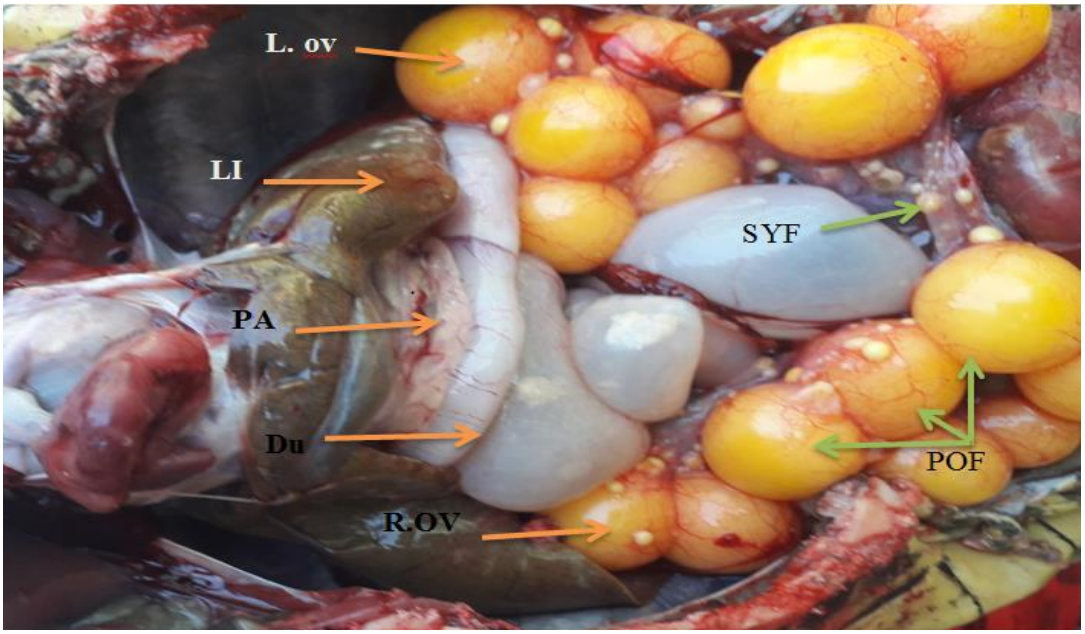

Figure 1. Anatomy of the reproductive system of Iraqi green freshwater turtle in the breeding season. L. OV: Left ovary, R. OV: Right ovary, LI: Liver, PA: Pancreas, DU: Duodenum, POF: Pre-ovulatory follicles, SYF: Small yellow follicles

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Figure 2. Anatomy of the reproductive system of Iraqi green freshwater turtle in the breeding season. IN: Infundibulum, IS: Isthmus, MG: Magnum, UT: Uterus, OV: Ovary

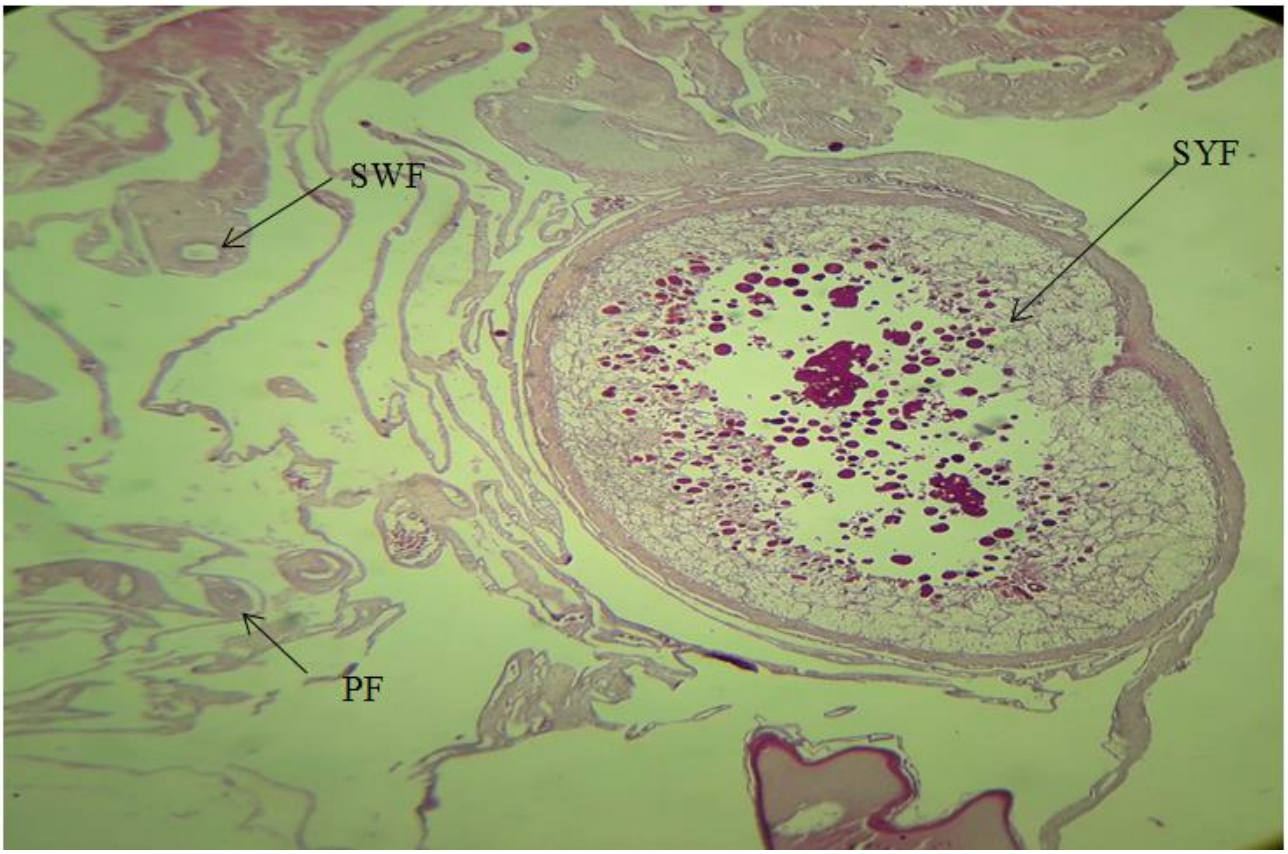

Figure 3. Histology of reproductive system of Iraqi green freshwater turtle in breeding season. SYF: Small yellow follicle, SWF: Small white follicle, PF: Primary follicle

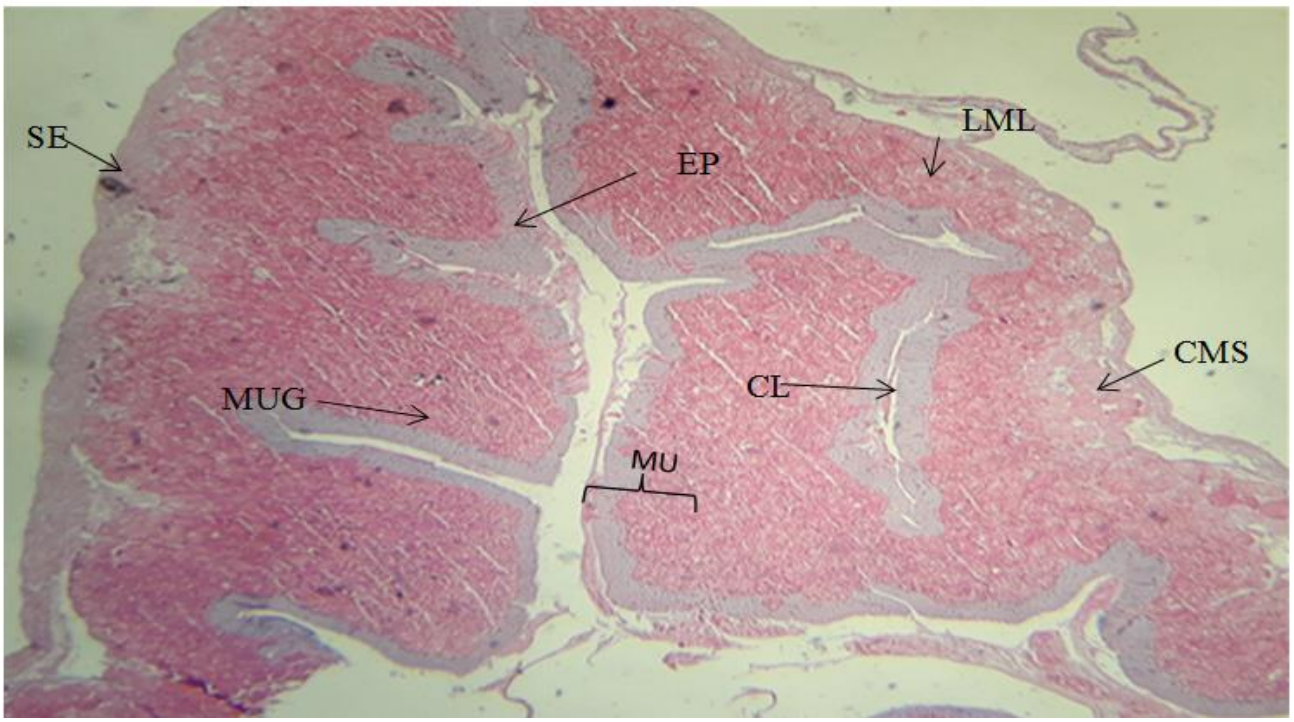

Figure 4. Histology of reproductive system of Iraqi green freshwater turtle in breeding season. MU: Mucosa, EP: Epithelium. CM: Circular muscular layer, LML: Longitudinal muscular layer, SE: Serosa, MUG: Mucosal gland, CL: Crept-like gland, H and E staining 10 X.

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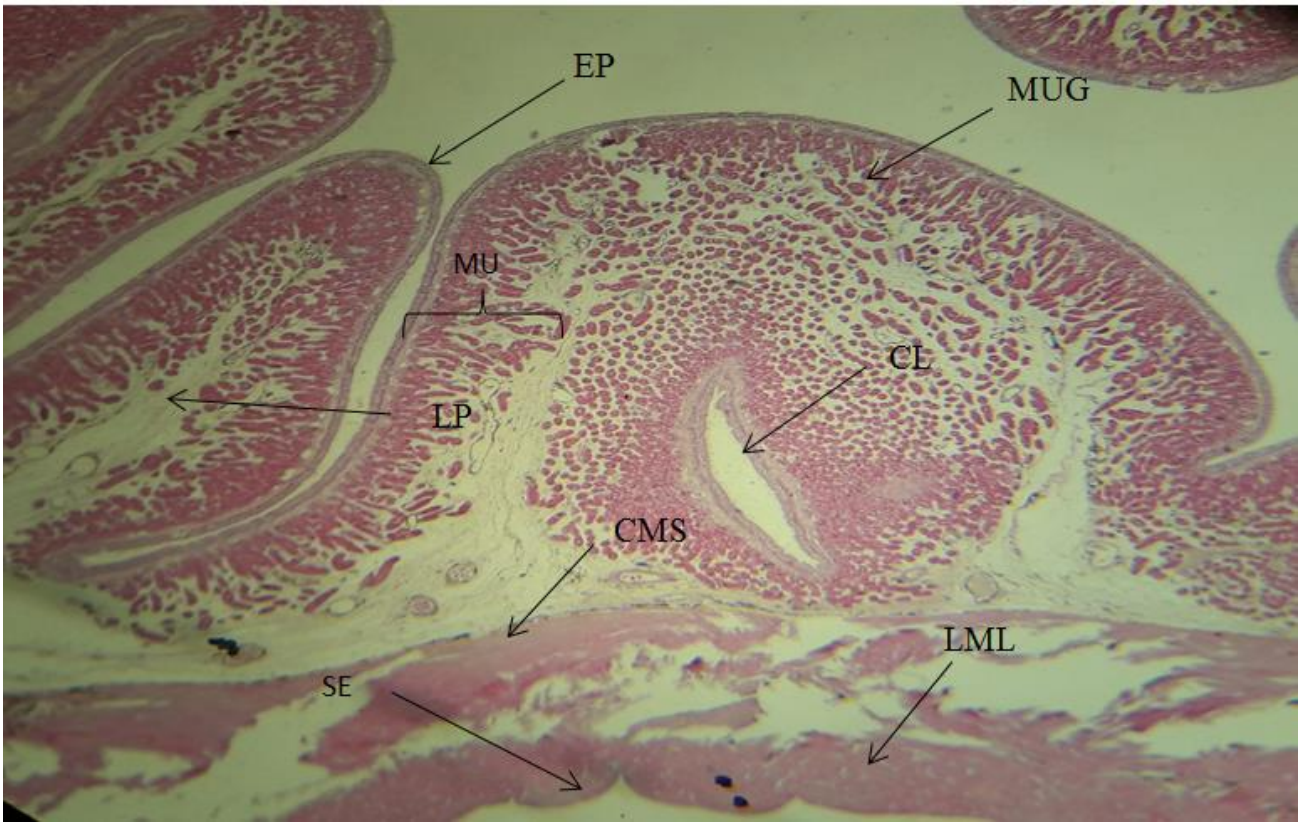

Figure 5. Histology of the reproductive system of Iraqi green freshwater turtle in the breeding season. MU: Mucosa, EP: epithelium, CM: Circular muscular layer, LML: Longitudinal muscular layer, SE: Serosa, MUG: Mucosal gland, CL: Crept-like gland, LP: Lamina propria, H and E staining 10 X.

DISCUSSION

The reproductive system of the female Iraqi green freshwater turtle (*Chelonia mydas*) during breeding season consists of two ovaries and oviducts. This observation aligns with findings from previous studies conducted on *Podocnemis lewyana* turtles by Yntema (1981), Callebaut et al. (1997), and Sánchez-Ospina et al. (2014). The findings revealed that ovaries occupied the entire abdominal cavity bilaterally, extending from the cranial aspect near the liver to the caudal end of the coelomic cavity. The ovaries exhibited a distinctive bunch-of-grapes morphology, featuring various sizes of follicles.

The mean weight of the left and right ovaries in female Iraqi green freshwater turtles during the breeding season was recorded as 19.5 ± 0.01 gm and 22 ± 0.022 gm, respectively. Concurrently, the mean SCL of the turtles was measured at 24 ± 0.08 cm. The mean lengths of the left and right ovaries were found to be 15.9 ± 0.01 cm and 17 ± 0.04 cm, respectively. These findings align with the results reported by Faillab et al. (2018) in *Chelonia mydas*, where the length of ovaries varied between 2.57 cm and 7.90 cm, and the width ranged from 0.14 cm to 1.14 cm. Interestingly, this study also corresponds with the observations made in *Podocnemis lewyana* turtles by Sánchez-Ospina et al. (2014), who found that the ovaries presented as extended tubular structures measuring 20 mm in length 2 mm in width, and 2 mm in thickness. Moreover, the length of the ovaries was found to be associated with the laying period, exhibiting elongation in more advanced stages.

The left and right oviducts indicated a mean weight of approximately 3.3 ± 0.05 gm and 4 ± 0.05 gm, with corresponding lengths of 13 ± 0.022 cm and 14 ± 0.056 cm, respectively. These oviducts were observed to be highly convoluted and composed of four distinct segments, as also noted by Girling (2002). The four segments identified were the infundibulum, tubal uterine or magnum, isthmus, uterus, and vagina. The infundibulum, characterized by a very transparent and thin funnel membrane, was further divided into two parts including the funnel and the tubular part. The magnum was a long, wide, and curvaceous tube with a white appearance, exhibited a robust blood supply on its external surface. Notably, the magnum was distinguished by its large diameter, longest coiled portion, and thick wall, contributing to its vital role in the reproductive process.

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Isthmus was shorter and less coiled than the magnum contrasts with the uterus, the widest, thickest, and less coiled dark tube that ended in the vagina. The uterus started as a tube and then posteriorly swollen in a cyst-like shape, sharing a thickness similar to that of a magnum. The vagina was a muscular, short, narrow, and straight segment, that leads to the cloaca.

Histologically, the epithelium is the outer layer of the cortex comprising cuboidal cells, and the cortex contains mature and immature follicles, supporting the findings of studies performed by Nainan et al. (2010) and Pérez-Bermudez et al. (2012). The oocyte inside the follicle has a central rounded poor nucleus and dense chromatin, the oocyte is encompassed by fibrillar cytoplasm, and the medulla of the ovaries is intractable to see due to the excessive activity of the cortex, as the process of laying eggs takes place simultaneously.

The magnum and uterus of the *Chelonia mydas* oviduct exhibit a three-layered structure, comprising mucosa, muscularis, and serosa from internal to external layers. The mucosa in both magnum and uterus consists of multiple longitudinal folds lined by ciliated secretory epithelium of pseudostratified columnar type. Interestingly, the mucosal folds in the uterus are narrower and taller than those in the magnum, with some folds displaying a leaf-like appearance. Both the magnum and uterus feature branched crypt-like depressions, lined by cuboidal cells, with a higher concentration observed at the caudal part of the oviduct. The lamina propria is densely populated with glands contributing to the production of albumin in the magnum and shell egg in the uterus. The number of glands increases significantly in the uterus, emphasizing its specialized role in reproductive processes. The tunica muscularis comprises circular muscle fibers as the inner layer and longitudinal smooth muscle fibers as the outer layer. Notably, the thickness of the muscle fiber layers intensifies from the magnum toward the end of the uterus. Finally, the serosa, forming a thin outer layer, consists of simple squamous cells underlining connective tissue.

CONCLUSION

The results of the current study indicate that the mean weights of left and right ovaries and left and right oviducts in the turtles were 19.5 ± 0.01 g, 22 ± 0.022 g, 3.3 ± 0.05 g, and 4 ± 0.05 g respectively. The mean lengths of carapace, left ovary, right ovary, left oviduct, and right oviduct were 24 ± 0.08 cm, 15.9 ± 0.01 cm, 17± 0.04 cm, 13 ± 0.022 cm, 14 ± 0.056 cm. Anatomically the oviducts include the infundibulum, magnum, isthmus, uterus, and vagina. The widespread distribution of this species in Iraqi rivers could be due to the activity of the ovaries and oviducts during the breeding season, which extends for 6 months. Therefore, more study needs to be conducted on green freshwater turtles during different seasons.

DECLARATIONS

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Availability of data and materials
The data of the current study are available with a reasonable request from the corresponding author.

Competing interests
There are no conflicts of interest for the submission and publication of this study.

Ethical considerations
The current research has followed all ethics during collecting the data, writing the article, and revising the draft of the manuscript. The final edition of the article is prepared originally for review and publication in this journal.

Authors' contributions
Salim Salih Ali AL-Khakani and M.S.H. Simawy were responsible for the research article proposal, experiment design, explaining the findings, and article writing. Mustafa Fadhil, Amina Imad Jawad, Sabreen M. Al-Janabi handled the preparation of materials, funding acquisition, and data curation. Dunia M. Al-Rubaie and Ranin S Hamad contributed to the review and editing process. All authors confirmed the last edition of the article before processing in the journal.

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Comparative Analysis of Lateral Flow Assay with Indirect ELISA for Detection of Anti-NSP Antibodies of Foot and Mouth Disease

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ABSTRACT

Foot-and-mouth disease (FMD) was an exceedingly infectious disease that spread to Indonesia in May 2022. A reliable diagnostic serologic test that can distinguish between infected and vaccinated animals was an important part of FMD (serotype O) control programs in affected areas in Indonesia. For this reason, a non-structural protein (NSP) serological test based on 3ABC proteins has been used. The indirect ELISA serological test requires time, skill, and specialized equipment. An alternative method that can be employed is the lateral flow assay (LFA), which offers the advantages of simplicity and portability, enabling rapid acquisition of results. The objective of this study was to validate the efficacy of a user-friendly anti-NSP antibody LFA for rapid diagnostic purposes. This was done by assessing its sensitivity and specificity in stored samples that had previously been tested using indirect ELISA. There were 32 preserved biological materials from dairy and beef cattle in three provinces in Indonesia that were examined with developed LFA. The results of each sample on LFA were compared to the ELISA result for its sensitivity and specificity according to positive and negative values on both tests. The test had a sensitivity of 95.2% and a specificity of 100%, compared to the indirect ELISA. The measured kappa value is also very good at 0.93, so LFA can be optionally used when examining anti-NSP FMD antibodies. Therefore, the LFA anti-NSP for detecting FMD is considered reliable because of its simplicity and the accuracy of the test results.

Keywords: Antibody, Bovine serum, Foot and mouth disease, Indirect ELISA, Lateral flow assay

INTRODUCTION

Foot-and-mouth disease (FMD) is a highly contagious disease that affects cloven-hoofed mammals (World Organization for Animal Health, 2022). The first FMD outbreak was observed in Indonesia in 1887, and the country has been declared FMD-free without inoculation since 1986. However, in 2022, there was a recurrence of FMD outbreaks in cattle and goats in Indonesia. The virus's serotype O/ME-SA/Ind-2001e sublineage was found and identified (Blacksell et al., 2019; Susila et al., 2022). The disease-causing virus, FMDV, is in the genus Aphthovirus and is in the family Picornaviridae. Its genome is 8.4 kb long and is made up of positive-sense, single-stranded RNA ([(+)] ssRNA). The virus produces ten non-structural proteins (Lpro, 2A, 2B, 2C, 3A, 3B1, 3B2, 3B3, and 3Cpro) from four precursor polypeptides including L, P1, P2, and P3 (Knowles et al., 2012). The virus contains four structural proteins (VP1–VP4).

In the oropharynx of some ruminant animals, such as cattle, the virus can persist for several months after recovering from infection, while pigs do not develop into carriers of the virus (WOAH, 2022). Detecting disease-carrying animals is crucial in controlling FMD, as they can serve as a reservoir of infection for other vulnerable animals. The majority of commercially available inactivated FMD vaccines only consist of the viral structural protein (SP). Uninfected animals, regardless of whether they have been vaccinated or not, should not possess antibodies to nonstructural protein (NSP) based on theoretical considerations. Hence, the presence of antibodies targeting NSP can serve as a means of distinguishing between animals that have received vaccination against FMDV and those that have not (Doel, 2003; WOAH, 2022). Some serological detection techniques include lateral flow assay (LFA), solid phase competition (SPC) ELISA, liquid phase blocking (LPB) ELISA, and virus neutralization assay (VNT, Wong et al., 2020).

The current serology test, the indirect ELISA, requires time, skill, and specialized equipment so this test can only be performed in select laboratories (Wong et al., 2020). The LFA strips that identify antibodies against NSPs are needed for

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differentiating infected from vaccinated animals (DIVA), just like they are for ELISA. The LFA is used in the current study to find NSPs. This assay may be a useful option for field FMD surveys as it can identify antibodies in cattle and goat serum. The purpose of this study is to determine how effective LFA is as a field substitute for indirect ELISA serological testing.

MATERIALS AND METHODS

Ethical approval

This study has complied with all relevant ethical guidelines of Universitas Airlangga, Indonesia.

Sera sample

The samples used in this research were collected from preserved biological materials and evaluated using indirect ELISA for monitoring purposes at the Tekad Mandiri Citra Laboratory, Indonesia. The study used 32 samples in total. The beef cattle and dairy that were chosen for sampling were from the province of East Java (14 samples), Banten (4 samples), and Lampung (14 samples) Indonesia, which historically had not received previous vaccination of FMD.

Indirect ELISA nonstructural protein

In this study, the commercial kits CHEKIT IDEXX FMD-3ABC bo-ov (IDEXX Laboratories, USA) were used. This kit detects antibodies against FMDV NSP 3ABC, which is a produced recombinant protein in the *Escherichia coli* expression system. This kit can only be used for bovine and ovine serum (Fukai et al., 2013). The test and control sera were diluted at a ratio of 1:100 using a diluent buffer. A microplate coated with FMD antigen is loaded with 100 µl of the diluted serum, followed by incubation at 37°C for 60 minutes. After washing, 100 µl of anti-ruminant IgG peroxidase conjugate was added to the wells of the microtitration plate. The plates were then incubated at 37°C for 60 minutes. After washing, each well was filled with 100 µl of TMB (3, 32, 5,52-tetramethylbenzidine) substrate, which was incubated at room temperature for 15 minutes in the dark before being filled with 100 µl of stop solution. Microplate read using an ELISA reader at 450 nm (Yousaf et al., 2021). The manufacturer recommended interpretation was < 20% is negative, 20–30% is ambiguous, and >30% is positive.

Lateral flow assays

The FMD commercial kit PMKit Ab (Tekad Mandiri Citra, Indonesia) was used for this assay. Bovine-ovine serum was put into a reagent tube (30 µl), homogenized, and left for 10 minutes. Then two drops of diluent were added and homogenized. The solution was dripped onto the test device marked "S" and the result was read in 10-15 minutes. The interpretation is negative if both the T line and the C line show a wine-red color reaction, and positive if there is no color reaction on the T line, just only on C line showing a wine-red color reaction.

Specificity and sensitivity tests

Compared to the gold standard, sensitivity and specificity are frequent statistical tools in evaluating the performance of alternative tests. This study compared the use of LFA as an alternative test to the gold standard of indirect ELISA (Table 1). In this study, "true positive" samples were determined using LFA and indirect ELISA. "False positive" samples were those that tested positive using LFA but negative using indirect ELISA. "False negative" samples were those that tested negative using LFA but positive using indirect ELISA. Both the "true negative" results obtained from LFA and indirect ELISA testing are negative. Sensitivity is the proportion of subjects with an actual positive outcome (true positives + false negatives) who are correctly assigned a positive assignment (true positives only). Sensitivity = TP/(TP+FN). Specificity is the proportion of subjects with an actual negative outcome (true negatives + false positives) who are correctly assigned a negative assignment (true negatives only). Specificity = TN/(TN+FP, Monaghan et al., 2021).

Table 1. The calculation for sensitivity and specificity of lateral flow assay

		Indirect ELISA	
		+	-
Lateral Flow Assay	+	TP	FP
	-	FN	TN
		Sensitivity: TP/(TP+FN)	Specificity: TN/(TN+FP)

*TP: True positive, FP: False positive, FN: False negative, TN: True negative

Statistical analysis

The collected data were analyzed using Microsoft Excel 2018 (US). The hypothesis of conditional independence was examined using the Kappa (κ) statistic (IBM SPSS 25, US).

RESULTS AND DISCUSSION

The samples used in this research were collected from preserved biological materials and evaluated using indirect ELISA for monitoring purposes at the Tekad Mandiri Citra Laboratory (Indonesia). The study used 32 samples in total. The cattle and dairy that were chosen for sampling were from the province of East Java (14 samples), Banten (4 samples), and Lampung (14 samples), which historically had not received previous vaccination of FMD. Serum samples tested using indirect ELISA resulted in 21 positive, 3 ambiguous, and 8 negative samples. Serum samples tested using lateral flow assay resulted in 20 positive and 12 negative samples. The details of each province can be seen in Table 2. All samples that were negative and ambiguous in the indirect ELISA, when tested with LFA were negative. This suggests that the negative cutoff of LFA includes both negative and ambiguous values in indirect ELISA. Only sample SL9 tested by indirect ELISA was positive, but tested by LFA was negative. The LFA demonstrated an overall sensitivity of 95.2% and a specificity of 100% when compared to the indirect ELISA. To determine the compatibility between standardized tests and new tests kappa statistical analysis is performed (Thrusfield, 2005). The tested LFAs had their kappa statistical values calculated with the indirect ELISA test as a comparison. The kappa value obtained was 0.93, indicating excellent agreement with the comparative test.

Table 2. Results of anti-nonstructural protein antibody testing by lateral flow assays and indirect ELISA on bovine serum samples from East Java, Banten, and Lampung provinces, Indonesia

Serum code	Breed	Province	Result		
			LFA	ELISA	
			(+/-)	(% Value)	Interpretation
JB1	FH	East Java	+	92.82	P
JB2	FH	East Java	+	81.75	P
JB3	FH	East Java	+	96.96	P
JB4	FH	East Java	+	99.38	P
JB5	FH	East Java	+	122.48	P
JB6	FH	East Java	+	60.92	P
JB7	SC	East Java	+	41.22	P
JB8	SC	East Java	-	23.26	S
JB9	SC	East Java	-	13.49	N
JB10	SC	East Java	+	68.31	P
JB11	OC	East Java	+	36.54	P
JB12	OC	East Java	-	25.34	S
JB13	OC	East Java	-	10.02	N
JB14	OC	East Java	-	3.81	N
BK1	OC	Banten	-	3.69	N
BK2	SC	Banten	-	10.51	N
BS1	SC	Banten	-	17.86	N
BS2	SC	Banten	-	15.97	N
SL1	SC	Lampung	+	116.53	P
SL2	SC	Lampung	+	121.22	P
SL3	SC	Lampung	+	104.99	P
SL4	SC	Lampung	+	132.06	P
SL5	SC	Lampung	+	153.94	P
SL6	OC	Lampung	+	109.8	P
SL7	OC	Lampung	+	70.77	P
SL8	OC	Lampung	+	47.61	P
SL9	OC	Lampung	-	35.11	P
SL10	OC	Lampung	+	110.59	P
SL11	SC	Lampung	+	60.26	P
SL12	SC	Lampung	+	44.94	P
SL13	SC	Lampung	-	23.85	S
SL14	SC	Lampung	-	14.66	N
Number of positives			20	21	

* FH: Friesian holstein, OC: Ongole, SC: Simmental-crossbreed, P: Positive, S: Suspect/ambiguous, N: Negative, LFA: Lateral flow assay

Since the FMD serotype O breakout in May 2022, Indonesia has been on high alert for the growth and spread of this case (Susila et al., 2022). In the event of an FMD outbreak, early and exact FMDV diagnosis allows for efficient FMD surveillance and response by implementing adequate controls and prevention measures. The availability of high-throughput machinery and highly skilled individuals is crucial for the diagnostic assays to check and diagnose FMD. Additionally, the poor samples that came from moving materials from a field to a lab may impede or postpone the early detection of the illness (Wong et al., 2020). Therefore, rapid LFA may serve as a promising on-site diagnostic method for rapid FMD detection and enable timely control measures.

In places where vaccination is used to reduce FMD, sero-surveillance should be carried out using a test that can distinguish between infected and vaccinated animals (Rout et al., 2014). The DIVA has effectively identified antibodies targeting specific NSPs of FMDV (Paton et al., 2006). To identify anti-NSPs antibodies in pigs, Chen et al. (2009) employed recombinant FMDV serotype O 3ABC protein. Later, Wu et al. (2011) developed an LFA strip based on FMDV serotype O recombinant 2C′3AB protein, in which 3C was removed due to low immunogenicity and substituted with a fragment of 2C protein fused to the N-terminus of 3AB. Despite the high sensitivity and specificity of the test, the serotypes of positive and tested vaccination serum samples were not disclosed. The use of LFA strip technology in DIVA has been hypothesized despite the absence of any reports to that effect.

The LFA kit in the assay operates in a competitive format. The competitive format of the assay is the target binds to the ligand and blocks the ligands from binding to the reporter (Qian and Bau, 2004). Because macromolecules are challenging to immobilize directly on solid phases, these competitive formats are frequently used for the detection of micromolecules. For macromolecules and whole-cell immunoassays, the competitive format can also be developed as an optional technique. The LFA kit used NSP of FMD as a ligand. Target analytes in this configuration interfere with the reporter's ability to bind to the test ligands. A reporter such as carbon black, dye-encapsulating liposomes, colored polystyrene, colloidal gold, and phosphor is combined with the target analyte in a solution that dissolves on the strip. Therefore, in the absence of any target analytes, a signal would be generated at the test line.

CONCLUSION

In this study, the results of the LFA analysis showed a sensitivity of 95.2% and a specificity of 100% compared to indirect ELISA. The compatibility of LFA with ELISA was demonstrated to be excellent, indicating its potential use as a differentiating infection from vaccinated animals test. Compared to ELISA, which requires time, equipment, and expertise, LFA is more practical and faster to use, and also easier to carry to the field. Further tests need more samples and groups to evaluate the effectiveness and reliability of the LFA in the field.

DECLARATIONS

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Availability of data and materials

The datasets generated during the current study are available from the corresponding author upon reasonable request.

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Authors' contributions

Akmal Jauhari did samples testing and manuscript preparation. Siti Munawaroh did the manuscript preparation. Wyanda Arnafia did manuscript preparation and revision. Denniswara Sibit did the analysis and funding. Jola Rahmahani designed the research and supervision. Suwarno Suwarno designed the research and manuscript revision. All authors read and approved the final version of the manuscript.

Competing interests

The authors declare that they have no conflicts of interest concerning the work presented in this article.

Ethical considerations

All the authors have thoroughly checked and confirmed the ethical concerns regarding the originality of collected data, analyzed data, and written sentences of this article before submission to the journal.

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Constraints to the Development of Turkey Farming in Southern Benin

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ABSTRACT

The turkeys are reared throughout the national territory of Benin, but their breeding is less developed than other poultry species, such as chickens and ducks. The current study aimed to characterize turkey farming in Southern Benin to identify the constraints associated with the farming practice that limit its development. A survey was performed in 104 turkey farms in the Atlantic, Ouémé, and Zou departments. The frequencies of qualitative variables and average quantitative variables were calculated and compared across departments. The investigated variables included turkey housing, feeding practices, reproduction management, health management, difficulties encountered, marketing of animals, and farm products. It was found that the housing, feeding, health monitoring, and constraints varied from one department to another. The turkeys were raised in fence-run buildings in the Ouémé (76.7%), modern poultry houses in the Atlantic (75%), and traditional habitats (42.9%) with a free range in the Zou. The free range prevented farmers from separating the turkeys from other poultry species. The poultry species present with turkeys on studied farms were chickens, ducks, and guinea fowl. The turkeys were fed more with commercial feed in the Atlantic (100%) and Ouémé (92.7%) regions and with cereals and agricultural by-products in Zou (82.1%). The prophylaxis consisted of deworming the birds, vaccinating them against Newcastle disease, controlling bacterial infections with antibiotics, and giving them vitamins in drinking water. The farmers vaccinated more turkeys in Zou than in Atlantic and Ouémé. The vaccination and administration of antibiotics do not prevent the introduction of disease into farms due to poor farm biosecurity, resulting in animal deaths. In conclusion, this study identified the obstacles that limit the development of turkey farming by region in Southern Benin. These barriers are primarily related to housing, feeding, mating, and marketing. Scientific research could potentially solve some of these issues, notably those concerning feeding and mating success. However, housing and marketing concerns would necessitate support from the authorities.

Keywords: Constraint, Feeding, Prophylaxis, Turkey

INTRODUCTION

Benin's poultry industry operates under two main systems, including traditional and modern, with local species being reared in the traditional system and exotic species in the modern system (Guezodje, 2009; FAO, 2015). In rural areas, traditional poultry farming is particularly prevalent as it serves various cultural, social cohesion, and economic purposes. These include the production of income for women, the use of local breed chickens and white-shelled eggs in traditional ceremonies and ethnopharmacology, and the production of meat and eggs for consumption and sale (Guezodje, 2009). The poultry species reared in Benin are chickens, guinea fowl, ducks, and turkeys. These birds' poor performance in this system prevents farmers from meeting customer demands. The relative studies have indicated that the poor performance of animals in this system is due to technical problems related to the lack of housing, the low genetic potential of the breeds reared, insufficient feed, and lack of health monitoring (Youssao et al., 2010, Boko et al., 2013; Houessionon et al., 2020). These mentioned studies have focused on chickens, ducks, and guinea fowl, and do not include turkeys. The results obtained have made it possible to improve the production methods of the species concerned, to provide farmers with many feed formulas, and to improve the performance of local breeds by crossbreeding with foreign breeds (Dahouda et al., 2009; Youssao et al, 2010; Boko et al., 2013). After the improvement of the birds' performance, the quality of their meat has been evaluated and improved to reassure consumers and facilitate their marketing (Tougan et al., 2013; 2018). Insufficient attention to turkey farming in Benin means that the meat production of this species decreases year after year, despite the efforts made by the farmers. It is therefore necessary to find ways and means to improve national turkey meat production to increase local production and productivity on farms. To achieve this, it is necessary to understand the characteristics of turkey farming. For this purpose, typology studies have been conducted in the north (Ouaké only) and south of the country (Dèdéhou et al., 2018; Dotché et al., 2021). These studies carried out in the north of the country (Benin), did not take regional variations into account, which prevented identifying problems by region and better organizing improvement work. This study aimed to investigate the regional variation of turkey farming in Benin and to identify the problems that hamper the development of turkey farming in the study area.

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MATERIALS AND METHODS

Ethical approval

The research protocol has been approved by the ethics committee of the Laboratory of Animal Biotechnology and Meat Technology of Benin (N°214 DPSA/LBATV/D).

Study area

Data were collected from August 2018 to August 2019 in the departments of Atlantic, Ouémé, and, Zou in Benin (Figure 1). The Atlantic department is located in the south of Benin and covers an area of 3233 km². It extends from Godomey to the edge of Sèhouè. It has eight communes and in the present study, data were collected in the communes of Abomey-Calavi and Allada. Atlantic Department has a four-season sub-equatorial climate (two rainy and two dry seasons) with an annual rainfall of 1060 mm (Dotché et al., 2021).

With nine communes, the department of Ouémé has a total area of 1865 km². This department has a four-season climate with 900-1500 mm of rainfall and is located in the sub-equatorial zone. Activities were conducted in this department's communes of Porto-Novo, Akpro-Missérété, Avrankou, and Sèmè-Podji (Dotché et al., 2021).

Zou covers an area of 5,243 km² with 9 communes and has a climate of transition between the sub-equatorial climate and the humid tropical Sudano-Guinean climate of northern Benin. The annual precipitation ranged from 900 mm to 1200 mm on average. There are two rainy seasons and two dry seasons in Zou. The research was conducted in this department's communes of Bohicon and Djidja (Dotché et al., 2021). The communes chosen in each department were those where several turkey farmers were located.

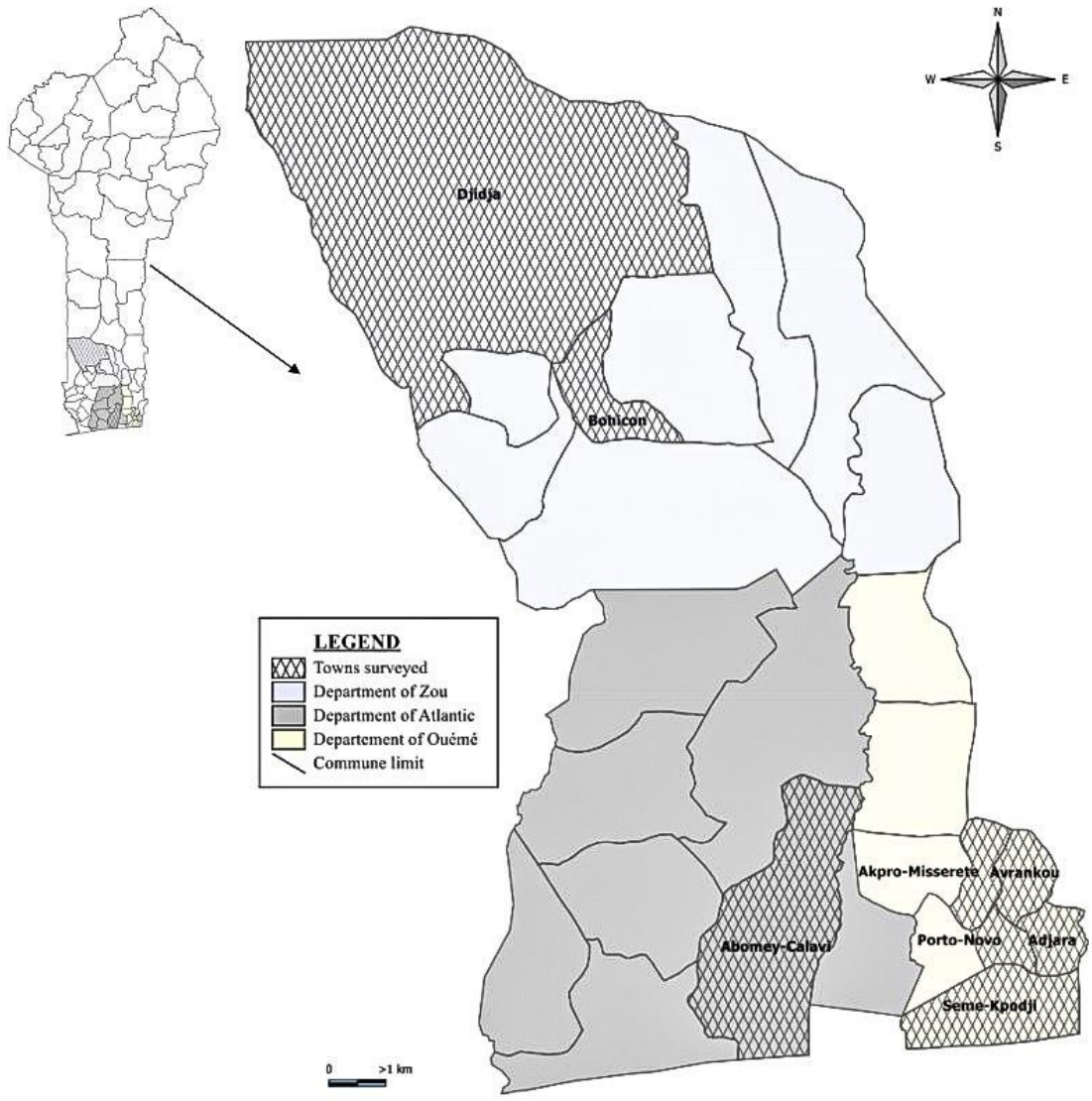

Figure 1. Study area in Benin (2018-2019)

Methodology

The methodology used for data collection was a retrospective survey through direct interviews with the farmers. This survey collected information on the breeders and the characteristics of their farms. The data were collected in 104 farms of the Ouémé, Atlantic, and Zou departments. In the absence of a list of breeders, it was necessary to contact the territorial agricultural development agencies to get into contact with the first farmers. Next, the "snowball" method was used to find the others (Goodman, 1961). All the farmers found by this method were interviewed. The number of turkeys per farm averaged 22.3 in the Atlantic, 14.3 in Ouémé, and 36.7 in Zou. A multiple-choice survey form was used for data collection from farmers. The questions were open-ended and included the identification and education of breeders, habitats, production objectives, modes of animal acquisition, utilization of livestock products, identification of birds, selection of reproducers, breeding constraints, and marketing of livestock products.

Statistical analysis

The data collected were analyzed using SAS software (SAS Institute Inc., Cary, NC, USA, 2013). The SAS Proc GLM procedure was used to conduct an analysis of variance for the quantitative variables (herd structure, product selling prices). The department impact was the only variation component taken into account in the analysis of the variance model. Where this factor (department) had an effect, comparisons between department averages were made two by two using the student t-test ($p < 0.05$).

The frequencies observed for the qualitative variables (study level, habitats, pathologies, and limitations) were determined using the SAS Proc Freq method. The bilateral Z test was used to compare the relative frequencies between the two departments and the Chi-square test was used to assess the department's impact on frequencies. For each relative frequency, a 95% confidence interval (CI) was calculated according to Formula 1 (Rousson, 2013).

$$IC = 1{,}96\sqrt{\frac{P(1-P)}{N}} \quad \text{(Formula 1)}$$

Where, P denotes the relative frequency and N is the sample size. The Correspondence Analysis (CA) function of the FactoMineR package of R4.1.3 was used for the Factorial Correspondence Analysis to explore the criteria used for the selection of reproducers by departments.

RESULTS

Profile of farms

The majority (95.29%) of the turkey farmers surveyed were married men. The farmers were composed of educated (81.4%) and uneducated (18.6%) people. Educated people have primary (21.18%), secondary (41.18%), and university (10.59%) levels (Table 1). The gender, education level, and marital status of respondents did not vary significantly from one department to another. The activities of the respondents were diversified. These activities were households, livestock, agriculture, fishing, handicrafts, trade, and state functions. Households were reported only in Atlantic (16.7%). The proportions of those engaged in animal husbandry as their main activity in the Atlantic (73.8%) and Zou (71.4%) were significantly higher than those in Ouémé (15.2%, $p < 0.001$). In contrast, the proportions of traders and artisans keeping turkeys were significantly lower in the Atlantic and Zou regions than in Ouémé. For these traders and artisans, turkey rearing was a secondary activity. Those holding state functions were met only in the Atlantic (14.3%) and Ouémé (18.2%) departments.

The main motivation of the breeders for this breeding was its profitability for the majority of the interviewed people in the three departments (Table 2). Other reasons that motivated respondents to invest in turkey breeding were the ease of rearing, its hardiness (they appreciate its rusticity), and the pleasure (only in the Zou) of the species. The people who rear turkeys for pleasure are those who do it because they love it, not for profit. The proportion of people motivated by the hardiness of the species in the Atlantic (36.8%) was significantly higher than in Ouémé (9.1%) and Zou (7.1%, $p < 0.001$). To start breeding, all the breeders in Atlantic and Ouémé purchased the turkeys (Table 2). In Zou, 96.4% bought the turkeys to start rearing and 3.6% inherited them from their parents. The turkeys raised by the respondents were animals of local genetic type. The production objective on the majority of the surveyed farms was meat production. The other production objectives were eggs and young turkey production. The proportion of farmers producing eggs for marketing in Atlantic (60.4%) and Zou (60.7%) was significantly higher than that of Ouémé (21.2%, $p < 0.001$). Young turkey production was more reported in Zou than in Atlantic and Ouémé ($p < 0.05$). The products resulting from breeding were sold by the majority of respondents in all three departments. Besides sales, some breeders used the products for family consumption. Those who used the products for family consumption were more encountered in Zou (50%) than in Atlantic (2.3%) and Ouémé (3%, $p < 0.001$).

Turkeys habitat

The majority (93.8%) of breeders had habitat for turkeys. The proportion of farmers who had habitat for turkeys in Zou (100%) was significantly higher than that in Ouémé (81.8%, $p < 0.05$). The habitats used were hen houses, buildings and runs, and traditional habitats. Chicken houses were more used in the Atlantic (75%) than in Ouémé (13.3%) and Zou (28.6%, $p < 0.001$). The Ouémé breeders (76.7%) used a fenced area (where the turkeys had a building with a run) more than the breeders in Zou (25%) and Atlantic (2.8%, $p < 0.05$). Traditional habitats were used more in Zou than in the Atlantic (15%, $p < 0.05$). Traditional habitats were not used in Ouémé farms (Table 3). These traditional habitats used in

Zou (42.9%) and Atlantic (25%) are constructed of clay, wood, straw, and mosquito netting. The turkeys were housed separately from other poultry species on the majority of surveyed farms in the Atlantic (88.2%) and Ouémé (55.7%) departments. The proportion of breeders performing this separation in the Atlantic was significantly higher than those in the Ouémé and Zou departments (35.71%, $p < 0.05$). The poultry species present with turkeys on studied farms were chickens, ducks, and guinea fowl (Table 3). Chickens were recorded in the majority of farms (97.6% in the Atlantic, 90.6% in Ouémé, and 95.4% in Zou). Ducks were recorded on the majority of farms in Ouémé (53.1%). The guinea fowl were more encountered in the Atlantic (70.7%) than in Ouémé (31.3%) and Zou (22.7%, $p < 0.001$).

Turkey feeding

The turkeys were fed with commercial feeds, cereals, agricultural by-products, kitchen waste, and forages (Table 4). The commercial feeds were used more in Ouémé (100%) and Atlantic (92.7%) than in Zou (67.9%, $p < 0.001$). On the other hand, cereals and agricultural by-products were more used in Zou (82.1%) than in Ouémé (51.5%) and Atlantic (53.7%, $p < 0.05$). The breeders in Ouémé used more kitchen leftovers to feed the turkeys than those in the Atlantic. The fodders were used only in Zou (17.9%) and Atlantic (7.3%, $p < 0.01$). Cereals used in turkey feed were corn and sorghum. The agricultural by-products used in turkey feed were corn bran, rice bran, palm kernel meal, and soybean bran. The fodder used to feed the birds was the leaves of *Ipomoea batatas*, *Moringa oleifera*, *Tridax procubens*, and *Manihot esculenta*. The feed was served twice a day (morning and evening). The quantity served to turkeys is estimated by the breeder. This quantity is not measured. The breeder estimates it by taking into account the number of animals available and their age.

Table 1. Profile of turkey farms surveyed in Southern Benin during 2018-2019

Variable	Atlantic (n = 43)		Ouémé (n = 33)		Zou (n = 28)		Significance
	(%)	CI	(%)	CI	(%)	CI	
Sex							
Men	93[a]	7.6	93.9[a]	8.1	96.4[a]	6.9	NS
Women	6.9[a]	7.6	6.1[a]	8.1	3.6[a]	6.9	NS
Level of education							
Out of school	18.6[a]	11.6	30.3[a]	15.7	21.4[a]	15.2	NS
Primary	30.2[a]	13.7	21.2[a]	13.9	17.9[a]	14.2	NS
Secondary	37.2[a]	14.4	36.4[a]	16.4	53.6[a]	18.5	NS
University	13.9[a]	10.4	12.1[a]	11.1	7.1[a]	9.5	NS
Main Activity							
Homemaker	16.7[a]	11.1	0[b]	0	0[b]	0	**
Breeder	73.8[a]	13.1	15.2[b]	12.2	71.4[a]	16.7	***
Farmer	21.4[a]	12.3	18.2[a]	13.2	25[a]	16.0	NS
Fishermen	2.4[a]	4.6	3.0[a]	5.8	0[a]	0	NS
Artisan	2.4[b]	4.6	30.3[a]	15.7	3.6[b]	6.9	***
Employee	14.3[a]	10.5	18.2[a]	13.2	0[a]	0	NS
Merchant	2.4[b]	4.6	18.2[a]	13.2	0[b]	0	**

n: Sample size, %: Percentage of surveys; **: $p < 0.05$; ***: $p < 0.001$; NS: Not significant; CI: Confidence Interval, [ab] the percentages of the same row followed by different letters differ significantly at the threshold of 5%.

Table 2. Production objective, origin of the turkey, and motivation of breeding in Southern Benin (2018-2019)

Variable	Atlantic (n=43)		Ouémé (n=33)		Zou (n=28)		Chi² Test
	(%)	CI	(%)	CI	(%)	CI	
Production target							
Meat	90.7[a]	8.7	100[a]	0	100[a]	0	NS
Egg	60.4[a]	14.6	21.2[b]	13.9	60.7[a]	18.1	***
Young turkeys	2.3[b]	4.5	0[b]	0	14.3[a]	12.9	**
Product uses							
Consumption of family	2.3[b]	4.5	3.0[b]	5.8	50[a]	18.5	***
Sale	97.7[a]	4.5	100[a]	0	96.4[a]	6.9	NS
Origin of animals at the start of the farm							
Purchase	100[a]	0.0	100[a]	0	96.4[a]	6.9	NS
Heritage	0[a]	0.0	0[a]	0	3.6[a]	6.9	NS
Motivation for turkey farming							
Ease of breeding	21.1[a]	12.2	9.1[a]	9.8	14.3[a]	12.9	NS
Rusticity	36.8[a]	14.4	9.1[b]	9.8	7.1[b]	9.5	**
Profitability	81.6[a]	11.6	96.9[a]	5.8	89.3[a]	11.4	NS
Pleasure	0[b]	0	0[b]	0	25[a]	16.1	***

n: Sample size, %: Percentage of surveys; ** : $p < 0.05$; *** : $p < 0.001$; NS: Not significant; CI: Confidence interval, [ab] the percentages of the same row followed by different letters differ significantly at the threshold of 5%.

Table 3. Turkey habitat in Southern Benin during 2018-2019

Variable	Atlantic			Ouémé			Zou			Significance
	n	(%)	CI	n	(%)	CI	n	(%)	CI	
Habitat for turkeys										
Available	36	94.4^{ab}	7.5	33	81.8^{b}	13.2	28	100^{a}	0	**
No habitats	36	5.6^{ab}	7.5	33	18.2^{a}	13.2	28	0^{b}	0	**
Types of housing										
Chicken house	36	75^{a}	14.1	30	13.3^{b}	12.2	28	28.6^{b}	16.7	***
Building and route	36	2.78^{c}	5.4	30	76.7^{a}	15.1	28	25^{b}	16.0	***
Traditional	36	25^{a}	14.1	30	0^{b}	0	28	42.9^{a}	18.3	***
Separation of turkeys from other birds										
Yes	34	88.2^{a}	10.8	29	55.7^{b}	18.1	28	35.7^{b}	17.7	***
No	34	11.8^{b}	10.8	29	44.3^{a}	18.1	28	64.3^{a}	17.7	***
Species present										
Duck	41	43.9^{a}	15.2	32	53.1^{a}	17.3	22	22.7^{a}	17.5	NS
Chicken	41	97.6^{a}	4.7	32	90.6^{a}	10.1	22	95.5^{a}	8.7	NS
Guinea fowl	41	70.7^{a}	7.5	32	31.3^{b}	16.1	22	22.7^{b}	17.5	***

n: Sample size; %: Percentage of surveys; ** : $p < 0.05$; *** : $p < 0.001$; NS: Not significant; CI: Confidence Interval, ^{ab} the percentages of the same row followed by different superscript letters differ significantly at the threshold of 5%.

Table 4. Types of feed used for turkey farms in Southern Benin during 2018-2019

Variable	Atlantic (n=41)		Ouémé (n=33)		Zou (n=28)		Significance
	(%)	CI	(%)	CI	(%)	CI	
Cereals and agricultural by-products	53.7^{b}	20.3	51.5^{b}	17.1	82.1^{a}	14.2	**
Commercial feed	92.7^{a}	8.3	100^{a}	0	67.7^{b}	17.3	***
Kitchen scraps	31.7^{b}	18.8	69.7^{a}	15.7	46.4^{ab}	18.5	**
Fodder	7.3^{ab}	0	0^{b}	0	17.9^{a}	14.2	**

n: Sample size, %: Percentage of surveys; ** : $p < 0.05$; *** : $p < 0.001$; CI: Confidence interval, ^{ab} the percentages of the same row followed by different superscript letters differ significantly at the threshold of 5%.

Management of reproduction in farms

Mode of reproduction

The mating was followed by the majority of farmers in Ouémé (67.74%) and Zou (57.14%). The proportions of breeders who followed matings in the Ouémé and Zou were significantly higher than those in the Atlantic (16.7%, $p < 0.001$). The number of eggs laid on Atlantic farms (13.89) was significantly higher than in Ouémé (11.97) and Zou (11.79). All the farmers in Ouémé, Zou, and the majority of those in the Atlantic (90.9%) practiced natural incubation. Besides natural incubation, some breeders practice artificial incubation (Table 5). The natural incubation was performed by a turkey or hen. Some breeders collect eggs from turkeys and give them to the hens to incubate. Incubation in turkey was practiced by the majority of respondents (87.5% in the Atlantic, 96.8% in Ouémé, and 85.7% in Zou). Incubation under the hen was more performed in Zou (57.1%) than in the Atlantic (21.9%) and Ouémé (3.23%, $p < 0.001$). The proportion of farmers using the hen to hatch eggs in the Atlantic was also significantly higher than that in Ouémé ($p < 0.05$). In comparison to Zou, the Atlantic, and Ouémé had significantly higher egg hatching rates ($p < 0.05$).

