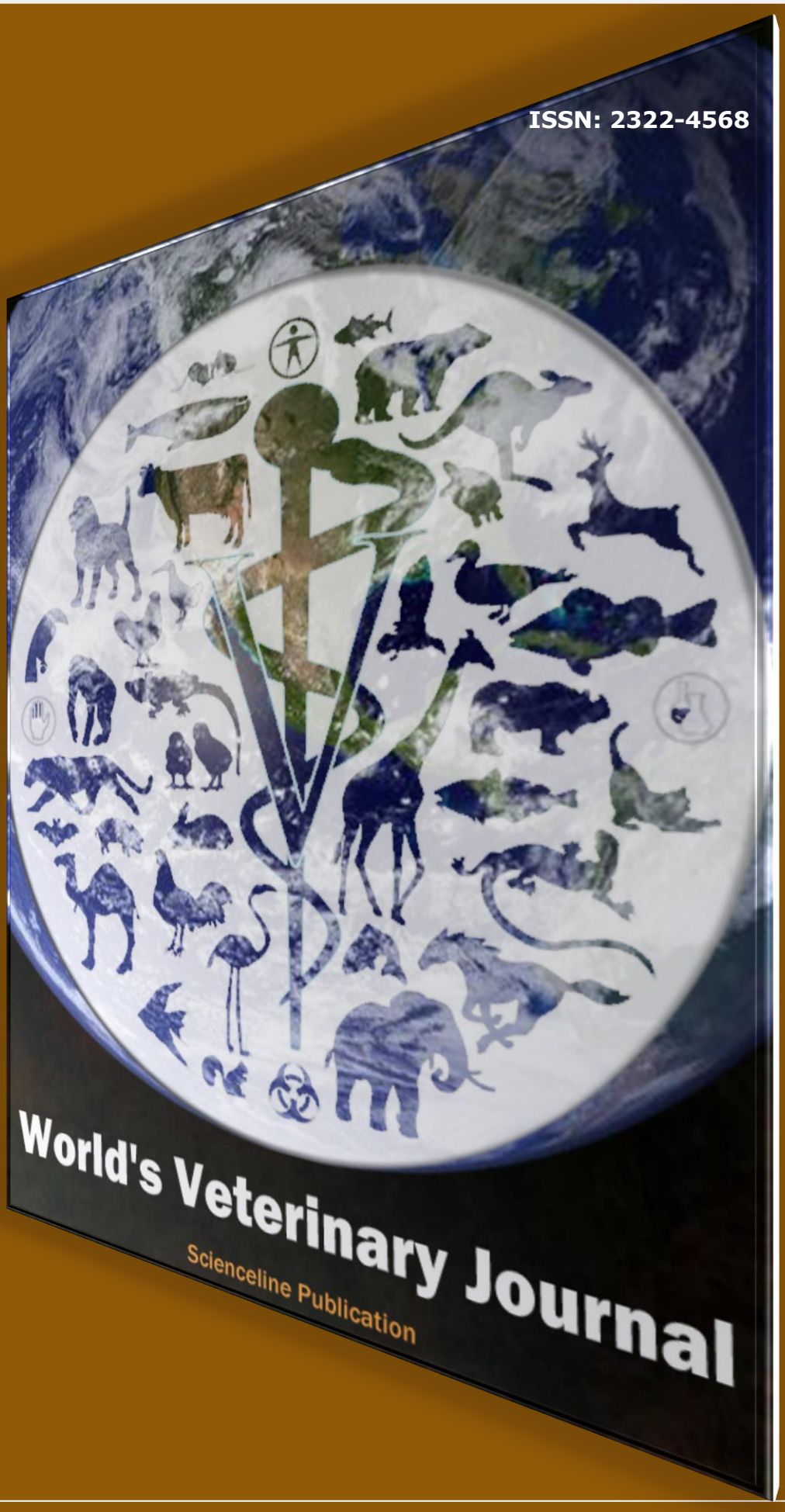
World's Veterinary Journal

BOOKLET



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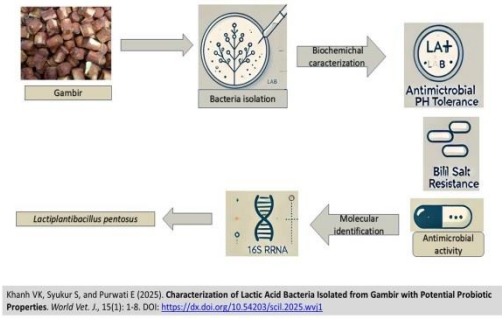
Volume 15 (1); March, 2025

Research Paper

Characterization of Lactic Acid Bacteria Isolated from Gambir with Potential Probiotic Properties

Khanh VK, Syukur S, and Purwati E.

World Vet. J. 15(1): 1-8, 2025; pii:S232245682500001-15
DOI: https://dx.doi.org/10.54203/scil.2025.wvj1



ABSTRACT: Gambir is commonly used as a key ingredient in betel quid and is one of Indonesia's major agricultural commodities. West Sumatra is the primary production region, contributing approximately 80-90% of the country's total gambir production. This study aimed to investigate the potential of lactic acid bacteria (LAB) isolated from gambir, assessing their potential probiotic qualities using biochemical and molecular approaches. Biochemical characterization included catalase activity testing and fermentation pattern analysis, while molecular identification was carried out through the *16S rRNA* gene sequence. The results revealed that the isolated LAB strains were Gram-positive, bacilli-shaped, catalase-negative, and exhibited hetero-fermentative behavior. Further biochemical analysis confirmed their ability to ferment a variety of sugars but not produce gas from glucose. Basic Local Alignment Search Tool analysis showed that the bacterial isolate from gambir, labeled with the sample code GM2, was closely related to *Lactiplantibacillus pentosus,* a species recognized for its probiotic potential. The isolates showed antimicrobial activity against common foodborne pathogens like *Salmonella* spp. and other pathogens, suggesting their potential use in food preservation. The present study also demonstrated the LAB isolates' tolerance to low pH and bile salts, which are key attributes for probiotic candidates. Thus, the findings of the current study suggest that LAB from gambir possess promising characteristics for application in probiotic products and as biocontrol agents in food safety.

Keywords: *16s rRNA*, Gambir, Lactic acid bacteria, Probiotic

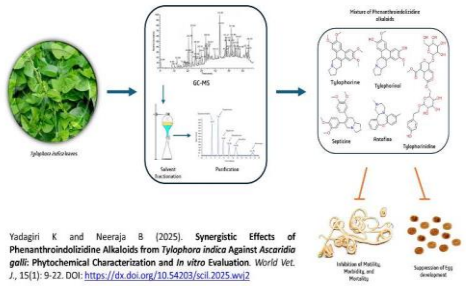
[Full text-PDF]

Research Paper

Synergistic Effects of Phenanthroindolizidine Alkaloids from *Tylophora indica* Against *Ascaridia galli*: Phytochemical Characterization and *In vitro* Evaluation

Yadagiri K and Neeraja B.

World Vet. J. 15(1): 9-22, 2025; pii:S232245682500002-15
DOI: https://dx.doi.org/10.54203/scil.2025.wvj2



ABSTRACT: The increasing resistance of helminths such as *Ascaridia galli* to conventional anthelmintics has necessitated the search for alternative treatments from natural sources. This study aimed to assess the anthelmintic properties of phenanthroindolizidine alkaloids derived from *Tylophora indica* leaves, a plant renowned for its medicinal value, including its traditional use in treating respiratory disorders, inflammation, and various infections. The leaves were subjected to sequential extraction with chloroform, ethanol, and water, which produced yields of 7.2%, 17.8%, and 11.2%, respectively. Phytochemical analysis revealed that the ethanol extract was rich in bioactive compounds, including significant amounts of alkaloids, flavonoids, phenols, and terpenoids. Quantitative analysis confirmed the ethanol extract's superiority, displaying the highest contents of phenolics (7.51 ± 0.62 mg/g), flavonoids (9.34 ± 1.63 mg/g), and alkaloids (17.65 ± 1.69 mg/g), underscoring its potential for various therapeutic applications. Further fractionation and High-Performance Liquid Chromatography (HPLC) purification isolated key phenanthroindolizidine alkaloids, including Tylophorinidine, Tylophorine, Septicine, Tylophorinol, and Antofine. Structural characterization via Nuclear Magnetic Resonance (NMR) and High-Resolution Mass Spectrometry (HRMS) validated these compounds. *In vitro* assays demonstrated significant dose-dependent anthelmintic activity against *Ascaridia galli* worms. Ethanol extracts exhibited the highest mortality rates, achieving 100% mortality within 24 hours at a concentration of 5 mg/mL. The mixture of all five alkaloids at 500 µg/mL showed a synergistic effect, leading to rapid and complete anthelmintic action. The egg embryonation assay further highlighted the efficacy of these alkaloids. The egg embryonation assay further demonstrated the potent efficacy of these alkaloids, with the mixture at 500 µg/mL inhibiting 92.67% of egg development, surpassing the positive control, i.e. piperazine citrate, which showed 87.25% inhibition. Among individual alkaloids, Tylophorinidine exhibited the highest inhibition of egg embryonation (80.76%), followed by Antofine (78.42%). These findings demonstrated the potent anthelmintic properties of phenanthroindolizidine alkaloids from *Tylophora indica*, particularly when used in combination (Tylophorinidine, Tylophorine, Septicine, Tylophorinol, and Antofine), compared to their individual effects. The study underscores the potential of these compounds as effective treatments for helminth infections and highlights the importance of further research to isolate specific mechanisms and optimize their therapeutic efficacy.

Keywords: Anthelmintic activity, *Ascaridia galli*, Phenanthroindolizidine alkaloids, Phytochemical analysis, *Tylophora indica*

[Full text-PDF]

Research Paper

Effects of Post-Therapy Changes on the Level of Immunoglobulin M in Dogs with Dermatitis

Pusparini NPDP, Suwiti NK, Suardana IBK, and Besung INK.
World Vet. J. 15(1): 23-30, 2025; pii:S232245682500003-15
DOI: https://dx.doi.org/10.54203/scil.2025.wvj3

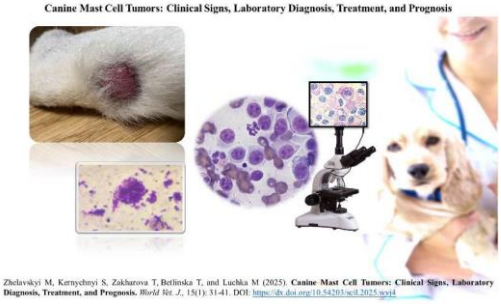
Pusparini NPDP, Suwiti NK, Suardana IBK, and Besung INK (2025). **Effects of Post-Therapy Changes on the Level of Immunoglobulin M in Dogs with Dermatitis**. *World Vet. J.*, 15(1): 23-30. DOI: https://dx.doi.org/10.54203/scil.2025.wvj3

ABSTRACT: Dermatitis is an inflammation of the skin characterized by itching, hair loss, lesions, and redness. Various agents can cause dermatitis, including *Sarcoptes scabiei, Demodex canis*, and *Microsporum canis*. Animals experiencing dermatitis undergo internal changes in their bodies, particularly in the immune system. The presence of an infection is usually preceded by the appearance of Immunoglobulin M (IgM). This study aimed to determine the differences in IgM levels in dogs with dermatitis before therapy (pre-therapy) and after therapy (post-therapy), as well as the differences in IgM levels between dogs with mild and severe dermatitis. The study involved 40 local dogs, divided into two groups, including 20 dogs with mild dermatitis and 20 dogs with severe dermatitis. Serum sampling was conducted in two phases: the first phase was pre-therapy, and the second phase was 14 days after therapy (post-therapy). The therapy administered to dogs with mild dermatitis consisted of diphenhydramine HCl and ivermectin, while the therapy for dogs with severe dermatitis included diphenhydramine HCl, ivermectin, amoxicillin, and dexamethasone. Serum samples from the dogs were then tested using the Enzyme-Linked Immunosorbent Assay method. The results of the study revealed that serum IgM levels in dogs with mild and severe dermatitis did not show any significant difference. In dogs with mild dermatitis, serum IgM levels before therapy were not statistically different compared to those after therapy. However, in dogs with severe dermatitis, serum IgM levels before therapy were significantly higher compared to after therapy. The results of this study indicate that therapy can impact serum IgM levels in dogs with severe dermatitis, while it does not significantly affect these levels in cases of mild dermatitis.
Keywords: Dermatitis, Dog, Enzyme-linked immunosorbent assay, Immunoglobulin M, Ivermectin, Therapy
[Full text-PDF]

Research Paper

Canine Mast Cell Tumors: Clinical Signs, Laboratory Diagnosis, Treatment, and Prognosis

Zhelavskyi M, Kernychnyi S, Zakharova T, Betlinska T, and Luchka M.
World Vet. J. 15(1): 31-41, 2025; pii:S232245682500004-15
DOI: https://dx.doi.org/10.54203/scil.2025.wvj4



Zhelavskyi M, Kernychnyi S, Zakharova T, Betlinska T, and Luchka M (2025). **Canine Mast Cell Tumors: Clinical Signs, Laboratory Diagnosis, Treatment, and Prognosis**. *World Vet. J.*, 15(1): 31-41. DOI: https://dx.doi.org/10.54203/scil.2025.wvj4

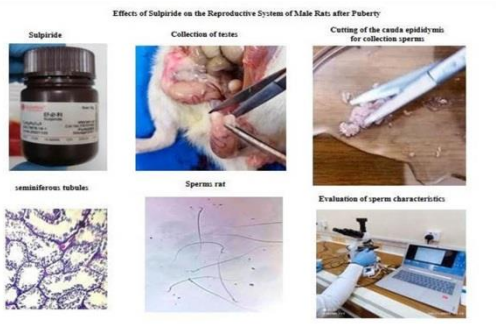
ABSTRACT: Canine mast cell tumors, a tumor originating from mast cells involved in allergic reactions and inflammation, are among the most common skin tumors in dogs. The present study aimed to explore the clinical features, diagnostic approaches, and prognosis of canine mastocytomas through a case study. A 5-year-old male Akita, weighing 35.8 kg, was brought to the Doctor VET veterinary clinic in Kamianets-Podilskyi, Ukraine, for evaluation. Upon initial examination, the dog had a body temperature of 38.5°C, a heart rate of 74 beats per minute (bpm), and a respiratory rate of 28 breaths per minute, all of which were within normal physiological limits. The animal was alert and responsive and displayed no signs of systemic distress. A detailed physical examination revealed a tumor located 35.2 mm below the plantar surface of the tarsal joint (art. tarsi). The tumor was round, mobile, and surrounded by a thin fibrous capsule, with no signs of pain or discomfort during palpation. Cytological analysis showed a high-cellularity smear with numerous mast cells scattered throughout the field. These cells were round to oval in shape with abundant cytoplasm containing dense, basophilic to metachromatic granules. The hematological evaluation indicated a systemic inflammatory or immune response triggered by the tumor, as evidenced by neutrophilic leukocytosis (73.1%; 8.89×10^9/L). Biochemical analysis revealed an elevated alkaline phosphatase activity level (4.45 µmol/L), suggesting systemic involvement. The tumor was surgically excised, ensuring complete removal with wide margins to minimize the risk of recurrence. Histological examination of the excised tissues confirmed a densely cellular neoplastic infiltrate composed predominantly of mast cells arranged in sheets and clusters. The mast cells displayed significant cellular and nuclear pleomorphism, characterized by moderate to marked anisocytosis and anisokaryosis. While no significant necrosis was observed, scattered apoptotic bodies were present, indicating ongoing cellular turnover. This case highlighted the critical importance of early diagnosis and comprehensive management of canine mastocytomas. Low-grade tumors often carry a favorable prognosis when treated promptly and appropriately. However, higher-grade or poorly differentiated tumors may require multimodal therapeutic approaches to achieve better outcomes.
Keywords: Canine, Diagnosis, Mast cell tumor, Mastocytoma, Skin tumor

[Full text-PDF]

Research Paper

Effects of Sulpiride on the Reproductive System of Male Rats after Puberty

Abd AA, Al-Juhaishi OA, and Jumma QS.
World Vet. J. 15(1): 42-48, 2025; pii:S232245682500005-15
DOI: https://dx.doi.org/10.54203/scil.2025.wvj5



Abd AA, Al-Juhaishi OA, and Jumma QS (2025). **Effects of Sulpiride on the Reproductive System of Male Rats after Puberty.** *World Vet. J.*, 15(1): 42-48. DOI: https://dx.doi.org/10.54203/scil.2025.wvj5

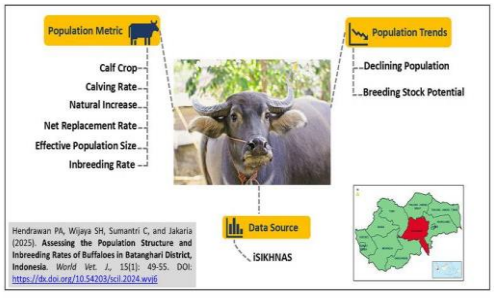
ABSTRACT: Sulpiride is an antipsychotic drug commonly used in humans to mitigate the effects of stress by selectively targeting central dopaminergic receptors. During male rat puberty, neurotransmitter systems, including the dopaminergic system, undergo significant development, playing a crucial role in the release of gonadal hormones and the regulation of reproductive function. The present study aimed to investigate the effects of sulpiride on reproduction parameters in adult male rats. This study used 30 adult male rats with an average body weight of 250-300g and an average age of 90-95 days. The rats were randomly divided into three groups of 10 each. Group 1 (G1) received 10 mg/kg sulpiride, Group 2 (G2) received 25 mg/kg sulpiride, and the control group (G3) received normal saline, all administered via gavage. This study evaluated hematological (testosterone, luteinizing hormone, prolactin ,and Follicle-stimulating hormone) and histopathological parameters) spermatogenesis, seminiferous tubules, and total sperm count). The histopathology result of the testes from treated rats revealed significant histological changes. In G1, the seminiferous tubules exhibited destruction, with disrupted spermatogenesis and reduced numbers of sperm in the lumen. These changes were more pronounced in G2 ,which received the higher dose of sulpiride (25 mg/kg). In contrast ,the control group (G3) displayed normal histological structures and spermatogenesis. Hormonal analysis showed a significant decrease in testosterone and luteinizing hormone (LH) levels in G2 compared to G1 and G3. The hematological results for blood serum showed that the concentration of the hormone prolactin was also significantly increased in G2 treated with 25 mg/kg sulpiride as compared with G1 and G3; the concentration of follicle-stimulating hormone (FSH) levels did not differ significantly across groups. Sperm motility and concentration were significantly reduced in G2 compared to G1 and G3, accompanied by a significant increase in the percentage of abnormal and dead sperm .Histological findings further confirmed severe destruction of the seminiferous tubules in G2 compared to G1 and the control group. In conclusion, administering sulpiride at concentrations of 10 mg/kg and 25 mg/kg in adult male rats caused significant structural and functional defects in the seminiferous tubules of the testes.
Keywords: Follicle-stimulating hormone, Luteinizing hormone, Male rat, Prolactin, Sulpiride, Testosterone

[Full text-PDF]

Research Paper

Assessing the Population Structure and Inbreeding Rates of Buffaloes in Batanghari District, Indonesia

Hendrawan PA, Wijaya SH, Sumantri C, and Jakaria.
World Vet. J. 15(1): 49-55, 2025; pii:S232245682500006-15
DOI: https://dx.doi.org/10.54203/scil.2025.wvj6



Hendrawan PA, Wijaya SH, Sumantri C, and Jakaria (2025). **Assessing the Population Structure and Inbreeding Rates of Buffaloes in Batanghari District, Indonesia.** *World Vet. J.*, 15(1): 49-55. DOI: https://dx.doi.org/10.54203/scil.2024.wvj6

ABSTRACT: Buffaloes are important in animal husbandry, agriculture, and sociocultural and religious activities in Indonesia. The buffalo population has decreased at the national and regional levels, including in the Batanghari District, Jambi Province, Indonesia. This study analyzed the population structure, effective population size, and inbreeding rate of buffalo populations in the Batanghari District, Jambi, Indonesia, based on secondary data. The data population of 3,149 buffaloes used in this study was sourced from the Integrated National Animal Health System (ISIKHNAS) in the Batanghari District in 2023. The results showed a calf crop of 21.71%, a calving rate of 16.61%, a natural increase of 14.74%, and a net replacement rate of 279.51%. The effective population size was 592 heads, and the inbreeding rate was 0.08%. It can be concluded that the natural increase rate of the buffalo population in the Batanghari District was low, but the number of young replacement animals was sufficient. The effective population size was 592 heads, and the level of inbreeding per generation remained within acceptable limits. Although the buffalo population in the Batanghari District exhibited a negative trend, it still had potential as a source of breeding stock, as indicated by the replacement rate.
Keywords: Buffalo, Effective population, Inbreeding rate, Population structure

[Full text-PDF]

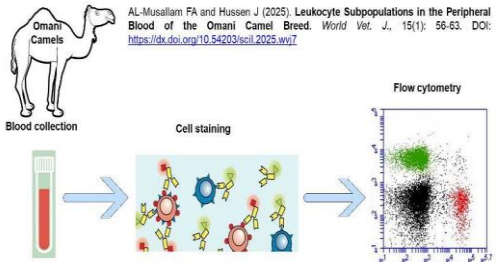
Research Paper

Leukocyte Subpopulations in the Peripheral Blood of the Omani Camel Breed

AL-Musallam FA and Hussen J.

World Vet. J. 15(1): 56-63, 2025; pii:S232245682500007-15

DOI: https://dx.doi.org/10.54203/scil.2025.wvj7



ABSTRACT: Breed-specific variations in immune responses have been studied across various species and breeds. The identification of camel breeds with high immune competence can enhance the breeding of camels with superior immune responsiveness. To date, no study has examined the immune cell composition in the blood of the Omani camel breed. The present study aimed to analyze the immunophenotype of blood leukocytes in the Omani camel breed and investigate the impact of age and gender on the tested immune parameters. To do so, blood samples were collected from 32 clinically healthy camels, randomly selected and comprising 17 camel calves (8 males and 9 females) and 15 adult camels (4 males and 11 females). The samples were tested using flow cytometry and membrane immune fluorescence. The results of the present study revealed a significantly lower count of white blood cells (WBC) in the Omani camel breed than the reference ranges reported for dromedary camels in the published literature. The leukogram was characterized by the dominance of neutrophils (54-60 %) in the blood, followed by lymphocytes (23-33 %). When compared to adult camels, the leukogram pattern in young Omani camels was characterized by elevated WBC and lymphocytes but low levels of eosinophilic granulocytes. The analysis of lymphocyte subsets revealed the dominance of gd T cells over helper T cells and B cells in the blood of young camel calves, confirming that camels belong to the gd T cell-rich species. In addition, lower numbers of B cells and helper T cells in young camels suggest lower cell-mediated and humoral immune functionality compared to adults. Although some differences were identified between male and female adult camels, these results are limited by the low numbers of male camels within the adult group. In conclusion, the distinct leukogram patterns observed in young and adult camels highlight the significant impact of age on the immune competence of Omani camels.
Keywords: Camel, Omani camel, Immunophenotype, Leukocyte

[Full text-PDF]

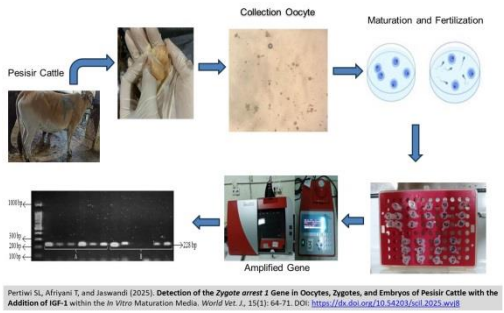
Research Paper

Detection of the Zygote arrest 1 Gene in Oocytes, Zygotes, and Embryos of Pesisir Cattle with the Addition of IGF-1 within the In Vitro Maturation Media

Pertiwi SL, Afriyani T, and Jaswandi.

World Vet. J. 15(1): 64-71, 2025; pii:S232245682500008-15

DOI: https://dx.doi.org/10.54203/scil.2025.wvj8



ABSTRACT: *Zygote arrest 1* (*ZAR 1*) is a maternal gene that plays a crucial role in the oocyte-to-embryo transition. The present study aimed to investigate the presence or absence of the *ZAR 1* gene in oocytes, zygotes, and embryos of Pesisir Cattle. Ovaries were collected from cattle at slaughterhouses, and oocytes were retrieved at the Biotechnology Laboratory. The collected oocytes were matured in a maturation medium supplemented with Insulin-like Growth Factor-1 (IGF-1) at a concentration of 10 µg/ml or without IGF-1 for 24 hours in a CO_2 incubator maintained at 38.5°C. Following maturation, the oocytes were fertilized for 18 hours, and the resulting embryos were cultured for 48 hours in a CO_2 incubator at 38.5°C. The samples were then subjected to PCR analysis. The amplification results revealed the presence of the *ZAR 1* gene band at the target size of 228 bp in oocytes matured with and without IGF-1. A comparative analysis of oocytes and embryos showed differences in the gene bands, particularly in samples supplemented with IGF-1. These findings suggest that IGF-1 supplementation during oocyte maturation significantly influences *ZAR 1* gene expression in embryos. The observed variations in *ZAR 1* gene expression across the oocyte, zygote, and embryo stages highlight the gene's pivotal role in reprogramming post-fertilization and maintaining early embryonic development.

Keywords: Embryo, Insulin-like Growth Factor-1, Oocyte, Zygote, *Zygote arrest 1* gene

[Full text-PDF]

Research Paper

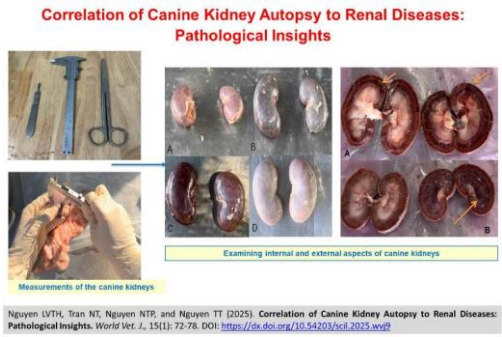
Correlation of Canine Kidney Autopsy to Renal Diseases: Pathological Insights

Nguyen LVTH, Tran NT, Nguyen NTP, and Nguyen TT.

World Vet. J. 15(1): 72-78, 2025; pii:S232245682500009-15

DOI: https://dx.doi.org/10.54203/scil.2025.wvj9

ABSTRACT: Kidneys play a vital role in regulating fluids, electrolytes, hormones, and metabolic waste in canines. This study aimed to enhance the understanding of the correlations between canine kidney autopsy findings and renal diseases. A total of 194 domestic dog samples, including 153 males and 41 females with an average age of 3.88 years (ranging from 1 to 7 years), were physically examined using post-mortem evaluations to understand the prevalence and characteristics of kidney diseases, focusing on both external and internal examinations of the kidneys. Key parameters such as kidney size, texture, and coloration were measured to provide insights into the overall kidney health of the canine population in Vietnam. Results indicated that 22.68% of the dogs had kidney cysts, 29.38% showed signs of external hemorrhage, and 52.06% of the cases exhibited internal hemorrhage, proving to be a condition linked to increased renal vascular resistance and further potentially contributing to renal dysfunction. No evidence of necrosis was detected, and the majority of renal capsules (90.98%) were easy to peel off for further analysis. Kidney size and weight varied obviously in dogs presenting with specific hemorrhagic conditions. This study emphasized the importance of external and internal kidney evaluations in diagnostic measurements and treatment protocols for canine renal diseases while also providing further insights into the current status of the canine population in Vietnam.



Keywords: Dog, Hemorrhage, Kidney, Post-mortem, Renal disease

[Full text-PDF]

Research Paper

Prevalence of Multi-Drug Resistance *Escherichia coli* in Broiler Chicken Meat in Jember, Indonesia

Suswati E, Pratama DR, and Hermansyah B.

World Vet. J. 15(1): 79-85, 2025; pii:S232245682500010-15

DOI: https://dx.doi.org/10.54203/scil.2025.wvj10

ABSTRACT: Antibiotic resistance has become one of the global health problems nowadays. Chicken meat is one of the largest food commodities in the world. *Escherichia coli* (*E. coli*) is one of the bacteria that is often found in chicken meat. These bacteria are capable of being pathogenic in both animals and humans. This study aimed to determine the prevalence of multidrug-resistant *E. coli* isolated from broiler chicken meat in the study location. The *E.* coli utilized in this study were derived from 25 grams of chicken meat obtained from 30 samples procured from six markets within the Jember district. The resistance test method used was Kirby-Bauer with Mueller-Hinton media. The results of the study showed that 100% of chicken meat was contaminated with *E. coli*. All isolated *E. coli* from samples in the study were multidrug-resistant. *E. coli* was 100% resistant to cotrimoxazole and cefixime, 96.67% resistant to chloramphenicol and amoxicillin-clavulanic, 93.3% resistant to tetracycline, 90% resistant to ceftriaxone, and 80% resistant to azithromycin and ciprofloxacin. The minimum resistance profile to 5 types of antibiotics with a multiple antibiotic resistance (MAR) index was between 0.625-1. Thus, the study revealed a high risk of infection associated with the consumption of uncontrolled chicken meat.

Keywords: Antibacterial agent, Chicken, *Escherichia coli,* Multi-drug resistance

[Full text-PDF]

Research Paper

In Vivo Evaluation of a Polyethylene Glycol-Based Cryoprotectant during Cold Stress in a Rat Model

Zazharskyi V, Zaslavskyi O, Sosnickyi O, Bilan M, Zazharska N, and Biben I.
World Vet. J. 15(1): 86-95, 2025; pii:S232245682500011-15
DOI: https://dx.doi.org/10.54203/scil.2025.wvj11

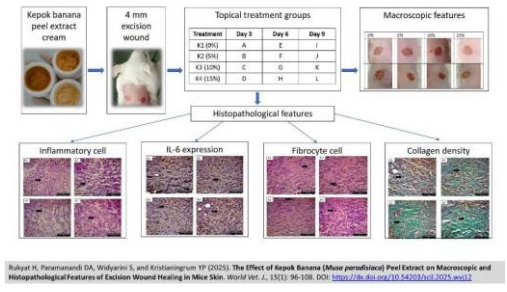
ABSTRACT: Cold stress is an environmental factor that impacts the viability of animals and humans. This study aimed to determine the effectiveness of a cryoprotectant based on polyethylene glycol in reducing cold stress in laboratory rats. For the experiment, 30 outbred Wistar rats (5 weeks) with an average body weight of 55.1 ± 5.3 g were used. Three groups of animals were formed (10 rats per group). The first group served as a positive control, kept at a room temperature of +18 – +20 °C and received 0.1 ml of 0.9% NaCl solution. The second group, the negative control, was kept in critically low temperatures (+2–+4°C) and administered 0.1 ml of 0.9% NaCl solution per experimental animal. Rats of the third group were subjected to cold stress and received 0.1 ml of the experimental preparation. Cold stress in laboratory rats was created using a cooling thermostat. Observation for 14 days included monitoring the dynamics of changes in the live weight of animals (before the start of the experiment, and on days 7 and 14 of the observation) as well as biochemical and haematological blood indicators. Fecal samples were collected from the rectum to determine the qualitative and quantitative state of the intestinal microbiota. The survival level of animals that received the experimental drug within fourteen days was 80.0%, compared to only 40% in the untreated group. When using the experimental drug in laboratory animals, an increase in body weight was noted. The number of full-fledged *Escherichia coli* in rats that received the drug was 3.4 times higher than the indicator of the group of animals that was kept at a critically low temperature without the drug. The prolonged low temperature in control rats had a negative effect on the animal's body as evidenced by increased leukocyte counts and ALT levels, as well as decreased ALT/AST ratio, and total bilirubin. The use of an experimental polyethylene glycol-based preparation had a positive effect on the weight of rats, blood parameters, and intestinal microbiota of rats under cold stress.
Keywords: Biochemistry, Blood morphology, Cold stress, Cryoprotectant, Intestinal microbiota, Rat

[Full text-PDF]

Research Paper

The Effect of Kepok Banana (*Musa paradisiaca*) Peel Extract on Macroscopic and Histopathological Features of Excision Wound Healing in Mice Skin

Rukyat H, Paramanandi DA, Widyarini S, and Kristianingrum YP.
World Vet. J. 15(1): 96-108, 2025; pii:S232245682500012-15
DOI: https://dx.doi.org/10.54203/scil.2025.wvj12



ABSTRACT: Kepok banana peel extract is known to have a bioactive content that can accelerate wound healing. The present study sought to evaluate the effects of Kepok banana peel extract on the macroscopic and histopathological features of excision wound healing in mouse skin. A total of 24 BALB/c mice were divided into four treatment groups, with each group consisting of six mice. The mice were further divided into three subgroups based on observation days, including days 3, 6, and 9. Each mouse received two excision wounds. The four treatment groups included K1 (control), K2 (topical therapy using Kepok banana peel extract ointment 5%), K3 (topical therapy using Kepok banana peel extract ointment 10%), and K4 (topical therapy using Kepok banana peel extract ointment 15%). The Kepok banana peel extract was obtained using the maceration method, and the ointment Kepok was prepared as a cream with extract concentrations of 5%, 10%, and 15%, using bio cream as the base. Wound healing activity was evaluated across three phases, including inflammatory, proliferative, and remodeling. The parameters observed in the current study included macroscopic and histopathological characteristics of the wound. Macroscopic observations involved wound size, while histopathological analysis included quantification of inflammatory cells, fibrocytes, collagen density, and interleukin-6 expression. Therapy using Kepok banana peel extract ointment was administered for 9 days in the treatment groups. Macroscopic features of the wounds were observed daily, and skin samples from each group were collected on days 3, 6, and 9. The results demonstrated that the 5%, 10%, and 15% concentrations of Kepok banana peel extract formed wound healing areas on mouse skin on days 3, 6, and 9, and were able to reduce the number of inflammatory cells on days 3, 6 and 9 able to reduce IL-6 expression on days 3, 6 and 9, unable to increase fibrocytes on day 3, 6, and 9 and able to increased collagen density on days 6 and 9. The 15% concentration of Kepok banana peel extract applied for 9 days showed the greatest potential to accelerate wound-healing.
Keywords: Histopathological feature, Kepok banana peel, Macroscopic feature, Ointment, Skin, Wound healing

[Full text-PDF]

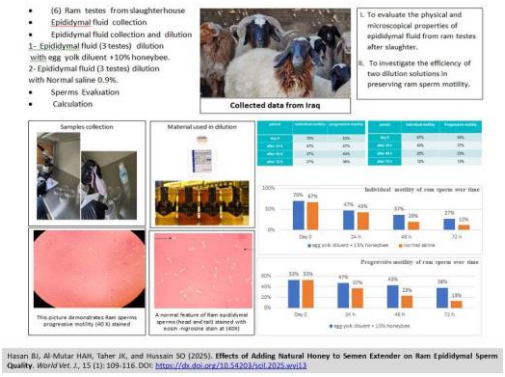
Research Paper

Effects of Adding Natural Honey to Semen Extender on Ram Epididymal Sperm Quality

Hasan BJ, Al-Mutar HAH, Taher JK, and Hussain SO.

World Vet. J. 15(1): 109-116, 2025; pii:S232245682500013-15

DOI: https://dx.doi.org/10.54203/scil.2025.wvj13



ABSTRACT: Numerous studies have indicated that male infertility is often associated with poor semen quality. The present study investigated the use of natural honey as an antioxidant and nutrient additive to semen extender to evaluate its effect on sperm parameters. A total of 16 fresh testes from sexually mature rams were collected for the investigation and immediately transported to the reproductive technology laboratory at the College of Veterinary Medicine, Baghdad University. For the first group, sperm was collected and diluted with an egg yolk extender mixed with 10% natural honey, while the second group consisted of epididymal sperm diluted with 0.9% normal saline. Epididymal fluid was collected and evaluated for both groups. The results showed significant differences in mean individual motility between the two groups after 48 and 72 hours, as determined by the comparison of proportions. Egg yolk plus honey diluent was significantly more effective than normal saline diluent in preserving sperm cell viability after 48 and 72 hours. The same finding applied to progressive motility; the egg yolk plus honey diluent was significantly more efficient than the normal saline diluent for the time frame after 48 and 72 hours, respectively. In conclusion, the findings demonstrated that the egg yolk extender supplemented with 10% honey was more effective in preserving ram sperm motility over time than normal saline. The addition of honey to the egg yolk extender improved the motility, the live-dead ratio, and the viability of the liquid storage of ram epididymal fluid. Furthermore, egg yolk plays a crucial role in protecting sperm from the detrimental effects of low temperatures.
Keywords: Epididymal sperm, Honey, Ram, Semen extender, Sperm motility

[Full text-PDF]

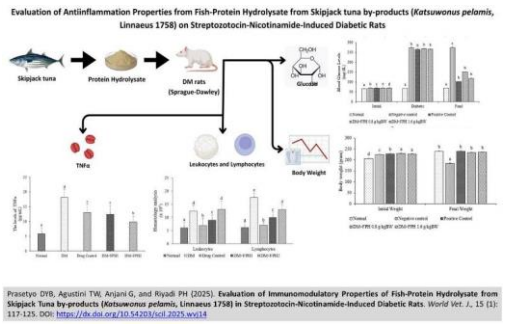
Research Paper

Evaluation of Immunomodulatory Properties of Fish-Protein Hydrolysate from Skipjack Tuna by-products (*Katsuwonus pelamis*, Linnaeus 1758) in Streptozotocin-Nicotinamide-Induced Diabetic Rats

Prasetyo DYB, Agustini TW, Anjani G, and Riyadi PH.

World Vet. J. 15(1): 117-125, 2025; pii:S232245682500014-15

DOI: https://dx.doi.org/10.54203/scil.2025.wvj14



ABSTRACT: Fish protein hydrolysate (FPH) is an alternative to managing fish by-products. Protein hydrolysis by proteolytic enzymes breaks down the protein into bioactive peptides (BP). Bioactive has a high-protein content and various beneficial health properties, including antioxidants, immunoregulatory, antibacterial, anti-inflammatory, and other activities. The current study aimed to investigate the anti-diabetic and immunomodulatory activities of FPH from skipjack tuna by-products. Male *Sprague Dawley* rats (n = 25) were equally divided into five groups: healthy group, diabetic mellitus (DM) group, DM + Imunos 0.8 g/ kg BW (drug control group), DM + 0.8 g/kg BW (FPH 1), DM + 1.6 g/kg BW FPH (FPH 2). Diabetic rats were induced by being fed with a high-fat diet (HFD) for 3 months, followed by nicotinamide (NA; 120 mg/kg BW)-streptozotocin (STZ) injection (60 mg/kg BW). The initial and final body weights before and after treatment were measured. The leukocyte and lymphocyte levels were measured using a hematology analyzer. The pro-inflammatory cytokine tumor necrosis factor α (TNFα) level was measured using enzyme-linked immunosorbent assay (ELISA). The result showed that the blood glucose levels after treatment using FPH significantly decreased compared with DM rats. Leukocyte and lymphocyte numbers also decreased significantly after treatment using FPH 1 than in DM rats. The pro-inflammatory cytokine TNFα in the FPH rat groups improved significantly compared with DM rats. These study results suggested that FPH from skipjack tuna by-product administration can be used as anti-diabetic and immunomodulatory candidates.
Keywords: Diabetes mellitus, Fish protein hydrolysate, Inflammation, Skipjack tuna by-product

[Full text-PDF]

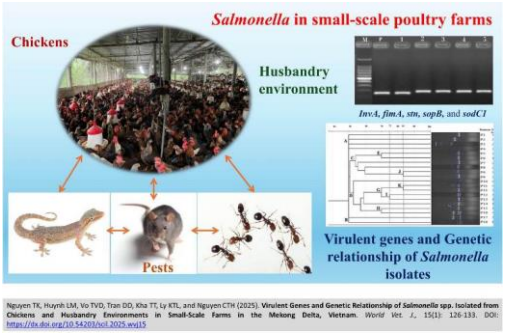
Research Paper

Virulent Genes and Genetic Relationship of Salmonella spp. Isolated from Chickens and Husbandry Environments in Small-Scale Farms in the Mekong Delta, Vietnam

Nguyen TK, Huynh LM, Vo TVD, Tran DD, Kha TT, Ly KTL, and Nguyen CTH.

World Vet. J. 15(1): 126-133, 2025; pii:S232245682500015-15

DOI: https://dx.doi.org/10.54203/scil.2025.wvj15



ABSTRACT: *Salmonella* is one of the most severe pathogens causing diseases in poultry and humans, and several factors could become transmission vectors in the husbandry environment. This study was conducted from April to July 2024 to clarify the prevalence of common *Salmonella* serovars in chickens and the husbandry environment and their pathogenicity and genetic relationship in small-scale farms in the Mekong Delta, Vietnam. A total of 279 samples were randomly collected from fresh chickens' feces (n = 54), husbandry environment (n=81), and pests (n=144), including rats, geckos, and ants, in four small-scale farms to examine the prevalence of *Salmonella* spp. By the conventional isolation method, 75 samples were positive for *Salmonella*, accounting for 26.88%. The prevalence of *Salmonella* in chicken feces, the environment, and pests were 27.78%, 12.35%, and 34.72%, respectively. Of 75 positive *Salmonella* isolates, two common serovars were identified, including *S. Gallinarum* (13.33%) and *S. Enteritidis* (10.67%); however, *S. Pullorum* and *S. Typhimurium* were not detected using PCR. These *Salmonella* isolates were detected virulent genes by using PCR, and found that these isolates harbored several virulent genes, including *InvA* (100%), *fimA* (100%), *stn* (93.33%), *sopB* (89.33%), and *sodC1* (54.57%). The ERIC-PCR method was used to determine the genetic relationship among *Salmonella* strains carrying virulent genes present in chickens, environment, and pests in these small-scale farms. The results showed diversity in phenotype and similarity in the genetic relationship (more than 75% similarity) among *Salmonella* strains isolated from chicken feces and the livestock environment. In conclusion, the study indicated that pathogenic *Salmonella* serovars could survive and be transmitted among sources, including chickens, the husbandry environment, and pests in small-scale poultry farms in the Mekong Delta.
Keywords: Chicken, Environment, Genetic relationship, Pest, *Salmonella*, Virulent gene

[Full text-PDF]

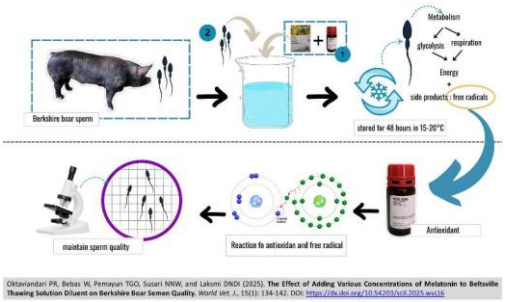
Research Paper

The Effect of Adding Various Concentrations of Melatonin to Beltsville Thawing Solution Diluent on Berkshire Boar Semen Quality

Oktaviandari PR, Bebas W, Pemayun TGO, Susari NNW, and Laksmi DNDI.

World Vet. J. 15(1): 134-142, 2025; pii:S232245682500016-15

DOI: https://dx.doi.org/10.54203/scil.2025.wvj16



ABSTRACT: Boar spermatozoa contain polyunsaturated fatty acids, rendering them susceptible to damage from free radicals. Oxidative stress in liquid semen can be prevented by modifying the diluent by adding antioxidants. Melatonin is an indoleamine compound that can be used as an antioxidant with high potential in the reproductive system. This study aimed to determine the effect of adding various concentrations of melatonin to Beltsville Thawing Solution (BTS) diluent on the quality of Berkshire boar semen. A Completely Randomized Design (CRD) method was employed, with the experiment divided into four treatment groups, each replicated six times. A total of 24 samples were used in this study. P0 was used as the control group, while groups P1, P2, and P3 were given the addition of melatonin to BTS diluent with different doses, namely 0.5 mM, 1.0 mM, and 1.5 mM. The samples were stored for 48 hours and then examined for boar semen quality. The variables examined included the percentage of motility, abnormality, viability, plasma membrane integrity, and malondialdehyde (MDA) levels. The results showed that the addition of a melatonin dose of 1.0 mM to the BTS diluent was the optimal concentration that could maintain motility, abnormalities, viability, plasma membrane integrity, and MDA levels in Berkshire boar semen compared to other treatment groups and the control group. This study indicated that melatonin functions as an effective antioxidant, neutralizing free radicals and thereby inhibiting oxidative stress in Berkshire boar semen.
Keywords: Beltsville thawing solution, Berkshire boar, Malondialdehyde, Melatonin, Semen quality

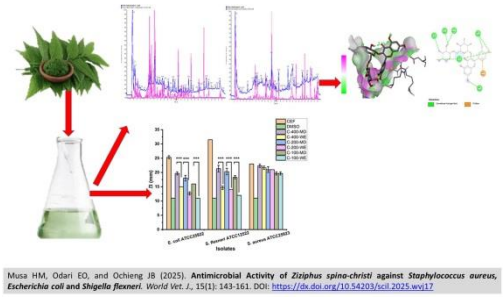
[Full text-PDF]

Research Paper

Antimicrobial Activity of *Ziziphus spina-christi* against *Staphylococcus aureus*, *Escherichia coli* and *Shigella flexneri*

Musa HM, Odari EO, and Ochieng JB.

World Vet. J. 15(1): 143-161, 2025; pii:S232245682500017-15
DOI: https://dx.doi.org/10.54203/scil.2025.wvj17



Musa HM, Odari EO, and Ochieng JB (2025). **Antimicrobial Activity of *Ziziphus spina-christi* against *Staphylococcus aureus*, *Escherichia coli* and *Shigella flexneri***. *World Vet. J.*, 15(1): 143-161. DOI: https://dx.doi.org/10.54203/scil.2025.wvj17

ABSTRACT: Antibiotic resistance remains a global concern, with up to 1.91 million deaths projected to occur due to resistant Gram-negative and Gram-positive bacteria by 2050. The study hence aimed to assess the antimicrobial activity of Ziziphus spina-christi leaf extracts in relation to specific bacterial strains and elucidate the molecular mechanisms to validate the in vitro findings. *Ziziphus spina-christi* leaf extracts were tested against *Staphylococcus aureus* (*S. aureus*), *Escherichia coli* (*E. coli*), and *Shigella flexneri* (*S. flexneri*). The leaf powder was subjected to both aqueous and methanol-dichloromethane extraction. Phytochemical products were determined by Liquid Chromatography-Mass Spectrometry and Gas Chromatography-Mass Spectrometry for water extract and methanol dichloromethane extract, respectively. The agar well diffusion method, broth microdilution, and minimum bactericidal concentration against three bacterial species, *S. aureus*, *E. coli*, and *S. flexneri*, were used to assess the antibacterial activity of extracts. The results have shown that both plant extract has a significant level of antibacterial activity at higher concentrations (400 mg/ml) against the gram-positive bacteria. In addition, the methanol-dichloromethane extract exhibited the highest antibacterial activity against Gram-negative bacteria (*S. flexneri*, and *E. coli*), conversely, the water extract demonstrated a lower activity against *S. flexneri* and *E. coli,* with inhibition zones of 15 ± 0 mm for both bacteria. At a lower concentration (100 mg/ml), the methanol-dichloromethane extract produced inhibition zones of 19.6 ± 0.5 mm against *S. aureus*, closely followed by S. flexneri and *E. coli*. The water extract exhibited high antibacterial activity against Gram-positive bacteria. However, exhibited reduced antibacterial activity against *S. flexneri* and *E. coli*, indicating a concentration-dependent antibacterial effect. Extraction methods were significantly different, with products generated from non-aqueous extraction demonstrating a higher potency against both Gram-negative and Gram-positive bacteria than the aqueous extract. Docking results demonstrated that water extract had a high binding activity against penicillin-binding proteins. Moreover, it serves as a potent beta-lactamase inhibitor as it binds to their active site, rendering them inactive and inhibiting the hydrolysis of Beta lactam antibiotics. In conclusion, the methanol-dichloromethane and water Ziziphus spina-christi leaves could be considered a promising source of antimicrobial ingredients.
Keywords: Antimicrobial resistance, Beta lactamase, Methanol-dichloromethane Extract, Molecular docking, *Ziziphus spina-christi* leave

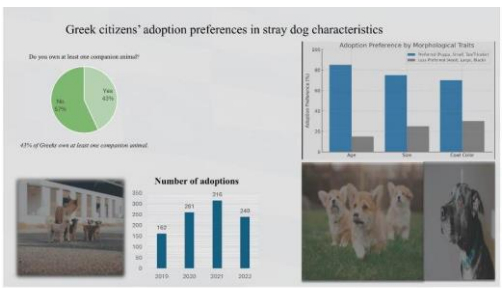
[Full text-PDF]

Research Paper

Adoption Preferences of Greek Citizens in Stray Dogs: The Role of Morphological Characteristics

Siettou AS, Theodoropoulou E, Karagianni V, Sossidou E, and Siettou C.