The age of entry into the reproduction of males in the Atlantic (9.15 months) was significantly higher than that reported in Ouémé (8.1 months), which was in turn higher than that observed in Zou (6.04 months, $p < 0.05$). The same observation was made for the age of entry into reproduction of females (Table 6). The number of eggs laid per hatching was greater in the Atlantic than in Ouémé and Zou ($p < 0.05$). The number of young turkeys alive at hatching in the Atlantic Department (12.4) was significantly higher than that of Ouémé (8.6), which was also higher than the number of young turkeys alive at hatching in Zou (3.1, $p < 0.001$). The same finding was made for the number of weaned turkeys.

Choice of reproducers

The breeders have the criteria to select the best reproducers on the farms (Table 7). The criteria used to select male reproducers were mating ability, size (larger than females), health status, age (older than females), and hardiness (rusticity). Size was the criterion used by the majority of breeders in the three departments. The proposition of the breeders using the ability to mount in Zou (60%) was significantly higher than that of Ouémé (18.2%) and Atlantic (0%, $p < 0.001$). The health and feather status (shiny) was used more in Atlantic (76.9%) than in Zou (32%) and Ouémé (4.5%, $p < 0.001$). Correspondence factor analysis (CA) indicated that Zou breeders mainly used the mating ability

criterion to select males; while Atlantic breeders employed health, feather, and hardiness criteria (Figure 2). The farmers of Ouémé considered mainly the age and size of animals (Figure 2).

The criteria used to select female reproducers were laying ability (good layer), incubation ability (good incubator), maternal ability (good mother), aplomb, health status, color, and acceptance ability of the male. According to poultry farmers, a good layer was a turkey that could lay more eggs per laying season, and a good incubator could hatch all the eggs laid. A good mother was a turkey who could bring all her young to weaning. She had to be able to defend her offspring against predators. The criteria, such as egg-laying, hatching, and maternal ability, were assessed through the performance of the mother of the subject to be selected, as these future mothers have not yet laid eggs to be judged on their own performance. Rearing ability and maternal ability were used more in the Atlantic and Ouémé than in Zou ($p < 0.001$). In contrast, hatchability, plumage color, and ease of acceptance of males during mating were more used in Zou than in Ouémé and Atlantic ($p < 0.05$). Health status was used only in the Atlantic. The results of the CA showed that Zou breeders mainly consider plumage color, aplomb, hatchability, and ease of male acceptance to select female reproducers (Figure 3). The Atlantic breeders mainly consider maternal ability and health status to selecting females and those of Ouémé consider egg-laying ability (Figure 3).

Table 5. Reproduction mode of turkey farms in Southern Benin (2018-2019)

Variable	n	Atlantic		Ouémé (n=31)		Zou (n=28)		Significance
		(%)	CI	(%)	CI	(%)	CI	
Mating assistance								
Yes	36	16.7^{b}	12.2	67.7^{a}	16,5	57.1^{a}	18.3	***
No	36	83.3^{a}	12.2	32.3^{b}	16.5	42.9^{b}	18.3	***
Type of incubation								
Artificial	33	18.2^{a}	13.2	3.2^{a}	6.2	10.7^{a}	11.5	NS
Natural	33	90.9 ^{a}	9.8	100^{a}	0	100^{a}	0	NS
Natural incubation								
Under turkey	32	87.5^{a}	11.5	96.8^{a}	6.2	85.7^{a}	12.9	NS
Under hen	32	21.9^{b}	14.3	3.2^{c}	6.2	57.1^{a}	18.3	***
Type of reproduction								
Seasonal	15	33.3^{a}	23.9	6.5^{b}	8.6	3.6^{b}	6.9	****
Non-seasonal	15	66.7^{b}	23.9	93.6^{a}	8.6	96.4^{a}	6.9	****

n: Sample size, %: Percentage of surveys; *** : $p < 0.001$; NS: Not significant; CI: Confidence interval, ^{ab} the percentages of the same row followed by different superscript letters differ significantly at the threshold of 5%.

Table 6. Age of breeders and laying performance of turkeys in Southern Benin (2018-2019)

Variable	Atlantic			Ouémé			Zou			Significance
	n	Mean	SE	n	Mean	SE	n	Mean	SE	
Age of male breeders (months)	23	9.15^{a}	0.36	24	8.13^{b}	0.36	28	6.04^{c}	0.33	***
Age of female breeder (months)	22	8.84^{a}	0.44	23	7.61^{b}	0.44	28	5.91^{c}	0.39	***
Number of eggs laid per turkey	19	13.89^{a}	0.72	32	11.97^{b}	0.55	28	11.79^{b}	0.59	**
Number of eggs hatching	10	12.37^{a}	0.78	32	9.72^{ab}	0.55	28	3.08^{b}	0.59	**
Number of series of laying per year	10	2.10^{b}	0.46	27	3.37^{a}	0.28	26	3.81^{a}	0.28	**
Number of turkeys at hatching	16	12.37^{a}	0.73	32	9.72^{b}	0.51	25	3.08^{c}	0.58	***
Hatching rate (%)	10	89.06^{a}	14.80	32	81.20^{a}	13.69	28	26.12^{b}	16.44	**
Number of turkeys weaned	12	11.67^{a}	0.79	32	8.63^{b}	0.48	26	2.19^{c}	0.54	***
Number of dead turkeys	12	1.75^{a}	0.34	32	1.09^{a}	0.23	25	0.84^{a}	0.26	NS
Age at culling of breeding stock (months)	10	28.20^{a}	2.79	23	20.70^{a}	1.83	25	21.84^{a}	1.76	NS

n: Sample size, %: Percentage of surveys; **: $p < 0.05$; *** : $p < 0.001$; NS: Not significant; SE: Standard Error; ^{abc} Means of the same row followed by different superscript letters differ significantly at the threshold of 5%.

Table 7. Criteria for selection of reproductive male and females turkeys in Southern Benin (2018-2019)

Variable	Atlantic		Ouémé		Zou		Chi² Test
	(%)	CI	(%)	CI	(%)	CI	
Criteria for choosing reproductive males							
n	13		22		25		
Skills for mating	0[b]	0	18.2[b]	16.1	60[a]	19.2	***
Large size	76.9[a]	22.9	72.7[a]	18.6	60[a]	19.2	NS
Feathers and health status	76.9[a]	22.9	4.5[c]	8.7	32[b]	18.3	***
Male older than female	0[a]	0	4.5[a]	8.7	0[a]	0	NS
Hardiness	7.7[a]	14.5	0[a]	0	0[a]	0	NS
Criteria for the selection of reproductive females							
n	12		15		22		
Good layer	66.7[a]	26.7	66.7[a]	23.9	0[b]	0	***
Good incubator	8.3[b]	15.6	6.7[b]	12.7	45.5[a]	20.8	**
Good mother	66.7[a]	26.7	33.3[a]	23.9	4.5[b]	8.7	***
Aplomb	8.3[a]	15.6	13.3[a]	17.2	40.9[a]	20.5	NS
Good health	16.7[a]	21.1	0[b]	0	0[b]	0	**
Color	8.3[b]	15.6	6.7[b]	12.7	45.4[a]	20.8	**
Ability to accept the male	0[b]	0	0[b]	0	27.3[a]	18.6	**

n: Sample size, %: Percentage of surveys; ** : $p < 0.05$; *** : $p < 0.001$; NS: Not significant; CI: Confidence interval. [ab] the percentages of the same row followed by different letters differ significantly at the threshold of 5%.

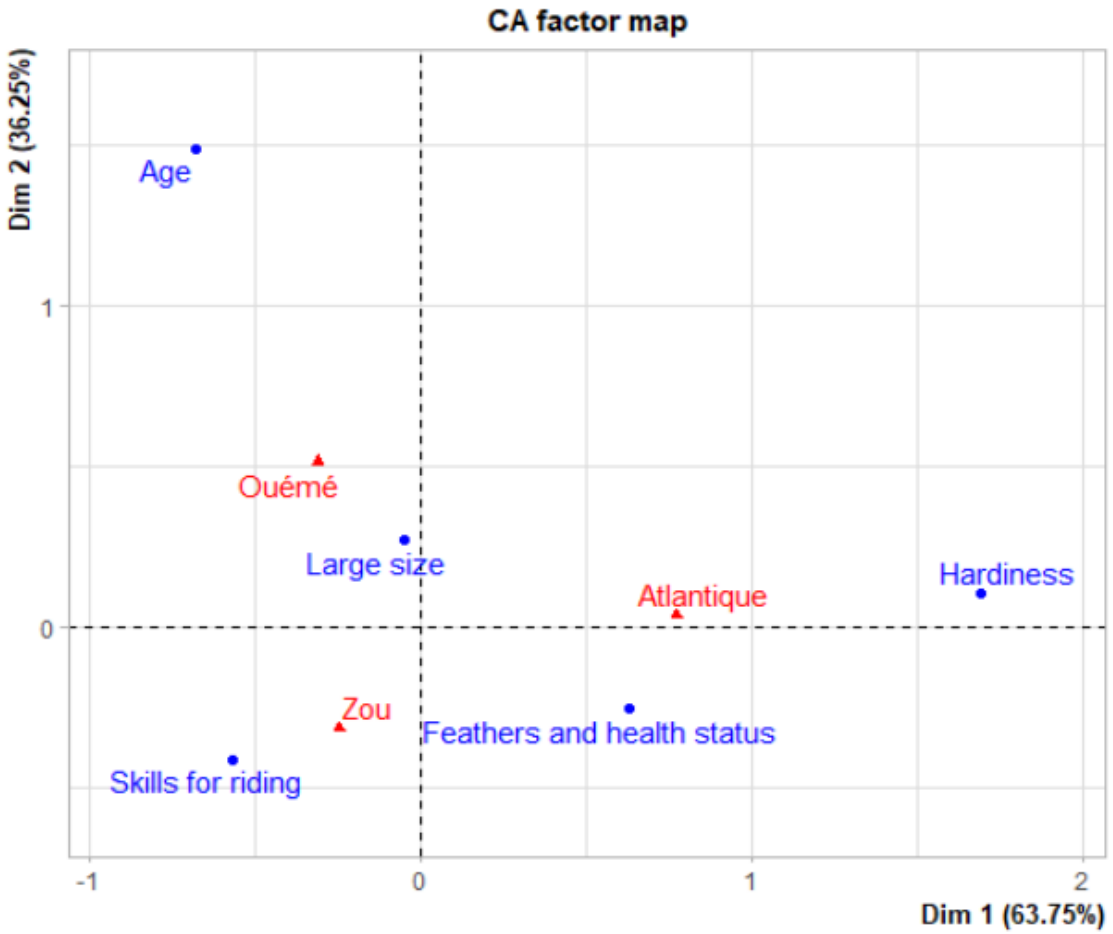

Figure 2. Distribution of selection criteria of reproducer males by region in Southern Benin (2018-2019). For the selection of males, breeders in the Zou region take their skills for riding into account. Breeders in the Atlantic region considered the feathers, health status and hardiness (rusticity) and breeders in the Ouémé region considered the large size.

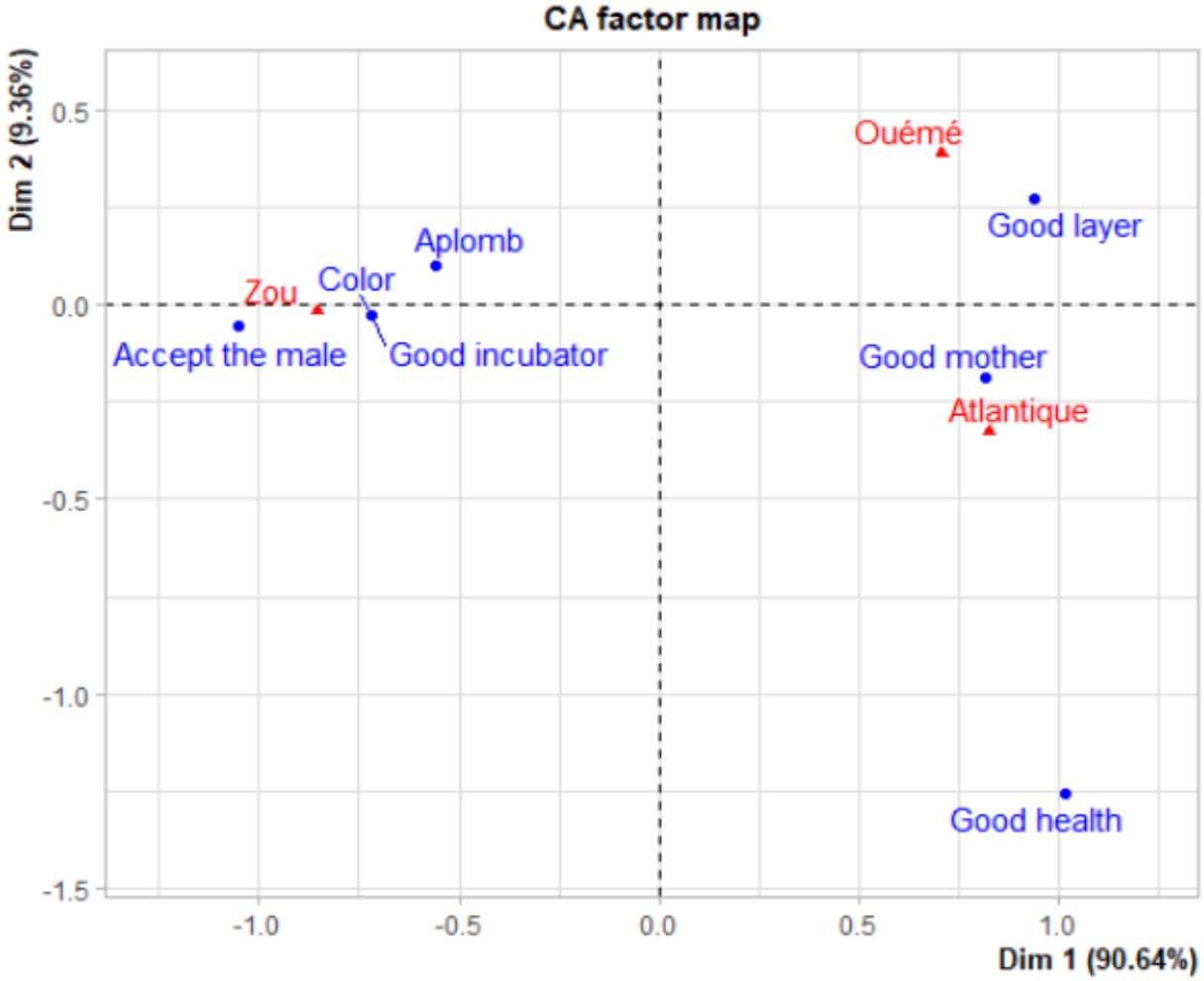

Figure 3. Distribution of selection criteria of reproducer females by region in Southern Benin. The criteria used to select reproductive females in Zou were included of color, aplomb, appropriate incubator and acceptance of male, good mother, and health in the Atlantic and good layer in Ouémé.

Health monitoring and pathologies encountered

The sanitary monitoring of the farm was focused on the cleaning of breeding materials and medical prophylaxis. The prophylaxis applied consisted of deworming the birds, vaccination against Newcastle disease, control of bacterial infections with antibiotics, and administration of vitamins (Table 8). The treatments for bacterial infections are not targeted at specific bacteria, as breeders do not have the necessary knowledge to make the diagnosis. The proportion of those who dewormed the birds did not differ significantly between departments. On the other hand, the administration of antibiotics and vitamins to birds was more common in Ouémé and Zou than in the Atlantic ($p < 0.05$). The farmers vaccinated more turkeys against the Newcastle disease in Zou (75%) than in Atlantic (34.5%) and Ouémé (35.3%, $p < 0.05$). These precautions did not stop diseases from entering farms. The pathologies encountered by farmers are Newcastle disease, smallpox (an infectious disease caused by *variola* virus), respiratory diseases, coccidiosis, scabies, Gumboro disease, and bronchitis (Table 8). Fowl plague was more reported in Ouémé (57.7%) than in Atlantic (11.5%) and Zou (3.8%, $p < 0.001$). Smallpox and scabies were more recorded in Zou farms than in the Atlantic and Ouémé ($p < 0.001$). Respiratory diseases were reported only in the Atlantic (15.4%).

The farmers treated diseases when they appeared with modern or traditional medicine. Some of them combine both treatments (modern and traditional). The majority of farmers in all departments used modern medicine for the treatment of diseases because of its high efficacy (Table 8). The reasons for using traditional medicine were its low cost in Atlantic, efficacy in Ouémé, and efficacy, low cost, and reduction of bacterial resistance in Zou.

Difficulties encountered

The difficulties encountered by the farmers were disease, mortality, theft, lack of a market for sale, and high production costs (Table 9). The proportion of farmers reporting high disease and mortality as difficulties in Ouémé (64.3%) and Zou (66.7%) was significantly higher than that of Atlantic farmers (15.8%, $p < 0.001$). High morbidity was recorded between hatching and the age of entry into reproduction in the majority of farms. The causes of morbidity do not vary from one department to another. These causes were lack of hygiene, pathogens, inadequate food, rain, wind, and coolness. The diseases sometimes lead to the deaths of the affected subjects. Other causes of bird deaths were accidents,

predators, and seasons (rainy seasons). The turkeys died much more in the rainy season, mainly in the Zou department. The mortality rate was highest in young turkeys that have not been weaned. The proportion of Atlantic farmers (100%) reporting mortality at this age was significantly higher than that of Ouémé (64.3%) and Zou (66.7%).

No Atlantic farmer has reported predators as a cause of death of turkey poults before weaning. The diseases are more reported as causes of mortality of turkey poults in the Atlantic (75%) and Ouémé (93.3%) than in Zou (36.4%, $p < 0.05$). Predators were more implicated in the mortality of turkey poults in the Ouémé (93.3%) than in Zou (50%, $p < 0.05$). These predators kill more weaned turkeys in Ouémé and Zou than in the Atlantic ($p < 0.05$). The cases of accidents of weaned turkeys were reported only in Ouémé (52.2%). Disease remains the only cause of mortality of turkeys at reproductive age in the Atlantic. Accidents and predators were the main causes of mortality at this age in Ouémé and Zou. Thefts were reported only in Ouémé (57.14%). The absence of a market for the sale was reported more by farmers in Zou (37%) than in the Atlantic (15.8%) and Ouémé (3.6%, $p < 0.05$). The high cost of production (especially feed) was more reported in the Atlantic (73.7%) than in the Ouémé (7.1%) and Zou (14.8%, $p < 0.001$).

Marketing of livestock products

The sales opportunities for livestock products were very diverse (Table 9). The end-of-year feast period was the period chosen by the majority of farmers to sell turkeys for slaughter. The proportion of Ouémé farmers who sell during this period in Ouémé (100%) was significantly higher than that of the Atlantic (64.3%) and Zou (74.1%, $p < 0.05$). Turkeys were sold more at the age of slaughter, during the children's back-to-school period, in the case of family care, and in the case of death in Ouémé than in the other departments (Table 11). The farmers in Ouémé (74.1%) and Atlantic (57.1%) sold more turkeys when they stopped laying than in Zou (14.8%, $p < 0.001$).

Turkey poults are sold at a higher price in the Ouémé (5904 F cfa [the franc of the financial community in Africa]) than in Zou (3722 F cfa) and Atlantic (3279 cfa ($p < 0.001$). On the other hand, adult males for slaughter are more expensive in the Atlantic (28,058 F cfa, the franc of the financial community in Africa) than in Ouémé (22,433 F cfa, $p < 0.05$). The selling price of adult females for slaughter follows the same trends as that of adult males, but the difference between the price of Zou and Ouémé was significant ($p < 0.05$). The price of reproducer males does not vary from one department to another (Table 10). On the other hand, reproducer females are more expensive in the Atlantic (23,708 F cfa) than in Ouémé (12,857 F cfa) and Zou (17,071 F cfa, $p < 0.001$). The selling price of breeding turkeys in Zou was also significantly higher ($p < 0.05$) than in Ouémé. The market price of turkeys is used by farmers in Ouémé (87.1%) and Zou (81.5%), while those in Atlantic (54%) use the customer's profile to set the selling price (Table 11). Thus, a customer who appears richer may buy a more expensive animal than one who appears poorer.

Table 8. Health monitoring and pathologies encountered on turkey farms in Southern Benin (2018-2019)

Variable	Atlantic			Ouémé			Zou			Significance
	n	(%)	CI	n	(%)	CI	n	(%)	CI	
Prophylaxis										
Internal parasites control	29	65.5^{a}	17.3	34	82.4^{a}	12.8	16	75^{a}	21.2	NS
Vaccination	29	34.5^{b}	17.3	34	35.3^{b}	16.1	16	75^{a}	21.2	**
Antibiotic	29	58.6^{b}	17.9	34	82.5^{a}	12.8	16	93.8^{a}	11.9	**
Vitamins	29	62.1^{b}	17.7	34	85.3^{a}	11.9	16	93.8^{a}	11.9	**
Diseases encountered										
Newcastle disease	26	11.5^{b}	12.3	26	57.7^{a}	18.9	26	3.8^{b}	7.4	***
Smallpox	26	46.2^{b}	19.2	26	46.2^{b}	19.2	26	92.3^{a}	10.2	***
Respiratory disease	26	11.5^{a}	12.3	26	0^{b}	0	26	0^{b}	0	**
Coccidiosis	26	42.3^{a}	19	26	3.9^{b}	7.4	26	11.5^{b}	12.3	**
Scabies	26	11.5^{b}	12.3	26	0^{c}	0	26	38.5^{a}	18.7	***
Gumboro, bronchitis	26	3.8^{a}	7.4	26	3.8^{a}	7.4	26	7.7^{a}	10.2	NS
Modes of treatment										
Traditional treatment	33	54.6^{a}	17.0	30	26.7^{b}	15.8	28	50^{a}	18.5	**
Modern treatment	33	78.8^{b}	13.9	30	96.7^{a}	6.4	28	78.6^{b}	15.2	**

n: Sample size, %: Percentage of surveys; **: $p < 0.01$; ***: $p < 0.001$; NS: Not significant; CI: Confidence interval, ^{abc} the percentages of the same row followed by different superscript letters differ significantly at the threshold of 5%.

Table 9. Causes of morbidity and mortality in Turkeys of Southern Benin during 2018-2019

Variable	Atlantic			Ouémé			Zou			Significance
	n	(%)	CI	n	(%)	CI	n	(%)	CI	
Difficulties encountered										
High diseases and mortality	19	15.8^{b}	16.4	28	64.3^{a}	17.7	27	66.7^{a}	17.8	***
Theft	19	0^{b}	0	28	57.1^{a}	18.3	27	3.7^{b}	7.1	***
Lack of market	19	15.8^{b}	16.4	28	3.6^{b}	6.9	27	37.0^{a}	18.2	**
Expensive breeding	19	73.7^{a}	19.8	28	7.1^{b}	9.5	27	14.8^{b}	13.4	***
High mortality period										
Before weaning	20	100^{a}	0	20	75^{b}	18.9	25	68^{b}	18.3	**
Between weaning and laying	20	5^{b}	9.6	20	65^{a}	20.9	25	40^{a}	19.2	**
From the age at first laying of the turkey	20	0^{a}	0	20	5^{a}	9.5	25	0^{a}	0	NS
Causes of pre-weaning mortality										
Accident	20	55^{b}	21.8	15	93.3^{a}	12.6	22	45.5^{b}	20.8	**
Predator	20	0^{c}	0	15	93.3^{a}	12.6	22	50^{b}	20.9	***
Disease	20	75^{a}	19	15	93.3^{a}	12.6	22	36.4^{b}	20.1	**
Season	20	30^{a}	20.1	15	13.3^{a}	17.2	22	22.7^{a}	17.5	NS
Causes of mortality between weaning and laying age of the turkey										
Accident	6	0^{b}	0	24	50^{a}	20	8	0^{b}	0	**
Predator	6	16.7^{b}	29.8	24	87.5^{a}	13.2	8	62.5^{a}	33.5	**
Disease	6	66.7^{a}	37.7	24	75^{a}	17.3	8	37.5^{a}	33.5	NS
Season	6	16.7^{a}	29.8	24	8.3^{a}	11.1	8	37.5^{a}	33.5	NS
Causes of mortality from the age at first laying of the turkey										
Accident	4	0^{ab}	0	24	54.2^{a}	19.9	13	23.1^{a}	22.9	**
Predator	4	0^{b}	0	24	87.5^{a}	13.2	13	76.9^{a}	22.9	***
Disease	4	100^{a}	0	24	0^{b}	0	13	0^{b}	0	***
Season	4	0^{a}	0	24	8.3^{a}	11.1	13	7.7^{a}	14.5	NS

n: Sample size, %: Percentage of surveys; **: $p < 0.05$; ***: $p < 0.001$; NS: Not significant; CI: Confidence interval, ^{ab} the percentages of the same row followed by different superscript letters differ significantly at the threshold of 5%.

Table 10. Sale period of turkeys in Southern Benin (2018-2019)

Variable	Atlantic			Ouémé			Zou			Significance
	n	(%)	CI	n	(%)	CI	n	(%)	CI	
Adult selling period										
Of slaughterable age	14	50^{b}	26.2	27	88.9^{a}	13.7	27	11.1^{c}	11.8	***
Back to school for children	14	7.1^{b}	13.5	27	48.1^{a}	18.9	27	0^{b}	0	***
Family Care	14	7.1^{b}	13.5	27	37.0^{a}	18.2	27	0^{b}	0	***
Bereavement	14	0^{ab}	0	27	14.8^{a}	13.4	27	0^{b}	0	**
Laying stop	14	57.1^{a}	25.9	27	74.1^{a}	16.5	27	14.8^{b}	13.4	***
End of the year party	14	64.3^{b}	25.1	27	100^{a}	0	27	74.1^{b}	16.5	**
Any time	14	0^{b}	0	27	0^{b}	0	27	22.2^{a}	15.5	**
Definition of the selling price										
Market price	21	42.7^{b}	21.2	31	87.1^{a}	11.8	27	81.5^{a}	14.7	***
Weight and size of the animal	21	0^{a}	0	31	9.7^{a}	10.4	27	0^{a}	0	NS
Client's head	21	54.14^{a}	21.3	31	9.7^{b}	10.4	27	62.9^{a}	18.2	***

n: Sample size; %: Percentage of surveys; **: $p < 0.05$; ***: $p < 0.001$; NS: Not significant; CI: Confidence interval, ^{ab} the percentages of the same row followed by different superscript letters differ significantly at the threshold of 5%

Table 11. Selling price in the African Financial Community (CFA france) for turkeys and eggs in Benin (2018-2019)

Variable	Atlantic			Ouémé			Zou			Significance
	n	Mean	SE	n	Mean	SE	n	Mean	SE	
Young turkeys	24	3279.2^{b}	357.7	26	5903.8^{a}	343.7	18	3722.2^{b}	322.4	***
Adult male	26	28057.7^{a}	1180.4	30	22433.3^{b}	1098.9	27	25055.6^{ab}	1158.34	**
Adult female	26	20292.3^{a}	837.9	30	11883.3^{c}	780.12	27	14703.7^{b}	822.3	***
Egg	22	943.2^{a}	51.9	8	912.5^{a}	86.2	24	1008.3^{a}	49.7	NS
Male reproducer	20	26500^{a}	2013	7	25428.6^{a}	2406	14	28428.6^{a}	1701.3	NS
Female reproducer	12	23708.3^{a}	951.6	7	12857.1^{c}	1245.9	14	17071.4^{b}	881	***

n: Sample size; **: $p < 0.05$; ***: $p < 0.001$; NS: Not significant; ES: Standard Error; ^{abc} Means of the same row followed by different superscript letters differ significantly at the threshold of 5%

DISCUSSION

Profile of farms

The majority of turkey farmers are men. Most male involvement in turkey farming has been previously reported in Cameroon and Nigeria (Ngu et al., 2014; Amao et al., 2017). In contrast to this study, Bakoji et al. (2012) report the majority involvement of women in turkey farming in Bauchi State, Nigeria. There are several reasons for the low involvement of women in turkey farming in Benin. These include a lack of resources and a lack of a market for the meat. Indeed, turkey meat is very expensive, which limits its consumption by the population, whereas women are often more active in the trade of products that are easily sold (Dotché et al., 2021). The majority of breeders are educated people and this finding contrasts with that reported by Dèdéhou et al. (2018) in the commune of Ouaké in northern Benin that some farmers are out of school as reported by the majority of farmers in this study. The lack of schooling among these farmers is an obstacle to controlling the performance of livestock. As a result, because farmers are not educated, they are unable to record weights, and calculate egg-laying and profitability rates for their activity. The main production objective of the farmers is meat production for consumption. The same observation was made by Ngu et al. (2014) in Nigeria. The turkey farming has social, cultural, and economic importance for the surveyed farmers. This economic and cultural importance of turkey farming in Benin has already been reported in Southern Benin by FAO (2015).

Constraints of the development of turkey farming

The farmers in the Atlantic used chicken housing and those in the Ouémé used buildings and fenced areas because they had less space to practice extensive farming characterized by free-range and traditional housing, as in Zou, where farmers are in a rural environment with a high availability of space. The traditional housing is built with precarious materials such as straw and rammed earth and prevents farmers in Zou from separating turkeys from other poultry species (chickens, ducks, and guinea fowl). This type of farming has already been reported in the commune of Ouaké in the north (Attakpa et al., 2011). An important number of farmers in Ouémé (44%) cannot separate turkeys from other poultry because the birds are kept on the range for a long period of the day, during which time they live with other species of birds such as local chickens, ducks, and guinea fowl, which are often reared on a free-range. This cohabitation of several species and age groups represents a biosecurity problem. Certain species have the capacity to harbor pathogens without becoming ill and spread them to other vulnerable species (Conan et al., 2012; Pauly et al., 2019; Correia-Gomes and Sparks, 2020). This is the case of H5N1 avian influenza, whose transmission increases with the mixing of several species (Conan et al., 2012). In the same sense, older birds that already have stronger immune systems can harbor pathogens and transmit them to younger birds (Conan et al., 2012). Therefore, farmers in Zou need to improve turkey housing to be able to separate them from other species and reduce the liberty of the birds as recommended by these authors (Conan et al., 2012). The improved housing in Zou would also provide more protection for the birds as traditional housing does not provide enough protection and exposes them to the weather (high wind and rain) and predators (Nyoni et al., 2019; Nyoni et al., 2021; Desta, 2021). This exposure is expressed in the high mortalities observed by farmers in younger and more fragile animals (Otte et al., 2021). The Ouémé farmers may focus their breeding on a single species, particularly turkeys, to reduce cohabitation between several species.

Constraints related to turkey feeding

Commercial feed is used more in Ouémé and Atlantic than in Zou, where farmers mainly use cereals and crop by-products; this is related to the accessibility of these resources by farmers. Thus, in the department of Zou, agriculture is more developed and farmers in this area have more access to these products than farmers in the Atlantic and Ouémé. The feed resources used in Zou have already been reported in turkey farms in Ouaké (Attakpa et al., 2011; Dèdéhou et al., 2018) because the breeders in this commune are also farmers who rear birds in a system like that in Zou. The two forms of feed (commercial feed and agricultural by-products) have insufficiencies in terms of quality and quantity.

The deficiencies associated with feed quality relate to the imbalance between the intake and the needs of the animals. The agricultural by-products used in Zou are often unbalanced feeds and do not cover all the needs of the turkeys. The consequences of using only such a feed resource in poultry are decreased zootechnical and laying performance (Markos and Abdela, 2016). Some of these feeds, like cereal bran, can become contaminated during handling and transmit pathogens to the birds, as they are not treated (heated, for example) before being fed to the turkeys (Abdisa and Tagesu, 2017). The pathogens that untreated agricultural by-products can transmit to birds are Newcastle disease, avian influenza, salmonellosis and parasitosis (Abdisa and Tagesu, 2017; Sun et al., 2021). The available commercial feeds in Benin are well treated to prevent the transmission of pathogens, but they are unbalanced because they are made for chickens and not for turkeys. The farmers could use commercial turkey feed. Unfortunately, commercial turkey feed is not available in Benin. This forces some farmers to use chicken feed for

turkeys. As a result, these chicken feeds do not cover the needs of the birds equally, forcing farmers in the Atlantic and Ouémé departments to give a very high quantity of feed to the birds, thus increasing their production costs. This solution fund by the farmers isn't the appropriate one because it increases the food costs. The best solution is to formulate feeds that consider the needs of the turkeys and their physiology. To achieve this, farmers need the assistance of researchers to have balanced formulas for the turkey, because these farmers often do not have the necessary qualifications for the formulation of feed.

The deficiencies in quantity are found in the lack of measurement of the quantities of feed provided to the turkeys. As a result, farmers cannot know if the quantity provided is appropriate or not. The lack of performance recording is a contributing factor in that the farmer cannot judge the effectiveness of the feed used. Thus, if the farmers kept accurate records of the performances, they would be able to determine how much the quantity or the quality of the feed used was inadequate. In fact, the nutrient composition and quantity of feed given to turkeys should vary according to the animal's status (reproducer, cull, fattened), age (young, adults) and weight.

Constraints related to the management of reproduction

Natural mating is the most common method of reproduction, in extensive and semi-extensive poultry farms in Benin (Youssao et al., 2013). This mating method's failure results from the females' frequent inability to bear the weight of the males, which forces them to move around a lot during mating and causes ejaculation outside of the female's genitalia. The same finding was reported by Chowdhury et al. (2014) in many Asian countries. These difficulties in successful natural mating have also been reported in turkey farming in Nigeria (Adebisi and Ewuola, 2019). To correct this problem, farmers in the Atlantic and Ouémé choose heavier males whose weight may prevent the females from making enough movements. Unlike these farmers, those in Zou and Ouémé assist the female during mating. The farmers' assistance consists of keeping the female in place to allow the male to perform a complete and effective mating. These two methods ensure mating but have negative consequences (aggression to females and biosecurity problems linked to assistance) for breeding. Thus, choosing heavier males results in terrifying the female and injuring her (Chowdhury et al., 2014; Ferrante et al., 2019). The female's assistance during mating could cause biosecurity issues because, in traditional poultry farms, hygiene is insufficient and farmers can contaminate females through their hands. In addition, the assistance of the female during mating increases labor time for the farmer. In order to solve the challenges associated with mating in Zou, farmers select males who are proficient mounters and females who readily accept males for reproduction. Artificial insemination is a method that could solve this problem (Chowdhury et al., 2014; Mohan et al., 2018; Adebisi and Ewuola, 2019). The semen of turkeys can be collected, analyzed, and used to inseminate females. In Southern Benin, incubation occurs naturally. The hatching rate in Atlantic and Ouémé is higher than in Zou, indicating that the farmers in this area do not provide adequate conditions for bird mating, resulting in infertile eggs. This finding agrees with the results of Adebisi and Ewuola (2019) who reported a low egg fertility rate in naturally mated turkeys compared to artificially inseminated turkeys. Indeed, after laying, only fertilized eggs can hatch following incubation (Leborgne et al., 2013). This fertility problem in Zou is confirmed by the very low number of mean young turkeys hatching (3 young turkeys) in this department compared to those in the Atlantic (12 young turkeys) and Ouémé (10 young turkeys). The farmers do not know the causes of this low fertility and attribute it to the incubation ability of the females, which leads them to choose good incubating females for reproduction and perform incubation under the hen. These efforts have not improved egg fertility in Zou. The farmers in the Atlantic and Ouémé departments, in contrast to those in Zou, were more focused on the quantity of poults hatching and weaning than on egg fertility. As a result, they selected females from mothers who lay a lot of eggs and wean a lot of poults.

Constraints related to health monitoring and mortality

The primary challenges faced by the farmers in Ouémé and Zou were diseases, as the animal housing in these two departments is insufficient to protect them. Thus, these animals are exposed to pathologies in the wild through contact with sick animals and contaminated objects (Conan et al., 2012; Samanta et al., 2018). The most common pathogens are viral diseases such as Newcastle disease (in Ouémé), smallpox (in Zou), and parasitic diseases (scabies). These diseases are already reported in poultry farms in Benin generally (Boko et al., 2012; Youssao et al., 2013; Houessionon et al., 2020) and especially in turkeys (Attakpa et al., 2011). Farmers in these two departments treat birds against bacterial diseases, deworm them, and vaccinate them (particularly in Zou) in an effort to reduce disease. The farmers in these two departments also deworm their animals. These dewormings (the fight against internal parasites) are also practiced in the more developed farms of the Atlantic because these farmers are also confronted with parasitic diseases.

The diseases that breeders face on their farms are the main reasons why the youngest birds die, especially before they are weaned, as their immune systems are still developing and cannot fight off the illnesses. The same observation has already been made in turkey farms in the north of the country (Attakpa et al., 2011). The vaccination would increase

the immunity of the birds (Samanta et al., 2018; Otte et al., 2021) but young turkeys are not vaccinated by the respondents for financial reasons. Farms need to implement biosecurity protocols in order to preserve poults. Apart from diseases, predators and accidents are responsible for the deaths of turkeys in free-range farms. The same finding has already been made in free-range poultry (Otte et al., 2021). The farmers who use this method of rearing birds must build housing to limit the birds' mobility because adult mortality is also linked to predators and accidents. Reducing bird mobility will actually result in fewer accidents, diseases, predations, and deaths because it will confine turkeys and prevent them from contracting diseases from other free-ranging animals or accidentally coming into contact with predators (Conan et al., 2012; Samanta et al., 2018; Otte et al., 2021), but this reduction must consider the financial capacity of the farmers to avoid the elimination of their activity. The best way to raise turkeys is not to transform all the farms over to the better system used in the Atlantic, which would require expensive feed and building costs for new housing. It is necessary to consider a semi-free-range system, similar to that practiced in the Ouémé Department, but exclusively for the rearing of turkeys. The system requires separating the animals based on their age.

Constraints related to the marketing

The main difficulty in marketing turkeys is the lack of an outlet market in Zou since turkeys are expensive for the population of the surveyed area, which is commonly rural. One strategy that could be used to facilitate the marketing of turkeys in this department is the installation of a slaughterhouse to sell turkey cuts. Currently, the main period for turkey sales in this department is the Christmas and New Year period, as the festive period is an occasion for high meat consumption. The sale of turkey during the year-end festive period has also been reported by FAO (2015) in Benin and by Ouedraogo et al. (2015) in Burkina-Faso. In the other two departments, the existence of demand means that turkeys are sold on various occasions. Selling during the children's school year, for family care, and at funerals shows that turkey farming plays an economic and social role for farmers.

The price of turkeys was higher in the Atlantic than in Ouémé and Zou because the cost of production is higher in this department due to investments in housing and feed. In the Atlantic, farmers use improved poultry houses and turkeys are better monitored, while in the Ouémé, turkeys are reared in small fences, and in the Zou in traditional housing.

CONCLUSION

The study performed from August 2018 to August 2019 on constraints to the development of turkey farming in Southern Benin shows that turkey farming is carried out with improved techniques in the Atlantic region, with traditional techniques in the Zou region, and with more or less improved techniques in the Ouémé region. There are several obstacles standing in the way of this farming sector's growth including the high cost of food in the Atlantic; pathological issues (diseases from contact with other poultry species); social problems (theft cases) in the Ouémé; and pathological issues and insufficient markets in the Zou. The improvement of turkey meat production should be by attention to these difficulties in the study regions. Improving the biosecurity of some farms and implementing it in others is necessary to reduce disease rates and young turkey mortality. To improve turkey production in Benin, the difficulties faced in each region must be addressed. Further studies are needed to focus on developing feed formulas specifically adapted to the needs of turkeys to rectify feeding issues. There is also a need for artificial insemination to overcome the mating difficulties identified by the breeders. Finally, the authorities should support this farming activity by providing financial assistance to breeders to enable them to build housing for their animals.

DECLARATIONS

Funding

This study received no financial support.

Aailability of data and materials

All data of the current study are available in this article.

Authors' contributions

Dotche Ogoudanan Ignace and Youssao Abdou Karim Issaka designed and planned the study, supervised data collection and analyzed the data. Adebo Nasser, Okambawa Lionel, and Koffi Monique collected data and drafted the first version of the manuscript. Agbokounou Aristide, Baba Loukyatou Issimouha, and Dotche Ogoudanan Ignace wrote the final version of the document and carried out the critical review. Youssao Abdou Karim Issaka corrected the document. All authors read and approved the final version of the article.

Competing interests

The authors declare that they have no conflict of interest.

Ethical considerations

The authors took ethical concerns and farmers' consent into account prior to the surveys. This article was originally written without copying from other articles.

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Molecular Detection of *Entamoeba* spp. in Monkeys (*Macaca* spp.) in Babylon Province, Iraq

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ABSTRACT

Amoebiasis is a widespread parasitic disease caused by *Entamoeba histolytic*a (*E. histolytic*a), affecting various hosts, such as humans, birds, and pigs. This study aimed to investigate *Entamoeba* spp. in monkeys (*Macaca* spp.) diagnose them using molecular methods. A total of 33 fecal samples were collected from monkeys (*Macaca* spp.) aged 3-5 years in Babylon province to investigate a common and zoonotic parasitic disease. Initially, microscopic examination was conducted on all samples, and those yielding positive results were preserved for molecular study. The DNA was extracted, and conventional PCR was carried out with a pair of primers to detect the 857 bp fragment of *E. histolytica* SSU rRNA gene. PCR results for 19 fecal samples, previously identified as positive by the direct smear method, from monkeys in the reserves of Babylon province indicated that the presence of the SSUrRNA gene with an 857 bp was 45% in only 15 samples. Sequencing of the SSUrRNA gene revealed 98-100% similarity with *E.histolytica* sequences deposited in International GenBank, which have the sequence numbers OP522013, OP522014, OP522015, OP522016, OP522017, Op626161, Op626162, Op626163, Op626164, and Op626165.

Keywords: *Entamoeba histolytica*, Gene, Monkey, SSU rRNA

INTRODUCTION

Nature reserves and public zoos play a crucial role in housing diverse animal species, ranging from pets to predators. These environments provide a secure habitat for animals, fostering their growth and reproduction. Additionally, these facilities serve as invaluable resources for researchers, facilitating easy access to specimens and species that are under study (Thawait et al., 2013).

Captive animals of various kinds are generally susceptible to infections by a multitude of parasites. This susceptibility is influenced by several factors, including nutrition, the management system in place for the animals, and environmental conditions, such as temperature, humidity, and the pollution levels in the surrounding environment (Kashid et al., 2003; Goossensa et al., 2005; Singh et al., 2006; Atanaskova et al., 2011). Some types of parasites may be harmless to some animals, but they threaten the lives of others. Contact between humans and captive animals increases the chance of the spread of zoonotic parasitic diseases, which pose a threat to the health of the animals as well as those working in those places (Panayotova-Pencheva, 2013).

Entamoeba histolytic causes an intestinal disease called Amebiasis. It attacks the intestinal wall of the host, causing intestinal symptoms, abdominal discomfort, and bloody or loose mucous stools (Guillén, 2023). The infection period lasts for approximately 1-3 weeks. In advanced cases of infection, the infection may spread to other organs of the body, causing liver abscesses, lung abscesses, or brain abscesses, which leads to severe symptoms and may be fatal (Shirley et al., 2020; lin et al., 2022; Guillén, 2023)

This study aimed to undertake a molecular investigation of a parasitic species, analogous to human parasites, with the objective of confirming the potential presence of shared parasite species, such as Entamoeba spp. The methodology involved the application of conventional polymerase chain reaction (PCR) and DNA sequencer technologies.

Material and methods

Ethical approval

All procedures conducted on animals were in accordance with the ethical standards of the institution. and the current study was approved by the Committee of the Department of Biology, Faculty of Education, University of AL-Qadisiyah, Al-Diwaniyah, Iraq (No.456).

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Sampling

A total of 33 fecal samples were collected from *Macaca* spp. monkeys situated in both governmental and private reserves in the central region of Babylon, including its districts. These monkeys, aged between 3 and 5 years, were imported from Japan and Afghanistan. The direct smear method was employed, utilizing iodine Lugol's dye, to examine the presence of Entamoeba spp. cysts. Positive smears were identified using a Compound light microscope (Olympus, Japan; 40x). Subsequently, the samples were preserved at -20°C for molecular confirmation.

Prepared DNA and primers

A pair of primers for the gene SSU rRNA [F:5'-GTCAGAGACCACATGAAC-3, R: 5'-GTTGTCCCGACCTAATCC-3], based on Al-Abodi et al. (2014) was designed to confirm the diagnosis of *Entamoeba* using conventional PCR technique with a molecular weight of 857bp. DNA was extracted using Stool Genomic DNA Extraction Kit (Bioneer, Korea) following the manufacturer's protocol and the concentration and purity of the extracted DNA were measured by a Nano-drop spectrophotometer (Thermo, USA).

The PCR mix was prepared using AccuPower® Premix Kit (Bioneer, Korea) according to the manufacturer's instruction and the PCR reaction was performed with initial denaturation for 5 minutes at 95°C, followed by 35 cycles of 95°C for 30 seconds (denaturation), annealing at 58°C for 30 seconds, extension at 72°C for 40 seconds, with a final extension at 72°C for 5 minutes. The PCR products were analyzed by electrophoresis in a 1% agarose gel. Then, PCR product was sent to Bioneer Company in South Korea for the purpose (by DHL fast and saving DNA samples at -20°C) of knowing the sequence of DNA fragments using the DNA sequencing system to determine the type of *Entamoeba* spp. through phylogenetic tree analysis and the National Center for Biotechnology Information Genbank-Primer-Blast database program.

Statistical analysis

The data was analyzed using the statistical program (SPSS 24) where the Chi-Square test was used to determine the significant differences under the probability level ($p \leq 0.05$). T-test was also used to analyze the data.

RESULTS

The PCR examination of 33 stool samples collected from the monkeys (*Macaca* spp.) showed that only 15 samples produced positive results, accounting for a rate of 45%. The results of the statistical analysis indicated no significant differences in the presence of the SSU rRNA gene in collected samples using the direct swab method followed by PCR technique, as shown in Figure 1 ($p > 0.05$). The results revealed that only 10 samples were identical to the global isolates registered in NCBI representing *Entamoeba histolytica* (*E. histolytica*). Upon comparing the local parasite sequences bearing the serial numbers OP522013, OP522014, OP522015, OP522016, OP522017, Op626161, Op626162, Op626163, Op626164, Op626165, with the global isolates, the percentage of congruence ranged 99-100%. Utilizing the MEGA 6 program, a genetic tree was constructed to illustrate the genetic relationship between local isolates of *E. histolytica* bacteria and global isolates registered in NCBI, as depicted in Figure 3. The results of the genetic tree analysis of local isolates showed the presence of common ancestors, where the local isolates of *E. histolytica*, which carry the serial numbers OP522013, OP522014, OP522015, OP522016, OP522017, op626161, op626162, op626163, op626164, op626165 isolates, showed a genetically close relationship with isolates L00636.1, KP233840.1, AB002793.1, ON724174.1 and AB608092.1 globally registered in NCBI.

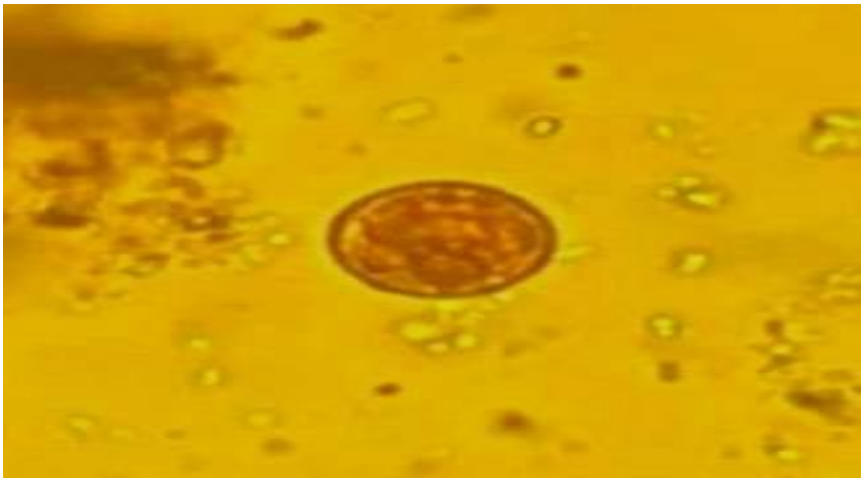

Figure 1. Cyst of *Entamoeba histolytica* (400x) isolated from the feces of monkeys (*Macaca* spp) in Babylon province

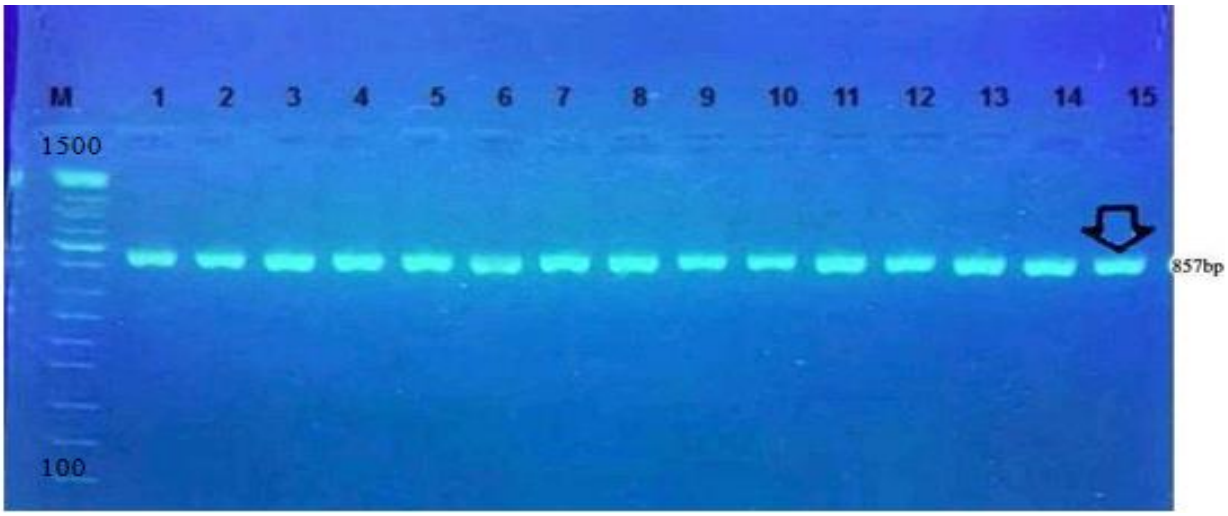

Figure 2. Amplified SSU rRNA gene electrophoresis in PCR. Columns 1-15 represent fecal samples (of monkeys at ages ranging between 3-5 years and both sexes) positive for polymerase chain reaction, showing the 857bp SSUr RNA gene of the *Entamoeba* spp. Column M represents a Ladder bearing molecular weight 100-1500 bp.

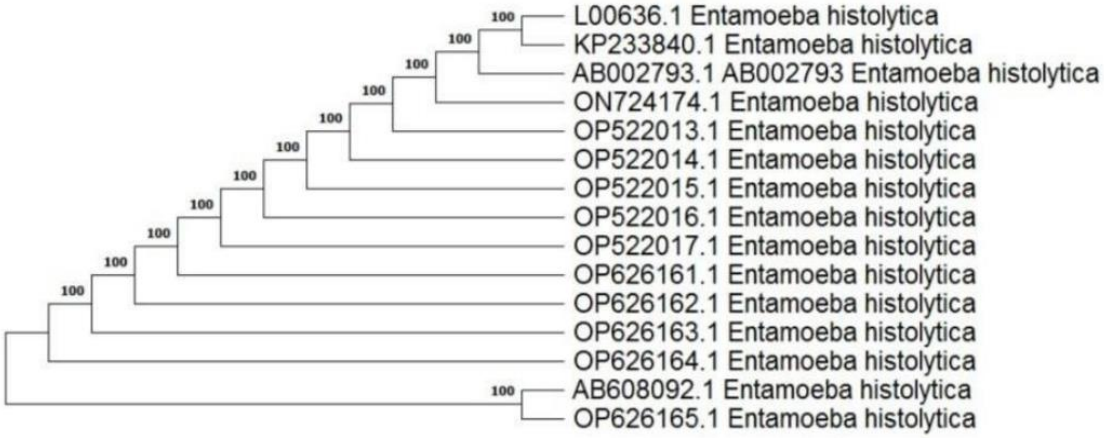

Figure 3. Genetic tree analysis using MEGA 6 program. The results show a common genetic relationship in local parasite samples taken from the feces of monkeys with *E. histolytica* parasite samples registered in NCBI-Genbank.

DISCUSSION

The results of the current study, utilizing PCR to detect the SSU rRNA gene of the *Entamoeba* species with a molecular weight of 857bp in 15 fecal samples from monkeys that tested positive by the direct smear method, revealed an overall presence rate of 45% in only 15 samples. The absence of a positive result for the PCR reaction in 4 samples may be attributed to potential uncontrolled laboratory conditions. The detection of *E. histolytica* in monkeys using molecular methods is consistent with some studies that indicated the presence of this parasite in different species of captive nonhuman primates. A study by Levecke et al. (2010) indicated that the tantalus monkey, greater spot-nosed monkey, Sunda pig-tailed macaque, olive baboon, and Bornean orangutan were infected with *E. histolytica* at a various rate. Amoebiasis has also been reported in other species of monkeys as mentioned in studies by Márquez-Monter et al. (1991) and Takano et al. (2005). Moreover, *E. histolytica* was not found in the patas monkey (Beaver et al.,1988), mandrill (Verweij et al., 2003; Mätz-Rensing, 2004), mantled guereza (Loomis et al., 1983; Suzuki et al., 2008), and Western gorilla (Sleeman et al., 2000).

The identification of the *E. histolytica* parasite in Iraqi monkeys, whether through the direct smear method or molecular methods, represents one of the first reports in Iraq concerning a parasite shared between monkeys and humans. In line with the current study, some international studies indicated the possibility of infecting species in different types of monkeys with *E. histolytica* (Márquez-Monter et al., 1991; Takano et al., 2005; Levecke et al., 2010).

It is noteworthy that 10 samples from the current study were identical to globally recorded *E. histolytica* species, demonstrating a genetic relationship with isolates registered in the National Center for Biotechnology Information. These include the German isolate recorded by Tannich et al. (1991), the Japanese isolate recorded by Tanaka et al. (1997), and Thailand isolates recorded by Koi et al. (2012), the Iraqi isolate recorded by AL-Abodi et al. (2014), and the Iraqi isolate

taken from livestock and recorded by ALseady et al. (2022), with a match rate of 100%. A recent study by Liu et al. (2022) in non-human primates in a Zoological Garden in Nanjing, China, further supports the findings of the current study, particularly in the Macaca species.

CONCLUSION

This study confirmed the presence of *Entamoeba histolytic* in monkeys of Iraq which is one of the first isolation reports in the Middle East. This study recommends conducting further studies on other zoonotic parasites in monkeys in the study area.

DECLARATIONS

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Authors' contributions

Zaman Turkey Abdul Abbas contributed to collecting samples and statistically analyzing data. Sadiya Aziz Anah contributed to the implementation of PCR. All authors discussed the results, commented on the manuscript, and gave final approval of the final version of the manuscript.