World Vet. J. 15(1): 162-166, 2025; pii:S232245682500018-15
DOI: https://dx.doi.org/10.54203/scil.2025.wvj18



Siettou AS, Theodoropoulou E, Karagianni V, Sossidou E, and Siettou C (2025). **Adoption Preferences of Greek Citizens in Stray Dogs: The Role of Morphological Characteristics**. *World Vet. J.*, 15(1): 162-166. DOI: https://dx.doi.org/10.54203/scil.2025.wvj18

ABSTRACT: A critical aspect of addressing the stray dog population issue in Greece involves the investigation of the likelihood of dog adoptions based on morphological traits. Recent statistics indicate that 43% of Greek households own at least one companion animal. This study aimed to explore some of the factors influencing the decision to adopt a stray and the morphological traits that are preferred by the adopters. To undertake this research, data spanning four years (2019-2022) were used; the data comprised information on 858 adoptions from a Greek animal welfare organization on the Island of Lesvos in the Aegean Sea. The analysis revealed that younger age, smaller size, and tan or tricolor coats were associated with faster adoption rates. In addition, when comparing the data of all four years (2019-2022), the year of adoption was found to be a statistically significant variable, which confirms that Greeks also followed the international trend of increased adoptions during the Covid-19 pandemic.
Keywords: Adopter preference, Greece, Morphological trait, Shelter dog, Stray companion animal

[Full text-PDF]

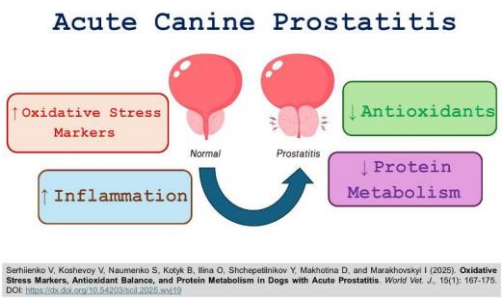
Research Paper

Oxidative Stress Markers, Antioxidant Balance, and Protein Metabolism in Dogs with Acute Prostatitis

Serhiienko V, Koshevoy V, Naumenko S, Kotyk B, Ilina O, Shchepetilnikov Y, Makhotina D, and Marakhovskyi I.

World Vet. J. 15(1): 167-175, 2025; pii:S232245682500019-15
DOI: https://dx.doi.org/10.54203/scil.2025.wvj19

ABSTRACT: The prostate gland in dogs is highly vulnerable to the action of negative pathogens due to its structure and topography. Among the numerous etiological factors in the development of prostatitis, inflammatory processes and oxidative stress play a predominant role, regardless of whether the condition is bacterial, viral, or autoimmune in origin. This study aimed to assess protein metabolism and redox balance indicators in the prostate tissue of dogs with acute prostatitis. For biochemical analyses, prostate tissue samples were taken from 24 mixed-breed dogs, including twelve animals that were considered healthy with no abnormalities of the genitourinary system (control group) and twelve animals with newly diagnosed acute prostatitis, from which samples were obtained via biopsy (experimental group). Following homogenization and sample preparation, all biochemical parameters in the prostate tissue were determined spectrophotometrically. The results of biochemical studies in dogs with acute prostatitis demonstrated a significant increase in the content of thiobarbituric acid-reactive compounds by 102.2% and the level of lipid hydroperoxides by 35.7% compared to healthy dogs in the control group. In contrast, the total protein content was 32.9% lower than in the control group, while reduced glutathione levels decreased by 76.5%. Similar changes to the dynamics of oxidative stress markers were indicated by the activity of antioxidant enzymes, with glutathione peroxidase and catalase activities increasing by 61.3% and 21.8%, respectively, relative to the control group. These findings indicate the presence of oxidative stress in dogs with acute prostatitis. The biochemical changes observed in prostate tissue provide a foundation for future research aimed at developing therapeutic methods that incorporate anti-inflammatory, antibacterial, and antioxidant agents for the treatment of acute prostatitis in dogs.
Keywords: Biochemistry, Dog, Inflammation, Oxidative stress, Prostate



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Case Report

Surgical Correction of Anal Atresia in a 4-Day-Old Brown Swiss Calf

Olivera-Calderon R, Meza-Miguel E, Ninahuanca J, Payano UI, García-Olarte E, Miranda-Torpoco C, Condor WG, Olivera-Acuña W, and Sarapura V.

World Vet. J. 15(1): 176-181, 2025; pii:S232245682500020-15

DOI: https://dx.doi.org/10.54203/scil.2025.wvj20

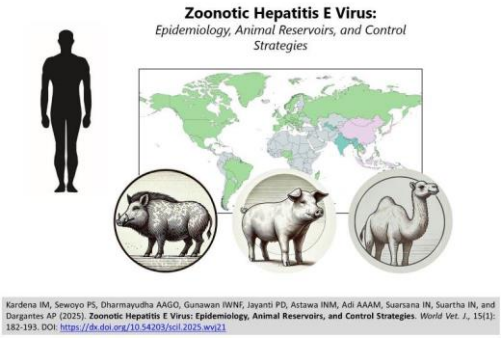
ABSTRACT: A 4-day-old Brown Swiss calf was presented to a veterinary clinic in Huancayo, Peru, with congenital anal atresia. The condition was characterized by an absence of defecation, progressive abdominal distension, and a blind rectal pouch confirmed radiographically. Clinical examination revealed no anal opening, moderate tympany, and normal neurological function. Surgical correction was conducted under pre-anesthetic sedation using xylazine (0.2 mg/kg IM) and sacrococcygeal epidural anesthesia with lidocaine (0.5 mL/kg). A 12-cm vertical incision in the intertubercular ischiatic region exposed the distended rectal pouch, allowing for an end-to-cutaneous anastomosis with polyglactin 910 (Vicryl® 2-0). Subsequently, a 2-3 cm distal rectal incision was created to form a neoanus. The mucosal edges were sutured to the skin margins with interrupted simple stitches (Vicryl® 3-0), a technique aimed at preventing stenosis and maintaining a neoanal diameter of approximately 1.5-2 cm. Postoperative management included prophylactic penicillin. Within three hours of surgery, the calf passed impacted meconium, indicating the functional patency of the neoanus. Although the prognosis was favorable, the patient was closely monitored for five days to detect any signs of stricture or infection. This case illustrated the effectiveness of early surgical intervention in reestablishing gastrointestinal continuity in congenital anal atresia.
Keywords: Anal agenesis, Clinical significance, Postoperative outcome, Surgical technique

[Full text-PDF]

Review

Zoonotic Hepatitis E Virus: Epidemiology, Animal Reservoirs, and Control Strategies

Kardena IM, Sewoyo PS, Dharmayudha AAGO, Gunawan IWNF, Jayanti PD, Astawa INM, Adi AAAM, Suarsana IN, Suartha IN, and Dargantes AP.
World Vet. J. 15(1): 182-193, 2025; pii:S232245682500021-15
DOI: https://dx.doi.org/10.54203/scil.2025.wvj21



ABSTRACT: Hepatitis E virus (HEV) is a leading cause of acute hepatitis transmitted through the enteric route, impacting millions of individuals worldwide annually. While HEV is generally self-limiting, it can lead to considerable illness and death, particularly in gestating women, immunocompromised individuals, and those with pre-existing conditions, such as chronic liver disease. Hepatitis E virus genotypes 1 and 2 infect solely humans and are prevalent in areas with poor sanitation, whereas genotypes 3 and 4 are zoonotic, infecting both animals and humans. Hepatitis E virus genotype 7 has been reported to infect both humans and animals; however, further research is needed to clarify its zoonotic potential. Zoonotic transmission occurs primarily through the consumption of contaminated meat and close contact with infected animals, posing significant public health risks. Epidemiological studies indicated an increasing seroprevalence of HEV in humans and animals across diverse regions, highlighting the need for strengthened public health measures. While HEV infections in animal reservoirs are generally asymptomatic, they represent a critical source of human infections. The present review aimed to highlight HEV's current classification, epidemiology, modes of transmission from animals to humans, prevention, and control measures, with a special focus on HEV zoonotic genotypes and their animal reservoirs.
Keywords: Control measure, Epidemiology, Hepatitis E virus, Public health, Zoonotic genotype

[Full text-PDF]

Review

The Association between the Global Threat of Ocean Pollution and Climate Change on the Distribution of Antibiotic Resistance: One Health Strategy

Bukha KK, Altayr NA, Shlayek SA, and Eldaghayes IM.
World Vet. J. 15(1): 194-214, 2025; pii:S232245682500022-15
DOI: https://dx.doi.org/10.54203/scil.2025.wvj22

ABSTRACT: Antibiotic resistance represents a fundamental issue affecting public health today. Antibiotic resistance occurs when bacteria alter their response to antibiotics. Pathogenic bacteria and their genes can diffuse throughout human and natural habitats. The complicated interactions between diverse bacterial communities that affect the health of people, aquatic animals, and the aquatic environment are an illustration of ecological issues. Pollution of the oceans by antibiotic-resistant bacteria (ARB) can disturb the natural equilibrium of the oceans and may spread to humans. Also, climate change (CC) significantly affects the health of marine environments. Rising temperatures, acidification, increased sea levels, an increasing number of invasive marine animals, changed biological systems, and a decrease in biodiversity are some of the major impacts on the oceans caused by CC. However, the elevated temperatures linked to CC facilitate the higher spread of bacterial infections in aquatic environments, aquatic animals, and humans with the emergence of antibiotic resistance. The present study aimed to provide a scientific understanding of the relationship between ocean pollution and CC, as well as their impacts on ocean health. Additionally, the present study presented the current status of ARB and its associated genes in the oceans, comparing to future projections based on previous studies. One Health (OH) concept strategies for reducing antibiotic pollution in the ocean were discussed. The present paper is a foundation for further studies to determine the prevalence of antibiotic resistance in the oceans, as well as to understand the current state and key highlights of ocean pollution.

Keywords: Climate change, Drug-resistant bacteria, Heavy metal resistance gene, Ocean pollution, One Health concept

[Full text-PDF]

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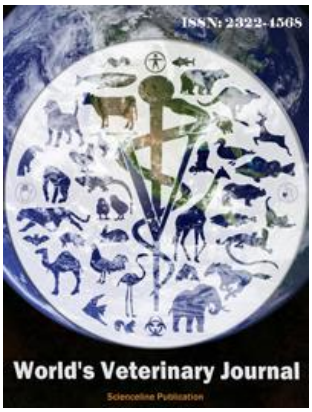

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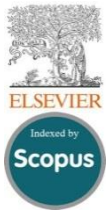

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Characterization of Lactic Acid Bacteria Isolated from Gambir with Potential Probiotic Properties

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ABSTRACT

Gambir is commonly used as a key ingredient in betel quid and is one of Indonesia's major agricultural commodities. West Sumatra is the primary production region, contributing approximately 80-90% of the country's total gambir production. This study aimed to investigate the potential of lactic acid bacteria (LAB) isolated from gambir, assessing their potential probiotic qualities using biochemical and molecular approaches. Biochemical characterization included catalase activity testing and fermentation pattern analysis, while molecular identification was carried out through the *16S rRNA* gene sequence. The results revealed that the isolated LAB strains were Gram-positive, bacilli-shaped, catalase-negative, and exhibited hetero-fermentative behavior. Further biochemical analysis confirmed their ability to ferment a variety of sugars but not produce gas from glucose. Basic Local Alignment Search Tool analysis showed that the bacterial isolate from gambir, labeled with the sample code GM2, was closely related to *Lactiplantibacillus pentosus*, a species recognized for its probiotic potential. The isolates showed antimicrobial activity against common foodborne pathogens like *Salmonella* spp. and other pathogens, suggesting their potential use in food preservation. The present study also demonstrated the LAB isolates' tolerance to low pH and bile salts, which are key attributes for probiotic candidates. Thus, the findings of the current study suggest that LAB from gambir possess promising characteristics for application in probiotic products and as biocontrol agents in food safety.

Keywords: *16s rRNA*, Gambir, Lactic acid bacteria, Probiotic

INTRODUCTION

Gambir is widely recognized as a key ingredient in betel quid and stands out as one of Indonesia's top agricultural commodities. West Sumatra plays a dominant role in its production, accounting for approximately 80-90% of the country's total gambir output. Within this region, Lima Puluh Kota Regency is the largest producer, contributing 70.39% of the total production (Nasution et al., 2015). In addition to its use in betel quid, gambir has applications in various industries, including batik, paint, and textiles, it is derived from the sap of the leaves and twigs of the *Uncaria gambir* plant, which is then processed and dried to produce the final gambir product (Aditya and Arianti, 2016). In Sumatra, Gambir is known by various local names, including gambee, gani, kacu, sontang, gambe, gambie, gambu, gimber, pengilom, and selet (Anggraini et al., 2011).

This plant has a subtle odor and a taste that begins as bitter and astringent but gradually becomes mildly sweet. Gambir is also recognized as a valuable source of antioxidants and belongs to the Rubiaceae family (coffee family). It contains several polyphenolic compounds, such as catechins (catechin acid), catechin tannate, and quercetin (Ndagijimana, 2013; Firdausni et al., 2020). These compounds have well-documented antioxidant properties and may play a crucial role in supporting the growth and viability of lactic acid bacteria (LAB). Specifically, catechins and other polyphenols have been shown to exert antimicrobial effects, selectively inhibiting the growth of harmful pathogens while potentially fostering the growth of beneficial LAB (Choe and David, 2009). Furthermore, these antioxidants can enhance LAB stress tolerance, protecting them from oxidative damage and improving their survival under challenging conditions, such as low pH and bile exposure (Choe and David, 2009). Thus, the bioactive compounds in gambir may contribute significantly to the effective isolation of LAB strains with potential probiotic properties, highlighting gambir as a promising natural source for probiotic applications. Gambir (*Uncaria gambir*) may possess several unique properties that make it a promising candidate for the isolation of LAB, particularly for probiotic applications. These properties include: rich polyphenolic content, antioxidant properties, natural antimicrobial activity, source of nutrients for lab growth, and

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potential prebiotic effect. This evaluation encompasses morphological identification, biochemical profiling, molecular DNA analysis, and purification to evaluate the probiotic potential of the subject for promoting overall health (Syukur et al., 2014). The LAB have been recognized for producing lactic acid, hydrogen peroxide, and carbon dioxide and for breaking down food into simpler components, such as glucose, fructose, amino acids, and short-chain fatty acids (Stanton et al., 2001).

Among the probiotics, LAB can inhibit and eliminate pathogenic bacteria from their surroundings (Trisna and Wahud, 2012). They are classified as safe microorganisms (food-grade microorganisms) and are generally recognized as safe (GRAS) for health. Probiotics, as live microorganisms, have been shown to provide beneficial effects by balancing the gut microbiota (Trisna and Wahud, 2012). Gambir has been used traditionally in Southeast Asia for its astringent. The astringent and anti-inflammatory properties of gambir could indirectly influence gut health by reducing inflammation in the digestive system and promoting overall digestive balance, which could create conditions favorable for LAB (Melia et al., 2015). The benefits of consuming probiotics include maintaining the balance of microorganisms in the digestive system (Dewi et al., 2021). A study demonstrated that supplementing *Lactobacillus bulgaricus* in both the feed and drinking water of quails resulted in positive effects on live weight, feed intake, and feed conversion ratio (FCR) (Vahdatpour and Babazadeh, 2016). Kusumawati (2002) outlines several beneficial health effects of probiotics, including enhancing immune defense against infectious diseases, particularly gastrointestinal infections, and diarrhea, lowering blood serum cholesterol levels, alleviating symptoms of lactose intolerance, exhibiting antihypertensive properties, supporting digestive processes, possessing anti-mutagenic and anti-carcinogenic activities, influencing immune system responses, and decreasing the risk of colon cancer and tumor development. Isolating and identifying LAB from gambir is crucial to determining and obtaining LAB species with potential probiotic properties, particularly concerning their ability to promote gut health by balancing the microbiota, inhibiting pathogenic bacteria, and providing beneficial health effects, such as anti-inflammatory, antimicrobial, and immune-modulating activities.

MATERIALS AND METHODS

Study design

This experimental laboratory research was designed to isolate and characterize LAB from gambir samples. The study was conducted at the Microbiology Laboratory, Department of Animal Product Technology, Faculty of Animal Science, Andalas University.

Sampling

The gambir sample taken is gambir sap that has been fermented or dried. Three different gambir samples were used in this study, labeled GM1, GM2, and GM3, which were sourced from different locations in Limapuluh Kota Regency, West Sumatra, Indonesia. The samples were stored at 4°C (refrigerated) in sterile containers to prevent the growth of unwanted microorganisms and maintain the integrity of the sample. All storage procedures followed standard microbiological protocols to prevent cross-contamination and preserve sample quality.

Isolation and identification of lactic acid bacteria

The bacterial isolates from Gambir were identified based on the guidelines of Purwati et al. (2005). A one-gram portion of the sample was mixed with 9 mL of MRS Broth in a test tube and vortexed until the mixture was fully homogenized, referred to as the 10^{-1} dilution. From this, 100 µL was transferred into an Eppendorf tube containing 900 µL of fresh MRS Broth, followed by vortexing to achieve uniformity, representing the 10^{-2} dilution. This serial dilution was carried out until the 10^{-6}dilution was obtained. For the 10^{-6}dilution, 100 µL of the solution was spread onto MRS agar plates using the spread plate method. The inoculum was evenly distributed using a sterile hockey stick. The plates were then placed in an anaerobic jar and incubated at 37°C for 48 hours.

Gram staining

A bacterial sample was spread onto a clean, alcohol-disinfected glass slide and allowed to air dry over a Bunsen burner or drying apparatus. A drop of crystal violet was applied for one minute, followed by rinsing with distilled water and drying again. Afterward, iodine was added for one minute, and the slide was rinsed and dried once more. The slide was briefly immersed in diluted alcohol to wash off the excess stain and then treated with safranin for 30 seconds. Following another rinse with distilled water, the slide was air-dried over the Bunsen flame before being examined under a microscope (Romadhon et al., 2012).

Catalase test

To perform the catalase test, 10 µL of the LAB isolate was placed on a clean glass slide. A 10 µL drop of 3% hydrogen peroxide (H_2O_2) was added, and the formation of bubbles was observed to detect the presence of catalase activity (Public Health England, 2014).

Fermentation type test for lactic acid bacteria

The LAB isolate was inoculated in 5 mL of MRS Broth (Neogen Culture Media), and an inverted Durham tube was placed in the test tube. After incubating at 37°C for 48 hours, the presence of gas bubbles in the Durham tube was recorded to determine the type of fermentation (Romadhon et al., 2012).

Acid tolerance test

The acid tolerance test followed the method of Sunaryanto and Marwoto (2013) to obtain LAB isolates with high tolerance to gastric conditions (pH 3). A 0.1 ml bacterial suspension from MRS Broth was added to a series of tubes containing 9 ml of sterile MRS Broth (Merck, Germany) and incubated at 37°C for 90 minutes. The pH was adjusted by adding HCl 5 N. The cultures were then grown on MRS agar for 24 hours at 37°C.

Bile salt tolerance test

Bile salt tolerance testing of LAB was carried out using the method of Sunaryanto and Marwoto (2013). The MRS Broth (Merck, Germany), which contains 0.3% and 0.5% oxgall, was inoculated with one ml of the test bacterial culture and incubated at 37°C for 5 hours. Then, 100 µl of the culture was transferred into 900 µl of MRS Broth in Eppendorf tubes for enrichment. The culture was subsequently grown on MRS agar for 24 hours at 37°C. The number of surviving bacteria was counted using the plate count method. Viability (%) was used to compare the total number of cells before and after incubation. Bacteria with higher viability percentages after bile salt exposure were considered more likely to survive.

Antimicrobial activity

The antimicrobial activity of LAB isolates was evaluated against *Escherichia coli*, *Staphylococcus aureus*, and *Listeria monocytogenes*. The tests were conducted in triplicate for accuracy. Ampicillin and kanamycin, both well-known broad-spectrum antibiotics, were used as positive controls due to their established antimicrobial properties. The LAB cultures were grown in MRS Broth for 18-22 hours. Fresh overnight cultures of the target bacterial pathogens were inoculated into 20 mL of nutrient agar at a final concentration of 0.2% (v/v). After the MRS agar solidified, wells with a diameter of 6 mm were created. Subsequently, 50 µL of the LAB supernatant was introduced into each well. The plates were incubated anaerobically at 37°C for 14-16 hours, and the inhibition zones were measured to assess antimicrobial activity (Kaushik et al., 2009).

Molecular identification

DNA extraction from LAB was carried out using a commercial kit (Promega, USA). Polymerase Chain Reaction (PCR) was performed with the forward primer F 16S-27F (5'-AGA GTT TGA TCC TGG CTC AG-3') and the reverse primer R 16S-1492R (5'-GTT TAC CTT GTT ACG ACTT-3'), following the protocol in the Table 1 provided by Promega (2010).

Molecular applications for analyzing bacterial diversity through *16S rRNA* gene analysis are increasingly used to overcome challenges in bacterial cultivation. The *16S rRNA* gene is particularly suitable for bacterial identification because it is present in all organisms (Suryani et al., 2010). Chun et al. (2007) further emphasized that the use of *16S rRNA* gene sequences for studying the phylogeny of bacteria has become widespread. The function of the *16S rRNA* gene remains relatively unchanged over time (mutations), suggesting that random sequence changes provide a more accurate measure of evolutionary time. Additionally, the *16S rRNA* gene, which is approximately 1,500 base pairs long, is sufficiently large to serve as a valuable tool for bioinformatics purposes. The *16S rRNA* gene sequence has been extensively used for prokaryotic organism identification. Sequence comparisons based on nucleotide similarity values and phylogenetic methods are effective for identifying bacterial isolates.

Table 1. Polymerase chain reaction program for *16S rRNA* gene

Process sequence	Temperature	Time
Pre-denaturation	95°C	2 minutes
Denaturation	95°C	45 seconds
Annealing	56°C	45 seconds
Extension	72°C	1 minute and 40 seconds
Final Extension	72°C	10 minutes
Cooling	4°C	~ (until completion)

Data analysis

Sequencing data were processed using the BioEdit software for analysis. To perform sequence alignment, the obtained sequences (query) were compared to those stored in GenBank by conducting database searches on the NCBI website using the BLAST Tool (Depson, 2012).

RESULTS AND DISCUSSION

Isolation of lactic acid bacteria

The total LAB in Table 2 count in gambir samples GM1, GM2, and GM3 were 48×10^8 CFU/g, 52×10^8 CFU/g, and 50×10^8 CFU/g, respectively. These results aligned with other studies using similar raw materials, such as *gulai paluik*. The total LAB colonies in this study were higher than those reported by Meriza (2021) and Pratama (2020), who found 11×10^7 CFU/g and 25×10^7 CFU/g, respectively. This higher count was attributed to the fact that gambir is a fermented product, which typically results in a higher LAB colony count. Based on these results, the LAB colonies identified in this study can be categorized as probiotic candidates, consistent with Wijayanto's (2009) assertion that probiotic bacterial cell viability should range between 10^7–10^9 CFU/g.

Table 2. Total colonies of lactic acid bacteria isolated from Gambir

Sample code	Total lactic acid bacteria ($\times10^8$ CFU/g)
GM1	48
GM2	52
GM3	50

Identification of lactic acid bacteria

Macroscopic observations in Table 3 reveal LAB isolates with characteristics including round shape, smooth texture, creamy color, shiny convex surface, and smooth, regular colony edges on MRS agar. This finding was consistent with the findings of Purwati et al. (2005), who reported similar colony characteristics for LAB on MRS agar. Additionally, LAB isolates from soursop fruit by Delfahedah et al. (2013) and from fermented *pisang kepok* (a type of banana) by Finanda et al. (2021) showed round, smooth-edged, convex colonies with milky white and creamy colors. The LAB in Table 4 isolates were catalase-negative, as evidenced by the absence of air bubbles caused by O^2 gas. LAB did not produce the catalase enzyme, which breaks down H^2O^2 into water and non-harmful oxygen. To convert hydrogen peroxide into oxygen, an enzyme called catalase is needed (Carroll et al., 2017). However, LAB isolated in this study could not produce this enzyme.

Table 3. Morphological characteristics of the lactic acid bacteria isolate

Sample	Colony form	Color	Size	Surface	Corner
GM1	Bacilli	White-beige	1.5 mm	Slippery, convex	Flat, smooth
GM2	Bacilli	White-beige	2.0 mm	Slippery, convex	Flat, smooth
GM3	Bacilli	White-beige	1.5 mm	Slippery, convex	Flat, smooth

Table 4. Biochemical properties of the lactic acid bacteria gambir isolate

Sample	Catalase test	Fermentation type
GM1	Negative	Hetero-fermentative
GM2	Negative	Hetero-fermentative
GM3	Negative	Hetero-fermentative

Resistance of lactic acid bacteria to stomach acid

A critical quality for probiotics was their survival in the acidic environment of the stomach, which was assessed by testing LAB resistance to low pH. It was essential to select probiotic strains that thrived in acidic conditions to maximize their efficacy.

Based on Table 5, the acidity of the stomach served as the first gate for selecting bacteria before they entered the intestines. The viability results of LAB under acidic conditions (pH 3) ranged from 66.8% to 88.5%. Higher viability levels indicated that the bacteria were more resilient to low pH conditions. The GM2 isolate showed the highest viability at 88.5%. Each isolate displayed varying viability due to differences in its ability to tolerate the acidic conditions of the stomach. Wijayanto (2009) observed that most microorganisms typically undergo cell death in highly acidic

environments or at low pH levels. This is because extreme acidity can damage cell membranes, causing the release of intracellular components, such as magnesium, potassium, and lipids, ultimately leading to cell death. Furthermore, resistance to stomach acid is a critical criterion for an isolate to be considered a probiotic (Khuruna and Kanawijaya, 2007).

Resistance of lactic acid bacteria to bile salts

This test was conducted to evaluate the survival ability of gambir LAB isolates in the intestinal environment, which contains a bile salt concentration of 0.3%.

Based on Table 6, the viability of LAB against bile salts ranged from 70.3% to 92.4%. The LAB isolate GM2 exhibited the highest viability among the other isolates. Higher percentages indicated greater resistance of LAB to bile salts. Leverrier et al. (2003) noted that bile salts could cause morphological changes in bacterial cells due to protein release from the cells. Wijayanto (2009) stated that the viability of bacterial cells, which were candidate probiotics, should range between 10^6 and 10^9 CFU/mL to survive in the gastrointestinal tract.

Antimicrobial activity

The highest viability of LAB against acid and bile salts was observed in LAB GM2, which was subsequently evaluated for its antimicrobial activity against pathogenic bacteria. Isolate LAB GM2 demonstrated the ability to inhibit pathogenic bacteria.

Based on Table 7, the inhibition zone for *E. coli* O157 measured 9.56 mm, which was comparable to the findings of Meriza (2021) regarding the antimicrobial activity of LAB isolates from *gulai paluik* against *E. coli* O157, which showed an inhibition zone of 11.51 mm. The inhibition zone diameter for *S. aureus* was 10.83 mm, while for *L. monocytogenes*, it measured 6.18 mm. According to Pelczar and Chan (2008), a larger clear zone indicates stronger inhibitory activity of LAB isolates against pathogenic bacteria. The inhibition activity can be categorized into four levels: weak activity (< 5 mm), moderate activity (5–10 mm), strong activity (10–20 mm), and very strong activity (> 20-30 mm, Morales et al., 2003).

Table 5. Resistance test of lactic acid bacteria isolated from gambir to acid pH

LAB isolate sample	Number of bacterial cells (CFU/ml) $\times 10^8$		LAB viability (%)
	Control	pH 3	
GM1	23.3	19.5	83.7
GM2	16.5	14.6	88.5
GM3	16.0	10.7	66.8

CFU: Colony forming unit, LAB: Lactic acid bacteria

Table 6. Test of the resistance of lactic acid bacteria isolated from gambir to bile salts

LAB isolate sample	Number of bacterial cells (CFU/ml) $\times 10^8$		LAB viability (%)
	Control	Oxgall 0.3%	
GM1	9.4	8.1	86.2
GM2	10.6	9.8	92.4
GM3	15.5	10.9	70.3

CFU: Colony forming unit, LAB: Lactic acid bacteria

Table 7. Clear zone diameter test antimicrobial activity test of GM2 isolate with antibiotics as a positive control

Sample code	Diameter of clear zone (mm)		
	E. coli O157	*S. aureus*	*L. monocytogenes*
GM2	9.56	10.83	6.18
Ampicillin	12.36	12.22	10.23
Kanamycin	15.23	13.14	11.08

E. coli O157: *Escherichia coli* O157; *S. aureus*: *Staphylococcus aureus*; *L. monocytogenes*: *Listeria monocytogenes*

Molecular identification

The sequencing results of the LAB isolated from Gambir, sample code GM2, were compared with Gene Bank data using the BLAST program on the NCBI website, as shown in Figure 1.

Based on Figure 2, the analysis results using BLAST, the LAB isolates from *gambir* GM2 had 99.33% similarity with *Lactiplantibacillus pentosus*, with a sequence length of 1492 bp. Hagstrom et al. (2000) suggested that isolates with

16S rRNA sequence similarity greater than 97% could represent the same species. In contrast, sequence similarities between 93-97% could represent bacterial identities at the genus level but differed at the species level. BLAST analysis showed that the bacterial isolate from gambir, labeled with the sample code GM2, was closely related to *Lactiplantibacillus pentosus*.

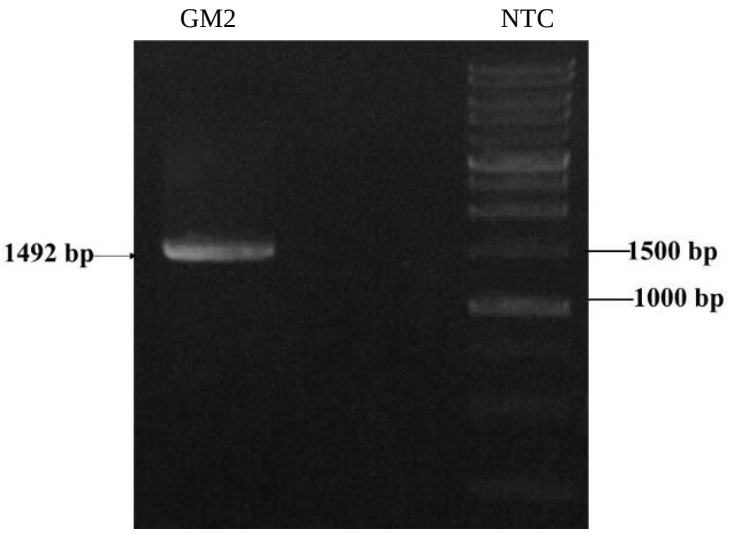

Figure 1. Results of electrophoresis of lactic acid bacteria gambir isolate. GM2: Gambier sample, NTC: Control without template

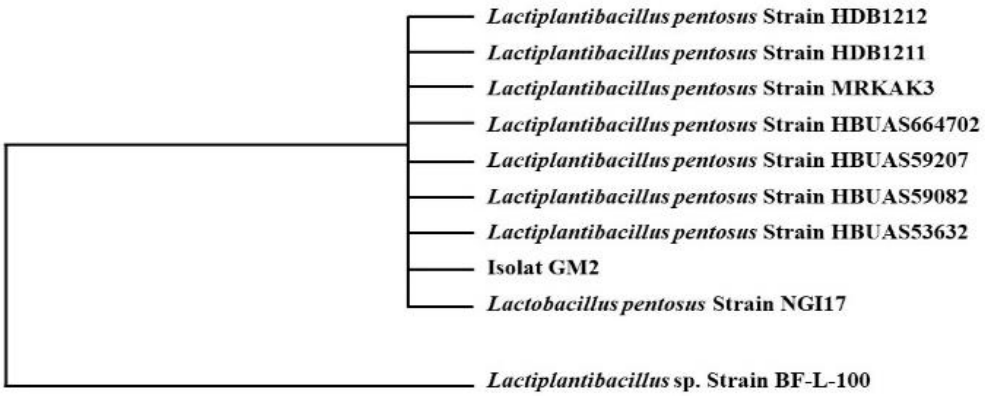

Figure 2. Phylogenetic tree of lactic acid bacteria GM2 isolated from Gambir. GM2: Gambir isolate

CONCLUSION

The LAB B isolated from gambir with sample code GM2 exhibited a bacillus morphology, white in color, and it was classified as a gram-positive bacterium. The isolate of GM2 was catalase-negative, demonstrated hetero-fermentative fermentation, and showed acceptable resistance to stomach acid and bile salts. Molecular identification confirmed that LAB GM2 was *Lactiplantibacillus pentosus*. It is recommended that future research be conducted *in vitro*, *in vivo*, and in clinical studies to evaluate the probiotic effects of *Lactiplantibacillus pentosus* GM2.

DECLARATIONS

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Authors' contributions

Varhanno Khallifhatul Khanh was responsible for the overall design and execution of the research, data collection, and analysis. Writing the manuscript and ensuring all necessary revisions were made by all authors. Muthia Sukma assisted in the conceptualization of the study and contributed to the experimental design. Additionally, Muthia Sukma

helped with data analysis and interpretation. Rahmi Fithria contributed to the literature review, interpretation of results, and final editing of the manuscript. All authors have read and approved the final version of the manuscript before publication in the present journal.

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Competing interests

The authors have not declared any conflict of interest.

Ethical considerations

This paper was originally written by the authors and has not been published elsewhere. The authors checked the text of the article for plagiarism index and confirmed that the text of the article is written based on their original scientific results.

Availability of data and materials

The data to support this study finding is available upon reasonable request to the corresponding author.

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Synergistic Effects of Phenanthroindolizidine Alkaloids from *Tylophora indica* Against *Ascaridia galli*: Phytochemical Characterization and *In vitro* Evaluation

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ABSTRACT

The increasing resistance of helminths such as *Ascaridia galli* to conventional anthelmintics has necessitated the search for alternative treatments from natural sources. This study aimed to assess the anthelmintic properties of phenanthroindolizidine alkaloids derived from *Tylophora indica* leaves, a plant renowned for its medicinal value, including its traditional use in treating respiratory disorders, inflammation, and various infections. The leaves were subjected to sequential extraction with chloroform, ethanol, and water, which produced yields of 7.2%, 17.8%, and 11.2%, respectively. Phytochemical analysis revealed that the ethanol extract was rich in bioactive compounds, including significant amounts of alkaloids, flavonoids, phenols, and terpenoids. Quantitative analysis confirmed the ethanol extract's superiority, displaying the highest contents of phenolics (7.51 ± 0.62 mg/g), flavonoids (9.34 ± 1.63 mg/g), and alkaloids (17.65 ± 1.69 mg/g), underscoring its potential for various therapeutic applications. Further fractionation and High-Performance Liquid Chromatography (HPLC) purification isolated key phenanthroindolizidine alkaloids, including Tylophorinidine, Tylophorine, Septicine, Tylophorinol, and Antofine. Structural characterization via Nuclear Magnetic Resonance (NMR) and High-Resolution Mass Spectrometry (HRMS) validated these compounds. *In vitro* assays demonstrated significant dose-dependent anthelmintic activity against *Ascaridia galli* worms. Ethanol extracts exhibited the highest mortality rates, achieving 100% mortality within 24 hours at a concentration of 5 mg/mL. The mixture of all five alkaloids at 500 µg/mL showed a synergistic effect, leading to rapid and complete anthelmintic action. The egg embryonation assay further highlighted the efficacy of these alkaloids. The egg embryonation assay further demonstrated the potent efficacy of these alkaloids, with the mixture at 500 µg/mL inhibiting 92.67% of egg development, surpassing the positive control, i.e. piperazine citrate, which showed 87.25% inhibition. Among individual alkaloids, Tylophorinidine exhibited the highest inhibition of egg embryonation (80.76%), followed by Antofine (78.42%). These findings demonstrated the potent anthelmintic properties of phenanthroindolizidine alkaloids from *Tylophora indica*, particularly when used in combination (Tylophorinidine, Tylophorine, Septicine, Tylophorinol, and Antofine), compared to their individual effects. The study underscores the potential of these compounds as effective treatments for helminth infections and highlights the importance of further research to isolate specific mechanisms and optimize their therapeutic efficacy.

Keywords: Anthelmintic activity, *Ascaridia galli*, Phenanthroindolizidine alkaloids, Phytochemical analysis, *Tylophora indica*

INTRODUCTION

Parasitic infections pose significant challenges to the poultry industry worldwide, with *Ascaridia galli* (*A. galli*) being a prevalent nematode that detrimentally affects poultry health and productivity (Permin et al., 1999). Ascaridiasis, the disease caused by *A. galli*, results in poor feed conversion, reduced growth rates, and significant economic losses (Mitiku et al., 2023). Conventional treatments for nematode infections in poultry have predominantly relied on synthetic anthelmintics (Coles et al., 2006). However, the overuse and misuse of these drugs have led to the development of resistant parasite strains, raising concerns about their long-term efficacy and safety (Kaplan, 2004). This situation underscores the urgent need for alternative, sustainable anthelmintic solutions. While several natural products have been investigated for their anthelmintic properties, the potential of plant-derived alkaloids as effective and safe alternatives to synthetic anthelmintics has gained significant attention in recent years (Katiki et al., 2019; Molgaard et al., 2021). Alkaloids, a diverse class of nitrogen-containing compounds found in various plants, have demonstrated broad-spectrum anthelmintic activity against a wide range of parasites (Waller et al., 2001). Their mechanisms of action often involve disrupting neuromuscular function, inhibiting enzymes crucial for parasite survival, or inducing oxidative stress (Katiki et al., 2019). Furthermore, their complex chemical structures contribute to reduced risks of drug resistance development, while their relatively low toxicity to hosts enhances their appeal as viable anthelmintic agents (Wink, 2012; Molgaard et al., 2021).

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Moreover, the utilization of plant-derived alkaloids aligns with the growing interest in sustainable and environmentally friendly pest control methods (Katiki et al., 2019). Despite the investigation of numerous natural products for anthelmintic activity, the potential of plant-derived alkaloids, particularly phenanthroindolizidine alkaloids (PAs) from *Tylophora indica*, remains largely untapped for combating *Ascaridia galli* infections. The existing gap in the literature regarding the synergistic effects of these alkaloids necessitates further investigation. *Tylophora indica*, a medicinal herb native to India, is well-known for its wide range of therapeutic properties, including anti-inflammatory, immunomodulatory, and anticancer activities (Sarma et al., 2011). Phenanthroindolizidine alkaloids derived from this plant, such as tylophorine and tylophorinine, have shown remarkable bioactivity in numerous studies (Wu et al., 2001; Choudhary et al., 2004). These alkaloids, however, have not been extensively studied for their anthelmintic potential against nematodes like *A. galli*. The primary objective of this study was to evaluate the anthelmintic efficacy of a combination of phenanthroindolizidine alkaloids isolated from *Tylophora indica* leaves against *Ascaridia galli*. This involved detailed phytochemical analysis and structural characterization of the alkaloids, followed by *in vitro* evaluation of their synergistic anthelmintic effects. It is hypothesized that the combination of phenanthroindolizidine alkaloids from *Tylophora indica* exhibits a synergistic anthelmintic effect against *Ascaridia galli*, enhancing efficacy compared to individual alkaloid treatments.

MATERIALS AND METHODS

Plant material

The leaves of *Tylophora indica* (Asclepiadaceae) were collected near Osmania University, Hyderabad, India, in March 2021. After inspection and thorough rinsing, they were air-dried in the shade to preserve phytochemicals and then ground into fine powder using a mixer grinder, preparing them for subsequent analysis and extraction.

Extraction

200 grams of powdered leaf material were sequentially extracted with chloroform, ethanol, and water using a Soxhlet apparatus to ensure thorough compound extraction. For each solvent (350 mL), the mixture was heated to 50°C and refluxed for 8 hours. This temperature was chosen to prevent the degradation of heat-sensitive compounds while ensuring efficient extraction. The resulting extracts were filtered to remove solid residues and then evaporated under reduced pressure at 40°C to obtain crude extracts. These extracts were subsequently stored at 4°C to preserve their integrity. This stepwise extraction process effectively utilized chloroform, ethanol, and water as solvents, maximizing the recovery of diverse compounds from the plant material (Handa et al., 2008).

Qualitative analysis of phytochemicals in *Tylophora indica* leaves

Tylophora indica leaf powder underwent qualitative analysis for the identification of alkaloids, flavonoids, phenols, saponins, steroids, terpenoids, glycosides, tannins, carbohydrates, and amino acids, following the protocol described by Harborne (1998).

Quantitative phytochemical analysis

Total phenolic content

The total phenolic content (TPC) of *Tylophora indica* leaf extracts was evaluated using the Folin-Ciocalteu assay (Prior et al., 2005). A solution containing 1 mL of the *Tylophora indica* extract (1 mg/mL) was combined with 2.5 mL of 10% Folin-Ciocalteu reagent and 2 mL of 2% sodium carbonate solution. After incubation for 15 minutes, the absorbance was recorded at 765 nm using a microplate reader. A calibration curve for gallic acid (ranging from 1 to 0 mg/mL) was utilized, and the results were reported as gallic acid equivalents (GAE) per milligram of dry extract weight. All measurements were conducted in triplicate to ensure consistent and reliable data.

Total flavonoid content

The total flavonoid content (TFC) of *Tylophora indica* leaf extracts was assessed using a colorimetric aluminum chloride method (Chun et al., 2003). A 0.5 mL aliquot of the extract was mixed with methanol, 10% aluminum chloride, potassium acetate, and water. The mixture was incubated for 30 minutes in a dark environment. The absorbance of the solution was then measured at 420 nm using a UV-1650 spectrophotometer (Shimadzu, Japan). A standard curve for quercetin (ranging from 1 to 0 mg/mL) was prepared, and the findings were expressed in quercetin equivalents (QE) per gram of dry extract weight. Each sample was analyzed in triplicate to ensure accuracy and reproducibility (Prior et al., 2005).

Total alkaloidal content

The quantification of alkaloids in *Tylophora indica* leaf extracts was achieved through a precise colorimetric assay. To prepare the samples, one gram of the extract was solubilized in a 2% sulfuric acid solution prepared with 50% ethanol, followed by filtration and sequential extraction with chloroform. The chloroform phase was evaporated to dryness, and the residue was reconstituted in 2% sulfuric acid. This solution was then reacted with bromocresol green in the presence of a phosphate buffer adjusted to pH 4.7. Further chloroform extraction was performed, and the absorbance of the solution was recorded at 470 nm using a UV-visible spectrophotometer. Atropine served as the calibration standard, and the results were represented as atropine equivalents (AE) in milligrams per gram of dry sample weight. All experiments were conducted in triplicate to ensure reproducibility and minimize error (Prior et al., 2005).

Total triterpenoid content

The determination of triterpenoid concentration was carried out according to the methodology proposed by Lei et al. (2015). In this procedure, 1 mL of the crude extract obtained from *Tylophora indica* leaf powder was combined with 1 mL of 5% (w/v) vanillin dissolved in glacial acetic acid and 2 mL of perchloric acid. The mixture was maintained at 60°C for 15 minutes and subsequently cooled in an ice bath for 20 minutes. Following this, 10 mL of acetic acid was introduced, and the solution was homogenized. After an additional incubation period of 10 minutes, the absorbance of the reaction mixture was measured at 538 nm using a spectrophotometer. Calibration was performed with oleanolic acid as the reference standard, and the results were expressed in terms of oleanolic acid equivalents (OAE) per milligram of dry extract weight.

Collection and maintenance of *Ascaridia galli*

Adult *Ascaridia galli* worms were obtained from the intestines of naturally infected chickens, sourced from local poultry farms and slaughterhouses in Hyderabad, India. Chickens were identified as infected based on clinical signs and post-slaughter visual inspection of intestines for *Ascaridia galli*. Upon collection, the worms were promptly transferred to Tyrode's solution or physiological saline, maintained at 37°C. To minimize temperature fluctuations, insulated containers were used during the transfer process, ensuring a stable environment for the worms. Continuous monitoring indicated that temperature variations were minimal, staying within a range of ± 2°C during collection and transfer. These measures were essential to preserve the physiological integrity and viability of the worms. The worms were stored in these solutions for a brief period to guarantee their suitability for subsequent experimental tests. Careful and precise handling of the worms was paramount for accurate in vitro experimentation (Brewer and Wehr, 2001).

Observation of motility, morbidity, and mortality of *Ascardia galli*

Ten live and active *Ascaridia galli* worms were placed in separate airtight sterile containers. Each container was filled with 25 mL of Ringer Locke (RL) solution mixed with *Tylophora indica* solvent extracts (chloroform, ethanol, and aqueous) at concentrations of 0.5, 1, 2, 3.5, and 5 mg/mL. A control group was maintained in the Hedon-Fleig solution without any extract addition. Piperazine citrate, serving as the positive control, was utilized at a concentration of 5 mg/mL. Behavioral responses of the worms were meticulously monitored at predetermined temporal intervals (5, 15, 30 minutes, and 1, 2, 4, 8, 15, and 24 hours) and systematically categorized based on motility: highly vigorous (++++), moderate (+++), reduced (++), minimal (+), or complete cessation of movement (-). The outcomes of this detailed observational framework provided the foundational data for subsequent in vitro experimental evaluations (Tavares et al., 2013).

Quantitative measure of mortality percentage

The reduction in motility of *Ascaridia galli* worms exposed to chloroform, ethanol, and aqueous leaf extracts of *Tylophora indica* was quantitatively assessed using an Electronic Micro-Motility Meter (EMM) or Optical Multimeter, as described by Gadelhaq et al. (2016). This instrument operates by detecting interruptions in a light beam caused by the locomotor activity of worms within a quartz chamber containing Ringer Locke (RL) solution. These interruptions are translated into electrical signals by a photodetector, enabling real-time quantification of motility inhibition, expressed as a percentage reduction. Experimental conditions were rigorously standardized with a temperature controller maintaining environmental stability. The three extracts (chloroform, ethanol, aqueous) were evaluated in groups of ten worms per extract, with each group tested in triplicate at concentrations of 1, 2, 3, 4, and 5 mg/mL in Petri dishes at ambient temperatures ranging between 25-30°C. Observations of worm motility were conducted at defined intervals of 1, 5, 10, 15, and 24 hours. Additionally, the experimental design incorporated a positive control (5 mg/mL piperazine citrate in RL) and a negative control (RL solution alone) to validate the outcomes. The quantitative analysis highlighted the extracts' potential anthelmintic activity by showing significant motility suppression in treated worms compared to controls, providing valuable insights into the efficacy of *Tylophora indica* leaf extracts against *A. galli*.

Gas chromatography-mass spectrometry analysis of *Tylophora indica* leaves

The ethanolic extract of *Tylophora indica* (100 mg) was dissolved in 1 mL of HPLC-grade methanol and spiked with 1 µL of 2,4,6-trimethylphenol (1 mg/mL) as an internal standard. After vortexing and filtration through a 0.45 µm PTFE syringe filter, 100 µL of the filtrate was derivatized with 50 µL of MSTFA (N-methyl-N-[trimethylsilyl] trifluoroacetamide) at 60°C for 30 minutes. The analysis was conducted using an Agilent 7890 GC system coupled to an Agilent 7000D Triple Quadrupole GC-MS/MS, fitted with an HP-5MS (High Performance-5 Methyl Silicone) capillary column (30 m x 0.25 mm, 0.25 µm film thickness; Agilent Technologies). Helium (99.999%) was used as the carrier gas at 1 mL/min. The oven temperature was programmed to start at 70°C (2 minutes), ramp to 150°C at 10°C/min (1 minute), and then to 280°C at 5°C/min (10 minutes). Mass spectrometry was performed in electron impact mode with anion source, quadrupole, and transfer line temperatures set at 230°C, 150°C, and 280°C, respectively. The instrument operated in MRM mode across an m/z range of 50–550. Peak identification relied on retention times and NIST library (v2017) spectra, while quantification used internal standard calibration. System performance was validated with blank methanol and standard solutions, and repeatability was confirmed via triplicate injections, ensuring precise and reproducible results. The mass range was 50–550 m/z, and data were collected in multiple reaction monitoring (MRM) mode. Data analysis involved calibration using standards and identifying molecular ion peaks based on retention times and mass spectra. Quantification was performed using the internal standard method, where the peak area ratios of analytes to the internal standard (2,4,6-trimethylphenol) were used to calculate the concentrations of the target compounds. The quality control included analyzing blank methanol and standard solutions to confirm system performance. Repeatability tests validated the reliability of results, ensuring robust analysis (Patel et al., 2011).

Solvent fractionation

The ethanolic extract of *Tylophora indica* leaf powder was fractionated systematically. Approximately 10 grams of crude extract were diluted in 100 mL of distilled water and placed in a separatory funnel. The aqueous solution was first extracted with 100 mL of hexane to isolate non-polar substances, with the hexane fraction collected separately. The aqueous layer was then extracted three times with 100 mL of ethyl acetate to capture ethyl acetate-soluble compounds. The ethyl acetate fractions were combined and concentrated via vacuum distillation at 40°C. Next, the residual aqueous layer was extracted three times with 100 mL of n-butanol. The n-butanol fractions were pooled and concentrated under reduced pressure at 40°C, yielding the n-butanol fraction. The final aqueous layer, devoid of hexane-, ethyl acetate-, or n-butanol-soluble compounds, was concentrated at 40°C under reduced pressure to produce the aqueous fraction till one-tenth of the original volume. All fractions-hexane, ethyl acetate, n-butanol, and aqueous were dried and stored individually at 4°C for subsequent analysis. This method effectively separated the ethanolic extract into distinct fractions of varying polarities, enabling detailed chemical and biological evaluations (Koleva et al., 2001).

Isolation of phenanthroindolizidine alkaloids from ethyl acetate fraction

The phenanthroindolizidine alkaloids were isolated from the ethyl acetate fraction of the *Tylophora indica* leaf extract through a systematic process. The ethyl acetate fraction was concentrated under a vacuum to a crude residue and purified via silica gel column chromatography (60 cm × 3 cm, 2 mL/min flow rate) using a chloroform/ethanol/diethylamine gradient (10:0:0.05 to 5:2:0.05 v/v/v). Fractions were analyzed by TLC with UV and alkaloid-specific stains, pooled, and further purified by preparative HPLC on a Waters XBridge C18 column (250 mm × 10 mm, 5 µm particle size). HPLC purification utilized a methanol/water gradient (50:50 to 80:20 v/v over 30 minutes) to separate alkaloids based on polarity and stationary phase affinity. Methanol optimized alkaloid solubility, while water maintained initial polarity. A 5 mL/min flow rate and 500 µL–1 mL injection volumes ensured efficient resolution and high-purity isolation for further analysis. UV detection at 254 nm was used for alkaloid identification. The entire process was conducted at room temperature. The purified phenanthroindolizidine alkaloids were identified and confirmed through ^{1}H-NMR, ^{13}C-NMR, and HRMS spectroscopy, with spectral data compared to established standards. This method effectively extracted and purified phenanthroindolizidine alkaloids from the ethyl acetate fraction of *Tylophora indica* leaf extract (Dhiman et al., 2013).