Competing interests

The authors report no conflicts of interest.

Ethical considerations

Ethical issues, such as data fabrication, double publication and submission, redundancy, plagiarism, consent to publish, and misconduct, have been checked by all the authors before publication in this journal

Availability of data and material

All data of the current study are available in the present article.

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Effects of Different Dosages and Methods of Saponin Preparation from *Mucuna pruriens* Leaves on *In Vitro* Feed Digestibility

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ABSTRACT

The *Mucuna pruriens* is commonly used in traditional medicine for anti-inflammatory, antibacterial, neuroprotector, antidiabetic, and anti-cancer purposes. The bioactive compounds, such as flavonoid, tannin, and saponin, could improve feed digestion efficiency in ruminants' rumen. The current study aimed to evaluate the effects of different dosages and the two methods of saponin preparation from *Mucuna pruriens* leaves on *in vitro* feed digestibility parameters. A randomized block design with nested arrangements (2×5×3) was used in this study. Two methods of obtaining saponins from *Mucuna pruriens* leaves, including meal (MPLM) and extract (MPLE) of *Mucuna pruriens* leaves, were compared. The nested treatments of the preparation methods were the dosages of the saponin as feed additives in feed samples, involving 0%, 0.025%, 0.050%, 0.075%, and 0.10%. There were 15 samples in each group (five-level dosage and three repetitions). The feed contained 40% forage and 60% concentrate. The obtained results indicated that saponin preparation from *Mucuna pruriens* leaves (MPLM and MPLE) significantly affected dry matter, organic matter, and crude fiber rumen degradability (r-DMD, r-OMD, r-CFD, respectively), as well as NH_3, volatile fatty acid, propionate, butyrate concentrations, acetate-to-propionate (A/P) ratio, acetate, and propionate percentage. However, there was no significant impact on protozoa population, acetate concentration, butyrate percentage, *in vitro* dry matter digestibility (IVDMD), and *in vitro* organic matter digestibility (IVOMD). The MPLM saponin revealed significantly higher values on digestibility parameters except for protozoa, A/P ratio, and acetate percentage. The MPLM saponin dosage of 0.05% showed the highest values for r-DMD (56.48%), r-OMD (56.51%), and r-CFD (54.64%), total Volatile fatty acid (77.71 mM), propionate (21.57 mM), propionate percentage (27.76%), IVDMD (65.95%), and IVOMD (65.86%), but lowest in A/P ratio (2.04). In conclusion, the findings of the present study suggest that the MPLM saponin at a dosage of 0.05% holds promising potential for enhancing the fermentation profile in ruminants.

Keywords: *In vitro*, *Mucuna pruriens*, Nutrient digestibility, Rumen fermentation, Saponin

INTRODUCTION

In commercial feedlots, where ruminants are raised for high productivity, feed antibiotics, such as monensin, have been traditionally used (Ogunade et al., 2018). Monensin reduces protozoa, fungi, and methanogen bacteria; however, its use is constrained due to increasing awareness of its impact on human health and concerns regarding the development of resistance (Shen et al., 2017). Current research endeavors focus on developing natural feed additives as alternative antibiotics. The ongoing research is primarily centered on the exploration of secondary metabolites, such as saponin (Unnawong et al., 2021), tannins (Patra and Saxena, 2011), flavonoids (Gohlke et al., 2013), and polyphenols, as rumen modifiers (Vasta et al., 2019). Klevenhusen et al. (2011) found that the secondary metabolites decreased the acetate and ammonia concentration, acetate-to-propionate ratio, and methane production while increasing propionate *in vitro*. Additionally, they have antimicrobial properties against bacteria, fungi, and protozoa.

The *Mucuna pruriens*, also known as the velvet bean, belongs to the *Fabaceae* family with approximately 150 species of annual and perennial legumes (Lampariello et al., 2012). *Mucuna* is a multipurpose legume that plays an essential role in soil fertility, soil structure improvement, soil protection against erosion, and weed control (Buckles et al., 1998), especially in smallholder farmers and when rotated with the maize, it contributes to improvements in water productivity (Masikati et al., 2014). *Mucuna pruriens* seeds are commonly used as raw materials for making tempeh, a traditional Indonesian food, especially in Wonogiri, Central Java (Handajani, 2001). The product is popularly called "*koro benguk tempeh*," when used as a dish, and some small and medium enterprises further process the tempeh to produce chips (Winarni and Dharmawan, 2017). *Mucuna* is not only used as tempeh raw materials but also as an ingredient for making nuggets, cookies for school-age children, and vegetable milk (Mang et al., 2016). Besides, *Mucuna pruriens* has the potential to serve as a fiber source in new dietary food products, function as an antioxidant (Encalada

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and Campos, 2021), have hypolipidemic potential (Dimitry et al., 2022), and could affect fertility (Daramola et al., 2015).

Mucuna is an essential medicinal plant used to remedy various diseases, such as diabetes, arthritis, dysentery, and cardiovascular diseases (Nadkarn, 2001). It has high concentrations of L Dopa (4-7%), making it a potential alternative for treating Parkinson's disease and offering an alternative to conventional medicine with long-term side effects (Katzenschlager et al., 2004). The L Dopa is present in roots, stems, leaves, and seeds (Lampariello et al., 2012). *Mucuna pruriens* leaves as an extract had secondary metabolites, such as flavonoids, tannins, saponins, anthraquinones, terpenoids, flavonoids, and cardiac glycosides (Agbafor and Nwachukwu, 2011). *Mucuna pruriens* leaf extract has a significant antimicrobial effect against fungal and bacterial species (Mastan et al., 2009) and the potential to control protozoa (Ekanem et al., 2004). Given the significant contribution of protozoa to carbohydrate breakdown in the rumen and their predatory capacity, the *Mucuna pruriens* would be useful as a feed additive for antiprotozoal agents in ruminant productions (Williams et al., 2020). Modifying microbial composition by adding some feed additive decreases methane production and fermentation efficiency (Castillo-González et al., 2014). Therefore, this study aimed to evaluate the effects of different dosages and the two methods of saponin preparation from *Mucuna pruriens* leaves on *in vitro* feed digestibility parameters.

MATERIALS AND METHODS

Ethical approval

The Ethics Committee has approved all research procedures of Brawijaya University Malang, East Java, Indonesia, with letter number 141-KEP-UB-2022.

Materials

Materials used in this experiment included meal (MPLM) and extract (MPLE) of *Mucuna pruriens* leaves prepared from fresh leaves collected from Wajak, Malang, East Java, Indonesia. For MPLM, 10 kg leaves were aerated in shading and then dried in the oven at 60°C for 24 hours. The oven-dried *Mucuna pruriens* leaves were milled into a fine powder using a mechanical grinder and then stored in an air-tight plastic bag. The preparation of MPLE saponin involved the extraction of fine powder from *Mucuna pruriens* using methanolic solvent by microwave-assisted extraction (MAE) method (Wang et al., 2012). The MAE procedure employed a modified microwave sharp type R21D0SIN with the power 450 W, voltage 220-240 volt/50 Hz, and dimensions 52 cm × 40.7 cm × 32 cm (length × width × height), equipped with a close vessel unit. The modified microwave featured a thermostat to control the temperature, which did not exceed 40°C. To initiate the extraction, 16.5 g of *Mucuna* leaves powder was placed in a 250 ml round flask and mixed with 100 ml methanolic solvent. The microwave was turned on for 15 minutes at 40°C. A chiller pump was used to flow the coolant liquid in and out of the condenser to keep the temperature in the flask at 40°C. The extract was filtered using the Whatman 1 filter paper. Then, another 100 ml methanolic solvent was added to the filtrate, and the same extraction process was done. The solvent is then evaporated and stored in a sealed bottle.

Methods

The experiment used a randomized block design of nested arrangement (2×5×3) for *in vitro* digestibility laboratory research, following the methodology outlined by Tilley and Terry (1963). The treatments were the saponin dosage level of *Mucuna pruriens* leaves, prepared through two different preparation methods, namely MPLM saponin and MPLE saponin. *Mucuna pruriens* leaves saponin were used at levels of 0%, 0.025%, 0.050%, 0.075%, and 0.10% in the basal diet based on Xu et al. (2010) and Castro-Montoya et al. (2011). The basal diet comprised 40% maize forage (*Zea mays*) and 60% concentrate in dry matter basis. The nutrient content of the feed ingredients and *Mucuna pruriens* leaves are presented in Table 1.

The saponin content of the leaf meal was 12.41 mg/g. To meet the dosage level of MPLM saponin the leaves meal was added as much as 0 mg, 10 mg, 30 mg, and 40 mg into a 500 mg basal diet in each fermentor tube. Moreover, to meet the dosage level of MPLE saponin, the liquid extract of *Mucuna pruriens* leaves was added as much 0 µL, 80 µL, 160 µL, 240 µL, and 330 µL into 500 mg basal diet in each fermentor tube. The extract had 1504.44 mg/L saponin content. All treatment diets were tested using one-and two-step *in vitro* feed digestibility tests in three runs as replication (Tilley and Terry, 1963). Each treatment diet was placed in a fermentation tube containing a mixture of 10 ml rumen fluid and 40 ml McDougall buffer solution, then incubated at 39°C for 48 hours (one step *in vitro* feed digestibility test) for dry matter (r-DMD), organic matter (r-OMD) and crude fiber (r-CFD) *in vitro* degradability measurement in the rumen.

After the incubation, the fluid or supernatant was carefully removed from the tube into a centrifuge tube without disturbing the solid part (sample residue) at the bottom of the tube. Subsequently, the supernatant in each centrifuge tube was homogenized, and then 5 ml of the supernatant was taken and used as a protozoa cell count sample. Another 5 ml of

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the supernatant was taken and used as the sample to analyze ammonia and volatile fatty acid concentration. The supernatant's sample protozoa cell count was added and homogenized with 5 ml formalsaline 10%. The protozoa cell count was done using a counting chamber (Hausser Scientific, catalog #3800) under a light microscope (Olympus CX 43, USA) at 100×magnification (Park et al., 2019). A supernatant sample for ammonia and VFA concentration analysis was added with a few drops of H_2SO_4 10% until it reached pH 2.5-3.0. The sample was then divided into two aliquots and stored at -20°C temperature in the refrigerator for ammonia and VFA concentration analysis.

The sample residue was centrifuged at 16,000 rpm for 10 minutes, the supernatant was discharged, and the pellet residue was oven-dried at 55°C for 24 hours and finally weighed to determine r-DMD, r-OMD, and r-CFD. Another set of samples underwent an additional 48 hours at 39°C incubation in a 50 ml mixture solution of 3 ml of 20% HCL and 1 ml of 5% pepsin (two steps *in vitro* feed digestibility test) for *in vitro* dry matter digestibility (IVDMD) and *in vitro* organic matter digestibility (IVOMD) measurements.

Table 1. The nutrient content of feed ingredients

Nutrient content (% dry matter basis)	Concentrate	Maize forage	*Mucuna* leave
Dry matter	88.68	91.10	87.68
Ash	7.09	11.85	10.43
Crude protein	15.78	10.00	27.00
Ether extract	3.74	2.10	2.80
Crude fiber	9.44	30.50	29.48
Nitrogen-free extract	57.61	45.55	30.29
Total digestible nutrient (TDN)*	69.59	59.27	70.70

* TDN: 1.6899 + 1.3844(CP) + 0.7526(NFE) – 0.8279(EE) + 0.3673(CF)

Analysis of rumen metabolite

Total VFA, including acetate, propionate, and butyrate concentrations, were analyzed based on Li et al. (2014) using gas chromatography (Agilent Technologies 7820A GC system. Santa Clara. USA). The concentrations of ruminal ammonia nitrogen (NH_3-N) concentrations were measured using Chaney and Marbach (1962) methods.

Statistical analysis

The data were analyzed using SPSS software version 25. The obtained data were subjected to the analysis of variance (ANOVA) for a randomized block design with a nested arrangement. Duncan's Multiple Range Test (DMRT) was conducted to compare the mean values between treatments, aiming to determine any significant effect of the treatments ($p < 0.05$).

RESULTS AND DISCUSSION

Effect of saponin dosage on rumen fermentation profiles

The effect of *Mucuna pruriens* leaves saponin dosages, either MPLM or MPLE saponin, on the parameters of the *in vitro* digestibility test are presented in Table 2.

As indicated in Table 2, the level of addition of *Mucuna pruriens* leaves saponin in the diet, either prepared as meal or extract, had significant effects on *in vitro* digestibility test parameters including r-CFD, NH_3 concentration, and protozoa population ($p < 0.05$). The addition of MPLE saponin in the diet showed a significant effect on r-DMD and r-OMD ($p < 0.05$). The supplementations *Mucuna pruriens* leaves saponin in the diet, either MPLM or MPLE, had insignificant effects on IVDMD ($p > 0.05$) but IVOMD of MPLM showed a significant effect ($p < 0.05$), while MPLE did not show a significant effect ($p > 0.05$). Wahyuni et al. (2014) reported that adding saponin from *Sapindus rarak* increased IVDMD and IVOMD.

The level of addition of *Mucuna* leaves saponin prepared as extract (MPLE) in the diets had a significant effect on r-DMD, r-OMD, and r-CFD ($p < 0.05$). The crude fiber rumen degradations (r-CFD) decreased with the increasing dosage level. This shows that *Mucuna* extract leaves saponin in the diets suppressed straight crude fiber degradability in the rumen. On the other hand, the addition of MPLM saponin to the diets did not significantly affect r-DMD and r-OMD ($p > 0.05$) but it had a significant effect on r-CFD ($p < 0.05$). The highest values of r-CFD were found in MPLM (54.64%) by 0.05% dosage level and tended to decrease when the dosage level increased. The higher crude fiber degradability in diets prepared with MPLM saponin compared to MPLE may be attributed to the fact that MPLM contains not only saponin but also 27% crude protein (Table 1). This leads to an increase in the crude protein content of the diets. Feed digestibility depends on the rumen microorganism's activity since it plays a role in fermentation, while the rumen microorganism was affected by the material feed substances. Goel and Makkar (2012) reported that saponins modify ruminal fermentation by suppressing ruminal protozoa and selectively inhibiting some bacteria and fiber

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degradation. The rumen microbial population in ruminants with regular diets containing sufficient crude fiber must be fiber-degrading bacteria or fibrolytic bacteria (Chen et al., 2022).

Table 2. Parameters of *in vitro* digestibility test of feeds supplemented with different saponin dosages in the forms of *Mucuna pruriens* leaves meal and extract

Variables	Saponin	Dosage of saponin in the diet					SD
		0%	0.025%	0.050%	0.075%	0.100%	
r-DMD (%)	MPLM	54.67	54.43	56.59	54.46	53.88	0.93
	MPLE	54.67^{b}	45.43^{a}	45.60^{a}	47.98^{a}	45.20^{a}	0.67
r-OMD (%)	MPLM	53.82	54.19	56.51	53.31	53.22	1.02
	MPLE	53.82^{b}	45.25^{a}	45.37^{a}	47.83^{a}	45.05^{a}	0.69
r-CFD (%)	MPLM	50.79^{bc}	52.05^{c}	54.64^{d}	49.12^{ab}	47.41^{a}	0.99
	MPLE	50.79^{b}	49.21^{ab}	48.99^{ab}	46.94^{a}	46.70^{a}	0.50
IVDMD (%)	MPLM	62.15	62.03	65.95	61.76	61.54	0.87
	MPLE	62.15	61.81	61.66	61.51	60.77	0.71
IVOMD (%)	MPLM	61.86^{a}	61.93^{a}	65.86^{b}	61.26^{a}	61.22^{a}	0.88
	MPLE	61.86	61.35	61.66	61.28	60.68	0.74
NH_3 (mM)	MPLM	6.68^{ab}	7.15^{b}	6.31^{a}	6.24^{a}	6.13^{a}	0.15
	MPLE	6.68^{b}	6.23^{b}	5.43^{a}	5.36^{a}	5.27^{a}	0.13
Protozoa (10^3 cell/ml liquid)	MPLM	79.92^{c}	77.64^{c}	76.83^{c}	60.50^{b}	47.06^{a}	3.77
	MPLE	79.92^{b}	63.69^{a}	62.56^{a}	58.06^{a}	56.86^{a}	1.92

Note: Different superscript letters in the same row mean the significantly different ($p < 0.05$). MPLM: *Mucuna pruriens* leaves meal, MPLE: *Mucuna pruriens* leaves extract, r-DMD: Dry matter, r-OMD: Organic matter, r-CFD: Crude fiber degradability in the rumen, IVDMD: *In vitro* dry matter digestibility, IVOMD: *In vitro* organic matter digestibility, NH_3: Rumen ammonia, SD: Standart deviation.

Based on Table 2, saponin supplementations either from MPLM or MPLE significantly decreased ammonia concentration and protozoa population ($p < 0.05$), where the lowest values were found at 0.1% dosage level. Saponin affects rumen fermentation by reducing protein degradations, thus decreasing rumen ammonia concentration (Demirtas et al., 2018). Lower ammonia concentration must also indicate the increase of rumen ammonia utilization to increase microbial growth, especially fibrolytic bacteria in the rumen (Jadhav et al., 2016).

The decrease in protozoa population as a result of saponin addition in the diets aligns with findings from Krisnawan (2011), Suhartati et al. (2011), and Hidayah (2016). Saponin is known to have functioned as a defaunation agent for rumen protozoa (Wina et al., 2006). Most researchers reported that saponin decreases the rumen protozoa population as saponins have an antiprotozoal effect in the rumen (Hu et al., 2006; Guo et al., 2008). Several factors influence the effectiveness of saponin use, including the source of saponins, level in the diet, time after saponins feeding or consumption, and types of protozoa (Wina et al., 2006). In addition, Tan et al. (2020) found the different genera of rumen protozoa ciliates appear to be selectively inhibited by tea seed saponins.

Saponin made a bond with the surface sterols protozoa membrane, leading to the rupture of their cell wall (Arum et al., 2013). Another mechanism through which saponins influence the rumen environment involves the modulation of microbial ruminal and ruminal metabolite, although this modulation depends on the basal diet (Wang et al., 2019). The decrease in rumen protozoa population in this research was accordingly followed by an increase in feed degradability and digestibility (Liu et al., 2019). The increase of the fermentations parameter in this research indicates feed efficiency digestions in the rumen. Saponins also could inhibit bacterial (*Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 6538, *Klebsiella pneumonia*, *Bacillus subtilis* ATCC 6633, *Pseudomonas aeruginosa* ATCC 14028) and a fungal strain (*Candida albicans*) growth (Mataalah et al., 2012). Wang et al. (2012) reported that some anti-nutritional factors, such as saponin in tea extract, inhibit methanogenesis. The decrease in the ruminal protozoa population decreased methane production (Morgavi et al., 2012). Saponins have a toxic effect on protozoa since some rumen bacteria can hydrolise saponin to their free glycoside fractions (Newbold et al., 1997). However, some bacteria degrads saponin into sapogenin so that it cannot affect protozoa (Ramos-Morales et al., 2014) or the protozoa produce extracellular polysaccharides around the membrane and avoid the degradations by saponin (Wina et al., 2006). This variability may explain why some studies found that saponins do not change the protozoa populations (Kang et al., 2016). It can be the explanation for the increasing protozoa populations on meal saponin supplementations.

Effects of saponin dosage on volatile fatty acids profiles

As indicated in Table 3, the addition of *Mucuna pruriens* saponin to the diet at different dosages either prepared as a meal or extract had a significant effect on total VFA, the concentrations of acetate, propionate, and butyrate, and A/P ratio, as well as the percentages of acetate, butyrate, and propionate ($p < 0.05$). Total VFA production was at its highest level in 0.05% saponin dosage either in the meal form (77.71 mM) or extract (75.76 mM). However, total rumen VFA decreased beyond the 0.05% dosage level.

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Table 3. Volatile fatty acids profile of feeds supplemented with different saponin dosages in the forms of *Mucuna pruriens* leaves meal and extract

Variables	Saponin	Dosage of saponin in the diet					SD
		0%	0.025%	0.050%	0.075%	0.100%	
VFA (mM)	MPLM	61.06^{a}	61.30^{a}	77.71^{b}	72.99^{b}	71.54^{b}	2.01
	MPLE	61.06^{a}	60.40^{a}	75.76^{b}	64.04^{a}	60.49^{a}	1.61
Acetate (mM)	MPLM	34.31^{a}	38.04^{ab}	43.64^{c}	42.51^{bc}	41.58^{bc}	0.93
	MPLE	34.31^{a}	35.60^{a}	46.85^{b}	37.45^{a}	37.20^{a}	1.32
Propionate (mM)	MPLM	16.35^{b}	14.43^{a}	21.57^{d}	19.04^{c}	18.94^{c}	0.82
	MPLE	16.35	15.60	15.93	16.50	15.17	0.38
Butyrate (mM)	MPLM	10.41^{b}	8.83^{a}	12.50^{c}	11.44^{bc}	11.02^{bc}	0.43
	MPLE	10.41^{c}	9.21^{ab}	12.98^{d}	10.10^{bc}	8.13^{a}	0.49
A/P Ratio	MPLM	2.10^{a}	2.65^{b}	2.04^{a}	2.23^{a}	2.21^{a}	0.08
	MPLE	2.10^{a}	2.29^{ab}	2.94^{c}	2.28^{ab}	2.45^{b}	0.13
Acetate (%)	MPLM	56.13^{a}	62.07^{b}	56.17^{a}	58.19^{a}	58.10^{a}	0.75
	MPLE	56.13^{a}	58.85^{b}	61.85^{c}	58.49^{ab}	61.46^{c}	0.74
Propionate (%)	MPLM	26.82^{b}	23.51^{a}	27.76^{b}	26.11^{b}	26.49^{b}	0.56
	MPLE	26.82^{b}	25.87^{b}	21.02^{a}	25.74^{b}	25.11^{b}	0.88
Butyrate (%)	MPLM	17.05^{c}	14.42^{a}	16.07^{bc}	15.69^{abc}	15.41^{ab}	0.26
	MPLE	17.05^{c}	15.28^{b}	17.13^{c}	15.77^{bc}	13.43^{a}	0.42

Note: Different superscript letters at the same row mean significant differences ($p < 0.05$), MPLM: *Mucuna pruriens* leaves meal, MPLE: *Mucuna pruriens* leaves extract, VFA: Volatile fatty acid, A/P ratio: Acetate-to-propionate ratio, SD: Standart deviation.

The normal range of VFA for optimum rumen microbial growth was 80-160 mM (McDonald et al., 2010). However, the VFA value was below the range in the current study. Besides the saponins, *Mucuna pruriens* leaves contained tannins and could form complexes binding with proteins. This process reduces the value of rumen-degradable protein, leading to a decrease in the total VFA concentration due to the decreased proteolysis and less oxidative deamination of feed proteins. There was sufficient ammonia to develop bacteria, but the branched-chain fatty acids were insufficient, so the total VFA decreased. Branched-chain fatty acids consisting of isobutyric acid, two methyl butyrate, and valeric acid are a source of carbon skeletons for bacteria, and these compounds are the result of decarboxylation and deamination of branched-chain amino acids (Nurhaita et al., 2010).

Based on Table 2, the addition of *Mucuna pruriens* saponin in the form of a meal at 0.05% dosage level resulted in high organic matter degradability and digestibility consistent with the findings in Table 3 showing high VFA concentrations. Volatile fatty acid concentrations correlated with dry matter and organic matter digestibility (Noziere et al., 2011). The increase in volatile fatty acids signifies enhanced fermentation of organic matter and higher rumen microbial activity (Madrid et al., 2002). Rumen volatile fatty acid and other carbon skeletons are the end products of organic matter fermentations, including carbohydrates, protein, and lipids that provide energy and carbon skeleton for rumen microbial growth (Dijkstra, 1994). The efficacy of saponins as rumen antiprotozoal is affected by many factors, including the source and the form of saponins (Patra and Saxena, 2009). The effect of *Mucuna pruriens* leaves saponin in the form of either MPLM or MPLE saponin on *in vitro* feed degradability, protozoa population, metabolic products of fermentation in the rumen as well as *in vitro* feed digestibility are presented in Table 4.

Table 4. Parameters of *in vitro* digestibility test of feeds supplemented with saponin in the forms of powder and extract from *Mucuna pruriens* leaves

Measurement	MPLM	MPLE
r-DMD (%)	54.81 ± 2.90^{b}	47.78 ± 4.29^{a}
r-OMD (%)	54.21 ± 3.03^{b}	47.46 ± 4.12^{a}
r-CFD (%)	58.80 ± 3.05^{b}	48.53 ± 1.87^{a}
NH_3 (mM)	6.50 ± 0.70^{b}	5.79 ± 0.70^{a}
Protozoa (10^3 cell/ml liquid)	68.39 ± 14.02	64.35 ± 6.68
VFA (mM)	68.92 ± 7.52^{b}	66.08 ± 2.49^{a}
Acetate (mM)	40.02 ± 4.16	38.28 ± 5.05
Propionate (mM)	18.07 ± 2.69^{b}	15.91 ± 0.80^{a}
Butyrate (mM)	10.84 ± 1.37^{b}	10.16 ± 1.78^{a}
A/P ratio	2.25 ± 0.26^{a}	2.41 ± 0.32^{b}
Acetate (%)	58.13 ± 2.57^{a}	59.36 ± 2.45^{b}
Propionate (%)	26.14 ± 1.77^{b}	24.91 ± 2.29^{a}
Butyrate (%)	15.73 ± 1.09	15.73 ± 1. 57
IVDMD (%)	62.69 ± 2.42	61.58 ± 2.24
IVOMD (%)	62.42 ± 2.37	61.37 ± 2.11

Note: Different superscript letters at the same row mean significant differences ($p < 0.05$). r-DMD: Dry matter, r-OMD: Organic matter, r-CFD: Fiber degradability in the rumen, NH_3: Rumen ammonia, VFA: Volatile fatty acid, IVDMD: *In vitro* dry matter digestibility, IVOMD: Organic matter digestibility, A/P ratio: Acetate-to-propionate ratio.

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As presented in Table 4, the addition of saponins from MPLM to the diet led to significant differences in r-DMD, r-OMD, r-CFD, NH_3, total VFA, propionate and butyrate concentrations, propionate, and acetate percentage, and A/P ratio ($p < 0.05$). However, there were non-significant differences in protozoa population, acetate concentration, butyrate percentage, IVDMD, and IVOMD ($p > 0.05$). The saponin preparations from MPLM tended to show higher values for the digestibility parameters, NH_3, protozoa populations, and VFA profile except for acetate percentage and A/P ratio. In a study by Wang et al. (1998), it was found that saponin from *Yucca scidigera* extract could affect proteolytic activity and reduce protozoal number but did not affect dry matter degradability and bacterial activity. The other study by Patra and Yu (2014) indicated that saponin from *Quilaja Saponaria* decreased the methane but had no effect on VFA concentrations. Furthermore, a study by Lu and Jorgensen (1987) revealed an increase in digestibility and propionate concentrations using saponin from extracted alfalfa.

CONCLUSION

Mucuna pruriens leaves prepared as meal had better rumen degradability and VFA profile, compared to the extract. The use of MPLM with a source of 0.05% saponin in the diet yielded more favorable outcomes in enhancing the fermentation profile of feed within the rumen compared to MPLE. It is necessary to carry out *in vivo* research to determine *Mucuna pruriens* leaves meal saponin as a ruminant feed additive.

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Authors' contributions
Triana Muhartatik wrote the manuscript and conducted the research, Siti Chuzaemi, Halim Natsir, and Marjuki conceptualized the research, supervised the research, and Marjuki revised the final form of the manuscript. All authors read and approved the final draft of the manuscript.

Competing interests
The authors have declared no conflicts of interest.

Ethical considerations
Before publication in this journal, all the authors conducted checks for ethical issues, such as data fabrication, double publication and submission, redundancy, plagiarism, consent to publish, and misconduct.

Availability of data and materials
All data related to the current study are available in this manuscript.

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The Relationship of Histamine Content in European Pilchard (*Sardina pilchardus*) with Freshness, Temperature, and Storage Duration

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ABSTRACT

Histamine food poisoning, stemming from the consumption of certain histamine-rich fish species, such as tuna, mackerel, European pilchards, and herring, is one of the major public health issues worldwide. The present study aimed to evaluate the histamine content in fresh European pilchard (*Sardina pilchardus* Walbaum, 1792) of Mehdia, a coastal city in the north of Morocco. Three randomly selected batches of fresh European pilchards, each weighing 20kg, were obtained from different boats upon landing. The evolution of histamine production was monitored every 8 hours for 6 days, with one batch stored at 0°C and the other at 10°C. The organoleptic characteristics were examined considering sensory evaluation according to the rating system of European Council Regulation No. 2406/96 as common marketing standards for certain fishery products and the quality index method (QIM). The histamine content in European pilchard flesh was determined using the fluorometric method. The results indicated that the average histamine content did not exceed 5 ppm during storage at 0°C. The freshness ratings were highest during the first 3 days, corresponding to QIM values of 0 to 10 at 0°C. On days 4 and 5, the freshness ratings were on quality A, corresponding to QIM values of 11 and 12, and on the last day, they were on quality B, corresponding to a QIM value of 15 with preservation of the organoleptic quality. Statistically, a significant correlation was found between the European pilchard's freshness and the storage duration. In contrast, this correlation between the histamine content and the storage duration was insignificant. At 10°C, the average histamine content exceeded the regulatory limit in force (100 ppm) after 32 hours of storage, and spoilage occurred on day 3. Statistical analysis revealed a strong correlation between the histamine content, storage temperature, the degree of freshness, and the duration of storage. The extra freshness quality index of European pilchard guarantees a very large margin of safety regarding histamine and can be consumed without risk.

Keywords: Degree freshness, European pilchard, Histamine, Sardine, Storage, Temperature

INTRODUCTION

Histamine is a biogenic amine responsible for several allergic and inflammatory phenomena (Oktariani et al., 2022; Bose et al., 2023). It is formed as a result of the enzymatic activity of histidine decarboxylase produced by some bacteria, such as *Morganella morganii*, *Raoultella ornithinolytica*, *Raoultella planticola*, *Proteus vulgaris*, *Proteus mirabilis*, *Klebsiella sp*., *Enterobacter cloacae*, *Enterobacter aerogenes*, *Citrobacter freundi*, *Serratia liquefaciens*, and *Serratia fonticola* (Tao et al., 2022; Ginigaddarage et al., 2023; Rachmawati et al., 2023), which is accentuated by poor storage conditions (Emborg and Dalgaard, 2008; Abuhlega and Ali, 2022). This leads to the decarboxylation of L-histidine, which is naturally present in the flesh of many species of fish (García-Ruiz et al., 2011). Consumption of fish containing high levels of histamine causes histamine or scombroid poisoning (Colombo et al., 2017). The World Health Organization (WHO) estimates that around 600 million people experience food intoxication each year, resulting in the annual loss of 420,000 lives (WHO, 2022). This significantly impedes socio-economic development as it negatively affects the health systems, national economies, tourism, and commercial exchanges (WHO, 2022). Internationally, Dalgaard et al. (2008) reported thousands of outbreaks, incidents, and cases of food poisoning had been reported in Japan, Denmark, the United Kingdom, and Taiwan between 1986 and 2005. In France, an average of 11 outbreaks of histamine food poisoning affected around 67 cases per year between 2008 and 2019 (ANSES, 2021). In Morocco, the Poison Control and Pharmacovigilance Center (CAPM) recorded 14344 outbreaks, incidents, and cases of food poisoning between 2013 and 2020, with an annual number of outbreaks varying from 226 in 2020 to 2,887 in 2015. Food-borne diseases occupy the second etiology, with 15.7% of all poisonings. Poisoning caused by fish accounts for 10.6% of these food poisonings (Poison Control and Pharmacovigilance Center of Morocco, 2021).

On the regulatory level, the toxic dose of histamine in fish is not yet known accurately (Emborg and Dalgaard, 2008; Hungerford, 2021). According to ANSES (2021), the histamine level below 50 ppm is generally considered non-toxic. From 50 to 200 mg/kg, the food can present a potential risk of toxicity, especially for sensitive people. Histamine

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levels within a range of 200-1000 mg/kg suggest a probable risk of toxicity. Beyond 1000 mg/kg, the fish is considered toxic.

International regulations define a maximal limit for histamine in fish to determine the suitability of fish consumption for humans (Debeer et al., 2021). The Moroccan Ministerial Decree n° 293-19 (Official Bulletin, 2019) and European Commission regulation n° 2073/2005 (EC, 2005) have aligned histamine as a safety microbiological criterion, setting concentration limits for nine fish samples. Accordingly, the average content must not exceed 100 mg/kg, two samples may exceed 100 mg/kg but not reach 200 mg/kg, and no sample should exceed 200 mg/kg. At the Codex Alimentarius Commission, histamine study has been extensively discussed across various committees. This includes the analysis of epidemiological data, the evaluation of public health risks caused by histamine, the review of sampling plans, and applied protection (FAO/WHO, 2013). In the United States, the Food and Drug Administration (FDA) has proposed a very severe lowering of the histamine limit from 50 to 35 ppm as a limit of decomposition in fish far exceeding internationally recognized Codex guidelines and standards (FDA, 2021), which is 100 ppm. This proposal limit may represent a major obstacle to international trade in fishery products.

Histamine in seafood is a very complex issue for the fisheries sector. Epidemiological and economic data, foreign exchange inputs generated by this sector and its contribution to food, economic and social security, discussions within the Codex Alimentarius, and the new regulatory requirements proposed by the FDA show that it is still important to study this health hazard. The first link in the value chain is storage, which is the intermediate step between harvesting and processing or consuming seafood products. The control of histamine during this stage is crucial to avoid any bacterial contamination that may increase the histamine level. In this context, this experimental study aimed to contribute to better control of the danger of histamine in the European pilchard (*Sardina pilchardus*, Walbaum, 1792) of Mehdia (a coastal city located in the north of Morocco) through monitoring of the freshness and kinetics of histamine production in the fresh European pilchard during 6 days of storage at 0°C and at 10°C. In this regard, the relationship of histamine with freshness, histamine content, temperature, and duration of storage, as well as the ability of the frosting to control histamine, were examined.

MATERIALS AND METHODS

Ethical approval

This study was conducted according to the guidelines of the Agronomic and Veterinary Institute Hassan II, Rabat, Morocco.

Study location

The study was carried out in the laboratory of the Food Safety Unit of the Agronomic and Veterinary Institute Hassan II, Rabat, Morocco.

Samples

The study focused on evaluating fresh European pilchard (*Sardina pilchardus*) landed in the fishing port of Mehdia in northern Morocco. For this purpose, three randomly selected batches of fresh European pilchards, each weighing 20 kg, were taken from the landing of three different boats. These batches were promptly placed into insulated boxes containing ice and transferred immediately to the laboratory. In the laboratory, each batch was divided into two groups (A and B). Hygienic and cold conditions (0°C) were ensured. Group A was preserved under melting ice in a refrigerator at 0°C, and group B was preserved under melting ice in a refrigerator at 10°C. The investigated European pilchard had an average weight of 25.60 ± 6.2 g and an average length of 17.67 ± 0.76 cm.

To comprehensively assess the alteration in each group, six fish per group were collected every 8 hours over 6 days, with the first sample collected 9 hours after the initial catch. This resulted in 102 samples, with 51 samples from the group stored at 0°C and 51 samples from the group stored at 10°C.

Temperature measurement

The core temperature of each sample was taken by a calibrated thermometer with an interchangeable probe (Testo 110, Forbach, France) on six fish pieces every 8 hours. The arithmetic average of the temperature values was calculated.

Organoleptic evaluation of freshness

The sensory evaluation was carried out on whole fish using two methods by a panel of three veterinary inspectors experienced in the matter. The rating system of European Council Regulation (EC) N^{o} 2406/96 (1996) establishes common marketing standards for certain fishery products. Hence, three classes of freshness are defined, namely first quality extra (E), second quality A, and third quality B or not admitted. To better interpret the results of this study, an appreciation score of freshness from 0 to 3 was assigned to each of the characteristics evaluated during its evaluation

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(skin and skin mucus, flesh consistency, gill covers, shape and color of eye, color, smell, and mucus of gills). The total sum of the appreciation scores was then calculated.

The quality index method (QIM) consists of assigning a score of demerits from 0 to 3 for each character (general appearance (Surface, stiffness, flesh firmness), eye (clarity cornea, pupil, shape), cover bloodiness, gills (color and smell), abdomen post girl, flesh appearance), the total sum of scores was calculated when the QIM value is high, the fish is spoiled (Triqui and Bouchriti, 2003).

Determination of histamine content

Lerke and Bell fluorimetry method is a quantitative histamine assay technique used in several laboratories and known for its accuracy, repeatability, simplicity, and speed. This method is based on the extraction, purification, and then determination of histamine using specific chemical solutions for each step (Lerke and Bell, 1976; Rachidi et al., 2011). Histamine was extracted from 10g of flesh well mixed with 90 ml of trichloroacetic acid (TCA), then separated by chromatography on an ion exchange column (Amberlite CG50) and eluted with hydrochloric acid (HCl, 0.7 Normality). The dosage was carried out by fluorometry after complexation with orthophthalaldehyde (OPA). Histamine concentrations were read using a Trilogy^{TM} fluorometer (Turner Designs Instrument, Model 7200-000, California, USA) by fluorescence at emission and excitation lengths wave of 450 and 360 nm, respectively.

Statistical analysis

The obtained results were analyzed by the R-Studio statistical software, a linear regression analysis software that allows the relationship existing between a dependent variable Y (average content of histamine), and independent variables (Freshness index [IF] and duration of storage) in each storage temperature. The intensity of the relationship given is appreciated by the determination index (R^2). This index is between 0 and 1. The relationship is weak when it is close to 0 and strong when it is close to 1. The significance was set at $p < 0.05$.

RESULTS AND DISCUSSION

The results showed a significant variation in the evolution of the mean histamine content and freshness index during storage for both protocols ($p < 0.05$). The average histamine content varied from 2.19 ppm on day 4 when stored at 0°C to 1988.32 ppm on day 3 when stored at 10°C. The freshness index evolved from extra quality to quality B on day 6 of storage at 0°C without crossing the organoleptic rejection threshold and towards organoleptic rejection on day 3 of storage at 10°C.

The first protocol for fish stored at 0°C

During storage at 0°C, the fish were stored under melting ice at 0°C for 6 days. Temperature monitoring results varied between 0.1°C and 1°C. The average temperature was 0.49°C. The mean histamine content and degree of freshness are represented in Table 1. Each value is the average of three different batches.

Table 1. Freshness index, quality index method, and histamine means contents for European pilchard stored at 0°C

Sampling time (hours)	Freshness index	Appreciation score of freshness	QIM	Histamine means contents (ppm)
0	E	27	0	2.77
8	E	27	0	4.22
16	E	26	0	3.86
24	E	26	0	3.14
32	E	25	4	2.26
40	E	25	4	3.72
48	E	25	9	4.35
56	E	25	9	4.09
64	E	25	10	3.22
72	A	23	11	3.21
80	A	23	12	4.22
88	A	21	12	2.19
96	A	21	12	3.2
104	A	20	12	3.6
112	A	18	12	3.31
120	B	14	15	5.02
128	B	14	15	4.95

E, A, B, Not admitted: Freshness index according to European Council Regulation (EC) No 2406/96: E (first quality extra), A (second quality), B (third quality). Not admitted: Spoiled, QIM: Quality index method

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The organoleptic evaluation of European pilchard stored at 0°C indicated that the loss of freshness was very slow, and it did not reach the level of organoleptic rejection after day 6. The organoleptic characteristics examined over time did not lose their quality simultaneously. The loss of abdomen integrity, shape, and loss of shine of the eyes began from day 3 by going through very distinct shades with the quality maintenance of the flesh, smell, and rigidity until day 6 of storage. Thus, the European Pilchard kept its freshness in class E (extra, the best one) for the first 3 days, with a corresponding QIM of 0 to 10. It then transitioned to class A on days 4 and 5 with a QIM between 11 and 12. Finally, class B had a QIM of 15 on the last day without reaching the organoleptic rejection threshold. There was a strong correlation between the QIM, the freshness index, and the duration of storage, with a determination coefficient of 0.85 for the freshness index and 0.9 for the QIM. Figure 1 shows a graphical representation of the evolution of the freshness index, the QIM, and the histamine level in European Pilchard stored at 0°C. QIM values have been reported in previous studies for organoleptic rejection from fish kept under ice (0°C); a QIM value of 15 was used for salmon and sea bream (Huidobro et al., 2000; Sveinsdottir et al., 2002). A lower QIM value was reported for hake (*Merluccius merluccius*) kept at 4°C and cod fillets under melting ice (Baixas-Nogueras et al., 2003; Cardenas et al., 2007). Considering the precision of prediction of the quality index methods, which are between days 1 and 3 (Baixas-Nogueras et al., 2003), it can be concluded that the Mehdia European Pilchard, stored under ice at 0°C can retain its organoleptic characteristics for 9 days. Comparable intervals between 9 and 12 days have been reported in other studies (Perera et al., 2020), and even longer shelf lives have been found in some cases, more than 16 days under salt-added ice (Losada et al., 2004).

Histamine content remained almost stable throughout 6 days of storage and varied between 2.19 and 5.02 ppm. This concentration was well below the regulatory limit in force. Moreover, the coefficient of determination value of 0.09 showed a very low relationship between the histamine content and the duration of storage when it was kept at 0°C. The statistical analysis yielded a linear regression equation in the form of Y (histamine content) = 1.55IFB+2.87. This equation showed that the duration of storage has no impact on the histamine content at this temperature. The coefficient of determination was equal to 0.09, near 0, reflecting the weak relationship between the two variables (Figure 2). The analysis reveals that when the fish maintained an Extra class freshness, there was no significant increase in histamine content. However, as the fish freshness transitioned from class A to class B, the histamine content exhibited a slight increase of 1.55 ppm, representing a minimal change.

The results of storage at 0°C suggested that histamine production in flesh European pilchard could be stopped by the fish frosting. This is consistent with a study conducted by Perera et al.(2020) and contrary to the study by Mohamed et al. (2022), indicating that the histamine production in flesh European pilchard was only delayed and histamine content could increase during storage in ice (0°C). These results are consistent with a study on the occurrence of histamine in canned European Pilchard marketed in Morocco, revealing that the average histamine content did not exceed 5.14 ppm. The study further indicated that canned sardines sold in the northern coastal area of Morocco, corresponding to the current area studied in this work, had the lowest risk with an average histamine content of 2.11 ppm (El Hariri et al., 2017). Mejrhit et al.(2018) previously found superior results in fresh Moroccan sardines with a concentration of up to 200 ppm, indicating a significant improvement in hygiene conditions, handling, and awareness-raising of professionals in the sector, as well as the evolution of fishing and processing techniques.

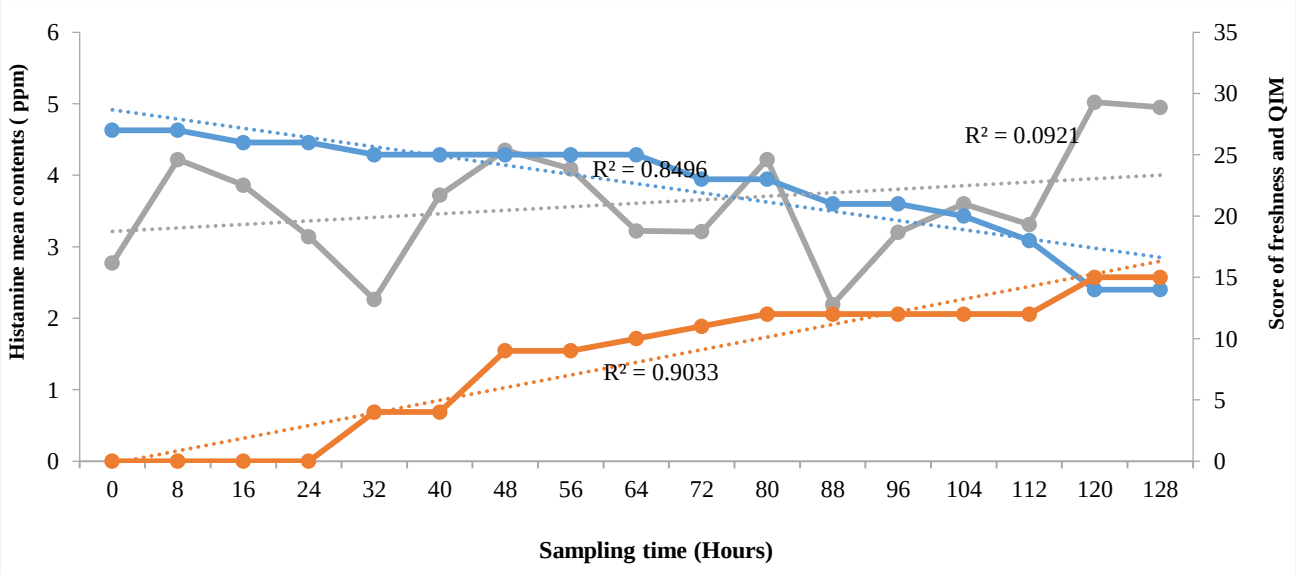

Figure 1. Evolution of freshness index, quality index method, and histamine mean contents of European pilchard stored at 0°C. Gray: Histamine mean content (ppm), Blue: Appreciation score of freshness, Orange: Quality index method (QIM)

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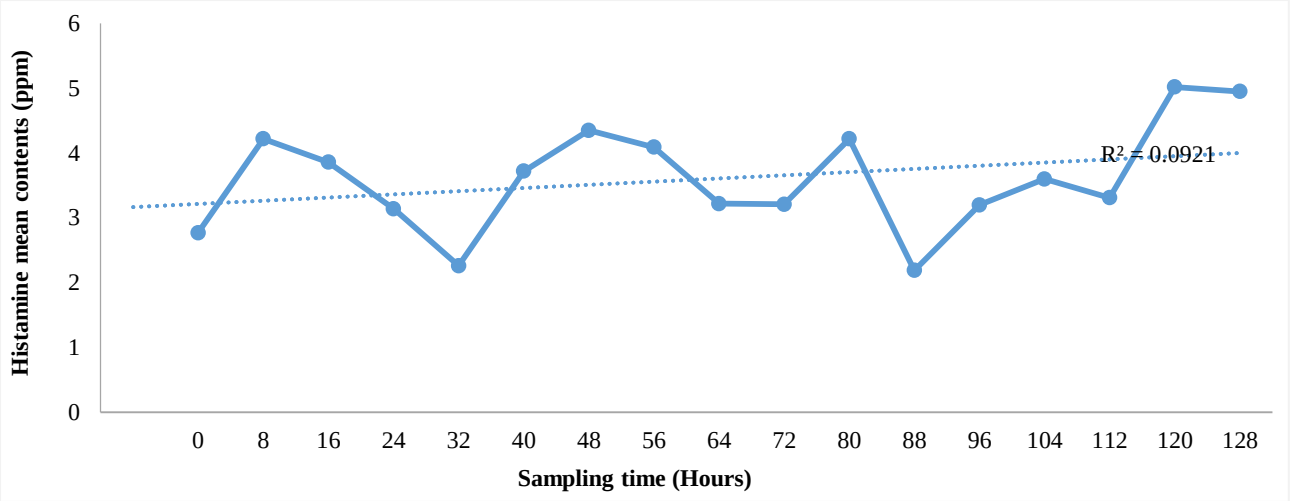

Figure 2. Histamine means contents of European pilchard stored at 0°C (ppm)

The second protocol for fish stored at 10°C

The fish were stored at 10°C for 6 days during this protocol. The temperature varied between 8.2°C and 10.7°C. The average temperature was 9.81°C. The results of the mean histamine content, freshness index, and QIM obtained are presented in Table 2. At storage at 10°C, the European pilchard began to lose its freshness from the 16th hour and passed rapidly to class A. Then, the fish reached the threshold of organoleptic rejection from the third day of storage, when there was a sour smell of putrefaction and an injury to the abdomen and eyes. The organoleptic rejection point corresponded to a QIM of 15. The evolution of the freshness index and QIM of the European pilchard stored at 10°C are represented in Figure 3. The evolution of histamine content of European pilchard stored at 10°C is represented in Figure 4. The graph shows a rapid change in the histamine content after 32 hours, reaching high concentrations of 824.74 ppm. The histamine level increased massively and reached an overwhelming value of 1988 ppm on the third day of storage.

Statistical analysis concluded a linear regression equation in the form Y = 554.57 IFB+30.11H (time). This equation showed that duration of storage has an impact on the histamine content. For each 8 hours, there was an increase of 30.11 ppm. As shown in Figure 5, the evolution of the histamine content as a function of duration of storage has a coefficient of determination equal to 0.87, which was close to 1, reflecting the strong relationship between the two variables. When the fish had an Extra freshness quality, the histamine content did not increase. When the fish freshness changed from class A to class B, the histamine content increased by 554.57 ppm, contrary to the 0°C storage temperature, where the histamine content increased by 1.55 ppm only. Thus, there was a strong association between histamine content and degree of freshness according to duration and storage temperature. This association was already reported by several studies involving European pilchard (Visciano et al., 2007; Mohamed et al., 2022).

Table 2. Freshness index, quality index method, and histamine mean contents for European pilchard stored at 10°C

Sampling time (hours)	Freshness index	Appreciation score of freshness	QIM	Histamine means contents. (ppm)
0	E	27	0	27.94
8	E	26	1	66.29
16	E	25	2	75.42
24	A	21	5	53.75
32	A	18	5	824.74
40	B	16	7	1022.72
48	B	15	8	2002.19
56	B	14	12	1852.42
64	B	14	15	1988.32
72	Not admitted	12	16	--
80	Not admitted	9	19	--
88	Not admitted	5	25	--
96	Not admitted	2	28	--
104	Not admitted	0	28	--
112	Not admitted	0	28	--
120	Not admitted	0	28	--
128	Not admitted	0	28	--

E, A, B, Not admitted: Freshness index according to European Council Regulation (EC) No 2406/96: E (first quality extra), A (second quality) B (third quality). Not admitted: Spoiled, QIM: Quality index method.

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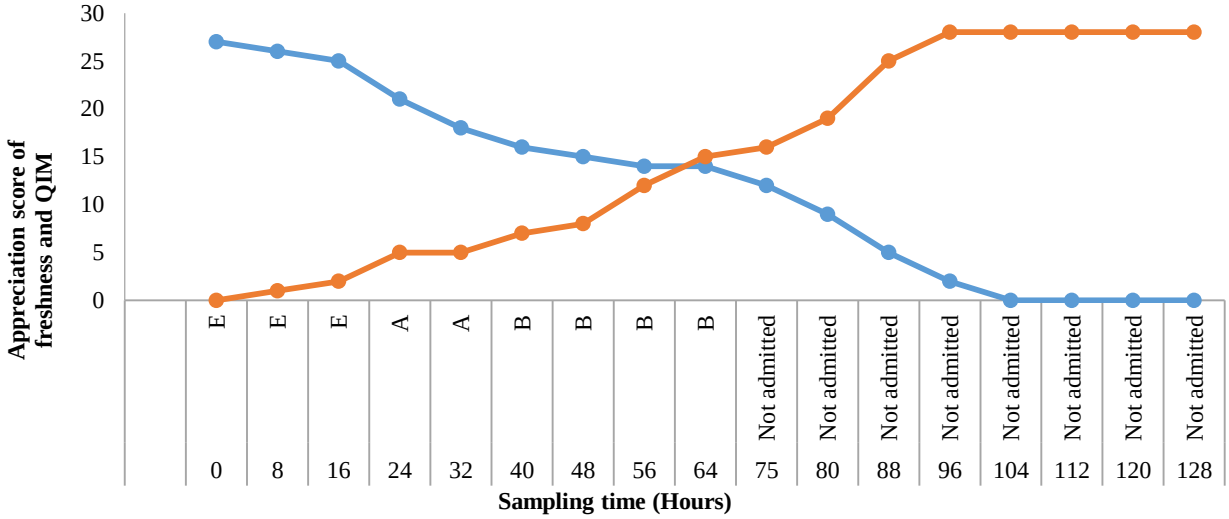

Figure 3. Evolution of the freshness index and quality index method of the European pilchard stored at 10°C. Blue: Appreciation of score of freshness, Orange: Quality index method. E, A, B, Not admitted: Freshness index according to European Council Regulation (EC) No 2406/96: E (first quality extra), A (second quality) B (third quality). Not admitted: Spoiled, QIM: Quality index method.

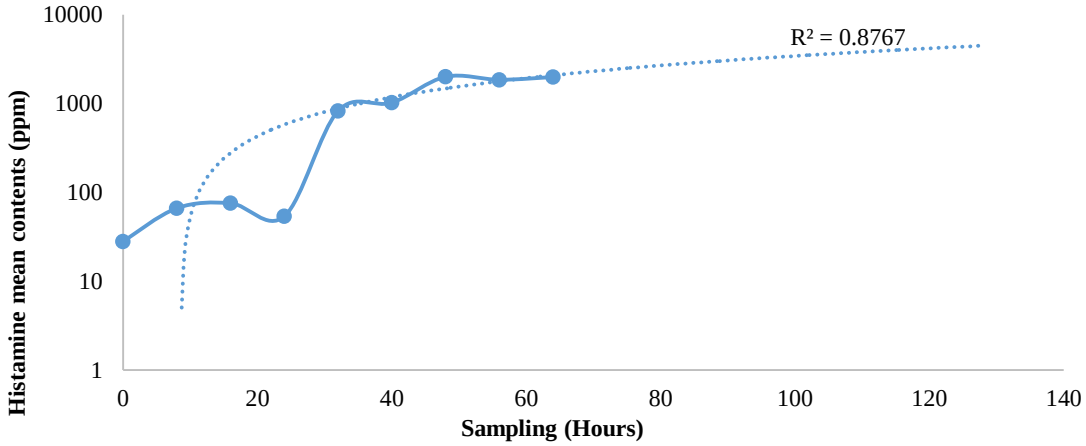

Figure 4. Histamine means contents of European pilchard stored at 10°C (ppm)

Comparative study of both protocols

The evolution of histamine and freshness is highly variable during storage for both protocols. Figure 5 indicates the difference in the evolution of the histamine content for each protocol. The average histamine content remained relatively constant at 0°C, while at 10°C, the average histamine content evolved rapidly between the 32 and 56 hours of storage and then slowed down to the end of storage.

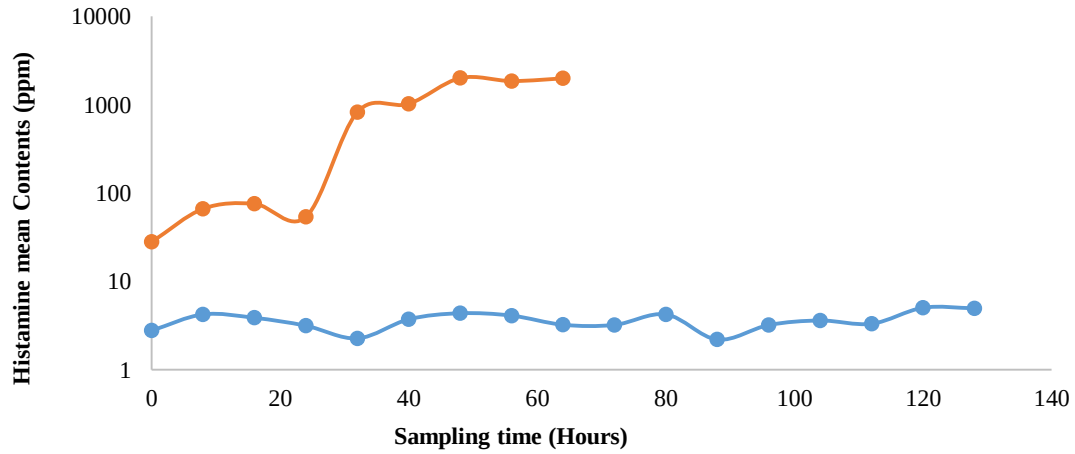

Figure 5. Histamine mean contents of European pilchard stored at 0 and 10°C (ppm). Blue: Storage at 0°C, Orange: Storage at 10°C.

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CONCLUSION

The storage at 0°C kept the organoleptic characteristics of the fresh European pilchard in very good condition for up to 6 days. The mean histamine content did not increase during storage at 0°C, unlike storage at 10°C, where the histamine content could easily exceed the acceptability threshold. A strong association was found between histamine content and degree of freshness according to duration and storage temperature. The storage temperature is critically important for the histamine content in flesh fish as well as for the degree of freshness over a given shelf life. When the fish has an extra freshness class, the histamine content does not increase. It can be concluded that fresh European Pilchard, which has an extra freshness quality, guarantees a very large margin of safety with regard to histamine and that it can be consumed without any risk. These results highlight the importance and suitability of frosting and the control of the storage phase in managing the risk of histamine in fishery products by involving all stakeholders in the sector, mainly the competent health authorities and food business operators. Further research is suggested to be conducted regarding the bacteria forming histamine in European pilchard, toxic doses of histamine, and the other risk management factors, such as the level of consumption specific to certain categories of consumers.

DECLARATIONS

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Authors' contributions

R. Khatouf conceptualized, conducted the research, and wrote the manuscript, Said Dahani, Oleya El Hariri, and N. Bouchriti conceptualized, analyzed data, supervised the research, and revised the final form. All authors read and approved the final version of the manuscript.

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Availability of data and materials

The data of the current study are available regarding the reasonable request from the authors.

Ethical considerations

Ethical issues (including plagiarism, consent to publish, misconduct, data fabrication and/or falsification, double publication and/or submission, and redundancy) have been checked by all the authors before publication in this journal.

Competing interests

The authors had no conflict of interest to declare.

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The Influence of Mannan Oligosaccharides and Beta Glucan Supplementation on Growth Performance, Blood Constituents, and Cecal Parameters of Broiler Chickens

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ABSTRACT

Growth promoters in poultry feed have been under severe attention since antibiotics were banned for use in animal diets by the European Union. Thus, it has been important for poultry researchers to find alternatives to antibiotic growth promoters (AGPs) to boost the health and production performance of poultry. This research was conducted to evaluate the effects of adding ALTIMOS® (cell wall of *Saccharomyces cerevisiae*; mannan oligosaccharides [MOS] + beta-glucan [BG]) to broiler diets on productive performance, blood parameters, intestine histopathology, and cecum microbiota of broiler chicken. A total of 252 one-day-old Ross chicks were randomly selected and divided into seven treatments, with six replicates of each treatment. The treatments were the control group (0% feed additives), and groups that received 0.05, 0.125, 0.250, 0.500, 1.0, and 2.0 g MOS+BG /kg basal diet for 35 days feeding trial. The results showed that during most trial periods, the group fed the basal diet supplemented with 1.0 g MOS+BG/kg had the highest body weight and weight gain, as well as the lowest feed consumption and best feed conversion ratio, compared to other treated groups. Moreover, this group had the best productive performance in the accumulative period. The inclusion of MOS+BG at 1.0 g/kg diet showed no significant effect on carcass percent compared to the control group. In addition, the inclusion of MOS+BG at 1.0 g/kg diet resulted in the lowest count of *Escherichia coli* and *Enterococcus* in the cecum, the highest *Lactobacillus* bacteria count among all experimental treatments, and a higher yeast count compared to the control group. The group fed 1.0 g MOS+BG/kg ration had the lowest blood cholesterol, whereas there were no significant differences among all experimental groups in the measured liver functions. Notably, the Hemoglobin percentage in the group fed MOS+BG at 1.0 g/kg feed was the highest. In the group fed 1.0 and 2.0 g MOS+BG/kg, the intestinal villi length was longer, and the histopathology revealed mild alteration. Overall, the supplementation of 1.0 g MOS+BG/kg diet improved growth performance, blood constituents, and cecum's beneficial bacteria counts of broilers.

Keywords: Beta-glucan, Blood constitute, Broiler chicken, Cecal parameter, Growth Performance, Mannan Oligosaccharide

INTRODUCTION

In poultry production, the main target is improving the broiler chickens' performance. To obtain optimal development, broiler chickens must receive diets that meet their needs to be healthy and ensure maximum output. Antibiotics have been used for decades to boost avian immune responses as well as growth promoters (AGP). As known, the supplementation of growth-promoting antibiotics in the animal diet has been banned by the European Union (EU) since 2006 (Anadón, 2006). Long-term use of antibiotics in poultry feed affects mainly the consumer due to the development of drug-resistant bacteria (Sweeney et al., 2018). Therefore, antibiotics are no longer used as promoters for growth in chickens' diets due to the harmful consequences on poultry or human health (Kovitvadhi et al., 2019). Prebiotics and probiotics have been used as alternative feed additives instead of antibiotics to improve intestinal health and chickens' performance (Karar et al., 2023). Potential prebiotics derived from the outer cell wall of yeast are now known as mannan-oligosaccharides (MOS). Mannan-oligosaccharides are used as an energy source by beneficial microorganisms such as *lactobacilli* and *bifidobacteria* (Leblebicier and Aydoğan, 2018). Broilers' growth parameters were enhanced by MOS. In the previous study, there was an increase in body weight, body weight gain, and feed conversion ratio when the broiler chickens were supplemented with 1.0 g MOS+ beta-glucan (BG)/kg of ration (Tufail et al., 2019). Otherwise, the chicks fed prebiotics such as MOS (0.2% of diet), had a lower level of cholesterol and creatinine compared to the control chickens (Biswas et al., 2019). In addition, MOS can reduce intestinal pathogenic microbes and it may improve the health of mucous membranes (Mahfuz et al., 2019). Therefore, this study was undertaken to assess the performance,

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blood biochemical parameters, relative organ weights, and cecal microbial content of broiler chickens fed different levels of a prebiotic compound (MOS+BG) as a growth promoter.

MATERIALS AND METHODS

Ethical approval

The ethical statement for all treated chickens was performed following the protocol of the Animal Use Committee of the Agriculture Research Centre, Ministry of Agriculture and Land Reclamation, Giza, Egypt.

Experimental design, management, and diets

The present study was conducted at the Regional Center for Food and Feed (RCFF), Agricultural Research Center (ARC), Giza, Egypt. A total of 252, one-day-old, straight-run Ross broiler chicks with an average initial body weight of 40.00 ± 1.00 g were adapted for three days. On the fourth day of age, with an average 100 g body weight/chick, they were randomly assigned to seven treated groups of six replicates of each with six chicks per replicate, using a completely randomized design. The treated groups are the control (basal diet), and groups that received 0.05, 0.125, 0.250, 0.500, 1.0, and 2.0 g of ALTIMOS® (24.5% MOS+27.7% BG, as yeast cell wall)/kg basal diet. Chicks were housed in wire-bottomed stainless-steel cages in a closed room with exhaust fans to keep normal ventilation. During the experimental period (35 days), feed and water were available *ad libitum*. In the first week, the temperature was adjusted at 31 ± 0.5˚C, and the relative humidity was approximately 60-70%. Then, the temperature decreased by 2˚C per week and the relative humidity reduced from the second week to a final temperature and relative humidity of 24 ± 0.5˚C and 50-60%, respectively, at the fifth week of the age.

Broiler chicks were subjected to continuous light for 24 hours daily during the first week of the experiment. From the second week up to the end of the trial, the daily light schedule was changed to 23 hours of light and 1 hour of darkness per day. According to the vaccination program followed by most Egyptian broiler chicken farms, all experimental broiler chicks were vaccinated against common diseases as shown in Table 1. According to the National Research Council (NRC, 1994)**,** diets were designed to meet the nutritional requirements of broiler chickens during production periods (Table 2).

Table 1. Vaccination program of broiler chickens in the present study

Age (day)	Vaccines	Method used
7	IB	Eye drop
10	H_5N_3	Subcutaneously injected into the lower back part of the neck
14	Infectious bursal disease (Gumboro D78)	Drinking water
24	Infectious bursal disease (Gumboro D78)	Drinking water

Corporation and country made of vaccines: CEVA company, France

Table 2. Composition and calculated chemical analysis of basal diets during the starting, growing, and finishing periods of broiler chickens

Ingredients (kg)	Starting (Day 1-14)	Growing (Day 15-28)	Finishing (Day 29-35)
Yellow corn (7.5% CP)	54.099	57.740	63.400
Soybean meal (46% CP)	34.380	28.480	23.920
Corn gluten meal (60% CP)	4.360	5.010	4.512
Soybean oil	3.179	4.705	4.390
Di-calcium phosphate	1.620	1.842	1.676
Limestone	0.936	0.701	0.697
Vitamin Mixture[1]	0.200	0.200	0.200
Mineral Mixture[2]	0.200	0.200	0.200
NaCl	0.400	0.400	0.400
Lysine-HCl	0.314	0.420	0.348
Methionine	0.237	0.227	0.182
Choline chloride	0.075	0.075	0.075
Total	100.000	100.000	100.000
Calculated values			
Metabolizable energy (KCal/kg)	2999.401	3160.462	3203.099
Crude protein (%)	23.003	21.074	18.989
Calcium (%)	0.960	0.900	0.850
Available phosphorus (%)	0.450	0.480	0.440
Lysine (%)	1.360	1.300	1.130
Methionine (%)	0.610	0.580	0.510
Methionine + cysteine (%)	0.980	0.940	0.850

[1]Vitamin mixture (IU or mg/kg diet): 12000 IU Vitamin A, 2000 IU Vitamin D3, 10 mg Vitamin E, 5 mg Vitamin K3, 3 mg Vitamin B1; 6 mg Vitamin B2; 5 mg Vitamin B6, 0.03 mg Vitamin B12, 40 mg nicotinic acid amine, 10 mg D-Ca-pantothenate, 0.075 mg folic acid, 375 mg choline, 80 mg.
[2]Mineral mixture (mg/kg diet): 60 mg Manganese, 80 mg Iron, 8 mg Copper, 0.5 mg Iodine, 0.2 mg Cobalt, 0.15 mg Selenium.

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Measurements and methods of interpreting results

Productive performance and relative organ weights

Body weight (BW), body weight gain (BWG), feed intake (FI), and the mortality rate of broilers were recorded weekly for each replicate during all periods of growth. The feed conversion ratio (FCR) was calculated by dividing feed intake by body weight gain. At 35 days of age, six chicks were randomly selected from each treated group weighed, slaughtered, blood filtered, feathered, and then eviscerated. The dressing, front part, part, liver, gizzard, heart, spleen, bursa of Fabricius, and abdominal fat were weighed, and the relative weight was calculated.

Microbiological, blood biochemical constituents, and histopathological study

The cecal contents were taken from the six chicks of each group that were slaughtered at 35 days of age, for cecal contents, and bacterial counting, including *Escherichia coli* (*E. coli)*, *Enterococcus*, *Lactobacillus*, Yeast, and *Salmonella* as colony-forming units, Cfu/g (Collin et al., 1995). As well as, at 34 days of age, blood samples were collected from the wing vein of six individuals of each treated group into 2 ml sterile vials and allowed to clot for 4 hours followed by serum separation using a centrifuge (10 minutes, 2000 rpm) before being stored at -20°C for later analysis. Serum measurements were made using commercially available kits (Biosystem S.A., Costa Brava, 30, Barcelona, Spain) following the manufacturer's instructions. Aspartate aminotransferase (AST) and alanine aminotransferase (ALT) as indicators of liver function, urea, and creatinine as signs of kidney function, as well as cholesterol and triglycerides were measured. Also, at the same time (34 days of age), other blood samples were aliquoted into 2 ml sterile vials with anti-coagulant and centrifuged for 10 min at 4000 rpm to measure hematological parameters including red blood cell count (RBCs), Hemoglobin content (Hb), white blood cells (WBCs), lymphocytes (L), neutrophil, monocytes, and eosinophils according to Sysmex software of automated hematology analyzer for animal, XT-2000iV analyzer (software version, 00-11, Sysmex, Kobe, Japan).

In all groups, the intestinal tissue samples were collected from the six chicks of each group that were slaughtered at 35 days of age and were preserved in 10% neutral buffered formalin for 72 hours. Then fixed tissue was processed using a paraffin embedding technique and cut into 4 µm thick sections using a microtome (Leica 2135, Germany), and stained by hematoxylin and eosin stain. The stained tissue was examined under a light microscope and photographed with an Olympus XC30 (Tokyo, Japan) digital camera. Intestinal villi length, width, and crypt depth of six intestinal villi were measured in captured images at 40x magnification using TS view software for morphometric analysis. Six images were analyzed to calculate an average for each chick (Mohamed et al., 2020).

Economic efficiency

The administration variables for broiler meat output in each group were assumed to be constant, but weight gain and feed consumption for each experimental group have been calculated to evaluate the economic efficiency of feeding (EEF). Then the following formula has been used to calculate the economic efficiency of feeding/Egyptian pound (EGP):

$$EEF = \frac{Net\ revenue\ (EGP)}{total\ cost\ \ (EGP)} \times 100$$

Statistical analysis

Data were analyzed by the least square procedure of the general linear model (GLM) of SAS software (SAS, 2010)**.** The separation of mean was done using Duncan's New Multiple Range Test (Duncan, 1955)**.** The fixed effects model used in the analysis was :$Y_{ij} = \mu + T_i + \varepsilon_{ij}$ Where Y_{ij} is the value of the respective variable, µ is the overall mean of the respective variable, T_i is the effect due to the i^{th} treatments where i = 1, 2 …and 7 (1 = Control, 2 = 0.05g MOS+BG, 3 = 0.125g MOS+BG, 4 = 0.250g MOS+BG, 5 = 0.5 MOS+BG, 6 = 1 g MOS+BG and 7 = 2g MOS+BG), ε_{ij} is a random error associated with the ij^{th} observation and is assumed to be independently and normally distributed. The significance level was set at ($p \leq 0.05$).

RESULTS

Productive performance

Performance parameters influenced by prebiotic supplementation (MOS+BG) are mentioned in Table 3. There were significant differences ($p \leq 0.05$) among treated groups during all periods from 0 to 35 d in body weight (BW), body weight gain (BWG), feed intake (FI), and feed conversion ratio (FCR).

The findings showed that the group fed a basal ration provided by 1.0 g MOS + BG/kg ration had the highest BW and BWG in growing and accumulative periods among all experimental groups ($p < 0.05$). The group fed MOS + BG (0.25 g/kg) had the highest BW and BWG in the starting period, whereas there was no significant difference ($p > 0.05$) between BW and BWG of MOS + BG (1.0g/kg) group and MOS + BG (0.25 g/kg) group. At the finishing period, MOS + BG (1.0 g/kg) group had the highest BW among all experimental groups and a higher BWG than that of the control group. As observed in every period, MOS + BG (0.5 g/kg) group had higher BW and BWG than that of the control group. Moreover, MOS+ BG (0.125 g/kg) group and MOS + BG (2.0 g/kg) group had significantly higher ($p < 0.05$)

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BWG as compared with the control group throughout the entire period. MOS+ BG (1.0 g/kg) group had the highest FI among all experimental groups at the growing and the entire period whereas it had a higher FI than the control group at the starting and finishing periods. Throughout the entire period, MOS + BG (0.125 g/kg), (0.5 g/kg), and (2.0 g/kg) groups had higher ($p < 0.05$) FI than the control group (Table 3). Similarly, MOS + BG (1.0 g/kg) group had the best FCR at growing, finishing, and accumulative periods. As noticed, MOS + BG (0.05 g/kg) group had the highest Feed conversion ratio (FCR) value throughout the entire period. It is worth noting that, MOS + BG 0.125 g/kg, and 0.5 g/kg groups had higher mortality rates than the control group in the accumulative period ($p < 0.05$).

Carcass characteristics

MOS + BG (1.0 g/kg) group had significantly ($p < 0.05$) similar relative carcass and liver weights as that of the control group. Moreover, MOS + BG (1.0 g/kg) group had the highest relative bursa, spleen, and heart weights followed by 0.05 g/kg group (Table 4). There were no significant differences among the relative spleen weights of the control group and MOS + BG (0.05 g/kg and 0.125 g/kg) groups; they had the lowest relative spleen weights compared with the remaining groups ($p < 0.05$). It was observed that there were no significant differences ($p > 0.05$) among relative liver, gizzard, and abdominal fat weights among all experimental groups (Table 4).

Table 3. Effects of diets supplemented by different levels of mannan oligosaccharides and beta-glucan (g/kg) on the performance of broiler chickens during 35 days of age

Productive parameters	Control	MOS+BG						SEM
		(0.05)	(0.125)	(0.250)	(0.5)	(1.0)	(2.0)	
Day 0-14								
Body weight (g)	477^{c}	485^{bc}	462^{d}	496^{a}	489^{b}	492^{ab}	480^{c}	6.00
Body weight gain (g)	377^{c}	385^{bc}	362^{d}	396^{a}	389^{b}	392^{ab}	380^{c}	6.00
Feed intake (g)	437^{a}	431^{b}	411^{c}	439^{a}	441^{a}	441^{a}	437^{a}	5.00
Feed conversion ratio (FCR)	1.16^{a}	1.12^{b}	1.13^{b}	1.10^{c}	1.13^{b}	1.12^{b}	1.15^{a}	0.01
Mortality	0^{b}	0^{b}	0^{b}	0.16^{a}	0^{b}	0.16^{a}	0.16^{a}	0.05
Day 15-28								
Body weight (g)	1744^{c}	1706^{d}	1731^{c}	1792^{b}	1730^{c}	1825^{a}	1778^{b}	17.00
Body weight gain (g)	1268^{c}	1221^{e}	1268^{c}	1295^{b}	1241^{d}	1333^{a}	1298^{b}	15.00
Feed intake (g)	1715^{c}	1686^{d}	1756^{b}	1798^{a}	1695^{d}	1796^{a}	1762^{b}	18.00
Feed conversion ratio (FCR)	1.35^{b}	1.38^{a}	1.38^{a}	1.39^{a}	1.36^{ab}	1.35^{b}	1.36^{ab}	0.02
Mortality	0.16^{b}	0.16^{b}	0.66^{a}	0.16^{b}	0.50^{a}	0.16^{b}	0^{b}	0.1
Day 29-35								
Body weight (g)	2321^{e}	2310^{e}	2359^{d}	2423^{b}	2382^{c}	2454^{a}	2369^{cd}	16.00
Body weight gain (g)	577^{d}	604^{c}	629^{b}	631^{b}	652^{a}	629^{b}	592^{cd}	15.00
Feed intake (g)	1153^{c}	1197^{b}	1193^{b}	1207^{ab}	1239^{a}	1185^{b}	1166^{bc}	24.00
Feed conversion ratio (FCR)	2.01^{a}	1.98^{b}	1.90^{cd}	1.92^{c}	1.90^{cd}	1.89^{d}	1.98^{b}	0.02
Mortality	0.16^{ab}	0.16^{ab}	0.33^{a}	0^{b}	0.16^{ab}	0^{b}	0.16^{ab}	0.09
Day 0-35								
Body weight gain (g)	2221^{e}	2210^{e}	2259^{d}	2323^{b}	2282^{c}	2354^{a}	2269^{cd}	16.00
Feed intake (g)	3305^{c}	3314^{c}	3361^{b}	3444^{a}	3374^{b}	3422^{a}	3364^{b}	27.00
Feed conversion ratio (FCR)	1.48^{b}	1.50^{a}	1.49^{ab}	1.47^{b}	1.47^{b}	1.45^{c}	1.48^{b}	0.01
Mortality	0.33^{c}	0.33^{c}	1.00^{a}	0.33^{c}	0.66^{b}	0.33^{c}	0.33^{c}	0.15

[a-e] Means, within a row with different superscripts, are significantly different ($p < 0.05$). SEM: Standard error of the means.

Table 4. Effects of diets supplemented by different levels of mannan oligosaccharides and beta-glucan (g/kg) on the relative organ weights of broiler chickens during 35 days of age

Treatment	Carcass	Liver	Bursa	Spleen	Gizzard	Heart	Abdominal fat
Control	77.03^{ab}	1.97	0.17^{e}	0.14^{c}	1.28	0.53^{b}	0.99
MOS+BG (0.050)	76.63^{b}	2.04	0.18^{e}	0.16^{c}	1.53	0.55^{ab}	1.01
MOS+BG (0.125)	76.78^{ab}	2.06	0.22^{d}	0.17^{c}	1.36	0.54^{b}	0.94
MOS+BG (0.250)	78.70^{a}	2.05	0.25^{c}	0.25^{b}	1.39	0.65^{ab}	0.97
MOS+BG (0.500)	77.40^{ab}	2.13	0.34^{a}	0.32^{a}	1.34	0.68^{ab}	1.14
MOS+BG (1.000)	76.71^{ab}	2.12	0.36^{a}	0.35^{a}	1.43	0.72^{a}	0.97
MOS+BG (2.000)	77.12^{ab}	2.01	0.28^{b}	0.25^{b}	1.40	0.67^{ab}	0.98
SEM	0.75	0.07	0.02	0.02	0.08	0.03	0.09

[a-c] Means, within a column with different superscripts, are significantly different ($p < 0.05$). SEM: Standard error of the means.

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Cecal microbiota

Regarding cecal microbiota presented in Table (5), there were significant increases ($p < 0.05$) in lactic acid bacteria in all experimental groups than that of the control group, except in the MOS + BG (2.0 g/kg) group which had the same value as that of the control group. It was observed that MOS + BG (0.5 g/kg) and MOS + BG (1.0 g/kg) groups had significantly lower *Escherichia coli* and *Enterococcus* counts than those of the control group ($p < 0.05$). All experimental treatments increased the yeast count significantly ($p < 0.05$) compared with the control group and the highest was obtained from MOS + BG (0.05 g/kg) group. Also, *Salmonell*a was not detected in all groups in the experiment (Table 5).

Table 5. Effects of diets supplemented by different levels of mannan oligosaccharides and beta-glucan (g/kg) on cecum's microbiota (Cfu/g) of broiler chickens during 35 days of age

MOS+BG / Parameters	Control	(0.05)	(0.125)	(0.250)	(0.5)	(1.0)	(2.0)
Lactic acid bacteria (Cfu/g)	12×10^4	$>10^5$	$>10^5$	$>10^5$	$>10^5$	$>10^5$	29×10^4
Escherichia coli (Cfu/g)	16×10^4	10×10^4	18×10^4	7×10^4	6×10^3	43×10^2	25×10^4
Enterococcus count (Cfu/g)	37×10^6	27×10^4	25×10^4	20×10^5	9×10^4	12×10^4	8×10^5
Total yeast count (Cfu/g)	20×10	27×10^4	49×10^3	28×10^2	33×10^2	39×10^3	39×10^3
Salmonella	ND	ND	ND	ND	ND	ND	ND

ND: Non-detected.

Blood constituents

As represented in Table 6, the group fed a 1.0 g MOS+BG/kg diet had a significant ($p < 0.05$) reduction in cholesterol, and urea levels compared to other groups. The data in Table 7 show the blood count (CBC) findings of broiler chickens fed a basal diet supplemented with different levels of MOS + BG. In comparison to the other groups, the group fed a 1.0 g MOS/kg had the greatest ($p < 0.05$) percent level of Hemoglobin (Hgb).

With increasing MOS+BG levels in the diet, the percentage of neutrophils gradually increased, while the percentage of monocytes gradually decreased. Moreover, there was no significance ($p > 0.05$) in the percentage of lymphocytes, RBCs, WBCs, and eosinophils among all experimental groups. There were no significant differences between groups that fed 0.5 and 1.0 g MOS+BG/kg diet in the percentage of neutrophils and monocytes ($p > 0.05$).

Table 6. Effects of diets supplemented by different levels of mannan oligosaccharides and beta-glucan (g/kg) on blood serum constituents of broiler chickens during 35 days of age

Treatment	Cholesterol (mg/dl)	Triglycerides (mg/dL)	Urea (mg/dl)	Creatinine (mg/dl)	AST (U/L)	ALT (U/L)
Control	130.20^{bc}	132.43^{a}	453.67^{ab}	76.00^{a}	4.23	0.2
MOS+BG (0.05)	132.80^{bc}	97.57^{b}	413.00^{ab}	74.00^{a}	4.4	0.2
MOS+BG (0.125)	152.03^{b}	75.27^{d}	316.00^{b}	51.67^{c}	4.06	0.2
MOS+BG (0.250)	181.43^{a}	130.27^{a}	415.67^{ab}	53.67^{c}	4.43	0.2
MOS+BG (0.5)	140.13^{bc}	96.87^{bc}	387.67^{ab}	57.33^{b}	4.50	0.2
MOS+BG (1.0)	117.73^{c}	91.87^{c}	311.33^{b}	57.00^{c}	4.06	0.2
MOS+BG (2.0)	123.73^{c}	90.40^{c}	443.33^{ab}	55.67^{c}	3.83	0.2
SEM	7.93	5.34	27.6	5.8	0.14	---

$^{a-c}$ Means, within a column with different superscript letters, are significantly different ($p < 0.05$). MOS: Mannan oligosaccharides, AST: Aspartate transaminase, ALT: Alanine aminotransferase, SEM: Standard error of the means.

Table 7. Effects of diets supplemented by different levels of mannan oligosaccharides and beta-glucan (g/kg) on blood serum count of broiler chickens during 35 days of age

MOS+BG / Variable	Control	(0.05)	(0.125)	(0.250)	(0.5)	(1.0)	(2.0)	SEM
Hgb (%)	8.43^{c}	8.50^{c}	8.62^{b}	8.57^{bc}	8.53^{bc}	9.70^{a}	8.55^{bc}	0.11
RBCs (Cells/µL)	2.88	2.55	2.60	2.52	2.80	2.57	2.63	0.17
WBCs (19-30×103/mm^3)	28.67	27.33	25.67	22.00	28.67	24.33	24.67	1.58
Lymphocytes (%)	63.00	64.00	65.00	61.67	66.33	63.00	65.00	1.75
Neutrophil (%)	25.33^{e}	26.67^{d}	27.33^{c}	27.33^{c}	28.33^{b}	28.00^{b}	30.33^{a}	0.06
Monocytes (%)	7.33^{a}	7.00^{b}	7.00^{b}	6.67^{c}	6.33^{d}	6.33^{d}	4.33^{e}	0.10
Eosinophils (%)	2.33	2.33	2.33	2.33	2.00	2.33	2.00	0.03

$^{a-e}$ Means, within a row, with different superscript letters are significant ($p < 0.05$). MOS+BG: Mannan oligosaccharides [MOS] + beta-glucan [BG], Hgb: Hemoglobin, RBCs: Red blood cells, WBCs: White blood cells, SEM: Standard error of the means.

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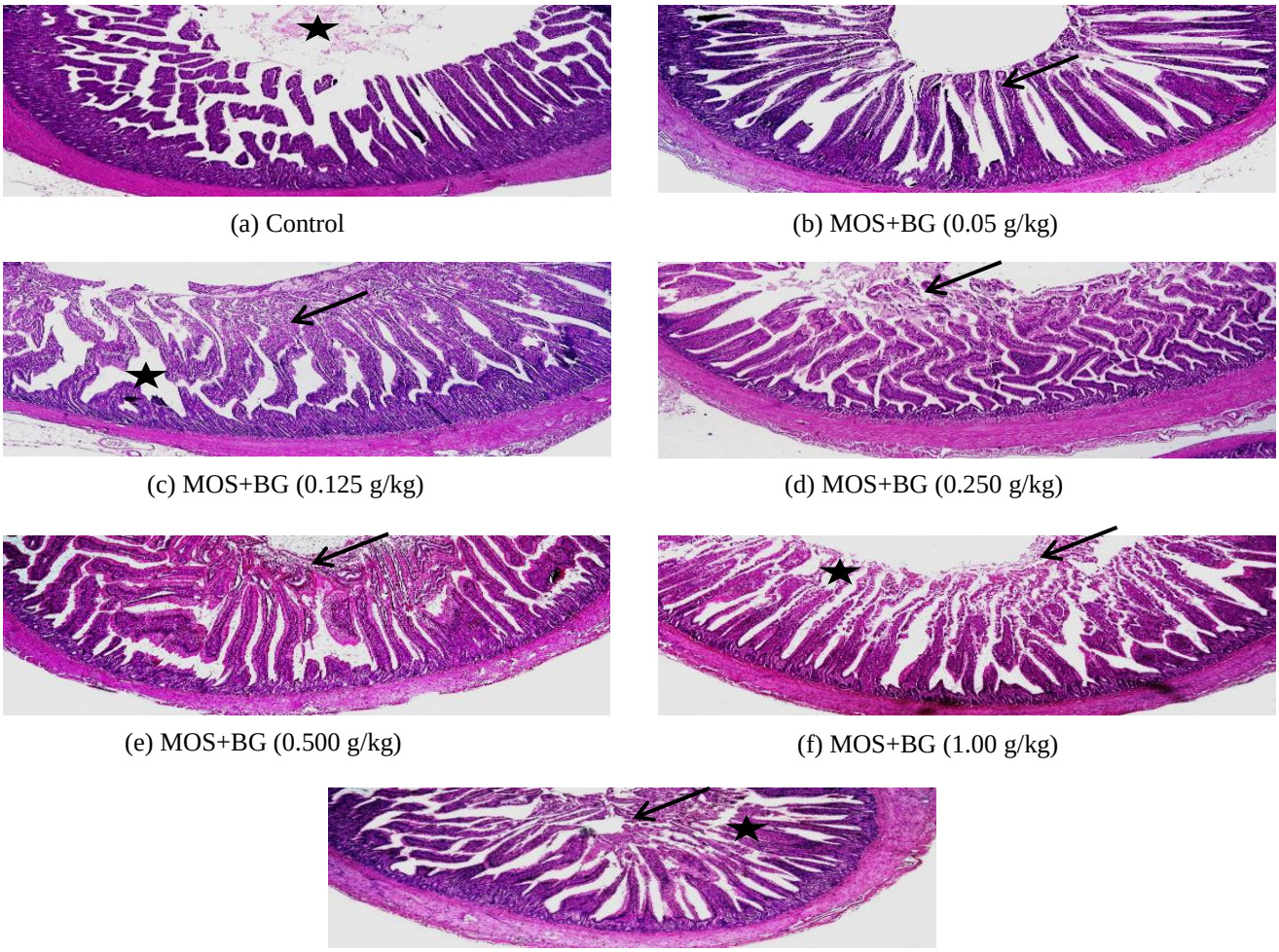
Intestinal histomorphometry and histopathology

A slight shortening of intestinal villi in the intestine of the control group chicks was observed under the microscope. In addition, mucous exudates, a few goblets cell hyperplasia in the lumen, and limited inflammatory cell infiltration in the lamina propria and submucosa (Figure 1a). In a group supplemented with 0.05 g MOS+BG/kg, the intestine had long intestinal villi with regression of the lesions observed (Figure 1b). In the group that fed 0.125 g MOS+BG/kg, the intestine had a mild histopathological change with a few mononuclear cell infiltrations and mucous exudates in the lumen (Figure 1c). Regarding the group treated with 0.25 g MOS+BG/kg, the intestine had a mild histopathological alteration with moderate goblet cell hyperplasia (Figure 1d). The intestine microscopy revealed severe goblet cell hyperplasia in the group supplemented with 0.5 g MOS+BG/kg (Figure 1e). In either group that fed a basal diet supplemented with 1.0 g MOS+BG/kg, or that supplemented with 2.0 g MOS+BG/kg, long intestinal villi, and mild epithelial hyperplasia were recorded (Figure 1f, g). The longest significant villi length was recorded in the 2.0 g MOS+BG/kg group followed by groups of 0.05 g MOS+BG/kg, 0.5 g MOS+BG/kg group, and 1.0 g MOS+BG/kg group. The highest crypt depth was recorded in the control group, and the lowest crypt depth appeared in the intestines of chicks that were fed 0.25 g MOS+BG/kg (Table 8).

Economic efficiency

According to Table 9, although the MOS+BG (1.0 g/kg) group showed higher ($p < 0.05$) cost/kg BW (31.365 EGP) than that of the control group (the lowest cost/kg BW; 30.135 EGP) among all experimental groups, it had the best value of net revenue and economic efficiency compared to other experimental groups ($p < 0.05$). The data revealed that the lowest cost/kg BW among all MOS+BG supplemented groups has resulted from the group fed 0.05 g/kg MOS+BG.



(a) Control (b) MOS+BG (0.05 g/kg)

(c) MOS+BG (0.125 g/kg) (d) MOS+BG (0.250 g/kg)

(e) MOS+BG (0.500 g/kg) (f) MOS+BG (1.00 g/kg)

(g) MOS+BG (2.00 g/kg)

Figure 1. The intestine of broiler chickens at 35 days of age. The mucous exudates in the lumen of the control group (**a**, star), long intestinal villi in MOS+BG (0.05 g/kg) group (**b,** arrow), luminal mucous exudates (**c**, star) with mild goblet cell hyperplasia in MOS+BG (0.125 g/kg) group (arrow), moderate goblet cell hyperplasia in MOS+BG (0.250 g/kg) group (**d**, arrow), severe goblet cell hyperplasia in MOS+BG (0.500 g/kg) group (**e**, arrow), long intestinal villi (**f** and **g,** star), and mild epithelial hyperplasia (**f** and **g** arrow), in MOS+BG (1.00 and 2.00 g/kg) group are shown. Haematoxylin and eosin staining, X40.

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Table 8. Effects of diets supplemented by different levels of mannan oligosaccharides and beta-glucan (g/kg) on intestine morphology of broiler chickens during 35 days of age

Treatment	Length (µm)	Width (µm)	Depth (µm)
Control	1089.53^{b}	159.05	279.44^{e}
MOS+BG (0.05)	1389.57^{cd}	187.37	221.36^{cd}
MOS+BG (0.125)	1029.08^{b}	174.26	240.18^{de}
MOS+BG (0.250)	818.43^{a}	153.45	120.71^{a}
MOS+BG (0.5)	1460.16^{bc}	182.56	153.67^{ab}
MOS+BG (1.0)	1341.31^{c}	210.68	251.5 ^{de}
MOS+BG (2.0)	1568.8^{d}	284.35	179.11^{bc}
SEM	40.63	10.45	9.41

$^{a-e.}$ Means, within a column, with different superscript letters differs significantly ($p < 0.05$). MOS+BG: Mannan oligosaccharides [MOS] + beta-glucan [BG], and SEM: Standard error mean.

Table 9. The economic efficiency of broiler chickens fed diets provided by different levels of mannan oligosaccharides and beta-glucan

Treatment	FI (g/chick)	Feed Cost L.E/chick	*Total Cost L.E/chick	BW (g/chick)	**Total Revenue L.E/chick	Net Revenue L.E/chick	EE	Relative E. E (%)
Control	3305	23.135	30.135	2321	88.198	58.063	1.93	100
MOS+BG (0.05)	3314	23.218	30.218	2310	87.780	57.562	1.90	99
MOS+BG (0.125)	3360	23.570	30.570	2359	89.642	59.072	1.93	100
MOS+BG (0.250)	3444	24.211	31.211	2423	92.074	60.863	1.95	101
MOS+BG (0.5)	3375	23.828	30.828	2382	90.516	59.689	1.94	100
MOS+BG (1.0)	3422	24.365	31.365	2454	93.252	61.887	1.97	102
MOS+BG (2.0)	3665	24.363	31.363	2369	90.022	58.659	1.87	97

* Including chick price which was 7 L.E, **assuming the price of 1 kg live weight was 38 L.E, ***assuming the economic efficiency of the control was 100, FI: Feed intake, BW: Body weight, EE: Economic efficiency

DISCUSSION

Productive performance

Throughout the experimental period, the higher body weight and body weight gain with the best feed conversion ratio were observed in the group that was given a 1.0 g MOS+BG/kg diet. This might be due to the MOS mechanism, which causes a reduction in the load of harmful bacteria and a rise in the production of helpful bacteria, leading to the creation of a healthy intestinal environment, which is reflected in the improvement of performance parameters as a result of better nutrients absorption in the gut. Furthermore, according to Sadeghi et al. (2013), the intestine-beneficial bacteria in the colon, such as *Lactobacillus* and *Bifidobacterium* spp. were developed by MOS+BG at 1.0 g/kg. The villi's surface area increased, and so did intestinal digestion and nutrient absorption (Chand et al., 2016).

The current study is in agreement with previous research (Kamran et al., 2013) which reported that broiler chickens given a basal diet supplemented with 1.0 g MOS+BG/kg showed a substantial improvement in BW, weight gain, and FCR. According to the dosage of MOS+BG in several previous studies, the finest level of MOS+BG for optimal growth performance is almost 1.0 g/kg diet (Abdel-Hafeez et al., 2017; Rehman et al., 2020). The results regarding BW and weight gain were in line with the findings of Ozpinar et al. (2010), who reported that the supplementation of 1.5 g MOS+BG /kg basal diet of the broiler chickens' ration increased the chickens' growth performance.

Carcass characteristics

The significant increase of lymphoid organs percentages (bursa and spleen) in this study due to MOS + BG supplementation, agreed with the results demonstrated by ELnaggar and Abdelkhalek (2017); they reported an increase in spleen relative weight significantly for hens supplemented with high levels of MOS (0.25 and 0.5 g of MOS /kg diet) compared to other experimental groups. Also, the indicated results in the current study, in contrast with results by Muhammad et al. (2020) who indicated no significant effect in relative weights of bursa and spleen between the broiler chickens fed MOS at either 0.5 g/kg or 1.0 g/kg diet, and that fed a basal diet, at 42 days of age. It was shown that carcass, and liver relative weights in broilers fed diets supplemented with MOS at 1.0 g/kg diet were not affected regarding the control group, which is following the results of the previous studies (Rehman et al., 2020; Karar et al., 2023). In disagreement with the present results, Habib et al. (2020) reported an increase in abdominal fat and a decline in the liver size of broiler chickens that were fed the diet supplemented with 4 g/kg MOS.

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Cecum microbial content

The groups that fed a basal diet with all different levels of MOS+BG exhibited a rise in yeast and *Lactobacillus* count while simultaneously showing a drop in *Enterococcus* count. According to Huyghebaert et al. (2011), MOS supplementation may increase the population of lactic acid bacteria in broilers' digestive tracts, improving their resistance to pathogenic bacteria like *E. coli* and *Enterococcus*. Additionally, *salmonella* spp. and *E. coli* fimbriae, which are sensitive to mannose, have unique receptors for the MOS supplements, which cause them to be removed with the digestive flow rather than adhering to intestinal receptors when the pathogen exposure is high. Also, MOS can reduce the number of pathogenic bacteria in the hindgut (Castillo et al., 2008).

According to the results of the current study, the gut microbial population's enhancement has a positive impact on growth performance. The present study's findings are consistent with those of Afrouziye et al. (2014), and Mostafa et al. (2015), who found an increase in *lactobacillus* and *bifidobacterial* spp. and a decrease in the number of *E. coli* in the cecum of broiler chickens fed a diet supplemented with MOS, compared to broilers on a basal diet. According to several studies, broilers fed a basal diet supplemented with MOS had lower cecum *E. coli* counts than those fed a basal diet without any supplements (Mostafa et al., 2015).

Blood parameters

The groups fed a basal diet supplemented with 1.0 g MOS+BG/kg diet and 2.0 g MOS+BG/kg diet had the lowest cholesterol levels. Biswas et al. (2019) found that the level of cholesterol decreased in broilers given a 0.2% MOS group compared to all treatment groups. The most important mechanism of prebiotics to reduce blood cholesterol levels is undoubtedly reduced intestinal lipid absorption by binding bile acids, which affects cholesterol excretion and hepatic production for new bile acids (Kumar et al., 2022).

In contrast with the present results, it was reported that the different quantities of MOS (0.5, 1.0, and 1.5 g/kg ration) showed no significant differences among the levels of blood biochemical indicators (Muhammad et al., 2020). Furthermore, there were no significant changes in AST and ALT levels across the experimental groups. These findings contradict the findings of Jameel et al. (2014) and Helal et al. (2015), who reported a substantial reduction in AST and ALT levels in chickens fed a prebiotic diet compared with chicks fed a basal diet. In contrast to the current results, Biswas et al. (2019) observed that 0.2% MOS significantly increased the AST of 42-day broiler chickens.

In the present study, creatinine levels were considerably lower in most of the experimental treated groups compared to the control group. This is consistent with the findings of Helal et al. (2015) and Muhammad et al. (2020), who reported that there was a decrease in creatinine in chicks fed a prebiotic diet compared to chicks fed a basal diet. The results indicated that the groups (0.125 g MOS+BG/kg diet) and (1.0 g MOS+BG/kg diet) had considerably lower blood urea levels than other experimental groups. These findings disagreed with those of Biswas et al. (2019), who reported a significant rise in blood uric acid concentration in broiler chickens, and with Muhammad et al. (2020) who showed no differences in the level of urea among all broiler chickens' groups. The hematological findings in this investigation revealed no significant difference in RBCs, WBCs, and lymphocytes across all treatment groups that are in agreement with Muhammad et al. (2020), who reported insignificant differences in RBCs, WBCs, and lymphocytes among broilers fed diets supplemented with MOS at levels of 0.5 and 1.0 g/kg basal diet.

Among all experimental groups, the current results confirmed that the Hemoglobin was considerably the highest in the treated group with a 1.0 g MOS+BG/kg diet. Furthermore, Hgb levels were considerably higher in all MOS experimental treated groups when compared to the control group, except the 0.05 g MOS+BG/kg treated group, which exhibited no significant change but was numerically higher than the control. The findings of the present study contradict previous research by Muhammad et al. (2020) who found no effect of MOS supplementation at 0.5, 1.0, and 1.5 g to the basal diet on Hgb levels of broilers compared to the control group.

Intestinal histopathology

Mannan supplementation substantially improved intestinal villi length and lowered crypt depth in the current investigation. Similarly, Karimian and Rezaeipour (2020) revealed that the dietary MOS 2.0 g/kg diet enhanced the height of the villus and reduced crypt depth in the broiler chicken's small intestine. However, some studies found no difference in the length of intestinal villi owing to MOS in diet (Baurhoo et al., 2009) and a rise in crypt depth (Oliveira et al., 2008). The rise in intestinal villi may have occurred as a result of higher *Lactobacillus* counts in the intestine of treated groups, which promote a healthy intestinal environment (Baurhoo et al., 2009). The surface area was increased by long villi and shallow crypts, which improves nutrient absorption (Yang et al., 2009). Stressors that impair the immune response can cause inflammation and damage to the host tissue (Berghman, 2016). In the current investigation, a little histological change in the intestine was seen in the control group; however, the MOS-supplemented groups showed fewer such alterations. These results are consistent with a prior study that found that MOS and live yeast supplementation reduced inflammation (Tarradas et al., 2020).

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Economic efficiency

According to the findings, the control group had the lowest feed costs of all experimental groups, and the group fed a ration with 0.05 g of MOS /kg of ration had lower costs than groups that fed higher MOS dosages. Except for MOS+BG (0.05 g/kg), all groups treated with MOS+BG had greater net revenue values as compared to the control. However, chickens were fed diets supplemented with MOS at 1.0 g/kg was the best. In Egypt, Mostafa et al. (2015) found no appreciable changes in the feed cost of 1.0 kg BWG across broiler chickens given diets supplemented with 0.5, 1.0, or 1.5 g of Bio-Mos/kg diet. The economic impact of using Bio-Mos in broiler diets is only of very limited interest to academics. So long as economic criteria are not compromised, a greater MOS dosage rate may be examined for improved outcomes. The increased body weight growth, enhanced feed conversion, and affordable MOS might be reasons for these improvements.

CONCLUSION

Conclusively, Mannan oligosaccharides plus beta-glucan supplementation, notably at level 1.0 g/kg feed, improved blood parameters without harming intestinal morphology and histology in broiler chickens. It is recommended to conduct further studies regarding the use of mannan oligosaccharides plus beta-glucan in broiler diets.

DECLARATIONS

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Authors' contributions

The study design, data collection, data analysis, writing, and manuscript review were all contributed equally by all authors. Additionally, the statistical results and the final edition of the manuscript were endorsed and agreed upon by all authors.

Competing interests

There are no stated conflicts of interest by the authors.

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Ethical considerations

All authors reviewed the manuscript for ethical issues such as plagiarism, consent to publish, misconduct, forgery and/or falsification of data, duplicate publication and/or submission, and redundancy.

Availability of data and materials

All data from the current study are available by request from the authors.

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Pig Slaughter Operators' Perception of Stunning Benefits: A Comparative Analysis of Electrical and Captive Bolt Effectiveness

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ABSTRACT

The pre-slaughter phase, which includes stunning, aims to reduce animal stress, ensuring a more compassionate and efficient process in the meat industry. Various methods are often used in slaughtering pigs, with electrical, mechanical, and chemical stunning being the most common techniques. Several studies have shown that selecting the appropriate method requires operators to comprehensively understand the slaughter process. Therefore, this study focused on evaluating the comprehension of pig slaughter operators regarding the benefits and effectiveness of electrical and captive bolt stunning methods. A total of 17 pigs slaughtered from seven slaughterhouses were selected as samples. Data collection was carried out using questionnaires, interviews, and direct observation. The results showed that operators clearly understood the benefits of stunning in terms of speed and ease. However, their comprehension regarding pig stress reduction before death remained limited. Although captive bolt stunning was known to have various benefits, such as shortening the duration of leg movements after slaughter, it required longer operation time, compared to the electrical method. Therefore, it can be concluded that there is no ideal stunning method as both methods of electrical and captive bolt stunning have their respective advantages and disadvantages.

Keywords: Captive bolt stunning, Effectiveness, Electrical stunning, Comprehension of operator

INTRODUCTION

Slaughter is an essential step in transforming animals into meat for human consumption, requiring compliance with established standards comprising hygiene, safety, working conditions, and welfare (Beageaud-Blacker, 2007). This step must be carried out sympathetically by minimizing the pain experienced by animals and recognizing their intrinsic value (Nakyinsige et al., 2013).

Stunning before slaughter is a legal requirement designed to induce unconsciousness and insensibility (inability to perceive stimuli), ensuring that the subsequent slaughter is performed without causing fear, anxiety, pain, suffering, and distress (EFSA, 2004). According to previous studies, stunning refers to any intentional process that induces painless loss of consciousness and sensitivity, even methods leading to instant death (EC, 2009). A comprehensive understanding of the unconsciousness and insensibility of animals before death holds significant importance, and the assessment of this parameter can be used to assess the effectiveness of various methods (EFSA, 2004). In practical settings, the evaluation of stunning effectiveness comprises observation of eye reflexes, reactions to painful stimuli, resumption of normal rhythmic breathing, and foot-righting reflexes (EFSA, 2004). This shows that it is important for all individuals associated with stunning and slaughter to be competent, adequately trained, and have a positive attitude toward the welfare of the animals (EFSA, 2004).

Pre-slaughter stress can affect postmortem muscle metabolism and meat quality, leading to increased levels of catecholamines and creatinine kinase in the body, rapid glycolysis, and lactic acid buildup in the meat (Bourguet et al., 2011; Pisestyani et al., 2015). Animal stress levels can be measured using neutrophil-lymphocyte ratio (Litmer et al., 2020). Previous studies have shown that pigs executed with electrical stunning had a lower ratio, and this condition showed reduced stress (Anugrah et al., 2022), further supported by a decrease in superoxide dismutase level (Prayoga et al., 2020).

Various stunning methods are commonly used in the pig slaughter process, including electrical, mechanical, and chemical methods (OIE, 2011). The electrical method relies on passing a large electrical current through the brain, thereby inducing generalized epilepsy and immediate loss of consciousness (EFSA, 2004). Meanwhile, the mechanical method is often carried out using a captive bolt or hitting the forehead with a wooden block (Goba et al., 2013). To avoid complications, the use of stunning must be informed by a proper understanding and knowledge, considering that the duration of unconsciousness and insensibility vary across different methods, species, and animals (EFSA, 2004).

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Although there are no ideal methods for stunning and killing farm animals, it is necessary to select procedures that offer more advantages in terms of welfare (EFSA, 2020). The choice of an inappropriate method can lead to significant losses (EFSA, 2004). In slaughterhouses of Bali, electrical and captive bolt methods are often used, with the captive bolt being a recent development. The Animal Australia Foundation introduced this current procedure in collaboration with the Faculty of Veterinary Medicine, Udayana University, and the Department of Agriculture and Food Crops, Badung Regency, Bali, Indonesia. Therefore, this study aims to assess the understanding level of pig slaughter operators and evaluate the effectiveness of electrical and captive bolt stunning methods.

MATERIALS AND METHODS

Ethical approval

This study was approved by the committee of the institution of Animal Care and use at the Faculty of Veterinary Medicine, Udayana University, with reference number B/233/UN14.2.9/PT.01.04/2021.

Experimental site

The experiment was carried out from May to July 2022 at the pig slaughterhouse located in Taman sub-village, Darmasaba village, Abiansemal subdistrict, Badung regency, Bali, Indonesia, as a pilot project of cooperation between the Faculty of Veterinary Medicine, Udayana University with Animal Australia Foundation and the Department of Agriculture and Food crops of Badung district, Bali-Indonesia.

Data collection

A total of 17 pig slaughter operators, all specializing in Landrace pig slaughter, were interviewed. These operators were selected from 7 different slaughterhouses at Taman sub-village, Darmasaba village, Abiansemal sub-district, Badung-Bali, Indonesia. The interviews aimed to gauge their comprehension level using a set of questionnaires, focusing on the benefits of stunning in the slaughter process and its correlation with meat quality. Furthermore, the electrical stunning method (230-volt, 60A, 50 Hz) in this study was carried out by operators. It involved attaching one electrode or stunning tongs to the head of the pig. In contrast, the treatment of the captive bolt stunning method was carried out using penetrating captive bolt guns, such as Matador Super Securit 3000. The process was performed when the pig was in a blangsung or iron restraint shaped like a block or tube. The effectiveness of electrical stunning 230 volt, 60A, 50 Hz, and captive bolt was evaluated by direct observation with several variables such as sound ends, leg movement ends, operational time in using the tool, and the value score of tool repetition to achieve fainting.

Data analysis

Data from the questionnaire, which assessed the operators' level of understanding, were processed descriptively. The relationship between variables was examined using the Chi-square test to determine the significance level. If $p < 0.05$ means significantly different, and $p > 0.05$ means not significantly different. Furthermore, data on the effectiveness of the pig stunning device were tested for normality using the Shapiro Wilk Test Subsequently, Wilcoxon Test was employed to find a mean difference between both methods. All data was analyzed using the SPSS-25 program (Santoso, 2018).

RESULTS AND DISCUSSION

Slaughter of pigs with the stunning method was performed at a slaughterhouse in Taman sub-village, Darmasaba village, Abiansemal sub-district, Badung-bali. The types of stunning methods used included electrical and captive bolt stunning, as well as hitting with a wooden stick (Table 1). The use of captive bolt was still relatively new and unique to Bali. The data of the study based on the interviews of all operators (17 operators) originating from all slaughterhouse businesses (7 locations) are tabulated in Table 1.

As can be seen in Table 1, of 17 operators, 13 (76.47%) had experience in performing stunning methods, while others had no experience (23.53%). Among the 13 participants with experience, the majority had been involved for 6-10 years and 11-15 years, accounting for 38.46%, followed by those with over 16 years at 23.08%. Furthermore, the type of stunning used was dominated by electrical (46.15%), followed by a combination of captive bolt and electrical (30.776%), and hitting with wood (23.08%). The profiles of pig slaughterer operators for each slaughterhouse are presented in Table 2.

Table 2 indicates that 7 (41.18%), 6 (35.92%), and 4 (23.53%) operators aged 51-60, 30-49, and 41-50 years old, respectively. According to the Central Statistics Agency of Indonesia, the productive age is between 15 and 64 years. Accordingly, the majority of operators were considered to be within the productive age, and this age range had a positive relationship with labor productivity (Suyono and Hermawan, 2013). This phenomenon was supported by the better

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knowledge and high responsibility for tasks exhibited by these individuals. At the productive age, the workforce could adapt quickly and easily adopt new technology. The results showed that the non-productive age often had problems with physical abilities and difficulty learning new technology, leading to suboptimal productivity (Ukkas, 2017).

A total of 7 (41.18%), 4 (23.53%), 4 (23.53%), and 2 (11.76%) respondents had worked as pig slaughter operators for 6-10, >16, 1-5, and 11-15 years, respectively. A study by Trijanuar revealed that a worker's experience level could be influenced by the length of service, level of knowledge, skills, and mastery of work and equipment. Experience is the basic capital in understanding and comprehending a job, and extended duration tends to provide maximum performance (Trijanuar, 2016).

Table 1. Profile of the seven pig's slaughterhouse business at Taman sub-village, Darmasaba village, Abiansemal sub-district, Badung-Bali, Indonesia during April-May 2022

Variables	Category	Percentage
Doing stunning	Yes	76.47% (13/17)
	No	23.53% (4/17)
Duration of the stunning device (years)	6-10	38.46% (5/13)
	11-15	38.46% (5/13)
	>16	23.08% (3/13)
The type of stunning	Electrical	46.15% (6/13)
	Captive Bolt and Electrical	30.77% (4/13)
	Hitting with a wooden	23.08% (3/13)

Table 2. Profile of pig slaughterer operators who work at Taman sub-village, Darmasaba village, Abiansemal sub-district, Badung-Bali, Indonesia during April-May 2022

Variables	Category	Percentage
Age (years)	30-40	35.29% (6/17)
	41-50	23.53% (4/17)
	51-60	41.18% (7/17)
Length of work (years)	1-5	23.53% (4/17)
	6-10	41.18% (7/17)
	11-15	11.76% (2/17)
	>16	23.53% (4/17)
Level of education	ES	5.88% (1/17)
	JHS	47.06% (8/17)
	SHS	41.18% (7/17)
	College	5.88% (1/17)

ES: Elementry school, JHS: Junior high school, SHS: Senior high school

The education level of pig slaughter operators predominantly consisted of individuals with junior high school education, accounting for 8 respondents (47.06%). This was followed by senior high schools, elementary schools, and colleges, with 7 (41.18%) and 1 (5.88%) participants, respectively. These findings underscore that education of the workforce at the pig slaughterhouse in Taman sub-village, Darmasaba village, was still relatively low due to the dominance of elementary and junior high school education among nine respondents (Table 2). These results were in line with a previous study indicating that abattoirs were one of the sectors where a level of education was not required for employment (Sidabalok et al., 2018). Table 3 presents the results of the data analysis concerning the operators' understanding of pig stunning benefits at Taman sub-village, Darmasaba village.