Effect of phenanthroindolizidine alkaloids on motility, morbidity, and mortality

All experimental procedures involving *Ascaridia galli* worms adhered to institutional and national ethical guidelines for animal research. Ethical approval for this study was obtained from PGP Life Sciences, with an approval number of PGP/LS/77/042/2023. Ten live and active *Ascaridia galli* worms were placed in sterile containers, which were tightly sealed and filled with 25 mL of Hedon-Fleig solution. The worms were treated with either Tylophorinidine (TLD), Tylophorine (TLP), Septicine (SPN), Tylophorinol (TNL), or Antofine (ANF) at concentrations of 250, 500, and 1000

µg/mL, or with a combination of all alkaloids in the ratio of 1:1:1:1:1 (500 µg/mL). A control group was maintained in a Hedon-Fleig solution without any alkaloids, and a positive control piperazine citrate was used at 2.5 mg/mL. The behavior of the worms was periodically observed at 5, 15, and 30 minutes, and at 1, 2, 4, 8, 15, and 24 hours. Activity levels were classified as very active (++++), moderately active (+++), somewhat active (++), sluggish (+), or deceased (-). The subsequent *in vitro* analyses were based on the findings of this observational study (Tavares et al., 2013).

Effect of phenanthroindolizidine alkaloids on mortality

An Electronic Motility Meter (EMM) was employed to quantitatively evaluate the mortality of *Ascaridia galli* worms treated with phenanthroindolizidine alkaloids TLD, TLP, SPN, TNL, and ANF at concentrations of 250, 500, and 1000 µg/mL, as well as a combined treatment of all five alkaloids in a 1:1:1:1:1 ratio (500 µg/mL), following the protocol outlined in Section 2.7 on quantitative percentage mortality measurement. Motility inhibition measurements were taken at 1, 5, 10, 15, and 24 hours post-treatment. A control group was maintained in a Hedon-Fleig solution without alkaloids, while piperazine citrate was used at 2.5 mg/mL. The EMM provided direct measurements of the inhibition percentage of motility for each group, facilitating a quantitative evaluation of the anthelmintic effects of the alkaloid mixture on both strains of *Ascaridia galli* (Gadelhaq et al., 2016).

Evaluation of phenanthroindolizidine alkaloids on *Ascaridia galli* egg embryonation

A modified *in vitro* egg embryonation assay was used to assess the effects of phenanthroindolizidine alkaloids on piperazine-sensitive and piperazine-resistant *Ascaridia galli* strains. Egg viability was confirmed microscopically by identifying intact eggs with clear shells and motile contents. Egg suspensions (100 viable eggs in 0.15 mL PBS) were prepared using flotation with a saturated salt solution, followed by PBS washing. The suspensions were mixed with phenanthroindolizidine alkaloids (TLP, TLD, SPN, TNL, or ANF) at 250, 500, and 1000 µg/mL or a combination of all five (500 µg/mL, 100 µg/mL each). Piperazine citrate (2.5 mg/mL) and PBS served as positive and negative controls, respectively. Mixtures were distributed into 96-well plates and incubated at 30°C ± 1°C for 12 days in a temperature-controlled incubator with daily monitoring. Each treatment was performed in triplicate for statistical reliability. Post-incubation, egg developmental stages were assessed microscopically using established morphological criteria to classify eggs as embryonated or unembryonated. The percentage of unembryonated eggs was recorded for each group up to day 12. This method, based on a modified assay from (Author, Year), quantified embryonation inhibition by observing the absence of cellular division or embryonic structures under 40× magnification, ensuring precise evaluation of treatment effects (Gauly et al., 2001).

Statistical analysis

Data were presented as mean ± SD. The statistical significance of differences between groups was analyzed using one-way ANOVA followed by the Turkeys post hoc test. GraphPad Prism software (GraphPad Software, Inc., La Jolla, CA, USA) was used for the analysis, and a significant difference was accepted at $p < 0.05$.

RESULTS AND DISCUSSION

Extraction and yield

Sequential Soxhlet extraction of *Tylophora indica* leaves resulted in three distinct crude extracts including a dark green, viscous chloroform extract; a brownish, gummy ethanol extract; and a dark brown, solid water extract. The yields of extracts from *Tylophora indica* leaves were calculated after rotary evaporation, resulting in 7.2% for chloroform, 17.8% for ethanol, and 11.2% for aqueous extraction.

Phytochemical analysis

The qualitative phytochemical analysis of *Tylophora indica* leaves identified various bioactive compounds in chloroform, ethanol, and aqueous extracts, with results summarized in Table 1. The ethanol extract was the richest in phytochemicals, suggesting it was a superior solvent for extraction. Alkaloids were the most abundant in the ethanol extract of *Tylophora indica*, while flavonoids and glycosides were the highest in the aqueous extract. Saponins were present in chloroform and ethanol but not in water, indicating solubility differences. Steroids preferred the non-polar chloroform solvent. Terpenoids and phenolic compounds, recognized for their extensive biological activities and potent antioxidant properties, were identified in the ethanol and aqueous extracts of *Tylophora indica*. Tannins were detected exclusively in the chloroform extract, while carbohydrates and amino acids, critical for their nutritional significance, were present across all extracts, with the highest concentrations observed in the aqueous fraction. Lipid content was predominantly associated with the chloroform extract. These findings highlight the intricate phytochemical composition

of *Tylophora indica* and emphasize the pivotal influence of solvent selection on the extraction efficiency of bioactive compounds. Among the solvents tested, ethanol demonstrated superior efficacy in extracting a broad spectrum of phytoconstituents, reflecting its versatility as an extraction medium.

Table 1. Phytochemical analysis of *Tylophora indica* leaves

Number	Phytochemical compounds	Chloroform	Ethanol	Aqueous
1	Alkaloids	-	+++	+
2	Flavonoids	-	++	+++
3	Glycosides	-	++	++
4	Saponins	+	+	-
5	Steroids	++	+	-
6	Terpenoids	-	+	+
7	Phenols	-	++	++
8	Tannins	+	-	-
9	Carbohydrates	+	+	+++
10	Amino acids	+	+	++
11	Fats	++	-	-

+++: Abundant; ++: Considerable; +: Present; -: Absent

Quantitative phytochemical analysis

The quantitative phytochemical analysis of *Tylophora indica* leaves revealed significant variations in the content of phenolics, flavonoids, alkaloids, and triterpenoids across different solvent extracts (Table 2). The total phenolic content (TPC) was quantified using the Folin-Ciocalteu reagent assay, revealing that the aqueous extract exhibited the highest TPC (8.13 ± 0.77 mg/g), followed by the ethanol extract (7.51 ± 0.62 mg/g) and the chloroform extract (3.21 ± 0.28 mg/g, $p < 0.05$). These findings highlight the superior efficacy of highly polar solvents in extracting phenolic compounds, consistent with previous studies (Harborne et al., 1999; Prior et al., 2005). Similarly, the total flavonoid content (TFC) was determined using an aluminum chloride-based colorimetric method, which indicated the aqueous extract had the highest TFC (13.24 ± 1.06 mg/g), followed by the ethanol extract (9.34 ± 1.63 mg/g) and chloroform extract (1.01 ± 0.11 mg/g, $p < 0.05$). These results suggest that the high phenolic and flavonoid concentrations in the aqueous extract may correlate with significant antioxidant potential (Harborne, 1998; Chun et al., 2003). The ethanol extract exhibited the highest total alkaloidal content (17.65 ± 1.69 mg/g, $p < 0.05$), highlighting its potential for pharmacological potential for therapeutic applications such as antimicrobial, anti-inflammatory, and anthelmintic effects (Thawabteh et al., 2019). The total triterpenoid content, quantified using the vanillin-glacial acetic acid method, was greatest in the aqueous extract (4.94 ± 0.25 mg/g), followed by ethanol (4.21 ± 0.26 mg/g) and chloroform (0.89 ± 0.05 mg/g), underscoring its therapeutic potential. These findings demonstrated that ethanol and aqueous extracts are particularly effective for extracting bioactive compounds from *Tylophora indica* leaves, with notable antioxidant and pharmacological properties.

Table 2. Quantitative phytochemical analysis of *Tylophora indica* leaves

Phytochemical class	Chf Ex.	EtOH Ex.	Aq. Ex
Total phenolic content (mg/g Gallic acid equivalent)	3.21 ± 0.28	7.51 ± 0.62	8.13 ± 0.77*
Total flavonoid content (mg/g Rutin equivalent)	1.01 ± 0.11	9.34 ± 1.63	13.24 ± 1.06*
Total alkaloidal content (mg/g atropine equivalents)	2.54 ± 0.18	17.65 ± 1.69$^{\#}$	5.64 ± 0.32
Total triterpenoids content (mg/g Oleanolic acid equivalent)	0.89 ± 0.05	4.21 ± 0.26*	4.94 ± 0.25*

The data are presented as mean ± standard deviation (SD) from three independent experiments. Different superscripts indicate significant differences between the extracts for each phytochemical class. Statistical significance was set at $p < 0.05$. Specifically, the superscript * denotes a significant difference compared to Chf. Ex., while $^{\#}$ denotes a significant difference compared to both Chloroform and Aqueous extracts.

Observation of motility, morbidity, and mortality of *Ascardia galli*

The observation of motility, morbidity, and mortality of *Ascaridia galli* worms treated with various extracts of *Tylophora indica* demonstrated significant differences in their observed effects based on the type and concentration of the extract as shown in Table 3. The control group maintained very high activity levels (++++) throughout the observation period. In contrast, the ethanol extract showed the highest efficacy in reducing worm motility, with complete inactivity (-) observed at concentrations of 3.5 mg/mL and above within 24 hours. Ethanol is superior for anthelmintic activity due to its intermediate polarity, enabling the extraction of both polar and non-polar bioactive compounds like

alkaloids, phenolics, and flavonoids. It effectively disrupts plant cell walls for efficient extraction and is safe and compatible with biological assays. The aqueous extract also demonstrated significant anthelmintic activity, with the worms becoming completely inactive at a concentration of 5 mg/mL within 24 hours., compared to chloroform extract. The chloroform extract was less effective compared to the ethanol and aqueous extracts, with partial inactivity observed at the highest concentration of 5 mg/mL. The positive control group treated with piperazine citrate showed a rapid reduction in worm activity, becoming completely inactive (-) within 8 hours, affirming the reliability of the assay. The data suggested that the ethanol extract of *Tylophora indica* is the most potent in terms of anthelmintic activity, followed by the aqueous extract. The results of this study are consistent with prior research demonstrating the efficiency of ethanol as a solvent for extracting bioactive constituents with notable pharmacological activities (Harborne, 1998). The observed outcomes highlight the potential of *Tylophora indica* extracts as potent anthelmintic agents, with the ethanol extract exhibiting the most pronounced efficacy. Further investigations are essential to isolate and structurally characterize the active constituents contributing to this activity and to elucidate their underlying mechanisms of action.

Table 3. Visual inspection of motility, morbidity, and mortality of *Ascardia galli* over the exposure to different solvent extracts of *Tylophora indica* leaves

Solvent extracts	Conc. in mg/ml	5 minutes	15 minutes	30 minutes	1 hour	2 hours	4 hours	8 hours	15 hours	24 hours
Control	0	(++++)	(++++)	(++++)	(++++)	(++++)	(++++)	(++++)	(++++)	(++++)
Chl. ex	0.5	(++++)	(++++)	(++++)	(++++)	(++++)	(++++)	(++++)	(++++)	(++++)
	1	(++++)	(++++)	(++++)	(++++)	(++++)	(++++)	(++++)	(++++)	(++++)
	2	(++++)	(++++)	(++++)	(++++)	(++++)	(++++)	(+++)	(+++)	(+++)
	3.5	(+++)	(+++)	(+++)	(+++)	(+++)	(+++)	(+++)	(+++)	(+++)
	5	(+++)	(+++)	(+++)	(++)	(++)	(++)	(++)	(++)	(++)
EtOH. ex	0.5	(++++)	(++++)	(++++)	(+++)	(+++)	(+++)	(+++)	(+++)	(++)
	1	(+++)	(+++)	(++)	(++)	(++)	(+)	(+)	(+)	(-)
	2	(++)	(++)	(++)	(+)	(+)	(+)	(-)	(-)	(-)
	3.5	(++)	(++)	(+)	(+)	(-)	(-)	(-)	(-)	(-)
	5	(++)	(+)	(-)	(-)	(-)	(-)	(-)	(-)	(-)
Aq. ex	0.5	(++++)	(++++)	(++++)	(++++)	(++++)	(++++)	(++++)	(++++)	(++++)
	1	(++++)	(++++)	(++++)	(+++)	(+++)	(+++)	(+++)	(+++)	(+++)
	2	(+++)	(+++)	(+++)	(+++)	(++)	(++)	(++)	(++)	(++)
	3.5	(+++)	(++)	(++)	(++)	(++)	(++)	(+)	(+)	(-)
	5	(++)	(++)	(++)	(+)	(+)	(+)	(-)	(-)	(-)
Piperazine	2.5	(+++)	(++)	(++)	(-)	(-)	(-)	(-)	(-)	(-)

Very active (++++); Moderately active (+++); Slightly active (++); Sluggish (+); Dead (-); Chl. Ex: Chloroform extract, MeOH: Methanolic extract; Aq: Aqueous extract of *Tylophora indica*.

Quantitative measure of mortality percentage

The data presented in Figure 1 indicate that the ethanol extract demonstrated superior efficacy in inducing mortality in *Ascaridia galli* worms compared to all other treatment groups. At a concentration of 5 mg/mL, the ethanol extract achieved complete mortality (100%) within 5 hours, with this effect remaining consistent over a 24-hours observation period ($p < 0.05$). Low concentrations of ethanol extract also showed substantial mortality, with 60% and 70% mortality observed at concentrations of 3 mg/mL and 4 mg/mL within an hour, eventually reaching 100% by 24 hours. The aqueous extract showed moderate efficacy, with the highest concentration (5 mg/mL) causing 50% mortality at 24 hours ($p < 0.05$). Lower concentrations resulted in lesser mortality, indicating a dose-dependent effect. The chloroform extract was the least effective, with only 20% mortality observed at the highest concentration (5 mg/mL) after 24 hours. Lower concentrations exhibited minimal or no mortality. The results indicated that the ethanol extract of *Tylophora indica* is the most potent in causing mortality of *Ascaridia galli* worms, followed by the aqueous extract, and the chloroform extract showing the least efficacy. The high mortality rate observed with the ethanol extract suggests it contains the most effective bioactive compounds for anthelmintic activity, consistent with previous studies demonstrating the superior extraction capabilities of ethanol for obtaining pharmacologically active compounds (Harborne, 1998).

GC-MS analysis of *Tylophora indica* leaves

The ethanolic extract of *Tylophora indica* leaves underwent GC-MS/MS analysis, with the resulting chromatogram and the list of identified compounds displayed in Figure 2 and Table 4, respectively. The analysis identified a total of 37 chemical constituents in the extract, including prominent compounds such as β-Sitosterol, Stigmasterol, Lupeol, and notable flavonoids like Quercetin and Kaempferol, as detailed in Table 1. These compounds indicated potential therapeutic applications of *Tylophora indica*, particularly in anti-inflammatory, antioxidant, and anti-cancer activities (Patel et al., 2011). Notably, significant quantities of phenanthroindolizidine alkaloids, including Tylophorine,

Tylophorinidine, Tylophorinol, Antofine, Cryptopleurine, and Septicine, were detected. Tylophorinidine, the most abundant alkaloid, constituted 80.36% of the area, highlighting its major presence in the ethanolic extract. This compound, along with Tylophorine and Tylophorinol, has been extensively studied for its potent anti-cancer properties (Saraswati et al., 2013). Antofine and Septicine also showed substantial presence, further contributing to the therapeutic potential of the extract. The identification of flavonoids, such as Quercetin and its derivatives, supports the antioxidant capabilities of the extract. These findings underscored the rich phytochemical composition of *Tylophora indica* leaves and highlighted the efficacy of ethanol as a solvent for extracting bioactive compounds, particularly phenanthroindolizidine alkaloids. This comprehensive profiling through GC-MS/MS provides a valuable foundation for further pharmacological studies and potential drug development. The presence of these compounds suggests that the ethanolic extract of *Tylophora indica* leaves could be a valuable source of therapeutic agents for treating inflammatory diseases and cancers. Further studies are warranted to isolate these alkaloids and investigate their specific mechanisms of action.

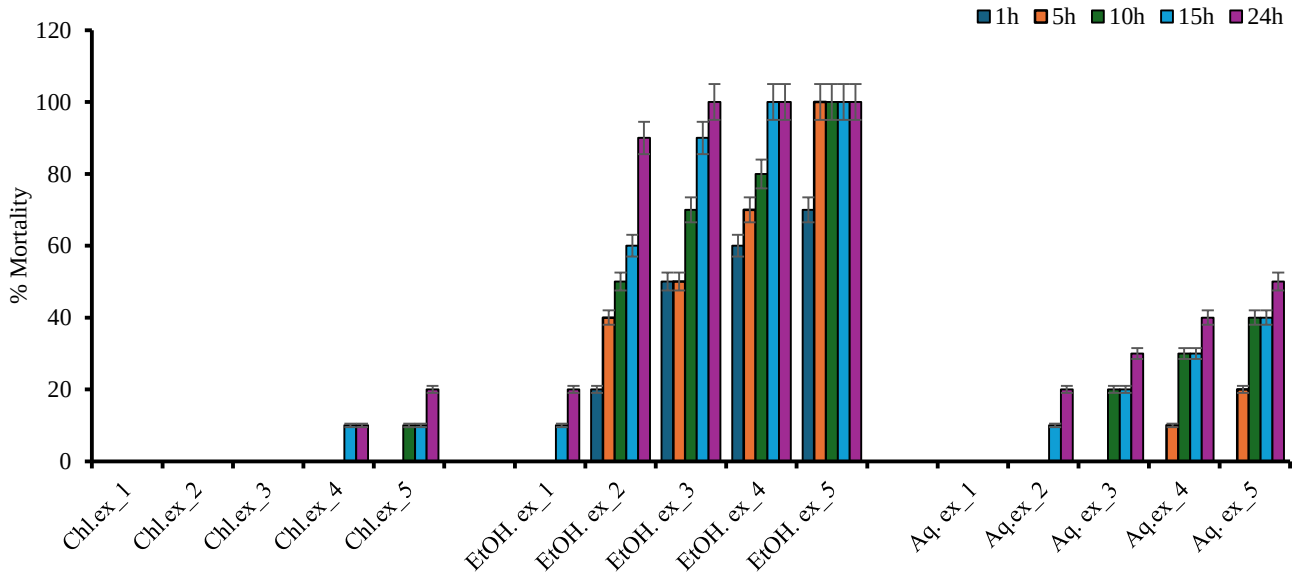

Figure 1. Mortality of *Ascaridia galli* after exposure to different concentrations (1-5 mg/ml) of *Tylophora indica* leaf extracts (chloroform, ethanol, and aqueous) at different time points (1h, 5h, 10h, 15h, and 24h). Data presented as mean percentage mortality. A statistically significant difference was observed between all groups, with a p-value of less than 0.05

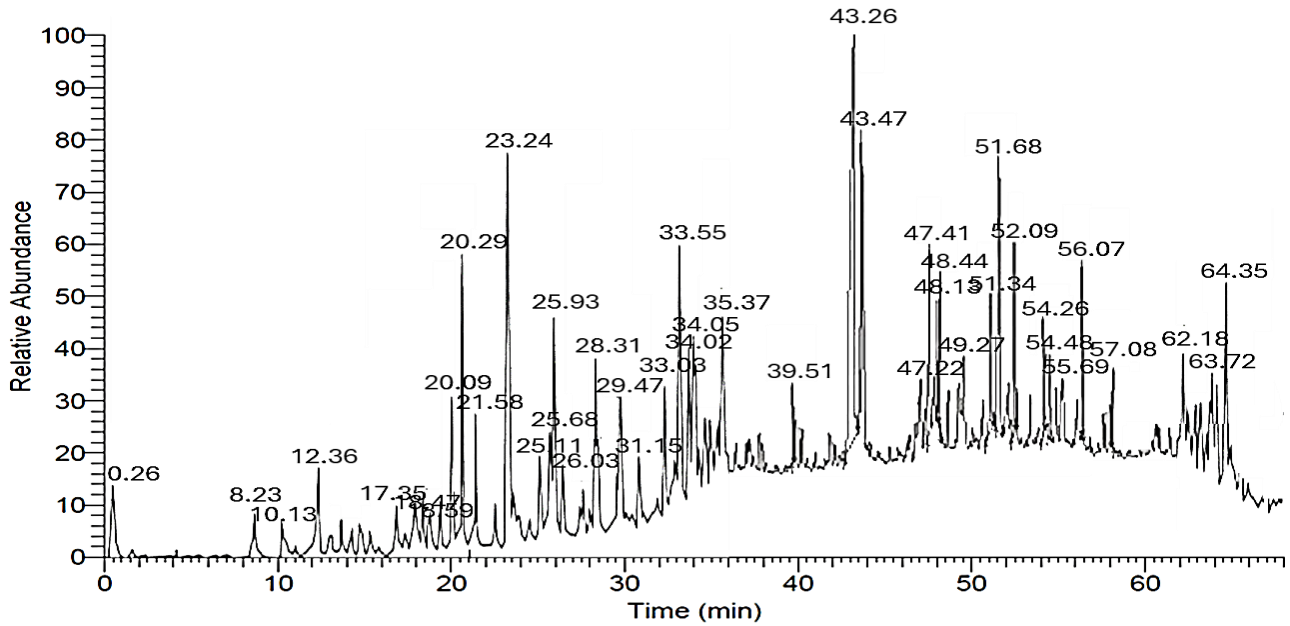

Figure 2. The GC-MS chromatogram of the ethanolic extract of *Tylophora indica* leaves illustrates the detection of diverse phytochemical constituents. The x-axis denotes the retention time (minutes), while the y-axis represents the relative abundance as a percentage. Each peak in the chromatogram corresponds to a distinct compound eluting at a specific retention time, with the relative abundance of the peaks reflecting the proportional representation of the respective compounds within the extract.

Table 4. GC-MS analysis of ethanolic extract of *Tylophora indica* leaves

Number	R_t	Name of a chemical compound	Molecular formula	M. Wt (g/mol)	Area (%)
1	0.26	Cyclotrisiloxane, Hexamethyl-	$C_6H_{18}O_3Si_3$	222.46	13.46
2	12.36	Tert-Butyl(5-Isopropyl-2-Methylphenoxy) Dimethylsilane	$C_{16}H_{26}O_2Si$	278.47	18.42
3	20.09	Benzene, 2-[(Tert-Butyldimethylsilyl) Oxy]-1-Isopropyl-4-Methyl-	$C_{16}H_{26}OSi$	262.46	30.24
4	20.29	1,4-Bis (Trimethylsilyl)Benzene	$C_{12}H_{22}Si_2$	238.48	58.54
5	21.58	2,4-Cyclohexadien-1-One, 3,5-Bis(1,1-Dimethylethyl)-4-Hydroxy-	$C_{17}H_{26}O_2$	262.39	26.68
6	23.24	Methyl β-Sitosterol	$C_{30}H_{53}O$	429.71	77.34
7	25.11	Methyl (Methyl 4-O-Methyl-Alpha-D-Mannopyranoside) Uronate	$C_{10}H_{18}O_8$	266.25	17.51
8	25.68	Phytol	$C_{20}H_{40}O$	296.53	24.19
9	25.93	n-Hexadecanoic Acid	$C_{16}H_{32}O_2$	256.42	45.63
10	26.03	Tocopherol	$C_{29}H_{50}O_2$	430.71	16.39
11	28.31	Lupeol	$C_{30}H_{50}O$	426.71	35.84
12	29.47	Campesterol	$C_{28}H_{48}O$	400.68	26.73
13	31.15	Stigmasterol	$C_{29}H_{48}O$	412.69	14.96
14	33.03	β-Sitosterol	$C_{29}H_{50}O$	414.71	33.14
15	33.55	α-Amyrin	$C_{30}H_{50}O$	426.71	58.42
16	34.02	β-Sitosterol glycoside	$C_{35}H_{60}O_6$	592.85	40.42
17	34.05	Epicatechin	$C_{15}H_{14}O_6$	290.27	41.27
18	35.37	Protocatechuic acid	$C_7H_6O_4$	154.12	42.61
19	39.51	Gallic acid	$C_7H_6O_5$	170.12	31.73
20	43.26	Tylophorine	$C_{24}H_{25}NO_2$	359.46	100
21	43.47	Tylophorinidine	$C_{23}H_{23}NO_2$	345.43	80.36
22	47.22	Ferulic acid	$C_{10}H_{10}O_4$	194.19	29.83
23	47.41	Tylophorinol	$C_{24}H_{26}N_2O_2$	374.48	55.08
24	48.13	Vanillic acid	$C_8H_8O_4$	168.15	48.97
25	48.44	Antofine	$C_{24}H_{27}NO_4$	393.48	51.66
26	49.27	p-Coumaric acid	$C_9H_8O_3$	164.16	33.15
27	51.34	Cryptopleurine	$C_{19}H_{21}NO$	263.37	44.62
28	51.68	Septicine	$C_{21}H_{23}NO$	305.41	74.69
29	52.09	Quercetin	$C_{15}H_{10}O_7$	302.24	54.38
30	54.26	Kaempferol	$C_{15}H_{10}O_6$	286.24	42.11
31	54.48	Rutin	$C_{27}H_{30}O_{16}$	610.52	39.42
32	55.69	Isorhamnetin 3-O-β-glucoside	$C_{22}H_{22}O_{12}$	462.4	33.71
33	56.07	Isorhamnetin 3-O-β-galactoside	$C_{22}H_{22}O_{11}$	446.4	50.44
34	57.08	Quercetin 3-O-β-glucuronide-4′-methylether	$C_{22}H_{20}O_{13}$	508.4	31.09
35	62.18	Kaempferol-3-O-glucoside	$C_{21}H_{20}O_{12}$	448.38	35.11
36	63.72	Kaempferol-3,7-dirhamnoside	$C_{27}H_{30}O_{14}$	594.53	28.74
37	64.35	Quercetin 7-O-β-D-glucopyranoside	$C_{21}H_{20}O_{12}$	464.38	45.36

R_t: Retention time; M.Wt: Molecular Weight

Solvent fractionation, HPLC purification, and structural analysis of phenanthroindolizidine alkaloids

The solvent fractionation of the ethanolic extract of *Tylophora indica* leaves successfully separated the extract into hexane, ethyl acetate, n-butanol, and aqueous fractions. Each fraction was collected, concentrated, and stored for subsequent analysis. The hexane fraction targeted non-polar substances, while the ethyl acetate and n-butanol fractions captured compounds with intermediate polarities. The remaining aqueous fraction contained the polar compounds. This fractionation method allowed for a detailed examination of the chemical and anthelmintic properties of each fraction, facilitating a comprehensive analysis of the phytochemical constituents of *Tylophora indica* (Koleva et al., 2001). Given the promising anthelmintic activity observed in the ethyl acetate fraction, phenanthroindolizidine alkaloids were successfully isolated from this fraction of the *Tylophora indica* leaf extract through a combination of column chromatography and preparative HPLC. The systematic isolation process began with concentrating the ethyl acetate fraction under reduced pressure to obtain a residue. This residue was subjected to column chromatography using a chloroform/ethanol/diethylamine gradient, and the fractions collected were monitored with TLC. Fractions indicating the presence of phenanthroindolizidine alkaloids were pooled and further purified using preparative HPLC. The HPLC chromatograms (Figures 3a and 3b) show distinct peaks corresponding to the isolated phenanthroindolizidine alkaloids, including Tylophorine, Tylophorinidine, Septicine, Tylophorinol, and Antofine. Figure 3a (Standard alkaloid Run Chromatogram) shows clear, distinct peaks for known phenanthroindolizidine alkaloids, including Tylophorinidine, Tylophorine, Septicine, Tylophorinol, and Antofine, with retention times (R_t) at 4.35 min, 7.38 min, 9.55 min, 12.29 min and 15.36 min, respectively. These retention times serve as reference points for comparing the sample run. Figure 3b (Sample Run Chromatogram) confirms the presence of the above phenanthroindolizidine alkaloids by displaying corresponding peaks at nearly identical retention times (Rt) to the expected elution order. Additionally, the sample run chromatogram includes an extra peak at 23.16 min, indicating the presence of an unknown compound. The consistency of retention times across both runs verifies the accurate identification of Tylophorinidine, Tylophorine, Septicine, Tylophorinol, and Antofine in the sample. The phenanthroindolizidine alkaloids were further identified and verified using ^{1}H-NMR, ^{13}C-NMR, and HRMS spectroscopy. The spectral data matched established standards, confirming the accurate identification of the isolated compounds. The major alkaloids identified included Septicine, Tylophorinol, and Antofine, which are known for their potent pharmacological activities, particularly anti-inflammatory and anti-cancer properties. Spectral data was illustrated here.

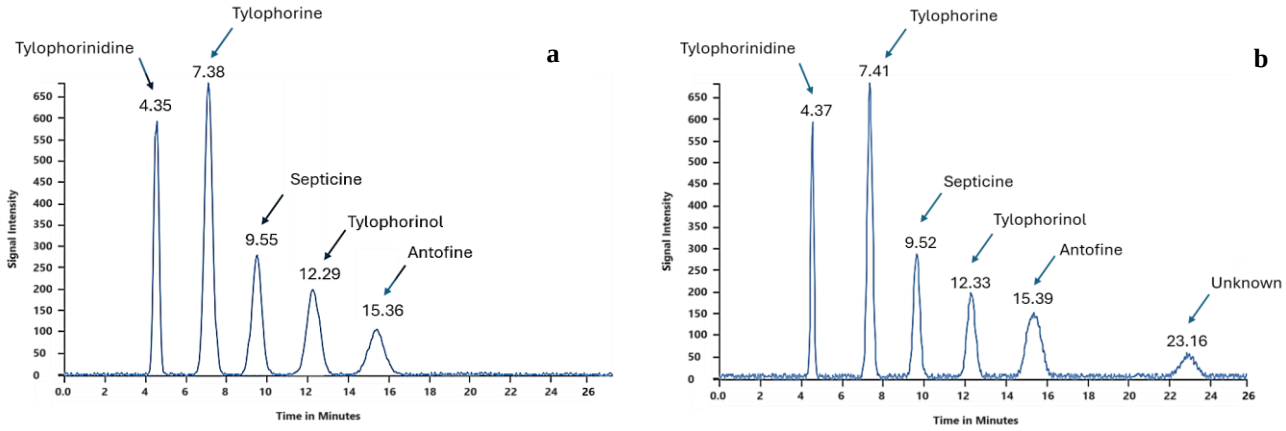

Figure 3. HPLC Chromatograms of Standard Phenanthroindolizidine Alkaloids and *Tylophora indica* Leaf Extract Fraction. **a**: HPLC chromatogram of standard phenanthroindolizidine alkaloids (Tylophorine, Tylophorinidine, Septicine, Tylophorinol, and Antofine) separated under defined chromatographic conditions. **b**: HPLC chromatogram of the ethyl acetate fraction of *Tylophora indica* leaf extract, further purified to isolate phenanthroindolizidine alkaloids.

Effect of phenanthroindolizidine alkaloids on motility, morbidity, and mortality

The study evaluated the impact of phenanthroindolizidine alkaloids from *Tylophora indica* on *Ascaridia galli* motility as shown in Table 5. The control group maintained high activity levels throughout the 24 hours. TLD and TLP exhibited dose-dependent motility inhibition, with TLD showing complete inactivity at 1000 µg/mL by 24 hours and TLP at 500 µg/mL and 1000 µg/mL showing rapid reduction in motility. SPN and TNL also demonstrated potent anthelmintic effects, achieving complete inactivity at 1000 µg/mL by 24 hours. ANF showed similar results with a significant reduction in motility at higher concentrations. The mixture of alkaloids at 500 µg/mL exhibited the strongest synergistic effect, with complete inactivity by 1 hour. The positive control group with piperazine citrate confirmed the assay's reliability, showing complete inactivity by 15 hours. These findings suggested that phenanthroindolizidine alkaloids, particularly Tylophorinidine and Tylophorine, possess significant anthelmintic activity and potential as effective therapeutic agents.

Table 5. Visual inspection of motility, morbidity, and mortality of *Ascardia galli* over the exposure of different phenanthroindolizidine alkaloids of *Tylophora indica*

Solvent extracts	Conc. in mg/ml	5 minutes	15 minutes	30 minutes	1 hour	2 hours	4 hours	8 hours	15 hours	24 hours
Control	0	(++++)	(++++)	(++++)	(++++)	(++++)	(++++)	(++++)	(++++)	(++++)
TLP	250	(++++)	(++++)	(++++)	(++++)	(+++)	(+++)	(+++)	(+++)	(++)
	500	(+++)	(+++)	(+++)	(++)	(++)	(+)	(+)	(-)	(-)
	1000	(++)	(++)	(++)	(+)	(+)	(+)	(-)	(-)	(-)
TLD	250	(++++)	(++++)	(++++)	(+++)	(+++)	(+++)	(+++)	(++)	(++)
	500	(+++)	(+++)	(++)	(++)	(+)	(+)	(+)	(-)	(-)
	1000	(++)	(++)	(+)	(+)	(-)	(-)	(-)	(-)	(-)
SPN	250	(++++)	(++++)	(++++)	(++++)	(++++)	(+++)	(+++)	(+++)	(+++)
	500	(+++)	(+++)	(++)	(++)	(++)	(++)	(+)	(+)	(-)
	1000	(++)	(++)	(+)	(+)	(+)	(-)	(-)	(-)	(-)
TNL	250	(++++)	(++++)	(++++)	(++++)	(++++)	(+++)	(+++)	(+++)	(+++)
	500	(+++)	(+++)	(++)	(++)	(++)	(++)	(+)	(++)	(+)
	1000	(++)	(++)	(+)	(+)	(+)	(-)	(-)	(-)	(-)
ANF	250	(++++)	(++++)	(++++)	(++++)	(++++)	(+++)	(+++)	(++)	(++)
	500	(+++)	(+++)	(++)	(++)	(++)	(+)	(+)	(-)	(-)
	1000	(+++)	(++)	(++)	(+)	(+)	(+)	(-)	(-)	(-)
Mixture of PA's	(1:1:1:1:1) 500	(+++)	(++)	(+)	(-)	(-)	(-)	(-)	(-)	(-)
Piperazine	2.5	(+++)	(+++)	(++)	(++)	(+)	(+)	(+)	(-)	(-)

Conc: Concentration; Very active (++++); Moderately active (+++); Slightly active (++); Sluggish (+); Dead (-).

Effect of phenanthroindolizidine alkaloids on mortality

The mortality data reveal that phenanthroindolizidine alkaloids exhibit significant anthelmintic activity against *Ascaridia galli*, with effectiveness being dose-dependent as shown in Figure 4. Higher concentrations resulted in greater mortality rates specifically, TLD at 250 µg/mL induced 20% mortality by 24 hours, achieving 100% mortality at 500 µg/mL and 1000 µg/mL by 15 hours and 10 hours, respectively, indicating high efficacy at higher concentrations. TLP showed a similar trend, with 10% mortality at 250 µg/mL by 24 hours and reaching 100% mortality at 1000 µg/mL by 10 hours. SPN and TNL demonstrated substantial efficacy, with SPN at 1000 µg/mL reaching 100% mortality by 15 hours and TNL achieving the same by 15 hours. Lower concentrations of these compounds were less effective but still caused notable mortality rates. Antofine (ANF) was similarly effective, with 100% mortality at 1000 µg/mL by 15 hours. The mixture of all five alkaloids at 500 µg/mL exhibited the most rapid and complete anthelmintic effect, achieving 100% mortality by 10 hours, suggesting a possible synergistic effect. The positive control, piperazine citrate (2.5 mg/mL), achieved 100% mortality by 15 hours, confirming the reliability of the assay. These findings indicated that phenanthroindolizidine alkaloids from *Tylophora indica* possess potent anthelmintic properties, with Tylophorinidine, Tylophorine, and the alkaloid mixture showing the most promise. The dose-dependent mortality observed underscores their potential as effective treatments for helminth infections. Further studies are recommended to isolate and characterize the specific mechanisms underlying their anthelmintic activity.

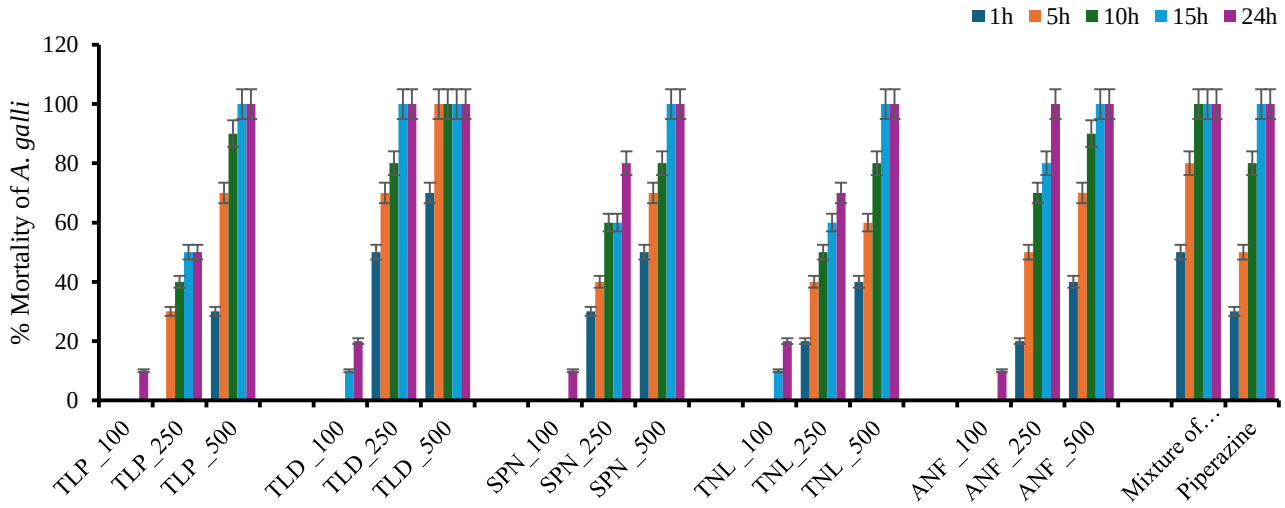

Figure 4. Percentage mortality of *Ascaridia galli* following exposure to varying concentrations (100-500 µg/mL) of TLP, TLD, SPN, TNL, and ANF, a mixture of all five alkaloids, or piperazine (positive control) at different time points (1, 5, 10, 15, and 24 hours). A statistically significant difference was observed between all groups, with a p-value of less than 0.05.

Evaluation of phenanthroindolizidine alkaloids on *Ascaridia galli* egg embryonation

The *in vitro* egg embryonation assay results demonstrated that phenanthroindolizidine alkaloids from *Tylophora indica* exhibited significant inhibitory effects on the embryonation of *Ascaridia galli* eggs (Figure 5, $p < 0.05$). TLD at 1000 µg/mL showed the highest inhibition among the individual alkaloids, with 80.76% undeveloped eggs, followed by ANF at 78.42%, TNL at 76.89%, SPN at 74.11%, and TLP at 72.81% ($p < 0.05$). The mixture of all five alkaloids at 500 µg/mL demonstrated the highest embryonation inhibition, with 92.67% undeveloped eggs, suggesting a synergistic effect that enhances their overall efficacy. The positive control, piperazine citrate (2.5 mg/mL), showed 87.25% inhibition, confirming the reliability of the assay and providing a benchmark for comparison. The results underscore the potential of phenanthroindolizidine alkaloids as promising anthelmintic agents, demonstrating dose-dependent inhibition of egg embryonation, which emphasizes their therapeutic applicability in controlling helminth infections. Further investigation is necessary to isolate and elucidate the specific mechanisms responsible for their inhibitory effects on embryonation, as well as to evaluate potential synergistic interactions among these alkaloids to enhance their efficacy.

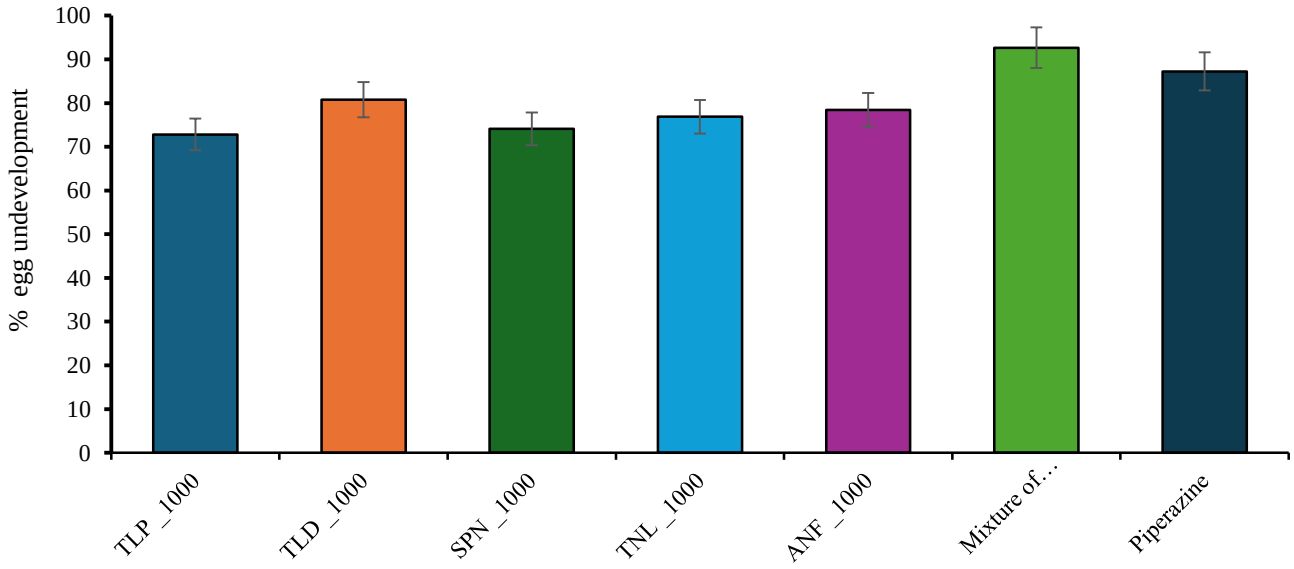

Figure 5. Percentage inhibition of egg development in *Ascaridia galli* following exposure to 1000 µg/mL of TLP, TLD, SPN, TNL, ANF, a mixture of all five alkaloids, or piperazine (positive control). A statistically significant difference was observed between all groups, with a p-value of less than 0.05.

CONCLUSION

Structural characterization identified five phenanthroindolizidine alkaloids: Tylophorinidine, Tylophorine, Septicine, Tylophorinol, and Antofine, all of which exhibited significant dose-dependent anthelmintic activity against *Ascaridia galli*. The combination of all five alkaloids demonstrated a synergistic effect, achieving the highest motility inhibition, mortality rates, and embryonation inhibition (92.67% at 500 µg/mL). Among the individual compounds, Tylophorinidine and Antofine were particularly potent. These findings establish the potential of *Tylophora indica* alkaloids as promising therapeutic agents for helminth infections. Future studies should focus on isolating specific active compounds, elucidating their mechanisms of action, and further exploring synergistic interactions to optimize their efficacy.

DECLARATIONS

Availability of data and materials

All data generated or analyzed during this study are included in the manuscript. Additional datasets, if required, are available from the corresponding author upon reasonable request.

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Competing interests

The authors have not declared any conflict of interest.

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Authors' contributions

Yadagiri Katti was responsible for conceptualization, methodology, investigation, data analysis, drafting, and revising the manuscript. Bethi Neeraja contributed to conceptualization, supervision, resource provision, and critical manuscript review and editing. Both authors have read and approved the final version of the manuscript before publication in the present journal.

Ethical considerations

All authors have contributed to the preparation of this original paper. The authors observed the final version of the finished paper and evaluated any corrections and updates. They also checked the similarity index of the article.

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Effects of Post-Therapy Changes on the Level of Immunoglobulin M in Dogs with Dermatitis

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ABSTRACT

Dermatitis is an inflammation of the skin characterized by itching, hair loss, lesions, and redness. Various agents can cause dermatitis, including *Sarcoptes scabiei*, *Demodex canis*, and *Microsporum canis*. Animals experiencing dermatitis undergo internal changes in their bodies, particularly in the immune system. The presence of an infection is usually preceded by the appearance of Immunoglobulin M (IgM). This study aimed to determine the differences in IgM levels in dogs with dermatitis before therapy (pre-therapy) and after therapy (post-therapy), as well as the differences in IgM levels between dogs with mild and severe dermatitis. The study involved 40 local dogs, divided into two groups, including 20 dogs with mild dermatitis and 20 dogs with severe dermatitis. Serum sampling was conducted in two phases: the first phase was pre-therapy, and the second phase was 14 days after therapy (post-therapy). The therapy administered to dogs with mild dermatitis consisted of diphenhydramine HCl and ivermectin, while the therapy for dogs with severe dermatitis included diphenhydramine HCl, ivermectin, amoxicillin, and dexamethasone. Serum samples from the dogs were then tested using the Enzyme-Linked Immunosorbent Assay method. The results of the study revealed that serum IgM levels in dogs with mild and severe dermatitis did not show any significant difference. In dogs with mild dermatitis, serum IgM levels before therapy were not statistically different compared to those after therapy. However, in dogs with severe dermatitis, serum IgM levels before therapy were significantly higher compared to after therapy. The results of this study indicate that therapy can impact serum IgM levels in dogs with severe dermatitis, while it does not significantly affect these levels in cases of mild dermatitis.

Keywords: Dermatitis, Dog, Enzyme-linked immunosorbent assay, Immunoglobulin M, Ivermectin, Therapy

INTRODUCTION

Dogs are among the animals that can live alongside humans. One of the health issues frequently encountered in dogs is skin disorders, such as dermatitis (Marsella and De Benedetto, 2017). The occurrence of dermatitis in dogs is often closely related to poor management practices. Several triggering factors for the emergence of dermatitis include cleanliness and skin care, improper nutrition, parasite control, and an uncomfortable environment. Stress and the mental health of dogs, such as a lack of playtime, exercise, and sufficient social interaction, can reduce their resistance to infections (Valenzuela et al., 2013).

Dermatitis is an inflammation or irritation of the skin characterized by symptoms such as itching, hair loss, sores, and redness (Ali et al., 2024). The causes of dermatitis can vary, including parasitic infections (ticks and mites), bacterial infections, fungal infections, nutritional deficiencies, physiological stress, and genetic factors (Trinh et al., 2024). Additionally, other factors, such as the environment, the cleanliness of dog handlers, and the immunity of each individual, influence the incidence of dermatitis (Kristianty et al., 2017). Dermatitis can cause inflammation or lesions on the skin, with varying degrees of severity. These lesions can be categorized as mild, moderate, or severe, and can be differentiated into primary and secondary lesions (Suwiti et al., 2022). Dogs experiencing mild dermatitis show mild itching, hair loss, and the formation of non-extensive primary lesions (Suwiti et al., 2022). Conversely, dogs with severe dermatitis experience intense itching, scratching, and continuous biting of their bodies, which can lead to skin damage and inflammation. This condition is often accompanied by open wounds due to excessive scratching, as well as primary and secondary lesions that extend throughout the body (Almutawa et al., 2024).

The treatment of dermatitis in dogs generally involves administering antihistamines to reduce itching or pruritus, using ivermectin for antiparasitic purposes, administering anti-inflammatory medications to alleviate skin inflammation, and prescribing antibiotics to prevent secondary infections (Cahyaniarta et al., 2019; Papich, 2023). The occurrence of dermatitis often occurs in the same dog. Dermatitis can lead to changes in the animal's internal body, particularly in the immune system (Ferreira et al., 2021). Immunoglobulin M (IgM) is one type of antibody produced by the animal's

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immune system. The IgM plays an important role in providing early protection against new infections before other types of antibodies are produced (Erkocoglu and Kocabas, 2015). It helps combat infections by triggering an inflammatory response. Parasitic infections that cause dermatitis can affect the circulation of IgM and IgG, even though an IgE response has already occurred in the body (Laksmi et al., 2019).

The occurrence of an infection by parasites can stimulate and increase the levels of IgE, IgM, and IgG in the body (Mabbott, 2018). Examining IgM levels in dogs suffering from dermatitis is very important, as this information can provide insight into the dog's immune system resilience. If there is an increase in IgM antibodies in a dog with dermatitis, it indicates that the animal's immune system is functioning well. The body responds to the presence of antigens by producing IgM antibodies as an initial line of defense (Ouchida et al., 2012). The administration of anti-inflammatory drugs in cases of dermatitis can influence IgM levels (Kasim et al., 2022). Therefore, it is important to assess IgM profile in dogs with dermatitis both pre-treatment and post-treatment. This study aimed to determine the difference in immunoglobulin M levels in dogs with mild and severe dermatitis and to compare immunoglobulin M levels in pre-therapy and post-therapy.

MATERIALS AND METHODS

Ethical approval

Ethical clearance for this study was approved by the Animal Ethics Committee of the Faculty of Veterinary Medicine Udayana University, Denpasar, Indonesia, No: B/210/UN14.2.9/PT.01.04/2024.