Based on Table 3, the majority of operators, comprising 9 individuals (69.23%), who employed the stunning method before slaughter expressed that it facilitated easier handling of pigs. In contrast, 4 operators (30.77%) showed the absence of a significant effect. These results indicated that most respondents were aware of the benefits of the method. According to a previous study, stunning could facilitate production work due to its quick operation time (Beageaud-Blacker, 2007).

The majority of operators at the abattoir (69.23%) were of the opinion that stunning did not reduce the stress of pigs. This opinion correlated with education level. Specifically, 83.33% of respondents with junior high school certificates (5 participants) expressed the belief that stunning did not alleviate stress, compared to 66.67% (4 participants) of those with senior high school education. Out of 13 operators (30.77%), only 4 stated that the method could reduce stress, consisting of 1, 2, and 1 participants with junior high school, senior high school, and college education levels (100%), respectively. These results suggest that a significant proportion of operators were unaware of the stress-reducing effects of stunning, and educational levels appeared to impact this understanding. Furthermore, a

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higher level of education affected better understanding of stunning in reducing stress. Based on Table 3, 7 operators (53.85%) agreed that the method positively affected the quality improvement of produced pigs and enhanced consumer acceptance, with others disagreeing due to their level of education.

In this study, operators' understanding of the benefits of pig stunning at the Taman sub-village, Darmasaba village, correlated with their education levels. Education is an attempt to develop individuals' thinking abilities (Franco et al., 2018). Education can increase the insight and knowledge of a workforce and improve work skills (Suyono and Hermawan, 2013). The understanding levels of pig slaughter operators in the Taman sub-village, Darmasaba Village, were in a good category. This could be seen from the high percentage of respondents' understanding level of the benefits of stunning to facilitate easier pig handling and the effect of the method on meat quality and duration of slaughter process or cutting.

Table 3. Analysis of understanding of operators of benefits of stunning of pigs at Taman sub-village, Darmasaba village, Abiansemal sub-district, Badung-Bali, Indonesia during April-May 2022

Variables \ Category		Yes	No
Easy to handle pig		69.23% (9/13)	30.77% (4/13)
Reducing pig stress		30.77% (4/13)	69.23% (9/13)
Influencing quality pig (better)		53.85% (7/13)	46.15% (6/13)
Repairing reception of pig consumers (better)		53.85% (7/13)	46.15% (6/13)
Shortening the pig-cutting process		100% (13/13)	- (0/13)
Operators will recommend pig stunning to other slaughterhouses		100% (13/13)	- (0/13)
Association of operators education's level in comprehension stress on pig	JHS	16.67% (1/6)	83.33% (5/6)
	SHS	33.33% (2/6)	66.67% (4/6)
	Collage	100% (1/1)	- (0/1)
Association of operators education's level in the comprehension effect of pig stunning increase of pig quality	JHS	50.0% (3/6)	50.0% (3/6)
	SHS	50.0% (3/6)	50.0% (3/6)
	Collage	100% (1/1)	- (0/1)
Association of education level of operators regarding the relation of pig stunning process on the consumer perceptions in acceptance of pig produced	JHS	50.0% (3/6)	50.0% (3/6)
	SHS	50.0% (3/6)	50.0% (3/6)
	Collage	100% (1/1)	- (0/1)

JHS: Junior high school, SHS: Senior high school

The effectiveness of stunning with the electrical and captive bolt

The use of electrical stunning and captive bolt stunning before slaughter at a slaughterhouse located in the Taman sub-village, Darmasaba, can be observed in Figures 1 and 2. The effectiveness of stunning with electrical and captive bolts based on several variables is presented in Table 4.

As can be seen in Table 4, the sound ends of the pig after electrical stunning was 3.8 seconds, while stunning with a captive bolt was 3.65 seconds. There was no significant difference between the two methods in terms of the sound ends of pigs ($p > 0.05$). According to the European Food Safety Authority (EFSA, 2020), vocalizations or sounds are expected to occur only in aware animals and are used to monitor the animals' consciousness levels. Some animals are likely not to produce sounds in consciousness. Based on the results, the absence of vocalizations did not necessarily show unconsciousness.

The leg movement for electrical stunning was 3.85 minutes and captive bolt stunning was 3.10 minutes, with significant differences ($p < 0.05$). Compared to electrical stunning, the captive bolt showed a better outcome due to the shorter time required to achieve fainting. However, the operational time for the electrical method was 2.8 minutes, which was significantly shorter than a captive bolt at 3.65 minutes ($p < 0.05$). Stunning process in animals must be carried out quickly to minimize the return of consciousness (EFSA, 2020). Procrastination or improper device sticking could lead to fear, pain, and discomfort (EFSA, 2004). This is an essential aspect of stunning as well as a concern in animal welfare (EFSA, 2020). The score of the device repetition to achieve fainting in pig for electrical stunning (3.80 times) was not significantly different from the captive bolt (3.85 times, $p > 0.05$). The repetition was carried out due to the failure or ineffectiveness of the device, which was marked by the level of return to consciousness in pigs. According to the European Food Safety Authority (EFSA, 2020), the assessment of the consciousness level had two possibilities, namely outcomes of consciousness and unconsciousness. The use of stunning equipment, use duration, placement of tools on animals, and storage and maintenance of equipment were factors affecting the effectiveness of the process (Edwards, 2018). Moreover, the European Food Safety Authority (EFSA, 2020) stated that the effectiveness of electrical stunning depends on two important factors. First, the electrodes must be placed on either side of the head, between the eyes and the base of the ear, to facilitate penetration to the brain. Second, the current delivered to the brain must be sufficient to induce an immediate epileptic seizure. According to the Humane Slaughter Association (HSA, 2016), electrical stunning

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could be effective in pigs at an electrical voltage of 250 Volts, with a current and resistance of 1.6 Ampere and 150 Ohms. When the tool is used beyond a reasonable timeframe, an issue with the equipment may occur, necessitating its replacement with a more suitable alternative.

Figure 1. Stunning pig with electrical at slaughterhouse located in Taman sub-village, and Darmasaba village, Abiansemal sub-district, Badung-Bali, Indonesia

Figure 2. Stunning pig using captive *bolt* at slaughterhouse located in Taman sub-village, Darmasaba village, Abiansemal sub-district, Badung-Bali, Indonesia

Table 4. The effects of electrical and captive bolt stunning methods at slaughterhouses of Taman sub-village, Darmasaba village, Abiansemal sub-district, Badung-Bali, Indonesia on several variables of fainting in pigs

Variables	Electrical stunning	Captive bolt stunning	P-value
Sound ends after stunning (seconds)	3.80 (0.41)	3.65 (0.75)	0.7384^{ns}
Leg movement ends after stunning (minutes)	3.85 (0.37)	3.10 (0.72)	0.0005*
Operational time in using the tool (minutes)	2.80 (041)	3.65 (0.49)	0.0001*
The value score of tool repetition to achieve fainting (times)	3.80 (0.41)	3.85 (0.37)	1.0000^{ns}

The numbers in the table show the mean value (SD). *significantly different ($p < 0.05$), ns: Not significantly different ($p > 0.05$)

CONCLUSION

In conclusion, the application of stunning methods during pig slaughter in Taman sub-village, Darmasaba village, Abiansemal sub-district, Badung-Bali, Indonesia, was perceived by operators as a means to enhance the efficiency and ease of the slaughter process and reduce the stress levels of the animal. Furthermore, the use of the captive bolt and electrical stunning presented a set of advantages and disadvantages. Captive bolts induced faster unconsciousness in animals than electrical stunning but required an extended operational time.

DECLARATIONS

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Availability of data and materials

The authors confirm that all data supporting this study has been used to write this article.

Authors' contributions

All authors contributed equally to the study. I Wayan Suardana conceived and designed the study. Putu Devidia Trisha Suciada conducted the trial and collected the samples. Romy Muhammad Dary Mufa analyzed the data. All authors have read and approved the final manuscript.

Ethical considerations

The authors written the article originally and also check the last draft manuscript for similarity index. This article is not submitted to anywhere else and the findings analysed and written under supervsions of all authors.

Competing interests

The authors declare that there are no conflicts of interest for this study.

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Production Performance and Some Biochemical Traits of Layer Hens Fed on Date Palm Kernel Supplementation

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ABSTRACT

To enhance the well-being and productivity of poultry, researchers have conducted investigations into various botanical sources, including date palm kernel, and their bioactive components. The present investigation was conducted to assess the productive performance and certain biochemical characteristics of layer hens that were administered date palm kernel supplementation in their diet. To this end, 40 ISA Brown laying hens (48 weeks old) were used in the current study. The adaptation phase for the chickens lasted for 10 days before the initiation of the experiment. The study lasted 8 weeks. The chickens were then randomly assigned into two groups of 20, with 4 replications (5 chickens per replication). Chickens in the treatment group received 5% of dry matter ground date palm kernel (GDPK), as an additive to the basal diet, and the control group (CL) chickens were fed the basal diet. The eggs were collected daily during the study. At the end of weeks 1, 2, 4, and 6 of the study, egg production percentage, egg weight, and the feed conversion ratio were measured. At the end of the experiment, blood samples were collected to measure the serum levels of glucose, total protein, cholesterol, creatinine, and uric acid. The study findings revealed significant increases in the daily egg production percentage and egg weight during the experiment in the GDPK group, compared to the CL group. The feed conversion ratio recorded significant decreases in the GDPK group when compared to the CL group. Furthermore, the findings indicated significant increases in the serum total protein and significant decreases in the glucose, cholesterol, creatinine, and uric acid concentrations in the GDPK group, compared to the CL group. In conclusion, the results indicated the positive effects of adding ground date palm kernel to the diet of layers on production performance, such as egg weight, conversion ratio, and some biochemical traits, including total protein, glucose, cholesterol, creatinine, and uric acid.

Keywords: Date palm, Egg production, Feed additive, Kernel, Laying hen

INTRODUCTION

The rapid growth of the world population has led to a sharp increase in the demand for egg production. To meet this demand, there is a critical need for substantial improvements in hen genetics, nutrition, and husbandry practices (Guerrero-Legarreta, 2010; Küçükyilmaz et al., 2012). In the poultry industry, it is essential to explore cost-effective methods to boost egg production while maintaining high quality. Therefore, there has been a demand from consumers for healthier eggs with high quality (Wang et al., 2017; Marelli et al., 2021).

The global chicken business is one of the most rapidly expanding agricultural sectors. There have been several recent threats to the poultry industry, including rising worldwide populations, altered climates, a lack of feedstuffs, a weak economy, and new illnesses (El-Sabrout et al. 2019). Chicken egg is a complete food since it has both organic and inorganic constituents and includes a significant amount of water (~75% water; El-Sabrout et al., 2022).

Liquid egg white, freeze-dried egg powder, and egg white protein are some innovative egg products that have gained popularity among customers (Perić et al., 2011). Despite the common belief that eating eggs can raise blood cholesterol, several clinical and epidemiological studies have established no such correlation (Alagawany et al., 2018; Réhault-Godbert et al., 2019).

In order to improve chicken health and production, scientists have studied some botanical sources, such as date palm kernel, and their bioactive constituents. Poultry farms are beginning to embrace the use of these compounds because of their nutritional significance, medicinal capabilities, and lack of residual effects. Date palm kernel, for instance, has long been used in chicken feeds to maintain chickens' wellness and maximize productivity (El-Husseiny et al., 2008; Tareen et al., 2017). Date palm kernel possesses beneficial activities, such as anti-inflammatory and antibacterial properties, attributed to its bioactive constituents that significantly influence physiological functioning (El-Husseiny et al., 2008; Saki et al., 2014; Abo Ghanima et al., 2020).

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The current study was performed to study the efficiency of production performance and some biochemical traits of layer hens fed with date palm kernel supplementation.

MATERIALS AND METHODS

Ethical approval

The current study was conducted according to the ethical guidelines College of Veterinary Medicine, University of Al-Qadisiyah, Iraq.

Date palm collection

The date palm kernel was collected during the flowering period at the end of March month before pollination from male palm trees growing in the Al-Qadisiyah agriculture department, Iraq.

Date palm chemical composition

Crude protein, crude fiber, crude fat, ash, and total sugars for date palm kernel grains were determined according to AOAC (Bardaa et al., 2016). The concentrations of calcium (Ca), potassium (K), iron (Fe), and magnesium (Mg) were determined using Atomic Absorption Spectrometry (AAS) according to Hosseinzadeh et al. (2015).

Animals and study procedure

The study included 40 ISA Brown laying hens (48 weeks old). The chickens were randomly assigned into two groups of 20 hens with 4 replicates of 5 chickens per each. Before the experiment, the chickens were left for 10 days to adapt to the experimental environment. These groups were the treatment group that received 5% of dry matter ground date palm kernel (GDPK), as an additive to a standard diet (Table 1), until the end of the experiment (8 weeks), and a control group (CL) that received basal diet only.

Table 1. The chemical analysis and composition of diet in layer chickens in present study

Ingredients	Percentage
Corn	40
Soybean meal	20
Rice	25
Wheat bran	10.7
Lime stone	2
Premix 1	0.3
NaCl	0.5
Mono-calcium phosphate	1.5
Total	100
Chemical analysis	
CP	18.16
EE	2.65
CF	3.44
Ash	12.2
Ca	3.82
Available P	0.28
Lysine	0.7
Methionine	0.2
ME (Kcal/kg)2	2630

Premix 1: Contained 4540 mg/kg of Fe, 5000 mg/kg of Cu, 3400 mg/kg of Mn, 43 mg/kg of Co, 6000 mg/kg of Zn, 140 mg/kg of Se, 3600 kIU/kg of vitamin A, 360 kIU/kg of vitamin D, and 3 kIU/kg of vitamin E; CP: Crude protein, CF: Crude fiber, EE: Ether extract. The diet was balanced according to the last commercial recommendation of ISA Brown laying chickens' catalog.

Sampling

The produced eggs (n = 20) were collected daily during the study. Eggs collected at the end of weeks 1, 2, 4, and 6 were used to measure the egg quantity, egg weight, and the efficiency of the feed conversion ratio (FCR). At the end of the experiment, blood samples (2 ml) were collected from the wing vein of all chickens in a tube without anticoagulant for measuring the serum levels of glucose, total protein, cholesterol, creatinine, and uric acid with a commercial kit according to manufacturer instruction.

Production parameters, egg quality traits, and feed conversion ratio

The initial and final body weights were measured using a digital balance with an accuracy of ±0.5 g at 78 and 84 weeks of age for each replicate within the treatment. The egg number and weight were recorded daily during the experimental period from 78 to 84 weeks of age. Egg weights were recorded daily, while egg mass (g/hen) was calculated by multiplying the laid eggs numbers and weight (g) for all replicates within each treatment. Egg weights were recorded on a daily basis, while egg mass (g/hen) was calculated by multiplying the laid egg numbers and their respective weights (g) for all replicates within each treatment. Feed consumption (g/hen/d) was recorded weekly for each replicate, and FCR was measured as the ratio of feed in grams to the weight of eggs produced. A total of 140 normal eggs were randomly collected from eggs laid in the last three days (4 groups × 7 replicates × 5 eggs) to determine the external and internal egg quality traits.

Statistical analysis

GraphPrism v7 and SPSS software were used to analyze and present data. The sample t-test was used to analyze the data. The standard deviation of the mean was utilized to display mean data in graphs and tables. P values less than 0.05 were considered for significant differences.

RESULTS

The study findings revealed significant increases in the daily egg production percentage for all examined weeks in the GDPK group, compared to the CL group ($p < 0.05$, Figure 1). Moreover, egg weight was significantly higher at all tested week points in the GDPK group than that of the CL group ($p < 0.05$, Figure 2). Moreover, the feed conversion rate recorded significant decreases in the GDPK group when compared with this finding from the CL group ($p < 0.05$, Table 4 and Figure 3). Furthermore, the findings indicated significant increases in the serum total protein ($p < 0.05$) and significant decreases in the glucose, cholesterol, creatinine, and uric acid concentrations in the GDPK group when compared with these findings from the CL group ($p < 0.05$; Table 5 and Figure 4).

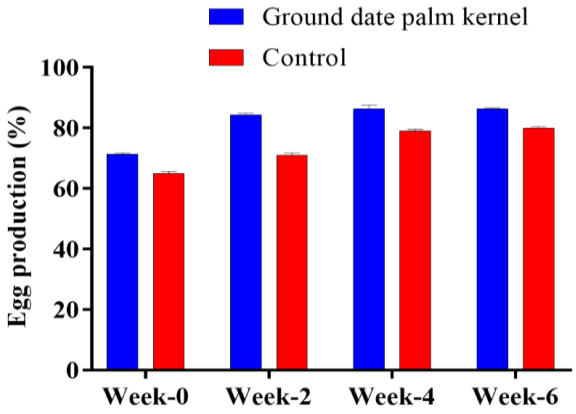

Figure 1. Daily egg production of laying hens fed ground date palm kernel

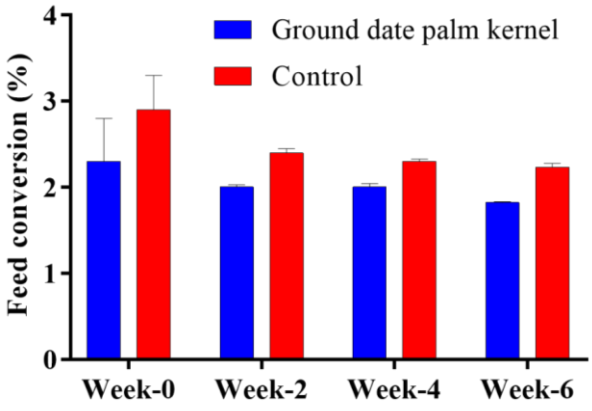

Figure 3. Feed conversion efficiency of laying hens fed ground date palm kernel

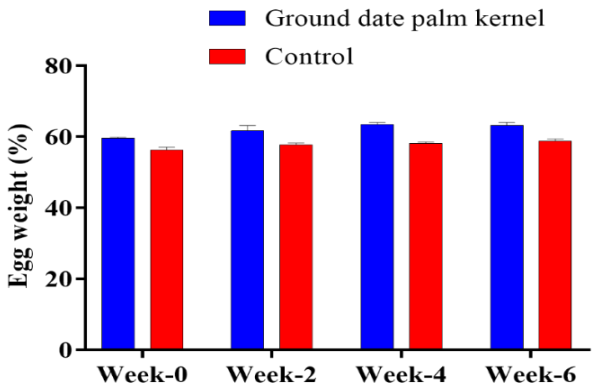

Figure 2. Egg weight of laying hens fed ground date palm kernel

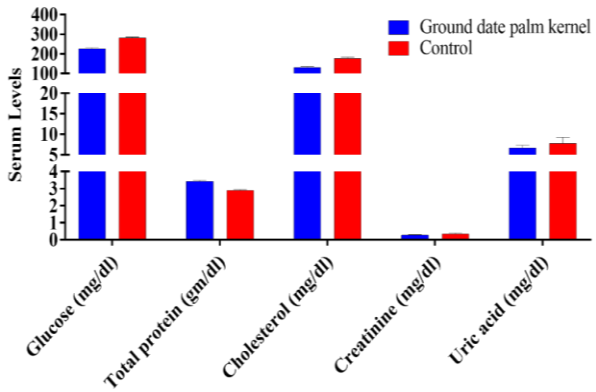

Figure 4. Serum biochemical parameters of laying hens fed ground date palm kernel

DISCUSSION

Contemporary poultry lines have been genetically engineered to optimize both meat and egg production. However, their heightened metabolic activity leads to an increased production of intracellular free radicals. These free radicals pose the risk of inducing oxidative stress, triggering inflammation, and disrupting various cellular processes (Hosseinzadeh et al., 2015). Numerous botanical items and their bioactive substances have been studied for their potential to eliminate free radicals and restore crucial cellular functions essential for poultry health and production. Plant medicinal seeds, such as date palm kernel, are examples of botanical constituents gaining attention in poultry farms for their nutritional quality, therapeutic capabilities, and residue-free nature (Hosseinzadeh et al., 2015; Chen et al., 2018).

The findings from the present study revealed a significant increase in both egg production and egg weight with the inclusion of GDPK as a feed additive. These effects became evident early in the experiment, specifically during the initial week of treatment. While limited information is available regarding the impact of the kernel on egg production, numerous researchers have explored various herbal, plant, and seed preparations in this context. These preparations often contain components known for their diverse health benefits, including antioxidative and anti-inflammatory activities (Alkhoori et al., 2022).

The seeds of the fenugreek plant have been employed for medicinal purposes for many years, primarily owing to their antimicrobial and anti-inflammatory characteristics (Adil et al., 2015). For laying hens mainly, Samani et al. (2020) found that 1% fenugreek increased feed intake and yolk color, especially in the latter cycle of egg production. Using 0.4% fenugreek in hen diets increased both egg output and egg quality. Ginger roots utilized as a medicinal plant all over the globe are another instance of a nutritional approach for increasing egg production. The gingerols, zingerone, and date palm kernel help beneficial microbiota and antioxidant defenses. Supplementing layer diets with ginger powder (10-15gm/kg) increased egg production and serum antioxidant capacity (Zhao et al., 2011). Several scientists and plant breeders throughout the globe have lately been interested in pumpkin seeds because of their potential as both a food source and medicine. They are abundant in saturated fatty acids and omega-3 fatty acids (Bardaa et al., 2016). According to Martínez et al. (2012), including 10% pumpkin into a layer hen diet improves beneficial fatty acids while lowering total cholesterol and detrimental fatty acids in the content of eggs.

In addition to the previously mentioned benefits, date seeds are rich in bioactive compounds with antioxidative and anti-inflammatory properties. Specifically, extracts from Ajwa date seeds have been recently discovered to exhibit exceptional antioxidant capabilities, comparable to those found in ascorbic acid (Anwar et al., 2022). This highlights the potential health-promoting attributes of date seeds and their significance as a source of natural antioxidants (Anwar et al., 2022). Because of their high phenolic component content, date seeds have also been studied for their potential antioxidant benefits. Salomón-Torres et al. (2019) observed that the polyphenolic composition of 'Medjool' date seeds is 10 magnitudes greater than that of the pulp. Research has demonstrated the antioxidant properties of date seeds, which have been assessed using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) method. The presence of various phenolic compounds, including tannins, saponins, flavonoids, coumarins, and anthraquinones, leads to these antioxidant characteristics. These bioactive molecules play a vital role in neutralizing free radicals and protecting cells from oxidative damage, making date seeds a promising natural source of antioxidants. Date palm kernel polyphenolic compounds could deliver significant antioxidants, helping to promote health and stave against illness in egg-laying hens. Employing the DPPH technique, Bentrad and Gaceb-Terrak (2020) discovered that extracts of date seeds had antioxidant properties. Compared to other antioxidant compounds, the extract of date palm kernel showed the greatest free radical inhibitory effectiveness. The phenolic chemicals found in date seeds are responsible for the antioxidant qualities of seeds since they efficiently donate hydrogen to DPPH radicals. These phenolic components include phenolic acids, flavonoids, and catechin tannins. Hilary et al. (2021) indicated the total polyphenol composition of date seed preparations and found significant increases in the phenolic compounds after consuming these preparations. Phenolic compounds prevent oxidative destruction of DNA, proteins, and lipids by neutralizing free radicals.

Due to the presence of these phenolic compounds in the date kernel, bird health and maximizing performance could have been improved in the current study, leading to an increase in the performance of the tested chickens. Date palm kernel contains bioactive constituents with beneficial impacts on physiological functioning and therapeutic qualities. Phytobiotics, tannins, phenols, flavonoids, and essential oils are found in eggs, and they serve several purposes in the birds' bodies. Feed additives such as date palm kernel have positive effects on egg weight, ovary features, and lowering yolk trimethylamine contents (López-Sobaler et al., 2017; Abolhasani Zadeh et al., 2022; Rohmah et al., 2022; Margiana et al., 2022; Arif et al., 2023).

CONCLUSION

The study results indicate the positive effects of adding ground date palm kernel to the diet of layers on production performance such as egg weight, conversion ratio, and some biochemical traits including total protein, glucose, cholesterol, creatinine, and uric acid. Therefore, more study is needed to evaluate the effects of date palm kernel on chicken production and biochemical traits.

DECLARATIONS

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Competing interests

The authors declare that they have no conflict of interest.

Authors' contributions

Zahira A. Al-Zuhairi, Afrah S.Mhyson, and Basima J. Mohammed, designed, drafted the article, and revised it critically for important intellectual content; Zahira A. Al-Zuhairi, and Afrah S.Mhyson, analysis, and interpretation. All the authors reviewed the final draft of article and agreed the content before submission.

Availability of data and materials

The authors will provide all necessary data to the editor upon request.

Ethical considerations

All authors have reviewed the ethical issues (including plagiarism, consent to publish, misconduct, data fabrication and falsification, multiple publishing and submission, and redundancy)

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Effects of Suture Implantation Using Different Suture Materials on the Skin Histopathology, Immune Expression of Interleukin-6, and Hematological Parameters in Rat

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ABSTRACT

Suture implantation is a procedure to promote rearrangement of the extracellular matrix. Various cellular responses of post-suture implantation affect the outcome of this procedure. The current study aimed to analyze the effects of suture implantation using polycaprolactone/polylactic acid/hyaluronic acid (PCL/PLA/HA) on skin histopathology, expression of IL-6, and hematological parameters in rat models. To conduct the study, 25 male Sprague Dawley rats, three months old were randomly divided into five groups, including G1 (control), G2 (sham, group injected using skin cannula), and G3-G5 (suture implanted groups). For the suture-implanted groups, a cannula was used using suture materials. Specifically, G3 received truglyde implants, G4 received PCL/PLA/HA implants, and G5 received polydioxanone (PDO) implants. The back skin and blood samples were collected on day 3. Histopathological analysis was conducted on the samples using H and E, Congo red, immunohistochemistry against IL-6, and hematology. The analysis of the data revealed that the group with suture implantation using PCL/PLA/HA had the smallest wound area, compared to the other implanted groups. Further, the PCL/PLA/HA group showed a significant decrease in eosinophils infiltration and IL-6 level on the skin samples after suture implantation. Moreover, there were no significant differences across the groups in most of the hematological parameters after suture implantation, including total erythrocytes, hemoglobin, eosinophil, basophil, and monocyte levels. The total neutrophils increased after suture implantation in all groups, while the total lymphocytes decreased. It can be concluded that the best material according to parameters evaluated in the current study for suture implantation was PCL/PLA/HA.

Keywords: Eosinophil, Histopathology, Hyaluronic Acid, Interleukin-6, Polycaprolactone, Polylactic Acid, Suture implantation

INTRODUCTION

Suture implantation in the skin tissue is a common procedure in cosmetic surgery, known to promote the rearrangement of the collagen of an extracellular matrix (Wang et al., 2023). The rearrangement of the extracellular matrix is expected to increase collagen deposition and repair tissue during the mechanism of aging (Adelman and Cornwell, 2020). Synthetic polymers have been developed for suture implantation, offering the ability to modulate tissue characteristics by promoting interactions between cells and the matrix, as well as facilitating revascularization (Cornwell et al., 2016). However, it is important to note that the diverse cellular responses elicited post-suture implantation can significantly influence the overall outcome of the procedure (Lovric et al., 2018).

A common cellular response following implantation is an increase in inflammation which is predominantly caused by eosinophils due to a hypersensitivity mechanism (Aronson et al., 2022). Eosinophils play significant roles in skin homeostasis and the regulation of T helper 1 (Th1) and Th2 balance. However, the excessive increase in eosinophils causes massive local inflammation, prolongs the duration of inflammation, and inhibits the process of tissue wound healing (Blanchard and Rothenberg, 2009). Moreover, eosinophil promotes a synthesis of pro-inflammatory cytokines, such as interleukin-6 (IL-6, Li et al., 2022). Interleukin-6 is essential during an acute inflammatory phase in wound healing; however, it must be decreased in the late processes to increase the matrix collagen deposition (Johnson et al., 2020). The suture material significantly affects the skin matrix rearrangement after suture implantation. A good suture material must be well tolerated by the skin with minimum inflammatory responses. Therefore, it is essential to develop suture materials for implantation using compounds that are well-tolerated by skin tissue, prevent delayed wound healing, and enhance clinical outcomes.

Polylactic acid (PLA) is a resorbable thermoplastic polyester compound, which is used in bone surgery because of its bio-resorbability and biocompatibility (Li et al., 2020). Hyaluronic acid (HA) is a component that can stimulate tissue

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adhesion after implantation (Corduff et al., 2021). Polylactic acid is commonly combined with HA and known as PLA/HA (Åkerlund et al., 2022), which is regularly used to increase tissue toughness and improve the tissue's mechanical properties. However, PLA/HA can be degraded in a short period after implantation and makes poor clinical responses. Hence, it can be combined with polycaprolactone (PCL) which is a bioresorbable polyester used in cosmetic surgery to improve matrix deposition. Although PCL is frequently utilized in bone applications, its use as an implantation material on the skin remains relatively unexplored (Ng et al., 2007). The present study aimed to analyze the effects of the implantation of different suture materials on skin histopathology, expression of IL-6, and hematological parameters in male rats.

MATERIALS AND METHODS

Ethical approval

The animal experimentation was approved by the ethical clearance committee from Lembaga Penelitian Universitas YARSI, Jakarta with registration number 134/KEP-UY/EA.10/VI/2023. This study was conducted from July until December 2023 in the Laboratory of Animal Models IratCo, Bogor, Indonesia.

Study design

This study involved 25 male Sprague Dawley rats (Laboratory of Animal Models IratCo, Bogor), weighing 250 grams, and aged 3 months old. The rats were housed in acrylic cages with access to water and feed *ad libitum*. The rats were randomly divided into five groups, including a control group without induction (G1), a sham-induced using cannula without suture implant (G2), suture implanted groups (G3-G5), while third group was implanted using truglyde (G3), fourth group was implanted using PCL/PLA/HA (G4), and fifth group was implanted using polydioxanone PDO (G5).

Before the suture implantation, the rat was intraperitoneally anesthetized using ketamine (10 mg/kg BW) in combination with xylazine (2 mg/kg BW, Sotoudeh and Namavar, 2022). The hair on the lateral side of the vertebrae was shaved using a clipper (Indonesia) and the area was disinfected using isopropyl alcohol. Subsequently, the cannula (Dermax, China) was implanted into the dermal part of the rat skin, as per the method outlined by Janhofer et al. (2018). The cannula (Nanumcompany, South Korea), truglyde (Truglyde, India), PCL/PLA/HA (APTOS, Georgia), and PDO (Whitemedience, South Korea) were used in this study. The suture was implanted as long as 2 cm on the lateral side of the rat vertebrae.

Sample collection

The rats were maintained for 3 days. On day 3, the rats were anesthetized by ketamine and xylazine using similar doses to the suture implantation procedure. After that, the rats' blood samples (1 ml) were collected from the retro-orbital plexus and stored using an EDTA tube (1 mL). Finally, they were euthanized using cervical dislocation. The skin samples were then collected and fixed using 10% neutral buffer formalin.

Laboratory tests

The blood samples were tested against several hematological parameters, including total erythrocytes, leucocytes, platelets, neutrophils, eosinophils, basophils, lymphocytes, and monocytes, and the level of hemoglobin. The blood samples were tested using an automated haemo-analyser (VetScan VS2, USA).

The skin samples were dehydrated using graded alcohol, and xylene and blocked using liquid paraffin. The samples were cut using a microtome. The skin slides were then stained by hematoxylin and eosin (H and E, Feldman and Wolfe, 2014), Congo red staining (Song et al., 2018), and immunohistochemistry (IHC) against antibody anti-IL-6 (Prakoso et al., 2020). The slide was dehydrated and applied with endogenous peroxidase and protein block. The slide was then incubated using antibody anti-IL-6 (Novocastra, USA) for 30 minutes. The slide was incubated using post-primary antibody and diethylaminobenzidine (DAB). The slide was counter-stained using hematoxylin and mounted using Entelan nue. The slides were then photographed using an Olympus microscope (CX33, Japan) at 400× magnification and analyzed using ImageJ software (NIH, USA). The H and E slide was applied regarding the wound tissue area, the Congo red for the number of eosinophils, and the IHC of IL-6 for the density of immunoreactivity of IL-6 were conducted.

Data analysis

The collected data was analyzed statistically using SPPS 26. The normality test was performed using the Shapiro-Wilk test. The normally distributed and homogenously data, especially hematological parameters were analyzed using

parametric analysis. The parametric analysis was the analysis of variance (ANOVA) followed by a post hoc test using the least significant difference (LSD). In contrast, the Congo red and IHC IL-6 data were analyzed using the Kruskal Wallis and Mann Whitney-U test. The significance value used in this study was 0.05 and the data were presented as mean and standard of deviation.

RESULTS AND DISCUSSION

The results indicated that the group implanted using PCL/PLA/HA had the smallest area of inflammation, compared to the other groups on day 3 ($p < 0.05$). This was followed by the groups using PDO and truglyde concomitantly. While the average number of eosinophils increased in the truglyde group, it decreased in the group using PDO and PCL/PLA/HA implants, compared to the truglyde group ($p < 0.05$). These findings were consistent with the immunoreactivity of IL-6 on the skin after suture implantation, which increased after implantation. The highest IL-6 expression was determined in the group truglyde. However, the minimum immune expression of IL-6 was PCL/PLA/HA ($p < 0.05$, Table 1).

There were no significant differences in several hematological parameters after suture implantation, including total erythrocytes, hemoglobin level, eosinophils, basophils, and monocytes ($p > 0.05$). The findings revealed that the total leucocytes and platelets increased in the truglyde and PDO groups, compared to others ($p < 0.05$). The total neutrophil count increased following the implantation of suture materials in all groups ($p > 0.05$). The neutrophils significantly increased in group truglyde, PCL/PLA/HA, and PDO, with no significant difference among the groups ($p > 0.05$). In contrast, the total lymphocytes indicated a trend of decreasing after suture implantation using truglyde, PCL/PLA/HA, and PDO ($p < 0.05$, Table 2).

Table 1. Inflammation area, number of eosinophils, and immunoreactivity of IL-6 in the rat skin after suture implantation

Parameter	Group (mean ± standard deviation)				
	G1 (Control)	G2 (Sham)	G3 (Truglyde)	G4 (PCL/PLA/HA)	G5 (PDO)
Wound tissue area	0.00 ± 0.00	0.00 ± 0.00	14.32 ± 4.24a	6.14 ± 4.00b	14.32 ± 4.24a
Number of eosinophils	0.30 ± 0.65	0.73 ± 0.86	18.00 ± 14.95a	4.20 ± 4.59b	11.10 ± 6.53c
Immunoreactivity of IL-6	0.25 ± 0.59	0.33 ± 0.72	9.25 ± 5.11a	0.74 ± 1.16b	1.94 ± 2.29c

[abc] different superscript indicated significant differences ($p < 0.05$). A control group without induction (G1), a sham-induced using cannula without suture implant (G2), suture implanted groups (G3-G5), while third group was implanted using truglyde (G3), fourth group was implanted using PCL/PLA/HA (G4), and fifth group was implanted using polydioxanone PDO (G5).

Table 2. Haematological profile of rat models after suture implantation

Parameter	Group (mean ± standard deviation)				
	G1 (Control)	G2 (Sham)	G3 (Truglyde)	G4 (PCL/PLA/HA)	G5 (PDO)
Erythrocytes ($\times 10^6$ mm^3)	7.19 ± 0.64	7.84 ± 0.38	8.05 ± 0.39	7.83 ± 0.70	7.60 ± 0.69
Haemoglobin (g/dL)	12.98 ± 0.81	13.48 ± 0.58	13.68 ± 0.35	13.50 ± 0.84	12.93 ± 0.78
Leucocytes ($\times 10^3$ mm^3)	14.93 ± 3.47	14.67 ± 3.39	17.94 ± 2.14a	14.00 ± 3.18	17.75 ± 2.90a
Platelets ($\times 10^3$ mm^3)	790.16 ± 115.84	832.33 ± 122.65	942.20 ± 144.21a	726.00 ± 117.90	930.00 ± 170.27a
Neutrophils ($\times 10^3$ mm^3)	0.24 ± 0.10	0.29 ± 0.67	0.72 ± 0.52a	0.61 ± 0.50a	0.68 ± 0.66a
Eosinophils ($\times 10^3$ mm^3)	0.01 ± 0.01	0.03 ± 0.01	0.02 ± 0.01	0.02 ± 0.03	0.04 ± 0.04
Basophils ($\times 10^3$ mm^3)	0.04 ± 0.01	0.02 ± 0.00	0.02 ± 0.00	0.02 ± 0.01	0.02 ± 0.00
Lymphocytes ($\times 10^3$ mm^3)	16.33 ± 3.03	16.38 ± 2.76	14.65 ± 2.64b	10.02 ± 3.50a	13.45 ± 2.32b
Monocytes ($\times 10^3$ mm^3)	1.61 ± 0.45	1.17 ± 1.16	1.71 ± 1.11	1.81 ± 1.30	1.61 ± 0.91

[abc]different superscript indicated significant differences ($p < 0.05$). A control group without induction (G1), a sham-induced using cannula without suture implant (G2), suture implanted groups (G3-G5), while third group was implanted using truglyde (G3), fourth group was implanted using PCL/PLA/HA (G4), and fifth group was implanted using polydioxanone PDO (G5).

The macroscopical signs, and microscopical findings include of histopathology, eosinophils, and immunoreactivity of the skin after suture implantation were shown in Figure 1 in a comprehensive detail.

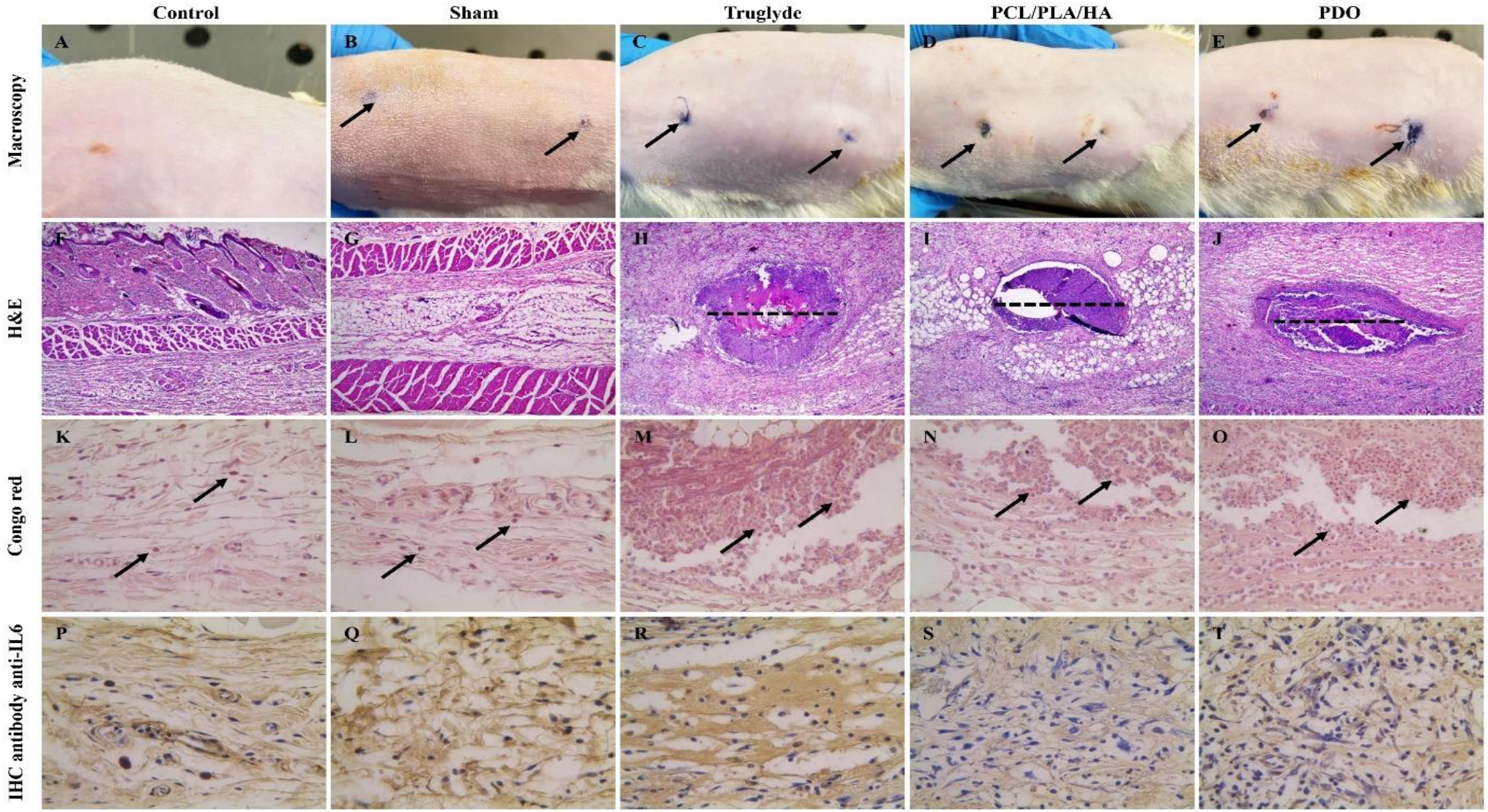

Figure 1. Macroscopy, histopathology, eosinophils, and immunoreactivity in the skin of rat models after suture implantation. There are no macroscopical (A) and microscopical (F) lesions of the skin in a normal rat, the skin also shows a mild expression of eosinophils (arrow; K) and IL-6 (P) in the dermal part; sham group showed the similar pattern to normal group (arrow; B, G, L, Q); however, the truglyde group indicated severe swollen (arrow; C) during the macroscopical examination, large wound area (dotted line; H), severe number of eosinophils (arrow; M), and strong immunoreactivity of IL-6 (brown color) in dermal part (R); the PCL/PLA/HA group showed a swollen (D) in one side of suture implant with a smaller wound area (I), minimum eosinophil numbers (arrow; N), and mild immunoreactivity of IL-6 (browm color; S); PDO group indicated a similar macroscopic lesion (arrow; E) to the PCL/PLA/HA group, but with wider wound area (dotted line; J), moderate eosinophil numbers (arrow; O), and moderate immunoreactivity of IL-6 (brown color; T). H and E, 40× (F-J); Congo red, 400× (K-O); IHC antibody anti-IL-6, 400× (P-T).

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The collagen deposition is expected to increase skin tension and strength. The success of collagen deposition depends on the suture types, site of implantation, and immune status of the patient. However, the suture types become the most eminent factor in matrix deposition (Kim et al., 2019). For several decades, PDO has been the most thread used for suture implantation in aesthetics. The utilization of PDO causes minimum histopathological effects and can be used to correct the nose and cheek shape, nose position, and skin tensile strength (Unal et al., 2021). However, a previous study reported that the utilization of PDO generates barbed sutures, secondary infection, thread palpability, abscess, and chronic pain (Surowiak, 2022). According to Wu (2019), PDO has a limited duration within the skin tissue and may eventually dissolve, resulting in the loss of its potential benefits as a dermal filler and collagen deposition promoter. However, the current study indicated that PDO implantation induced significant skin tissue lesions and triggered an escalation in inflammatory responses within 3 days. The histopathological changes were especially severe infiltration of eosinophils and increased density of immunoreactivity of IL-6.

However, the area of inflammation, eosinophil infiltration, and immunoreactivity of IL-6 in a group using PCL/PLA/HA is lower than in the PDO group. These mechanisms are influenced by the several compounds inside the suture material. Besides PDO, the most common type of suture ingredient consists of PLA/HA. PLA is an inert component that can be used to stimulate the synthesis of collagen (DeStefano et al., 2020). As a biodegradable microparticle, the PLA promotes collagenesis within the dermal part through its potency to activate fibroblast activity (Nethi et al., 2019). Moreover, the potency of PLA as a dermal filler and matrix collagen promoter can be increased with the combination using HA. Hyaluronic acid consists of glycosaminoglycan that can increase skin hydration and collagen expression, especially type 1 collagen (Bartus et al., 2013). The previous study by Zhao et al. (2024) described PLA which is combined with HA potential for dermal filler, especially preventing oedema, swelling, and redness on the skin tissue. Chen et al. (2023) described in their study that a combination of HA and PLA promotes wound healing without hypertrophic scar generation.

In addition, PCL combined with PLA/HA is a new model of suture implant combination. Polycaprolactone is a biopolymer which is essential to increase skin tensile strength and can decrease thermal transition effects post-implantation (Pitjamit et al., 2020). Polycaprolactone is also applicable for hard tissue surgery, such as bone injuries (Dehghani Firoozabadi et al., 2022). The combination of PCL with PLA/HA has been shown to prolong the biodegradation of suture material post-implantation and minimize brittleness (Moura et al., 2019). The decrease in brittleness and biodegradation improves collagen density and also decreases inflammatory responses. This mechanism is marked in the current study by the minimum expression of IL-6 and eosinophils. The minimum of eosinophils in PCL/PLA/HA group indicates that the hypersensitivity and rejection response was lower than the other materials. Furthermore, IL-6 is a pro-inflammatory cytokine, and its expression changes following external insults (Uciechowski and Dempke, 2020). However, the minimum immune expression of IL-6 after suture implantation is essential to increase tissue regeneration and matrix collagen rearrangement (Johnson et al., 2020).

The suture implantation causes a systemic immune response which is marked by a change of leucocyte, neutrophil, and lymphocyte after three days. This is a typical cellular immune response in the body of the host. The leucocytes increase as the first lineage of cellular response (Selvi et al., 2016), additionally, the neutrophil and lymphocytes increase in responding to inflammatory stimulation (Cai et al., 2021) and tissue repair (Prakoso and Kurniasih, 2018), concomitantly.

CONCLUSION

This study revealed that the implantation of suture materials using PCL/PLA/HA has better clinical outcomes regarding wound tissue area, number of eosinophils, and IL-6 during an acute phase of post-implantation. This finding was indicated by the decrease of the wound area, eosinophils infiltration, and immunoreactivity of IL-6, rather than the hematological profile. Therefore, advanced studies regarding the utilization of PCL/PLA/HA against a longer observation period using more complex parameters such as COX-2, CD4+, CD8+, VEGF, and FGF should be conducted to prove its safety and potency as a dermal filler.

DECLARATIONS

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Authors' contributions

Muhammad Hafid Ernanda designed, performed an experiment, data analysis, and drafted of manuscript. Ndaru Anri Damayanti designed monitored, evaluated the data analysis, and revised the draft of the manuscript. Wening Sari performed data analysis and corrected the paper. The last edition of the manuscript was read and approved by all authors.

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Availability of data and materials
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The authors have no conflict of interests.

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Dry Matter Intake, Digestibility, and Growth Performance of Peulh Breed Lambs Fed Millet Silage Treated with NaCl

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ABSTRACT

Livestock feeding is a major challenge in Niger. The aim of this study conducted at the International Crops Research Institute for the Semi-Arid Tropics (ICRISAT) experimental station in Sadoré, Niger, was to assess the effects of adding 1% NaCl to millet stover silage on the dry matter intake, digestibility, and weight performance of Peulh-bred lambs. Four treatments were tested, consisting of millet stover silages of two cultivars (Siaka Millet and Local Sadoré) with or without adding NaCl. The biological material included 32 lambs of Peulh breed Niger aged around 15 months with an average weight of 28.64 kg. They were divided into four blocks of homogeneous average weight and for each block, there were eight lambs. Each treatment was randomly assigned to a block. The trial lasted 75 days, including 15 days of adaptation and 60 days of data collection. Weight evaluation of animals was recorded, and bromatological analyses were carried out. Results indicated that there were significant differences between silages, according to NaCl addition and treatment, for some parameters of chemical composition, feed value, and zootechnical parameters of lambs. Depending on the treatment, moderately high significant differences were recorded for ash, organic matter, and crude fiber while low significant differences were recorded for digestibility coefficient and organic matter digestibility. Regarding NaCl addition, highly elevated significant differences were recorded for ash and organic matter. These differences were moderately significant for crude fiber and organic matter digestibility. Low significant differences were recorded for dry matter, nitrogen-free extract, digestibility coefficient, feed value, total weight gain, and average daily gain. It is concluded that the addition of 1% NaCl negatively affects the weight development of lambs although it improves the quality of silage parameters such as dry matter, ash, and digestibility coefficient.

Keywords: Millet residue, Salt, Sheep, Silage, Ruminant feed, Zootechnical performance.

INTRODUCTION

Livestock plays a key role in the economies of Sahelian countries and the food security of rural households (Cissé, 2015). In Niger, livestock is the second most important economic activity after agriculture and thus plays a key role in the country's economy. Livestock is practiced by 87% of the population and has contributed to 11-16% of the national Gross Domestic Product (GDP) and 25-40% of the agricultural Gross Domestic Product over the past decade (Rhissa, 2010; INS-Niger, 2019; MAG/EL-Niger, 2020). In the past, livestock was mainly concentrated in the north, in pastoral areas. However, it is now becoming increasingly important in agricultural areas in the southern parts of the country (Sourabie et al., 1995; Rhissa, 2010). The livestock population, including cattle, sheep, goats, horses, and donkeys was estimated at 59,809,696 heads in 2023. Small ruminants account for around 60.73% of this total, with 15,139,186 heads of sheep (MEL-Niger, 2022). Despite the numerical importance of small ruminants, the food shortage remains a major constraint limiting the development of their breeding in Niger. It is noteworthy that animal feed is essentially based on natural pastures. These are becoming increasingly scarce with the spread of crops, the disappearance of fallow land, and human pressure. This situation was further exacerbated by insufficient rainfall, leading to the disappearance of several palatable forage species (Alhassane et al., 2017). All these factors contribute to a decline in available fodder, which is estimated at 21,441,980 tonnes of dry matter in 2022, compared to the estimated needs of 33,873,786 tonnes of dry matter, giving a fodder deficit of around 36.70%, due to the decrease of pastures and the increase of need for food (MEL-Niger, 2022). Given these numerous constraints, which considerably reduce the productivity of livestock in Niger, other alternatives need to be found to feed animals properly (Dan Gomma et al., 2017). Millet is used as a dual-purpose crop (Moussa et al., 2017). Millet stovers are well appreciated by ruminants. Millet fodder could therefore be used as a basal diet for livestock such as sheep to boost their zootechnical performance (Umutoni et al., 2021). However, it has been

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observed that the main constraint of traditional storage of crop residues was the gradual loss of their nutrients, their level of ingestibility, and their digestion potential (Kanwé et al., 1997). To optimize the utilization of these residues, it is important to explore the technical possibilities for preserving or increasing their nutritional value, digestibility, and ingestibility. According to Rêgo et al. (2010), preserving fodder in the form of silage helps to preserve its initial quality. This initial quality could be improved by adding various additives. According to Masui et al. (1979) adding NaCl to forage during ensiling improved the nutritional value of the product.

The aim of this study was to assess the effects of adding NaCl to stover silages of two millet cultivars (Siaka Millet and Local Sadoré) on the zootechnical performance of lambs. Specifically, the study aimed to determine the chemical composition and feed value of the silages according to the treatments and the addition of NaCl. Additionally, it sought to compare parameters such as dry matter intake, apparent digestive utilization coefficient, feed conversion ratio, body weight, average daily gain, and total weight gain of the lambs. Furthermore, changes in average daily gain, dry matter intake, and refused dry matter over time were to be assessed in relation to the treatments and NaCl addition.

MATERIALS AND METHODS

Ethical approval

This study was conducted in accordance with the ethical rules relating to animal welfare. The experimental procedure was validated at the Faculty of Agronomy of the Abdou Moumouni University in Niamey, Niger and has received the approval of the Ministry of Agriculture and Livestock through the General Direction of Veterinary Services (DGSV) under authorization number MAG/EL/DGSV-002.

Experimental site

The study was carried out in Niger, at the Sadoré experimental station of the International Crops Research Institute for the Semi-Arid Tropics (ICRISAT Sahelian Centre) situated between 13° 14' N and 2° 16' E (Korombé et al., 2023a). The Sadoré station is characterized by a rainy season that starts late in June and ends in October. The dry season runs from November to May, with a hot dry period between March and May. Average annual rainfall is 562 mm and temperatures generally vary between 12°C and 44°C, with an average of 29.4°C (Umutoni et al., 2021).

Animals and trial duration

The trial was conducted from 25 December 2020 to 09 March 2021. It lasted 75 days, including 15 days of adaptation to the environment, conduct, treatments, and 60 days of data collection according to the previous study of Dan Gomma et al. (2017). The animals were bought at local livestock markets (Torodi and Balleyara). On arrival, they were identified (numbered ear tags), weighed (Pi) and their ages estimated. The trial was conducted with 32 young male Peulh-bred sheep with an average age of 15.56±1.03 months and an average initial weight of 28.64±1.66 kg (Table 1). The diet included wheat bran as a supplementary feed for all the animals at a rate of 300 g/day per lamb. This quantity was selected based on the findings of Chermiti (1999), who indicated that increasing the amount of wheat bran beyond this threshold could lead to a decrease in the voluntary intake of fodder by sheep. Therefore, in order to avoid any negative impact on the consumption of silages, this specific quantity was chosen. All the animals received the same quantities and types of wheat bran. The only thing that varied in the diet was the type of silage, which was distributed *ad libitum*, as the aim was to assess the ingestibility of these silages. The animals had free access to supplements of mineral salts.

Table 1. Weight before the adaptation period and age of Peulh breed lamb according to treatment and NaCl addition at the start of the 75-day trial

Factors	Modalities	Pi (kg)	Age (Month)
Treatment	T1	28.60 ± 1.44	16.25 ± 1.26
	T2	28.63 ± 1.35	15.00 ± 0.00
	T3	28.73 ± 2.38	16.00 ± 1.41
	T4	28.60 ± 2.05	15.00 ± 0.00
		1.00	0.179
NaCl	With NaCl	28.61 ± 1.61	15.00 ± 0.00
	Without NaCl	28.66 ± 1.82	16.12 ± 1.25
	Mean	28.64 ± 1.66	15.56 ± 1.03
	P-value	0.954	0.023

Pi: Initial weight before the adaptation period, T1: Siaka millet silage without NaCl, T2: Siaka millet silage with NaCl, T3: Local Sadoré silage without NaCl, and T4: Local Sadoré silage with NaCl.

Treatments and experimental design

The treatments consisted of silage from the stovers of two millet varieties, namely an improved variety (Siaka Millet) and a local variety (Local Sadoré). These varieties were chosen based on their favorable performance in initial trials, indicating their suitability for silage (Korombé et al., 2023a). Furthermore, the widespread geographical distribution of the local variety Sadoré in the study area and the high level of interest among farmers in the Siaka millet variety during participatory evaluations of millet varieties in the Torodi area were additional factors influencing their selection (ICRISAT, 2018). Thus, four treatments consisting of the combination of the 2 millet varieties and 2 levels of salt addition were tested. The treatments included T1 (Siaka millet silage without NaCl), T2 (siaka millet silage with NaCl), T3 (local Sadoré silage without NaCl), and T4 (local Sadoré silage with NaCl). In addition to this basic diet, each lamb received 300 g/day of wheat bran by supplementation and this quantity could not be increased. According to Chermiti (1999), the quantities of fodder voluntarily ingested by sheep decreased beyond this quantity of concentrated feed in the ration.