Study design

A total of 40 dogs with dermatitis were used as research samples. The dogs in this study were obtained from owners who had dogs with dermatitis who came to the Taman Griya Pet Care animal clinic, Jimbaran, Badung, Bali for treatment and grouped based on the severity of their condition. The severity was assessed through clinical examination using a standard dermatological scale, which includes the size of the lesion, the intensity of inflammation, and its impact on the dog's behavior. The dogs were then divided into two groups: a group with mild dermatitis (Group 1) and a group with severe dermatitis (Group 2). Each group consisted of 20 dogs. The classification of mild and severe dermatitis in this study was that dogs experiencing mild dermatitis show mild itching, hair loss, and the formation of non-extensive primary lesions. Conversely, dogs with severe dermatitis experience intense itching, scratching, and continuous biting of their bodies, which can lead to skin damage and inflammation. Serum samples for all groups were collected twice, including before treatment (pre-therapy) and 14 days after treatment (post-therapy). Dogs in Group 1 received antiparasitic treatment with ivermectin (Intermectine, PT. Tekad Mandiri Citra, Bandung, Indonesia) at a dose of 0.2 mg/kg BW with subcutaneous route (Pusparini et al., 2023) and antihistamine treatment with diphenhydramine HCL (Dimedryl, PT. Bernofarm, Surabaya, Indonesia) at a dose of 1 mg/kg BW with subcutaneous route (Pusparini et al., 2023). Dogs in Group 2 received the same antiparasitic treatment: ivermectin (0.2 mg/kg BW) and, diphenhydramine HCL (1 mg/kg BW), along with antibiotic amoxicillin (Betamox LA®, Norbrook, Northern Ireland) at a dose of 0.1 ml/BW with intramuscular route and anti-inflammatory dexamethasone (PT. Meprofarm, Bandung, Indonesia) at a dose of 0.5 mg/kg BW with intramusculus route. All treatments were administered by injection on the first day, and followed by oral dexamethasone therapy (Dexaharsen, PT. Harsen, Indonesia) at a dose of 0.5 mg/kg BW for 5 days for dogs with severe dermatitis (Plumb, 2008). Monitoring of dermatitis recovery is done online by asking about the dog's condition, whether the itching has reduced, or whether the redness has reduced. Then, 14 days after therapy, the dog owner brought his dog back to the clinic so we could check the condition of his skin and take blood for post-therapy testing.

Serum sample

Samples were collected in December 2024. A total of three mL of blood was taken from the Vena Cephalica and placed into a non-EDTA tube for serum isolation. The serum samples were stored in a cooler box and transported to the laboratory. The examination of IgM levels in dog serum was conducted at the Veterinary Immunology Laboratory, Faculty of Veterinary Medicine, Udayana University, Bali, Indonesia.

Enzyme-linked immunosorbent assay

Examination of serum IgM levels in dogs with mild and severe dermatitis using the Enzyme-Linked Immunosorbent Assay (ELISA) method with the Canine IgM ELISA kit (Wuhan Feiyue Biotechnology Co., Ltd., China, Catalog: FY-EC6352). The ELISA test begins by preparing a 32 µg/mL stock solution. Six Eppendorf tubes are filled with 150 µL of standard diluent. In the first tube, 150 µL of the stock solution is added and mixed to achieve a 16 µg/mL concentration. Subsequently, 150 µL is transferred from one tube to the next, halving the concentration each time (16, 8,

4, 2, 1 µg/mL). The last tube contains only the standard diluent as a control, yielding a concentration of 0 µg/mL. Additionally, 20 mL of wash buffer concentrate is diluted with 580 mL of deionized water to prepare 600 mL of ready-to-use wash buffer. Next, the wells are prepared for samples, standards, and blanks. In the blank well, no sample or Horseradish Peroxidase-Conjugate reagent is added. In the standard well, 50 µL of standard solution is added, while in the sample well, 40 µL of diluent and 10 µL of sample are added, resulting in a final dilution of 5 times. After all wells are prepared, the plate is sealed with a plate sealer. The plate is then incubated for 30 minutes at 37°C. After incubation, the plate was opened, the liquid in the wells was discarded, and each well was washed with wash buffer. The wells are left for 30 seconds and then dried. This washing process is repeated five times. Next, 50 µL of HRP-conjugate reagent is added to each well, except the blank well. The plate is re-covered and incubated for another 30 minutes at 37°C (Laksmi et al., 2019).

After 30 minutes of incubation, the liquid in the wells was discarded again, and the washing process was repeated five times as before. Then, 100 µL of a mixture of substrate reagents A and B was added to each well, followed by a 10-minute incubation at 37°C in the dark. The reaction was stopped with 50 µL of stop solution, and absorbance at 450 nm was measured within 15 minutes using a spectrophotometer. The IgM concentration was determined by comparing the sample's O.D. value with a standard curve plotted on graph paper. The calculation used the equation $Y = a + bx + cx^2$, where X is the IgM concentration, Y is the O.D. value, and a, b, and c are constants.

Statistical analysis

The data obtained were analyzed using SPSS for Windows, version 25 (SPSS Inc., Chicago, IL, USA). The difference in mean serum IgM levels of dogs with dermatitis was analyzed using the Independent T-test. The level of significance measured was 5% ($p < 0.05$).

RESULTS

The IgM levels in 80 serum samples of dogs showed varying results, ranging from 10.435 µg/mL to 13.761 µg/mL. Serum IgM levels are an important indicator in assessing a dog's immune response, especially in dermatological conditions, such as dermatitis (Pan et al., 2021). The IgM levels studied can vary due to several factors, such as exposure time and type of pathogen (Zhang et al., 2022).

The mean IgM level in mild dermatitis was 12.2885 ± 0.6345 µg/mL, while in severe dermatitis it was 12.0531 ± 0.5374 µg/mL. Although the mean IgM level in mild dermatitis was slightly higher than in severe dermatitis, statistical analysis showed no significant difference ($p > 0.05$, Table 1).

This study showed that the average serum IgM levels of dogs with mild dermatitis at pre-therapy were 12.2885 ± 0.6355 µg/mL. Meanwhile, the average IgM levels at post-therapy were slightly higher than pre-therapy; however, this difference was not statistically significant ($p > 0.05$, Table 2).

In this study, the mean serum IgM levels of dogs with severe dermatitis before therapy were 12.0531 ± 0.5374 µg/mL. After therapy, the mean serum IgM levels decreased by 11.6302 ± 0.7131 µg/mL. The decrease in IgM levels post-therapy showed a significant difference ($p < 0.05$, Table 3).

Table 1. The mean of Immunoglobulin M levels in dogs suffering from mild dermatitis and severe dermatitis

Sample type	Mean serum IgM level (µg/mL)
Mild Dermatitis	12.2885 ± 0.6345[a]
Severe Dermatitis	12.0531 ± 0.5374[a]

[a]: Not significantly different ($p > 0.05$).

Table 2. The mean of Immunoglobulin M levels in dogs with mild dermatitis at pre-therapy and post-therapy

Mild dermatitis	Mean serum IgM level (µg/mL)
Pre-therapy	12.2885 ± 0.6355[a]
Post-therapy	12.3750 ± 0.8254[a]

[a]: Not significantly different ($p > 0.05$).

Table 3. The mean of Immunoglobulin M levels in dogs with severe dermatitis at pre-therapy and post-therapy

Severe dermatitis	Mean serum IgM level (µg/mL)
Pre-therapy	12.0531 ± 0.5374[a]
Post-therapy	11.6302 ± 0.7131[b]

[b]: Real difference ($p < 0.05$).

DISCUSSION

Skin disorders in dogs are a major animal health problem worldwide (Zahri et al., 2024). Dermatitis in dogs is a common condition that can be caused by various factors, such as allergies, bacterial infections, fungi, and parasites (Marsella and De Benedetto, 2017). The IgM is one type of antibody that is first produced by the immune system when exposed to antigens. Serum IgM concentrations in 63 stray dogs with dermatological conditions ranged from 0.59 to 2.08 g/L (Maden et al., 2013). The difference in IgM levels from the results of this study and previous studies is thought to be due to differences in the amount of antigen entering each dog. This condition affects the levels of IgM produced (Laksmi et al., 2019). A study conducted at the Animal Hospital of the Faculty of Veterinary Medicine, Konya, Turkey, showed that IgM levels in 20 healthy dog samples ranged from 0.79 to 2.6 g/L (Maden et al., 2013). Increased IgM levels usually indicate that the immune system is responding to infection or antigen invasion. This is especially relevant in cases of dermatitis, where bacterial or parasitic infections may be the cause of this increase (Maden et al., 2013).

Initially, the immune response is dominated by TH2 cells and involves cytokines, such as IL-4, IL-5, IL-6, IL-13, and IL-31 (Drechsler et al., 2024). IL-4, IL-5, IL-6, IL-13, and IL-31 influence IgM production by regulating B cell activity and adaptive immune pathways. IL-6 plays a major role in supporting IgM production during the early phase of the immune response by inducing B cell activation. In contrast, IL-4 and IL-13 promote isotype switching from IgM to other antibodies, such as IgG or IgE, thereby reducing IgM production in the later phase. IL-31, although associated with chronic inflammation, has an indirect effect on IgM production through the regulation of Th2 cells. TH2 cells are known to play a role in supporting antibody production and the process of immunoglobulin class switching (Nutta et al., 2019). After activation, T lymphocytes will stimulate B lymphocytes by releasing cytokines that trigger the proliferation and differentiation of B lymphocytes. B lymphocytes that recognize antigens through the B cell receptor (BCR) will continue their function with support from T lymphocytes. At this stage, B lymphocytes will differentiate into plasma cells that produce IgM antibodies. Several components of the humoral immune system, such as IgA, IgG, IgE, and IgM, as well as elements of the cellular immune system, including interleukin-2 (IL-2) and IL-10, are also known to contribute to the development of dermatitis (Bou Zerdan et al., 2021).

In mild dermatitis, the immune system works efficiently to manage local antigens, combat pathogens, and protect against infection. IgM, one of the first immunoglobulins produced in response to antigens, plays a role in supporting the initial defense against microorganisms that enter through mildly damaged skin (Xia et al., 2023). In mild dermatitis, inflammation is typically localized and well-regulated, allowing B lymphocytes responsible for IgM production to function normally. In contrast, severe dermatitis involves a more complex and chronic inflammatory response. This response is regulated by cytokines, which play a pivotal role in mediating both pro-inflammatory effects, such as interleukins IL-1, IL-6, IL-8, tumor necrosis factor (TNF), and interferon (IFN)-γ, as well as anti-inflammatory effects such as IL-10 (Tosi et al., 2024). Increased levels of pro-inflammatory cytokines, such as IL-6 and TNF-α can interfere with B cell activity, potentially reducing IgM production (Liu et al., 2021). Severe inflammatory conditions are often accompanied by increased apoptosis or dysfunction of immune cells, including plasma cells responsible for immunoglobulin production (Szymanski et al., 2021). In addition, excessive immune response in severe dermatitis can trigger immunosuppressive mechanisms, such as the release of IL-10 and the activation of regulatory T cells (Tregs), which aim to reduce inflammation and indirectly suppress the production of antibodies, such as IgM (Agrawal et al., 2011). In mild dermatitis pre-therapy, although the immune response was stimulated by local inflammation, the initial IgM levels may not be too high. This is because, in the early phase of inflammation, the immune system may not be fully active in producing antibodies to combat potential infection or irritation, as mild dermatitis is generally associated with lower levels of inflammation severity (Helen et al., 2021). In mild dermatitis, the immune system is only exposed to a small number of antigens, so the immune response tends to be weaker. As a result, B-cell activation and IgM production are limited. Therapeutic management for dogs with mild dermatitis usually involves the use of antiparasitic drugs, such as ivermectin and antihistamines. Ivermectin is often used to treat a variety of parasitic infestations in dogs, including mites that cause conditions such as demodectic mange and scabies. However, its use should be approached with caution due to the potential for serious side effects, especially in certain breeds (Hermawan et al., 2024). Antihistamines are often prescribed to reduce the itching (pruritus) caused by allergic reactions in canine dermatitis. However, their use should be tailored to the needs of each case, and other treatment options may be considered depending on the individual dog's response (Outerbridge and Jordan, 2021). After therapy, these drugs reduce immune and inflammatory activity, which reduces the production of immune cells involved in the initial inflammatory response. Once the inflammation is under control, the body begins to produce more IgM as an additional protective measure, helping the body ward off possible infections or allergies that arise after the skin repairs (Wilson et al., 2019).

Before therapy, dogs with severe dermatitis usually have widespread inflammation throughout their body. This causes an increase in IgM production, as the immune system attempts to respond to tissue damage or the presence of

large amounts of antigen. In cases of severe dermatitis, secondary infections or exposure to large amounts of antigen occur, stimulating B cell activation. This condition causes high levels of IgM production as an initial response to the antigen. Elevated IgM levels pre-therapy reflect an active inflammatory reaction, where the dog's body fights to combat the factors causing severe dermatitis, such as bacteria, stronger allergens, or severe irritation. Treatment for this condition often involves a combination of several drugs, including ivermectin, diphenhydramine HCl, dexamethasone, and amoxicillin (Farhan et al., 2024). In severe dermatitis, corticosteroids work by interacting with glucocorticoid receptors in cells, which in turn affect the expression of genes involved in inflammation and immune system function (Strehl et al., 2019). These mechanisms include inhibition of the production of proinflammatory cytokines, such as IL-6 and TNF-α, as well as regulation of molecular signals important for lymphocyte differentiation and activity (Chen et al., 2023).

The decrease in IgM levels after therapy for severe dermatitis is due to the mechanism of action of the drugs used. Ivermectin, as an antiparasitic, works by eradicating parasites that trigger the immune response (Fahmy et al., 2021). With the elimination of parasites, antigenic stimulation is reduced so that B cell activation and IgM production also decrease. Corticosteroids have potent immunosuppressive properties. This drug suppresses the activity of the immune system, including the process of B cell differentiation into plasma cells that are responsible for producing IgM. The combined effect of these two drugs reduces inflammation and suppresses excessive immune responses so that IgM levels in circulation are significantly reduced after therapy (Indayani et al., 2024). Diphenhydramine HCl is a first-generation antihistamine that functions as an inverse agonist, binding to the H1 receptor to inhibit inflammation caused by histamine (Linton et al., 2023). By blocking histamine, diphenhydramine helps reduce the activation of excessive immune responses. Ivermectin is an antiparasitic that works by interfering with the parasite's nerve function. It works by releasing Gamma Amino Butyric Acid (GABA), which prevents neurotransmitters, causing paralysis in parasites (Pandit and Tarkeshwar, 2023). In addition, ivermectin can have an immunomodulatory effect, potentially affecting IgM levels by reducing immune reactivity (Lotfalizadeh et al., 2022). Diphenhydramine HCl and amoxicillin can also affect the decrease in IgM levels in severe dermatitis through different mechanisms. Diphenhydramine HCl, as an antihistamine, helps reduce symptoms of inflammation, such as itching and swelling, by blocking histamine receptors (Doniec et al., 2024). Although it indirectly affects IgM levels, the decrease in inflammation due to the antihistamine effect can reduce antigenic stimulation that plays a role in B cell activation and IgM production. Amoxicillin, as an antibiotic, works by eliminating bacterial infections that may worsen dermatitis. By reducing the infectious load, the antigenic source is reduced, thereby reducing excessive immune responses, including IgM production (Maslakah and Kusumarini, 2023). By treating the infection, amoxicillin can help reduce high IgM levels due to the immune response to infection. A significant decrease in serum immunoglobulin G titers has been observed following amoxicillin administration (Dufour et al., 2005). The decrease in serum IgM levels after therapy for severe dermatitis also indicates that the therapy is effective in reducing the inflammatory process. Immunoglobulin M is usually produced as an initial response to infection or inflammation, so this reduction or decrease in IgM levels may indicate that the inflammatory process underlying dermatitis has been controlled (Saghazadeh and Rezaei, 2020). Some therapies for dermatitis, such as the use of corticosteroids, are known to affect the composition of immunoglobulins (Mustafa, 2023). Administration of corticosteroids can cause a decrease in IgM levels in the body because corticosteroids have an immunosuppressive effect, which can inhibit the immune response and antibody production, including IgM (Hussain and Khan, 2022). This finding indicates that treating dermatitis with various drugs can affect the formation of IgM.

The limitation of this study was the absence of a control group because the use of a control group requires more resources, including time, cost, and access to healthy dogs without dermatitis. It is difficult to ask permission from healthy dog owners to have their dogs' blood taken for research. The second was the passive sample collection method, namely waiting for the owner of a dermatitis dog to come to the clinic; this caused the samples used in this study to be small. This study only focuses on IgM levels alone, not using other immune parameters because researchers want to focus on the results of IgM levels in dermatitis as an initial picture, and it is hoped that in the future, there will be further research on dermatitis by measuring other immune parameters, such as interleukins and cytokines. The decrease in IgM is regarded as an indicator of effective therapy for controlling inflammation. However, the specific mechanisms linking changes in IgM levels to clinical improvement in dermatitis have not been thoroughly evaluated. Additional studies are needed to clarify and strengthen the causal relationship between IgM reduction and clinical recovery.

CONCLUSION

IgM levels in dogs with mild dermatitis were not significantly higher than those with severe dermatitis. In dogs with mild dermatitis, serum IgM levels pre-therapy were not statistically different compared to post-therapy. However, in dogs with severe dermatitis, serum IgM levels in pre-therapy were significantly higher compared to post-therapy. Further

research is needed, including the use of dermatitis drugs that do not suppress the immune response, and also to check the management practices to prevent dermatitis cases.

DECLARATIONS

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Authors' contributions

Ni Ketut Suwiti designed and analyzed the study. Ni Putu Dyah Prashanti Pusparini collected samples, wrote the manuscript, and analyzed the data. Ida Bagus Kade Suardana and I Nengah Kerta Besung supported the implementation of the study. All authors have read and approved the data and the final draft of the manuscript.

Competing interests

The authors have not declared any conflict of interest.

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Ethical considerations

The authors confirm that all authors have reviewed and submitted the manuscript to this journal for the first time. Additionally, all authors checked the originality of data and sentences via plagiarism checkers.

Availability of data and materials

The original data presented in the study are included in the article. For inquiries, please contact the corresponding author.

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Canine Mast Cell Tumors: Clinical Signs, Laboratory Diagnosis, Treatment, and Prognosis

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ABSTRACT

Canine mast cell tumors, a tumor originating from mast cells involved in allergic reactions and inflammation, are among the most common skin tumors in dogs. The present study aimed to explore the clinical features, diagnostic approaches, and prognosis of canine mastocytomas through a case study. A 5-year-old male Akita, weighing 35.8 kg, was brought to the Doctor VET veterinary clinic in Kamianets-Podilskyi, Ukraine, for evaluation. Upon initial examination, the dog had a body temperature of 38.5°C, a heart rate of 74 beats per minute (bpm), and a respiratory rate of 28 breaths per minute, all of which were within normal physiological limits. The animal was alert and responsive and displayed no signs of systemic distress. A detailed physical examination revealed a tumor located 35.2 mm below the plantar surface of the tarsal joint (art. tarsi). The tumor was round, mobile, and surrounded by a thin fibrous capsule, with no signs of pain or discomfort during palpation. Cytological analysis showed a high-cellularity smear with numerous mast cells scattered throughout the field. These cells were round to oval in shape with abundant cytoplasm containing dense, basophilic to metachromatic granules. The hematological evaluation indicated a systemic inflammatory or immune response triggered by the tumor, as evidenced by neutrophilic leukocytosis (73.1%; 8.89×10⁹/L). Biochemical analysis revealed an elevated alkaline phosphatase activity level (4.45 µmol/L), suggesting systemic involvement. The tumor was surgically excised, ensuring complete removal with wide margins to minimize the risk of recurrence. Histological examination of the excised tissues confirmed a densely cellular neoplastic infiltrate composed predominantly of mast cells arranged in sheets and clusters. The mast cells displayed significant cellular and nuclear pleomorphism, characterized by moderate to marked anisocytosis and anisokaryosis. While no significant necrosis was observed, scattered apoptotic bodies were present, indicating ongoing cellular turnover. This case highlighted the critical importance of early diagnosis and comprehensive management of canine mastocytomas. Low-grade tumors often carry a favorable prognosis when treated promptly and appropriately. However, higher-grade or poorly differentiated tumors may require multimodal therapeutic approaches to achieve better outcomes.

Keywords: Canine, Diagnosis, Mast cell tumor, Mastocytoma, Skin tumor

INTRODUCTION

Mast cell tumors (MCTs) are among the most prevalent cutaneous neoplasms in dogs, arising from mast cells, which play a key role in allergic responses and inflammatory processes (Blackwood et al., 2012; Gerasimos et al., 2023; Zhelavskyi et al., 2024a). These tumors exhibit a wide range of clinical behaviors and morphological features, complicating diagnosis and therapeutic management. MCTs represent approximately 7-21% of all cutaneous malignancies in canines (Gómez et al., 2020). MCTs are frequently diagnosed in middle-aged to older dogs, with certain breeds, such as Boxers, Labrador Retrievers, Boston Terriers, and Pugs, showing a higher predisposition (Warland and Dobson, 2013). Despite ongoing research, the exact mechanisms underlying the development of mast cell tumors remain unclear. However, mutations in the *c-KIT* gene have been identified as a significant factor contributing to their pathogenesis (MacDonald et al., 2023), which encodes the tyrosine kinase receptor, and plays a key role in the development of mastocytomas (Watson et al., 2020; Coelho et al., 2023; Zmorzynski et al., 2024). Mast cells grow and multiply uncontrollably as a result of these mutations. Clinical signs of mastocytoma can range from small nodules on the skin to large, ulcerated, and infiltrative masses (Cino et al., 2023). Tumors often appear on the trunk, limbs, or head. Symptoms may include itching, ulcers, swelling, and systemic reactions, such as vomiting and anorexia due to histamine release from tumor cells (Kimura et al., 2021; Bhanpattanakul et al., 2025). Cytological examination reveals characteristic mast cells with granules (Berlato et al., 2021; Oberholtzer et al., 2024). Histological analysis allows for the

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determination of the tumor's grade according to Patnaik (Grade I-III), which is crucial for prognosis (Rassnick et al., 2010; Horta et al., 2018; Roberts et al., 2022).

The excision should include sufficient healthy tissue around the tumor to minimize the risk of recurrence (Hoshino et al., 2020). The primary treatment method for mastocytomas is surgical removal with wide margins. Chemotherapy serves as an adjuvant therapy in cases with a high risk of recurrence or metastasis (London et al., 2009; Korbelik et al., 2021). Medications, such as vincristine, cyclophosphamide, and prednisolone, help reduce residual tumor cells (Wilson et al., 2020; Roberts et al., 2022).

Targeted therapies, particularly tyrosine kinase inhibitors (imatinib, toceranib), show promising results in treating mastocytomas with *c-KIT* mutations (Cilloni et al., 2021; Bertola et al., 2024). The prognosis depends on the tumor grade, the presence of metastasis, and treatment effectiveness. Well-differentiated tumors (Grade I) have a favorable prognosis with high survival rates after surgical removal (Kiupel et al., 2011; Ribatti, 2025). Tumors of intermediate and low differentiation (Grade II-III) have a poor prognosis and require comprehensive treatment (Horta et al., 2018; Roberts et al., 2022; Larsen et al., 2023). The present study aimed to investigate canine mastocytoma, including its clinical features, diagnosis, treatment, and prognosis.

MATERIALS AND METHODS

Ethical approval

The clinical investigations were carried out in compliance with the Law of Ukraine "On Protection of Animals from Cruel Treatment" (21/02/2006, 3447-IV) and followed the European Commission's guidelines on the treatment of vertebrates, ensuring protection from thirst, hunger, malnutrition, discomfort, fear, pain, and suffering.

Study design

A 5-year-old male Akita weighing 35.8 kg was brought to the Doctor VET veterinary clinic in Kamianets-Podilskyi, Ukraine, for evaluation. Upon initial examination, the dog had a body temperature of 38.5°C, a heart rate of 74 beats per minute (bpm), and a respiratory rate of 28 breaths per minute, all of which were within normal physiological limits. The animal was alert and responsive and displayed no signs of systemic distress. The owner's primary concern was the presence of a well-defined, palpable mass on the left hind limb, which had gradually increased in size over time. A detailed physical examination revealed the location of the tumor: 35.2 mm below the tarsal joint (art. tarsi) in the plantar surface (Figure 1). The tumor had smooth borders, was round, 35.2 mm in diameter, hard to palpation, and surrounded by a thin fibrous capsule. The surface was hilly, pink in color, without hair and damage (ulcer, exudation). The tumor was mobile and not attached to deep tissue structures. During palpation, no pain reaction or discomfort was noted in the animal. External examination and palpation revealed no changes in the regional lymph nodes. During ultrasonography of the internal organs of the abdominal cavity (SonoSite 180 Plus FUJIFILM, Sonosite, USA), C60/5-2 Transducer (5-2 MHz), Ultrasound gels (Parker Laboratories, USA) and X-ray (Vet Ray Digital DR [Vet Ray Technology, IDEXX, USA]) of the chest, no signs of metastasis were found.

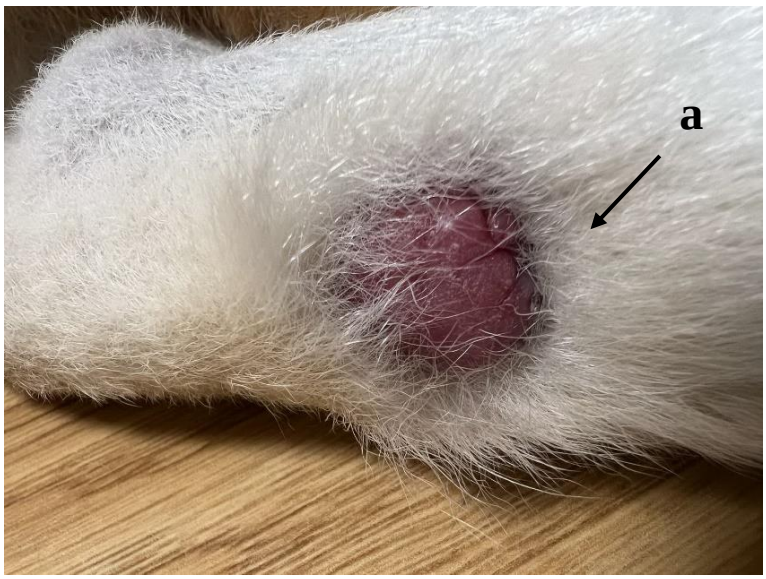

Figure 1. Mastocytoma in in a 5-year-old male Akita dog. The tumor (35.2 mm) is located below the tarsal joint (art. tarsi) on the plantar surface of the left hind leg (**a**).

Anesthetic protocol and surgical procedure

In the beginning, a preoperative clinical examination was performed. The fur around the tumor was shaved, and the skin was cleaned with a 5% alcohol solution of iodine (B. Braun Medical Inc., USA). Methadone hydrochloride (Lannett Company, Inc., USA) was used for premedication at a dose of 0.2 mg/kg intravenously (vena cephalica). The use of propofol for general anesthesia ensures rapid induction of anesthesia, smooth awakening, and minimal side effects with proper monitoring (Johnson et al., 2022).

Anesthesia was induced with propofol (Propofol 200mg/20ml emulsion for injection vials, Baxter International Inc., USA). The basic dose was 4 mg/kg intravenously (vena cephalica). The drug was administered slowly until the required level of anesthesia was reached (within 30-60 seconds). Anesthesia was maintained using propofol at a dose of 0.5 mg/kg, administered intravenously to ensure a stable anesthetic state and minimize discomfort during the procedure. Continuous monitoring of vital signs was performed throughout anesthesia (temperature of 38.3°C, a heart rate of 71 beats per minute (bpm), respiratory rate of 31 breaths per minute; the arterial blood pressure: systolic pressure of approximately 131 mmHg and a diastolic pressure of 95 mmHg: Dawei HD10-VET (Dawei Veterinary Medical, China)) to maintain patient safety and optimal physiological conditions. For local anesthesia, 2% lidocaine hydrochloride (Lidocaine, Mylan Pharmaceuticals, Italy) was utilized to ensure adequate pain management during the procedure (Kudnig and Séguin, 2022; Duffee et al., 2023).

An incision was made in the skin and tissues above the mastocytoma during the operation. An incision was performed at an adequate margin, maintaining a distance of 0.5 cm (not mm, which is typically insufficient in surgical oncology) from the visible tumor borders to ensure the removal of all potentially affected tissues and minimize the risk of residual disease.

After the removal of the tumor, a thorough examination of the operating area was carried out to detect possible remnants of tumor cells. The excised mastocytoma, measuring 38.2 mm in diameter, was immediately placed in a sterile container and transported to the laboratory for histopathological evaluation to verify the diagnosis and assess the tumor's malignancy grade. Intraoperatively, the surgical site was extensively irrigated with 0.9% sodium chloride solution (B. Braun Melsungen AG, USA) to reduce the risk of infection and remove cellular debris. Hemostasis was carefully managed to prevent excessive bleeding, and the wound was closed in multiple layers using an atraumatic suturing technique to enhance tissue recovery and minimize postoperative complications. The deeper layers of tissues were closed using absorbable sutures, followed by the closure of the skin with non-absorbable sutures to ensure wound strength and minimize the risk of dehiscence. Appropriate aseptic techniques were employed throughout the procedure to prevent postoperative infections. For this, absorbable (size: 3-0, Ethicon, Johnson and Johnson, USA) and non-absorbable suture material (size: 3-0, Surgipro II, Medtronic, USA) were used (Johnson et al., 2022). During the operation, physical indicators were monitored on the patient's monitor (Digicare Animal Health DigiVet, Life Window Lite Series, USA). After the operation, the dog was transferred to the postoperative ward for condition monitoring and recovery from anesthesia. Postoperative care included the use of the antibiotic 15% amoxicillin (Amoxicillin Bioveta 150 mg/ml LA injectable suspension) at a dose of 1.0 ml per 15 kg of body weight, intramuscularly, and painkillers meloxicam (0.5% Metacam, Boehringer Ingelheim Vetmedica GmbH, Boehringer Ingelheim, Germany), on the first day-0.4 ml/10 kg of body weight, subcutaneously, then in the dose on the first day-0.2 ml /10 kg of body weight, subcutaneously. Regular examinations were also carried out to monitor wound healing and detect possible complications (Wustefeld-Janssens et al., 2021). Pain assessment and infection monitoring were conducted using a multimodal approach beyond routine medication administration. The Glasgow Composite Measure Pain Scale (CMPS; Kudnig and Séguin, 2022) was utilized to evaluate the dog's discomfort postoperatively, while wound inspection was performed daily to check for signs of infection, such as erythema, swelling, discharge, or increased local temperature. The follow-up period lasted 14 days, during which the wound healing process was monitored, and the sutures were removed at the appropriate time. No significant postoperative complications were observed.

Conducting a biopsy for cytological examination

During the clinical examination, a cytological diagnosis was established: mastocytoma. For this, the field of operation was prepared (Berlato et al., 2021; Bellamy and Berlato, 2022). The skin at the biopsy site was treated with 80% ethyl alcohol (Decon Laboratories, Inc., USA). An aspiration fine needle (ABTG, Argon Chiba Biopsy Needle, USA) was used for the biopsy (Valenciano and Cowell, 2019).

The collected material was carefully placed onto a clean glass slide. It was then evenly smeared to create a thin, uniform layer suitable for microscopic examination. The smear was immediately fixed using 70% methyl spirit (BASF SE, Germany) to preserve cellular morphology and prevent artifacts. This preparation ensured the sample's integrity for subsequent staining and analysis. Staining was performed using Quik-Diff (Siemens Healthineers, Germany). The puncture site was treated with 80% ethyl sprite (Decon Laboratories, Inc., USA).

The sample was stained using the Romanowsky method (Raskin and Meyer, 2016). Initially, the sample was fixed in 70% methanol (BASF SE, Germany). Then, it was immersed in a mixture of dyes containing azure II and eosin (Merck, MilliporeSigma, Germany). After staining, the sample was washed with water to remove excess dye and dried before microscopic examination (Axioscope 5 Zesis, Germany).

Sample selection and drug preparation

The biopsies were promptly immersed in a container with 10% aqueous formaldehyde (Sigma-Aldrich, Merck KGaA, Germany), appropriately labeled, and dispatched to the histology laboratory (Kamianets-Podilskyi, Ukraine). A sterile adhesive plaster was applied over the biopsy site (Berlato et al., 2021; Bellamy and Berlato, 2022). The skin biopsy was fixed in 10% formaldehyde for at least 24-48 hours. The samples were then processed using the hematoxylin and eosin (H&E) staining protocol provided (Leica Biosystems, Germany).

Blood collection and analysis

At the start of the study, a 50.0 µL blood sample was collected from the cephalic vein. A thorough hematological assessment was performed to analyze several key parameters. These included the erythrocyte count ($\times 10^{12}$/L), which measures the number of red blood cells; the leukocyte counts ($\times 10^{9}$/L), indicating the total white blood cell count; and the leucogram values ($\times 10^{9}$/L, %), which detail the distribution of different white blood cell types. The analysis also evaluated hemoglobin concentration (µmol/L), a critical measure of the blood's oxygen-carrying capacity; hematocrit (L/L), representing the proportion of blood volume occupied by red blood cells; mean corpuscular volume (MCV, fl); mean corpuscular hemoglobin (MCH, fmol); mean corpuscular hemoglobin concentration (MCHC, mmol/L); and thrombocyte count ($\times 10^{9}$/L), which quantifies platelets essential for blood clotting. The analysis was performed using the Abaxis Vetscan HM5 Hematology Analyzer (USA), a device known for its precision in evaluating blood components. In addition to hematological analysis, blood chemistry was assessed using the VetScan VS2 Biochemistry Analyzer (USA). This instrument was employed for the in vitro quantitative measurement of various biochemical markers, such as albumin (ALB, mmol/L), alkaline phosphatase (ALP, µmol/L), alanine aminotransferase (ALT, µmol/L), amylase (AMY, µmol/L), total bilirubin (TBIL, µmol/L), blood urea nitrogen (BUN, mmol/L), calcium (Ca, mmol/L), phosphorus (P, mmol/L), creatinine (CRE, µmol/L), glucose (GLU, mmol/L), sodium (Na^+, mmol/L), potassium (K^+, mmol/L), total protein (TP, g/L), and globulin (GLOB, g/L). These parameters provide critical insights into metabolic, renal, hepatic, and electrolyte status, ensuring a comprehensive evaluation of the subject's physiological condition.

Statistical analysis

Statistical analysis was performed to evaluate the data collected during the study. All computations and analyses were conducted using the Statistica® 12.6 software (StatSoft, USA), a comprehensive tool for statistical and data visualization tasks.

RESULTS

Hematological findings

The RBC count (8.22×10^{12}/L) had remained within the reference range (Table 1). A mild leukocytosis had been observed, with WBC values (12.17×10^{9}/L) slightly exceeding the upper reference limit, which could have been attributed to a systemic inflammatory or immune response triggered by the tumor. Neutrophil levels (NEU, 73.1%; 8.89×10^{9}/L) had fallen within the normal range for both percentage (52.0-81.0%) and absolute count ($3.0\text{-}12.0 \times 10^{9}$/L), indicating a well-regulated inflammatory response without excessive neutrophil activation. The lymphocyte percentage and absolute count had also been within normal limits, suggesting an appropriate immune response without signs of lymphopenia or lymphocytosis. Monocyte levels had remained within the expected range. Basophils (BAS, 0.1%) had been at the upper limit of the normal range (0-0.1%), while the absolute count had stayed within acceptable limits ($0\text{-}1.0 \times 10^{9}$/L), suggesting no significant basophilia despite potential mast cell involvement. Hemoglobin levels had exceeded the reference range, indicating possible hemoconcentration, likely due to tumor-associated metabolic changes. The hematocrit value (HCT, 0.44 L/L) had been at the upper limit of normal (0.35-0.45 L/L). The mean corpuscular volume (MCV) had been below the normal range (60.0-76.0 fl), indicating microcytosis, while the mean corpuscular hemoglobin concentration (MCHC) had been elevated above the reference range (18.61-23.58 mmol/L), suggesting hyperchromasia. The platelet count (PLT, 134.0×10^{9}/L) had been at the lower end of the normal range. These hematological findings highlighted a mild systemic response to mastocytoma, characterized by leukocytosis and specific alterations in red blood cell and platelet parameters.

Table 1. Hematological parameters in a 5-year-old Akita male dog with mastocytoma

Parameter	Units	Measured values*	Results
Erythrocytes (RBS)	$\times 10^{12}$/L	5.4-8.50	8.22
Leukocytes (WBS)	$\times 10^{9}$/L	6.0-12.0	12.17
Neutrophil (NEU)	%	52.0-81.0	73.1
	$\times 10^{9}$/L	3.0-12.0	8.89
Lymphocyte (LYM)	%	15.0-25.0	21.3
	$\times 10^{9}$/L	1.0-4.80	2.59
Eosinophils (EOS)	%	0.5-3.0	2.1
	$\times 10^{9}$/L	0-1.0	0.2
Monocytes (MON)	%	0-6.0	3.4
	$\times 10^{9}$/L	0.2-1.50	0.4
Basophils (BAS)	%	0-0.1	0.1
	$\times 10^{9}$/L	0-1.0	0
Hemoglobin (HGB)	G/L	95.0-150.0	178
Hematocrit (HCT)	L/L	0.35-0.45	0.44
MCV	fl	60.0-76.0	53.6
MCH	fmol	1.24-1.67	1.34
MCHC	mmol/L	18.61-23.58	25.0
Thrombocyte (PLT)	$\times 10^{9}$/L	117.0-490.0	134.0

MCV: Mean corpuscular volume, MCH: Mean corpuscular hemoglobin, MCHC: Mean corpuscular hemoglobin concentration, Source of control data: Harvey (2012)

Biochemical findings

Amylase levels were 20.48 µmol/L, within the normal range, showing no pancreatic dysfunction (Table 2). Total bilirubin remained low (at 0.71 µmol/L), excluding hyperbilirubinemia. Blood urea nitrogen was 5.58 mmol/L, indicating normal renal function. Calcium levels were 2.54 mmol/L. Phosphorus was slightly below the normal range at 1.46 mmol/L (1.50-2.50 mmol/L), suggesting potential dietary or metabolic factors affecting phosphorus balance. Creatinine was 98.08 µmol/L (53.0-125.0 µmol/L), confirming preserved kidney filtration. Glucose levels were slightly elevated at 6.20 mmol/L (3.9-6.7 mmol/L), possibly reflecting stress hyperglycemia. Sodium and potassium levels were 147.0 mmol/L (143.0-150.0 mmol/L) and 4.80 mmol/L (4.1-5.4 mmol/L), respectively, indicating normal electrolyte balance. Total protein was at a normal range of 66.87 g/L (54.0-75.0 g/L), with globulin levels at 3.21 g/L (1.9-3.7 g/L), suggesting no significant protein loss or immune abnormalities. These results highlighted minor deviations in alkaline phosphatase and glucose levels, which may reflect the systemic impact of mastocytoma, while most parameters indicated stable metabolic and organ functions.

Table 2. Biochemistry parameters in a 5-year-old Akita male dog with mastocytoma

Parameter	Units	Measured values*	Results
Albumin	mmol/L	0.36-0.67	0.56
Alkaline phosphatase	µmol/L	0.2-2.01	4.45
Alanine aminotransferase	µmol/L	0.31-1.16	0.35
Amylase	µmol/L	5.0-25.0	20.48
Total bilirubin	µmol/L	0-3.42	0.71
Blood urea nitrogen	mmol/L	3.5-10.4	5.58
Ca	mmol/L	2.34-2.76	2.54
P	mmol/L	1.50–2.50	1.46
Creatinine	µmol/L	53.0-125.0	98.08
Glucose	mmol/L	3.9-6.7	6.20
Na^+	mmol/L	143.0–150.0	147.0
K^+	mmol/L	4.1-5.4	4.80
Total protein	g/L	54.0–75.0	66.87
Globulin	g/L	1.9–3.7	3.21

Source of control data: Bonagura and Twedt (2013)

Cytological findings

The cytological smear obtained from the fine-needle aspiration of a mastocytoma in a 5-year-old Akita dog showed numerous round to oval cells with distinct cytoplasmic granules (Figure 2). These cells exhibited moderate to high cellularity with round nuclei, some of which are eccentrically located, and a finely granular chromatin pattern. Occasional binucleated cells and anisokaryosis (variation in nuclear size) were observed, indicating mild nuclear atypia. The cytoplasm was abundant and filled with metachromatic granules, staining prominently purple with the cytological dye. The basis background was scattered mast cell degranulation, contributing to a granular extracellular appearance. Few eosinophils and scattered inflammatory cells were present, consistent with a reactive or inflammatory component associated with mast cell tumors. Overall, the findings supported a diagnosis of mastocytoma, with cytological features suggesting a well-differentiated to moderately differentiated grade.

The cytological preparation demonstrated a high cellularity smear with numerous mast cells dispersed throughout the field (Figure 3). The mast cells were round to oval in shape, with moderate to abundant cytoplasm filled with dense, basophilic to metachromatic granules. The nuclei were round to oval and centrally to eccentrically located, with a fine to moderately coarse chromatin pattern. Occasional cells exhibited binucleation, and mild anisokaryosis was observed, reflecting nuclear pleomorphism. The cytoplasmic granules are uniformly distributed, although some areas show evidence of degranulation in the background, producing a granular extracellular matrix. Additionally, small clusters of eosinophils and rare lymphocytes were present, indicating a mild inflammatory response. The smear background appeared clean, with minimal proteinaceous material and a lack of significant necrosis. No evidence of mitotic figures or overtly malignant characteristics, such as marked pleomorphism or nuclear atypia, was noted, which was consistent with a low-to intermediate-grade mast cell tumor.

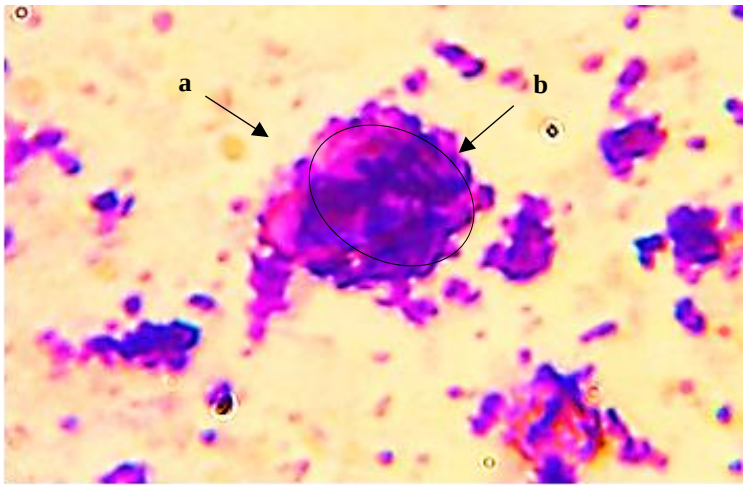

Figure 2. Mastocytoma in a 5-year-old male Akita dog. **a**: Mast cell, **b**: Intracellular granules stained dark blue. Magnification x2000, stained with Quik-Diff.

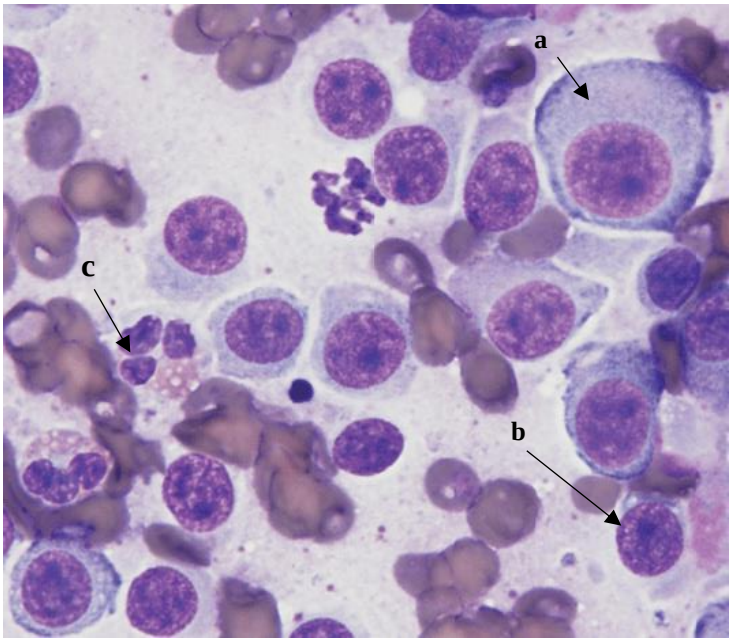

Figure 3. Mastocytoma in a 5-year-old male Akita dog. **a**: Mast cell, **b**: lymphocytes, and neutrophils (**c**). Magnification x3000, stained with aqueous Romanowsky.

Histological findings

The tissue showed a densely cellular neoplastic infiltrate composed predominantly of mast cells arranged in sheets and clusters. The mast cells display cytoplasm was abundant, containing numerous metachromatic granules typical of mast cells (Figure 4). Notable cellular and nuclear pleomorphism was observed, characterized by moderate to significant anisocytosis and anisokaryosis. The surrounding stroma was moderately fibrous, with evidence of infiltration by small numbers of eosinophils and occasional lymphocytes, suggesting a reactive inflammatory response. The presence of mast cells extending into deeper dermal layers was observed, and the margins of the tumor appeared infiltrative, consistent with locally invasive behavior. There was no significant necrosis within the examined field, although scattered apoptotic bodies were visible.

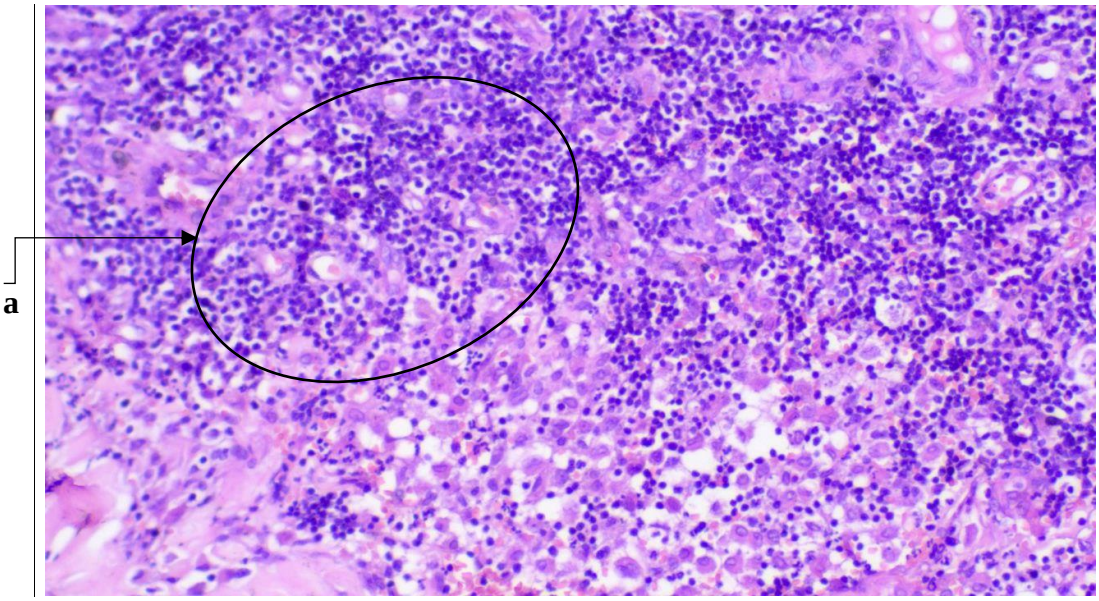

Figure 4. Mastocytoma in a 5-year-old male Akita dog. Active cellular (**a**): Infiltration (lymphocytes, plasma cells, neutrophils) is visualized in the histological preparations, magnification x160, stained with H and E.

DISCUSSION

According to previous studies, mast cell tumors account for approximately 20-25% of all skin neoplasms in dogs. They are most frequently observed in dogs over the age of 5, with an average age of onset around 9 years. It is noted that MCTs can present as solitary lesions, but in 11-14% of cases, multiple tumors may occur (Blackwood et al., 2012; Harshbarger and Meinkoth, 2017; Bellamy and Berlato, 2022). Certain breeds exhibited a higher predisposition to developing mast cell tumors, including Boston Terriers, Boxers, Labradors, and Shar Peis. There was no significant evidence of gender predisposition for MCTs (Berlato et al., 2021; Gerasimos et al., 2023).

Classification is primarily determined by the histological zone exhibiting the highest number of mitotic or nuclear variations. A study by Kiupel (2020) demonstrated a principled approach and correlation between tumor grade and clinical outcome (Kiupel et al., 2021). The 2013 Oncology and Pathology Working Group (OPWG) consensus statement recommended the use of both the Kiupel (2011) classification schemes for cutaneous MCTs (Kiupel et al., 2011). In 2021, the OPWG suggested incorporating two classification systems to better predict tumor behavior and study survival rates. Therefore, MCTs are classified into several categories (grades), including low grade (I), medium grade (II), and high grade (III, Berlato et al., 2021).

New data have emerged on the association of histological nodule grades with clinical outcomes in dogs with MCT exposed to radiopharmaceuticals (Stefanello et al., 2024). It is possible for mast cell tumors in dogs to be differentiated at a low to a high grade.