To allocate the animals to each treatment, four blocks of eight lambs (eight replicates) of homogenous weight ($p > 0.05$) were formed. Each block received one treatment at random (Table 1). It should be noted that the animals were kept in individual boxes measuring 1.5 m × 1m (1.5 m^2), throughout the trial.

Silage making

The stovers of the two millet cultivars (Siaka Millet and Local Sadoré) were produced at the Sadoré experimental station. They were cut at maturity after removing the ears and chopped into small pieces of 2 to 5 cm using a chopper (Morales et al., 2015; Trevisoli et al., 2017). Then, 30 kg of chopped millet stovers, according to the cultivar, were placed in plastic bags. This operation was carried out in compacted layers using a manual compactor with or without adding NaCl at a rate of 10 kg per tonne of stover depending on the type of treatment (Tamboura et al., 2005). The first bag of properly compacted chopped residues was placed into a second one to promote an anaerobic environment (Korombé et al., 2023a; 2023b). The bags were then hermetically sealed with string and tape and kept anaerobically at room temperature for at least 60 days before the start of the trial in a closed, well-ventilated building (de Pinho Costa et al., 2012; Lyimo, 2017). It should be noted that for each treatment, the quantities required to conduct the trial were produced.

Animals' management

On arrival, all animals were vaccinated against the three main diseases of small ruminants in the study area, namely pasteurellosis, small ruminant plague (SRP), and sheep and goat pox. The animals were also dewormed with Albendazole 300 (Manufacturing company was Lobs International Health, 121 Aguesseau Street, 92100 Boulogne-Billancourt, France) at a dosage of 7.5 mg per kg of body weight with a repeat dose one week later and ivermectin (Manufacturing company was Boehringer Ingelheim, Lyon and Reims, France) at a dose of 0.02 ml per kg of body weight). Albendazole 300 is administered by oral route and ivermectin by subcutaneous injection. In addition, the animals received multivitamins (Introvit-B-Complex manufactured in the Netherlands, contains vitamins B1, B2, B6, B12, and Nicotinamide, D panthenol, Ascorbic acid, Biotin, Choline chloride) at a dosage of 5ml/sheep. Regarding the vaccination, vaccine for pasteurellosis was Pastovac (Central Livestock Laboratory, Niamey, Niger). Vaccine for sheep and goat pox was Dermovac (Central Livestock Laboratory Niamey, Niger). The vaccine for the small ruminant plague was Perivac (Central Livestock Laboratory, Niamey, Niger).

The silage was distributed in two daily meals at fixed times, in the morning at 9 am and in the afternoon at 4 pm. The lamb also received 300g of wheat bran per day at 1.30 pm. Drinking water and lickstones (Yellow Rockies contains Sodium, Magnesium, Cobalt, Manganese, Selenium, Zinc, Iodine, and Iron) were available *ad libitum* to all animals throughout the trial.

Animals' weight and age determination

Animals were weighed before and after the adaptation period and every 10 days thereafter until the end of the trial. The age of animals was determined by examining their teeth (Landais and Bassewitz, 1982).

Feed offered and refused

After the adaptation period, the quantities of feed offered and refused were weighed daily throughout the trial. The silages were distributed in such a way as to constitute at least 10% refusals because, according to Cinq-Mars (2008), refusals can easily reach 10 to 15% with good forage. Samples of 100 g of the different silages were taken at each distribution and placed in an oven at a temperature of 65°C for 72 hours to determine the Dry Matter (DM) content (Trevisoli et al., 2017). The rejects were put in cotton bags and air-dried for 5 or 7 days to determine the DM content. All samples were crushed through a 1 mm sieve and kept in well-sealed plastic bags for later analysis.

Faeces collection

Two periods of feces collection of 5 continuous days were considered during the trial. The first collection was carried out 25 days after the start of the trial, including a three-day period of adaptation to fecal bags, and the second 25 days after the end of the first still including three days of adaptation to the fecal bags. A period of adaptation to the fecal bags of 3 days was observed before the start of each collection. Collections were made in the morning and evening. The fresh feces collected were weighed per animal and a mixed sample of 100 g was taken and put in a cotton bag then air-dried for 5 to 7 days to determine the DM content (Umutoni et al., 2021). Samples were also crushed through a 1 mm sieve and kept in well-sealed plastic bags for later analysis at the animal production Laboratory in the Faculty of Agronomy at Abdou Moumouni University in Niamey, Niger.

Bromatological analyses

Bromatological analyses were performed at the animal production laboratory of the Faculty of Agronomy at the Abdou Moumouni University in Niamey, Niger using AOAC (1990) procedures. The analysis was used to measure the levels of DM, Nitrogen (N), crude protein (CP), crude fiber (CF), ash, ether extract (EE), and nitrogen free extract (NFE).

Parameter determination

The variables were calculated according to the mathematical formulas developed by Rivière (1991). They were as follows, quantity of dry matter ingested (QDMI), quantity of dry matter refused (QDMR), apparent digestive utilization coefficient (ADUC), average daily gain (ADG), feed conversion ratio (FCR), fodder value (FV), digestibility coefficient (DC), digestible organic matter (DOM), digestible nitrogen matter (DNM). The formulas were as follows:

QDMI = QDMD – QDMR (Formula 1)

Where, QMSD is the quantity of dry matter distributed and QMSR denotes the quantity of dry matter refused.

$$\text{ADUC}(\%) = \frac{[I-F]}{I} \text{ X } 100 \quad \text{(Formula 2)}$$

Where, I determines the quantity of feed ingested and F signifies the quantity of feces.

$$\text{ADG } (g/d) = \frac{\text{Body weight 2} - \text{Body weigth 1}}{\text{Age 2} - \text{Age 1}} \quad \text{(Formula 3)}$$

Where, g/d is gram per day

$$\text{FCR } (\text{Kg of DM}/\text{Kg of body weight}) = \frac{\text{QDMI during a period}}{\text{Weight gain over the same period}} \quad \text{(Formula 4)}$$

FV (FU) = (DOM (% of DM)X K)/100

Where, FU defines the fodder unit, DOM is digestible organic matter, % of DM denotes the percentage of dry matter, K determines the coefficient calculated on the basis of DC, fat content in % of DM, and the table for calculating the fodder value of feed (Rivière, 1991). The digestibility coefficient was determined as a function of the crude cellulose content in % of DM and the table for calculating the fodder value of feed (Rivière, 1991).

DOM (%) = (OM (% of DM)X DC)/100 (Formula 5)

Where, OM is organic matter, DOM denotes digestible OM, DC signifies digestibility coefficient determined as a function of the crude cellulose content in % of DM, and the table for calculating the fodder value of feed (Rivière, 1991).

DNM (%) = (CP (%)X DC)/100 (Formula 6)

Where, CP is the crude protein and DC shows a digestible coefficient.

Data analysis

The SPSS 20.0 software (United States of America) was used to analyze the data, using the general linear model (GLM) method with the least significant difference (LSD) test to compare the means of the various parameters at the 5% significance level. The parameters studied were considered as dependent variables and treatment and NaCl addition were used as fixed factors for all analyses.

RESULTS

Silage chemical composition

The chemical composition of silages varied according to treatment and within each cultivar according to the addition of NaCl (Table 2). Thus, depending on the treatment, only Ash, OM, and CF varied significantly ($p < 0.05$). Treatments T2 and T4 were characterized by higher levels of Ash, while the opposite results were recorded for OM and CF. Analysis based on the NaCl addition indicated significant differences ($p < 0.05$) between the means of DM, Ash, OM, and NFE for Siaka's millet stover silages. Silages with NaCl were characterized by higher DM and Ash, while the opposite results were obtained with CF and NFE. For the Local Sadoré variety, the Ash, OM, CF, and NFE rates varied significantly ($p < 0.05$) according to the addition of NaCl. The addition of NaCl resulted in silages with higher levels of ash and lower

levels of OM, CF, and NFE. Overall, the addition of NaCl significantly ($p < 0.05$) increased DM and Ash levels, while OM, CF, and NFE levels decreased significantly ($p < 0.05$), compared to silages without NaCl (Table 2).

Table 2. Comparison of the chemical composition of silages offered to Peulh breed lambs according to treatment and the addition of NaCl in a trial lasting 75 days

Factors	Modalities	DM (%)	Ash (% DM)	OM (% DM)	CP (% DM)	CF (% DM)	EE (% DM)	NFE (%DM)
Treatment	T1	32.76±1.24^{bA}	7.87±1.24^{bB}	88.08±1.54^{aA}	4.18±0.73^{aA}	39.37±2.09^{aB}	2.16±1.01^{aA}	42.38±0.45^{aA}
	T2	35.67±1.08^{aA}	12.97±0.82^{aA}	82.13±0.48^{bB}	3.78±0.80^{aA}	38.60±1.04^{aB}	2.02±1.08^{aA}	37.73±2.59^{bA}
	T3	34.39±1.29^{aA}	7.22±1.83^{bB}	90.05±3.03^{aA}	3.72±0.65^{aA}	42.71±0.53^{aA}	1.52±0.42^{aA}	42.09±3.48^{aA}
	T4	35.25±1.73^{aA}	12.39±0.51^{aA}	82.36±2.04^{bB}	4.07±0.60^{aA}	37.62±0.20^{bB}	1.17±0.49^{aA}	39.49±1.92^{bA}
	p-value	0.116	0.001	0.002	0.825	0.004	0.460	0.126
NaCl	without NaCl	33.57±1.44^{b}	7.54±1.44^{b}	89.07±2.40^{a}	3.95±0.67^{a}	41.04±2.28^{a}	1.84±0.77^{a}	42.23±2.23^{a}
	With NaCl	35.46±1.31^{a}	12.68±0.69^{a}	82.24±1.33^{b}	3.92±0.65^{a}	38.11±0.86^{b}	1.60±0.88^{a}	38.61±2.26^{b}
	p-value	0.043	0.000	0.000	0.946	0.003	0.616	0.030

In each column, depending on the treatment, the averages with at least the same superscript capital letter are not significantly different between them. In each column, depending on the cultivar, the means with the same lower-case superscript letter are not statistically different from each other. T1: Siaka millet silage without NaCl, T2: Siaka millet silage with NaCl, T3: Local Sadoré silage without NaCl and T4: Local Sadoré silage with NaCl, DM: Dry matter, OM: Organic matter, CP: Crude protein, CF: Crude Fiber, EE: Ether extract, NFE: Nitrogen free extract, SEM: Standard error of mean, % DM: Percentage of DM.

Table 3. Comparison of the nutritional value of silages offered to Peulh breed lambs according to treatments and salt addition in a trial lasting 75 days

Factors	Modalities	DC (% DM)	DNM (% DM)	DOM (% DM)	FV (FU)
Treatment	T1	48.23±0.90^{aAB}	2.02±0.39^{aA}	42.48±0.13^{aA}	0.33±0.01^{aA}
	T2	48.43±0.46^{aAB}	1.83±0.39^{aA}	39.78±0.37^{bB}	0.31±0.00^{bA}
	T3	47.17±0.12^{bB}	1.76±0.31^{aA}	42.48±1.45^{aA}	0.32±0.01^{aA}
	T4	48.83±0.23^{aA}	1.99±0.29^{aA}	40.22±1.17^{aAB}	0.31±0.01^{aA}
	p-value	0.023	0.764	0.013	0.126
NaCl	without NaCl	47.70±0.82^{b}	1.89±0.34^{a}	42.48±0.92^{a}	0.33±0.01^{a}
	With NaCl	48.63±0.39^{a}	1.91±0.32^{a}	40.00±0.81^{b}	0.31±0.01^{b}
	p-value	0.015	0.921	0.002	0.038

In each column, depending on the treatment, the averages with at least the same superscript capital letter are not significantly different ($p > 0.05$). In each column, depending on the cultivar, the means with the same lower-case superscript letter are not significantly different from each other ($p > 0.05$). T1: Siaka millet silage without NaCl, T2: Siaka millet silage with NaCl, T3: Local Sadoré silage without NaCl and T4: Local Sadoré silage with NaCl, DC: Digestibility coefficient, DNM: Digestible nitrogen matter, DOM: Digestible organic matter, FV: Fodder value, SEM: Standard error of mean, % DM: Percentage of DM, FU: Fodder unit.

Silage feed value

The feed value components studied varied between treatments and within the same cultivar as a function of NaCl addition (Table 3). Analysis by treatment indicated lower significant differences ($p < 0.05$) in the means of the DC and DOM. Treatment T4 had the highest DC, while treatments T1 and T3 gave the highest DOM values. For the Siaka's Millet variety, the DOM and FV averages decreased significantly with the addition of NaCl ($p < 0.05$). On the other hand, in the Local Sadoré variety, only DC increased significantly with the addition of NaCl, compared to silages without NaCl ($p < 0.05$). In general, the addition of NaCl to silage significantly reduced ($p < 0.05$) the values for DOM and VF, while significantly improving CD, compared to silages without NaCl ($p < 0.05$).

Zootechnical performance

The zootechnical performance of lambs was evaluated according to treatment and NaCl addition (Table 4). Analysis of Table 4 indicated that there were no significant differences between treatments for any of the parameters studied ($p > 0.05$). For silages of Siaka's Millet variety, only the final weight (Pf) decreased significantly ($p < 0.05$) with the addition of NaCl. As for the local Sadoré variety, no significant difference was noted depending on the addition of NaCl ($p > 0.05$). Overall, the addition of NaCl to silage resulted in a significant decrease ($p < 0.05$) in total weight gain (TWG) and average daily gain (ADG), compared to silages without NaCl.

Evolution over time of average daily gain

The variations in average daily gain (ADG) over time, as a function of treatment (Figure 1a) and NaCl addition (Figure 1b), indicated a sawtooth evolution. For the treatments, only the ADG means for the first decade varied

significantly ($p < 0.05$), T1 gave the best ADG. Within each treatment, the evolution of the ADG as a function of trial duration indicated significant variations ($p < 0.05$) only in treatments T1 and T3. In both cases, the best ADG were recorded in order during the first, fifth, and sixth decades. On the other hand, the lowest ADG was recorded in the second and fourth decades for T1 and T3 respectively. Analysis according to the addition of NaCl revealed significant variations ($p < 0.05$) in ADG during the first and sixth decades. The silages without NaCl recorded the best ADG. For the silages without NaCl, a significant variation ($p < 0.05$) between the ADG means was also noted according to the duration of the trial. Thus, the ADG obtained during the first decade was higher than those of the other periods (Table 5).

Evolution over time of the total quantity of ingested dry matter

Analysis of the total quantity of dry matter intake (TQDMI) over time, as a function of treatment (Figure 2a) and NaCl addition (Figure 2b), indicated an overall upward trend. However, there was a slight fall in the quantities ingested in the fourth and sixth decades for all treatments (Figure 2a) and for silages with and without NaCl (Figure 2b). It should be noted that there was no significant difference among the groups for treatments and for NaCl addition. Similarly, on the one hand, for each treatment (Figure 2a) there were no significant differences between the averages according to the duration of the trial and, on the other hand, in silages with or without NaCl ($p > 0.05$; Figure 2b) the differences were also non-significant according to the duration of the trial ($p > 0.05$; Table 6).

Evolution over time of refused dry matter rates

The rates of dry matter refused (RDMR) as a function of treatment (Figure 3a) and NaCl addition (Figure 3b) indicated a general downward trend. However, a slight increase in RDMR was recorded in the fourth decade for treatments T1, T3, and T4 (Figure 3a) and as a function of NaCl addition (Figure 3b). There was no significant difference ($p > 0.05$) between the groups for the treatments or for the addition of NaCl. Also, within each treatment (Figure 3a) and within silages with and without NaCl (Figure 3b), there were no significant differences between the means according to the duration of the trial ($p > 0.05$; Table 7).

Table 4. Comparison of zootechnical parameters of Peulh breed lambs fed NaCl-treated silages according to the treatments and NaCl addition in a trial lasting 75 days

Factors	Modalities	$Pi_{2\ (kg)}$	Pf (kg)	TWG (kg)	ADG (g/d)	TQDMI (g/d)	FCR	ADUC (%)
Treatments	T1	28.04±1.41^{aA}	31.60±0.58^{aA}	3.56±1.83^{aA}	59.38±35.82^{aA}	682.28±33.30^{aA}	11.49±14.64^{aA}	88.45±0.87^{aA}
	T2	27.49±1.73^{aA}	28.59±1.89^{bA}	1.10±1.38^{aA}	18.33±27.04^{aA}	601.88±128.84^{aA}	32.83±14.45^{aA}	88.22±1.22^{aA}
	T3	27.90±2.16^{aA}	30.13±2.06^{aA}	2.23±0.73^{aA}	37.08±14.24^{aA}	675.64±68.13^{aA}	18.22±13.87^{aA}	89.47±1.57^{aA}
	T4	29.30±2.63^{aA}	30.43±2.63^{aA}	1.13±1.27^{aA}	18.75±24.99^{aA}	664.29±29.38^{aA}	35.43±10.85^{aA}	89.26±0.39^{aA}
	p-value	0.577	0.120	0.302	0.100	0.112	0.345	0.112
NaCl	Without NaCl	27.97±1.37^{a}	30.86±1.21^{a}	2.89±1.71^{a}	48.23±38.05^{a}	678.96±30.05^{a}	14.08±14.51^{a}	88.96±1.27^{a}
	With NaCl	28.39±2.43^{a}	29.51±2.44^{a}	1.11±1.28^{b}	18.54±26.16^{b}	633.09±99.08^{a}	34.14±14.76^{a}	88.74±1.07^{a}
	p-value	0.669	0.178	0.022	0.022	0.266	0.319	0.695

In each column, depending on the treatment, the averages with at least the same superscript capital letter are not significantly different ($p > 0.05$). In each column, depending on the cultivar, the means with the same lower-case superscript letter are not statistically different from each other ($p > 0.05$). T1: Siaka millet silage without NaCl, T2: Siaka millet silage with NaCl, T3: Local Sadoré silage without NaCl, and T4: Local Sadoré silage with NaCl, Pi_2: Initial weight after the adaptation period, Pf: Final weight; TWG: Total weight gain, ADG: Average daily gain, TQDMI: Total quantity of dry matter ingested, FCR: Feed conversion ratio, ADUC: Apparent digestive utilization coefficient; SEM: Standard error mean.

Table 5. Comparison of average daily gain of Peulh breed lambs fed NaCl-treated silages as a function of treatment and addition of NaCl according to the duration of the trial

Factors	Modalities	10	20	30	40	50	60	p-value
Treatments	T1	194.50±136.62^{aA}	14.25±12.61^{bA}	46.43±34.26^{abA}	19.20±39.54^{bA}	125.89±93.92^{abA}	69.85±35.82^{abA}	0.018
	T2	30.63±55.58^{aB}	15.62±53.83^{aA}	39.29±37.12^{aA}	11.61±60.57^{aA}	57.14±57.74^{aA}	21.57±27.04^{aA}	0.803
	T3	153.13±50.06^{aAB}	7.62±10.12^{bA}	-1.07±4979^{bA}	21.43±26.88^{bA}	62.50±41.80^{bA}	43.63±14.24^{bA}	0.000
	T4	62.25±38.86^{aB}	63.75±58.37^{aA}	0.36±81.65^{aA}	-1.79±61.20^{aA}	-1.79±95.19^{aA}	22.06±24.99^{aA}	0.475
	p-value	0.045	0.235	0.486	0.907	0.184	0.077	
NaCl	Without salt	173.81±97.80^{aA}	10.94±11.18^{bA}	22.68±47.01^{bA}	20.31±31.32^{bA}	94.20±75.35^{abA}	56.74±28.87^{bA}	0.000
	With salt	46.44±47.48^{aB}	39.69±58.03^{aA}	19.82±62.30^{aA}	4.91±56.82^{aA}	27.68±79.40^{aA}	21.81±24.10^{aB}	0.744
	p-value	0.008	0.182	0.918	0.543	0.105	0.022	

In the rows, within each treatment according to the trial period, the means which have at least one lowercase letter in common as a superscript are not significantly different at the 5% threshold. In the columns, for each trial period, the means with at least one capital letter in common are not significantly different at the 5% threshold. T1: Siaka millet silage without NaCl, T2: Siaka millet silage with NaCl, T3: Local Sadoré silage without NaCl and T4: Local Sadoré silage with NaCl.

Table 6. Comparison of total quantity of dry matter ingested averages of Peulh breed lambs fed NaCl-treated silages as a function of treatment and addition of NaCl according to the duration of the trial

Factors \ Number of days	Modalities	10	20	30	40	50	60	p-value
Treatments	T1	659.65±23.61^{aA}	676.5±38.02^{aA}	698.95±51.31^{aA}	654.65±27.76^{aA}	709.13±34.90^{aA}	694.8±49.32^{aA}	0.305
	T2	556.44±138.53^{aA}	575.11±109.49^{aA}	606.84±137.10^{aA}	620.58±135.17^{aA}	627.59±133.50^{aA}	624.74±187.03^{aA}	0.97
	T3	622.15±71.14^{aA}	668.65±77.07^{aA}	685.5±77.60^{aA}	680.9±61.62^{aA}	694.00±58.71^{aA}	702.68±46.87^{aA}	0.601
	T4	643.05±49.69^{aA}	669.15±22.42^{aA}	674.30±36.47^{aA}	660.55±32.29^{aA}	671.35±19.97^{aA}	667.33±17.74^{aA}	0.763
	p-value	0.351	0.187	0.455	0.742	0.478	0.692	
NaCl	Without salt	640.90±53.01^{aA}	672.58±56.42^{aA}	692.23±61.33^{aA}	667.78±46.42^{aA}	701.56±45.44^{aA}	698.74±44.74^{aA}	0.252
	With salt	599.75±106.90^{aA}	622.13±88.77^{aA}	640.57±99.63^{aA}	640.57±93.45^{aA}	649.47±91.41^{aA}	646.04±125.08^{aA}	0.942
	p-value	0.339	0.178	0.247	0.495	0.194	0.312	

In the rows, within each treatment according to the trial period, the means which have at least one lowercase letter in common as a superscript are not significantly different at the 5% threshold. In the columns, for each trial period, the means with at least one capital letter in common are not significantly different at the 5% threshold. T1: Siaka millet silage without NaCl, T2: Siaka millet silage with NaCl, T3: Local Sadoré silage without NaCl, and T4: Local Sadoré silage with NaCl.

Table 7. Comparison of refused dry matter rates fed NaCl-treated silages as a function of treatments and addition of NaCl in Peulh breed lambs according to trial duration

Factors \ Number of days	Modalities	10	20	30	40	50	60	p-value
Treatments	T1	25.54±4.60^{aA}	22.26±7.41^{aA}	17.88±10.00^{aA}	26.52±5.41^{aA}	16.80±5.94^{aA}	22.74±4.61^{aA}	0.265
	T2	49.67±25.02^{aA}	46.30±19.78^{aA}	40.57±24.76^{aA}	38.09±24.41^{aA}	36.82±24.11^{aA}	37.34±33.77^{aA}	0.970
	T3	36.00±13.22^{aA}	27.36±14.32^{aA}	24.22±14.42^{aA}	25.08±11.45^{aA}	22.64±10.91^{aA}	21.03±8.71^{aA}	0.601
	T4	26.76±9.96^{aA}	21.52±4.49^{aA}	20.49±7.31^{aA}	23.25±6.47^{aA}	21.08±4.00^{aA}	21.89±3.56^{aA}	0.763
	p-value	0.146	0.062	0.219	0.469	0.24	0.533	
NaCl	Without salt	30.77±10.73^{aA}	24.81±10.90^{aA}	21.05±11.98^{aA}	25.80±8.33^{aA}	19.72±8.71^{aA}	21.89±6.52^{aA}	0.176
	With salt	38.21±21.47^{aA}	33.91±18.75^{aA}	30.53±20.02^{aA}	30.67±18.34^{aA}	28.95±18.08^{aA}	29.61±23.72^{aA}	0.924
	p-value	0.346	0.185	0.248	0.504	0.203	0.399	

In the rows, within each treatment according to the trial period, the means which have at least one lowercase letter in common as a superscript are not significantly different at the 5% threshold. In the columns, for each trial period, the means with at least one capital letter in common are not significantly different at the 5% threshold. T1: Siaka millet silage without NaCl; T2: Siaka millet silage with NaCl, T3: Local Sadoré silage without NaCl and T4: Local Sadoré silage with NaCl.

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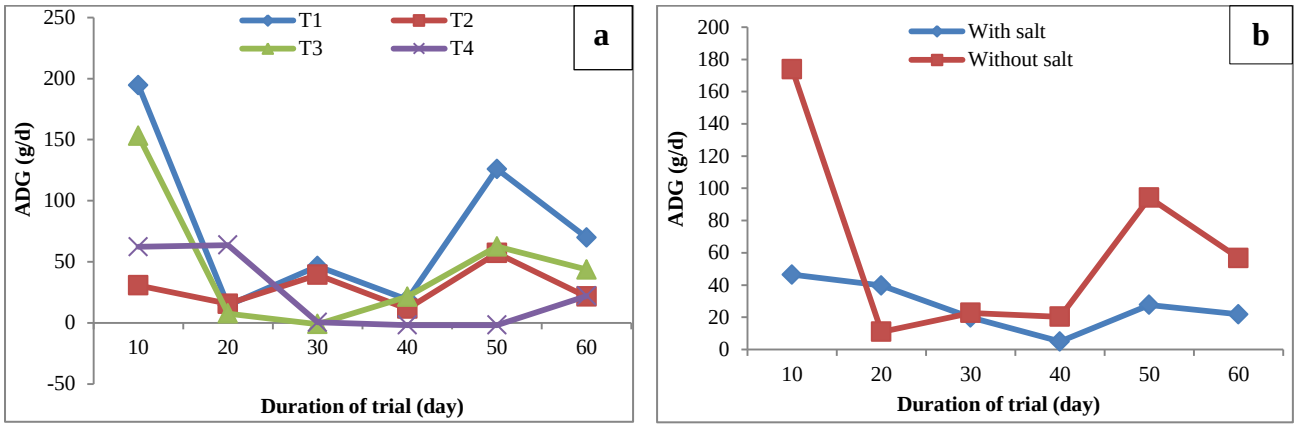

Figure 1. Evolution of average daily gain over time fed NaCl-treated silages, as a function of treatments (**a**) and NaCl addition (**b**) in Peulh breed lambs in a trial lasting 75 days

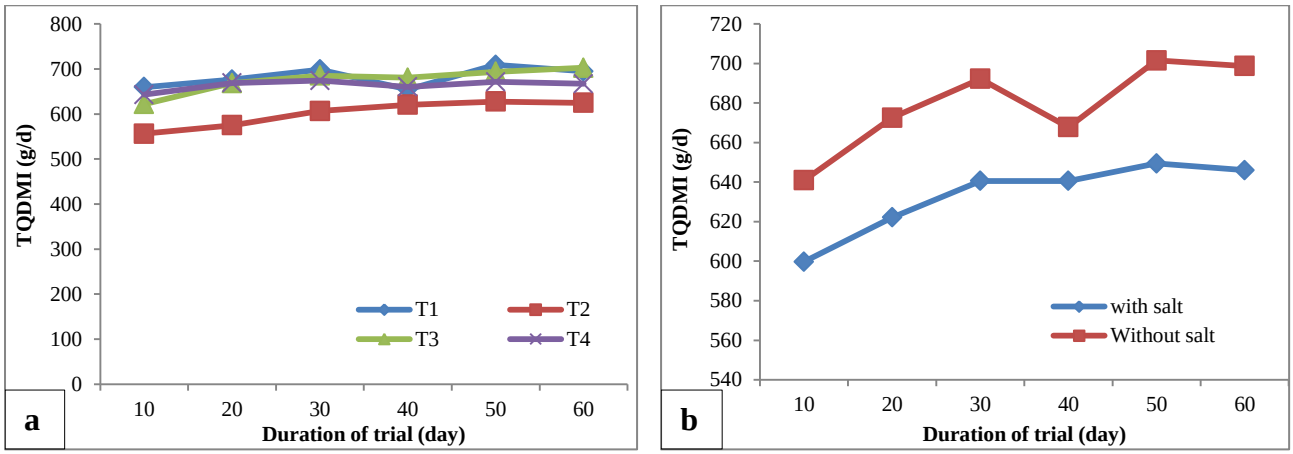

Figure 2. Evolution over time of total quantity of dry matter ingested fed NaCl-treated silages as a function of treatments (**a**) and NaCl addition (**b**) in Peulh breed lambs in a trial lasting 75 days. QMSTI: Total quantity of dry matter ingested

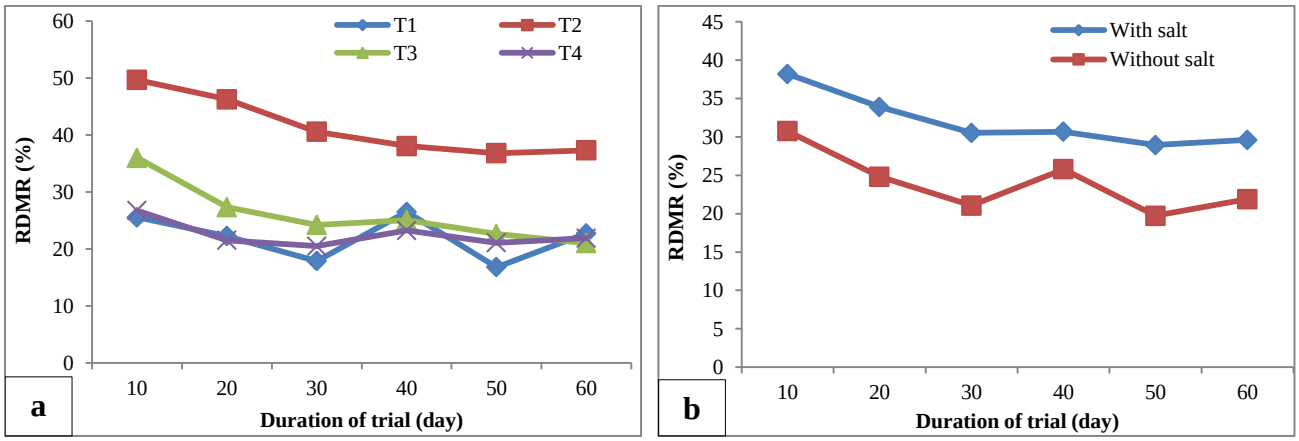

Figure 3. Evolution over time of refused dry matter rates fed NaCl-treated silages as a function of treatments (**a**) and NaCl addition (**b**) in Peulh breed lambs in a trial lasting 75 days

DISCUSSION

The chemical composition of silages

The addition of NaCl increased the DM and ash contents and decreased the OM, CF, and NFE contents. Silage additives can play several roles in the silage preservation process. Thus, a classification is made according to their properties, such as stimulants of fermentation, inhibitors of fermentation, inhibitors of aerobic deterioration, nutritional value improvers, and absorbents (Muck et al., 2018). NaCl can then be considered as a fermentation stimulant by limiting the spoilage of silage through the drop in pH (McLaughlin et al., 2002; Korombé et al., 2023a) and as a nutritive value improver and absorbent, in that it provides the minerals Na (39%) and Cl (61%) and absorbs the moisture from the

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stovers before silage by increasing the DM rate (Johansson, 2008; Korombé et al., 2023a). Ergin and Gumus (2020) also indicated that the ash content increased after the addition of NaCl to alfalfa silage, compared with the controls without NaCl.

The decrease in OM, CF, and NFE with the increase in DM and ash contents can be explained through the various relationships that exist between these parameters. According to Mahaman (2018), the DM percentage is calculated as the OM percentage to which the ash percentage has been added. It is also well known that CF and NFE were components of OM. From these relationships, it is clear that an increase in Ash content can lead to an increase in DM rate, a decrease in OM rate, and, indirectly, a decrease in CF and NFE contents. This decrease in CF content after the addition of NaCl was recorded by Ergin and Gumus (2020) on alfalfa silage. This can be explained by the fact that NaCl contributes to the solubilization of cellulose and the breaking of chemical bonds between cellulose and hemicellulose or lignin. So, when the chemical bonds are broken, the polymers are converted into monomers (Jiang et al., 2015). This is well confirmed by the results reported by Ergin and Gumus (2020), who noted high levels of hemicellulose in silages with NaCl.

In this study, the addition of NaCl had no effect on the CP content. This result supports that of Ergin and Gumus (2020) who recorded no significant difference for this parameter between alfalfa silage with NaCl and that without NaCl.

The nutritional value of silages

NaCl addition increased overall the value of the digestibility coefficient (DC). This situation was probably linked to the reduction in CF in silages with NaCl. According to Rivière's table (1991), when the CF value decreased, the DC value increased. The same type of relationship between CF content and DC was reported by Andrieu and Baumont (2000) and Peyrat et al. (2014) when studying maize silages. These results further confirm the role of NaCl in cellulose solubilization reported by Jiang et al. (2015).

The addition of NaCl globally decreased the OMD value. This was due to the significant decrease in OM content in silages with NaCl. Indeed, this decrease could not be compensated by the positive effect of NaCl on DC, since OMD was a function of these two parameters. Fodder value also decreased with the addition of NaCl to the silage. This parameter was closely linked to DOM, DC, and EE. The latter was higher in silages without NaCl. Digestible Nitrogen Matter (DNM) was not changed with the addition of NaCl. As this parameter was obtained from the CP and DC, this result can be explained by the fact that, on the one hand, a decrease in CP content was observed with the addition of NaCl and, on the other hand, an increase in DC with the addition of NaCl. It was as though there was compensation on both sides, i.e. in the silages with NaCl and without NaCl.

Weight performances

The means of live weights obtained for the lambs at 16.1 months (after the adaptation period) and 18.1 months (at the end of the trial) were 28.6 kg and 30.19 kg respectively. These results were within the range of 22.53 to 33.95 kg initial weight and 27.29 to 41.72 kg final weight reported by Tensaba et al. (2023) for lambs averaging 18 months old. The average initial weight of the animals in this study, however, is lower than that reported by Abdou et al. (2011), which is 27 kg for Oudah lambs aged 12 months on average.

In this study variations in ADG over time, as a function of treatments and NaCl addition, indicated a general trend with irregular patterns of the curves. This result was similar to that obtained by Simian (2017), who recorded irregular patterns of the curves for ADG evolution over time, with Djallonké sheep aged around 18 months and fed dual-purpose crop stovers. However, the shape of the curves differs according to the study.

According to Mahaman (2018), there was a positive correlation between the quantity of diet and the weight gains of the animals, so the evolution of the ADG of lambs in irregular trend could be explained through the quantities of diet which was a function of the state of health of the animals and the environmental conditions.

Furthermore, the results of this study indicated a variation in ADG from 18.33 to 59.38 g/d with an average of 33.39 g/d. These ADG values were lower than those obtained by (Abdou, 1997; Somda, 2001; Simian, 2017; Sana et al., 2020; Umutoni et al., 2021; Tensaba et al., 2023). Thus, on the one hand, it was observed that the minimum value of ADG (18.33 g/d) obtained in this study is higher than that reported by Amuda (2013), which was -19.05 g/d, but lower than that recorded by Ayano (2013) of the order of 30.56 g/d, and on the other hand that the opposite results were obtained for the maximum values of ADG.

These differences and similarities between the results of this study and those of others can be explained by differences between the diets used, the characteristics of the sheep (breed, age, initial weight), and the duration of the trials.

Overall, NaCl addition reduced the ADG of lambs. This result can be explained by the fact that NaCl seems to have a negative effect on the digestibility of organic matter (White et al., 2019; Korombé et al., 2023b). White et al. (2019) concluded that high NaCl diets affect rumen performance. However, the results of this study are opposite to those reported by Rabelo et al. (2013) who recorded higher ADG than the control with NaCl doses of 0.5%, 1%, and 2%.

Average Daily Gain varied significantly over time, but at a lower rate only in silages without NaCl (T1 and T3). The highest ADG values were obtained in the first decade of the trial, after which they evolved overall without significant differences (T3). These results can be explained by the duration of the data collection (2 months) because, according to Simian (2017), sheep showed their best weight performance during the first 2 months. This would suggest that this duration is relatively short to produce significant differences in weight performance.

Ingested dry matter

The total quantities of dry matter ingested (TQDMI) varied in this study from 601.88 to 682.28 g/d, with an average of 656.03 g/d. These results were higher than those reported by Amuda (2013) and Ayano (2013), who recorded TQDMI ranging respectively from 242.26 to 592.41 g/d and from 459.28 to 530.01 g/d in West African dwarf lambs averaging 12 to 14 months of age and diet based on maize silage and mixed cassava haulm and panicum maximum grass silage. However, the results of this study were lower than those reported by de Carvalho et al. (2017), who recorded TQDMI between 740 and 1080 g/d in 6-month-old lambs fed 50% tropical forage silages and 50% a concentrate based on maize and soybean meal.

These differences with results of this study were due to differences in the characteristics of the sheep (initial weight, age, breed), in the nature of the diet, and in the duration of the trials. Overall, the analysis of TQDMI over time showed an upward trend. This result was reported by Keles et al. (2018), who observed a linear increase in TQDMI over time in lambs with an average weight of 21.6 kg, fed buckwheat silage or maize silage. This can be explained by the fact that feed intake capacity was linked to the weight or more to the size of the animals during their growth (Jarrige, 1988).

In this study, TQDMI did not vary significantly with NaCl addition, although there was a general downward trend with the addition of NaCl. The results of this study were contrary to those of Rabelo et al. (2013) who recorded significantly higher DM intake levels than the control (387 g/d) with NaCl doses of 0.5% (667 g/d) and 2% (516 g/d). It should be noted that feed refusal rates moved in the opposite direction to TQDMI, with a minimum of 16.80% and a maximum of 49.67%. Overall, for all the treatments concerned, the refusal rate was higher than 10%, which was the minimum feed refusal threshold to be allowed in the trials, especially for good forages. This feed refusal rate can reach 30 or 40% for some forage categories (Bougouma-Yameogo, 1995; Zoungrana et al., 1999; Kirilov et al., 2006; Cinq-Mars, 2008).

Feed conversion ratio

The feed conversion ratio (FCR) was in this study from 11.49 to 35.43 kg of feed per kg of weight gain, with an average of 24.11 kg of feed per kg of weight gain. The results of this study differ from those reported by Amuda (2013) and Ayano (2013) who recorded FCR ranging from -5.32 to 18.72 kg feed per kg weight gain and 12.96 to 16.04 kg of feed per kg of weight gain in West African dwarf lambs of an average age of 12 to 14 months, fed maize silage and mixed silage of cassava tops and panicum maximum grass. Variations in FCR between periods may be due to the stage of growth and the type of tissue deposited (Sangaré et al., 2005). The FCR did not significantly with the addition of NaCl, although there was a trend towards an increase in the FCR for lambs fed silage with NaCl. This can be explained by the negative effect of NaCl on digestibility mentioned by White et al. (2019); Korombé et al. (2023b). Diets with a high NaCl content alter the efficiency of digestion. Feed conversion ratio will therefore not be efficient.

Apparent digestive utilization coefficient

The apparent digestive utilization coefficient (ADUC) values recorded in this study ranged from 88.22% to 89.47%. These results were higher than those reported by Wilkins et al. (1971), who estimated that the apparent digestibility of silage dry matter for sheep ranged from 55.3% to 80.0%. The results of this study were also higher than those obtained by Mahaman (2018) who varied from 65 to 75% in lambs fed cowpea hay pellets. These differences with the results of this study can be explained by differences in the characteristics of the animals (age, breed, and initial weight), the type of diet (physical characteristics, chemical composition, Nutritive value), the duration of the trials and the fecal collection methods (Khan et al., 2003). The addition of NaCl did not significantly influence ADUC in this study. However, there was a general downward trend with the addition of NaCl. This result was in accordance with that reported by White et al. (2019) who concluded, that diets with high NaCl content alter rumen function.

CONCLUSION

The effects of millet stover silages, depending on NaCl addition, on the dry matter intake, digestibility, and growth performance of Peulh breed lamb of Niger, revealed differences in the chemical composition and feed value of the silages. Thus, overall, silages with NaCl were characterized by a high digestibility coefficient and high DM and ash contents and by low values for DOM, FV, OM, CF, and NFE compared with silages without NaCl. The evaluation also

indicated that the treatments were equivalent in terms of zootechnical performance, but that the addition of NaCl reduced the total weight gain and ADG of the lambs. The results indicated that the addition of NaCl to millet stover silages, despite some differences in chemical composition and nutritional value such as dry matter, ash and digestibility coefficient, but did not positively change the zootechnical performance such as daily weight gains, feed conversion ratio, digestibility, feed intake of the lambs. NaCl seems to had a negative effect on average daily weight gain. However, further investigations are needed to assess the effects of different doses of NaCl in millet stover silages on the zootechnical performance of lambs or to extend the study to other ruminant species.

DECLARATIONS

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Ethical considerations

The authors declare that all ethical aspects of the publication of an original article have been considered in the preparation of this paper.

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Authors' contributions

All authors participated in protocol development, Hamza Seydou Korombé, Amadou Maman Manouga, Abdoussalam Ibrahima contributed to trial conduct and data collection. All authors participated in data analysis. Hamza Seydou Korombe has written the first draft of the manuscript. All authors have reviewed the draft of the manuscript. All authors checked and approved the final version of the manuscript.

Competing interests

The authors declare that no conflict of interest has been presented.

Availability of data and materials

The authors declare that they are willing to provide the data relating to this study if reasonably requested.

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Effects of Different Methods of Ovulation Induction on Sex Hormones in Serum, and Meat of Rabbit Does

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ABSTRACT

High indicators of reproductive function in rabbits can be achieved using hormonal inducers of ovulation, in particular analogs of gonadotropin-releasing hormone, serum, and chorionic gonadotropins. Therefore, the aim of this study was to evaluate the dynamics of sex hormones in the blood serum and meat of rabbit does during ovulation stimulation over 5 consecutive pregnancies. For this purpose, 60 *Hyla* rabbit does were randomly divided into five groups of 12, ensuring four animals per group with three replicates. Animals of the first and second experimental groups, animals received intramuscular injections of serum gonadotropin, 40 IU and 25 IU respectively, three days prior to artificial insemination. Females of the third and fourth groups were administered combined doses of serum and chorionic gonadotropins (40 IU and 24 IU, respectively) during the same period. Rabbits of the control group were stimulated to ovulate by subcutaneous injection of 0.2 ml analog of gonadotropin-releasing hormone after artificial insemination. Long-term administration of gonadotropins revealed dose-dependent effects. Hyperprogesteronemia was detected in rabbit does (40 IU), while hyperandrogenia was noted in females (24 IU) during the combined administration of gonadotropins. The use of serum gonadotropin at a dose of 25 IU contributed to an increase in the level of follicle-stimulating, luteinizing hormone and progesterone while decreasing 17β-estradiol. A high dose (40 IU) in rabbit does did not cause significant fluctuations of hormones in blood serum, but decreased luteinizing hormone and progesterone. Long-term use of a gonadotropin-releasing hormone analog was accompanied by a pronounced decrease in the level of luteinizing hormone, as well as 17β-estradiol. However, the meat of all experimental animals did not increase the content of steroid hormones (testosterone and 17β-estradiol). It can be concluded that ovulation stimulation in rabbit does using a serum gonadotropin dose of 25 IU and the recommended dose of a gonadotropin-releasing hormone analog does not negatively impact the hormonal balance.

Keywords: Analogue of gonadotropin-releasing hormone, Artificial insemination, Equine chorionic gonadotropin, Human chorionic gonadotropin, Rabbit does

INTRODUCTION

Modern rabbit breeding is an industry that not only provides humanity with a useful product and valuable fur but is also widely used in scientific research, particularly for testing and evaluating assisted reproductive technologies (Viudes-de-Castro et al., 2017; Casares-Crespo et al., 2018; Bakeer et al., 2022). One such technology is artificial insemination (AI), which has become widely used in the practice of rabbit farms as a highly effective reproductive technology (Vicente et al., 2012; Gardela et al., 2020). The effectiveness of AI depends on both the physiological state of the female and the quality of males' ejaculate, especially its redox status (Casares-Crespo et al., 2016; Sanchez-Rodriguez et al., 2020; Koshevoy et al., 2021). The use of AI in rabbits is physiologically justified since the rabbit needs induced ovulation after insemination. In addition, the effectiveness of hormonal treatment in rabbit does has been demonstrated to increase induction of ovulation and stimulation of estrus by administration of gonadotropin-releasing hormone (GnRH), its analogues, serum or chorionic gonadotropins (Arias-Alvarez et al., 2013; Viudes-de-Castro et al., 2019). Ovulation should be induced with the hormone drugs exogenous administration, which can be performed intramuscularly, subcutaneously, or intravaginally. In addition, the effectiveness of hormone drugs is improved by the simultaneous use of organic phytoremedies (such as *Yucca schidigera*) and stimulants (Štochmal'ová et al., 2015; Elkomy et al., 2021; Viudes-de-Castro et al., 2023). The use of this group of drugs is limited by their negative effects (high levels of hormones in meat) on the health of rabbits and offspring, as well as the negative impact on humans when consuming products obtained from processed animals. It has become especially relevant given the "One Health" concept (Hughes

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and Watson, 2018; Miller and Leach, 2023). A high probability of developing side effects and morphological damage to the functional tissue of human ovaries are associated with incorrectly defined dosage and/or long-term use of gonadotropins (Herkert et al., 2022). The resulting negative consequences of gonadotropin use are related to the difference between recombinant forms of gonadotropins and endogenous hormones, which not only affects their pharmacodynamics but also modifies the cellular response (Banker & Garcia-Velasco, 2015; Casarini and Simoni, 2021). In rabbits does, there is evidence of the negative effect of different doses of intramuscular injections of lecirelin during insemination on the fertilization frequency, the total number of rabbits per litter, the number of stillbirths, and abortion rates (Zapletal and Pavlik, 2008). Although common, the effectiveness of AI with the introduction of GnRH analogs to the sperm dose, such as buserelin or [des-Gly10, D-Ala6]-LH-RH ethylamide, is limited due to the proteolytic activity of enzymes in sperm, which reduces the efficacy of added hormones and necessitates doses 15-25 times higher (Quintela et al., 2004; Gogol, 2016). High doses or long-term use of hormonal ovulation inducers cause negative side effects on the reproductive system of females and a decrease in offspring growth rates due to the lack of feeding when rabbits are weaned early (Karsch et al., 1997; Rebollar et al., 2008).

The balance of sex hormones, follicle-stimulating, luteinizing, estradiol, progesterone, and testosterone, in the body of rabbits, plays an important role in the implementation of sexual function and full fertilization, pregnancy, and childbirth. The follicle-stimulating hormone has a chemical nature, it is a complex glycoprotein, which stimulates the follicles' development until ovulation, enhances the synthesis of estrogens, and increases the sensitivity of the gonads to lutropin. This gonadotropic hormone is secreted by basophilic cells of adenohypophyses. The synthesis of follicle-stimulating hormone (FSH) is regulated by releasing hormones from the hypothalamic area of the brain (foliberin) as well as by the principle of feedback involving the content of androgens and estrogens in the blood (Laborde et al., 1981; Moore and Hasler, 2017). Rabbit does can not produce a sufficient amount of progesterone (P4) in the first reproductive cycle due to insufficient development of the corpus luteum. It is formed in the ovary from a ruptured tertiary (Graafian) follicle after ovulation and secretes P4 to support pregnancy. The corpus luteum will degenerate closer to the end of the estrous if pregnancy does not occur (Salem et al., 2020). Low concentrations of progesterone before artificial insemination change dynamically with the onset of pregnancy (Ubilla et al., 2001). It is known that human chorionic gonadotropin (hCG) can provoke additional synthesis of P4 after binding to LH receptors (Salem et al., 2020). According to Stevenson et al. (2007), hCG induces additional natural synthesis of P4 from accessory luteal cells after binding to LH receptors.

The use of hormonal stimulation and synchronization of oestrus increases fertility and reduces the amount of IA necessary for pregnancy. Correct hormonal stimulation provides a prolonged effect on ovulation during 3-4 reproductive cycles, after which repeated use of inducers is necessary (Rebollar et al., 2006). In addition, the fertility of rabbit does increases after the injection of equine chorionic gonadotropin (eCG) with hCG or gonadotropin-releasing hormone (GnRH), despite their different biological effects and specific actions (De Rensis and López-Gatius, 2014; Hassanein et al., 2021). The hormonal agents commonly used for ovulatory stimulation in rabbit does include buserelin at a dose of 0.2 ml per animal administered subcutaneously, and chorionic gonadotropin at doses ranging from 20-25 IU administered intravenously (Arias-Alvarez et al., 2010; El-Ratel et al., 2020).

Therefore, the current study aimed to investigate the dynamics of sex hormone levels in blood serum and meat of rabbit does following the administration of serum gonadotropins (at doses of 40 IU and 25 IU) and chorionic gonadotropins (at doses of 40 IU and 24 IU), effects of a gonadotropin-releasing hormone analog. This investigation sought to evaluate the effects of their long-term use.

MATERIALS AND METHODS

Ethical approval

Experiments on rabbit does with the use of hormonal drugs were reviewed and approved by the Bioethics Committee of the State Biotechnological University (ethical permit No. 4-05 dated May 5, 2019). Treatment of females and the necessary manipulations were carried out in accordance with the European Convention for the Protection of Vertebrate Animals Used for Experimental and Scientific Purposes (2006) and the General Ethical Principles of Animal Experiments adopted by the 1st National Congress on Bioethics (Kyiv, Ukraine, 2001).

Animals and experimental design

The experimental animals included 60 multiparous *Hyla* rabbit does, aged between 7 and 8 months, with weights ranging from 3.2 to 3.9 kg. During the research period, the experimental animals did not receive any medicines, vaccine prophylaxis, and antiparasitic treatments. To form five groups of animals, their total number was randomly divided into

groups of 12 animals each. Thus, four animals and three repetitions were used in each group, providing the necessary amount of data for statistical processing. The experimental design is shown in Figure 1. To determine the levels of hormones in rabbits, blood samples (1 ml in sodium citrate tube) were taken on day 7 of pregnancy, in the morning before feeding. This sampling was repeated consecutively over the course of five pregnancies (during the experiment, it was important to study the dynamics of changes during each pregnancy that occurred after the use of various ovulation inducers). Notably, animals of all experimental groups (1-4) received intramuscular injections of gonadotropins 3 days before AI. In contrast, rabbit does of the control group were subcutaneously injected with a GnRH analogue immediately after AI.

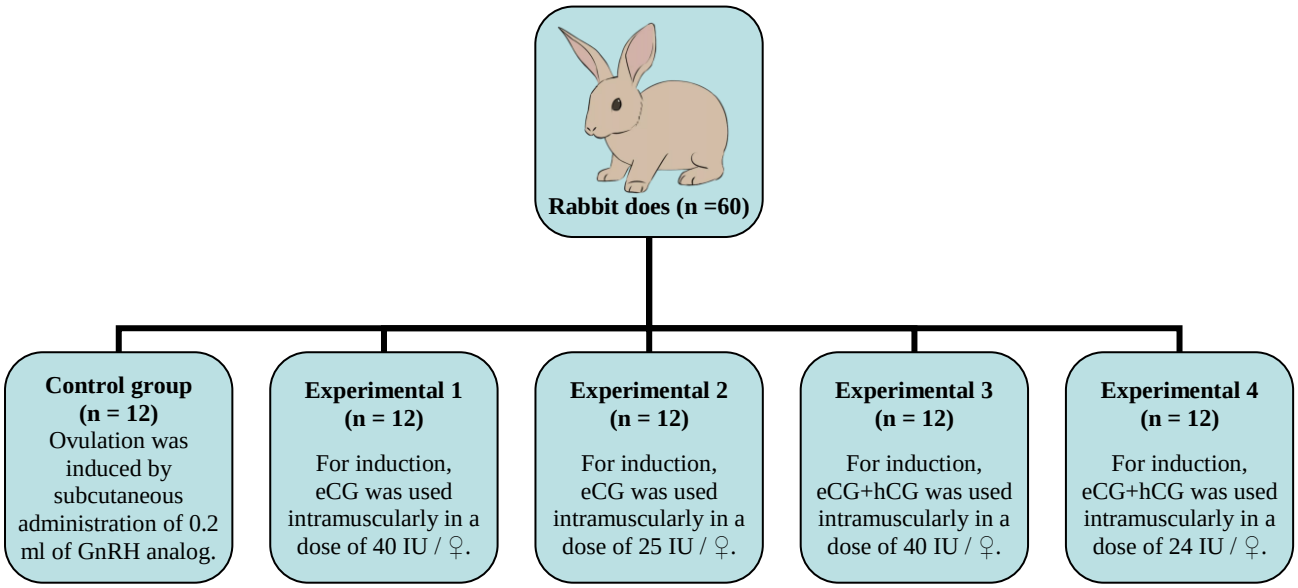


Figure 1. Groups of animals in the experiment and used dosages of ovulation inducers. GnRH: Gonadotropin-releasing hormone; eCG: Equine chorionic gonadotropin; hCG: Human chorionic gonadotropin.

Feeding and housing of rabbit does

All rabbit does were fed a ration containing ingredients and chemical composition as in Table 1. Each doe was fed *ad libitum* on this commercial diet throughout the experiment. Water was available through automatic drinkers attached to the galvanized wire cages. A complete feed diet (2750 kcal ME/kg, 18.5% CP, and 12.5% CF) was used as a commercial diet for feeding rabbits ad-libitum according to their physiological stage (El-Desoky et al., 2021).

The ration indicated in Table 1 included nitrogen-free extract 59.45 g/kg dry matter (DM), crude protein 17.54 g/kg DM, crude fiber 12.53 g/kg DM, ash 9.43 g/kg DM, and ether extract 2.05 g/kg DM. The humidity in the rooms where the rabbits were kept was 55.0 ± 5.0%, and the air temperature was 25.0 ± 1.0ºC. All animals were individually housed in galvanized wire batteries (70 × 50 × 40 cm) located in a naturally ventilated and lighted (12 hours of light: 12 hours of dark) room. About 5 days pre-kindling wooden nest boxes (containing straw or hay) with dimensions of 50 cm (length) ×30 cm (width) × 30 cm (height) attached to the dam's cages). The bedding was replaced daily by a new one to avoid any contamination from urine or fecal material.

Table 1. Composition of diet ingredients for rabbit does

Ingredient	Content
Alfalfa hay (g/kg)	280
Wheat bran (g/kg)	250
Barley (g/kg)	180
Soybean meal (g/kg)	180
Yellow corn (g/kg)	60
Molasses (g/kg)	30
Barley grain (g/kg)	10
Di-calcium phosphate (g/kg)	10
NaCl and premix* (g/kg)	10

* 1 kg of premix (minerals and vitamins mixture) contains Vitamin A (20,000 IU), Vitamin D3 (15,000 IU), Vitamin B1 (0.33), Vitamin B2 (1.0 g), Vitamin B6 (0.33 g), Vitamin B5 (8.33 g), Vitamin B12 (1.7 mg), pantothenic acid (3.33 g), biotin (33 mg), folic acid (0.83 g), choline chloride (200 g), Vitamin E (8.33 g) and Vitamin K (0.33 g).

Blood sample collection and sex hormone assay

No sedation or anesthesia medications were administered to the animals during blood sampling procedures. Blood for the study was collected on day 7 of the experiment from the lateral saphenous veins, following the generally accepted method. Blood samples were taken simultaneously in the morning into tubes with anticoagulant (sodium citrate; BD Vacutainer®, Russia). The levels of follicle-stimulating and luteinizing hormones, 17β-estradiol, progesterone, and testosterone were determined in the obtained blood serum samples of rabbits using standard sets of ELISA Kit reagents (LifeSpan BioSciences Inc., USA) with the Stat Fax 303 plus enzyme immunoassay (Awaraness Technology, USA).

Sex hormones in meat assay

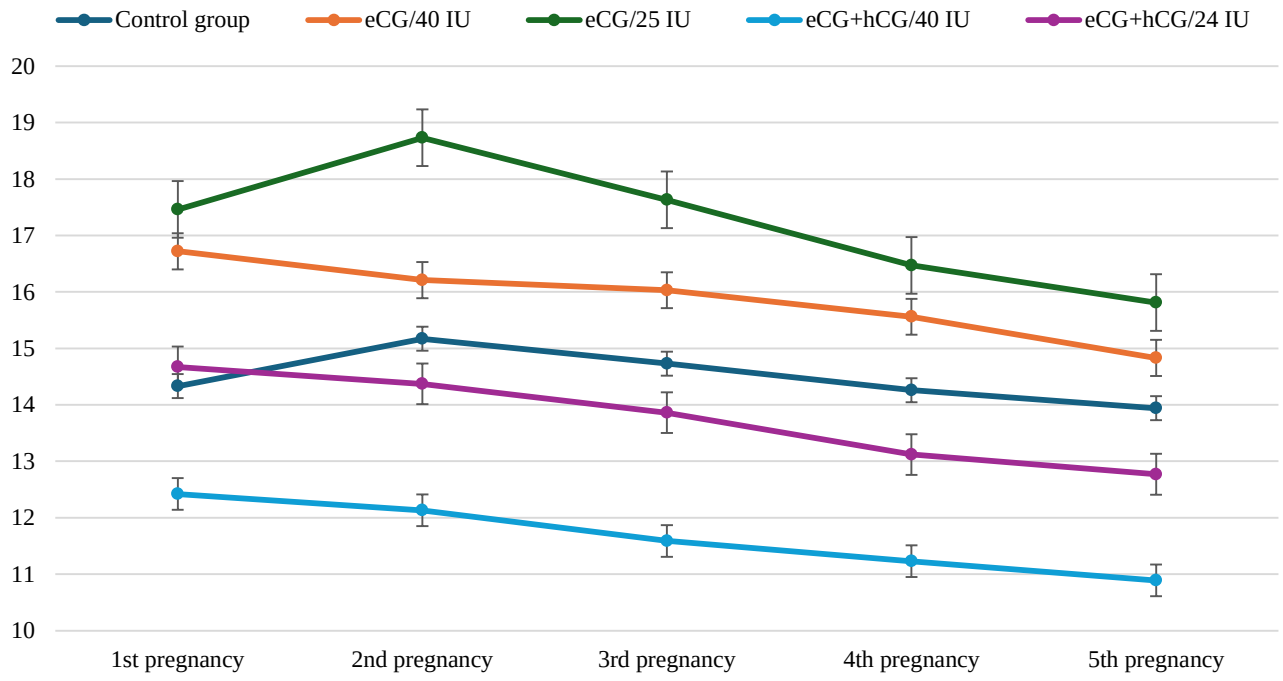
After euthanizing rabbits, 0.5 kg of muscle tissue from four animals in each group was collected and divided into three samples. These samples were then placed in polyethylene bags and stored at -18ºC. The surfaces of the muscle were cleaned from all fat and connective tissues. Testosterone and 17β-estradiol were determined using radioimmunoassay on the 411 analyzer (Germany). The analytic sensitivity of the testosterone assay was 0.005 mg/kg, and 17β-estradiol was 0.003 mg/kg (Rebaz et al., 2019).

Statistical analysis

All calculations were performed using Statistical Package for Social Science (SPSS), version 22 (SPSS Inc., USA). One-way analysis of variance (ANOVA) was performed to compare the data of control and experimental groups of rabbit does. The data in the tables were presented as mean ± standard deviation. The normality of the quantitative variables was tested with the Shapiro-Wilk test. Since all variables were not normally distributed, the Mann-Whitney test was used to compare quantitative variables. The significant differences between the treatments were confirmed by Tukey as a post-hoc test. P value less than 0.05 was considered statistically significant.

RESULTS AND DISCUSSION

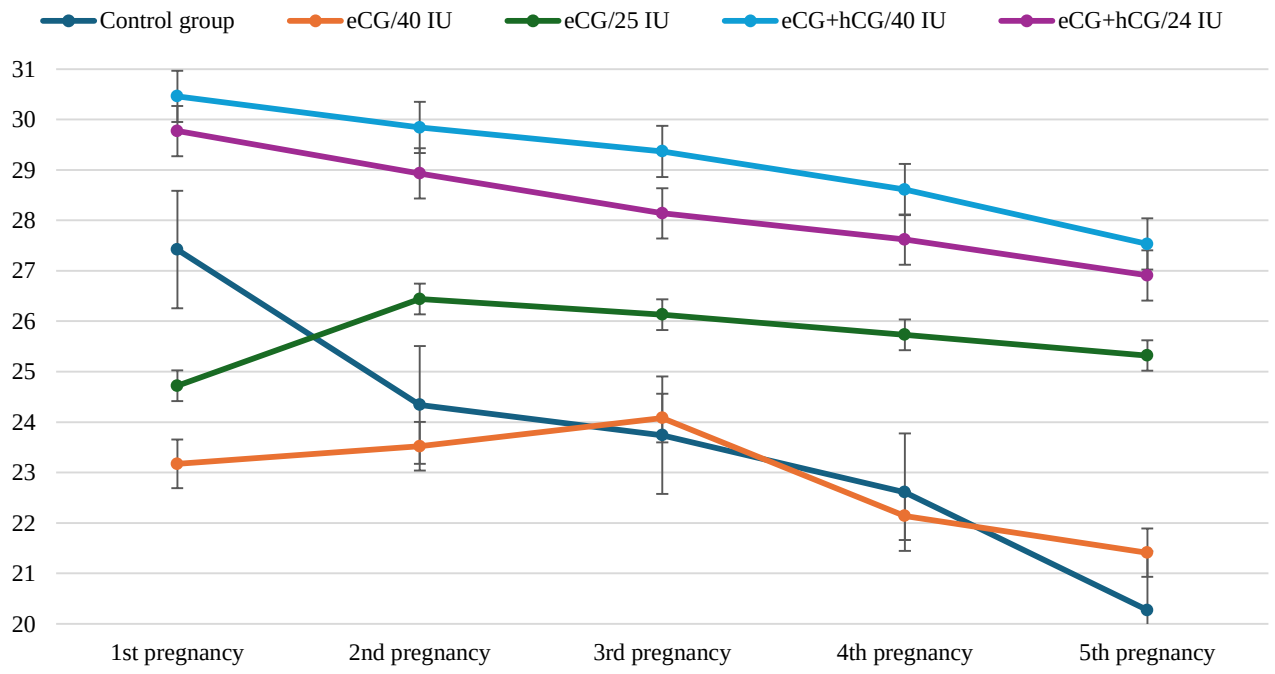
Changes in the dynamics of hormone levels of rabbits showed the specific effects of gonadotropins, compared to the GnRH analog ($p < 0.05$). The dynamics of FSH content in the blood serum of pregnant rabbit does during the experiment is shown in Graph 1. Thus, in the first pregnancy, the level of FSH was higher than the control indicators by 16.72 ± 0.38 IU/L and 17.46±0.42 IU/L in rabbits of experimental groups 1 and 2, respectively ($p < 0.05$). Subsequently, the level of FSH in animals of the first experimental group was characterized by slight upward fluctuations ($p < 0.05$). Positive dynamics of high activity of FSH in blood serum were observed in rabbits of experimental group 2.



Graph 1. Dynamics of follicle-stimulating hormone in blood serum of pregnant rabbit does (n=12, lU/L).

During the study, there was a decrease in FSH levels in animals of experimental group 3, compared to control rabbits. In particular, in females during the first pregnancy, FSH levels were lower than the control indicators by 12.42 ± 0.28 IU/L ($p < 0.05$). Similar changes were observed in experimental group 4. While there was a tendency to increase FSH levels during the first pregnancy, in subsequent pregnancies (second and third), there was a tendency for FSH levels to decrease. Additionally, during the fourth and fifth pregnancies, FSH levels were lower than the control data by 13.12 ± 0.27 IU/L and 12.77 ± 0.24 IU/L, respectively ($p<0.05$).

It should be noted that in rabbits of the control group, the level of FSH during the five pregnancies was accompanied by minor fluctuations, and at the end of the experiment, it had a tendency to decrease. The dynamics of the content of luteinizing hormone (LH) in the blood serum of rabbits during the experiment is summarized in Graph 2.

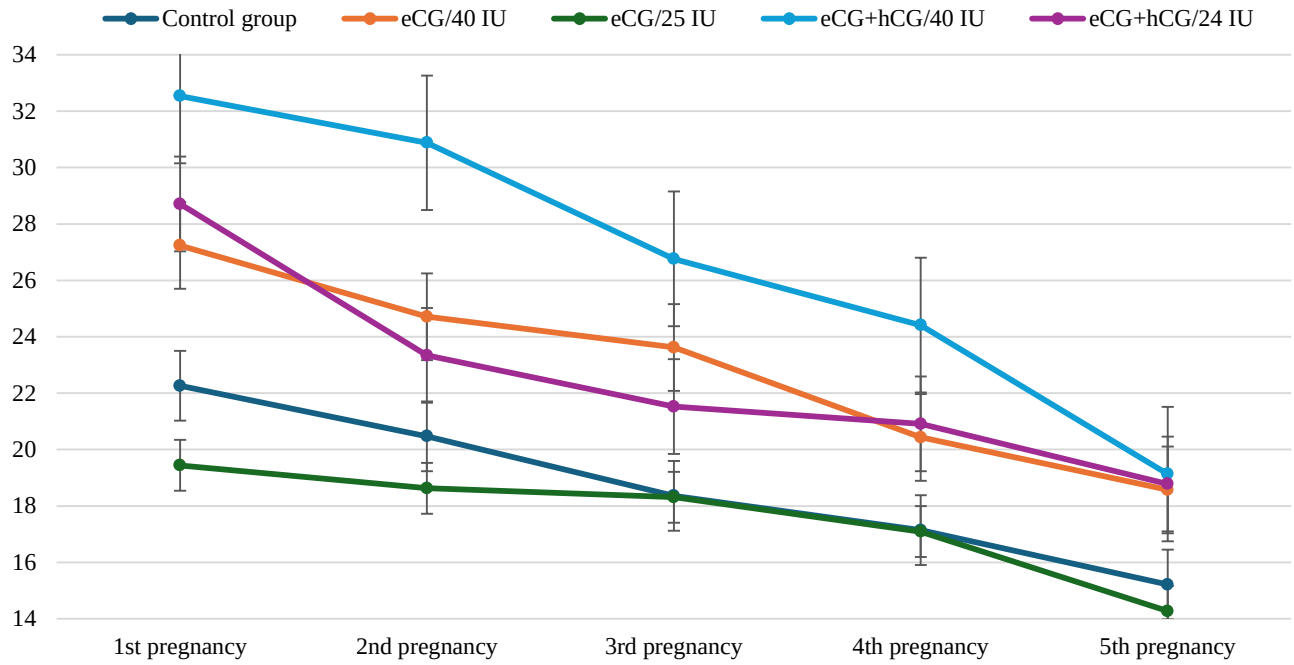


Graph 2. Dynamics of luteinizing hormone in blood serum of pregnant rabbit does (n=12, lU/L).

In females of experimental group 3, there was an increase in the LH level during the experiment ($p < 0.05$). The changes observed in rabbits of experimental group 4 were less pronounced in terms of LH level dynamics compared to the control data. In the first pregnancy, LH levels were higher in experimental group 4 rabbits by 29.77 ± 0.86 IU/L, and in the fifth pregnancy by 26.91 ± 0.73 IU/L ($p < 0.05$). For instance, in rabbits of experimental group 1 during the first pregnancy, LH levels were lower than those of the control group by 23.17 ± 0.71 IU/L ($p < 0.05$), with a tendency to decrease in the second and fourth pregnancies. However, an upward trend was observed in the third and fifth pregnancies. Similarly, in females of experimental group 2, LH levels were initially lower than the control data by 24.72 ± 0.74 IU/L ($p<0.05$) but showed an increasing trend in the second pregnancy. Subsequently, in the third, fourth, and fifth pregnancies, LH levels were significantly higher, by 26.13 ± 0.69 IU/L, 25.73 ± 0.72 IU/L, and 25.32 ± 0.70 IU/L, respectively ($p < 0.05$). Differences in LH levels between the first and fifth pregnancies were evident. Towards the end of the study, LH levels were likely lower than those of the first pregnancy by 20.27 ± 0.61 IU/L ($p<0.05$), as seen in Graph 2. A similar trend of LH level decrease, akin to FSH, was observed in rabbits of the control group throughout the entire study period.

The level of 17β-estradiol in the blood serum of rabbit does from different reproductive cycles exhibited dynamic changes during the experiment (Graph 3). The authors of the current study observed an increase in 17β-estradiol levels in the blood serum of rabbits in experimental groups 1, 3, and 4, whereas in animals of experimental group 2, the level of this hormone did not exceed the control data. In rabbits of experimental group 1, during the first pregnancy, the level of 17β-estradiol exceeded that of the control group by 27.24 ± 0.74 pmol/L, and in the fifth pregnancy by 18.57 ± 0.39 pmol/L, respectively ($p < 0.05$). Conversely, in animals of experimental group 2, the level of this hormone was lower during the first and second pregnancies by 19.44 ± 0.51 pmol/L and 18.63 ± 0.42 pmol/L, respectively ($p < 0.05$). In the

third and fourth pregnancies, it almost corresponded to the control data, while during the first pregnancy, it was reduced by 14.27 ± 0.24 pmol/L ($p < 0.05$).



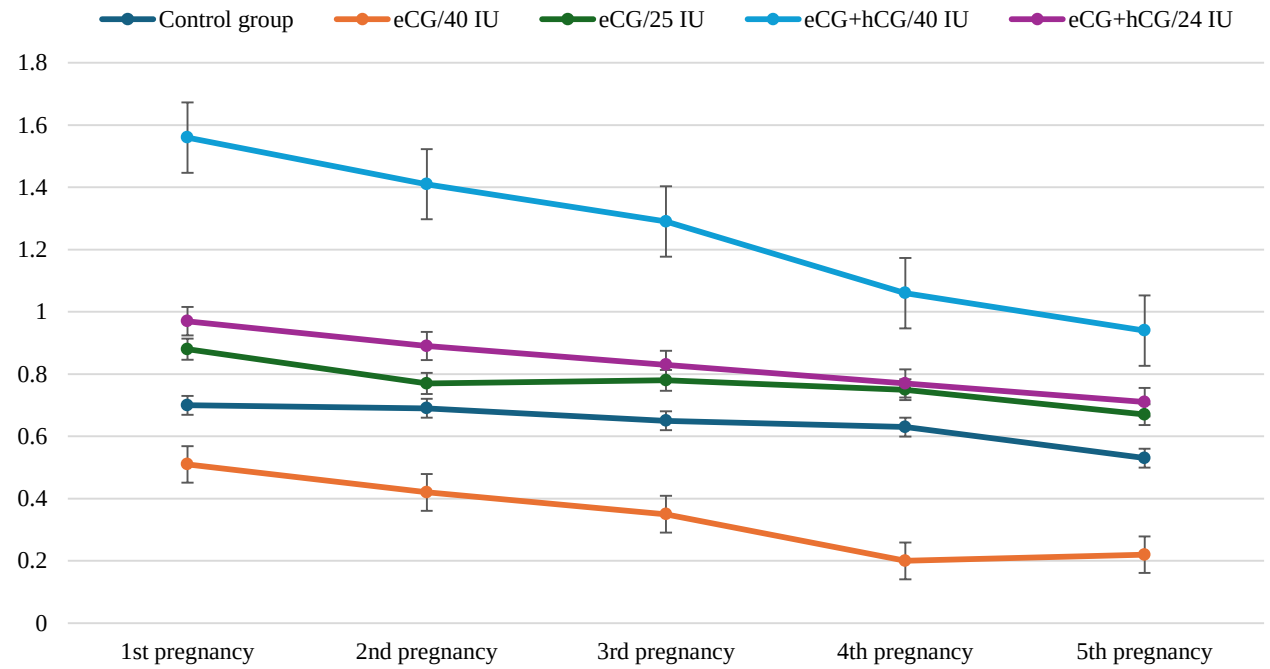
Graph 3. Dynamics of 17β-estradiol in pregnant rabbit does' blood serum (n=12, pmol/L).

The dynamics of changes in the level of 17β-estradiol (17β-E) in the blood serum of rabbit does after hormonal treatments revealed dose-dependent effects in experimental groups 3 and 4. In experimental group 3, the level of 17β-E increased by 32.54 ± 1.17 pmol/L in the first pregnancy compared to the control, while in the fifth pregnancy, it increased by only 19.13 ± 0.41 pmol/L ($p < 0.05$). Similarly, in experimental group 4, the level of 17β-E increased by 28.71 ± 0.77 pmol/L in the first pregnancy and by 18.78 ± 0.38 pmol/L in the fifth pregnancy ($p < 0.05$). Notably, significant changes in the level of 17β-E occurred in the control group, where the hormone level decreased steadily throughout the 1st-5th pregnancies. Specifically, in the fifth pregnancy, the level of 17β-E (22.26 ± 0.67 pmol/L) was significantly lower than that of the first pregnancy by 15.21 ± 0.27 pmol/L ($p < 0.05$). Generally, the values of the 17β-E level in the five pregnancies decreased constantly.

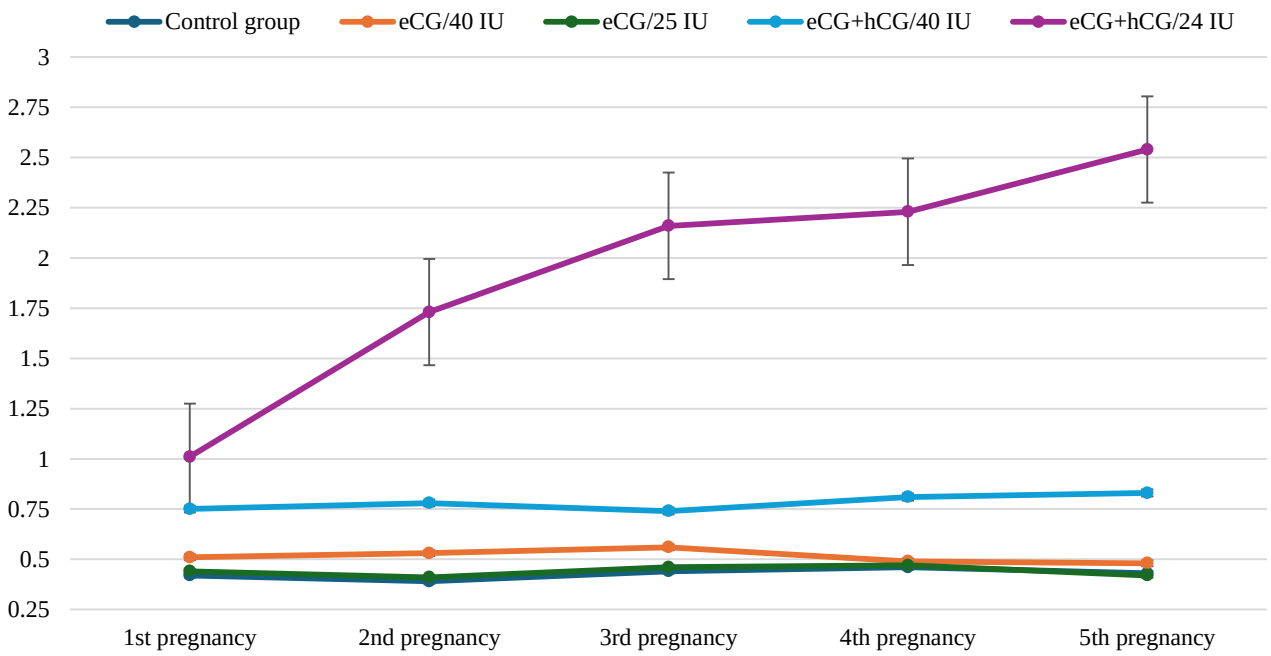
The dynamics of changes in the level of progesterone (P4) in the blood serum of rabbit does after hormonal treatments are illustrated in Graph 4. Females of experimental groups 2 and 4 exhibited a moderate effect on the level of P4 in blood serum, while rabbits of experimental group 3 showed a significant increase, and those of experimental group 1 showed a decrease in P4 levels compared to the control ($p < 0.05$). Specifically, in the first pregnancy, the level of progesterone was lower by 0.51 ± 0.02 nmol/L, in the second and third pregnancies by 0.42 ± 0.01 nmol/L and 0.35 ± 0.01 nmol/L, respectively, reaching its maximum decrease in the fourth and fifth pregnancies by 0.20 ± 0.01 nmol/L and 0.22 ± 0.01 nmol/L, respectively ($p < 0.05$). In females of the control group, only minor fluctuations in the level of P4 were detected during the experiment, but by the end of the study, this indicator was lower by 0.53 ± 0.01 nmol/L compared to the value of the first pregnancy ($p < 0.05$).

In the first pregnancy, the level of progesterone exceeded the control value by 0.88 ± 0.04 nmol/L in rabbits of experimental group 2 and by 0.97 ± 0.05 nmol/L in experimental group 4 ($p < 0.05$). Similarly, in the second cycle, there was a tendency for an increase in progesterone levels in animals of experimental group 2, while in females of experimental group 4, it was significantly higher by 0.89 ± 0.04 nmol/L ($p < 0.05$). Further, in experimental group 2, an increase of 0.78 ± 0.04 nmol/L in the third pregnancy and 0.67 ± 0.03 nmol/L in the fifth pregnancy was observed ($p < 0.05$). Meanwhile, the level of progesterone was higher by 0.83 ± 0.04 nmol/L in the animals of experimental group 4 in the third pregnancy, and by 0.77 ± 0.04 nmol/L and 0.71 ± 0.03 nmol/L in the fourth and fifth pregnancies, respectively ($p < 0.05$). In experimental group 3, the level of progesterone exceeded the control data by 1.56 ± 0.07 nmol/L in the first pregnancy, and by 1.41 ± 0.07 nmol/L in the second pregnancy ($p < 0.05$). Subsequently, the growth weakened somewhat, with the level of progesterone being higher by 1.29 ± 0.06 nmol/L in the third pregnancy, 1.06 ± 0.05 nmol/L in the fourth, and 0.94 ± 0.04 nmol/L in the fifth pregnancy ($p < 0.05$). Considering these changes, the dynamics of testosterone levels in the blood serum of rabbits during the experiment were determined, as shown in Graph 5. In

experimental group 1, an increase in testosterone level was observed in the first, second, and third pregnancies, by 0.51 ± 0.03 ng/dL, 0.53 ± 0.04 ng/dL, and 0.56 ± 0.04 ng/dL, respectively ($p < 0.05$). However, in the fourth and fifth pregnancies, animals of this group showed only a small tendency to increase the studied indicator. Similar slight fluctuations in testosterone levels compared to the control values were found in the females of experimental group 2.



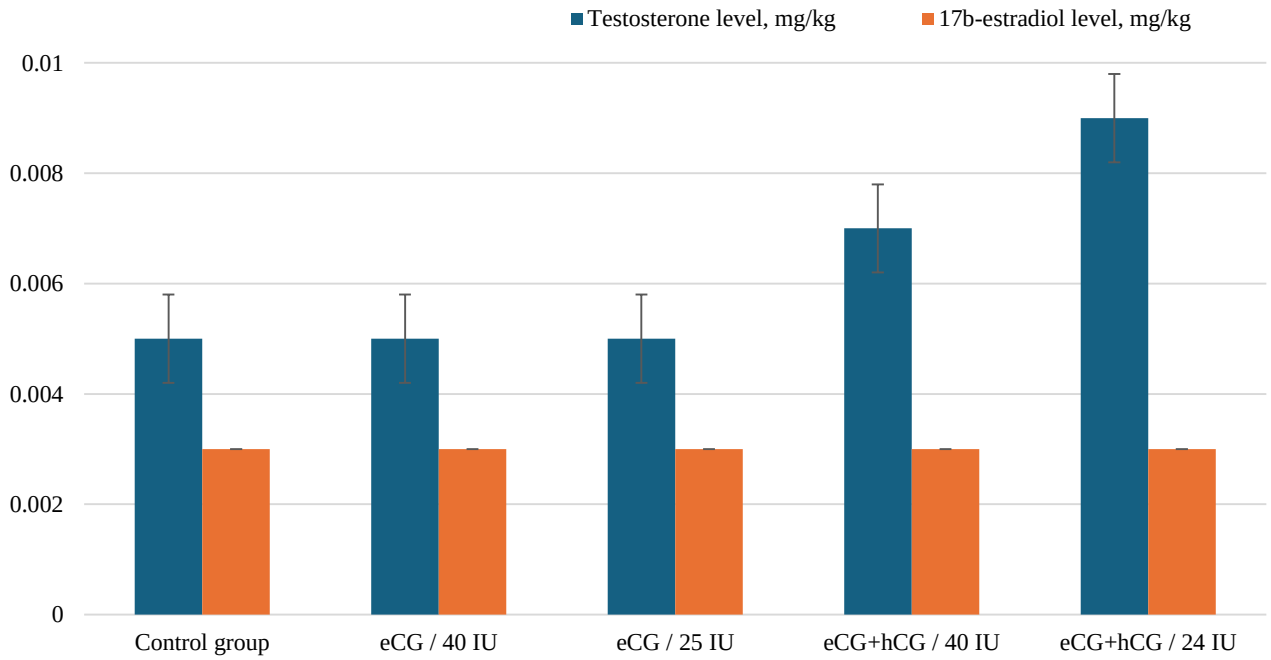
Graph 4. Dynamics of progesterone in the blood serum of pregnant rabbit does (n=12, nmol/L).



Graph 5. Dynamics of testosterone in blood serum of pregnant rabbit does (n=12, ng/dL).

A significant increase in androgenesis in the bodies of females in experimental group 3 was confirmed by a substantial rise in testosterone levels during the experiment, from 0.75 ± 0.09 ng/dL in the first pregnancy to 0.83 ± 0.11 ng/dL in the fifth pregnancy ($p < 0.05$). On the other hand, in the animals of experimental group 4, consistent development of hyperandrogenemia was observed, with testosterone levels in blood serum ranging from 1.01 ± 0.12 ng/dL in the first pregnancy to 2.54 ± 0.21 ng/dL in the fifth pregnancy, significantly higher than the control data ($p < 0.05$). The safety of the long-term use of hormonal means for the stimulation of ovulation in rabbits was evaluated by

assessing the content of sex hormones in the products of rabbit breeding, specifically rabbit meat, at the end of the experiment (during the 1st-5th pregnancies). The obtained data are presented in Graph 6. According to the data in Graph 6, the level of sex hormones (testosterone and 17β-estradiol) did not exceed the detection level. At the same time, there were no significant changes in the level of 17β-estradiol in the experimental groups, and the testosterone content showed an increasing trend, which confirms their safe content in rabbit products.



Graph 6. Sex hormone levels in the meat of rabbit does in the fifth pregnancy (n=4).

DISCUSSION

An important aspect of the manifestation of the reproductive ability of animals is the full course of folliculogenesis in the process of ovulation (Arias-Alvarez et al., 2010; Barker et al., 2012; Burow et al., 2019). To improve ovulation and induce superovulation in various species of animals, the use of gonadotropins has been proposed (Cole, 2012; De Rensis and López-Gatius, 2014; Li et al., 2021). Particularly, a large number of studies are devoted to the induction of ovulation in rabbits using serum and/or chorionic gonadotropins, as well as a gonadotropin-releasing hormone analog (Dal Bosco et al., 2011; Sun et al., 2017; El-Ratel et al., 2020). Studies have insicated a large number of complications that can arise from incorrect dosage or long-term administration (Brouillet et al., 2012; Albu et al., 2014; Chai et al., 2017). Therefore, this study is devoted to determining the hormonal balance in rabbits during pregnancy. The obtained data indicate the safety of the proposed protocols of ovulatory stimulation of rabbit does, which corresponds to the results obtained by Sirotkin et al. (2014). It is confirmed by the level of hormones in their blood and meat, which is consistent with the data of other researchers (Arias-Alvarez et al., 2013; Rebaz et al., 2019; Abdel-Khalek et al., 2022).

With the introduction of serum gonadotropin (eCG) in both dosages, an increase in the level of FSH was noted in rabbits during the five pregnancies. These data are consistent with those obtained by other authors (González-Mariscal et al., 2007; Rosell et al., 2020). On the contrary, the combined use of serum gonadotropin with chorionic gonadotropin (hCG) caused negative dynamics of the level of this hormone, in particular its decrease, especially at doses of 40 IU. A decrease in the level of FSH can lead to a decrease in the production of offspring from rabbits and low efficiency of artificial insemination (Hashem and Aboul-Ezz, 2018).

At the same time, the level of luteinizing hormone (LH) in the animals of experimental group 1 was lower than that of the control group. Therefore, a higher dosage of eCG negatively affects the dynamics of LH. On the contrary, in rabbits of experimental group 2, the level of this hormone was characterized by an increase during the experiment. The obtained results are correlated with the data established by other researchers (Hassanein et al., 2021). The combined use of gonadotropins (eCG + hCG) in both dosages contributed to a significant increase in the level of LH in the blood serum of rabbits, thus during pregnancy, they showed the full functioning of the corpora lutea pregnancy (Quintela et al., 2001; Viudes-de-Castro et al., 2019).

The level of estrogens under the influence of ovulation inducers also underwent dynamic changes. Rabbits of experimental groups 1 and 4 had a moderate increase in the level of 17β-estradiol in blood serum. Similar results are obtained by other researchers (Zhang et al., 2017; Jolivet et al., 2022). It is worth noting that the introduction of eCG at a dose of 25 IU had no effect on the level of estradiol, and the combined use of gonadotropins (eCG + hCG) at a dose of 40 IU caused its excessive synthesis as shown by Mebes et al. (2015).

Progesterone (P4) as a leading hormonal factor during pregnancy, had a salient value in the performed experiments (Abd-Elkareem, 2017; Kowalewski et al., 2020). Thus, a high dosage of eCG (40 IU) contributed to a significant decrease in the level of P4 in rabbits, while a lower dose (25 IU) increased its content compared to the data of the control group. The same results are shown in several studies (Peiró et al., 2010). Good results were also obtained with the combined administration of gonadotropins (eCG + hCG) at a dose of 24 IU during the experiment, as rabbits had a higher level of it, compared to the control. However, a high dose of 40 IU caused a permanent state of hyperprogesteronemia, which had negative consequences on the health of rabbits and their offspring (Bréard et al., 1998; Hoffman et al., 2009).

Contradictory data have been obtained regarding the level of testosterone in experimental animals. In the rabbits of experimental group 2, no significant changes were found, compared to the control group. However, with a higher dosage of eCG in experimental group 1, there was a slight increase in testosterone levels during the first, second, and third pregnancies. The combined administration of gonadotropins (eCG + hCG) caused a state of hyperandrogenism in the animals, which was especially pronounced in experimental group 4. These findings indicate the negative consequences of long-term administration of gonadotropins, as shown earlier (Garcia-Garcia et al., 2009; Rebaz et al., 2019).

The obtaining results are consistent with the data reported by El-Ratel et al. (2020). They found that the introduction of eCG with the injection of hCG or GnRH analogs before AI can synchronize estrus/ovulation to improve embryo production *in vivo*. In addition, fertility outcomes may be improved in rabbit does in which ovulation is induced by a single dose of eCG or hCG on day 5 after AI. As can be inferred, it is better to use hormonal ovulation inducers once or twice to avoid disturbances in the hormonal background and the health of rabbits. It should be noted that the obtained data on the content of sex hormones in rabbit meat correspond to the results obtained by Rebaz et al. (2019) in animals without hormonal treatment. Therefore, the proposed ovulatory stimulation protocols are safe for the consumer of rabbit breeding products.

The search for measures to replace hormonal inducers of ovulation and stimulation of estrus in rabbits by other means is an urgent problem of modern animal husbandry (El-Desoky et al., 2022). Alternative ways to stimulate ovulation in rabbit does. For example, it has been experimentally demonstrated that 24-hour temporary weaning can be an alternative non-hormonal method for oestrus synchronization during lactating in rabbit does of the second reproductive cycle, which was inseminated in the early postpartum period (Arias-Alvarez et al., 2010). controlling the lighting conditions of premises housing rabbit does presents an alternative approach to reducing the number of hormonal treatments. Thus, the study by Quintela et al. (2001) has revealed that a controlled lighting regime can be used to synchronize oestrus in lactating females instead of applying eCG treatment. Other researchers have shown the absence of reliable changes at different light intensities and a negative effect on body weight between the time of the first fertilization and the second period of parturition (Sun et al., 2017). Therefore, a promising direction for improving the reproductive health of rabbit does is the combination of hormones with metal nanoparticles that have antioxidant properties (Koshevoy et al., 2022; Naumenko et al., 2023). Moreover, it is promising to test the addition of herbal remedies, encapsulated hormonal inducers, etc. to the diets of rabbits (El-Desoky et al., 2021).

CONCLUSION

Different means of ovulation induction in rabbits, depending on the dosage, have a versatile effect on the level of hormones in blood serum but do not result in their accumulation in animal meat. Thus, products obtained from rabbits with long-term administration of hormonal drugs to stimulate ovulation are safe for the consumer. On the other hand, the obtained results indcated the presence of a negative effect of serum (eCG) and chorionic gonadotropins (hCG) on the hormonal balance of experimental females. The use of eCG at a dose of 40 IU contributed to an increase in the level of follicle-stimulating hormone (FSH), 17β-estradiol (E2), however, in this group of animals, a slight decrease in the level of luteinizing hormone (LH) and progesterone (P4) was noted. A lower dose of eCG (25 IU) contributed to an increase in FSH, LH, and P4 levels. The combined use of gonadotropins (eCG+hCG) was characterized by a negative effect on the hormonal background of rabbits including a high dose (40 IU) caused hyperprogesteronemia in experimental animals against the background of an increase in the level of testosterone and a decrease in FSH, and a dosage of 24 IU caused hyperandrogenia. It is worth noting that the long-term use of a gonadotropin-releasing hormone analog to stimulate ovulation is also not without negative changes – a significant decrease in the level of LH and a decrease in E2 was found in animals. Further research will be aimed at evaluating metabolic processes in rabbit does and developing ways to correct negative hormone dynamics.

DECLARATIONS

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Authors' contributions

Svitlana Naumenko, Vsevolod Koshevoy, and Yuliya Tverdokhlib developed the research design and substantiated its methodology. Yuliya Tverdokhlib organized and conducted experimental studies, and Kateryna Sinyagovska and Ludmila Kovaleva took part in the selection of blood and meat samples of rabbits. Vsevolod Koshevoy and Gennadiy Hryshchuk carried out statistical processing of the research results. Olga Miroshnikova, Yuliya Tverdohlib and Svitlana Naumenko analyzed the obtained data, and wrote draft of manuscript. All authors took part in discussing the results, checking the analysed data, writing the article and agreed on the final version of manuscript for submission.

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Competing interests

All the authors of the ancient manuscript unanimously state that there is no conflict of interest.

Availability of data and materials

The authors of this study are ready to send all data supporting the findings of the research upon reasonable request.

Ethical considerations

The authors of this article, while performing the work and preparing the manuscript, complied with the requirements of current regulations to prevent ethical violations, including plagiarism, double posting and/or submission and redundancy, fabrication or falsification of data.

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Prevalence and Antimicrobial Susceptibility of *Escherichia coli* Isolated from Goats in the Mekong Delta, Vietnam

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ABSTRACT

Escherichia coli is one of the severe pathogens causing severe diarrhea and resistance to antibiotics in domestic animals, including goats. From April to June 2023, 122 fresh feces of hybrid Boer goats of different ages and genders were collected randomly in the small-scale farms in the Mekong Delta, Vietnam, to clarify the prevalence and antibiotic resistance of *E. coli* isolated from those feces. By the traditional culture method, of 122 samples, 87 fecal samples were positive for *E. coli* (71.31%). There were no statistically significant differences in the prevalence of *E. coli* among male or female goats and ages (< 6 months and ≥ 6 months). *E. coli* was detected in goats over 6 months and under 6 months at 76.56% and 65.52%, respectively, while 88.20% and 85.42% in male and female goats. The antimicrobial susceptibility of *E. coli* strains to 7 examined antibiotics was conducted using the Kirby-Bauer disk diffusion method. The results indicated that *E. coli* was sensitive 100% to colistin (10 µg), amoxicillin/clavulanic acid (20/10 µg), cefuroxime (30 µg), doxycycline (30 µg), ciprofloxacin (5 µg), and 87.50% to ampicillin (10 µg) and bactrim (trimethoprim/sulfamethoxazole, 1.25/23.75 µg), respectively. However, those *E. coli* strains were highly resistant to streptomycin (93.75%), and 93.67% of *E. coli* strains were resistant to one to three antibiotics. Among them, the resistant pattern of Ge+Sm (gentamycin + streptomycin) was the most frequent detection (43.75%). The prevalence rate of antibiotic resistance genes (*blaampC*, *tetA*, *qnrA*, *strA*, and *sulII*) in *E. coli* strains isolated from goat feces was detected by PCR. Among them, gene *blaampC* was the most predominant (96.88%), followed by *qnrA* (68.75%). Furthermore, 81.25% of *E. coli* strains harbored two to five antibiotic-resistance genes, and the gene pattern of *blaampC + tetA + qnrA* was the most popular (21.88 %). The antibiotic resistance and harbored antibiotic resistance genes in *E. coli* strains isolated from goat feces increase animal and public health concerns.

Keywords: Antibiotic resistance gene, Antimicrobial susceptibility, *E. coli*, Goat, Small-scale farm

INTRODUCTION

Goat farming has been increasingly developing and accounting for an increasing structure in the livestock industry in the Mekong Delta, Vietnam, because goats can adapt to climate change's effects (Van Thu, 2018). However, several pathogens can infect goats during raising, and diseases caused by *Escherichia coli* often occur frequently (Begum et al., 2016). If not treated promptly, animals susceptible to this pathogen could die or be stunted or grow slowly; from there, it reduces economic efficiency in livestock farming. *Escherichia coli* is a typical habitat in the mammalian gut flora, especially in the lower intestine of mammals. Meshram et al. (2009) reported that *E. coli* causing diarrhea was an opportunistic disease associated with sloppy environmental conditions, poor sanitation, and poor management practices. Islam et al. (2016) in Bangladesh recorded a positive rate of *E. coli* in the rectum of goats at 52.0%, and young goats were very susceptible. *E. coli* present in the feces of goats can become a source of animal disease and contamination for the farm environment.

On the other hand, previous studies revealed that livestock ruminants are the primary reservoirs of this crucial foodborne pathogen (Bosilevac et al., 2015; Al-Ajmi et al., 2020; Gonzalez and Cerqueira, 2020). Goats have also emerged as critical reservoirs of *E. coli* transmission into humans through food contamination by animal feces (La Ragione et al., 2009; Carlos et al., 2010; Al-Ajmi et al., 2020). In previous reports, *E. coli* was detected rapidly from skin leather, fecal samples, and meats in goats. More than 90% of goat meat samples in Tanzania have reported positive for *E. coli* (Mwanyika et al., 2016). The goat skin leather and fecal samples revealed the presence of *E. coli* contamination at the rates of 1.7% and 19.7%, respectively, in the United States and Mexico (Hanlon et al., 2018), while there was at least 2.4% in goat's feces in Saudi Arabia (Bosilevac et al., 2015).

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Furthermore, the overuse of antibiotics in livestock has caused an increase in antibiotic-resistant pathogens, especially *E. coli*. Even though humans could be infected with those *E. coli* strains through consuming contaminated animal products, a risk of transmission of drug-resistant genes between different strains is also presented (Mellata, 2013; Kumar et al., 2020; Rahman et al., 2021). *Escherichia coli* isolated from domestic animals could be resistant to several antibiotics, such as erythromycin, tetracycline, ampicillin, gentamicin, sulfamethoxazole/trimethoprim, chloramphenicol, kanamycin, and streptomycin (Yamamoto et al., 2014; Abbassi et al., 2017; Massé et al., 2021; Pascu et al., 2022). The resistance phenomenon causes economic loss for farmers because of costs incurred in treatment failure and a prolonged period of treatment of bacterial infections (Bengtsson and Greko, 2014; Wu et al., 2021). Moreover, multi-drug resistance has become a critical global issue, especially *E. coli* (Loayza et al., 2019; Abdalla et al., 2021). Manishimwe et al. (2021) reported that 5.9% of goat fecal samples containing *E. coli* and *Salmonella* strains exhibited a multidrug-resistant (MDR) phenotype in the east province of Rwanda, Africa. This reveals a significant potential hazard to the health of humans and animals in those provinces of Rwanda.

Therefore, the present study aimed to clarify the prevalence and antibiotic resistance of *E. coli* isolated from the feces of goats raised in small-scale farms in the Mekong Delta, Vietnam.

MATERIALS AND METHODS

Ethical approval

This study was conducted by collecting samples according to the guidelines outlined in the Helsinki Declaration and the animal welfare and safety procedures of Can Tho University, Vietnam.

Sample collection

A total of 122 healthy goat fecal samples of all ages and genders were collected randomly at a total of six small-scale goat meat farms (< 30 herds/farm) in O Mon district, Can Tho City, and Chau Thanh district, Hau Giang province in the Mekong Delta, Vietnam, from April to June 2023. Those goats were hybrid Boer goats, including male goats (n = 74) and female goats (n = 48). In addition, those goats were at < 6 months of age (n = 58) and ≥ 6 months of age (n = 64). Those goats were fed different diets on those farms, including fresh grass and commercial feed. In this cross-sectional study, feces (25 grams) were collected directly in the morning after goats had shed feces on the sterilized plastic covers put under cages when collecting samples. Those goats were randomly selected at each row of cages in those farms. After that, the feces samples were kept in separate sterilized plastic bags in cool conditions (4^{o}C) for transport to the laboratory to detect *E. coli* within 24 hours at the Veterinary Food Hygiene Lab, Faculty of Veterinary Medicine, College of Agriculture, Can Tho University, Vietnam.

Insolation and identification

The isolation of *E. coli* was carried out according to Vietnamese National Standard TCVN 5155-90 and the guidelines of Barrow and Feltham (2003). The feces samples were incubated in buffered peptone water broth (BPW, Merck, Germany) to enrich *E. coli* in samples. After incubating at 37^{o}C for 24 h, one loop of enrichment broth of each sample was cultured on MacConkey agar (MC, Merck, Germany) for further incubation at 37^{o}C for 24 h. All suspicious colonies of *E. coli* were subculture on nutrient agar (NA, Merck, Germany) for further incubation at 37^{o}C for 24 h to examine biochemical tests following the guidelines of Barrow and Feltham (2003). Those confirmed *E. coli* strains were cultured on trypticase soy agar (TSA, Merck. Germany) and incubated at 37^{o}C for 24 h. Then, those *E. coli* strains were examined for antimicrobial susceptibility, and DNA was extracted for PCR to detect antibiotic-resistance genes.

Antimicrobial susceptibility

After identifying *E. coli*, 32 representative strains were selected to examine the antibiotic sensitivity. Those strains represented the genders and ages of goats in the surveyed farms. The Kirby-Bauer's agar plate diffusion method was used to examine the sensitivity of bacteria to antibiotics (Bauer et al., 1966). The results were compared with CLSI standards (2022) to evaluate the sensitivity level of bacteria to antibiotics. *Escherichia coli* ATCC 25922 was used as control quality, and the results were compared to the standards of CLSI (2022). Those strains, which were intermediate susceptibility, were accounted as susceptible strains.

The antibiotic discs were used in this study, including ampicillin (Am, 10 µg), amoxicillin/clavulanic acid (Ac, 20/10 µg), cefuroxime (Cu, 30 µg), gentamycin (Ge, 10 µg), colistin (Co, 10 µg), streptomycin (Sm, 10 µg), doxycycline (Dx, 30 µg), ciprofloxacin (Ci, 5 µg), and trimethoprim/sulfamethoxazole (Bt, 1.25/23.75 µg). Those antibiotic discs were supplied by Nam Khoa Biotek Ltd., Vietnam.

Prevalence of antibiotic-resistance genes

Thirty-two *E. coli* strains were examined for antimicrobial susceptibility and used to detect antibiotic-resistance gene prevalence. The DNA of 32 *E. coli* strains was extracted using the heat-shock method and stored at -20^{o}C for use in this experiment (Ahmed and Dablool, 2017). The PCR reaction used Mastermix 2X (Bioline, Canada) in a total of 25 µl: Mastermix 2X (12.5 µl), forward primer (0.5 µl), reverse primer (0.5 µL), distilled water (9.5 µL), and DNA template (2.0 µL). The primer sequences and PCR conditions were conducted following the guidelines for *blaampC* (Caroff et al., 1999), *qnrA* (Cattoir and Nordmann, 2009), *tetA* (Randall et al., 2004), *strA* (Carattoli et al., 2002), and *sulII* (Sáenz et al., 2004). In this study, the negative control was distilled water without DNA and RNA, while the positive controls were *E. coli* strains, which harbored these genes, isolated from cattle (cows, beef cattle) previously in the Mekong Delta and kept in Veterinary Food Hygiene Lab., Faculty of Veterinary Medicine, College of Agriculture, Can Tho University.

Statistical analysis

The Chi-square test was used to determine the difference in the prevalence of *E. coli* in goats and antibiotic resistance among those strains. The Pearson chi-square statistic was used at the significance level of 95% in the Minitab 17.0 software (Minitab Pty Ltd, Australia).

RESULTS

Of 122 goat fecal samples (Table 1), *E. coli* was detected in 87 samples at a high rate (71.31%). Moreover, there were no significant differences in the prevalence of *E. coli* in goat feces between genders and ages ($p > 0.05$). *E. coli* was detected at 88.20% and 85.42% in male and female goats, respectively, while at 65.52% and 75.56% in goats under 6 months and over 6 months.

The antimicrobial susceptibility test indicated that those *E. coli* strains were still susceptible to most examined antibiotics (Table 2), such as amoxicillin-clavulanic acid (100%), cefuroxime (100%), doxycycline (100%), ciprofloxacin (100%) and colistin (100%). However, these *E. coli* strains showed significant resistance to aminoglycoside antibiotics, including streptomycin (93.75%) and gentamycin (43.75%). Of 32 examined *E. coli* strains, 93.67% resisted one to three antibiotics (Table 3). Among them, the pattern of Ge + Sm (gentamycin + streptomycin) was the most frequent (43.75%), and Sm was present in all antibiotic-resistance patterns.

Moreover, those *E. coli* strains harbored various antibiotic-resistance genes (Table 4). Gene *blaampC* (96.88%) was the most detected from those *E. coli* strains, followed by *qnrA* (68.75%), *tetA* and *sulII* (40.63%), and *strA* (18.75%). Of 32 *E. coli* strains, 81.25% harbored multiple antibiotic-resistance genes from two to five genes (Table 5). The *blaampC* + *tetA* + *qnrA* pattern was the most predominant (21.88%).

Table 1. Prevalence of *Escherichia coli* isolated from feces of meat goats in small-scale farms in the Mekong Delta, Vietnam, from April to June 2023

Variable		No. of examined samples	No. of positive samples	Percentage
Gender	Male	74	66	88.20
	Female	48	41	85.42
				($p > 0.05$)
Age	< 6 months	58	38	65.52
	≥ 6 months	64	49	76.56
				($p > 0.05$)
Total		122	87	71.31

No: Number

Table 2. Antimicrobial susceptibility of *Escherichia coli* strains isolated from feces of meat goats in the Mekong Delta, Vietnam, from April to June 2023 (n = 32)

Antibiotic group	Antibiotic	Code	Sensitive		Resistant	
			No. of positive strains	Percentage	No. of positive strains	Percentage
Beta-lactam	Ampicillin	Am	28	87.50	4	12.50
	Amoxicillin-clavulanic acid	Ac	32	100.00	0	0.00
	Cefuroxime	Cu	32	100.00	0	0.00
Aminoglycoside	Gentamycin	Ge	18	56.25	14	43.75
	Streptomycin	Sm	2	6.25	30	93.75
Tetracycline	Doxycycline	Dx	32	100.00	0	0.00
Polypeptide	Colistin	Co	32	100.00	0	0.00
Quinolone	Ciprofloxacin	Ci	32	100.00	0	0.00
Sulfonamide	Bactrim*	Bt	28	87.50	4	12.50

*Trimethoprim/sulfamethoxazole; No: Number

Table 3. Antibiotic-resistance patterns of *Escherichia coli* isolated from feces of meat goats from April to June 2023 in Vietnam (n = 32)

No. of antibiotic	Pattern	No. of positive strains	Percentage
1	Sm	11	34.38
2	Am + Sm	1	3.13
	Ge + Sm	14	43.75
	Bt + Sm	1	3.13
3	Am + Bt + Sm	3	9.38
			($p < 0.05$)
Total		30	93.67

Am: Ampicillin; Bt: Bactrim; Ge: Gentamycin; Sm: Streptomycin; No: Number

Table 4. Prevalence of antibiotic-resistance genes in *Escherichia coli* isolated from feces of meat goats from April to June 2023 in Vietnam (n = 32)

Antibiotic group	Gene	No. of positive strains	Percentage
Beta-lactam	bla_{ampC}	31	96.88
Aminoglycoside	*strA*	6	18.75
Quinolone	*qnrA*	22	68.75
Tetracycline	*tetA*	13	40.63
Sulfonamide	*sulII*	13	40.63
			($p < 0.05$)

No.: Number

Table 5. Multiple antibiotic-resistance gene patterns of *Escherichia coli* strains isolated from feces of meat goats from April to June 2023 in Vietnam (n = 32)

No. of resistant genes	Pattern	No. of positive strains	Percentage
2	*blaampC+tetA*	5	15.63
	blaampC+sulII	1	3.13
	blaampC+qnrA	2	6.25
	blaampC+strA	1	3.13
3	*blaampC+tetA+qnrA*	7	21.88
	blaampC+tetA+sulII	5	15.63
4	*blaampC+tetA+qnrA+sulII*	4	12.50
5	*blaampC+tetA+qnrA+strA+sulII*	1	3.13
Total		26	81.25

No: Number

DISCUSSION

In this study, *E. coli* was isolated from goat feces in small-scale farms at a relatively high rate (71.31%). The previous reports indicated that *E. coli* was an enteropathogen frequently isolated from fecal samples of small ruminants, such as in sheep (34.7%) in Trinidad, goats in Germany and Egypt at 75.3% and 30.7%, respectively (Zschock et al., 2000; Adesiyun et al., 2001; Osman et al., 2013). Shabana and Al-Enazi (2020) also reported that *E. coli* was detected at a high rate (92.1%) in goat feces in Al-Madinah, Saudi Arabia. On the other hand, Adesiyun et al. (2001) and Shabana et al. (2017) reported that age was a significant factor affecting the occurrence of diarrhea caused by enteropathogens, including *E. coli*, and the prevalence of enteropathogens in young animals was higher than in older animals. However, this study showed that *E. coli* was present in the feces of meat goats and did not depend on gender or age. This difference could be due to the number of samples and the age of animals at the collecting times.

The antibiotic resistance of bacteria, including *E. coli*, was a considerable challenge in treating and preventing diseases in animals and humans (Bengtsson and Greko, 2014; Loayza et al., 2019; Abdalla et al., 2021; Wu et al., 2021). Although *E. coli* strains isolated from meat goats in this study were still sensitive to several antibiotics, they showed significant resistance to streptomycin (93.75%) and gentamycin (43.75%). Those antibiotics were commonly used to treat goat diseases in those examined farms, and the farmers used antibiotics mainly depending on their experiences. Besides, the hygiene status in those surveyed small-scale farms was poorly managed, the feces were not cleaned up, and

other animals could enter the farms. Pathogens, including antibiotic-resistant bacteria from feces or the environment, such as *E. coli*, could contaminate and spread in those farms. Pehrsson et al. (2016) reported that antibiotic-resistant *E. coli* and resistance genes could be transmitted between pathogens and benign microbes from diverse habitats through environments contaminated with feces. Obaidat et al. (2017) reported that *E. coli* isolated from sheep and goat farms in Jordan highly resisted tetracycline (45.5%), ampicillin (35.4%), and streptomycin (32.30%) but were sensitive to gentamycin (93.80%). *E. coli* strains isolated from goats in Bangladesh showed high resistance to ampicillin (65.38%), amoxicillin-clavulanic acid (60.26%), trimethoprim–sulfamethoxazole (52.56%), tetracycline (51.28%), streptomycin (47.44%), and gentamicin (37.18%) (Islam et al., 2016). Ndegwa et al. (2019) reported that most *E. coli* strains isolated from pastured goats in Virginia (USA) were resistant to tetracycline (51.00%), streptomycin (30.00%), and they also resisted ampicillin (19.00%) which had never been used on the farm. Moreover, *E. coli* isolated from goats in this region was highly resistant to tetracycline because of a history of previous use of tetracycline for treatment on the farm. Thus, the difference in antibiotic resistance levels could be due to the characteristics of using antibiotics in husbandry in each region.

Moreover, this study exhibited that *E. coli* strains isolated from the feces of meat goats in small-scale farms of the Mekong Delta could be multi-drug resistant to three antibiotics used frequently in this region. The pattern of Ge + Sm (gentamycin + streptomycin) was the most popular, consistent with the high resistance performance to gentamycin and streptomycin in the antimicrobial susceptibility test in this study. This could be due to the frequency of using those antibiotics in goats in the surveyed farms; thus, *E. coli* strains have established a high resistance to those examined antibiotics. The multi-drug resistance phenomenon of *E. coli* isolated from small ruminants was recorded in previous research in other regions. *E. coli* strains, originating from goats in Bangladesh, were multidrug-resistant to three to eight subclasses of antimicrobials (Islam et al., 2016). Obaidat et al. (2017) found that approximately one-third of both *E. coli* and *Salmonella enterica* isolated from small ruminant herds of rural Jordan, which were less used antimicrobials, were also multidrug resistant. Nsofor and Iroegbu (2012) indicated that the average number of antibiotic-resistance phenotypes of *E. coli* was significantly higher for goat and poultry than for cattle and swine. It revealed a significant public health concern in Southeast Nigeria that multidrug-resistant *E. coli* strains might become a potential reservoir of resistance genes to be transferred to other pathogenic bacteria. Prapasawat and Intarapuk (2021) showed that *E. coli* isolated from the feces of dairy goats in Thailand were resistant to at least one antimicrobial agent by disc diffusion method, especially streptomycin (65.6%), and 23.9% of those isolated *E. coli* strains were multidrug resistant. In addition, dairy goats in farms could become a reservoir and possibly spread antibiotic-resistant isolates to farmers and consumers via animals and their products in Thailand.