A significant factor is the presence of mutations in the *c-KIT* gene, which are found in approximately 13.6% of cases (Bellamy and Berlato, 2022; Song et al., 2025). The studies indicate that *c-KIT* mutations are more frequently associated with low-grade tumors (66.7%) and rarely found in high-grade tumors (Camus et al., 2016; De Ridder et al., 2021). Activating mutations in the *c-KIT* gene lead to constitutive activation of the *KIT receptor*, promoting uncontrolled cell proliferation and survival of mast cells. These mutations are commonly found in exon 11, but can also occur in exons 8, 9, and 17. *c-KIT* mutations are often associated with higher-grade MCTs and more aggressive tumor behavior (Korbelik

et al., 2021; De Nardi et al., 2022). Detection of *c-KIT* mutations can aid in the diagnosis and prognostication of MCTs. Molecular testing for these mutations is increasingly used to guide treatment decisions (Roberts et al., 2022). The presence of *c-KIT* mutations makes MCTs potential candidates for targeted therapy with tyrosine kinase inhibitors (*TKIs*), such as toceranib or masitinib, which specifically inhibit the *KIT receptor* and can improve outcomes in affected animals (Larsen et al., 2023). This finding suggested that histological classification may influence the prognosis of the disease (Iamone et al., 2024).

Cytology is a quick and cost-effective method commonly used for diagnosing MCT. These tumors typically exfoliate numerous cells containing many small, round, purple granules, facilitating diagnosis (Shaw et al., 2018; Zhelavskyi, 2024). Cellular features central to Kiupel's classification system can be evaluated using cytological preparations. Several recent studies have explored the correlation between cytologic characteristics and histologic grade.

Cytology is a rapid and cost-effective diagnostic method widely used for detecting mast cell tumors (MCT) in animals. Due to the high exfoliation rate of tumor cells, this technique provides valuable material for analysis (Paes et al., 2021). A key characteristic of mast cells is the presence of numerous small, round, purple-stained granules in the cytoplasm, which significantly facilitates their identification (Shaw et al., 2018). One of the main advantages of cytological examination is the ability to assess key morphological features used in Kiupel's classification system to predict the biological behavior of the tumor (Sabattini et al., 2018). Recent studies have identified a correlation between cytological features and histological grading of mast cell tumors (Del Río-Sancho and Christen-Zaech, 2025). Parameters, such as the number and characteristics of intracellular granules, nuclear atypia, mitotic count, and the presence of inflammatory infiltrates have been recognized as predictive markers of tumor malignancy (Cilloni et al., 2024). Moreover, cytological analysis allows for the rapid determination of the need for further histopathological evaluation, which is crucial for developing an effective therapeutic strategy. Current research focuses on improving cytological assessment methods, particularly through the use of immunohistochemical markers and molecular approaches, to enhance diagnostic accuracy and prognosis of MCTs (Larsen et al., 2023).

Currently, there are promising opportunities for diagnosticians to refine methods for selecting cytological samples and optimizing staining techniques, as well as in the development of specific tumor markers (Vicente et al., 2024). Advances in cytological techniques aim to enhance the accuracy of mast cell tumor (MCT) diagnosis, improve the differentiation between low- and high-grade tumors, and facilitate early detection, which is crucial for effective treatment planning (Vicente et al., 2024). Immunobiological aspects of oncogenesis are being increasingly studied, providing deeper insights into the mechanisms driving tumor progression, including the role of inflammatory mediators, immune evasion, and genetic mutations contributing to malignancy. Researchers are focusing on identifying molecular markers that could serve as reliable prognostic indicators, guiding personalized treatment approaches (Wilson et al., 2020; Gianni et al., 2024). Additionally, new data are emerging on the use of effective pharmacological treatments for cutaneous mastocytosis, contributing to the advancement of targeted therapeutic approaches (Oberholtzer et al., 2024). Recent studies have highlighted the potential of tyrosine kinase inhibitors, monoclonal antibodies, and other novel pharmacological agents in managing mast cell-related disorders. These therapeutic advancements not only improve patient outcomes but also provide a foundation for further exploration of combination therapies that could enhance treatment efficacy and minimize adverse effects (Green et al., 2023).

The ongoing integration of cytology, molecular diagnostics, and targeted therapies underscores the importance of a multidisciplinary approach in the diagnosis and management of mast cell tumors. Future research will likely focus on various criteria, developing non-invasive biomarkers, and expanding treatment options to improve the prognosis and quality of life for affected animals and humans (Oberholtzer et al., 2024). The drug cyclosporine is commonly used in the treatment of autoimmune disorders in dogs and to prevent the rejection of transplants (Wustefeld-Janssens et al., 2021). While effective, cyclosporine use can lead to a range of side effects, including an increased risk of neoplastic conditions, such as mastocytoma (Kudnig and Séguin, 2022). The immunosuppressive effects of cyclosporine help to manage autoimmune diseases but can also impair immune surveillance, increasing the risk of tumor development (Gómez et al., 2020; Kimura et al., 2021; Zhelavskyi et al., 2024b). Common side effects include vomiting, diarrhea, and anorexia, which are often dose-dependent and may decrease with dose adjustment (Walker et al., 2025). Long-term use of cyclosporine can cause gingival overgrowth, which may require dental care or surgical intervention (London et al., 2009). Though less common in dogs than in humans, nephrotoxicity remains a concern, particularly with prolonged use at high doses (Roberts et al., 2022; Brown et al., 2023). Increased incidence of different cancers, including mast cell tumors, has been observed in dogs receiving long-term cyclosporine therapy (De Ridder et al., 2021; Deng et al., 2024). Specific cases document the development of mastocytomas in dogs treated with cyclosporine, suggesting a potential causative relationship (De Andrade et al., 2023). Analyses of veterinary records indicate a higher frequency of mastocytomas in dogs that received cyclosporine compared to those not receiving the drug (Radia, 2025). Routine veterinary check-ups, including blood evaluation and physical examinations, are crucial for early detection of potential

side effects or neoplastic developments (De Ridder et al., 2021). Combining cyclosporine with other immunosuppressive drugs may allow for lower dosages and reduced side effects (Wustefeld-Janssens et al., 2021).

CONCLUSION

Canine mast cell tumors are among the most prevalent skin tumors in dogs, arising from mast cells involved in allergic and inflammatory responses. The current study underscores the importance of early detection and a comprehensive management approach. Cytological and histological analyses revealed characteristic mast cell features and moderate pleomorphism. Surgical removal was successful, with histology showing no significant necrosis, suggesting a favorable prognosis. However, advanced cases with metastasis or high-grade malignancies require a multidisciplinary approach, including surgery, chemotherapy, and targeted therapy. Regular veterinary check-ups are crucial for early detection, timely intervention, and improved quality of life for dogs.

DECLARATIONS

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Availability of data and materials

Data generated from this research are available for readers upon a well-founded request to ensure transparency and reproducibility.

Authors' contributions

Mykola Zhelavsky developed the study concept, reviewed the clinical records, collected the data, and performed the experimental procedures. Serhii Kernychnyi and Tatiana Zakharova led the development of the research base and supervised clinical studies. Tamara Betlinska and Maksym Luchka performed laboratory analyses and related research work. All authors actively participated in the critical evaluation and revision of the manuscript, ensuring its scientific rigor and consistency. They jointly revised and improved the final version of the manuscript, confirming that it accurately reflected the study's findings and their shared opinions. All authors approved the manuscript for submission, affirming their agreement with her findings and their commitment to the accuracy and integrity of the entire work.

Ethical considerations

Authors check and admit to ensure originality, maintain high ethical standards, and avoid fabrication of data, falsification, plagiarism, or improper publication.

Competing interests

The authors declare that they have no financial, professional, or personal conflicts of interest that could influence the content, outcomes, or interpretation of this research.

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Effects of Sulpiride on the Reproductive System of Male Rats after Puberty

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ABSTRACT

Sulpiride is an antipsychotic drug commonly used in humans to mitigate the effects of stress by selectively targeting central dopaminergic receptors. During male rat puberty, neurotransmitter systems, including the dopaminergic system, undergo significant development, playing a crucial role in the release of gonadal hormones and the regulation of reproductive function. The present study aimed to investigate the effects of sulpiride on reproduction parameters in adult male rats. This study used 30 adult male rats with an average body weight of 250-300g and an average age of 90-95 days. The rats were randomly divided into three groups of 10 each. Group 1 (G1) received 10 mg/kg sulpiride, Group 2 (G2) received 25 mg/kg sulpiride, and the control group (G3) received normal saline, all administered via gavage. This study evaluated hematological (testosterone, luteinizing hormone, prolactin, and Follicle-stimulating hormone) and histopathological parameters (spermatogenesis, seminiferous tubules, and total sperm count). The histopathology result of the testes from treated rats revealed significant histological changes. In G1, the seminiferous tubules exhibited destruction, with disrupted spermatogenesis and reduced numbers of sperm in the lumen. These changes were more pronounced in G2, which received the higher dose of sulpiride (25 mg/kg). In contrast, the control group (G3) displayed normal histological structures and spermatogenesis. Hormonal analysis showed a significant decrease in testosterone and luteinizing hormone (LH) levels in G2 compared to G1 and G3. The hematological results for blood serum showed that the concentration of the hormone prolactin was also significantly increased in G2 treated with 25 mg/kg sulpiride as compared with G1 and G3; the concentration of follicle-stimulating hormone (FSH) levels did not differ significantly across groups. Sperm motility and concentration were significantly reduced in G2 compared to G1 and G3, accompanied by a significant increase in the percentage of abnormal and dead sperm. Histological findings further confirmed severe destruction of the seminiferous tubules in G2 compared to G1 and the control group. In conclusion, administering sulpiride at concentrations of 10 mg/kg and 25 mg/kg in adult male rats caused significant structural and functional defects in the seminiferous tubules of the testes.

Keywords: Follicle-stimulating hormone, Luteinizing hormone, Male rat, Prolactin, Sulpiride, Testosterone

INTRODUCTION

Sulpiride is primarily marketed as an antipsychotic treatment and acts as a selective antagonist of dopaminergic receptors (Mohameda et al., 2010). In rodents, treatment by sulpiride has been shown to produce behavioral effects, such as anti-aggressive actions, and to induce ovulation in female rats (Martín-López et al., 1993; Ali, 2024).

The administration of sulpiride in rats has been associated with a significant increase in serum prolactin concentration. Hyperprolactinemia, caused by elevated prolactin levels, diminishes gonadotropin secretion, reduces gonadotropin-releasing hormone (GnRH) secretion, and impairs luteinizing hormone (LH) responses to GnRH (Jafarpour et al., 2019). Hyperprolactinemia causes infertility in mice due to its effect on the release of GnRH (Zheng et al., 2020). The regulated release of hypothalamic GnRH ensures the normal functioning of the hypothalamo-hypophysio-gonadal axis through the secretion of gonadotropins and testosterone in the systemic circulation, necessary for spermatogenesis, maturation of spermatozoa, and reproductive behavior (Nira et al., 2008). Studies have shown that dopamine antagonists synapse on GnRH terminals in the median eminence, where dopamine application inhibits GnRH release (Okamura et al., 2013; Al-Mousawe and Ibrahim, 2024). The hypothalamus secretes GnRH, which stimulates the pituitary gland to release follicle-stimulating hormone (FSH) and luteinizing hormone (LH; Smith, 2012; Ali and Najlaa, 2023). GnRH influences serum testosterone concentration and can inhibit testicular development and spermatogenesis over time (Pan et al., 2023). Male fertility in animals is regulator by two adenohypophysial hormones, including follicle-stimulating hormone (FSH), and luteinizing hormone (LH), through testosterone synthesis in Leydig cells and its aromatization to estradiol in Sertoli cells (Wang et al., 2021; Ramya et al., 2023). Regulation of gonadotropin-releasing hormone (GnRH) release ensures proper functioning of the hypothalamic-pituitary-gonadal axis by releasing

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gonadotropins and testosterone into the systemic circulation, which is necessary for reproductive behavior, spermatogenesis, and sperm maturation (Acevedo-Rodriguez et al., 2018; Pan et al., 2024). The testes are the primary reproductive organ in males, responsible for sperm production and the synthesis of testosterone, a key male hormone (Pan et al., 2024). There is growing global concern regarding the impact of persistent organic pollutants on reproductive health. Various environmental toxicants have been shown to adversely affect spermatogenesis in both rodents and humans, leading to reduced sperm count, abnormal sperm morphology, and diminished semen quality (Guerrero-Limón et al., 2024). The present study aimed to evaluate the effects of sulpiride on hormone levels, sperm parameters, and testes in male rats.

MATERIALS AND METHODS

Ethical approvals

The animal experimentation was approved by the Ethical Clearance Committee of the College of Veterinary Medicine, Tikrit University, under registration number Tu. Vet. 28 (2024). This study was conducted from February to April 2024 at the animal house of the College of Veterinary Medicine, Tikrit University, Tikrit, Iraq.

Animals and treatment

The study included 30 adult male rats, with an average body weight ranging from 250 to 300 g and an average age of 90 to 95 days (from February to April of 2024). The animals were randomly divided into three groups, each consisting of 10 male rats. Five rats were housed in standard plastic cages measuring 46×28×13 cm, under controlled conditions of lighting, ventilation, and temperature (20-25℃). To maintain hygiene, the cage floors were coated in sawdust and replaced two or three times a week. Food and water were provided ad libitum, and artificial lighting was maintained. Group 1 received a daily oral dose of 10 mg/kg sulpiride (Solarbio Life Science, China), Group 2 received 25 mg/kg sulpiride, and Group 3 (control) received normal saline. Treatments were daily administered by gavage for four weeks (Mereu et al., 1983).

Blood collection

Within 24 hours after the completion of the treatment, blood samples (0.5 mL) were collected from the tail vein of all rats (experimental and control groups) to measure serum concentrations of testosterone, prolactin, FSH, and LH. Blood collection was performed under anesthesia. Anesthesia was induced using isoflurane via a vaporizer in a chamber with 3-5% isoflurane for five minutes, and maintenance was achieved with 1–3% isoflurane via a nose cone. The isoflurane concentration was adjusted based on continuous monitoring of the respiratory rate. Sedation was confirmed by a toe pinch before the procedure (Charlès et al., 2023).

Collection of testes and spermatozoa

The testes and epididymis from three male rats in each group were collected. The left testis was collected for histological study, while the right testis and epididymis were frozen at -20°C for the evaluation of sperm parameters.

Sperm parameters

Evaluation of sperm characteristics

After the treatment period, the epididymis tail was removed and placed in a Petri dish containing 10 ml of normal saline at 37℃. The cauda of the epididymis was incised into pieces (at least 200 slices), and the sperm were discharged (Tajik and Hassan, 2008; Naji et al., 2022).

Sperm motility was assessed by placing a drop of liquefied semen on a microscope slide, covering it with a coverslip, and observing it under a microscope at ×400 magnification. The motile and immotile spermatozoa in different fields of view on the slide were counted. A total of 200 spermatozoa were counted, and the mean of the counts for each sample was calculated (Khan, 2022).

Sperm vitality was estimated by assessing the integrity of the cell membrane. The percentage of live sperm was determined by dye exclusion to identify sperm with intact cell membranes. One drop of sperm was mixed with two drops of 1% eosin solution on a clean microscope slide. After 30 seconds, three drops of 10% nigrosine solution were added to the slide and mixed. One drop of the sperm-eosin-nigrosine mixture was placed on a clear slide, and a smear was prepared. The slides were air-dried and counted for sperm under a microscope to count the unstained (live) and stained (dead) cells (Khan, 2022).

Sperm concentration was determined using a 100 µm deep hemocytometer (Neubauer chamber). The sperm were diluted 1:10 with a diluent containing 50 g of sodium bicarbonate (NaHCO3), 10 ml of formaldehyde solution (36%-

40%) [v/v], 0.25 g of trypan blue, and 1000 ml of distilled water. The hemocytometer sides were filled with sperm suspension and covered by a cover slide; the sperm were counted in twenty-five small squares of the chamber (Naji et al., 2022).

To identify and count sperm shape abnormalities, a fine epididymal sperm suspension was prepared and stained with 0.2 ml of 1% eosin in water. A drop of the stained suspension was placed on a clean microscope slide and allowed to dry. The slide was inspected for abnormalities in the shape of the sperm head and tail. A total of 1000 spermatozoa were examined per animal according to the method introduced by Bairy and Shivananda (2001), and abnormalities were expressed as the total number of abnormal spermatozoa per 1,000 spermatozoa (Khan, 2022; Naji et al., 2022).

Histological study of the testis

After the treatment period, three male rats from each group were sacrificed by cervical dislocation. The left testis was excised and cleared of the attached fat and connective tissue for histological study.

Histological sections were prepared following Luna's (1968) method, as described below: Specimens were immediately fixed in 10% buffered neutral formalin for 48 hours. After fixation, the specimens were washed with water. The specimens passed through ascending grades of ethanol (70%, 80%, 90%, 96%, and 100%). Tissues were cleared by immersing them in xylene. Specimens were immersed in a mixture of xylene and paraplast wax at 54-56°C in an electric oven for 15 minutes, followed by transfer to melted paraffin for 2-3 hours with periodic replacement of the paraffin. The tissue blocks were placed in fresh, melted paraffin to solidify and encase the tissues. Paraffin blocks were trimmed and sectioned into 5-10 µm thick slices using a microtome. Tissue sections were mounted on glass slides using Mayer's albumin. Sections were stained according to the method of Bancroft and Stevens (1982), and paraffin was removed from sections using xylene in two 10-minute steps. The sections were rehydrated through descending ethanol concentrations and washed with distilled water. The sections were stained with hematoxylin for 15 minutes, washed with tap water for 10 minutes, and then washed with distilled water. The sections were put in Eosin stain for 2-7 minutes and then washed with tap water. The sections were passed through ascending concentrations of ethanol (70%, 80%, 90%, 100%, and 100%). The slides were then placed in xylene for 2 minutes. The slides were removed from xylene, a mounting medium (Balsam) was placed over the sections to harden them, and a cover glass was applied. Finally, the histological sections were examined under a light microscope to assess the histological changes in the sections prepared from the testes of the treated and control animals.

Statistical analysis

The analysis of data was performed using the Statistical Analysis System (SAS, Version 9.1). Differences between groups were assessed using one-way and two-way ANOVA. The least significant difference (LSD) test was applied to determine statistical significance at $p < 0.05$ (SAS, 2010).

RESULTS

Serum hormonal profiles of testosterone and prolactin

The serum hormonal profiles for testosterone, prolactin, LH, and FSH are shown in Table 1. Testosterone and LH concentrations were significantly decreased in Group 2 (25 mg/kg sulpiride) compared to the control group ($p < 0.05$). Conversely, prolactin concentration was significantly increased in Group 2 compared to the control group ($p < 0.05$). However, no significant differences were observed in FSH concentrations across all groups ($p < 0.05$).

Semen evaluation

Sperm count

As shown in Table 2, sperm count was significantly reduced in Group 2 (25 mg/kg sulpiride) compared to Groups 1 (10 mg/kg sulpiride) and 3 (control group, $p < 0.05$).

Sperm motility

Sperm motility was significantly decreased in Group 2 (25 mg/kg sulpiride) compared to Groups 1 and 3 ($p < 0.05$, Table 2).

Sperm abnormality

Sperm abnormalities and the proportion of dead spermatozoa were significantly higher in Groups 2 (25 mg/kg sulpiride) compared to Groups 1 and 3 ($p < 0.05$, Table 2).

Table 1. Hormone concentrations (ng/ml) in the blood of male rats

Groups	Number of sample rats	Follicle-stimulating hormone	luteinizing hormone	Testosterone	Prolactin
G1 (10 mg/kg Sulpiride)	6	56.60 ± 1.04[a]	28.03 ± 1.03[b]	0.77 ± 0.6[b]	18.03 ± 0.23[b]
G2 (25 mg/kg Sulpiride)	6	55.07 ± 1.13[a]	18.25 ± 0.99[c]	0.63 ± 0.8[a]	20.13 ± 0.73[a]
G3 (normal Saline)	6	53.65 ± 0.71[a]	34.96 ± 1.78[a]	1.45 ± 0.5[c]	11.76 ± 0.55[c]

[abc] Means that a different capital superscript letter in the same column are significantly different ($p < 0.05$)

Table 2. Characteristics of sperm obtained from the epididymis tail of rats

Groups	Sperm Motility (%)	Concentration ($\times 10^4$/ml)	Sperm abnormality (%)	Dead sperms (%)
G1 (10 mg/kg Sulpiride)	80.66 ± 0.6[b]	20.21 ± 2.5[b]	11.10 ± 0.32[b]	12.40 ± 0.43[b]
G2 (25 mg/kg Sulpiride)	70.05 ± 0.55[c]	18.55 ± 1.18[c]	17.40 ± 0.48[a]	15.62 ± 0.28[a]
G3 (normal Saline)	85.92 ± 1.33[a]	23.24 ± 1.53[a]	9.91 ± 0.55[c]	8.11 ± 0.56[c]

[abc] Means that a different capital superscript letter in the same column are significantly different ($p < 0.05$).

Histological study of the testis

The histological examination of seminiferous tubules is presented in Figures 1-3. In the control group (Group 3), the seminiferous tubules were healthy and appeared normal (Figure 1). In Group 1 (10 mg/kg sulpiride), partial destruction of the seminiferous tubules was observed (Figure 2). The damage was more severe in Group 2 (25 mg/kg sulpiride), where extensive destruction of seminiferous tubules was noted (Figure 3).

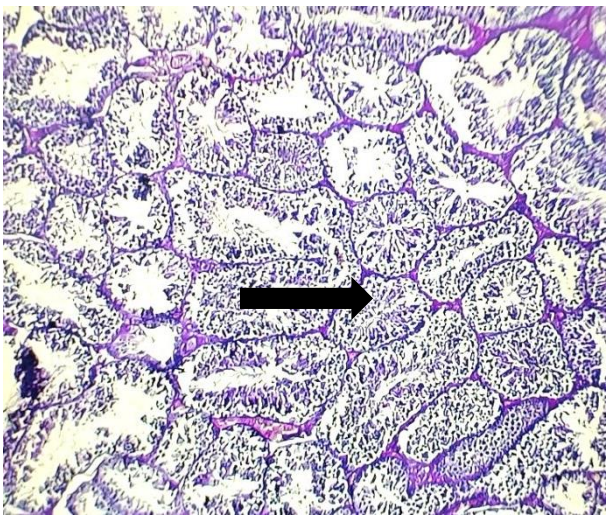

Figure 1. The normal seminiferous tubules of male rats in the control group (black arrow). ×100 H&E staining

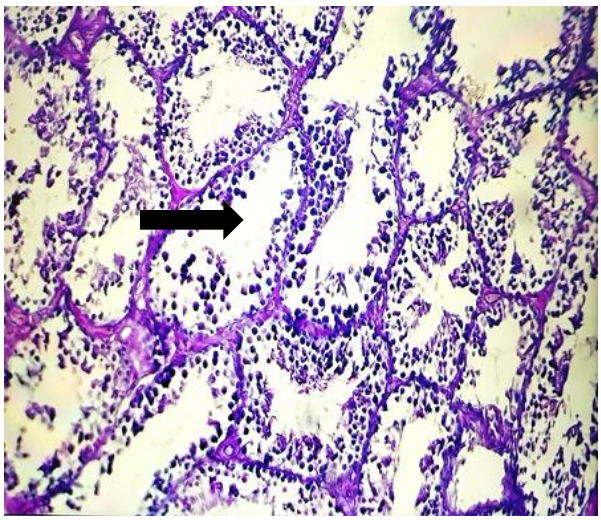

Figure 2. The mild seminiferous tubules degeneration of male rats treated with 10 mg/kg sulpiride (black arrow). ×100 H&E staining

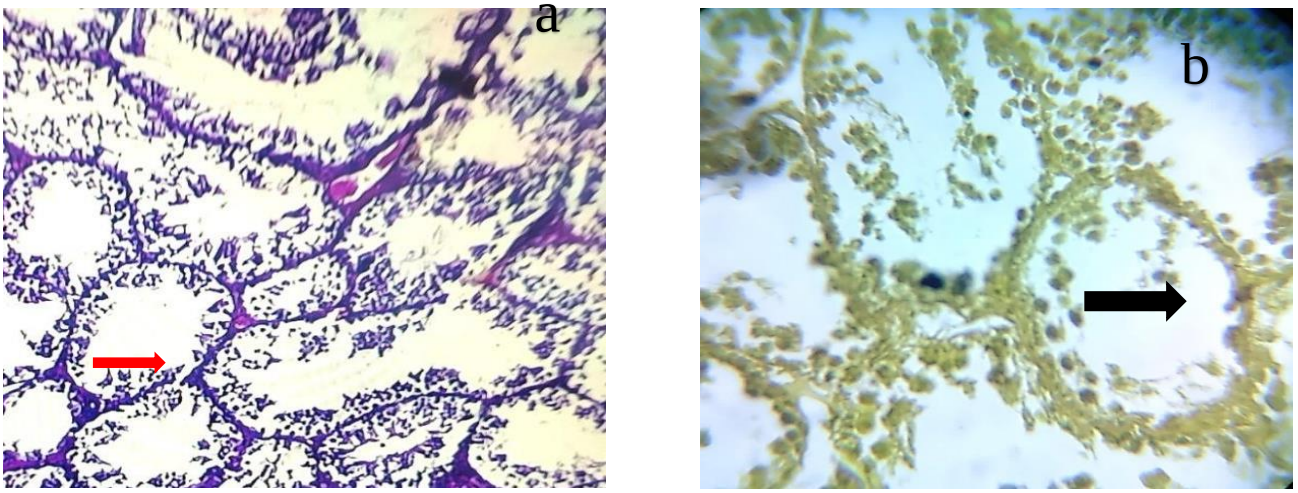

Figure 3. The increasing destruction of seminiferous tubules of male rats (red and black arrows) treated with 25 mg/kg sulpiride. **a**: ×100 H&E staining, **b**: ×400 H&E staining

DISCUSSION

The findings of this study revealed significant decreases in testosterone and luteinizing hormone (LH) concentrations, alongside increased prolactin levels in male rats treated with sulpiride (10 mg/kg and 25 mg/kg). These results align with previous studies reporting elevated serum prolactin and reduced testosterone and LH levels following sulpiride administration (Amiri et al., 2020; Zheng et al., 2020). The mechanism behind this effect involves sulpiride-induced stimulation of prolactin secretion from the pituitary gland, as previously demonstrated in Brown‐Norway rats (Zheng et al., 2020). Mahmoodi and Babaei (2021) similarly observed a significant reduction in testosterone levels in sulpiride-treated rats, corroborating the present study's findings. Taketa et al. (2011) reported that sulpiride treatment at higher doses (100 mg/kg) significantly increased prolactin levels, which aligns with the present study, where male rats were exposed to sulpiride in G1 (10 mg/kg) and G2 (25 mg/kg). Also, the results of this study are in agreement with the increasing levels of prolactin, where male rats were exposed to sulpiride (40 and 120 mg/kg, Zheng et al., 2020). Additionally, Ahmadi et al. (2015) demonstrated that sulpiride (40 mg/kg) acts on hypothalamic tuberoinfundibular dopaminergic neurons, increasing prolactin release in mice, consistent with the present findings. Ahmadi et al. (2013) reported the sulpiride treatment (40 mg/kg) in male mice due to a significant increase in serum prolactin levels found in the treated mice which were along with a significant decrease in LH and testosterone levels, which aligns with the findings of the present study. Elevated prolactin inhibits gonadotropin-releasing hormone (GnRH) secretion from the hypothalamus, leading to reduced LH secretion via negative feedback regulation, as also reported by Miki et al. (2016). In line with this study, Anderson et al. (2008) reported that chronic hyperprolactinemia induced by sulpiride (40 mg/kg) acts to suppress LH pulse in rats. Moreover, the findings of this study align with those of Szukiewicz (2024) which showed that hyperprolactinemia lowers the secretion of gonadotropin-releasing hormone, thereby decreasing the release of pulsation LH, and to a lesser degree, the FSH level in mammals. However, FSH concentrations remained unchanged, consistent with prior studies that reported no significant changes in FSH levels following sulpiride administration (Jafarpour et al., 2019). Jafarpour et al. (2019) who reported increased serum prolactin concertation and no changes in concertation of FSH when treatments used sulpiride (4 mg/kg) in male rats.

The results of sperm parameters align with findings that low dopamine levels increase sperm motility and viability through the stimulation of the D2 receptor, while higher levels of dopamine decrease sperm motility (Semet et al., 2017). The present study reported a significant reduction in sperm motility, viability, and concentration rates in rats administered sulpiride, along with a significant increase in sperm abnormalities and dead sperm. These findings are consistent with results reporting a significant decrease in sperm viability, motility, and count, as well as an increase in abnormal sperm, observed in treatment groups administered sulpiride (40 mg/kg, Ahmadi et al., 2012; 2015).

The histological study revealed the destruction of seminiferous tubules in rats receiving sulpiride. The extent of destruction significantly increased as the dose of sulpiride increased compared to the control group. These results align with the findings reported by Jafarpour et al. (2019). Vieira et al. (2013) also reported that treatment of male rats with sulpiride (25 mg/kg) caused changes in the volume and histopathology of the seminiferous tubules, such as an increased proportion of abnormal seminiferous tubules, as well as increased percentages of abnormal sperm head morphology and immobile sperm. Mahmoodi and Babaei (2021) reported that sulpiride (4 mg/kg) administration led to the destruction and disruption of the epithelium of seminiferous tubules, and increased interstitial space of the testicular tissue. Their findings are in agreement with the present study. Amiri et al. (2020) reported that sulpiride (4 mg/kg) administration led to a significant decrease in the number of spermatogenic cells (spermatogonia and spermatocytes) in seminiferous tubules, consistent with the results of the present study.

CONCLUSION

In conclusion, sulpiride (10 mg/kg) treatment negatively affects the male reproductive system, with these harmful effects increasing in severity with higher doses of sulpiride (25 mg/kg) treatment. Further researches are needed to focus on the sperm DNA damage during the use of sulpiride for treatment.

DECLARATIONS

Availability of data and materials

The data supporting the findings of this study are available upon reasonable request from the corresponding author.

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Competing interests

The authors declare that there is no conflict of interest.

Author's contributions

Ali Aziz Abd conducted the research, secured resources, prepared materials, and reviewed the manuscript. Qusia Saleh Jumma interpreted the findings and contributed to the experimental design. Oday Alawi Al-Juhaish performed statistical analyses and contributed to editing the manuscript. All authors have read and approved the final version of the manuscript before publication in the present journal.

Ethical considerations

This paper was originally written by the authors and has not been published elsewhere. The authors checked the text of the article for plagiarism index and confirmed that the text of the article is written based on their original scientific results.

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Assessing the Population Structure and Inbreeding Rates of Buffaloes in Batanghari District, Indonesia

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ABSTRACT

Buffaloes are important in animal husbandry, agriculture, and sociocultural and religious activities in Indonesia. The buffalo population has decreased at the national and regional levels, including in the Batanghari District, Jambi Province, Indonesia. This study analyzed the population structure, effective population size, and inbreeding rate of buffalo populations in the Batanghari District, Jambi, Indonesia, based on secondary data. The data population of 3,149 buffaloes used in this study was sourced from the Integrated National Animal Health System (ISIKHNAS) in the Batanghari District in 2023. The results showed a calf crop of 21.71%, a calving rate of 16.61%, a natural increase of 14.74%, and a net replacement rate of 279.51%. The effective population size was 592 heads, and the inbreeding rate was 0.08%. It can be concluded that the natural increase rate of the buffalo population in the Batanghari District was low, but the number of young replacement animals was sufficient. The effective population size was 592 heads, and the level of inbreeding per generation remained within acceptable limits. Although the buffalo population in the Batanghari District exhibited a negative trend, it still had potential as a source of breeding stock, as indicated by the replacement rate.

Keywords: Buffalo, Effective population, Inbreeding rate, Population structure

INTRODUCTION

Buffaloes are adaptable to marginal environments and low nutrition. They can provide high-quality protein through meat and milk, organic fertilizer and fuel in the form of dung, mechanical or draught power (plowing fields and pulling wood), and hides and skins for industrial use. These characteristics enable small, marginal farmers to support their families. Farmers also preserve buffaloes for cultural, religious, and societal reasons (Maulana et al., 2023; Prihandini et al., 2023).

Buffaloes play a notable role in farming in Indonesia; however, their national and regional populations are insufficient. According to the Indonesian Statistics Agency (Badan Pusat Statistik [BPS]) report, the buffalo population in Jambi Province, Indonesia, was 70,154 heads in 2003 and decreased to 47,567 in 2023. Hence, increasing the buffalo population in this region is important to prevent extinction. Carrying capacity, farming skills, and population structure influence buffalo productivity (Reswati and Putra, 2023). The buffalo population in Jambi decreased ten years ago and required mitigation by the government. According to the Indonesian Statistics Agency (BPS, 2024) the buffalo population dynamics in the Batanghari District have decreased over the past five years. In 2019, the buffalo population in Batanghari was 11,221; by 2023, it had decreased to 7,343 (Figure 1). The decline in the buffalo population increases inbreeding risk and reduces productivity traits. The population decline can be attributed to a lack of management systems, diseases such as *Septicaemia epizootica* (SE), foot and mouth diseases, and reduced farmland (Firmansyah et al., 2023).

Analyzing the structure of the livestock population is an effort to address the issue of mitigating the decline in the buffalo population in the Batanghari district. Many studies have examined the population structure of livestock, including cattle (Widyaningrum et al., 2021), buffaloes (Yendraliza et al., 2021a), ducks (Rusfidra et al., 2012), and rhinoceroses (Putra et al., 2020). However, studies of population structure, inbreeding rate, and effective population size in buffalo populations in the Batanghari district have not been reported. The present study aimed to determine buffaloes' population structure, inbreeding rate, and effective population size in the Batanghari District, Jambi Province, Indonesia.

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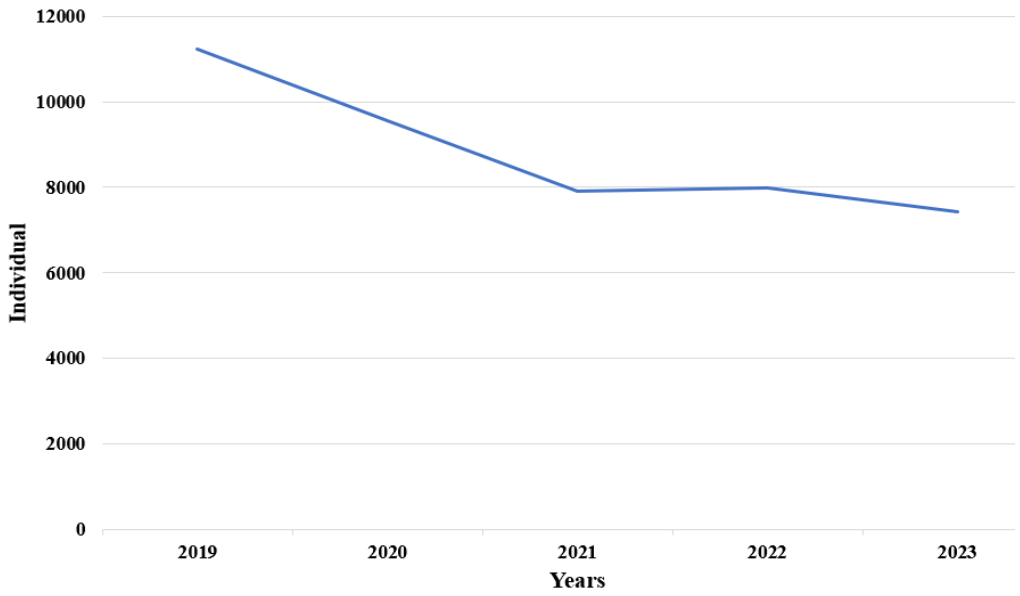

Figure 1. The population dynamics of Buffalo at Batanghari District, Jambi, Indonesia, from 2019 to 2023

Figure 2. Research location at Batanghari District, Jambi, Indonesia

MATERIALS AND METHODS

Ethical approval

This study was approved by the Animal Ethics Commission of the Faculty of Medicine, Universitas Andalas, Indonesia, with license number 588/UN.16.2/KEP-FK/2023.

Data collection

The population data of 3,149 buffaloes were obtained from a secondary source, the Batanghari District ISIKHNAS database in 2023, including data at the beginning and end of the year in Batanghari District, Jambi, Indonesia (Figure 2). These data were collected to calculate the calf crop, the calving rate based on population, natural increase, requirement of animal replacement, remains of young animals, the number of culled animals, the net return rate, output estimation, effective population size, and inbreeding rate per generation. Additionally, population dynamics from 2019 to 2023 were analyzed based on BPS data from 2019 to 2023. The data collection included buffalo calves (0-1 year), young buffalo (1-3 years), and adult buffalo (> 3 years).

Data determination

These data were used to calculate the calf crop, calving rate based on population, natural increase, requirement of animal replacement, remains of young animals, number of culled animals, net return rate, and output estimation, according to the methods described by Samberi et al. (2010) using the following formulas (Formulas 1-8).

(Formula 1) $$\text{Calf crop (\%)} = \frac{\text{Number of calf (heads)}}{\text{Number of adult female (heads)}} \text{ X } 100\%$$

(Formula 2) $$\text{Calving rate (\%)} = \frac{\text{Number of calf (heads)}}{\text{Number of population (heads)}} \text{X } 100\%$$

(Formula 3) $$\text{Natural increase (\%)} = \text{Calving based on population (\%)} - \text{Mortality (\%)}$$

(Formula 4) $$\text{Requirement of animal replacement} = \frac{\text{Number of adult animal (\%)}}{\text{Breeding length (years)}}$$

(Formula 5) $$\text{Remains of young animal} = \text{Number of young animal (\%)} + \text{Requirement of animal replacement (\%)}$$

(Formula 6) $$\text{Number of culled animal (\%)} = \text{Requirement of animal replacement (\%)}$$

(Formula 7) $$\text{Net return rate} = \frac{\text{Number of young animal (heads)}}{\text{Remains of young animal (heads)}} \text{ X } 100\%$$

(Formula 8) $$\text{Output estimation (\%)} = \text{Remains of young animal (\%)} + \text{Number off culled animal (\%)}$$

The Effective population size and inbreeding rate per generation were calculated using Hamilton (2009) according to the following formulas (Formulas 9 -10), the obtained data were then analyzed using descriptive statistics.

(Formula 9) $$Ne = \frac{(4\ Nm\ Nf)}{Nm+Nf}$$

(Formula 10) $$\triangle F = \frac{1}{2Ne}$$

In this equation, Ne represents the effective population, F represents the inbreeding rate per generation, Nm represents the number of adult males, and Nf represents the number of adult females.

RESULTS AND DISCUSSION

The population structure of buffalo in the Batanghari District in 2023 consisted of 3,149 heads (Table 1), with the following proportions: Calves 523 heads (230 males and 293 females), young buffalo 932 heads (346 males and 586 females), and adults 1,694 heads (164 males and 1,530 females). The results showed that the natural increase (NI) in Batanghari buffaloes was 14.74%, as presented in Table 2. A natural increase refers to the growth of a population over time, calculated as the difference between the number of live births and the number of deaths within that population. The natural increase observed in this study was classified as a low category. According to Samberi et al. (2010), natural increase (NI) is categorized as high if NI > 30%, medium if 15.01% < NI ≤ 30%, and low if NI < 15%. The results of this study were lower than the natural increase in the buffalo population in the Malang District, reported as 16.1% (Budiarto and Ciptadi, 2018), 20.18% buffaloes in Sumbawa (Putra and Lestari, 2022), and 55.59% for Kuntu buffalo (Yendraliza et al., 2021a). The natural increase in the buffalo population in the Batanghari District was low, consistent with the decline observed in the buffalo population in the Batanghari District over the past five years. A large number of slaughters of productive females to meet consumer demand and mortality due to diseases such as *Septicaemia epizootica* (SE) can reduce the buffalo population (Marsudi et al., 2017). Additionally, the low natural increase (NI) value of buffaloes may be attributed to the presence of non-productive buffaloes on the farm (Kgosikoma and Bastisani, 2014).

Table 1. Population structure of buffalo in Batanghari District, Jambi, Indonesia, 2023

Sex \ Group	Calf	Young	Adult	Total (N)
Male	230	346	164	740
Female	293	586	1,530	2,409
Total (heads)	523	932	1,694	3,149

N: Individual

Table 2. The technical coefficient in population structure analysis of buffaloes in Batanghari District, Jambi, Indonesia, 2023

Parameters		Value
Variable	Calf crop (%)	21.71
	Calving rate (%)	16.61
	Mortality (%)	1.87
	Natural increase (%)	14.74
Adult animals (%)	Male	5.21
	Female	76.50
Breeding length (years)	Male	2
	Female	8
	Sex ratio (Male/Female)	1/2
	Number of population observed (N)	3,149

N: Individual

Table 3. The analysis of the buffaloes population structure in Batanghari District, Jambi, Indonesia, in 2023

Parameters		N	(%)
Young animal	Male	346	10.99
	Female	586	18.61
	Total	932	29.60
Requirement of animal replacement	Male	82	2.60
	Female	191	9.56
	Total	273	12.17
Remains of young animals	Male	264	8.38
	Female	395	9.05
	Total	549	17.43
Culled animal	Male	82	2.60
	Female	191	9.56
	Total	273	12.17
Output estimation	Male	346	10.99
	Female	586	18.61
	Total	932	29.60
Net replacement rate	Male	-	131,06
	Female	-	148,45
	Total	-	279,51

N: Individual

Table 4. The Effective population and inbreeding rate of buffaloes in Batanghari District, Jambi, Indonesia, in 2023

Sex	Total
Number of males breeding (*NM)*	164
Number of females breeding (*NF*)	1,530
Total	1,694
Effective population	592
Inbreeding Rate (%)	0.08

Table 5. Population dynamics of buffaloes in Batanghari District, Jambi, Indonesia, from 2019 to 2023

Actual				Prediction			
Year	**N**	**Deviation**	**Percentage**	**Year**	**N**	**Deviation**	**Percentage**
2019	11,221	0	0	2024	6,770	664	9.808477
2020	9,560	-1661	-17.3745	2025	6,105	-664	-10.8814
2021	7,921	-1639	-20.6918	2026	5,441	-664	-12.2101
2022	7,991	70	0.875985	2027	4,777	-664	-13.9083
2023	7,434	-557	-7.4926	2028	4,112	-664	-16.1552
-	-	-757,4	-8.94	-	-	-399	-8.67

N: Individual

Table 6. The output estimation of buffaloes in Batanghari District, Jambi, Indonesia, from 2024 to 2028

Component \ Years		2024	2025	2026	2027	2028
Number of animals (N)	Male	2,675	2,912	3,129	3,346	2,478
	Female	5,350	5,824	6,259	6,693	4,956
	Total	8,025	8,737	9,388	10,039	7,434
Number of young animals (N)	Male (10.99%)	882	960	1,032	1,103	817
	Female (18.61%)	1,493	1,626	1,747	1,868	1,383
	Total	2,375	2,586	2,779	2,972	2,200
Number of culled animals (N)	Male (4.94%)	396	432	464	496	367
	Female (13.13%)	1,054	1,147	1,233	1,318	976
	Total	1,450	1,579	1,696	1,814	1,343
Remains of young animals (N)	Male (6.72%)	539	587	631	675	500
	Female (13.86%)	1,112	1,211	1,301	1,391	1,030
	Total	1,652	1,798	1,932	2,066	1,530
Output estimation (N)	Male	936	1,019	1,095	1,171	867
	Female	2,166	2,358	2,534	2,710	2,006
	Total	3,102	3,377	3,628	3,880	2,873

N: Individual

The net replacement rate (NRR) was 279.51, comprising 131.06 males and 148.45 females, as shown in Table 3. The Net Replacement Rate (NRR) is calculated by comparing the number of young replacement cattle to the annual replacement livestock requirements, multiplied by 100%. The NRR is used to assess whether the number of livestock births is sufficient to meet the replacement needs and maintain a stable population (Kusuma et al., 2017). Batanghari District is one of the areas that can be used as a source of feedstock owing to its high NRR value. Samberi et al. (2010) classify an area as a seed source area if the net replacement rate (NRR) is greater than 100%. Previous research has reported higher NRRs than the results of this study. Previous studies reported the NRR of buffalo in Sumbawa was 414.41, with 259.54 females and 154.88 males (Putra and Lestari, 2022), in Kuantan Regency, it was 455.71 with 310.60 females and 145.10 males (Yendraliza et al., 2021b), and in Ulukan Regency it was 279.02, with 121.97 females and 157.31 males (Putra et al., 2018). The effective population size of the buffalo population in the Batanghari District was 596 individuals (Table 4). The effective population size was considered adequate if it exceeded 100 Individuals. It is an important parameter in many population genetic models (Elsadina et al., 2021). The effective population size represents the number of breeding individuals in an ideal population that exhibits the same allele frequency distribution under the pressure of random genetic drift (Setiawati et al., 2020). Table 4 presents that the inbreeding rate of Batanghari buffaloes was 0.08%. The results of this study indicated that inbreeding did not yet occur at the crossbreeding level in buffaloes in the Batanghari District. A population is considered to be in good condition when the inbreeding rate is ≤ 1% per generation (Steensma et al., 2024). However, an increase in the inbreeding rate of 1% per generation can reduce productivity traits (Rusfidra et al., 2012).

Data from the Central Bureau of Statistics of Batanghari District from 2019 to 2023 showed that the average annual growth rate of the buffalo population in Batanghari District was -8.94% (Table 5). The growth rate in population size can be determined by estimating the net increase based on the data collected over the last five years (Widyaningrum et al., 2021). This population growth value can be used to predict the buffalo population. Based on the same coefficient calculation, the estimated buffalo population in 2028 will be 4,112 heads, and the average remains of young animals is -24 per year (Table 6). It can be concluded that the growth rate of the buffalo population in the Batanghari District showed a negative trend. Therefore, various mitigation efforts and policies must be implemented by the government as policymakers and by farmers as business actors. These efforts should address issues such as the slaughtering of productive females, improved reproductive management, health management, and farm management to reduce mortality and increase birth rates.

CONCLUSION

The natural rate of increase in the buffalo population in the Batanghari District was low (14.74%), with an effective population size of 592 individuals. The inbreeding rate per generation was 0.08, indicating that the population was in relatively good condition. Nonetheless, the buffalo population in the Batanghari District showed a negative trend.

However, the animals under study have potential as a breeding stock resource based on their NRR value. Therefore, the government and farmers should implement policies to increase the buffalo population and prevent this livestock from becoming extinct.

DECLARATIONS

Availability of data and materials

The data used in this study can be accessed through the Department of Agriculture and Livestock of the Batanghari District in 2023 (unpublished data).

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Authors' contributions

This research was made possible by the contributions of all authors. Panca Andes Hendrawan collected information and drafted the manuscript. Jakaria, Cece Sumantri, and Sony Hartono Wijaya revised the manuscript. All authors have read and approved the final version of the manuscript.

Competing interests

The authors have not declared any conflict of interest.

Ethical considerations

The authors confirmed that plagiarism checks have been carried out, and there is no copy and data falsification.

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Leukocyte Subpopulations in the Peripheral Blood of the Omani Camel Breed

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ABSTRACT

Breed-specific variations in immune responses have been studied across various species and breeds. The identification of camel breeds with high immune competence can enhance the breeding of camels with superior immune responsiveness. To date, no study has examined the immune cell composition in the blood of the Omani camel breed. The present study aimed to analyze the immunophenotype of blood leukocytes in the Omani camel breed and investigate the impact of age and gender on the tested immune parameters. To do so, blood samples were collected from 32 clinically healthy camels, randomly selected and comprising 17 camel calves (8 males and 9 females) and 15 adult camels (4 males and 11 females). The samples were tested using flow cytometry and membrane immune fluorescence. The results of the present study revealed a significantly lower count of white blood cells (WBC) in the Omani camel breed than the reference ranges reported for dromedary camels in the published literature. The leukogram was characterized by the dominance of neutrophils (54-60 %) in the blood, followed by lymphocytes (23-33 %). When compared to adult camels, the leukogram pattern in young Omani camels was characterized by elevated WBC and lymphocytes but low levels of eosinophilic granulocytes. The analysis of lymphocyte subsets revealed the dominance of γδ T cells over helper T cells and B cells in the blood of young camel calves, confirming that camels belong to the γδ T cell-rich species. In addition, lower numbers of B cells and helper T cells in young camels suggest lower cell-mediated and humoral immune functionality compared to adults. Although some differences were identified between male and female adult camels, these results are limited by the low numbers of male camels within the adult group. In conclusion, the distinct leukogram patterns observed in young and adult camels highlight the significant impact of age on the immune competence of Omani camels.

Keywords: Camel, Omani camel, Immunophenotype, Leukocyte

INTRODUCTION

The dromedary camel (*Camelus dromedarius*) is well-adapted to the desert, with the ability to grow, reproduce, and produce milk under harsh environments. As far as scientific research is concerned, camels have lagged behind other livestock species for a long time. However, in recent years, research on camels has gradually gained increased interest (Hoter et al., 2019; Tibary and El Allali, 2020).

Within the dromedary camel species, several breeds have been characterized based on morphological characteristics, geographical distribution, and genomic structure (Alhaddad and Alhajeri, 2019). Mainly characterized camel breeds include Al-Mujaheem, Al-Mugateer, Shaele, Homor, and the Omani camel breed (Alhaddad and Alhajeri, 2019; Almathen et al., 2022). The Omani camel breed, primarily found in the eastern regions of Oman, Saudi Arabia, and some regions across the United Arab Emirates is particularly notable as a racing camel population in the Arabian Peninsula (Almathen et al., 2022).