In this study, *E. coli* strains isolated from the feces of meat goats harbored genes *blaampC* and *qnrA* at a high rate. However, those *E. coli* strains did not show much resistance to beta-lactam and quinolone antibiotics in the antimicrobial susceptibility test. Whereas *E. coli* strains had high resistance to aminoglycoside antibiotics, *strA* was detected at the most minor rate (18.75%). Thus, the antibiotic resistance performance of *E. coli* strains might be affected by other factors, such as the pressure of using antibiotics, a combination of several genes, environmental factors, etc. (Bengtsson-Palme et al., 2017; Chen et al., 2019; Liu et al., 2020). Resistance genes could be acquired through natural mutations and transferred to the next generation or due to conjugation, transduction, or mutation of resistance genes between bacteria species (Sommer et al., 2017). Moreover, antibiotic-resistance genes are silent resistance genes that do not usually express or express at low levels, even when exposed to antibiotics (Stasiak et al., 2021). Hasan et al. (2014) reported that *E. coli* harbored antimicrobial-resistant genes, could transmit among species, and confer resistance to common antibiotics like penicillins, tetracycline, gentamicin, cephalosporins, and carbapenems. Shabana and Al-Enazi (2020) indicated that genes *rmtB*, *CTX-M*, and *qnr* were detected in healthy, diarrheic sheep and goats in Saudi Arabia. Those genes were present in all aminoglycoside-resistant *E. coli*, ESBL-producing *E. coli*, and fluoroquinolone-resistant *E. coli*, respectively.

In addition, those *E. coli* strains isolated from the feces of meat goats in this study also harbored multiple antibiotic-resistance genes. It indicated that those strains could highly resist several antibiotics and combine antibiotic-resistant effects, such as beta-lactam and quinolone antibiotics. Moreover, the multiple antibiotic resistance of *E. coli* strains could cause failure in treatment for goats and humans in the Mekong Delta. Even though bacterial populations that are exposed to various antibiotics can develop tolerance to these antibiotics, it is possible for one antibiotic in a combination to counter resistance to another antibiotic and offer effective treatment. However, if tolerance has already emerged for one antibiotic, the combination may inadvertently facilitate the spread of resistance to the partner antibiotic (Liu et al., 2020). Zhao et al. (2020) also reported that certain antibiotics, such as cephalexin, chloramphenicol, kanamycin, sulfamethazine, and tetracycline, enhanced the co-selection of antibiotic-resistance genes related to other antibiotic classes. Tenover (2006) indicated that multidrug resistance could be due to combined multiple resistance mechanisms. Multidrug resistance could be expressed in pathogens due to continuous exposure to an antibiotic or the acquisition of

genetic resistance elements through plasmids or transposons. The antibiotic resistance of *E. coli* via resistance genes can be obtained through horizontal gene transfer to make multi-drug resistant strains (Huddleston, 2014). The research of Van Hoek et al. (2023) showed that Shiga-toxin-producing *E. coli* (STEC) isolated from dairy goats and sheep farms in the Netherlands could harbor various antibiotic-resistance gene patterns, including genes *blaTEM*, *sul*, *aadA1*, *strA*, *tetA*, and *dfrA*. A few strains shared the same genetic pattern with STEC isolates from humans; it revealed that those *E. coli* strains could infect and cause severe diseases in humans.

CONCLUSION

The prevalence of *E. coli* in the feces of meat goats in small-scale farms in the Mekong Delta, Vietnam, was high and has no relation to gender and age. Although *E. coli* strains were still susceptible to most examined antibiotics, those isolated *E. coli* strains showed significant resistance to aminoglycoside antibiotics, and several antibiotic-resistant patterns were obtained. Moreover, those *E. coli* strains isolated from goat feces harbored antibiotic-resistance genes, especially *blaampC*, at a high rate. Further research should be conducted to clarify the antibiotic-resistance characteristics of *E. coli* in goats of the study area and control multi-drug-resistant *E. coli* strains to protect animals and public health.

DECLARATIONS

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

Thuan K. Nguyen, Binh C. Tran, and Trung T. Truong conceptualized, designed, and supervised the research. Thuan K. Nguyen critically reviewed the study. Binh C. Tran, Vy L.P. Nguyen, and Trung T. Truong collected samples and processed the data. Binh C. Tran analyzed and interpreted the data generated. All authors revised and approved the final edition of the manuscript.

Availability of data and materials

The authors of this article confirm that all data supporting the findings of this research are available upon reasonable request.

Ethical considerations

The authors considered farmers' ethical concerns and consent before conducting the study. This article was written originally without any copy from data of published articles and books.

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The Role of Neutrophils and NETosis in Local Immunity of Feline Inflammatory Aural Polyps

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CASE REPORT
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ABSTRACT

Feline inflammatory aural polyps are abnormal growths that can occur in the ear canals of cats, particularly in the middle ear. These polyps are frequently linked to persistent inflammation and can result in a range of ear-related complicated pathologies. The etiology is multifactorial. The purpose of the research was to study the cytology of an inflammatory polyp in a cat and to study the role of neutrophils and their mechanisms on the formation of extracellular protective traps by neutrophils (NETs). A 4-year-old, female spayed, Scottish fold cat, weighing 3.5 kg sent to a veterinary clinic (Mirra-Vet, Kyiv, Ukraine). Clinical, otoscopic methods, and laboratory methods of cytological diagnostics were used for the research. At the onset of the clinical investigation, exudate discharge from the ear and a painful response were observed. Upon detailed otoscopy, a polyp in the ear canal was diagnosed. An increase in the number of leukocytes (23.2×10^9/L), their absolute content, and an increase in the percentage of neutrophils (48.2 %) in the leukogram. Assessing the capacity of neutrophils to generate NETs (Neutrophil Extracellular Traps) was determined after samples were collected using a cytologic brush. Cytological analysis of samples from the inter-tragic incisure area highlighted a significant presence of neutrophils, forming extracellular protective traps. The results revealed free NETs in separate areas of the slides. The findings indicated the formation of cooperative groups among neutrophils, other phagocytes, and epithelial cells, along with slender nuclear streaks. During the treatment (Otoflox, 2 drops per ear), the inflammatory reaction disappears, polyp size decreases, exudative reactions decrease, and neutrophil activity decreases. After 3 days of treatment, the animal's condition improved. The ear was clean without sulfur and lesions. The complete treatment course spanned 7 days. During the treatment, the inflammatory reaction disappeared, polyp size decreased, exudative reactions decreased, and neutrophil activity decreased. Experimental studies have shown that during the inflammatory reaction in the ear, protective mechanisms of local immune defense are activated. Activated neutrophils perform their function through phagocytosis and the formation of NETs. These studies contribute to supplementing the data on the immunopathological mechanisms of feline inflammatory polyps.

Keywords: Ear polyps, Feline inflammatory, Local immunity, Neutrophils

INTRODUCTION

Feline inflammatory aural polyps are epithelial formations with signs of inflammation that can be localized in the external ear canal, the tympanic cavity, or on the mucous membrane of the nasopharynx (Greci et al., 2014; Momota et al., 2016; Hoppers et al., 2020). Feline inflammatory aural polyps can affect 7 to 12% of cats, breed predisposition has also been identified in Siamese and Himalayan cat breeds. Bilateral ear polyps in cats may account for up to 24% (Greci and Mortellaro, 2016; Hoppers et al., 2020). They typically occur unilaterally, with etiological factors encompassing both genetic and acquired aspects (Sula et al., 2014; Mutua and Gershwin, 2021).

Bacteria, viruses, and pathogenic fungi play an important role in the onset and development of feline inflammatory aural polyps (Hariharan et al., 2011; Mascarenhas et al., 2019). Recurrence is common in feline inflammatory ear polyps. Researchers report the prevalence of this condition among cats (McAullife et al., 2020). In dogs, inflammatory polyps statistically occur less frequently, and they are mostly localized on the nasal mucous membrane (Bizikova and Burrows, 2019). Currently, there are several diagnostic and treatment methods developed for animals with feline inflammatory aural polyps (Ginel et al., 2002; Moore et al., 2019). However, the study of local immunity is of particular interest among scientists (Wainberg et al., 2019; Tyler et al., 2020).

Inflammation is a fundamental and complex biological response that serves as a protective mechanism against infections and tissue damage (Mutua and Gershwin, 2021). Within the diverse elements of the immune system, neutrophils stand out as crucial participants in coordinating the inflammatory process (Zhelavskyi et al., 2020). In the dynamic landscape of inflammation, neutrophils play intricate roles, utilizing their unique extracellular protective traps to contribute to the complex immune response (Adrover et al., 2020; Qi et al., 2021).

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Neutrophils, a subtype of white blood cells, act as the initial responders to areas of infection or injury (Zhang et al., 2021). Their primary mission is to neutralize invading pathogens and facilitate tissue repair. Equipped with a diverse array of weapons, including enzymes, antimicrobial peptides, and reactive oxygen species, neutrophils are highly effective in combating microbial threats (Adrover et al., 2020; Demkow, 2021). One of the initial steps in the inflammatory response involves chemotaxis, where neutrophils are attracted to the site of infection or tissue damage by chemical signals (Qi et al., 2021; Zhang et al., 2021). These signals guide the neutrophils through the bloodstream, allowing them to quickly reach the affected area. This recruitment process is crucial for the timely deployment of neutrophils to contain and eliminate potential threats (Dömer et al., 2020).

The role of phagocytes, and their defense mechanisms in the development of acute and chronic otitis, remains insufficiently understood (Ginel et al., 2002; Moore et al., 2019). Further research is needed on the immunopathological mechanisms of feline inflammatory aural polyps and the utilization of effective treatment modalities (Bollez et al., 2018). Neutrophils can induce oxidative stress in various tissues of the animal body (Adrover et al., 2020; Qi et al., 2021). The build-up of free radicals contributes to the breakdown of cells and tissues. Moreover, it can serve as a stimulus for initiating apoptosis in immune cells. These disruptions are accompanied by alterations in cellular homeostasis (Adrover et al., 2020; Qi et al., 2021). Therefore, a detailed understanding of the role of neutrophils in the formation of local immunity is of paramount importance in clinical immunology and pathology. A thorough investigation of immunopathogenic mechanisms will allow researchers to gain new insights into the development of this pathology in animals. Novel approaches will find a place in developing effective methods and means for treating feline inflammatory polyps (Ginel et al., 2002; Tater et al., 2003; Greci and Mortellaro, 2016; Zhelavskyi et al., 2020). The purpose of this study was to survey of an inflammatory polyp cytology in a cat and to study the role of neutrophils and their mechanisms on the formation of extracellular protective traps by neutrophils (NETs).

MATERIALS AND METHODS

Ethical approval

The cats were in the possession of private individuals, and written consent was acquired from these owners. The clinical investigations were conducted in accordance with the Law of Ukraine "On Protection of Animals from Cruel Treatment" (21/02/2006, 3447-IV), and adhered to the guidelines set by the European Commission regarding the treatment of vertebrates, ensuring protection against thirst, hunger, malnutrition, discomfort, fear, pain, and suffering.

Clinical signs

A 4-year-old, spayed female Scottish Fold cat, weighing 3.5 kg, experiencing skin problems on the inner surface of the right ear, reached out to the veterinary clinic (Mirra-Vet, Kyiv, Ukraine). During the clinical examination, the discharge of fluid from the right ear with an unpleasant odor was diagnosed. During the clinical examination, body temperature was 38.7°C, heart rate was 177 bmp, and respiratory rate was 28 breaths per minute. The cat had depression and a decrease in appetite. The animal was depressed, during palpation of the auricle it had a painful reaction.

During a detailed clinical examination and otoscopy (Gima, Italy), inflammation and the presence of a polyp were diagnosed in the right ear canal. During the clinical examination, redness, epithelial proliferation in the recess of the polyp (diameter 3 mm) in the area of intertragic incisure, and accumulation of gray-colored, thick exudate with an unpleasant odor were found in the ear. Samples were taken from the ear using sterile swabs for antibiotic susceptibility testing and cytological examination.

Blood collection and analyses

At the beginning of the study, a blood sample (50.0 µL) was taken. The blood sample was taken at 9:15 AM from the cephalic vein. During the hematological analysis, various parameters were evaluated, including erythrocyte count ($\times 10^{12}$/L), leukocyte count ($\times 10^{9}$/L), leucogram values ($\times 10^{9}$/L, %), concentrations of hemoglobin (µmol/L), hematocrit (L/L), Mean Corpuscular Volume (MCV, fl), mean corpuscular hemoglobin (MCH, fmol), mean corpuscular hemoglobin concentration (MCHC, mmol/L), and thrombocyte count ($\times 10^{9}$/L). The Abaxis Vetscan HM5 Hematology Analyzer (USA) was employed by the laboratory for the analysis.

Cytology study

Assessing the capacity of neutrophils to generate Neutrophil Extracellular Traps (NETs). The diagnostic specimens were collected using a cytologic brush premoistened with 1/15 mol/l phosphate buffer solution NeoGalin18 (15 M NaH_2PO_4 + $2H_2O$ [11.8 g] + KH_2PO_4 [68.0 g] + C_6H2O_6 [10.0 g], pH 7.2). Following this, a smear was created and left to air-dry at room temperature (20 °C). The microslide was then fixed with methanol (90°) and stained using a 1%

phosphate-buffered saline solution (pH 7.2) for 2–3 minutes. Following this, the microslide was rinsed with phosphate buffer and stained with May-Grünwald dye-fixative eosin methylene blue (Sigma-Aldrich®, USA). Neutrophils from NETs were microscopically evaluated at x2500-3000 magnification (Zhelavskyi, 2021).

Treatment

For treatment, the drug Otoflox (LLC BIOTESTLAB, Ukraine) was prescribed, 2 drops per ear. 1 ml of Otoflox contains the following components including ivermectin 1 mg, clotrimazole 10 mg, florfenicol 2.5 mg, betamethasone (in the form of uniformidipropionate) 1 mg, as well as auxiliary substances (dimethyl sulfoxide, ethanol, polyethylene glycol, propylene glycol). The full course of treatment was 7 days.

RESULTS

Upon clinical examination, it was established that the cat periodically shakes its right ear and scratches its paw. During the examination of the external ear, a gray thick exudate with an unpleasant odor was established, which was most localized in the area. After cleaning with sterile cotton swabs, an epithelial thickening in the form of a polyp was revealed. The polyp had a diameter of 3 mm and a thickness of 4 mm, and the color was red (Figure 1).

Since the beginning of the treatment, positive dynamics have been observed. By the third day, there was a reduction in exudative reaction and hyperemia, the size of the polyp was decreasing, pain reactions disappeared, and body temperature normalized. Complete resolution of inflammatory signs was noted on the 7th day of treatment.

Based on the microbiological study, *Staphylococcus aureus* (x 10^8 colony-forming units CFU/ml), *Pantoea* spp. (x10^8 CFU/ml), *Enterococcus faecium* (x10^7 CFU/ml) microbes were identified. The highest sensitivity of microbes was established to the antibiotics (Ukrmediasnab, Ukraine), including enrofloxacin (concertation 30 mcg, 34 mm radial growth inhibition on agar medium), tetracycline (concertation 30 mcg, 31 mm), and doxycycline (concertation 30 mcg, 28 mm). When touched, the animal had a painful reaction. An increase in the number of leukocytes, their absolute content, and an increase in the percentage of neutrophils in the leukogram was established during the morphological examination of the blood (Table 1).

Cytological examination of samples from the area of intertragic incisure revealed a significant number of neutrophils. Neutrophils formed extracellular protective traps in which microorganisms were fixed. Free and unfixed NETs were diagnosed in separate areas of the slides. It was found that neutrophils formed cooperative groups with other phagocytes, epithelial cells, and slender nuclear streaks (Figure 2). During the treatment (Otoflox, 2 drops per ear), the inflammatory reaction disappears, polyp size decreases, exudative reactions decrease, and neutrophil activity decreases (Figure 3).

Table 1. Hematological parameters with Feline inflammatory aural polyps for 4-year-old, spayed female Scottish Fold cat

Parameter	Units	Measured values	Reference ranges*
Erythrocytes	$\times10^{12}$/L	5.0-10.0	8.3
Leukocytes	$\times10^{9}$/L	5.5-19	23.2
Neutrophil	%	35.0-37.0	48.2
	$\times10^{9}$/L	2.5-14.0	11.1
Lymphocyte	%	20.0-50.0	45.5
	$\times10^{9}$/L	1.5-7.0	10.5
Eosinophils	%	2.0-12.0	4.0
	$\times10^{9}$/L	0-1.0	0.9
Monocytes	%	1.0-4.0	2.3
	$\times10^{9}$/L	0-1.50	0.5
Basophils	%	0-0.05	0
	$\times10^{9}$/L	0-1.0	0
Hemoglobin	G/L	80.0-150.0	92.4
Hematocrit	L/L	0.24-0.45	0.52
MCV	fl	12-17	14.3
MCH	fmol	0.78-1.08	15.1
MCHC	mmol/L	18.62-22.34	21.4
Thrombocyte	$\times10^{9}$/L	300-600	537.0

MCV: Mean corpuscular volume; MCH: mean corpuscular hemoglobin; MCHC: mean corpuscular hemoglobin concentration; *****Harvey (2012).

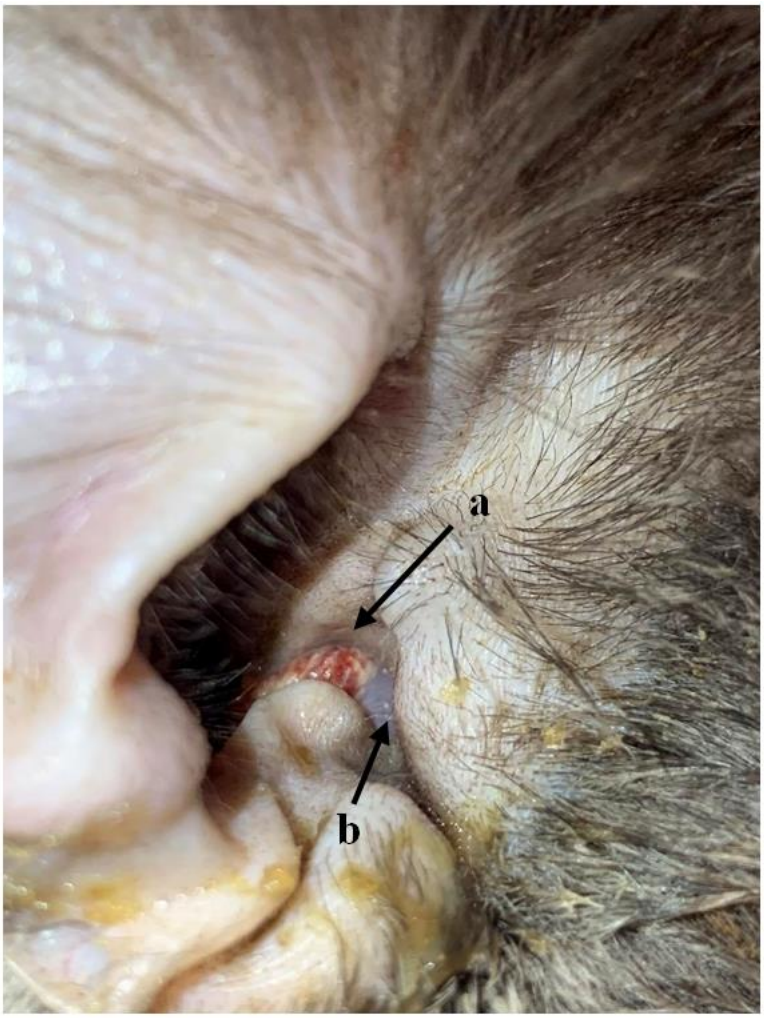

Figure 1. Clinical characteristics of feline inflammatory aural polyps of a 4-year-old, female spayed, Scottish fold cat (right ear). **a:** Visualization of a polyp. **b**: Inflammatory exudate in area *intertragic incisure*

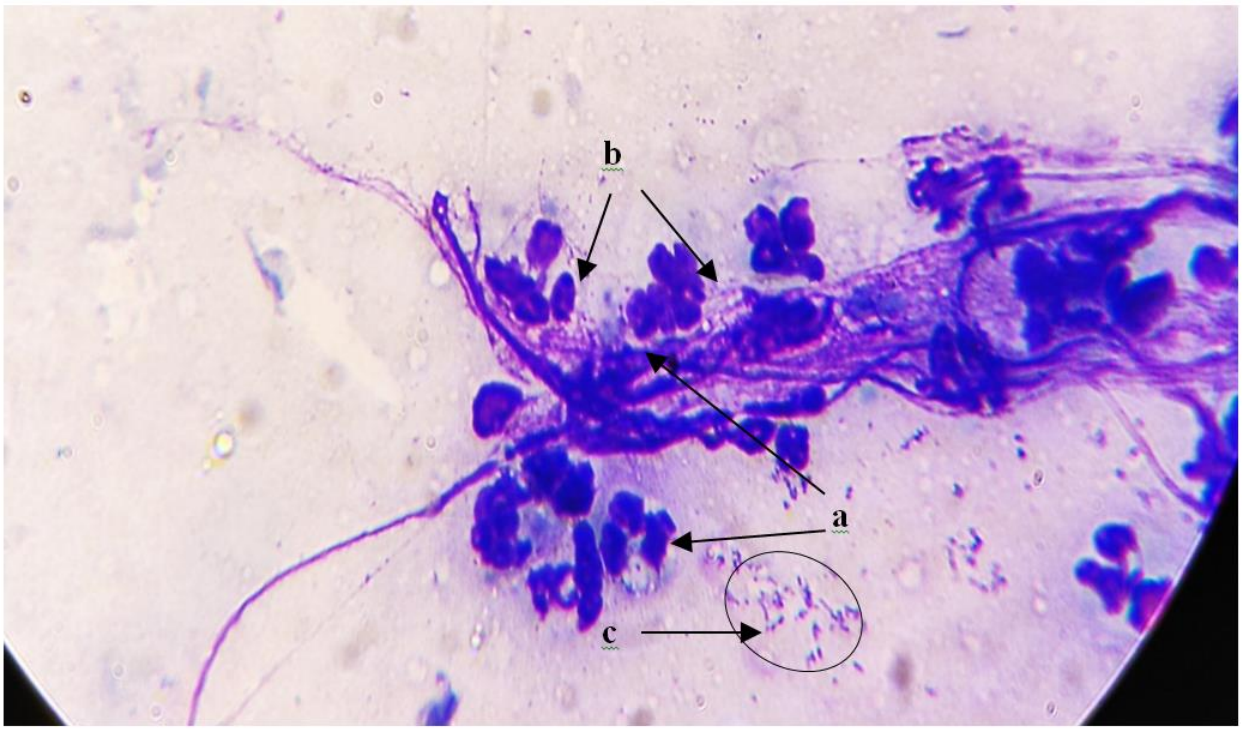

Figure 2. Cytology diagnostic of feline inflammatory aural polyps of a 4-year-old, female spayed, Scottish fold cat. **a**: visualization neutrophils. **b:** formed extracellular protective traps, in which microorganisms were fixed (**c**). Magnification x2000, stained with May-Grünwald

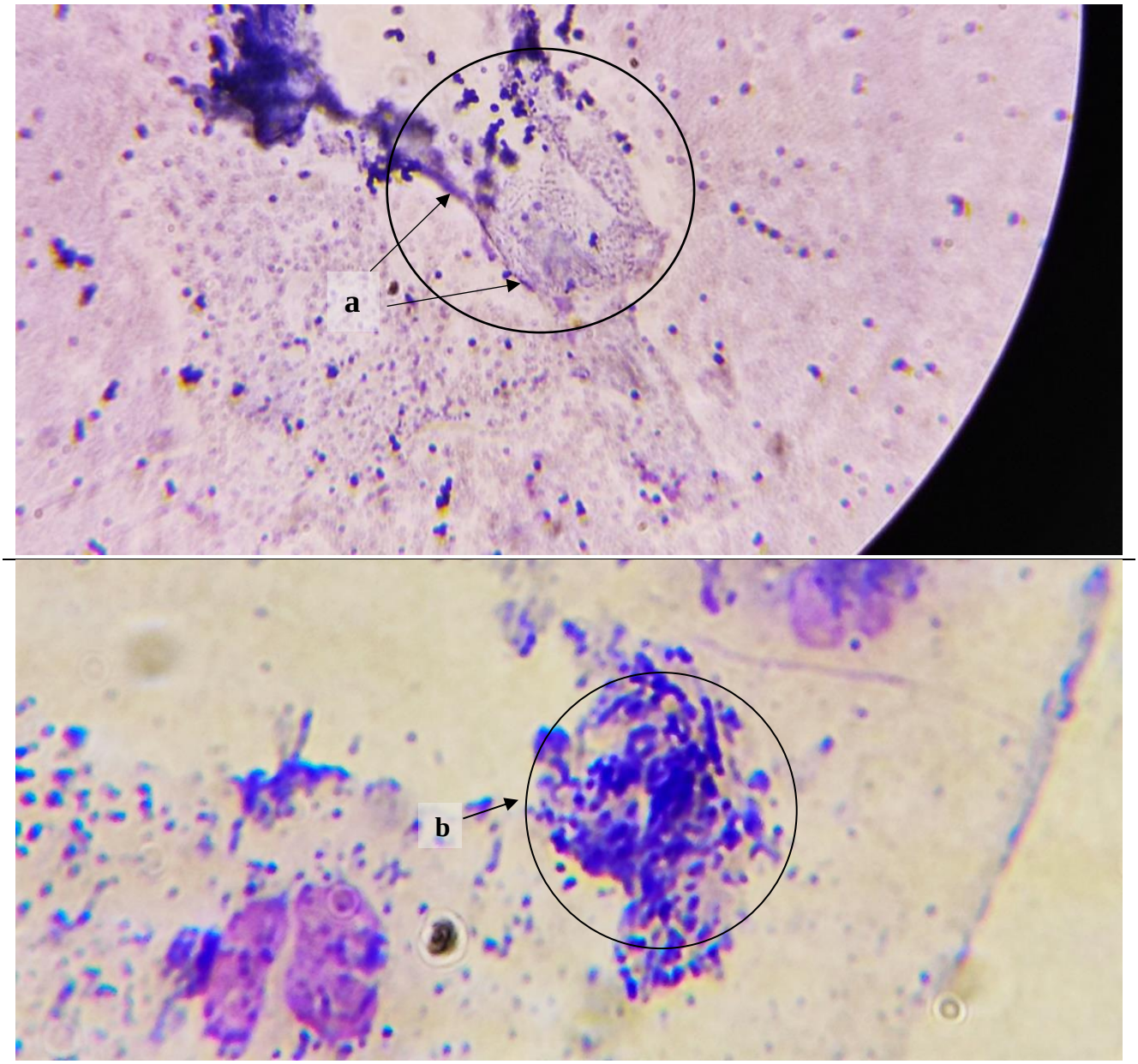

Figure 3. Cytology diagnostic of feline inflammatory aural polyps of a 4-year-old, female spayed, Scottish fold cat. **a:** Visualization unfixed NETs (magnification × 2500); and NETs which microorganisms were fixed (**b**), magnification x 3000), stained with May-Grünwald

DISCUSSION

Feline inflammatory aural polyps are abnormal growths in the ear canal of cats, often associated with chronic inflammation (Ginel et al., 2002; Tater et al., 2003). Feline inflammatory aural polyps may exhibit symptoms such as head shaking, scratching of the affected ear, and the presence of gray-colored, thick exudate with an unpleasant odor. Clinical examination often reveals the polyps, which may vary in size and color. Studies have demonstrated a breed predisposition in certain cat breeds, notably Siamese and Himalayan (Greci et al., 2014; Greci and Mortellaro, 2016; Mutua and Gershwin, 2021).

While local immunity plays a role in various aspects of the body's defense against infections and inflammation, specific information about the role of local immunity in feline inflammatory ear polyps may be limited (Adrover et al., 2020; Zhelavskyi, 2021). A remarkable aspect of neutrophil function in inflammation is the release of extracellular traps, known as NETs. In response to certain stimuli, such as bacterial or fungal infections, neutrophils undergo a unique form of cell death called NETosis (Hidalgo et al., 2021). During NETosis, the neutrophil releases its chromatin, adorned with antimicrobial proteins and enzymes, creating a web-like structure that entraps and neutralizes pathogens extracellularly (Vidémont and Pin, 2010). NETs not only immobilize microbes but also contribute to the amplification of the immune response by signaling other immune cells (Hidalgo et al., 2021; Zhelavskyi et al., 2023).

While extracellular traps play a vital role in microbial defense, their release can have both beneficial and detrimental consequences in the context of inflammation. On the positive side, NETs aid in pathogen containment and stimulate the immune system. However, excessive NETs formation or impaired clearance can lead to tissue damage and contribute to the pathogenesis of various inflammatory disorders (Dömer et al., 2021).

Neutrophils, having completed their tasks, undergo apoptosis, a programmed cell death. This controlled death allows for the removal of neutrophils by phagocytic cells, facilitating the resolution of inflammation. Failure in the timely removal of neutrophils may lead to chronic inflammation, emphasizing the importance of a balanced and regulated immune response. Neutrophils, with their versatile weaponry and unique ability to form extracellular traps, emerge as pivotal contributors to the inflammatory process (Hariharan et al., 2011; Greci et al., 2014; Hidalgo et al., 2021). Understanding the delicate balance between the protective and potentially harmful aspects of neutrophil function provides insights into the development of therapeutic strategies for inflammatory disorders. From chemotaxis to NETs formation, neutrophils exemplify the complexity of the immune system's response, underscoring their indispensable role in maintaining the delicate equilibrium between protection and pathology in inflammation (Moore et al., 2019).

Inflammatory polyps in felines pose a complex challenge for both veterinarians and cat owners. Understanding the underlying factors contributing to their development, recognizing the clinical signs, and applying a comprehensive diagnostic and treatment strategy are critical to ensuring the well-being of affected felines (Sauvé, 2019). Ongoing studies of etiology, study of immune mechanisms, the role of neutrophils, and phagocytosis will deepen knowledge of pathogenesis. The study of immunopathological mechanisms in the pathogenesis of feline inflammatory aural polyps will serve as a foundation for the development of informative diagnostic tools and effective treatment methods (de Bont et al., 2019). Considering that feline inflammatory ear polyps may have recurrences, immunomodulatory therapy should be applied. It is important to emphasize that the destruction of epithelial cells, neutrophil apoptosis, extracellular trap migration, the release of inflammatory mediators, and apoptosis promoters require new approaches in treating this pathology (Hidalgo et al., 2021; Zhelavskyi et al., 2023).

An intriguing facet of neutrophil activity in inflammation is the generation of extracellular traps, referred to as NETs. When exposed to specific triggers like bacterial or fungal infections, neutrophils undergo a distinctive type of cell death termed NETosis. In the course of NETosis, the neutrophil releases its chromatin, adorned with antimicrobial proteins and enzymes, forming a mesh-like structure that captures and neutralizes pathogens outside the cell. NETs not only immobilize microorganisms but also play a role in enhancing the immune response by signaling to other immune cells (Moore et al., 2019; Hidalgo et al., 2021).

CONCLUSION

Feline inflammatory aural polyps can occur against the background of recurrent inflammation. Experimental studies have shown that during the inflammatory reaction in the ear, protective mechanisms of local immune defense are activated. Activated neutrophils perform their function through phagocytosis and the formation of NETs. These studies contribute to supplementing the data on the immunopathological mechanisms of feline inflammatory polyps. Further studies on the mechanisms of phagocytosis will enable a deeper understanding of the role of immune cells in the system of cellular homeostasis and dysfunctions of local immunity.

DECLARATIONS

Availability of data and materials

Data from the study can be provided upon a reasonable request.

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Authors' contributions

Mykola Zhelavskyi conceptualized the presented idea, validated the medical history, contributed to data collection, and conducted the experiment. Mykola Maryniuk designed the study and conducted clinical research. Maryna Drobot performed laboratory research. The final version of the manuscript was reviewed and approved by all authors.

Competing interests

The authors state that they do not have any competing interests.

Ethical considerations

The authors have diligently examined various ethical considerations, including but not limited to aspects such as plagiarism, obtaining consent for publication, preventing misconduct, avoiding data fabrication and/or falsification, ensuring against double publication and/or submission, and eliminating redundancy.

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The Effects of Different Concentrations of Vitamin D3 on Immunological Parameters of Immunosuppressed Rats Induced

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ABSTRACT

Vitamin D3 receptor is expressed in several types of immune cells suggesting that Vitamin D3 could have immune regulatory roles. The current study was conducted to investigate the role of Vitamin D3 in reducing the toxicity of the cisplatin on some Immunological parameters in the rat model. The current experiment was conducted on 80 adult white male rats within the age range of 9-12 weeks. The animals were divided into eight groups (10 animals in each group). The control group was dosed with the physiological solution until the end of experiment (C). Rats in the second treatment were injected with cisplatin (2 mg/kg, T1). Rats in the third (T2), fourth (T3), and fifth (T4) groups were injected with cisplatin at a concentration (2 mg/kg) and received Vitamin D3 at levels of 5000 IU, 10,000 IU, and 15,000 IU, respectively. The rats in the sixth (T5), seventh (T6), and eighth (T7) groups were subjected to Vitamin D3 at concentrations of 5000 IU,10,000 IU, and 15,000 IU, respectively. At the end of the experiment, which lasted 21 days, the animals were anesthetized, their weights were recorded, and blood samples were collected. The findings revealed a significant elevation in the levels of interleukin-12, tumor necrosis factor-alpha, C-reactive protein, lymphocyte percentage, monocyte percentage, and eosinophil percentage within group T1 compared to the control and other treatment groups that received Vitamin D3. The average percentage of white blood cells and neutrophils in group T1 was significantly lesser than other groups. It can be concluded that supplementation of different Vitamin D3 levels (5000-10,000 IU) have positive influences on the immunological parameters of immunosuppressed rats.

Keywords: Cisplatin, Immunosuppressant, Interleukin, Vitamin D3

INTRODUCTION

Vitamin D is an essential fat-soluble vitamin with multiple functions. Vitamin D receptor is expressed in several types of immune cells suggesting its immune regulatory roles. Vitamin D insufficiency has been suggested to increase the risk of autoimmune diseases (Ao et al., 2021). However, little is known regarding its immunomodulatory effects in the condition of immune suppression. The demand for nutritional supplements has been increasing dramatically worldwide, encompassing a wide range of products including vitamins, protein supplements, herbal supplements, mineral supplements, and essential fatty acids (Abdul Aziz et al., 2020).

Vitamin D is stored in the body tissue as a steroid hormone 25-hydroxycholecalciferol, called calciferol. Vitamin D is the only vitamin that the human body can make. Vitamin D can be produced in the skin when exposed to sunlight. Other sources of vitamin D include supplements and food (Ao et al., 2021). Vitamin D may also act as an antioxidant, anti-inflammatory, immunoprotect, immune regulator, cellular oncogenic signaling, and apoptosis regulator, as well as cell-cycle and angiogenesis controllers (Balasa et al., 2014). A study indicated that the immune response of patients with type 2 diabetes and spinal tuberculosis who receive long-term drug therapy can be improved by supplementation with 1,25(OH)2D3 (Abdul Aziz et al., 2020).

Cisplatin (Cl2H6N2Pt) is a powerful chemotherapy medication comprising platinum, widely utilized to address various cancer types affecting different tissues (Nasiri et al., 2020). This drug necessitates specific precautionary measures due to its serious side effects when used in hospitals. Despite its adverse effects, it remains the primary treatment for many cancer types. Cisplatin toxicity arises from cross-linking within and between nuclear strands, leading to various effects, including nephrotoxicity, hepatotoxicity, cardiotoxicity, thrombocytopenia, anemia, and dysfunction in the peripheral nervous system (Nasiri et al., 2020). Although the mechanism of cisplatin toxicity is well understood, there is still a lack of effective treatments and preventive measures to mitigate these changes.

Hence, there is a pressing need to develop a substance that can enhance the safe usage of cisplatin (Sun et al., 2019). Recent studies have explored the use of potent natural plant-based antioxidants and nutritional supplements

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contain Vitamin D, to prevent or reduce oxidative stress and inflammation caused by cisplatin. These approaches have shown promising effects on the pathophysiological status (Abdel-Daim et al., 2019).

This study aimed to investigate the role of Vitamin D3 in reducing the toxicity of the chemical drug (cisplatin) on some immunological parameters in rat model.

MATERIALS AND METHODS

Ethical approval

All research methods and practices and the use of experimental animals have been approved by the Animal Care and Use Committee (ACUC), Faculty of Veterinary Medicine, College of Education, University of Al-Qadisiyah, Iraq.

Experimental animals

The study was conducted in the animal laboratory of the College of Education / University of Al-Qadisiyah, under standard conditions of temperature (22-28°C), ventilation, and lighting duration (14 hours of light and 10 hours of darkness). The animals were given free feeding (metabolizable energy of 1850 kcal/kg, and crude protein 14 %) and water for the duration of the experiment. All the mice were weighed and sacrificed by cervical dislocation and blood samples (2 ml) were collected immediately at the end of the study (8 weeks of age; Florea and Büsselberg, 2011).

Experimental design

The experiment included 80 male white rats (average weight 180-200 gr) distributed into 8 groups (10 in each groups). The rats were obtained from the laboratory of the University of Al-Qadisiyah, Iraq. Each group included 10 animals for a period of 21 days.

The experiment consisted of eight different treatment groups. The control group (C) received normal saline physiological solution daily for 21 days. The first treatment group (T1) received weekly injections of cisplatin at a concentration of 2 mg/kg of body weight for 21 days. The second treatment group (T2) received the same cisplatin injections and a daily dose of Vitamin D3 at a concentration of 5000 IU for 21 days. In the third treatment group (T3), animals received cisplatin injections along with a daily dose of Vitamin D3 at a concentration of 10,000 IU for 21 days. The fourth treatment group (T4) received cisplatin injections and a daily dose of Vitamin D3 at a concentration of 15,000 IU for 21 days. The fifth treatment group (T5) solely received a daily dose of Vitamin D3 at 5000 IU of body weight for 21 days. The sixth treatment group (T6) received a daily dose of Vitamin D3 at 10,000 IU of body weight for 21 days. Lastly, the seventh treatment group (T7) received a daily dose of Vitamin D3 at 15,000 IU of body weight for 21 days.

Cisplatin dosage

Cisplatin was obtained from drug stores (Iraq) in the form of a liquid bottle with a concentration of (50 mg/100 ml) and the dose was prepared as described by Florea and Büsselberg (2011) at a concentration of 2 mg/kg of body weight by dissolving the required concentration depending on the average body weight of the animal. Inject each animal weekly under the peritoneum for 21 days.

Serum interleukin-12

The level of interleukin-12 in the serum was determined using the ELISA test and the Direct ELISA Sandwich method according to the instructions contained in the examination kit supplied by the Chinese company BT Lab (Florea and Büsselberg, 2011).

Serum C-reactive protein

The CRB-Latex assay is a rapid-slide stacking assay based on the modification of the latex fixation method and was used for the direct detection of C-reactive protein (CRP) and its semi-quantitative estimation in serum (Desoize and Madoulet, 2002).

Platelets, total and differential numbers of white blood cells

Platelets, total and differential numbers of white blood cells were examined using Auto Blood Analyzer Sysmex-XP300 (Germany) according to instruction of the device (Desoize and Madoulet, 2002).

Statistical analysis

After data collection and tabulation, statistical analysis program SPSS version 25 (USA) was used. Where the data were statistically analyzed according to the one-way ANOVA test, and the Least Significant Difference (LSD) was used for the posthoc test at the 0.05 level of significance (Daniel and Cross, 2018). Mean data are expressed with standard deviation (SD).

RESULTS AND DISCUSSION

Table 1 presents the interleukin-12, TNF-α, C-reactive protein, and platelet count concentrations in different treatment groups. In the first treatment (T1), there was a significant increase in the concentration of interleukin-12 compared to the

other groups ($p < 0.05$). Regarding TNF-α, the T1 group showed a significant increase compared to the control and other treatments ($p < 0.05$).

The elevated levels of TNF-α observed could potentially be linked to immune cells present within the tumor microenvironment, including Kupffer cells and macrophages (Titov et al., 2022). The findings align with prior researches of Florea and Büsselberg (2011), Ito et al. (2012), and Erbas et al. (2014) which emphasized the existence of diverse receptor types, such as TNF-α, IL-6, and IL-12 in hepatocytes.

One of the important effects of high concentration of TNF-α is to stimulate the accumulation of reactive oxygen species (ROS) in the epithelial cells, and this accumulation causes damage to the DNA of the epithelial cell and cause mutation in the cells (Laird et al., 2014).

The improvement was observed in the patients diagnosed with hepatic steatosis that administered with Vitamin D3 in their treatment regimen (Ito et al., 2012). It was demonstrated that Vitamin D3 possesses the capacity to mitigate the inflammatory process through various mechanisms. These mechanism is due to diminish the T1 helper 1 (Th1) cell response while augmenting the Th2 response, resulting in reduced levels of TNF-α and interleukin-1. Furthermore, Vitamin D3 was found to decreases the concentration of interleukin-12 by impacting both monocytes and B cells, as indicated in studies by Mirhosseini et al. (2017) and Mohammed et al. (2019).

The platelet count indicated a significant reduction in group T1 compared to the other treatments ($p < 0.05$). The cisplatin binds with the DNA of bone marrow cells, which leads to the destruction of the bone marrow and thus reduces the production of platelets (Mu et al., 2005; Perry, 2008).

Vitamin D3 demonstrated positive effects on the blood platelet count, which is consistent with findings from a previous study by Papapostoli et al. (2016). The results of current study indicated that vitamin D may influence the process of megakaryocytopoiesis, and the formation of platelet precursor cells. This process involves calcium-dependent events mediated by the non-genomic activity of Vitamin D receptors (VDR) within mitochondria (Weir et al., 2011). Moreover, the relationship between platelet count and oxidative stress is closely linked, highlighting the role of vitamin D as a well-known antioxidant (Wilson et al., 2007). Furthermore, vitamin D demonstrates anticoagulant and anti-inflammatory properties, which additionally enhance its positive effects on platelet function.

The data depicted in Table 1 regarding C-reactive protein revealed an increase in group T1 compared to the control group and other treatment regimens ($p < 0.05$). These results were agreed with the study by Wu et al. (2014). C-reactive protein is an inflammatory biomarker and a strong indicator of kidney abnormalities and functions in humans and animals (Stuveling et al., 2003). C-reactive protein production is assumed to be restricted by the liver, but a recent study suggested that the kidney may be a second site for C-reactive protein formation (Zoair, 2021). The observed effect of Vitamin D3 on platelet count can be attributed to its anti-inflammatory properties. Several studies including Mohammed and Qasim (2021), Abolhasani Zadeh et al. (2022), and Huldani et al. (2022) have reported that Vitamin D3 supplementation can lead to reduced levels of inflammatory markers, including TNF-α and C-reactive protein in rats. Another study highlighted that decreased levels of Vitamin D3 in circulation were associated with increased inflammation, marked by elevated levels of IL-6 and C-reactive protein (Hafsan et al., 2022). The Vitamin D3 supplementation helps to reduce C-reactive protein levels and increase IL-10, which possesses potent anti-inflammatory effects in rats. IL-10 can inhibit monocyte activation and suppress the production of inflammatory mediators (Zhang et al., 2010; Zakharova et al., 2019; Ansari et al., 2022).

Table 1. The effects of different concentrations of Vitamin D3 on immune parameters of immunosuppressed rats with cisplatin

Group	IL-12 (pg/ml)	Tumor necrosis factor alpha (pg/ml)	Platelet count (x103/mm)	C-reactive protein (mg/L)
C	6.28±0.88^{B}	0.16±1.34^{B}	2.31±341.80^{A}	7.06±0.03^{B}
T1	1.10±11.63^{A}	0.68±3.36^{A}	4.22±263.40^{B}	8.27±0.07^{A}
T2	0.76±10.04^{B}	±2.930.28^{B}	2.83±309.50^{A}	7.51±0.08^{B}
T3	±8.160.75^{B}	±1.800.27^{B}	±320.402.96^{A}	7.45±0.08^{B}
T4	±7.610.71^{B}	±1.380.20^{B}	2.81±334.00^{A}	7.41±0.03^{B}
T5	±6.690.83^{B}	0.12±1.27^{B}	±336.402.44^{A}	7.08±0.04^{B}
T6	±6.970.8^{B}	1.310.16^{B}	3.13±335.80^{A}	7.15±0.04^{B}
T7	±7.200.83^{B}	±1.320.15^{B}	2.94±333.00^{A}	7.20±0.04^{B}

^{ABC} Different superscript letters indicate significant differences in the columns between the treatments ($p < 0.05$). IL-12: Interleukin 12. C: The control group that received the physiological saline solution for the duration of the experiment (21 days). T1: The first treatment represents a group of rats that were immunosuppressed with cisplatin at a concentration of 2 mg/kg bw. T2: The second treatment represented a group of rats that were immunosuppressed and dosed with Vitamin D3 at a concentration (5000 IU). T3: The third treatment represents a group of rats that were immunosuppressed and dosed with Vitamin D3 at a concentration of (10,000 IU). T4: The fourth treatment represents a group of rats that were immunosuppressed and dosed with Vitamin D3 at a concentration (15,000 IU). T5: The fifth treatment represents the group of rats that received Vitamin D3 at a concentration (5000 IU). T6: The fifth treatment represents the group of rats that were dosed with Vitamin D3 at a concentration of (10,000 IU). T7: The fifth treatment represents the group of rats that were dosed with Vitamin D3 at a concentration of (15,000 IU)

Table 2 presents the results of the total number of white blood cells and the percentage of lymphocytes, neutrophils, monocytes, and eosinophils in different treatment groups. In group T1, there was a significant decrease in the total number of white blood cells, compared to the control group and other treatments ($p < 0.05$). Regarding the percentage of lymphocytes, group T1 displayed a significant increase, compared to the control group and other treatments ($p < 0.05$).

The results of the current study indicated a significant decrease in the average percentage of neutrophils in group T1, compared to the control and other treatment groups ($p < 0.05$). The results showed a significant increase in the percentage of monocyte cells in group T1, compared with the control and other treatment groups ($p < 0.05$). The results indicated a significant increase in the average percentage of eosinophils in the treatment group 1 compared to the control group and other treatment groups ($p < 0.05$).

The reduction in white blood cell count was similar to the study of Mackall et al. (1994). The reason for the decrease in the white blood cells in treatment 1 is attributed to the fact that cisplatin causes side effects when used, including the decrease of white blood cells, and it is associated with immune suppression. These effects may lead to a significant decrease in white blood cells in peripheral blood. Additionally, cisplatin affects T and B lymphocytes in the spleen and lymph nodes (Florea and Busselberg, 2011). Chemotherapy is contributed to decline in white blood cell, by influencing the liver, and kidneys (Ito et al., 2012; Jhaveri et al., 2013). According to a study by Zhang et al. (2010), chemotherapy-induced reduction in white blood cell and it could be due to its impact on bone marrow, and it is reducing the ability of bone marrow to produce cells and compromising the immune system, thereby reducing overall body protection. This could be the results of an increase in the levels of IL-10, known for its immunosuppressive properties, and inhibiting IL-10 could potentially restore immunity function (Florea and Büsselberg, 2011). IL-10 is used therapeutically to inhibit the proliferation and cytokine production of Th1 lymphocytes (Nguyen et al., 2021). As a consequence of using cisplatin, there was a decrease in the levels of neutrophils, those results in weakening the body's defense mechanisms, and causing a deficiency of macrophages.

The expressing vitamin D receptors and 1α-hydroxylase, leading to anti-inflammatory effects (Ao et al., 2021). Vitamin D3 reduces the differentiation and secretion of inflammatory cytokines (IL-2, IFNγ, and TNF-α) by Th1 cells while enhancing the differentiation and secretion of anti-inflammatory cytokines (IL-4, IL-5, and IL-10) by Th2 cells. Furthermore, Vitamin D3 enhances T-cell differentiation and regulation, preventing an exaggerated stress response (Florea and Büsselberg, 2011).

Chemotherapy-induced bone weakening and immune system defects result in increased IL-12 levels, while Vitamin D3's role in immune regulation helps to counteract inflammation and maintain immune balance. The results of current study indicated that vitamin D supplementation can increase the level of immune cells such as monocytes, lymphocytes, and eosinophils.

Table 2. The effects of different concentrations of Vitamin D3 on the differential number of white blood cells in rats immunosuppressed with cisplatin.

Group	W.B.C Count	LYM (%)	NEU (%)	MONO (%)	Eosino (%)
C	9.96 ± 0.06^{A}	65.99 ± 3.11^{B}	25.00 ± 1.92^{A}	5.29 ± 0.22^{BC}	3.90 ± 0.16^{B}
T1	4.76 ± 0.04^{C}	74.04 ± 2.16^{A}	11.89 ± 0.98^{B}	7.22 ± 0.30^{A}	7.01 ± 0.26^{A}
T2	6.33 ± 0.19^{B}	63.64 ± 1.88^{B}	23.44 ± 0.57^{A}	6.42 ± 0.21^{B}	4.52 ± 0.21^{B}
T3	6.59 ± 0.27^{B}	68.76 ± 1.61^{B}	20.10 ± 0.74^{A}	6.11 ± 0.09^{B}	4.17 ± 0.17^{B}
T4	8.12 ± 0.13^{A}	67.99 ± 1.80^{B}	19.73 ± 2.21^{A}	5.98 ± 0.33^{B}	4.2 ± 0.18^{B}
T5	8.96 ± 0.13^{A}	66.34 ± 2.31^{B}	24.18 ± 1.16^{A}	5.26 ± 0.20^{BC}	3.90 ± 0.12^{B}
T6	8.10 ± 0.08^{A}	67.35 ± 2.07^{B}	23.01 ± 1.29^{A}	5.19 ± 0.25^{BC}	4.02 ± 0.10^{B}
T7	8.06 ± 0.09^{A}	68.51 ± 1.14^{B}	22.64 ± 1.19^{A}	5.84 ± 0.19^{B}	4.01 ± 0.21^{B}

^{ABC} Different superscript letters indicate significant differences in the columns between the treatments ($p < 0.05$). C: The control group that received the physiological saline solution for the duration of the experiment (21 days). T1: The first treatment represents a group of rats that were immunosuppressed with cisplatin at a concentration of 2 mg/kg bw. T2: The second treatment represented a group of rats that were immunosuppressed and dosed with vitamin D3 at a concentration (5000 IU). T3: The third treatment represents a group of rats that were immunosuppressed and dosed with vitamin D3 at a concentration of (10000 IU). T4: The fourth treatment represents a group of rats that were immunosuppressed and dosed with vitamin D3 at a concentration (15,000 IU). T5: The fifth treatment represents the group of rats that received vitamin D3 at a concentration (5000 IU). T6: The fifth treatment represents the group of rats that were dosed with vitamin D3 at a concentration of (10,000 IU). T7: The fifth treatment represents the group of rats that were dosed with vitamin D3 at a concentration of (15,000 IU). WBC: White blood cells, LYM: Lymphocyte, NEU: Neutrophile, MONO: Monocyte, EOSINO: Eosinophile.

CONCLUSION

It can be concluded that different levels of Vitamin D3 (5000-15,000 IU) have positive influences on immunological parameters in immunosuppressed rats. There is a need to evaluate the effects of Vitamin D supplementation at other different doses on the other immunosuppressed animal species and human.

DECLARATIONS

Competing interests

The authors declare that they have no conflict of interest.

Authors' contribution

All authors contributed to the conceptualization and design of the study. Material preparation, data collection, and analysis were performed by Safa Masser Kmosh and Ahmed J. Al-Naely. The first draft was written by Safa Masser Kmosh. The analysis of data was conducted by Ahmed J. Al-Naely. All authors read and approved the final manuscript.

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Availability of data and materials
Data from the study are available according to a reasonable request.

Ethical considerations
The study was conducted originally and all analyzed data are prepared based on the experiment results. The text of the article is written originally without any unpermitted used from other published articles.

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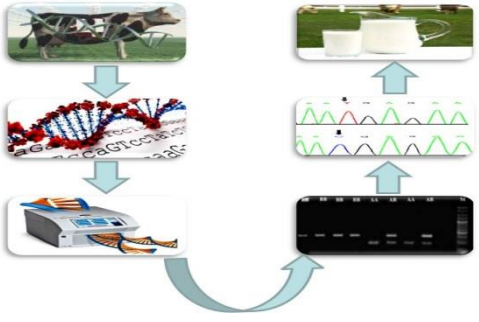

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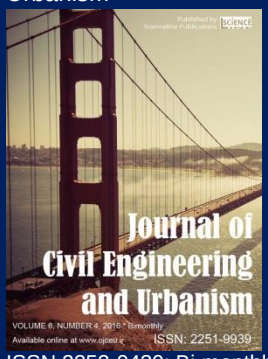

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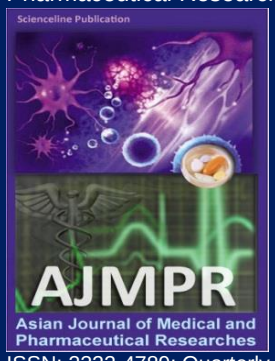

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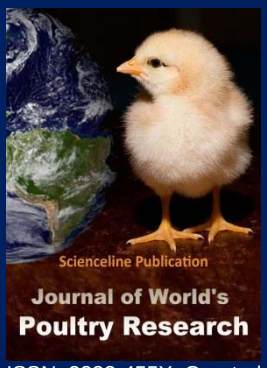

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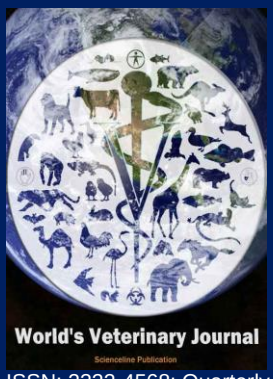

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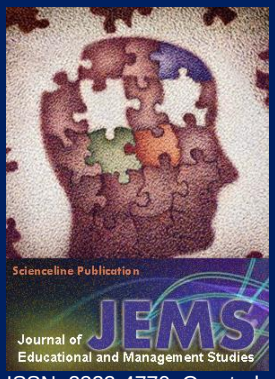

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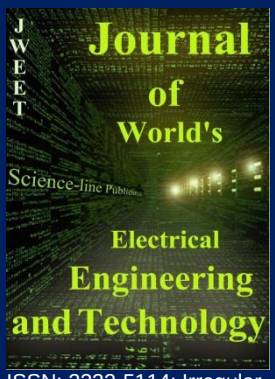

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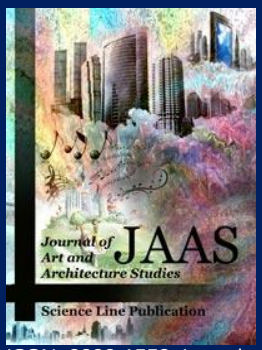

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