In both human and veterinary medicine, total and differential leukocyte counts, collectively referred to as the leukogram or leukon, have proven to be valuable and cost-effective tools for the evaluation of the physiological and pathological status, and these assessments aid in diagnosis, therapy, and prognosis (Ajadi et al., 2018; Balmant et al., 2018; Braun et al., 2021). In dromedary camels, the main leukocyte populations as well as several lymphocyte subsets - including CD4+ ab T cells, WC1+ γδ T cells, and B cells - have been characterized (Hussen and Schuberth, 2020). Previous studies show that camels, like other species from the artiodactyls group such as cattle and pigs, are classified as γδ-high species, meaning γδ T cells constitute a significant proportion of T cells in the blood of newborns and young animals. This contrasts with γδ-low species like humans and mice, where γδ T cells represent only a minor fraction of blood T cells (Guzman et al., 2014; Hussen and Schuberth, 2020). Previous studies have also explored the influence of several physiological parameters, such as age, gender, and pregnancy on camels' cellular immune system (Gaashan et al., 2020; Hussen et al., 2020).

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Despite these efforts, limited data are available on the immune cell composition in the blood of the Omani camel breed. This study was conducted to investigate the immunophenotype of blood leukocytes in Omani camels and evaluate the impact of age and gender on leukocyte distribution patterns.

MATERIALS AND METHODS

Ethical approval

This study was performed in line with the principles of the Declaration of Helsinki. Approval was granted by the Ethics Committee of King Faisal University, Saudi Arabia (KFU-REC-2024-JUN-ETHICS1843)

Animals and blood sampling

The study was conducted on an Omani camel breed reared on a private camel farm located in Hofuf City (Eastern Province of Saudi Arabia, Al-Ahsa Region). Blood sampling was performed on October 19, 2024, at 11:00 AM. A total of 32 camels were randomly selected from a population of 55 camels on the farm. The study population included 17 camel calves (aged 5-18 months) and 15 adult camels (aged 3-9 years). The young group consisted of 8 male and 9 female calves, while the adult group comprised 4 male and 11 female camels (Table 1). All adult female camels were nonpregnant and non-lactating at the time of sampling. The camels were kept under a traditional management system, where they grazed on grazing plants such as *Aeluropus lagopoides, Anabasis setifera, Atriplex halimus, Calligonum comosum* during the daytime and were housed in a fen barn at night. Water was provided *ad libitum*. All camels were clinically healthy based on clinical examination performed by a trained veterinarian. Blood samples (5 mL) were collected from the jugular vein using vacutainer tubes containing EDTA (Guangzhou Improve Medical Instruments Co., Ltd., Guangzhou, China) and transported to the laboratory within one hour of collection.

Table 1. Age (year) and gender of the investigated Omani camels sampled in October 2024 in Saudi Arabia

Age	Calf camel		Adult camel	
	Male	**Female**	**Male**	**Female**
Number	**8**	**9**	**4**	**11**
Minimum	0.5	0.5	3.0	5.0
25% Percentile[1]	1.0	0.5	3.8	5.0
Median	1.0	1.0	6.0	8.0
75% Percentile[2]	1.7	1.3	7.5	10.0
Maximum	1.8	1.5	8.0	10.0
Mean	1.2	0.9	5.8	7.6
Std. Deviation	0.4	0.4	2.1	2.0
Std. Error[3]	0.1	0.1	1.0	0.6

[1]: The value at which 25% of the values lie below that value, [2]: The value at which 75% of the values fall below this value, [3]: Standard error.

Cell separation and flow cytometry

Leukocytes were separated using hypotonic lysis of erythrocytes followed by centrifugation, as previously described by Alhafiz et al. (2022). Briefly, 1 mL of EDTA-treated blood was incubated with 5 mL distilled water in a 15 mL sterile falcon tube for 20 seconds to induce red blood cell lysis. Subsequently, tonicity was restored by the addition of 5 mL of 2x phosphate buffered saline (PBS), and the tube was centrifuged at 3000 RPM for 10 min at 10ºC. The lysis step was repeated twice with centrifugation at 2200 and 1500 RPM for 10 min until complete erythrolysis was achieved. Finally, the cells were suspended in cold PBS (1×10^6 cells / mL). Cell vitality was measured by the addition of propidium iodide, consistently exceeding 90% (Alhafiz et al., 2022). Total white blood cell (WBC) counts were determined using a Neubauer cell counter and light microscopy after diluting blood samples with Türk's solution (1:10). The absolute cell count of each cell subset was calculated by multiplying cell percentage by the total WBC count.

Flow cytometric analysis of leukocyte subsets

Monoclonal antibodies (mAbs) specific to cell markers BAQ44A (B cell), WC1 (γδ T cell), and CD4 (helper T cell) were used for cell labeling (Hussen et al., 2023). Cells were first incubated in a 96-well plate (1×10^5 cells / well) with the primary mAbs for 15 min at 4°C. After washing with PBS/BSA buffer, the second staining step was done by adding fluorochrome-labeled antibodies (10 µL of each mAb diluted 1: 100 in PBS/BSA buffer) specific to mouse IgM, IgG1, and IgG2a (Invitrogen) followed by incubation for 15 min at 4°C. Following a final wash, the cells were analyzed using an Accuri C6 flow cytometer (Becton Dickinson Biosciences, San Jose, California, USA).

Statistical analyses

Mean values, standard deviation (SD), and standard error of the mean (SEM) were calculated using the column statistics function in the Prism software (GraphPad). Data normality was assessed using the Shapiro-Wilk test. Comparisons between means were performed using paired student's t-test for normally distributed data or Mann-Whitney test for non-normally distributed data, with p values less than 0.05 indicating significant effects.

RESULTS AND DISCUSSION

Relative percentages and absolute counts of leukocyte subpopulations

Omani camel calves showed significantly ($p < 0.05$) lower percentages of neutrophils (54.9%) and eosinophils (3.2%) compared to adult camels (60.7% for neutrophils and 7.8% for eosinophils) ($p < 0.05$). In contrast, the percentage of lymphocytes was significantly higher in young camels (33.3 %) than in adults (23.9%; $p < 0.05$). The percentage of monocytes did not differ significantly between the two age groups ($p > 0.05$). Compared to adult camels, camel calves showed significantly higher numbers of total leukocytes (6176 versus 4753 cells/µL blood in adult camels) and lymphocytes (2076 versus 1120 cells/µL blood in adult camels), but lower numbers of eosinophils (204 versus 360 cells in adult camels) ($p < 0.05$). No significant differences were observed between the two groups regarding the absolute numbers of neutrophils and monocytes ($p > 0.05$) (Figure 1A-B).

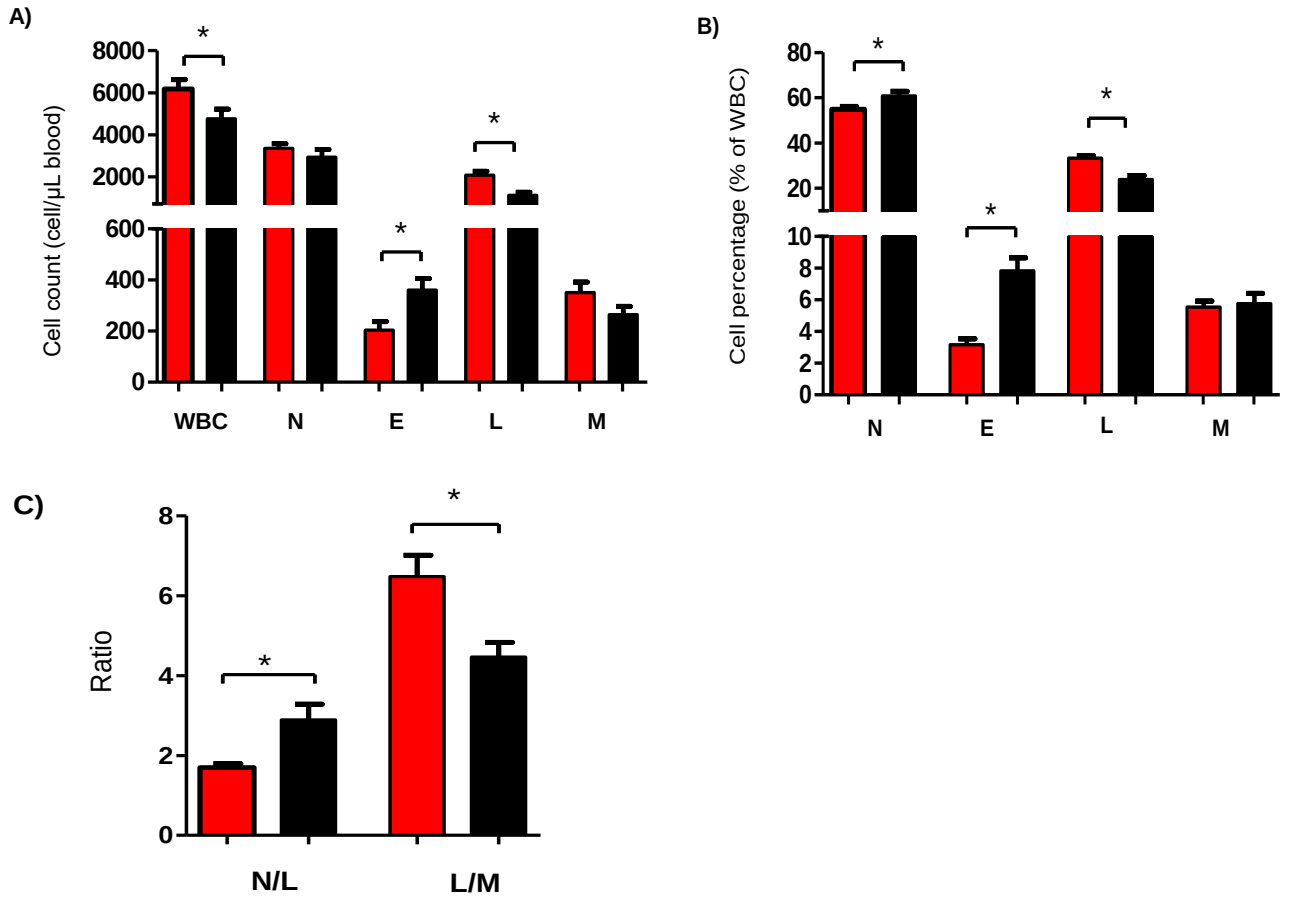

Figure 1. Leukogram patterns in young (red color) and adult (black color) Omani camels. **A**: Absolute count of neutrophils (N), eosinophils (E), lymphocytes (L), and monocytes (M) as quantified by flow cytometry. **B**: Relative percentages of total white blood cells (WBC) and the different leukocyte subpopulations in young and adult Omani camels. **C**: The neutrophil-to-lymphocyte (N/L) and lymphocytes-to-monocytes (L/M) ratios in young and adult Omani camels. *: $p < 0.05$.

Neutrophils to lymphocyte and lymphocyte to monocyte ratios

The neutrophils-to-lymphocyte ratio (N/L) was significantly ($p < 0.05$) lower in young camels (1.7) than in adult camels (2.9), while the lymphocyte-to-monocyte ratio (L/M) was significantly higher in the young camel group (6.5) than in the adults (4.5) ($p < 0.05$, Figure 1C).

Lymphocyte subsets in camel blood

The analysis of camel lymphocyte subsets revealed a significantly ($p < 0.05$) higher percentage of WC1+ T cells within total lymphocytes in young camels (23.4% of lymphocytes) compared to adult camels (7.4%). Conversely, the percentages of CD4 + T cells (13.3 versus 18.6% in adult camels) and B cells (8.4 versus 17.0% in adult camels) were significantly ($p < 0.05$) lower in camel calves than in adult camels (Figure 2A).

Absolute counts of lymphocyte subsets showed only significantly ($p < 0.05$) higher numbers of WC1+ T cells in young (476 cells/µL blood) than in adult (88 cells/µL) camels. However, no significant differences were observed in the absolute counts of CD4+ T cells and B cells between the two age groups ($p > 0.05$, Figure 2B).

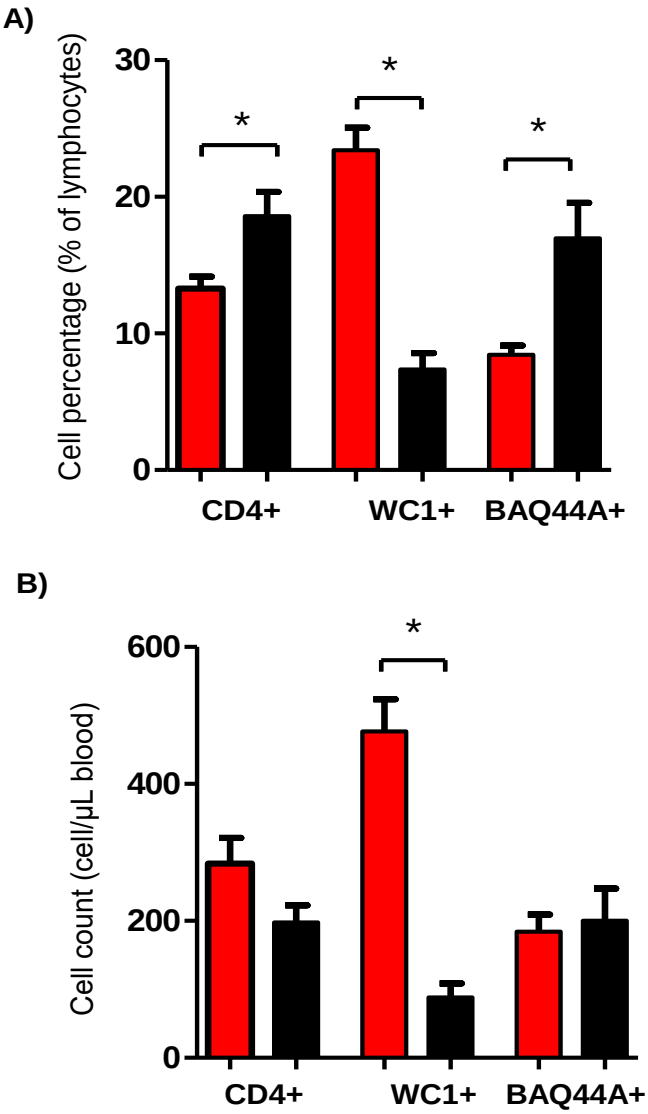

Figure 2. Lymphocyte subsets in young (red color) and adult (black color) Omani camels. **A:** Relative percentages of CD4 + T cells, WC1 + T cells, and BAQ44A+ cells (B cells) as quantified by flow cytometry. **B:** Absolute counts of CD4 + T cells, WC1 + T cells, and B cells in young (red color) and adult (black color) Omani camels. *: $p < 0.05$.

Impact of gender on the immune cell composition

The distribution of leukocyte subpopulations, their absolute counts, and the ratios in male and female camels are shown in Tables 2, 3, and 4. Comparisons between males and females within the two age groups revealed significant differences in the percentage of lymphocytes, the neutrophils-to-lymphocyte ratio, and the number of eosinophils ($p < 0.05$). The percentage of lymphocytes was significantly higher in female (26.1 % of WBC) than in male adult camels (17.5 % of WBC; Table 2). In contrast, the neutrophils-to-lymphocyte ratio was significantly lower in females (2.3) than in (4.3) adult males (Table 2). Male adult camels (525 cell/µL) showed significantly higher ($p < 0.05$) numbers of eosinophils than female adult camels (301 cell/µL; Table 3).

Table 2. Relative percentages of leukocyte subpopulations in blood samples collected from Omani camels (October 2024 in Saudi Arabia)

		Calf camel (5 – 18 months)		Adult camel (3 to 9 years)	
		Male (n =8)	**Female (n = 9)**	**Male (n = 4)**	**Female (n = 11)**
Neutrophils (% of WBC)	Minimum	49.6	47.3	59.2	45.2
	Maximum	62.2	64.2	79.2	66.4
	Mean	54.9	54.8	66.4	58.6
	SD	4.1	6.2	9.0	6.4
Eosinophils (% of WBC)	Minimum	0.8	1.3	6.5	2.8
	Maximum	4.9	6.9	14.6	11.1
	Mean	2.7	3.5	10.0	7.0
	SD	1.3	1.6	3.4	2.8
Lymphocytes (% of WBC)	Minimum	27.9	25.8	10.2	19.3
	Maximum	40.2	41.6	22.4	39.9
	Mean	33.5	33.1	17.5	26.1*
	SD	4.1	5.2	5.3	5.9
Monocytes (% of WBC)	Minimum	3.9	3.5	3.6	3.5
	Maximum	8.5	7.2	5.2	12.4
	Mean	5.8	5.3	4.3	6.2
	SD	1.6	1.4	0.7	2.7
Neutrophils/Lymphocytes	Minimum	1.2	1.1	2.7	1.1
	Maximum	2.2	2.4	7.8	3.4
	Mean	1.6	1.7	4.3	2.3*
	SD	0.3	0.4	2.3	0.6
Lymphocytes/Monocytes	Minimum	4	3.6	2.8	2.1
	Maximum	9.4	10.5	5.2	7.7
	Mean	6.2	6.7	4	4.6
	SD	2.0	2.3	1.1	1.5

*: Indicates significant impact of gender, SD: Standard deviation, n: Number. * indicates a significant difference at $p < 0.05$ in a row.

Table 3. Absolute cell counts of leukocyte subpopulations in blood samples collected from Omani camels (October 2024 in Saudi Arabia)

		Calf camel (5 – 18 months)		Adult camel (3 to 9 years)	
		Male (n =8)	**Female (n = 9)**	**Male (n = 4)**	**Female (n = 11)**
WBC (cell/µL)	Minimum	4000	3700	3500	2500
	Maximum	8800	8500	9100	8000
	Mean	6413	5967	5500	4482
	SD	1854	1913	2551	1543
Neutrophils (cell/µL)	Minimum	2024	1994	2125	1540
	Maximum	4733	4484	7209	4363
	Mean	3503	3216	3798	2609
	SD	943	925	2321	877
Eosinophils (cell/µL)	Minimum	43	47	344	124
	Maximum	356	521	803	487
	Mean	179	227	525	301*
	SD	114	155	216	122
Lymphocytes (cell/µL)	Minimum	1254	1013	665	483
	Maximum	3195	2786	1128	2617
	Mean	2165	1999	876	1209
	SD	739	764	199	616
Monocytes (cell/µL)	Minimum	192	157	167	135
	Maximum	747	598	331	548
	Mean	374	330	229	277
	SD	170	176	71	141

*****: Indicates significant impact of gender, SD: Standard deviation, n: Number. * indicates a significant difference at $p < 0.05$ in a row.

Table 4. Lymphocyte subsets in blood samples collected from Omani camels (October 2024 in Saudi Arabia)

		Calf camel (n = 17) (5 – 18 months)		Adult camel (n = 15) (3 to 9 years)	
		M (n =8)	**F (n = 9)**	**M (n = 4)**	**F (n = 11)**
CD4+ T cells (% of lymphocytes)	Minimum	9	7.2	9.1	10.4
	Maximum	18.9	21.5	28	30
	Mean	13.58	12.98	21.6	17.42
	SD	3.17	3.989	8.588	6.236
gd T cells (% of lymphocytes)	Minimum	12.1	15.4	4.1	2.5
	Maximum	30.9	37.1	11.3	18.9
	Mean	20.7	25.82	9	6.773
	SD	6.116	6.764	3.317	4.995
B cells (% of lymphocytes)	Minimum	4.89	4.7	6.33	7.3
	Maximum	12.56	12.12	32.61	38.9
	Mean	8.129	8.714	14.79	17.76
	SD	3.021	2.722	12.2	9.807
Helper T cells (cell/µl)	Minimum	160	113	84	100
	Maximum	603	594	297	433
	Mean	304.4	265.4	187.8	201.4
	SD	161.7	154.8	90.85	102.9
γδ T cell (cell/µl)	Minimum	181	256	27	13
	Maximum	668	826	121	314
	Mean	445.8	504.7	82.25	90.64
	SD	181.6	211.3	39.56	91.14
B cell/µl	Minimum	68	64	59	64
	Maximum	374	335	302	725
	Mean	182.9	185.1	129	225.8
	SD	104	110.2	115.8	199.3

gd: Gamma delta T cells, B cell: B lymphocytes, n: Number, M: Male, F: Female.

Breed-specific differences in the immune response have been studied for several species and breeds (Hadfield et al., 2018; Lawrence et al., 2013). In the present study, the total WBC counts in the Omani camel breed ranged between a minimum value of 2500 and a maximum value of 9100 cells/µL blood (mean ± SD: 5509 ± 1940), which is lower than the reference ranges reported for the dromedary camels in previous studies (Al-Busadah, 2007; Al-Mujalli et al., 2011; Martin-Barrasa et al., 2023). Whether these results represent a characteristic range for the Omani camel breed or not requires further comparative studies with other camel breeds. For instance, Martin-Barrasa et al. (2023) reported total WBC counts for the Canary Island dromedary camels ranging from 7.35 to 18.36 $X10^3$ cells/µL. Similarly, Fey and Bengoumi (2018) reported WBC values between 9.7 and 20.1 $X10^3$ cells/µL. The dominance of neutrophils (58 ± 7 % of WBC) followed by lymphocytes (29 ± 7 % of WBC) in blood aligns with previous literature on dromedary camels (Zongping, 2003; Vap and Bohn, 2015).

The influence of age on the immune system has been addressed in several previous studies (Romanyukha and Yashin, 2003; Elghetany and Lacombe, 2004). In a recent study, Martin-Barrasa et al. (2023) compared leukograms between young and adult dromedary camels in the Canarian Islands and reported higher total WBC counts, along with higher neutrophils, lymphocytes, and monocytes counts in calves compared to adults. A similar leukogram pattern was also observed in camel calves in Saudi Arabia (Gaashan et al., 2020). The results of the present study corroborate with the literature reports regarding the elevated numbers of WBC and lymphocytes and lower numbers of eosinophils in camel calves (Gaashan et al., 2020). However, while previous studies reported higher frequencies of neutrophils and monocytes in young than in adult camels (Martin-Barrasa et al., 2023), the present study found comparable numbers of neutrophils and monocytes between young and adult Omani camels. Whether this observation is specific to the Omani camel breed requires further investigation, including comparisons with other camel breeds.

Newborn and young artiodactyls, including camels, cattle, goats, sheep, and pigs, are characterized by high percentages of γδ T cells in their blood. In newborn camel calves, γδ T cells account for up to 35 % of blood lymphocytes (Hussen and Schuberth, 2020). In the present study, the dominance of γδ T cells over helper T cells and B cells is in agreement with previous studies. In the present study, the lower numbers of B cells and helper T cells, which are known for their central

role in humoral and cell-mediated immune responses, respectively, further confirm the reported difference in immune functionality between young and adult camels (Hussen and Schuberth, 2020).

CONCLUSION

The present study identified reference values for some immune cell populations in blood from the Omani camel breed. The results indicated the significant impact of age on the analyzed parameters. Specifically, the different frequencies of B cells and T cells suggest lower immune competence in terms of both humoral and cell-mediated immune responses in young camels as opposed to adult camels. Further studies on camels from other breeds could focus on functional differences in the immune system between different camel breeds. Although some differences were observed between male and female adult camels, these findings are limited by the smaller number of male camels in the adult group (n = 4). Therefore, future studies with larger sample sizes are needed to investigate gender-related differences in immune cell composition.

DECLARATIONS

Authors' contributions

Jamal Hussen designed the study, analyzed the data, and wrote the first draft of the manuscript. Fathi Ahmed AL-Musallam collected the samples, counted cells, and revised the manuscript. All authors read and approved the final version of the manuscript.

Ethical considerations

All authors have been screened for ethical issues, including plagiarism, consent for publication, misconduct, fabrication of data, and duplicate publication or submission.

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Competing interests

The authors have no relevant financial or non-financial interests to disclose.

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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Detection of the *Zygote arrest 1* Gene in Oocytes, Zygotes, and Embryos of Pesisir Cattle with the Addition of IGF-1 within the *In Vitro* Maturation Media

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ABSTRACT

Zygote arrest 1 (*ZAR 1*) is a maternal gene that plays a crucial role in the oocyte-to-embryo transition. The present study aimed to investigate the presence or absence of the *ZAR 1* gene in oocytes, zygotes, and embryos of Pesisir Cattle. Ovaries were collected from cattle at slaughterhouses, and oocytes were retrieved at the Biotechnology Laboratory. The collected oocytes were matured in a maturation medium supplemented with Insulin-like Growth Factor-1 (IGF-1) at a concentration of 10 µg/ml or without IGF-1 for 24 hours in a CO_2 incubator maintained at 38.5°C. Following maturation, the oocytes were fertilized for 18 hours, and the resulting embryos were cultured for 48 hours in a CO_2 incubator at 38.5°C. The samples were then subjected to PCR analysis. The amplification results revealed the presence of the *ZAR 1* gene band at the target size of 228 bp in oocytes matured with and without IGF-1. A comparative analysis of oocytes and embryos showed differences in the gene bands, particularly in samples supplemented with IGF-1. These findings suggest that IGF-1 supplementation during oocyte maturation significantly influences *ZAR 1* gene expression in embryos. The observed variations in *ZAR 1* gene expression across the oocyte, zygote, and embryo stages highlight the gene's pivotal role in reprogramming post-fertilization and maintaining early embryonic development.

Keywords: Embryo, Insulin-like Growth Factor-1, Oocyte, Zygote, *Zygote arrest 1* gene

INTRODUCTION

Pesisir cattle are a local breed from West Sumatra with significant potential as meat producers. They demonstrate strong adaptability to coastal environments, even in regions with limited forage availability. This adaptability offers opportunities to expand their development across the coastal areas of Indonesia. However, the primary challenges in developing Pesisir cattle include low productivity and declining genetic quality. Genetic improvement efforts have been implemented to enhance the productivity of Pesisir cattle (Adrial, 2010). One approach to genetic improvement involves the application of reproductive technologies (Suryana, 2009). The ovary, a vital reproductive organ, produces fertilized oocytes with optimal developmental potential and secretes steroid hormones necessary for preparing the reproductive tract for fertilization and implantation (Palermo, 2007). Dysfunction in the ovary can disrupt the reproductive system, and numerous studies have explored using bioactive compounds to enhance reproductive performance (Abdullah et al., 2018). One such bioactive compound is Insulin-like Growth Factor-1 (IGF-1), which plays a crucial role in the female reproductive system. IGF-1 regulates growth, cell differentiation, cell metabolism, and apoptosis, contributing significantly to early embryonic development (Byrne et al., 2002).

According to Abdullah et al. (2018), the supplementation of Insulin-like Growth Factor-1 (IGF-1) in oocyte maturation and culture media stimulates and enhances oocyte maturation, improves *in vitro* fertilization (IVF) outcomes, and increases the number of embryos reaching the blastocyst stage in various livestock species, including pigs and cattle (Neira et al., 2010) as well as buffaloes (Singhal et al., 2009). In livestock, particularly cattle, IVF is one approach to utilizing ovarian waste from female cows slaughtered in abattoirs. The process of IVF is expected to produce cattle embryos that can be transferred to recipient cows, thereby enhancing reproductive efficiency and genetic improvement efforts (Kaiin et al., 2005).

The development of an embryo through fertilization, cleavage, blastocyst formation, and implantation are highly dependent on the quality of the oocytes. The role of the oocyte is particularly critical during the maternal-embryonic transition (MET), the interval between fertilization and the activation of the embryonic genome's transcriptional activity.

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During this period, embryonic development is supported by maternal RNA and proteins synthesized during oogenesis (Telford et al., 1990; Brevini et al., 2004). The duration of the MET varies among species. In mammals, this process occurs at different developmental stages depending on the species (Brevini et al., 2004). In mice, MET occurs at the late 2-cell stage, while in pigs, it occurs at the 4-cell stage. In human embryos, it happens between the 4-cell and 8-cell stages, in rabbits at the 8-cell stage, and in sheep and cattle embryos between the 9-cell and 16-cell stages (Telford et al., 1990; Brevini et al., 2004).

The quest for enhanced reproductive efficiency in cattle has increasingly focused on optimizing *in vitro* fertilization (IVF) techniques. A pivotal factor in IVF's success is oocyte maturation, which directly influences embryo quality (Yang et al., 2022). Recent studies have highlighted the significant role of Insulin-like Growth Factor-1 (IGF-1) in promoting the developmental competence of bovine oocytes (Spicer et al., 2002). IGF-1 facilitates various cellular processes, including proliferation, differentiation, and metabolic regulation, improving maturation outcomes (Spicer et al., 2002; Yang et al., 2022).

One of the techniques for assessing the success of pre-implantation development in animals is highly dependent on maternal RNA and proteins synthesized during oogenesis in the early stages of embryo development. An important oocyte-specific maternal effect gene for early embryo development is *Zygote arrest 1* (*ZAR 1*, Brevini et al., 2004). *Zygote arrest 1* is a maternal gene that plays a crucial role in the oocyte-embryo transition, potentially providing new insights into the initiation of embryonic development and the control of mammal fertility. The *ZAR 1* gene is evolutionarily conserved in vertebrates, and the *ZAR 1* protein is characterized by the presence of an atypical plant homeobox zinc finger domain, highlighting its role in transcriptional regulation. Furthermore, the *ZAR 1* gene is considered one of the key regulators of successful pre-implantation development in pigs and cattle (Brevini et al., 2004; Uzbekova et al., 2006).

The *ZAR 1* gene is expressed in oocytes, zygotes, and all stages of embryonic development up to blastocyst formation. Reverse Transcription Polymerase Chain Reaction (RT-PCR) analysis was performed on oocytes and pre-implantation embryos produced *in vitro* (Brevini et al., 2004). Brevini et al. (2004) stated that the *ZAR 1* gene is the most recently identified maternal effect gene in mice and is also the most specific. The mRNA of the *ZAR 1* gene is present in oocytes and 1-cell embryos, with a significant decline observed at the 2-cell stage. In cattle, however, the *ZAR 1* gene is expressed across all stages of cell division but shows a decrease after the 2-cell stage. Meanwhile, studies by Pennetier et al. (2005) and Uzbekova et al. (2006) indicate that the *ZAR 1* gene is present in oocytes and 4-cell embryos but decreases notably at the 8-cell stage.

The *ZAR 1* gene is crucial for regulating the timing of early cell divisions during embryogenesis. Dysregulation of *ZAR 1* gene expression can lead to *Zygote arrest*, negatively impacting embryo viability and overall reproductive success (Michailidis et al., 2009). Thus, understanding how IGF-1 influences *ZAR 1* gene expression during oocyte maturation and subsequent embryonic development is essential for refining *in vitro* maturation (IVM) protocols. Recent findings indicate that adding IGF-1 to the maturation medium enhances oocyte quality and alters the expression of essential genes associated with embryo development, including the *ZAR 1* gene (Brevini et al., 2004).

Gene expression studies during embryogenesis in cattle are still limited, as they have only been carried out in a single species. Therefore, they are inadequate and need to be further elucidated to provide a model for controlling gene expression during early development in mammals, as well as zygotic or embryonic gene activation in cattle. A detailed analysis of gene expression during cattle embryogenesis is essential for understanding the basic cellular and molecular mechanisms of gene expression control, developing better embryo culture systems, and improving strategies for transgenic and cloning studies. This gene expression analysis method can be valuable for researchers engaged in animal and livestock genetics at the cellular and molecular levels. Insights into oocyte maturation with the addition of IGF-1 inform strategies to improve embryo quality and reproductive outcomes in cattle, contributing to advancing reproductive technologies in livestock production. The present study aimed to determine the presence or absence of the *ZAR 1* gene in oocytes, zygotes, and embryos of Pesisir Cattle.

MATERIALS AND METHODS

Materials

The Pesisir cattle are native to the coastal areas of West Sumatra. 16 pairs of ovaries from Pesisir Cattle were obtained from the Padang slaughterhouse (RPH). At the same time, semen samples were sourced from Friesian Holstein (FH) bulls produced by the Artificial Insemination Center in Lembang, Indonesia.

The materials used included NaCl, penicillin (100 IU/ml, Meiji, Japan), streptomycin (0.1 µg/ml, Meiji, Japan), Tissue Culture Medium (TCM)-199 (Sigma, M4530, UK), Pregnant Mare Serum Gonadotropin (PMSG), Insulin-like Growth Factor-1 (IGF-1, Sigma, UK), Bovine Serum Albumin (BSA, 0.3%, Sigma, A2153, UK), gentamicin (50 µg/ml,

Sigma, G1397, UK), Brackett-Oliphant (BO) medium, PBS (phosphate-buffered saline) solution, Aquabides, and mineral oil (Sigma, USA), 2% agarose, primers, master mix, Taq DNA polymerase, and 1-TAE buffer. The tools used included a stereo microscope (Nikon, Japan).

Methods

Collecting ovaries and oocytes

The procedure was carried out according to Nanda et al. (2019). The cattle ovaries obtained from the slaughterhouse were transported to the laboratory in a physiological NaCl medium supplemented with 100 µg/ml streptomycin (Meiji, Japan) and 100 IU/ml penicillin (Meiji, Japan) and stored in a thermos at 30-35°C. The ovaries were then thoroughly rinsed, and the oocytes were collected no later than 6 to 8 hours after incision. The collection of oocytes from the ovaries was performed by making incisions at surgical sites on the ovaries. The ovaries were placed in a petri dish containing 5 ml of collection medium, including PBS solution, and held using tweezers. Follicles on the surface of the ovaries were then incised using a scalpel. The follicular fluid and the PBS solution caused the oocytes to be released and collected. The oocytes in the petri dish were then selected under a stereomicroscope.

The selected oocytes were evaluated for quality and classified based on morphology according to the criteria described in previous studies (de Loos et al., 1989; Blondin and Sirard, 1995; Yuan et al., 2005). G1 represented the highest quality oocytes, characterized by five or more layers of compact cumulus cells. G2 included good-quality oocytes, identified by a uniform structure and fewer than five layers of cumulus cells. G3 oocytes were identified by scattered cumulus cells. The oocytes used in the current study were classified as grades A, B, and C.

The selected oocytes were then matured using TCM-199 medium, 10 µg/ml PMSG, BSA, without IGF-1, 10 µg/ml IGF-1 (Sigma, 1-5500), and 50 µg/ml gentamicin. The selected oocytes were rinsed thrice and then matured in a petri dish for 24 hours in a 5% CO_2 incubator at 38.5°C. The oocytes were cultured in 100 µL drops containing 10-15 oocytes and covered with mineral oil (Sigma-Aldrich, Inc., M-8410). All media used in the present study were equilibrated for at least 2 hours in a 5% CO_2 incubator at 38.5°C before use. After the oocytes had matured for 24 hours, two groups of oocytes were taken to observe *ZAR 1* gene expression by PCR, and the remaining oocytes were used for the *in vitro* fertilization stage.

In vitro fertilization

The procedure was carried out according to Nanda et al. (2019). Oocyte fertilization was performed using frozen semen from FH cattle. The frozen semen was thawed in warm water at 30-37°C for 30 seconds, then placed into a sterile tube and centrifuged at 700 g for 8 minutes. After centrifugation, the supernatant was removed, and the spermatozoa were diluted with BO medium to achieve a final concentration of 2×10^6 spermatozoa/mL. The spermatozoa and BO medium mixture were made into 100 µL petri dish drops for 10-15 oocytes, covered with mineral oil (Sigma, USA). The matured oocytes were washed twice in BO medium, then transferred to the respective drop according to treatment, and incubated in a 5% CO_2 incubator at 38.5°C for 18 hours (Nanda et al., 2019). After 18 hours of fertilization, two groups of fertilized oocytes were taken to observe *ZAR 1* gene expression by PCR, while the remaining fertilized oocytes were observed for *ZAR 1* gene expression in embryos after 48 hours of fertilization.

Zygote arrest 1 gene amplification

Polymerase chain reaction (PCR) was performed with cDNA equivalents corresponding to at least two batches of oocytes, zygotes, or embryos. The master mix reaction mixture consisted of 1x $MgCl_2$, 25 U Taq DNA polymerase, 1x PCR buffer (Tris-HCl, KCl), 2.5 mM dNTPs, primers, and sterile water.

Zygote arrest 1 amplification was performed in an automatic thermal cycler using the following conditions, including 2 minutes at 94°C (denaturation temperature), 10 seconds at 60°C (annealing temperature), and 2 minutes at 72°C (extension temperature) for 35 cycles. The primers were L-ACGTCGTCCTGGATGGTTAC and R-GCTGGTAGCTGTGGACGTACT, which encoded a 228 bp product.

PCR products were electrophoresed on a standard 2% agarose gel in 1x TAE buffer (40 mM Tris-acetate, 1 mM EDTA) with a total volume of 350 mL, using a 9 mm gel tray. Electrophoresis was performed at 80 V for 45 minutes, after which the fragments were visualized using a UV transilluminator at 312 nm. Each gel was then documented with a camera (Brevini et al., 2004; Uzbekova et al., 2006).

Variables

Determining whether there are differences in *ZAR 1* gene expression in oocytes, zygotes, and embryos of Pesisir cattle with and without IGF-1 treatment.

Statistical analysis

The data obtained from the current study are qualitative, in the form of band images of *ZAR 1* gene expression in oocytes, zygotes, and embryos of Pesisir cattle with and without IGF-1 treatment during maturation. For descriptive data, frequency and percentage were used to visualize the results. Data analysis was conducted using the Chi-Square test, with a p-value of less than 0.05, which was considered statistically significant. The analyses were performed using SPSS Statistics version 25.

RESULTS AND DISCUSSION

The influence of cumulus cells on the *ZAR 1* gene

Oocytes with cumulus cells used in the present study (Table 1) were grouped into three categories.

From Table 1, oocytes supplemented with IGF-1 during the maturation medium exhibited more significant cumulus cell swelling than those without IGF-1. According to Da Broi et al. (2018), cumulus cells support oocyte maturation through metabolic substances. Cumulus cells are crucial in providing nutrients to the oocyte and assisting in protein synthesis for zona pellucida formation. *In vitro*, oocyte maturation is a process influenced by individual oocyte characteristics, stemming from differences in the development and growth of oocytes collected from the ovaries (Conti and Franciosi, 2018).

During *in vitro oocyte* maturation, changes in cumulus cell morphology and interactions between follicular cells and the oocyte occur. Cumulus cells are essential for enhancing cytoplasmic maturation, which is necessary for pronucleus formation and the ability to continue development (Muhajir, 2018). Oocytes with cumulus cells have a higher fertilization capacity compared to those that mature without them. This is attributed to cumulus cells reducing the degree of *zona pellucida* hardening during culture. Hardening of the zona pellucida decreases the fertilization capacity of oocytes matured without cumulus cells (Kusindarta, 2009). Sato et al. (2018) demonstrated that insulin promoted oocyte maturation in cattle and the swelling of cumulus cells, and it could inhibit apoptosis. Insulin also enhanced *in vitro* oocyte maturation, fertilization, and embryonic development to the blastocyst stage in mice (Kiapekou et al., 2005). Adding insulin to the IVM medium improved pig models' maturation rates and increased IVF outcomes (Xia et al., 1994). Cumulus cells play a vital role in oocyte maturation and fertilization by releasing and mediating signals to the oocyte (Auclair et al., 2013).

Table 1. Matured results of oocytes from Pesisir cattle were used for PCR

Treatment	Amplification	Cumulus cell		
		G1	G2	G3
A (without IGF-1)	1	-	3	12
	2	-	9	6
	3	3	10	2
	4	5	10	-
B (IGF-1 10 µg/ml)	1	-	10	5
	2	4	7	4
	3	7	8	-
	4	10	5	-

Note: G1: oocyte lined with > 5 layers of compact cumulus cells, G2: With only 1-5 layers of compact cumulus cells, G3: With scattered cumulus cells.

Detection of the *Zygote arrest 1* gene

Detection of the *ZAR 1* gene using primer pairs L ACGTCGTCCTGGATGGTTAC and R-GCTGGTAGCTGTGGACGTACT. As many as 120 samples were amplified in Table 2, and the amplification can be seen in Figure 1.

The amplified samples (Table 2) without IGF-1 showed the presence of the *ZAR 1* gene in 50% of oocytes, 75% of zygotes, and 25% of embryos. In contrast, with the addition of 10 µg/ml IGF-1, the *ZAR 1* gene was detected in 75% of oocytes, zygotes, and embryos. These results indicated that adding IGF-1 to the maturation medium provides more significant benefits than its absence. However, the results showed no significant effect between the addition of IGF-1 and its absence on the *ZAR 1* gene in oocytes and zygotes ($p > 0.05$), although the addition of IGF-1 had a significant effect on *ZAR 1* gene detection in Pesisir cattle embryos ($p < 0.05$).

According to Bonilla et al. (2011), preimplantation embryo conditions depended on the endocrine environment that regulated its developmental program. Growth factors such as insulin were one endocrine signal influencing the embryo's

ability to develop in optimal and suboptimal environments. In cattle, insulin increased the proportion of preimplantation embryos developing to the blastocyst stage, altered blastocyst gene expression, enhanced the resilience of preimplantation embryos on days 4-6 against heat shock, and improved embryo survival after transfer to recipients. Boucher et al. (2014) noted that insulin and IGF-I regulated different biological processes, such as cell metabolism, proliferation, differentiation, and apoptosis, which are mediated by activating intrinsic tyrosine kinase activity at their receptors. The loss of insulin and IGF-I receptors in cells significantly decreased the expression of several genes, both maternally and paternally expressed.

According to Sánchez et al. (2009) and Wasielak et al. (2016), exogenous growth factors like insulin *in vitro* influenced the RNA of developmental genes, including the *ZAR 1* gene, which played a role during the early stages of embryonic development. Racedo et al. (2009) reported that *ZAR 1* mRNA levels were influenced by maturation stages in isolated oocytes, showing a decrease during the maturation of oocytes isolated from larger follicles while remaining relatively stable in those from smaller follicles.

Table 2. Amplification results of oocytes, zygotes, and embryos from Pesisir cattle

Treatment	Samples	Early development of embryo (%)			P-value
		Oocyte (20 samples)	Zygote (20 samples)	Embryo (20 samples)	
A	60	($^{10}/_{20}$) 50%	($^{15}/_{20}$) 75%	($^{5}/_{20}$) 25%	
B	60	($^{15}/_{20}$) 75%	($^{15}/_{20}$) 75%	($^{15}/_{20}$) 75%*	$p < 0.05$

Note: A: Without IGf-1, B: IGF-110 µg/ml, n: Sample size, the numbers in parentheses indicate the number of samples.* indicates a significant difference in a column.

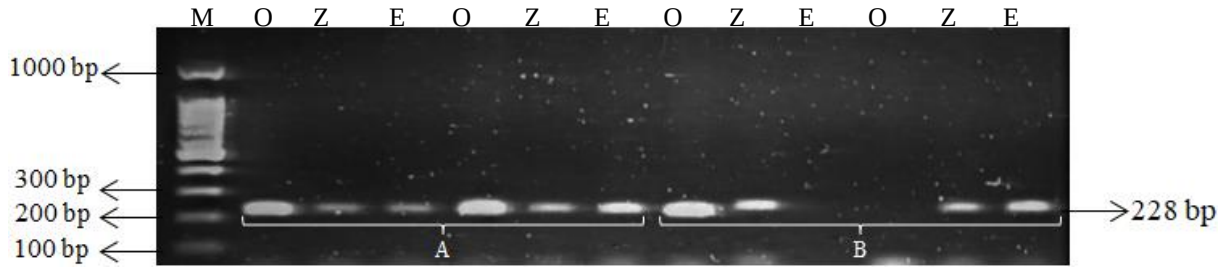

Figure 1. ZAR gene detection 1. **A:** IGF-1 addition to the maturation media, **B:** Without addition of IGF-1 to the maturation media. M (Marker), O (Oocytes), Z (Zygote), E (Embryo).

The *ZAR 1* gene was amplified explicitly because only one DNA band in each sample was electrophoresed according to the expected size of 228 bp (Figure 1). The amplification results from Figure 1A indicated that samples matured with the addition of IGF-1 showed a band for the *ZAR 1* gene in oocytes, zygotes, and embryos. In contrast, the amplification results from Figure 1B showed a difference in samples without adding IGF-1, as there was no *ZAR 1* gene band in oocytes and embryos, although a band for the *ZAR 1* gene was present in the zygotes.

The detection of the *ZAR 1* gene in Pesisir cattle embryos showed differences compared to the *ZAR 1* gene in FH cattle. In the study by Brevini et al. (2004), *ZAR 1* was detected up to the blastocyst stage with an increase in mRNA at the four-cell stage. Wasielak et al. (2016) indicated differences in *ZAR 1* mRNA levels produced by *in vivo* and *in vitro* embryos. *In vivo*, *ZAR 1* mRNA decreased starting from the four-cell stage, whereas *in vitro*, the decrease occurred earlier at the two-cell stage. The *ZAR 1* protein participated in interactions related to chromatin-mediated transcription regulation during the oocyte-embryo transition.

As noted in the previous study by Shirazi and Motaghi (2013), *ZAR 1* was a maternal-specific ovarian factor that played a crucial role during pregnancy. The transition from oocyte to embryo in mice showed that its expression was highly restricted to oocytes, zygotes, and, to a lesser extent, two-cell embryos. In *ZAR 1* knockout mice, embryos failed to develop beyond the first cleavage stage. Other studies found that less than 20% of embryos from *ZAR 1* (-/-) females progressed to the two-cell stage, showing a significant reduction in the synthesis of complexes requiring transcription, with no embryos developing to the four-cell stage (Wu et al., 2003a; Shirazi and Motaghi, 2013). According to the study by Brevini et al. (2004), the *ZAR 1* gene was observed up to the 8-cell stage.

Maternal genes were expressed in oocytes and embryos and played a crucial role in activating the embryonic genome. Abnormalities in the expression of these genes could lead to halted embryonic cleavage or transcriptional changes responsible for further embryonic development (Wasielak et al., 2016). The *ZAR 1* gene was important during the first stages of embryonic development, and the absence of *ZAR 1* protein in oocytes directly impacted cleavage potential and the development of embryos beyond the blastocyst stage (Sánchez et al., 2009). These results indicated that

maternal genes played a significant role in reprogramming after fertilization or maintenance during early pre-implantation embryos (Tsunemoto et al., 2008; Racedo et al., 2009).

The role of the *ZAR 1* gene in early embryonic development was essential, particularly in the context of bovine reproduction (Wasielak et al., 2016). Investigation into the expression of *ZAR 1* in oocytes, zygotes, and embryos in the presence of IGF-1 during *in vitro* maturation (IVM) revealed significant insights into its regulatory mechanisms and potential applications in enhancing reproductive efficiency (Brevini et al., 2004).

According to Yang et al. (2022), IGF-1 supplementation during *in vitro* maturation significantly increased the maturation rates of bovine oocytes, which was essential for successful fertilization and subsequent embryonic development. This improvement was attributed to IGF-1's role in promoting metabolic activity and enhancing the physiological conditions necessary for oocyte maturation. In the findings, *ZAR 1* expression was notably elevated in oocytes matured with IGF-1, supporting the hypothesis that this growth factor played a vital role in the transcriptional regulation of genes critical for early embryogenesis (Spicer et al., 2002).

The expression patterns of *ZAR 1* during the zygotic stage also warranted attention. Analysis indicated that *ZAR 1* remained expressed post-fertilization, which aligned with findings highlighting its importance in regulating the timing of embryonic cleavage divisions. Adding IGF-1 appeared to stabilize *ZAR 1* expression in zygotes, potentially facilitating a more robust transition from maternal to embryonic development control (Wu et al., 2003b). This stabilization mitigates the effects of *Zygote arrest*, a common issue that can lead to developmental failures (Wu et al., 2003a).

Furthermore, the impact of IGF-1 extended into the later stages of embryonic development. The presence of IGF-1 enhanced the initial stages of maturation and fertilization and supported progression to the blastocyst stage. The present results indicated that embryos developed with IGF-1 exhibited increased *ZAR 1* expression, correlating with higher blastocyst rates. This finding aligned with previous studies that underscored the role of *ZAR 1* in maintaining embryonic viability (Brevini et al., 2004; Michailidis et al., 2009).

However, it was crucial to recognize that while IGF-1 supplementation appeared beneficial, the timing and concentration of its application required careful optimization. Excessive levels may have altered gene expression patterns and unintended effects on embryonic development (Neira et al., 2010). Thus, further studies are warranted to elucidate the optimal conditions for IGF-1 use during IVM and its long-term implications for embryo quality and reproductive success.

CONCLUSION

The *ZAR 1* gene was detected in oocytes, zygotes, and Pesisir cattle embryos. The study revealed differences in the detection of the *ZAR 1* gene in oocytes, zygotes, and embryos of Pesisir cattle with or without adding IGF-1 in the *in vitro* maturation medium. The presence of the *ZAR 1* gene at the oocyte, zygote, and embryo stages indicated that maternal genes played important roles in reprogramming after fertilization or in maintaining early embryonic development. Based on the study results, further research should concentrate on the effects of IGF-1 supplementation in embryo culture media and detecting the *ZAR 1* gene during cleavage stages, from the four-cell to the blastocyst stage.

DECLARATIONS

Authors' contributions

All authors contributed to the study's conception, design, statistical analysis, and practical duties in the study. The first draft of the manuscript was written by Sedrisa Lidya Pertiwi, and Tinda Afriyani and Jaswandi commented on previous versions of the manuscript and reviewed the study. All authors read and approved the final version of the manuscript.

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Ethical considerations

This paper was originally written by the authors and has not been published elsewhere. The authors checked the text of the article for plagiarism index and confirmed that the text of the article is written based on their original scientific results.

Availability of data and materials

The data to support this study's findings is available upon reasonable request to the corresponding author.

Competing interests

The authors have no competing interests to declare.

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Correlation of Canine Kidney Autopsy to Renal Diseases: Pathological Insights

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ABSTRACT

Kidneys play a vital role in regulating fluids, electrolytes, hormones, and metabolic waste in canines. This study aimed to enhance the understanding of the correlations between canine kidney autopsy findings and renal diseases. A total of 194 domestic dog samples, including 153 males and 41 females with an average age of 3.88 years (ranging from 1 to 7 years), were physically examined using post-mortem evaluations to understand the prevalence and characteristics of kidney diseases, focusing on both external and internal examinations of the kidneys. Key parameters such as kidney size, texture, and coloration were measured to provide insights into the overall kidney health of the canine population in Vietnam. Results indicated that 22.68% of the dogs had kidney cysts, 29.38% showed signs of external hemorrhage, and 52.06% of the cases exhibited internal hemorrhage, proving to be a condition linked to increased renal vascular resistance and further potentially contributing to renal dysfunction. No evidence of necrosis was detected, and the majority of renal capsules (90.98%) were easy to peel off for further analysis. Kidney size and weight varied obviously in dogs presenting with specific hemorrhagic conditions. This study emphasized the importance of external and internal kidney evaluations in diagnostic measurements and treatment protocols for canine renal diseases while also providing further insights into the current status of the canine population in Vietnam.

Keywords: Dog, Hemorrhage, Kidney, Post-mortem, Renal disease

INTRODUCTION

The kidneys play an important role in maintaining a dog's well-being through the regulation of fluids, electrolytes, and waste management. Healthy kidneys ensure that homeostasis is achieved in both humans and animals (Mitrakou, 2011). Additionally, kidneys release certain hormones such as renin, which aid in blood pressure regulation and fluid volume control (Castrop et al., 2010). Furthermore, erythropoietin has been proven to accelerate wound healing processes (Haroon et al., 2003) and promote the brain's response in the presence of neural injury (Sirén et al., 2001). However, with such complex and critical roles, kidneys are also susceptible to various renal diseases that severely compromise an animal's well-being. Changes such as cortical echogenicity or loss of corticomedullary differentiation are indicative of renal diseases (Bragato, 2017).

Some of the most common diseases occurring in old canine patients are chronic kidney disease and acute kidney injury (Bartges, 2012). Chronic kidney disease is characterized by the gradual loss of kidney function and is one of the leading causes of morbidity (Kalantar-Zadeh et al., 2021). Acute kidney injury, on the other hand, is defined as the sudden onset of loss of renal function (Ross, 2011). In Vietnam, renal diseases account for 42.11% of urinary tract complications (Nguyen et al., 2020). These diseases not only affect a patient's quality of life but also lead to life-threatening complications if not properly diagnosed and treated. Physical examination of the kidneys has been shown to provide critical findings regarding chronic kidney disease (Polzin, 2011). Additionally, research has established correlations between the effects of hemorrhage on renal blood flow (Kennard et al., 2024) and the ratio of kidney length to lumbar vertebrae (Barella et al., 2012). Access to accurate and detailed information is crucial for understanding the current health status of a population, as demonstrated in this study, which focuses on the canine population in Vietnam.

Therefore, the present study aimed to collect and analyze data to interpret the status of renal health in Vietnamese canines and its correlation with renal diseases.

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MATERIALS AND METHODS

Ethical approval

The study was conducted through post-mortem examination, kidney observation, and measurements according to the procedures of the Animal Welfare Council, Hutech University, Ho Chi Minh City, Vietnam. The authors considered ethical concerns and consent, animal welfare, and safety procedures before conducting the study.

Sample collection and macroscopic examination

A post-mortem examination was performed on 194 dogs aged 1–7 years, comprising 153 males (78.87%) and 41 females (21.13%). The gender imbalance was acknowledged as a factor influencing the generalization of findings and correlations to gender. Afterward, a macroscopic examination of lesions was conducted, which recorded kidney measurements, texture, appearance, and status. After opening the abdominal cavity, the location and the signals of the kidneys were checked. The left kidney is slightly more caudal than the right kidney (Nyland et al., 2015). The kidneys were then removed from the body by cutting the renal vessels. To differentiate the left and right kidneys, a small transverse cut was made into the parenchyma of the right kidney. The kidneys were immediately evaluated after sectioning. Firstly, the renal capsule was removed to examine the external aspects of both kidneys (McDonough and Southard, 2016). The adhesion of the renal capsule was evaluated based on the difficulty of peeling the capsule. The appearance (smooth or spotty) and color (light, dark, or blue-gray) of the kidney surfaces were documented. Then, the consistency of the kidneys was assessed through gentle palpation of the kidneys. Additional signs, such as hemorrhage, necrosis, and tumors, were recorded before the examination of the internal aspects of the kidneys. Longitudinal sectioning was performed to examine the internal features of the kidneys. Abnormal findings on the cut surface of the kidneys, internal hemorrhage, and necrosis were also recorded.

Measurement protocols

The thickness of the renal cortex and medulla of each kidney was measured. Renal cortical thickness was defined as the maximal distance from the cortex-perirenal fat interface to the cortex-pyramidal base (Su et al., 2019). The thickness of the cortex varies depending on whether it is measured at the top or a lower point of the scallop. However, the cortex is thicker than the outer medulla zone beneath it (Bulger et al., 1979). The inner medulla is thicker than both the cortex and the outer medulla in healthy kidneys. The length, width, and weight of each kidney were separately recorded. Renal length was measured as the longest distance between the superior and inferior poles on the longitudinal section. Renal width was determined as the maximum transverse diameter at the hilum under healthy conditions (Su et al., 2019). Renal medulla thickness was defined as the distance from the corticomedullary junction to the tip of the medullary papilla (Beuchat, 1990). Gender and hemorrhage status were noted in all cases to evaluate their impacts on kidney measurements. A digital caliper was used for all measurements, with the unit of measurement being centimeters (cm).

Statistical analysis

Data were analyzed using mean values and standard deviations (SD) calculated in Microsoft Excel, version 2016.

RESULTS

When examining externally, there were only two main differentiations, whether the layer was smooth or not. Of the 194 dogs, 150 had a smooth layer on the kidney's surface (77.32%). Meanwhile, 44 of the 194 dogs had spots on them (22.68%, Table 1). Regarding the color of kidneys, there were three types of coloring recorded: 184 (94.85%) cases were reported to be light-colored, 9 (4.64%) cases were reported to be deep-colored, and 1 (0.52%) case was reported to be blue-gray. The samples provided showed three distinct types of consistency: 191 cases showed normal consistency (98.45%), 2 were tough cases (1.03%), and 1 was a soft case (0.52%). Other lesions that could be observed were the presence of hemorrhage on the outer shell, with 57 cases showing hemorrhage (29.38%). However, the other 137 (70.62%) cases did not present any signs of bleeding outside the kidney (Table 1).

While external factors may provide insights into the rough outline of underlying conditions, internal factors provide insights into the main roots of the complications. When evaluating bleeding conditions in the samples, 52.06% of the cases were found to have internal hemorrhage in the kidneys, while the other 47.94% showed no signs of bleeding during the dissection process. Necrosis is a kidney disorder involving damage to the cells of the kidneys. Necrosis is also a major factor in renal health: 100% of the cases showed no signs or progression of necrosis when examined. Out of 194 pairs of kidneys, 178 right kidneys and 175 left kidneys were reported to be easy to peel off, while the rest proved to be difficult to peel off for further inspection (Table 2).

Based on the results, the average thickness of the renal cortex was 0.47 ± 0.11 cm on the right kidney, while the average thickness of the renal cortex on the left kidney was 0.45 ± 0.12 cm. The renal medulla had an average thickness of 0.76 ± 0.23 cm on the right kidney and 0.74 ± 0.22 cm on the left kidney. When measuring the length of both kidneys, the average length was found to be 4.72 ± 0.50 cm on the right kidney and 4.81 ± 0.52 cm on the left kidney. The same right and left difference also applies to measuring the width of the kidneys, averaging 2.27 ± 0.38 cm on the right kidney while having an average of 2.28 ± 0.39 cm on the left kidney. When weighing individual kidneys, the average weight was 23.70 ± 4.97 g on the right kidney and 23.73 ± 5.29 g on the left kidney. It should also be noted that a total of 30 out of 194 cases (15.46%) had not been weighted properly due to unforeseen circumstances (Table 3).

Table 1. Examining external aspects of canine kidneys in domestic dogs (153 males and 41 females).

Variable	Criteria	Number of cases	Percentage
Appearance	Smooth	150	77.32
	Spots	44	22.68
Color	Light colored	184	94.85
	Deep colored	9	4.64
	Blue-gray	1	0.52
Consistency	Normal	191	98.45
	Hard	2	1.03
	Soft	1	0.52
Other problems	Hemorrhage	57	29.38
	None	137	70.62

Table 2. Examining internal aspects of the canine kidney in domestic dogs (153 males and 41 females).

Variable	Criteria	Number of cases	Percentage
Bleeding	Internal hemorrhage	101	52.06
	None	93	47.94
Necrosis	Absent	194	100.00
	Present	0	0.00
Renal capsule	Easy to peel (Right)	178	91.75
	Easy to peel (Left)	175	90.21
	Difficult to peel (Right)	16	8.25
	Difficult to peel (Left)	19	9.79

Table 3. Average measurements of the canine kidney in domestic dogs (153 males and 41 females).

Renal parameters	Number of samples	Mean ± SD (cm)	Mean ± SD (g)
Renal Cortex (Right)	194	0.47 ± 0.11	-
Renal Cortex (Left)	194	0.45 ± 0.12	-
Renal Medulla (Right)	194	0.76 ± 0.23	-
Renal Medulla (Left)	194	0.74 ± 0.22	-
Length (Right)	194	4.72 ± 0.50	-
Length (Left)	194	4.81 ± 0.52	-
Width (Right)	194	2.27 ± 0.38	-
Width (Left)	194	2.28 ± 0.39	-
Weight (Right)	164	-	23.70 ± 4.98
Weight (Left)	164	-	23.73 ± 5.29

SD: Standard deviation

Table 4. Gender difference in average measurements of canine kidneys in domestic dogs (153 males and 41 females).

Mean ± SD (cm)	Renal cortex		Renal medulla		Length		Width	
	Right	Left	Right	Left	Right	Left	Right	Left
Male	0.47 ± 0.11	0.44 ± 0.11	0.77 ± 0.25	0.76 ± 0.24	4.74 ± 0.47	4.82 ± 0.48	2.26 ± 0.36	2.27 ± 0.37
Female	0.48 ± 0.11	0.49 ± 0.13	0.73 ± 0.13	0.69 ± 0.14	4.67 ± 0.61	4.76 ± 0.67	2.30 ± 0.46	2.33 ± 0.48

SD: Standard deviation

Table 5. Correlation of hemorrhage to average measurements of the canine kidney in domestic dogs (153 males and 41 females).

External Hemorrhage	Internal hemorrhage	Number of cases	Renal cortex (cm)		Renal medulla (cm)		Length (cm)		Width (cm)	
			Right	Left	Right	Left	Right	Left	Right	Left
No	No	75	0.50	0.49	0.75	0.75	4.67	4.78	2.28	2.33
Yes	No	16	0.44	0.41	0.83	0.73	4.81	4.84	2.35	2.29
No	Yes	60	0.46	0.44	0.80	0.77	4.76	4.81	2.28	2.29
Yes	Yes	41	0.45	0.40	0.72	0.71	4.71	4.82	2.23	2.26

The thickness of the renal cortex showed slight differences between males and females. In males, the right and left renal cortices measured 0.47 ± 0.11 cm and 0.44 ± 0.11 cm, respectively, while in females, these values were reported as 0.48 ± 0.11 cm and 0.49 ± 0.13 cm, respectively (Table 4). Conversely, the renal medulla thickness was greater in the male canines. For males, the right and left medulla were 0.77 ± 0.25 cm and 0.76 ± 0.24 cm, respectively. For females, the measurements of the renal medulla were recorded as 0.73 ± 0.13 cm and 0.69 ± 0.14 cm for the right and left kidneys, respectively. Kidney length was, on average, greater in males, measuring 4.74 ± 0.47 cm on the right and 4.82 ± 0.48 cm on the left, as compared to females, whose kidneys measured 4.67 ± 0.61 cm on the right and 4.76 ± 0.67 cm on the left. Conversely, females exhibited slightly wider kidneys compared to males. The right and left kidney widths in females were 2.30 ± 0.46 cm, respectively, while in males, the measurements were reported as 2.26 ± 0.36 cm (right) and 2.27 ± 0.37 cm (left).

Upon further inspection, the study isolated cases of bleeding for deeper analysis, which led to four different results: No hemorrhage present in either the external or internal regions of the examined kidneys; external hemorrhage present but no internal hemorrhage; internal hemorrhage present but no external hemorrhage; and both external and internal hemorrhage present. When external hemorrhage was present, the average thickness of the right and left renal cortex was lower than the average, at 0.47 cm and 0.45 cm, respectively (Right: 0.44 cm without internal hemorrhage and 0.45 cm with internal hemorrhage present, Left: 0.41 cm without internal hemorrhage and 0.40 cm with internal hemorrhage present). In cases where internal hemorrhage was observed without the presence of external hemorrhage, kidney dimensions were generally larger than the average measurements. The thickness of the renal medulla increased, with the right medulla measuring 0.80 cm compared to the average of 0.75 cm and the left medulla measuring 0.77 cm compared to the average of 0.74 cm. Additionally, kidney length showed slight variations, with the right kidney measuring 4.76 cm, surpassing the average of 4.72 cm, while the left kidney remained consistent at 4.81 cm, aligning with the average. Moreover, kidney width displayed marginal increases, with the right kidney measuring 2.28 cm compared to the average of 2.27 cm and the left kidney measuring 2.29 cm compared to the average of 2.28 cm (Table 5). These results highlight subtle differences in kidney dimensions under these specific conditions.

DISCUSSION

Kidney's appearance

Evaluation criteria included external aspects such as appearance, color, texture, and other problems with the kidney, as well as internal aspects such as bleeding, necrosis, and the renal capsule. Recognizing these external and internal conditions is crucial for understanding the kidney's function and structure. Of 194 dogs, 144 were found to have "spots" on their kidneys. Kidney cysts are responsible for these "spots" appearing externally on the kidneys. Kidney cysts are complex, requiring careful diagnosis and early detection of symptoms to manage them effectively (Bergmann et al., 2018). Timely identification is crucial for developing an appropriate treatment plan and improving disease management outcomes. In particular, 22.68% of dogs had spots on their kidneys from the results (Van Dyck et al., 2018). This is alarming when taking into account the total population and its local implications. Abnormal colors were also observed, which indicate underlying chronic kidney disease conditions (Chetan et al., 2019). However, it should be noted that these dissections were performed post-mortem, so the color of the kidney might not reflect the overall health when the dog was alive. On the other hand, texture is also an important criterion to evaluate when dissecting, as it can indicate whether or not chronic renal failure is developing (Sebastian, 2009).

Internal functions of the kidney

Whereas external factors may provide a general understanding of underlying conditions, internal factors reveal the root causes of the complications. When examining, 52.06% of the samples were recorded to have experienced bleeding. This is a concerning percentage, as the presence of renal hemorrhage can also contribute to an increase in renal vascular

resistance (Sordi et al., 2024). Furthermore, increased renal vascular resistance correlates with a higher prevalence of renal dysfunction (Derchi et al., 2005).

Kidney sizes and measurements

Other aspects of the kidney, such as the thickness of the renal cortex and renal medulla, kidney length and width, and kidney weight, were measured, and the averages were calculated (Table 3). Accurate kidney size measurements are essential for assessing renal health (Hoey et al., 2016). In humans, kidney size has been found to correlate with kidney disease in some cases, especially when these parameters are correlated with other important diagnostic criteria such as serum creatinine concentration and creatinine clearance (Jovanović et al., 2013). Whether or not these findings can be applied to canine patients will depend on more thorough research with larger sample sizes to eliminate any irregularities and subclinical renal disease.

Correlation of gender with kidney size

When analyzing the differences between both genders, no definitive conclusion about kidney sizes could be drawn based on gender factors alone in this canine population. However, one notable finding was that males had longer kidneys on average as compared to females. This finding aligns with those of Kalucki et al. (2020), which were previously observed in humans but appear to apply to the canine population sample as well. Further research is required to draw more definitive conclusions.

Hemorrhage and its effects on renal measurements

Further analysis of external hemorrhage suggests that the difference in measurements may be because hemorrhage can lead to hypovolemia (Mattson et al., 2006), which decreases blood supply and results in less vascularized tissue, leading to reduced thickness. Conversely, in cases of internal hemorrhage, it is hypothesized that fluid accumulation leads to kidney enlargement, eventually resulting in measurements that are greater than average.

Accurate practices and understanding of renal diseases

Further investigation acknowledges that additional tests, such as biomarker analysis (Lippi et al., 2021), could have provided a more detailed overview and additional insights. Nevertheless, this study demonstrates that canine renal disease in Vietnam is a significant issue that requires attention. It is important to acknowledge that some of the data was compromised due to inadequate practices during the collection process. Therefore, it is essential to recognize this limitation and make improvements in future efforts.

CONCLUSION

After performing a physical examination of the kidneys, it is clear that all criteria and aspects are interconnected, forming a complex web of diseases and dysfunctions that negatively impact a dog's well-being. To tackle renal diseases, it is important to pay attention to all symptoms and abnormalities of the kidney. This is the key to accurate renal disease diagnosis and treatment. Future studies are encouraged to better understand and provide updates regarding this matter.

DECLARATIONS

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Author contributions

Loan Vu Thuy Hong Nguyen and Thuong Thi Nguyen conceptualized, designed, and supervised the research. Loan Vu Thuy Hong Nguyen, Thuong Thi Nguyen, and Nhung Thi Tran collected samples and conducted experiments. Thuong Thi Nguyen, Loan Vu Thuy Hong Nguyen, and Nguyen Tran Phuc Nguyen analyzed and interpreted the data generated. Thuong Thi Nguyen, Loan Vu Thuy Hong Nguyen, Nhung Thi Tran, and Nguyen Tran Phuc Nguyen critically reviewed the study. All authors revised and approved the submitted manuscript.

Competing interests

The authors declare no conflicts of interest.

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

This article was written originally without copying from previously published manuscripts, such as articles and books.

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Prevalence of Multi-Drug Resistance *Escherichia coli* in Broiler Chicken Meat in Jember, Indonesia

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ABSTRACT

Antibiotic resistance has become one of the global health problems nowadays. Chicken meat is one of the largest food commodities in the world. *Escherichia coli* (*E. coli*) is one of the bacteria that is often found in chicken meat. These bacteria are capable of being pathogenic in both animals and humans. This study aimed to determine the prevalence of multidrug-resistant *E. coli* isolated from broiler chicken meat in the study location. The *E.* coli utilized in this study were derived from 25 grams of chicken meat obtained from 30 samples procured from six markets within the Jember district. The resistance test method used was Kirby-Bauer with Mueller-Hinton media. The results of the study showed that 100% of chicken meat was contaminated with *E. coli*. All isolated *E. coli* from samples in the study were multidrug-resistant. *E. coli* was 100% resistant to cotrimoxazole and cefixime, 96.67% resistant to chloramphenicol and amoxicillin-clavulanic, 93.3% resistant to tetracycline, 90% resistant to ceftriaxone, and 80% resistant to azithromycin and ciprofloxacin. The minimum resistance profile to 5 types of antibiotics with a multiple antibiotic resistance (MAR) index was between 0.625-1. Thus, the study revealed a high risk of infection associated with the consumption of uncontrolled chicken meat.

Keywords: Antibacterial agent, Chicken, *Escherichia coli*, Multi-drug resistance

INTRODUCTION

Antibiotic resistance is one of the global health problems nowadays. This is because antibiotic resistance has a reasonably high morbidity and mortality rate (Akova, 2016). The 2022 report of the Global Antimicrobial Resistance and Use Surveillance System (GLASS) draws attention to the worrying prevalence of antibiotic resistance among common bacterial pathogens. The average reported rates of resistance across 76 countries are 42% for third-generation cephalosporin-resistant *Escherichia coli* (*E. coli*) and 35% for methicillin-resistant Staphylococcus aureus, which is a matter of serious concern (WHO, 2022). Antibiotic resistance is also one of the health problems faced by the Indonesian state. A study proved that 43% of *E. coli* bacteria taken from 2,494 Indonesians are resistant to various types of antibiotics, including ampicillin, cotrimoxazole, chloramphenicol, ciprofloxacin, and gentamicin (Dirga et al., 2021). Antibiotic resistance makes antibiotics less effective in the fight against infection. One of the leading causes of the onset of antibiotic resistance in the community is the inappropriate use of antibiotics. The incidence of antibiotic resistance is rapidly increasing, and this is because antibiotics are not only used directly by humans but are also commonly given to animal food sources (WHO, 2014). The use of antibiotics in animal food sources increases livestock production. Doses of antibiotics given to farm animals to increase growth tend to be given under the recommended therapeutic dose. Antibiotic resistance arising from the inappropriate use of antibiotics in farm animals can cause the onset of antibiotic resistance in humans (Van et al., 2020).

Chicken is one of the largest food commodities in the world. Chicken meat is a dietary necessity for humans due to its high protein content (Wahyono and Utami, 2018). *Escherichia coli* is one of the most common bacteria in chicken meat. These bacteria are capable of being pathogenic both in chickens and in humans. Bacterial contamination in chicken meat can be transmitted to humans during production, packaging, sales, and when consumed (FAO, 2013). The high need for chicken meat makes this commodity one of the causes that have the potential to cause antibiotic resistance in the community (Uddin et al., 2019). Several studies conducted in Indonesia have demonstrated the presence of high levels of multidrug resistance (MDR) in *E. coli* isolated from poultry. The MDR rates observed ranged from 45% to 92.7% across all samples tested (Wibawati, 2023; Febrilianti et al., 2024; Sirindon et al., 2024).

Jember Regency has become one of the areas with a considerable poultry population in East Java, Indonesia. According to data from the Central Statistics Agency of East Java, in 2016, the poultry population in Jember Regency reached more than 14 million. The poultry population in Jember Regency is dominated by broilers, with a population of

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more than 11 million (JATIM, 2016). Multidrug resistance is a condition in which a bacterium is resistant to at least one type of antibiotic from ≥3 antibiotic groups (Sweeney et al., 2018). *Escherichia coli* can become resistant to antibiotics by producing enzymes to damage antibiotics and modify their metabolic processes. *Escherichia coli,* which has been resistant, can transfer resistant genes to other groups of enterobacteria (Poirel et al., 2018).

To determine further policies in dealing with the incidence of antibiotic resistance, monitoring and supervising the incidence of bacterial resistance in the food chain should be conducted (WHO, 2014). The objective of the study was to investigate the prevalence of multidrug-resistant *E. coli* in broiler chicken meat isolates in the Jember Regency.

MATERIALS AND METHODS

Ethical approval

The Medical Faculty of Ethics Commission at the University of Jember authorized this study with no objections (1562/H25.1.11/KE/2022).

Sample collection and transportation

The sample comprised 30 units of 25 grams of broiler raw chicken meat obtained from six traditional markets in the Jember Regency. The sampling locations were selected from a list of chicken markets supplied by the Indonesian Department of Veterinary Services. The multistage random selection method was employed to identify markets. Samples were taken from the market to the microbiology laboratory of the medical faculty of Jember University using sterile polythene bags to prevent contamination during transport.

Isolation and identification of *Escherichia coli*

A total of 25 grams of chicken meat from the upper thighs was extracted from each sample and soaked in 225 milliliters of sterile aquadest for 15 minutes. Once the soaking process was complete, the aquadest and chicken meat were blended until a homogenous mixture was achieved (Kaihena and Ferdinandus, 2009). A suspension of broiler chicken meat was plated onto Salmonella Chromogenic Agar media (Chromagar, Paris, France). By a streaking method. Suspension that has been planted on the medium is incubated for 24 hours at 37°C. The blue colony that grows in the media could be *E. coli* (Oktavianto et al., 2016). Blue colonies in *Salmonella* chromogenic agar media suspected to be *E. coli* bacteria are then implanted in Eosyn methylene blue (EMB) media (Merck KGaA, Darmstadt, Germany) for confirmation that the bacteria are accurate to be *E. coli*. Colonies are planted on EMB media using the streaking method and incubated for 24 hours at 37°C. In EMB media, *E. coli* grows as colonies with a green metallic sheen color (Lal and Cheeptham, 2016).

Bacterial culture

Before being planted on Mueller-Hinton media, colonies of *E. coli* were first cultured on nutrient agar media (Oxoid, UK). Planting on a densely tilted nutrient agar (NA) medium using the continuous strike method. After being planted on NA media, the bacterial colony is incubated for 24 hours at 37°C.

Antibiotic susceptibility testing

Antibiotic susceptibility tests are performed using the Kirby-Bauer method using Mueller-Hinton media (Oxoid, UK). Before planting on Mueller-Hinton (MH) media, colonies of *E. coli* in NA media are diluted first using aquadest with a concentration equal to a standard solution of 0.5 McFarland. The suspension of *E. coli* is further implanted on MH media using a sterile cotton swab. Mueller-Hinton media that contained *E. coli* were then implanted with antibiotic discs. Antibiotics to be tested in this study are tetracycline, amoxicillin-clavulanic, ceftriaxone, cefixime, cotrimoxazole, azithromycin, ciprofloxacin, and chloramphenicol. All antibiotics used are Oxoid (Oxoid, UK) brands that are under Clinical and Laboratory Standards Institute (CLSI) guidelines. In accordance with the CLSI guidelines, the Kirby-Bauer method for the detection of antibiotic inhibition zones is categorized into three distinct groups: sensitive, resistant, and intermediate (CLSI, 2020). Mueller-Hinton media is then incubated for 24 hours at a temperature of 37°C.

Observation

After incubation for 24 hours, the diameters of inhibition growth zones on MH media were observed and measured using a vernier caliper with an accuracy of 0.05 mm. The results of the diameters of inhibition growth zones are then interpreted based on guidance from CLSI. The data obtained on antibiotic resistance will then be collated to create the Multiple Antibiotic Resistance (MAR) index. The multiple antibiotic resistance index is calculated by dividing the number of resistant antibiotics in samples by the entire number of antibiotics tested in the study (Adzitey, 2018). Data obtained from subsequent resistance tests are categorized into MDR criteria.

Statistical analysis

The evaluation's post-incubation results indicated the presence of inhibition zones, categorized as susceptible, intermediate, and resistant, in accordance with CLSI guidelines. The data analysis in the current study was conducted descriptively (Febrilianti et al., 2024).

RESULTS

The findings of the current investigation revealed that 30 out of 30 samples (100%) tested positive for *E. coli* from six traditional markets in Jember. The antimicrobial resistance and susceptibility profiles of the examined *E. coli* isolates against eight chosen antimicrobial drugs were assessed using the agar disc diffusion method, with the findings presented in Table 1. All isolates (100%) have shown resistance to two antimicrobial agents: cotrimoxazole and cefixime, 96.67% resistant to chloramphenicol and amoxicillin-clavulanic, 93.3% resistant to tetracycline, 90% resistant to ceftriaxone, and 80% resistant to azithromycin and ciprofloxacin. Most *E. coli* isolates exhibited numerous drug profiles (≥3 antimicrobial classes) and a MAR index of ≥0.2 (Table 2). Moreover, all *E. coli* isolates exhibited resistance to four and five distinct classes of antibiotics, respectively (Table 3).

Table 1. Antibiotic resistance profile of 30 isolated *E. coli* from meat samples in Jember, Indonesia, 2022

Antibiotic	Sensitive		Intermediates		Resistant	
	n	%	n	%	n	%
Azithromycin	6	20%	0	0.00%	24	80%
Cotrimoxazole	0	0.00%	0	0.00%	30	100.00%
Ciprofloxacin	3	10%	3	10%	24	80%%
Chloramphenicol	1	3.33%	0	2.22%	29	96.67%
Cefixime	0	0.00%	0	0.00%	30	100,00%
Tetracycline	0	0.00%	2	6.67%	28	93.33%
Amoxicillin-clavulanic	1	3.33%	0	0.00%	29	96.67%
Ceftriaxone	2	6.67%	1	3.33%	27	90%

Table 2. Multiple antibiotic resistance index of isolated *E. coli* from meat samples in Jember, Indonesia, 2022

Sample code	Resistant antibiotics	Number of resistant antibiotics	MAR index
D1	AZMCXTCIPCCFMTAMCCRO	8	1
D2	AZMCXTCIPCCFMTAMCCRO	8	1
D3	AZMCXTCIPCCFMTAMCCRO	8	1
D4	AZMCXTCIPCCFMTAMCCRO	8	1
D5	AZMCXTCIPCCFMTAMCCRO	8	1
E1	AZMCXTCCFMAMCCRO	6	0.75
E2	AZMCXTCIPCCFMTAMCCRO	8	1
E3	AZMCXTCIPCCFMTAMCCRO	8	1
E4	AZMCXTCIPCCFMTAMCCRO	8	1
E5	AZMCXTCIPCCFMT	6	0.75
F1	AZMCXTCCFMTAMCCRO	7	0.875
F2	AZMCXTCIPCCFMTAMCCRO	8	1
F3	AZMCXTCIPCCFMTAMCCRO	8	1
F4	AZMCXTCIPCCFMTAMCCRO	8	1
F5	CXTCIPCCFMTAMCCRO	7	0.875
G1	AZMCXTCIPCCFMTAMCCRO	8	1
G2	CXTCIPCCFMTAMCCRO	7	0.875
G3	AZMCXTCIPCCFMTAMCCRO	8	1
G4	AZMCXTCCFMTAMC	6	0.75
G5	AZMCXTCIPCCFMTAMCCRO	8	1
H1	CXTCIPCCFMTAMCCRO	7	0.875
H2	CXTCIPCCFMTAMCCRO	7	0.875
H3	AZMCXTCCFMTAMCCRO	7	0.875
H4	AZMCXTCIPCCFMTAMCCRO	8	1
H5	CXTCIPCCFMTAMCCRO	7	0.875
I1	AZMCXTCIPCCFMAMC	6	0.75
I2	AZMCXTCIPCCFMTAMCCRO	8	1
I3	CXTSFMTAMCCRO	5	0.625
I4	AZMCXTCIPCCFMTAMCCRO	8	1
I5	AZMCXTCIPCCFMTAMCCRO	8	1

MAR: Multiple antibiotic resistance, AZM: Azithromycin, CXT: Cotrimoxazole, CIP: Ciprofloxacin, C: Chloramphenicol, CFM: Cefixime, T: Tetracycline, AMC: Amoxicillin-clavulanic, CRO: Ceftriaxone.

Table 3. Classification of isolated *E. coli* from chicken meat samples by multi-drug resistance category

Sample code	Number of resistant antibiotics	MDR category
D1	8	MDR
D2	8	MDR
D3	8	MDR
D4	8	MDR
D5	8	MDR
E1	6	MDR
E2	8	MDR
E3	8	MDR
E4	8	MDR
E5	6	MDR
F1	7	MDR
F2	8	MDR
F3	8	MDR
F4	8	MDR
F5	7	MDR
G1	8	MDR
G2	7	MDR
G3	8	MDR
G4	6	MDR
G5	8	MDR
H1	7	MDR
H2	7	MDR
H3	7	MDR
H4	8	MDR
H5	7	MDR
I1	6	MDR
I2	8	MDR
I3	6	MDR
I4	8	MDR
I5	8	MDR

MDR: Multiple-drug resistance.

DISCUSSION

This study found that 100% of the 30 samples tested positive were contaminated by *E. coli*. The contamination of *E. coli* in this study tended to be higher than previous studies also conducted in Jember. In that study, *E. coli* contamination from 6 tested was 66.67% (Putri et al., 2018). The results of *E. coli* contamination in this study are aligned with other studies conducted in Pringsewu Regency in 2015. The study revealed that bacterial contamination was present in 100% of the samples tested, despite the colony-forming unit remaining low (Utari et al., 2016). *Escherichia coli* bacteria are generally present in the digestive tract of chickens (Mourand et al., 2020). Contamination of *E. coli* in chicken meat occurs most during the chicken slaughter process. During the slaughter process, the ruptured digestive tract causes *E. coli* initially present in the digestive tract to contaminate chicken meat (FAO, 2013). The variability in *E. coli* recovery rates noted in this study may be associated with farm biosecurity protocols and the hygiene practices of personnel. Farm hygiene is essential for minimizing pathogen infection. This study corroborates the report by Olopade et al. (2022) which indicated that animal handlers and equipment contributed to the ongoing contamination of the farm environment (Olopade et al., 2022).

In chicken meat, contamination of *E. coli* can also occur during transportation or processing. *Escherichia coli* contained in chicken meat has the potential to be able to transmit to the human body and cause serious public health problems. The emergence of drug-resistant *E. coli* has led to the accumulation of resistance genes in both human and animal populations. This phenomenon has the potential to complicate the management of infections caused by *E. coli*, which are often amenable to antibiotic treatment. The resultant challenges can impose a significant financial burden on patients (Mensah et al., 2022). The significant prevalence of potentially clinically important *E. coli* identified in this study is unsurprising, given *E. coli* is a prominent member of the Enterobacteriaceae family known to inhabit the gastrointestinal tract of chickens. Moreover, *E. coli* serves as a recognized indicator organism for investigating the dissemination of antibiotic-resistance genes (Al Azad et al., 2019).

In this study, the level of resistance of *E. coli* to macrolide (azithromycin), tetracycline, and cotrimoxazole was higher than in previous studies conducted in Blitar City, Indonesia. That study found resistance levels at 73.9% in macrolide, 45.8% in tetracycline, and 67% in cotrimoxazole (Wibisono et al., 2020). Alternative investigations presented disparate data, with the research conducted in Bangladesh. The resistance levels to tetracycline, cotrimoxazole, and ciprofloxacin were 100% (Al Azad et al., 2019). The level of resistance to the antibiotic chloramphenicol found in this study was lower than in other studies conducted in India. In that study, *E. coli* resistance to chloramphenicol was 100% (Joshi et al., 2012). The level of resistance to ceftriaxone in this study was also higher when compared to previous studies conducted in Bangladesh in 2019. The study obtained a level of resistance to chloramphenicol of 56.67% (Sarker et al., 2019). The level of resistance to amoxicillin-clavulanic antibiotics in this study was also higher when compared to previous studies conducted in Algeria by 43.3% (Halfaoui et al., 2017). The difference in the prevalence of *E. coli* resistance to antibiotics between regions and countries can be attributed to a multitude of factors. The educational background of farmers is a factor that influences the prevalence of antibiotic resistance in *E. coli* strains isolated from broiler chickens. *Escherichia coli* isolates from livestock owned by farmers with higher levels of education tend to demonstrate lower levels of antibiotic resistance (Wibisono et al., 2020). Poor sanitation in livestock areas is also one factor that affects the level of resistance of *E. coli* to antibiotics. Other factors that can affect the level of antibiotic resistance include the absence of antibiotic delivery programs, the type of feed used, and the absence of support from veterinarians during the process of animal management (Wibisono et al., 2020).

The prevalence of MDR-*E.coli* obtained in this study was 100%. These results tend to be higher than other studies that have been done before in Blitar City in 2019. In that study, the prevalence of MDR in broiler chickens was 83.75% (Wibisono et al., 2020). The prevalence of MDR *E. coli* from broiler chickens in Jember Regency had similar results as a study conducted in Bangladesh in 2019. In the study, the prevalence of *E. coli* is MDR by 100% (Al Azad et al., 2019). A series of studies conducted in Portugal between 2014 and 2019 revealed a prevalence of MDR *E. coli* from food-producing animals in the country ranging from 70% to 90% (Costa et al., 2022). The prevalence of MDR-*E. coli* in broiler chickens varies considerably across different regions and countries, and this variation is shaped by several different factors. Factors that can affect the prevalence rate of MDR in a region include the diversity of poultry production systems, geographical conditions, the type of antibiotics used, and government policies against the use of antibiotics in the livestock sector (Bywater et al., 2004; WHO, 2014).

Bacterial resistance in broiler chicken meat can occur due to improper antibiotics in the livestock sector. The use of antibiotics on livestock is used not only to prevent the spread of infection in livestock animals but also as a growth trigger. Antibiotics used to trigger growth are usually given at doses below the therapeutic dose (Mehdi et al., 2018). Administration with doses under therapy continuously will cause the accumulation of antibiotic residue in chicken meat. This causes bacteria that contaminate chicken meat to be exposed to low doses of antibiotics for a long time, which will

cause bacterial resistance to antibiotics (Mund et al., 2017).

Resistance to *E. coli* arising from improper use of antibiotics on livestock can affect general health conditions. Elevated resistance to antibiotics may result from the frequent consumption of animal products containing substantial antibiotic residues. Antibiotic resistance arises from the transfer of plasmids from resistant bacteria to susceptible bacteria, occurring when initially susceptible bacteria are exposed to antibiotics. This antibiotic is frequently utilized within the community and as an addition to animal feed to stimulate growth. This is a contributing factor to antibiotic resistance in Indonesia (Wibisono et al., 2020).

Infections in humans caused by resistant *E. coli* will be harder to cure (FAO, 2013). Antibiotics commonly used as therapeutic options against bacteria become less efficient (Mund et al., 2017). The inappropriate use of antibiotics in animal livestock has been identified as a contributing factor to the rise in antibiotic resistance. The utilization of antibiotics as food additives, particularly in low and middle-income countries, has been documented as a prevalent practice. To prevent the increasing bacterial resistance to antibiotics, it is necessary to establish policies from the government to regulate and monitor the inappropriate use of antibiotics in society. In addition to preventing the transmission of *E. coli* that have been antibiotic-resistant to the human body, broiler chicken meat should be cooked first at a temperature above 100°C before consumption (Adeyanju and Ishola, 2014; Ajayi et al., 2024).

CONCLUSION

The findings of this study indicate the presence of antibiotic resistance in *E. coli* isolates from broiler chicken meat in Jember City. All isolates exhibited 100% resistance to cotrimoxazole and cefixime, with high levels of resistance to other antibiotics. The minimum resistance profile was to 5 types of antibiotics with a MAR index between 0.625 and 1. All isolated *E. coli* from broiler chickens in the study were multidrug-resistant. Thus, the study revealed a high risk of infection associated with the consumption of chicken meat. Consequently, more molecular investigations employing random amplified polymorphic DNA analysis are advised to elucidate the clonal relationships of MDR-*E. coli* isolates from both animal and human sources.

DECLARATIONS

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Authors' contributions

Enny Suswati served as the lead investigator and was the catalyst for the study's conceptualization. Dava Rizky Pratama engaged in data collection, conducted the analysis of the data, and composed and finished the text for publication. Bagus Hermansyah contributed to the data analysis and played a crucial role in developing and finalizing the text for publication. All authors have reviewed and endorsed the final version of the study for publication in the current journal.

Competing interests

The authors declare that there is no conflict of interest.

Ethical consideration

This document was initially authored by the individuals responsible. The authors were not submitting this paper to any other journal or publisher.

Availability of data and materials

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

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In Vivo Evaluation of a Polyethylene Glycol-Based Cryoprotectant during Cold Stress in a Rat Model

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ABSTRACT

Cold stress is an environmental factor that impacts the viability of animals and humans. This study aimed to determine the effectiveness of a cryoprotectant based on polyethylene glycol in reducing cold stress in laboratory rats. For the experiment, 30 outbred Wistar rats (5 weeks) with an average body weight of 55.1 ± 5.3 g were used. Three groups of animals were formed (10 rats per group). The first group served as a positive control, kept at a room temperature of +18 – +20 °C and received 0.1 ml of 0.9% NaCl solution. The second group, the negative control, was kept in critically low temperatures (+2–+4°C) and administered 0.1 ml of 0.9% NaCl solution per experimental animal. Rats of the third group were subjected to cold stress and received 0.1 ml of the experimental preparation. Cold stress in laboratory rats was created using a cooling thermostat. Observation for 14 days included monitoring the dynamics of changes in the live weight of animals (before the start of the experiment and on days 7 and 14 of the observation) as well as biochemical and haematological blood indicators. Fecal samples were collected from the rectum to determine the qualitative and quantitative state of the intestinal microbiota. The survival level of animals that received the experimental drug within fourteen days was 80.0%, compared to only 40% in the untreated group. When using the experimental drug in laboratory animals, an increase in body weight was noted. The number of full-fledged *Escherichia coli* in rats that received the drug was 3.4 times higher than the indicator of the group of animals that was kept at a critically low temperature without the drug. The prolonged low temperature in control rats had a negative effect on the animal's body as evidenced by increased leukocyte counts and ALT levels, as well as decreased ALT/AST ratio and total bilirubin. The use of an experimental polyethylene glycol-based preparation had a positive effect on the weight of rats, blood parameters, and intestinal microbiota of rats under cold stress.

Keywords: Biochemistry, Blood morphology, Cold stress, Cryoprotectant, Intestinal microbiota, Rat

INTRODUCTION

Cold stress occurs when the ambient temperature falls below optimal levels. Symptoms of cold stress include loss of coordination, difficulty maintaining balance, slowed heart rate and breathing, loss of consciousness, and, in extreme cases, death. Environmental temperature is an important abiotic factor that influences the adaptation processes of animals and determines the composition and structure of their communities. In addition, temperature changes affect the composition and function of the gut microbiota, an important regulator of host physiological processes. These effects can have significant consequences for the ability of populations to adapt to climate changes (Berg et al., 2016; Bestion et al., 2017). Studies conducted on various animal taxa, such as chordates, arthropods, and Mollusca, confirm stable relationships between temperature, community composition, and functional characteristics of the gut microbiota (Barbian et al., 2015; Carey and Assadi-Porter, 2017; Hammer et al., 2019). The intestinal microbiota of many animals is closely linked to the metabolic, immune, and neuroendocrine systems of the host organism, ensuring their mutual interaction and regulation (Kau et al., 2011; Chevalier et al., 2015). For instance, in mammals and insects, germ-free (axenic) organisms of some species exhibit a number of phenotypic differences compared to those with gut microbiota (Neufeld et al., 2010; Ridley et al., 2012; Li et al., 2019). Experiments transplanting gut microbiota into axenic organisms have demonstrated that changes in microbiota composition can lead to phenotypic changes in hosts (Faith et al., 2014; Gould et al., 2018; Fontaine et al., 2018). Researchers suggest that changes in host phenotype caused by gut microbiota contribute to the evolution of host populations (Chevalier et al., 2015; Rudman et al., 2019) and species (Moeller et al., 2019). The presence of certain microorganisms in the gut is believed to play a key role in maintaining the host's fitness to its environment (Moeller et al., 2019).

The low thermal tolerance of metazoans, compared to unicellular eukaryotes and bacteria, is attributed to a complex systemic process. Aerobic processes in animals are the first to be affected by low and high temperatures, which is associated with impaired circulation and ventilation (Zare et al., 2018; Zhang et al., 2019). Oxygen levels in body fluids may decrease, reflecting either

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increased oxygen demand at high temperatures or inadequate mitochondrial aerobic capacity at low temperatures. Aerobic capacity decreases at temperatures beyond the thermal optimum and ceases at extreme temperatures when the transition to anaerobic mitochondrial metabolism occurs. Changes in mitochondrial density, along with molecular and membrane changes, play a key role in maintaining aerobic capacity and adapting to changes (Smits et al., 2017; Orkin et al., 2019). Oxygen delivery capacity is sufficient to meet all aerobic capabilities only within the thermal optimum. Beyond this range, survival is possible only through time-limited aerobic metabolism, and later through anaerobic metabolism, molecular protection by heat shock proteins, and antioxidant activity. In the hierarchy of causes and effects, the progressive reduction in oxygen availability at extreme temperatures can lead to increased oxidative stress and protein denaturation. Thus, limitations in the efficiency of the oxygen delivery system at a complex level of organization define the limits of thermal stability, which can lead to disruption of molecular functions (Pörtner, 2001; Zhu et al., 2019). Some adaptations, such as the synthesis of low-molecular-weight cryoprotectants that have more specific mechanisms of action, provide direct stabilizing effects on membranes and proteins. The mechanisms used by animals offer insights and alternative approaches that can be effectively applied in the cryopreservation of cells and tissues, a critical requirement in reproductive technologies (Horváthová et al., 2019; Carnaghi et al., 2021; Kolchyk et al., 2024).

Low temperatures adversely affect physical health, causing both general hypothermia and local damage. Among local manifestations, the peripheral areas of the human body are especially vulnerable - feet, hands, and ears. This can lead to cold injuries such as frostbite and trench foot (Heil et al., 2016; Zaneveld et al., 2017; Kokornaczyk, 2021). Preventing and treating such injuries allows one to maintain combat effectiveness and complete the assigned mission during difficult conditions (Sullivan-Kwantes et al., 2021; Plavina, 2023). The ongoing full-scale invasion of Ukraine by Russia has further highlighted the importance of research in this area. Searching for cryoprotectants to prevent and treat hypothermia in humans and animals is a pressing issue. Unfortunately, there is limited data on the effects of polyethylene glycol on cold stress in different animal species. A review of the literature revealed that polyethylene glycol is used in medicine and animal husbandry without adverse effects (Mansoori and Modirsanei, 2011; Lyseng-Williamson, 2018; Wang et al., 2023).

The present study aimed to evaluate the effectiveness of a cryoprotectant based on polyethylene glycol for the correction of cold stress in laboratory animals (rats) under low-temperature conditions (+2–4 °C).

MATERIALS AND METHODS

Ethical approval

The experiment was approved by the Commission for Bioethical Expertise of the Dnipro State Agrarian and Economic University. Experimental studies on laboratory rats were conducted in compliance with the principles of the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes (WorldLII, 1986).

Study design

The study aimed at investigating the effectiveness of an experimental preparation based on polyethylene glycol as a cryoprotectant for the treatment and prevention of hypothermia in animals. The research was carried out in the laboratory of the Department of Animal Infectious Diseases and the Biosafety Center of the Dnipro State Agrarian and Economic University, Ukraine. In the vivarium (36 m^2) where the animals were housed, the temperature was maintained at 23°C, humidity at 22%, and lighting was controlled on a 12-hour light/dark cycle (lights on at 07:30). Laboratory rats were kept in cages with 350 cm^2 of floor space per animal. They were fed CARE+ Rat super-premium complete food (energy value 3000 kcal/kg, protein 14%, Beaphar, the Netherlands), and tap water was provided *ad libitum*.

For the experiment, 30 male outbred Wistar rats (age 5 weeks) with an average body weight of 55.1 ± 5.3 g were used. The animals were obtained from the central vivarium of the Dnipropetrovsk Regional State Laboratory of the State Service of Ukraine for Food Safety and Consumer Protection, Ukraine. Before the experiment, laboratory animals were trained for about five days. To do so, the rats were placed in heat-resistant glass boxes and received food rewards from the experimenter for 10-20 minutes per day. During this time, the temperature of the animals was measured five times using a rectal thermometer. The thermometer was lubricated with petroleum jelly and inserted 2 cm from the anal sphincter. This criterion was considered fulfilled if two standard deviations from the average initial temperature (37.5 ± 0.5°C) were determined when the first two measurements were within 0.3-0.5°C of each other (Klune et al., 2020).

The animals were randomly assigned to three groups (10 rats each). The first group, which was a positive control (I, K+) kept at a room temperature of +18- +20 °C, received 0.1 ml of 0.9% NaCl solution. The second group was a negative control (II, K-) kept in critically low temperatures (+2-+4°C). They received 0.1 ml of 0.9% NaCl solution per experimental animal. Rats of the third group (III, experimental drug) were under cold stress and received experimental preparation of one concentration (2 drops [0.1 ml] per experimental animal) via forced oral administration. Cold stress in laboratory rats was created using a cooling thermostat (TSO-80 MIKROmed, 2020, Ukraine). To determine the dynamics of rat mass in experimental research models, mathematical calculations of linear dynamics were used, which take into account the rate of mass gain or loss depending on time (Brygadyrenko et al., 2019).

The experimental preparation

The experimental preparation consisted of the following components included ionol - 25.0 g/l, dimethyl sulfoxide - 37.5 g/l, polyethylene glycol PEG 400 - 230.0 g/l, PEG 1500 - 540.0 g/l in the form of a soluble drug with a gel-like consistency. This composition of the experimental drug has already been successfully used in previous studies (Zazharskyi et al., 2024a).

Morpho-biochemical analyses

Observation for a period of 14 days included monitoring changes in the live weight of animals (before the start of the experiment, and on 7 and 14 days of observation), as well as the biochemical and haematological indicators of blood. Blood samples (up to 0.5 ml) were collected from the tail vein in Eppendorf microtubes during decapitation for biochemical and morphological analyses. The number of erythrocytes and leukocytes in stabilized rat blood was determined using an automatic hematology analyzer (BC-2800Vet, Mindray, China). To calculate the leukogram, blood smears were prepared according to the Pappenheim method. The biochemical indicators of blood were examined by photometric and spectrophotometric methods with photometersMicrolab-200, (International Microlab, Shenzhen, China, 2021) and Vitalab Eclipse (Merck, the Netherlands, 2011). Analyses were performed using the respective software after setting the reaction with appropriate diagnostic test kits from Lachema (Erba Lachema, Karásek, the Czech Republic, 2021).

Study of microbiota

At the end of the experiment, animals that survived were euthanized via carbon dioxide inhalation (Qin and Meng, 2006). Fecal samples were collected from the rectum after the death of the animal to determine the qualitative and quantitative composition of the intestinal microbiota, taking into account the parietal microflora and the microflora of the intestinal lumen. Fecal samples (1 g) were collected in a sterile container by cutting the rectum following the rules of asepsis, after which serial dilutions in physiological solution (1:9) to 10^{-11} were carried out (Kaminska, 2015).

From each dilution tube, 1 ml of suspension was taken with a sterile pipette and introduced into selective nutrient media (bifidum medium [Himedia, India]), lactobact agar, enterococcus agar, Endo agar, bismuth sulfite agar, Wilson-Blair agar, Byrd-Parker agar, and Saburo agar (Himedia, India). It was then rubbed on the surface with a sterile spatula. Most representatives of the intestinal microflora (cocci, enterobacteria) are able to grow on nutrient media for 24 hours, but some microorganisms (staphylococci, enterococci, bifidobacteria, clostridia, yeast-like fungi) are cultured for 48 hours and sometimes even 72 hours. Cultivation was carried out for 24-72 hours at 24-43 °C (mesophilic aerobic and facultative anaerobic microorganisms were cultivated at a temperature of 37 °C, yeast - 24-28-30 °C, hemolytic Escherichia coli - 42-44 °C). Anaerobic bacilli were isolated using GENbox anaer bags (Biomerieux, France). Anaerobic conditions were monitored using the Anaer indicator (Biomerieux, France). The number of viable microorganisms was counted in Petri dishes and expressed in CFU/g (colony-forming units in 1 g of intestinal contents). Morphological signs and tinctorial properties of the selected microorganisms were studied after staining smears according to the Gram and Romanovsky-Giemsa methods under the immersion microscope system MICROmedXS-3330 (Ukraine). Differentiation was carried out by studying their biochemical properties on Hiss media with various sugars, Olkenytskyi, Christensen, Simmons, and malonate agar (Pharmaktiv, Ukraine). Identification was carried out taking into account their biological properties according to Bergey's identifier of bacteria.

Statistical analysis

The collected data were compared using ANOVA with the Statistica 6.0 package (StatSoft Inc., USA). Data are presented as mean ± standard deviation ($x \pm SD$). Differences between values in the groups were determined using the Tukey test, where the differences were considered significant at $p < 0.05$ (subject to the Bonferroni Amendment).

RESULTS

During the experiment, the number of dead rats in groups II and III was compared during the period of 7 and 14 days of observation (Table 1). In the experimental drug group (III), two animals died during the first seven days, while in the group of animals that were in critically low-temperature conditions without treatment (II), the death of four animals was detected in the first week of observation, and two more animals died in the following week.

The dynamics of rat weight during the observation period indicated the inhibition of animals under the influence of cold stress (Table 2). After 7 and 14 days of the experiment, the weight of rats in group II (cold maintenance without treatment) lagged behind the indicators of the comfort group (I) by 26.0% ($p < 0.05$) and 24.0% ($p < 0.05$), respectively. In the experimental drug group (III), the weight lag was 15.5% and 6.2%. However, a positive dynamic weight gain was noted in animals that were administered the experimental drug.

Cold stress over 14 days in laboratory rats influenced the morpho-biochemical indicators of blood, as presented in Table 3. A decrease in the level of globulin in animals of group II by 6.8% was revealed, which may be a result of cold stress. In contrast, when the experimental drug was used (group III), the level of urea increased by 38.6% and 34.0% compared to animals of the control groups I and II ($p < 0.05$). It was found that the creatinine level in the group exposed to the experimental drug was within the comfort group of 50.2 μmol/L. In group II (K-), creatinine was 16.1% higher than in the control group (K+), which may indicate acute renal failure. However, a sharp decrease in the level of total bilirubin in animals in the experimental group II by 9.6 times is noticed compared to rats of the control group I ($p < 0.05$).

It was established that the experimental drug does not have a negative effect on the level of AST or ALT. These indicators are within the limits of the comfort group (I, K+). There was an increase in the ALT level (252.3 U/L; $p < 0.05$) with a decrease in the ALT/AST ratio (1.0 unit; $p < 0.05$) in animals of group K- compared to comfort group K+. An increase in amylase activity was observed in animals of group III (experimental drug) by 10.2% compared to the comfort group. Notably, glucose levels were elevated in Groups II and III by 3.9-fold and 2.1-fold, respectively, compared to Group I ($p < 0.05$). The authors of the current study consider the positive dynamics of lowering the cholesterol level in animals of group III (experimental drug) to groups K+ and K- by 40.7% ($p < 0.05$) and 23.8%, respectively. An increase in the number of leukocytes in animals of the K- K-group was found in comparison with comfort (I) and group III by 3.6 and 2.2 ($p < 0.05$) due to an increase in the level of neutrophils with segmented nuclei by 50.0% and 22.4%, respectively.

It was established that the number of obligate and facultative microorganisms of genera *Bifidobacterium* and *Lactobacillus* during exposure to cold stress in both of the groups of animals with and without the drug decreased by half or more compared to control animals that were kept under comfortable conditions, but no statistical difference was detected (Table 4).

The total number of full-fledged *Escherichia coli* in rats exposed to cold conditions decreased by 19.7 times compared to those kept in comfortable conditions ($p < 0.05$). Despite the fact that there was a decrease in the total number of full-fledged *Escherichia coli* (by 5.9 times) in the group of animals that were injected with the drug and kept in the cold, compared to the group that was in comfortable conditions ($p < 0.05$), this indicator was 3.4 times higher ($p < 0.05$) than the rate in animals that were kept in cold conditions without the drug.

The number of lactose-negative *Escherichia coli* when rats were kept in cold conditions increased 6.2 times compared to animals under comfortable conditions ($p < 0.05$) and 2.3 times compared to animals receiving the drug ($p < 0.05$). During the experiment, bacteria of the genus *Citrobacter* were not detected in any group. As for other representatives of the intestinal microbiota of rats, the number of *Enterobacter spp., Proteus spp.*, and fungi of the genus *Candida* decreased under the influence of cold in both experimental groups, but no statistical difference between the indicators was found ($p > 0.05$). On the contrary, the number of *Escherichia coli* with altered enzymatic properties and lactose-negative *E. coli*, *Enterococcus spp.*, *Clostridium spp.*, *Klebsiella spp*., as well as *Staphylococcus epidermidis*, and *Staphylococcus aureus*, increased during the experiment in cold conditions, yet no statistical difference between the indicators was detected ($p > 0.05$).

Table 1. Survival of rats during the cold stress administered with cryoprotectant based on polyethylene glycol (n=10)

Observation period (day)	Number of live animals / (%) in groups		
	I*	II*	III*
1	10/100.0	10/100.0	10/100.0
7	10/100.0	6/60.0	8/80.0
14	10/100.0	4/40.0	8/80.0

* I: The first group, positive control (I, K+), was in comfortable conditions at a room temperature of +18 – +20 °C and received 0.1 ml of 0.9% NaCl solution per rat; II: The second group, negative control (II, K-), was in critically low temperatures (+2–+4°C) and received 0.1 ml of 0.9% NaCl solution per rat; III: The third group (III experimental drug) was critically in low temperatures (+2.0-+4.0°C) and were administered the experimental preparation.

Table 2. Dynamics of rat weight (g ± SD) in groups during the observation period (14 days) administered with cryoprotectant based on polyethylene glycol

Groups / Observation period (day)	I*	II*	III*
1	54.2 ± 4.7	55.1 ± 5.3	55.7 ± 4.2
7	66.9 ± 4.1^{a}	49.5 ± 3.7^{b}	56.5 ± 5.3^{ab}
14	76.4 ± 4.7^{a}	58.0 ± 4.2^{b}	71.1 ± 6.5^{ab}

Note: ^{ab} Different letters indicate selections that significantly ($p < 0.05$) within the row differ from each other. * I – The first group, positive control (I, K+), was in comfortable conditions at a room temperature of +18 – +20 °C and received 0.1 ml of 0.9% NaCl solution per rat; II – The second group, negative control (II, K-), was in critically low temperatures (+2–+4°C), receiving 0.1 ml of 0.9% NaCl solution per rat; III – The third group (III experimental drug) was critically in low temperatures (+2.0-+4.0°C), administered with the experimental preparation.

Table 3. Changes in blood biochemical parameters (mean ± SD) of rats at a critically low temperature during 14 days of the experiment (administering a cryoprotectant based on polyethylene glycol)

Parameters	Groups		
	I*	II*	III*
Total protein, (g/L)	67.2 ± 7.4	63.1 ± 6.9	66.2 ± 8.7
Albumins, (g/L)	29.1 ± 3.4	28.6 ± 2.7	30.2 ± 3.2
Globulins, (g/L)	38.2 ± 3.9	35.6 ± 3.7	36.1 ± 4.2
The albumin-globulin ratio, (units)	0.8 ± 0.1	0.8 ± 0.2	0.8 ± 0.1
Urea, (mmol/L)	8.8 ± 0.2^{a}	9.1 ± 0.4^{a}	12.2 ± 0.3^{b}
Blood urea nitrogen, (mg/100 g)	16.8 ± 2.3	17.4 ± 1.6	23.3 ± 2.5
Creatinine, (μmol/L)	50.3 ± 6.4	58.4 ± 5.9	50.2 ± 5.1
Aspartate aminotransferase (AST), (U/L)	171.3 ± 22.7	240.1 ± 19.8	256.6 ± 23.6
Alanine aminotransferase (ALT), (U/L)	68.1 ± 5.7^{a}	252.3 ± 21.9^{b}	97.4 ± 8.7^{a}
De Ritis Index (AST/ALT), (units)	2.5 ± 0.1^{a}	1.0 ± 0.1^{b}	2.6 ± 0.2^{a}
Alkaline phosphatase, (U/L)	305.6 ± 29.8^{a}	236.0 ± 31.7^{a}	181.4 ± 23.4^{b}
Alpha amylase, (U/L)	594.3 ± 48.6	624.5 ± 59.1	655.0 ± 57.3
Total bilirubin, (μmol/L)	10.6 ± 1.2^{a}	1.1 ± 0.2^{b}	7.4 ± 1.4^{a}
Glucose, (mmol/L)	2.1 ± 0.4^{a}	8.2 ± 1.3^{b}	4.4 ± 0.5^{c}
Calcium, (mmol/L)	2.3 ± 0.1	2.0 ± 0.2	2.5 ± 0.2
Phosphorus, (mmol/L)	4.3 ± 0.5	4.6 ± 0.3	3.9 ± 0.6
Ca/P, (units)	0.5 ± 0.1	0.4 ± 0.1	0.6 ± 0.1
Cholesterol, (mmol/L)	2.7 ± 0.1^{a}	2.1 ± 0.2^{ab}	1.6 ± 0.1^{b}
Gamma-glutamyl transferase (GGT), (U/L)	9.2 ± 1.8	6.3 ± 0.9	9.1 ± 1.2
Hemoglobin, (g/L)	106.4 ± 12.4	127.1 ± 24.2	128.7 ± 21.7
Hematocrit, (%)	23.5 ± 3.3	30.8 ± 4.6	28.2 ± 2.9
Erythrocytes, (10^{12}/L)	3.1 ± 0.5	3.7 ± 0.5	3.7 ± 0.2
MCV (mean corpuscular volume), (10^{-15} L)	75.8 ± 8.2	83.2 ± 9.3	76.2 ± 8.6
MCH (mean corpuscular haemoglobin), (10^{-12} g)	34.9 ± 4.8	34.3 ± 7.4	34.5 ± 5.1
MCHC (mean corpuscular haemoglobin concentration), (%)	45.1 ± 4.7	41.2 ± 3.8	45.9 ± 6.4
Color indicator, (units)	1.03 ± 0.02	1.03 ± 0.01	1.04 ± 0.01
Erythrocyte sedimentation rate (ESR), (mm/h)	1.1 ± 0.1	6.0 ± 0.2	1.0 ± 0.2
Platelets, (10^{9}/L)	366.4 ± 42.5	401.6 ± 37.7	454.2 ± 41.8
Leukocytes, (10^{9}/L)	3.2 ± 0.4^{a}	11.4 ± 2.2^{b}	5.3 ± 0.7^{a}
Leukocyte formula, (%):			
Basophils	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
Eosinophils	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
Lymphocytes	74.3 ± 5.4	64.1 ± 7.9	72.6 ± 8.4
Monocytes	9.2 ± 1.3	10.4 ± 2.2	7.7 ± 2.1
Myelocytes	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
Neutrophils:			
– young	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
– stick-core	1.0 ± 0.1	2.2 ± 0.3	1.2 ± 0.2
– segmented-nucleus	16.4 ± 2.1	24.6 ± 3.7	20.1 ± 2.9

Note: [abc] Different superscript letters indicate significant differences ($p < 0.05$) within each row. * I: The first group, positive control (I, K+), was in comfortable conditions at a room temperature of +18 – +20 °C, and received 0.1 ml of 0.9% NaCl solution per rat; II: The second group, negative control (II, K-), was in critically low temperatures (+2–+4°C), received 0.1 ml of 0.9% NaCl solution per rat; III: The third group (III experimental drug) was critically in low temperatures (+2.0-+4.0°C) with the use of experimental preparation.

Table 4. Qualitative and quantitative composition of the intestinal microbiota (lg 10 CFU/gram of feces) in groups of rats under cold stress (Mean ± SD) during 14 days of experiment administered with a cryoprotectant based on polyethylene glycol

Gut microbiota	I*	II*	III*
Bifidobacterium spp.	7.81 ± 7.34	3.20 ± 7.26	7.39 ± 7.28
Lactobacillus spp.	7.66 ± 7.34	4.40 ± 7.32	7.42 ± 7.32
E. coli (normal enzymatic properties strains)	7.65 ± 7.11[a]	6.35 ± 6.02[b]	6.88 ± 6.18[c]
E. coli (weakly fermenting strains)	2.90 ± 2.00	8.42 ± 4.28	4.88 ± 4.62
E. coli (lactose-negative strains)	2.41 ± 2.05[a]	7.45 ± 2.49[b]	2.73 ± 2.34[a]
Enterococcus spp.	4.64 ± 4.36	2.85 ± 6.51	6.0 ± 5.76
Enterobacter spp.	3.49 ± 3.17	5.93 ± 2.48	3.18 ± 2.68
Citrobacter spp.	0 ± 0	0 ± 0	0 ± 0
Klebsiella spp.	3.83 ± 3.23	3.92 ± 3.92	4.09 ± 3.73
Proteus spp.	3.45 ± 3.26	3.33 ± 3.02	3.35 ± 3.14
Staphylococcus epidermidis	3.40 ± 2.19	4.16 ± 3.19	2.97 ± 2.49
Staphylococcus aureus	2.85 ± 3.27	3.10 ± 3.59	4.15 ± 3.94
Clostridium spp.	4.39 ± 1.90	3.31 ± 2.89	3.45 ± 3.22
Candida spp.	2.30 ± 4.28	4.24 ± 3.58	4.08 ± 3.76

Note: [abc] Different superscript letters indicate significant differences ($p < 0.05$) within each row. * I: The first group, positive control (I, K+), was in comfortable conditions at a room temperature of +18 – +20 °C, and received 0.1 ml of 0.9% NaCl solution per rat; II: The second group, negative control (II, K-), was in critically low temperatures (+2–+4°C), received 0.1 ml of 0.9% NaCl solution per rat; III: The third group (III experimental drug) was critically in low temperatures (+2.0-+4.0°C), administered the experimental preparation.

DISCUSSION

Hypothermia is defined as a decrease in body temperature below 35°C (Brodeur et al., 2017). It is widely used for therapeutic purposes, including cardiopulmonary bypass, craniocerebral injuries, organ transplantation, and neonatal encephalopathy. It is classified as either primary (accidental) or secondary. Prolonged exposure of an animal with normal heat production in cold conditions leads to the development of primary hypothermia. Secondary hypothermia occurs when heat production and thermoregulation are disrupted due to diseases, injuries, or exposure to medications (Brodeur et al., 2017). Based on duration, hypothermia can be acute (several hours) or chronic (days or weeks) (Tveita and Sieck, 2022).

Cold stress is a significant environmental factor affecting animal viability and productivity (Hao and Wang, 2017; Worthmann et al., 2017; Zazharska et al., 2024). Scientists believe that long-term exposure of animals to low temperatures is closely associated with pathogens and infectious diseases that can cause harm to animal health and significant economic damage to livestock enterprises (Landin and Bonastre, 2018).

Despite extensive research on cold stress, no data were found on the effects of polyethylene glycol (PEG) under such conditions. Polyethylene glycol (PEG), through its properties as an osmotically active substance, creates osmotic pressure in the intercellular environment, retaining water inside the cells and preventing them from drying out (Mansoori and Modirsanei, 2011; Lyseng-Williamson, 2018; Wang et al., 2023). PEG acts as a membrane stabilizer, maintaining its liquid structure even at low temperatures. PEG is biocompatible and non-toxic, which makes it safe for use in plants, animals, and cell cultures (Barer, 2015).

PEG is a multifunctional polymer that has a wide range of applications, including its use as a food and cosmetic additive and as a carrier in PEGylated therapeutic agents (Ibrahim et al., 2022). It is administered orally to humans (Lyseng-Williamson, 2018). In Japan, for instance, treatment with PEG 3350 was well tolerated in patients with chronic constipation, which resulted in sustained improvements in bowel function (Nakajima et al., 2019). In another study, PEG was fed to chickens without negative effects (Mansoori and Modirsanei, 2011).

The authors of this study suggest that polyethylene glycol may mitigate cold stress due to its physicochemical properties, its role in maintaining the water balance of cells, its ability to preserve membrane structures, and its activation of protective mechanisms. Therefore, the use of the experimental drug in the present study ensured the survival of twice as many animals until the end of the experiment than in the group without the drug. Hematological and biochemical blood parameters are often the main diagnostic criteria (Zazharskyi et al., 2024a). Their fluctuations may indicate adverse effects of substances on organs and systems.

The number of leukocytes in the blood of animals under cold stress increased 3.6 times compared to the group at a comfortable

temperature. However, the use of cryoprotectant based on polyethylene glycol led to an increase in the number of leukocytes by only 1.7 times compared to the control group of rats (K+), where no statistical difference was found. A positive effect on blood components and a wound-healing action have already been reported in previous studies in the treatment of burns in laboratory rats by the experimental drug containing ionol - 25.0 g/l, dimethyl sulfoxide - 37.5 g/l, polyethylene glycol PEG 400 - 230.0 g/l, and PEG 1500 - 540.0 g/l. The protein concentration in the blood of guinea pigs receiving the experimental drug remained within physiological norms. Total bilirubin is a product of heme metabolism, which is part of hemoglobin in red blood cells and is responsible for transporting oxygen to tissues. After the destruction of red blood cells, bilirubin is released and goes to the liver, where it is metabolized and excreted from the body with bile. On the third day of burn treatment using the experimental drug, a significant decrease in bilirubin concentration by 65.2% ($P < 0.01$) was observed, which is probably due to a decrease in the level of intoxication of the body (Zazharskyi et al., 2024a).

Keeping animals in a cold environment (group K-) negatively affected many morpho-biochemical parameters of blood (Table 3). A sharp decrease in the level of total bilirubin in animals in experimental group II by 9.6 times may indicate irreversible pathological processes and the transition to chronic renal failure. There was an increase in the ALT level with a decrease in the ALT/AST ratio in animals of group K-, compared to comfort group K+, which is associated with signs of liver disease and myocardial dysfunction. The decrease in Ca loss in the blood of rats in the control group (K-) occurred by 15% compared to the comfort group. Research has documented various substances affecting blood parameters (Zazharskyi et al., 2024b; 2024c). For instance, the use of alcohol tincture of *Aralia elata* in rats leads to a decrease in the levels of creatinine, glucose, urea, cholesterol, bilirubin, and total calcium (Brygadyrenko et al., 2019).

In the present study, the level of total protein increases due to albumins, urea, and urea nitrogen in the blood of rats under cold stress when using an experimental preparation based on polyethylene glycol. After 14 days, alkaline phosphatase levels normalized to those of the comfort group. The level of Erythrocyte sedimentation rate (ESR) corresponds to the animals of the comfort group, with a significantly higher indicator in the K- group (6 times), which indicates the presence of an inflammatory process in the body of intact rats. Long-term use of low temperature in rats of the control group without treatment in the second week of the experiment led to a sharp decrease in the level of Gamma-glutamyl transferase (GGT), the enzyme which is localized in the cells of the liver and biliary tract and plays the role of a catalyst in specific biochemical reactions.

Environmental changes can impact animals by influencing the composition of their gut microbiota. Fluctuations in temperature can reduce the diversity of the microbiome, leading to a loss of key functions and potentially having negative consequences for the health and survival of animals (Fotina et al., 2018; Horváthová et al., 2019; Borovuk and Zazharska, 2022).

Bilan et al. (2019) found that co-exposure to glyphosate and common food additives had a significant impact on the gut microbiota composition of rats without changing the number of *Escherichia coli*, *Bifidobactrium*, and *Lactobacillus spp*. According to the present data, cold stress leads to a sharp decrease in these microorganisms. The number of lactose-negative *Escherichia coli* for the rats kept in cold conditions increased 6.2 times compared to animals under comfortable conditions and 2.3 times compared to animals receiving the drug.

Mixtures of glyphosate and food additives (saccharin and sodium benzoate) were found to allow microorganisms of the genera *Klebsiella*, *Enterobacter*, and *Pseudomonas*, as well as opportunistic yeast-like fungi *Candida* to spread more widely in the intestines of rats (Bilan et al., 2019). Based on the present findings, keeping rats at low temperatures contributes to a wider reproduction of the number of microorganisms of the genera *Klebsiella* and *Staphylococcus*, but to a decrease in the genera *Enterobacter* and *Candida* fungi.

In the present study, fluctuations in the number of probiotic strains of *Bifidobacteri*a and *Lactobacilli* (downward from the reference value of intestinal content after 14 days of the experiment) were observed in rats against the background of drug use. The number of *Escherichia coli* with normal enzymatic properties decreased due to an increase in the number of lactose-negative *Escherichia coli* and *Escherichia coli* with altered enzymatic properties, leading to fluctuations in the number of other *enterobacteria* and anaerobic *clostridia*.

CONCLUSION

The experimental cryoprotectant drug based on polyethylene glycol exhibits a markedly positive general biological effect on laboratory animals, resulting in a markedly higher survival rate of animals compared to the control group. The use of the experimental preparation has a positive effect on the morpho-biochemical parameters of the blood and intestinal microbiota of rats under cold stress. In the blood of rats exposed to cold stress using an experimental drug, the level of total protein increases due to albumin, urea, and urea nitrogen. Moreover, alkaline phosphatase normalizes to the level of the comfort group after 14 days of the experiment. The number of full-fledged *E. coli* in rats that received the drug was 3.4 times higher than in the group of animals kept in the cold without the drug. Future research can explore the effectiveness of polyethylene glycol-based cryoprotectants in combination with *Echinacea* extract during cold stress in laboratory animals.

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.

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.

Authors' contributions

Olexyi Zaslavskyi and Ivan Biben conceived and designed the experiment, and Olexandr Sosnickyi and Volodymyr Zazharskyi conducted experiments on animals. Marina Bilan conducted bacteriological studies. Volodymyr Zazharskyi and Nadiia Zazharska analyzed the obtained results, interpreted and wrote the manuscript, and edited and reviewed the article. All authors have read and approved the final draft of the manuscript for publication in the journal.

Conflicts of interests

The authors declare that there is no conflict of interest.

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The Effect of Kepok Banana (*Musa paradisiaca*) Peel Extract on Macroscopic and Histopathological Features of Excision Wound Healing in Mice Skin

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ABSTRACT

Kepok banana peel extract is known to have a bioactive content that can accelerate wound healing. The present study sought to evaluate the effects of Kepok banana peel extract on the macroscopic and histopathological features of excision wound healing in mouse skin. A total of 24 BALB/c mice were divided into four treatment groups, with each group consisting of six mice. The mice were further divided into three subgroups based on observation days, including days 3, 6, and 9. Each mouse received two excision wounds. The four treatment groups included K1 (control), K2 (topical therapy using Kepok banana peel extract ointment 5%), K3 (topical therapy using Kepok banana peel extract ointment 10%), and K4 (topical therapy using Kepok banana peel extract ointment 15%). The Kepok banana peel extract was obtained using the maceration method, and the ointment Kepok was prepared as a cream with extract concentrations of 5%, 10%, and 15%, using bio cream as the base. Wound healing activity was evaluated across three phases, including inflammatory, proliferative, and remodeling. The parameters observed in the current study included macroscopic and histopathological characteristics of the wound. Macroscopic observations involved wound size, while histopathological analysis included quantification of inflammatory cells, fibrocytes, collagen density, and interleukin-6 expression. Therapy using Kepok banana peel extract ointment was administered for 9 days in the treatment groups. Macroscopic features of the wounds were observed daily, and skin samples from each group were collected on days 3, 6, and 9. The results demonstrated that the 5%, 10%, and 15% concentrations of Kepok banana peel extract formed wound healing areas on mouse skin on days 3, 6, and 9, and were able to reduce the number of inflammatory cells on days 3, 6 and 9 able to reduce IL-6 expression on days 3, 6 and 9, unable to increase fibrocytes on day 3, 6, and 9 and able to increased collagen density on days 6 and 9. The 15% concentration of Kepok banana peel extract applied for 9 days showed the greatest potential to accelerate wound-healing.

Keywords: Histopathological feature, Kepok banana peel, Macroscopic feature, Ointment, Skin, Wound healing

INTRODUCTION

The skin is the largest organ in the body that plays an important role in protecting body against external agents, maintaining body temperature and detecting sensory information from the outside environment (Wosgrau et al., 2015). Physical injuries resulting from surgical procedures, falls, burns, infectious diseases, or other pathological conditions cause damage or loss of skin structure and its function (Ahmad et al., 2021). The prevalence of skin wound in the population is relatively high, with this condition often being associated with various diseases and posing large socioeconomic burdens on patients and healthcare systems (Atzingen et al., 2013). Wounds can range from simple epithelial damage to the skin or extend deeper, reaching the subcutaneous tissue, potentially affecting underlying structures such as tendons, muscles, blood vessels, nerves, parenchymal organs, and bones (Velnar et al., 2009).

Wound healing in the skin is a multifaceted process characterized by interrelated and overlapping mechanisms, such as cell migration and proliferation, extracellular matrix synthesis, and the roles of growth factors and cytokines, which work together to promote tissue repair (Gushiken et al., 2021). A proper wound healing process is essential for the restoration of the anatomical and functional stability of impaired skin (Murthy et al., 2013). Various modern commercial topical drugs are commonly used to accelerate wound healing and reduce the risk of infection in the wound, but the use of these drugs has functional limitations and causes several side effects (D'abadia et al., 2022). Thus, alternative therapies are needed to accelerate wound healing, one of which is the use of traditional medicine that utilizes natural ingredients derived from plants (Maulidya et al., 2020).

Kepok banana peels, often regarded as bio-agricultural waste, are discarded in large quantities, contributing to environmental pollution. The decomposition of banana peels generates toxic byproducts, including methane, which has a global warming potential that is 21 times greater than that of carbon dioxide (CO2), thereby contributing to an increase

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in temperature and causing climate change (Syakri, 2019). The use of banana peels is expected to help overcome these environmental problems. Banana peels have been used as a traditional medicine across various countries, including several Asian countries, due to their rich bioactive content (Savitri et al., 2022). For instance, a study by Achmad et al. (2021) examined the effects of banana peel extract on the healing of incision wounds in the gingiva of mice. The findings demonstrated that banana peel extract could effectively accelerate the wound healing process by preventing bacterial infections and mitigating excessive inflammation. Banana peels are rich in flavonoids, saponins, and tannins, which are recognized for their potential to enhance the wound healing process (Syakri, 2019). The current study aimed to investigate the effects of Kepok banana peel extract on macroscopic and histopathological aspects of excision wound healing in mice.

MATERIALS AND METHODS

Ethical approval

This research has received ethics approval from the Research Ethics Commission of the Faculty of Veterinary Medicine, Universitas Gadjah Mada, Yogyakarta, Indonesia (Number: 129/EC-FKH/Int/2023).

Study period

This research was conducted in January 2024 at the Department of Pathology, Faculty of Veterinary Medicine, Universitas Gadjah Mada, the Department of Histology, Faculty of Dentistry Medicine, Universitas Gadjah Mada, and the Pathology Laboratory of Prof. Dr. Sardjito Hospital, Yogyakarta, Indonesia.

Experimental designs

Twenty-four 2-month-old female BALB/c mice were randomly assigned to four treatment groups, with each group consisting of six mice further divided into three subgroups based on days, including day 3, day 6, and day 9. Each mouse received two excision wounds. The four treatment groups in the study included K1 (control), K2 (topical therapy using Kepok banana peel extract ointment 5%), K3 (topical therapy using Kepok banana peel extract ointment 10%), and K4 (topical therapy using Kepok banana peel extract ointment 15%). The mice were housed in individual cages and acclimatized for 7 days before treatment. The excision wounds were made using a 4 mm biopsy punch in the dorsal area with a wound diameter of 4 mm. The procedure was performed under general anesthesia, using a combination of ketamine (100 mg/kg body weight, intramuscular) and xylazine (10 mg/kg body weight, intramuscular; Plumb, 2008). The therapy using banana peel extract ointment was carried out for 9 days in the treated groups (K2, K3, and K4), and the observations of wound macroscopic morphology and wound diameter measurements were carried out on a daily basis. Necropsy and wound skin sampling of each group were carried out on days 3, 6, and 9. The collected wound skin samples underwent histopathological preparations by hematoxylin-eosin staining to observe the number of inflammatory cells and fibrocytes, Trichrome Masson staining to see collagen density, and immunohistochemical staining to examine the expression of Interleukin 6 (IL-6).

Wound-healing activity

Wound healing comprises three overlapping phases, including the inflammatory phase, the proliferative phase, and the remodeling/maturation phase (Ionita et al., 2022). The inflammatory phase was characterized by hemostasis and inflammation. The proliferative phase involved epithelialization, angiogenesis, and collagen deposition. In the remodeling phase, the wound undergoes contraction, reducing the visible scar tissue (Das, 2013).

Histological study

Sample collection was performed across four treatment groups, with each group consisting of six mice. Two wound skin samples were taken from each mouse, resulting in a total of 48 wound skin samples prepared for histopathological examination. The histopathological analysis in the present study included the quantification of inflammatory cell counts and fibrocyte numbers observed in the wound tissue using hematoxylin-eosin staining. Moreover, Masson's trichrome staining was employed to evaluate collagen density, and immunohistochemical staining was performed to analyze IL-6 expression in wound tissues.

Macroscopic analysis

The macroscopic analysis involved determining the excisional wound area in each mouse. The wound area was measured using a digital caliper to record the diameter, which was then used to calculate the wound area.

Statistical analysis

Data from macroscopic and histopathological analyses were statistically analyzed using IBM SPSS Statistics 26 (2019) software with α=0.05. One-way ANOVA was used for parametric data, while the Kruskal-Wallis test was employed for nonparametric data, with statistical significance set at $p < 0.05$. If significant differences were observed ($p < 0.05$) in the one-way ANOVA test, a follow-up analysis was conducted using Tukey's test. Similarly, if significant differences were found ($p < 0.05$) in the nonparametric Kruskal-Wallis test, a post hoc Kruskal-Wallis test was performed.

RESULTS AND DISCUSSION

Macroscopic features

Quantitative data from the macroscopic features for each treatment group were obtained from measuring the area of the wound using a digital caliper. Macroscopic observations of excisional wounds in mice treated with Kepok banana peel extract on days 3, 6, and 9 were shown in Figures 1,2, and 3. The results of the analysis of macroscopic feature data obtained from each group on days 3, 6, and 9 did not show any significant differences, as seen in Table 1. The lack of a significant difference in wound area among groups indicates that the wound healing process in the therapy groups (K2, K3, K4) proceeded in parallel with that of the normal healing group (K1/control).

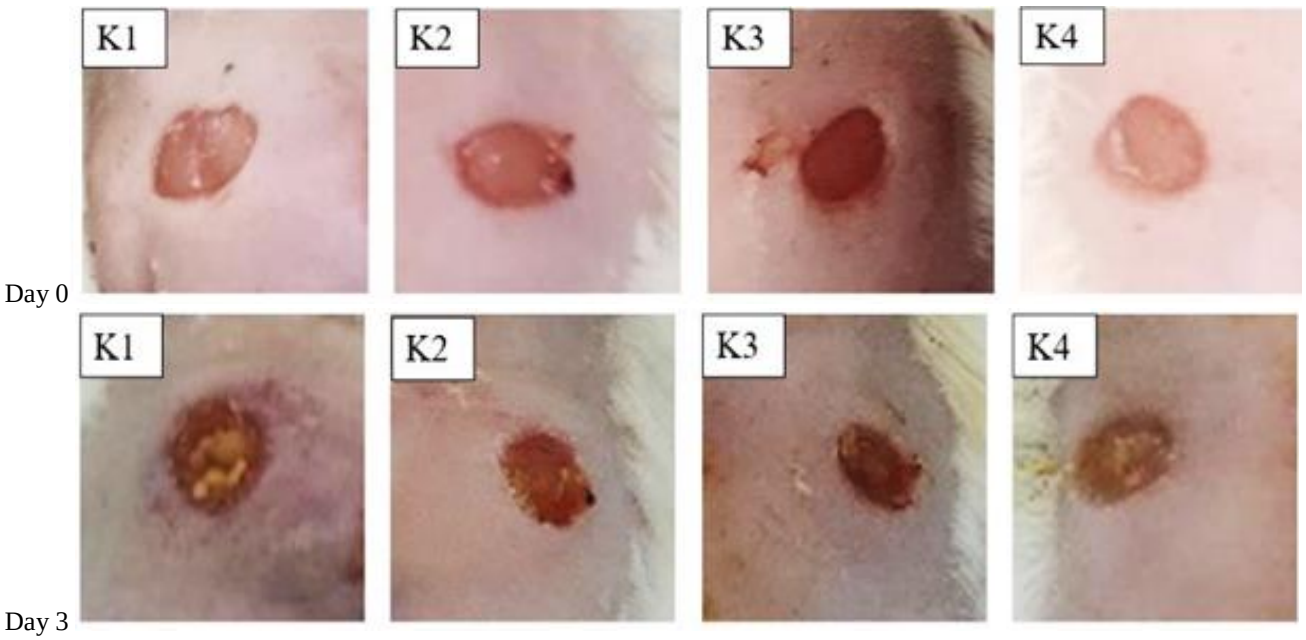

Figure 1. Macroscopic observations of excisional wounds in mice treated with Kepok banana peel extract on day 0 and day 3. **K1**: Control, **K2**: 5% Kepok banana peel extract ointment therapy, **K3**: 10% Kepok banana peel extract ointment therapy, **K4**: 15% Kepok banana peel extract ointment therapy.

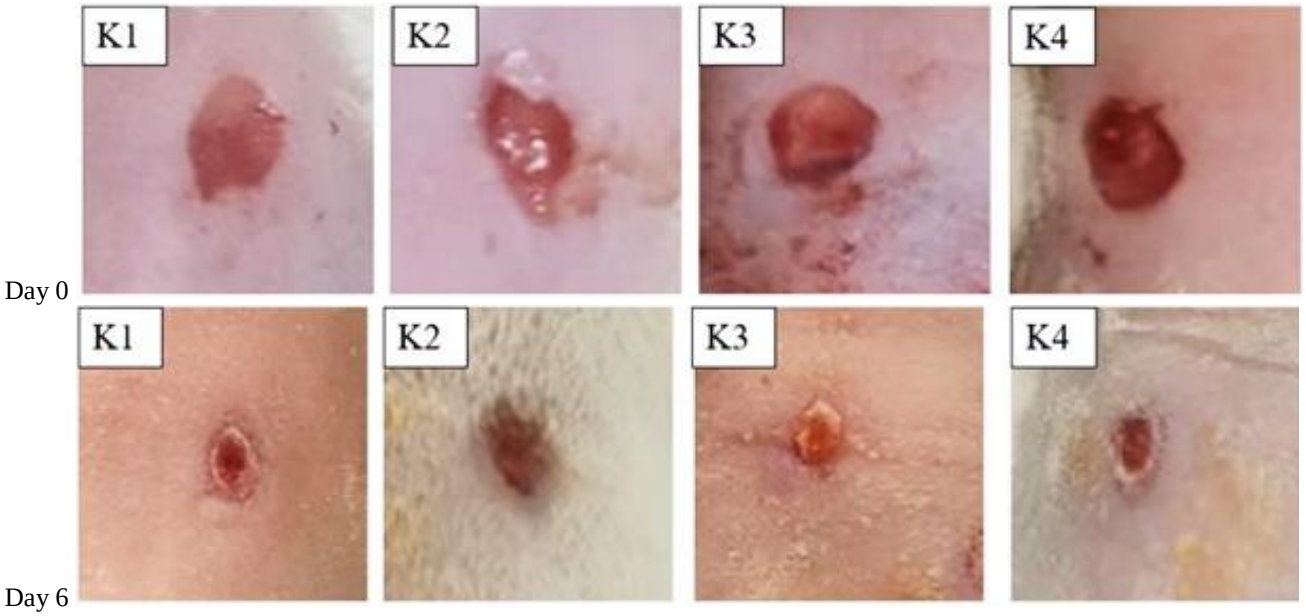

Figure 2. Macroscopic observations of excisional wounds in mice treated with Kepok banana peel extract on day 0 and day 6. **K1**: Control, **K2**: 5% Kepok banana peel extract ointment therapy, **K3**: 10% Kepok banana peel extract ointment therapy, **K4**: 15% Kepok banana peel extract ointment therapy.

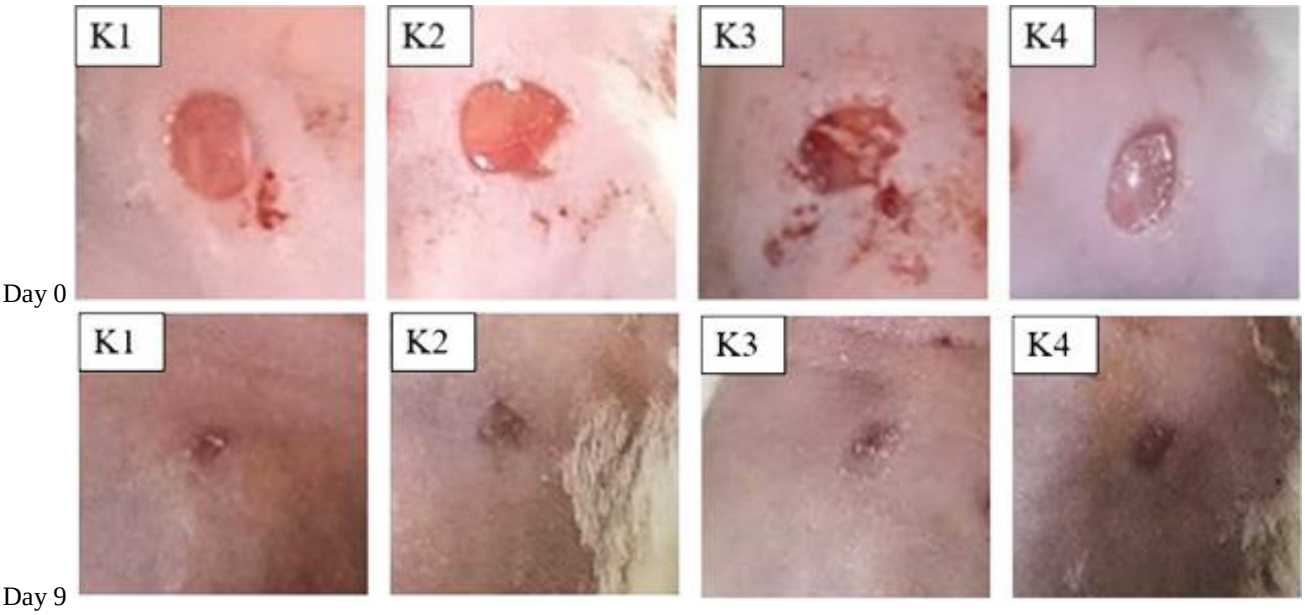

Figure 3. Macroscopic observations of excisional wounds in mice treated with Kepok banana peel extract on day 0 and day 9. **K1**: Control, **K2**: 5% Kepok banana peel extract ointment therapy, **K3**: 10% Kepok banana peel extract ointment therapy, **K4**: 15% Kepok banana peel extract ointment therapy.

Table 1. The results of excisional wound area measurements in Kepok banana (*Musa paradisiaca*) peel extract ointment groups on days 3, 6, and 9 after treatment

Groups \ Subgroups (Days)	Day 3	Day 6	Day 9
K1(0%)	12.52±2.84^{a}	2.86±2.05^{e}	0,00±0,00^{i}
K2(5%)	11.87±2.16^{a}	5.53±5.30^{e}	0,40±0,81^{i}
K3(10%)	10.71±1.84^{a}	3.88±2.78^{e}	0,05±0,10^{i}
K4(15%)	11.20±3.40^{a}	2.67±1.59^{e}	0,53±0,61^{i}
P value	0.783	0.603	0.471

Note: The absence of different notations indicates no significant difference between treatment groups within the same column ($p > 0.05$). K1: Control, K2: 5% concentration Kepok banana peel extract ointment therapy, K3: 10% concentration Kepok banana peel extract ointment therapy, K4: 15% concentration Kepok banana peel extract ointment therapy.

According to Rhea and Dunnwald (2020), the wound healing process in mice progresses through three phases included the inflammatory phase (days 0-5), the proliferative phase (days 3-14 days), and the remodeling/maturation phase (day 7-1 year). Therefore, the wound healing process that occurs on day 3 includes the inflammatory phase and the beginning of the proliferative phase. The inflammatory phase was characterized by the presence of hemostasis and inflammation. The proliferative phase was the phase of epithelialization, angiogenesis, and collagen deposition (Das, 2013). Kepok banana peel extract can heal wounds because it contains flavonoid compounds, saponins, and tannins (Syakri, 2019). While flavonoids can work as anti-inflammatory (Budiawan et al., 2023), tannins are known to have antibacterial activity to prevent infection in wounds, which can accelerate the contraction of fibrous tissue in the wound healing process (Budiawan et al., 2023). Fibrous contraction by fibroblasts triggers the formation of scar tissue, which will protect the formation of cells at the wound site (Budiawan et al., 2023). Saponins, as demonstrated in a study by Kim et al. (2011), inhibit inflammatory responses, promote epithelialization, and stimulate matrix synthesis, which was crucial for effective wound healing.

By day 6, the wound healing process advances into the proliferative phase, which was characterized by cell migration and proliferation, tissue synthesis granulation, and re-epithelialization. The granulated tissue was composed of a temporary extracellular matrix, macrophages, endothelial cells, and fibroblasts that provide strength to the skin. The re-epithelialization process plays a key role in closing the wound and restoring the skin's barrier function (York, 2022). Flavonoid compounds, tannins, and saponins contained in Kepok banana peel extract were compounds that can aid in healing wounds by helping in the proliferative phase by enhancing the vascularization during this phase (Meliawaty et al., 2021). Saponins can trigger the production of extracellular matrix and re-epithelialization, helping the wound healing process, especially in the proliferative phase (Kim et al., 2011). Moreover, tannins and saponins play a role in promoting fibroblast migration and proliferation (Marlinawati et al., 2023). Tannins also support the formation of new blood vessels by enhancing cell proliferation (Meliawaty et al., 2021).

On day 9, the wound healing process enters the proliferative and remodeling phases. The proliferative phase was marked by heightened migration and proliferation of keratinocytes, fibroblasts, endothelial cells, and leukocytes at the wound site. This phase also involved increased synthesis of extracellular matrix components, enhanced angiogenesis, and re-epithelialization, which together promote wound closure and restore the skin's barrier function. The remodeling phase was marked by the restructuring of the extracellular matrix, where collagen III was replaced with collagen I

(Gushiken et al., 2021). KepokSaponin compounds can stimulate matrix formation and re-epithelialization to accelerate wound closure (Kim et al., 2011). According to Meliawaty et al. (2021), saponins can help the formation of collagen I, which plays a role in stabilizing tissues formed in the *remodeling*/maturation phase.

Histopathological features

Inflammatory cell

Data on the number of inflammatory cells in each treatment group were obtained through microscopic observation at 400x magnification (Figure 4) in three fields of view. The average numbers of inflammatory cells in each treatment group for days 3, 6, and 9 are presented in Table 2. The statistical analysis conducted using the Kruskal-Wallis test revealed a significant difference among groups ($p < 0.05$) regarding the number of inflammatory cells on day 3, as indicated by the presence of different notations in the post-hoc Kruskal-Wallis test. Furthermore, the statistical analysis using the Kruskal-Wallis test for the number of inflammatory cells on day 6, along with the One-Way ANOVA for day 9, indicated significant differences among groups ($p < 0.05$), as evidenced by the different notations in the post hoc Kruskal-Wallis test (for day 6) and the subsequent One-Way ANOVA using Tukey's test (for day 9).

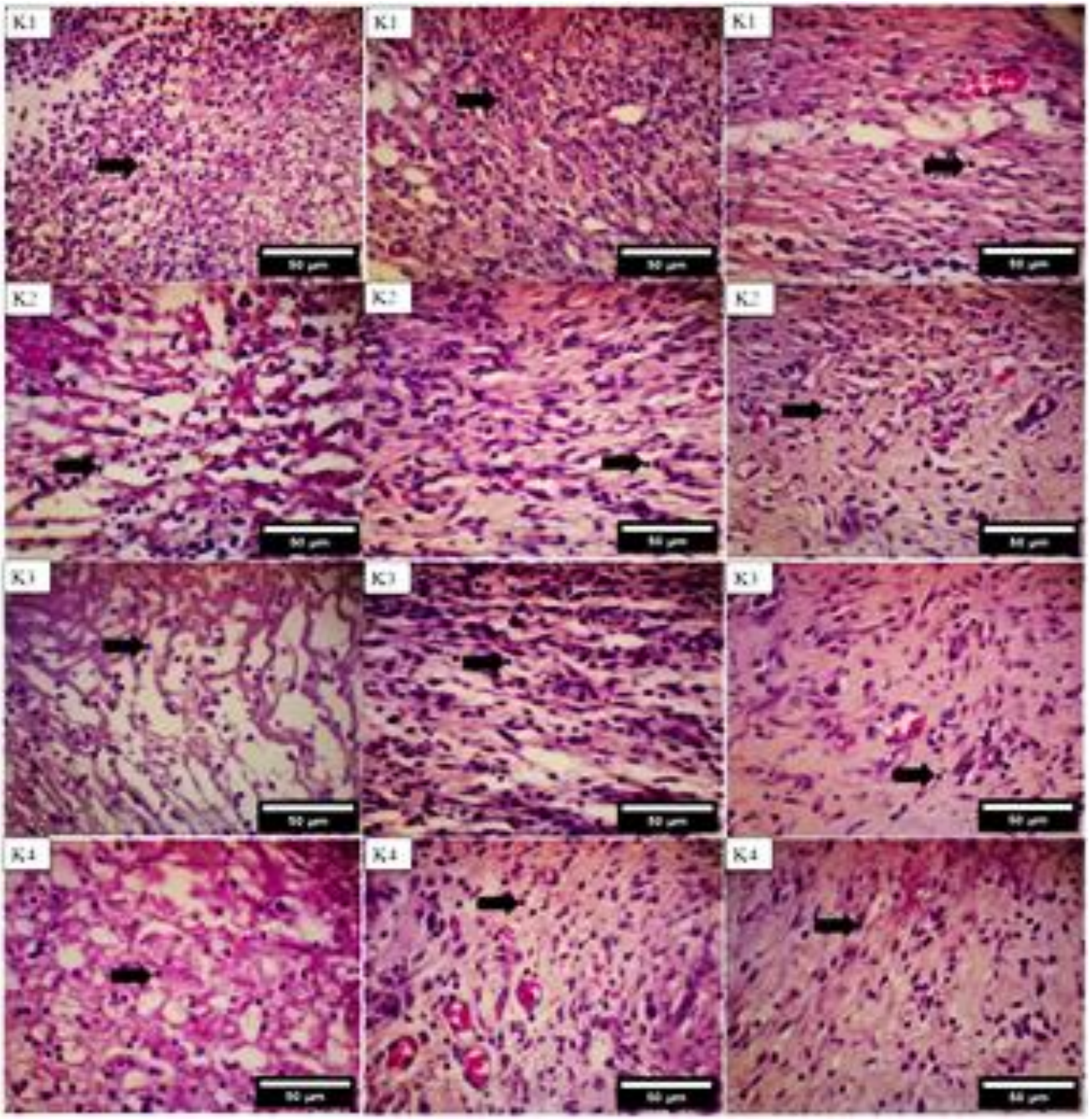

Figure 4. Histopathological characterization of inflammatory cells during wound healing in mice treated with Kepok banana peel extract (stained with hematoxylin-eosin, magnification 400x). Inflammatory cells are indicated by black arrows. **K1**: Control, **K2**: 5% concentration Kepok banana peel extract ointment therapy, **K3**: 10% concentration Kepok banana peel extract ointment therapy, **K4**: 15% concentration Kepok banana peel extract ointment therapy.

Table 2. Average number of inflammatory cells in Kepok banana (*Musa paradisiaca*) peel extract ointment groups on days 3, 6, and 9 after treatment

Groups \ Subgroups (Days)	Day 3	Day 6	Day 9
K1(0%)	251.08±108.46^{a}	159.92±22.95^{e}	115.83±12.56^{i}
K2(5%)	226.67±62.16^{a}	120.17±5.69^{e}	88.75±24.05^{ij}
K3(10%)	165.58±66.61^{ab}	119.75±9.34^{e}	69.58±23.22^{j}
K4(15%)	121±16.34^{b}	76.17±2.10^{f}	75.33±13.34^{j}
P value	0.036	0.023	0.022

Note: The presence of different notations indicates a significant difference among treatment groups within the same column ($p < 0.05$). K1: Control, K2: 5% concentration Kepok banana peel extract ointment therapy, K3: 10% concentration Kepok banana peel extract ointment therapy, K4: 15% concentration Kepok banana peel extract ointment therapy.

According to Geissler et al. (2022), inflammation was an integral part of the physiology of wound healing that aimed to clean the wound site from debris and pathogens. However, the excessive or prolonged inflammatory reaction can hinder the wound healing process and prevent complete recovery. The present study aligned with observations by Gunawan et al. (2019) that the infiltration of inflammatory cells during wound healing naturally decreases over time. The findings demonstrated that topical application of Kepok banana extract ointment on wounds can reduce the number of inflammatory cells, as indicated by the fewer inflammatory cells observed in the treatment groups (K2, K3, and K4) compared to the control group (K1). The large number of inflammatory products found indicates the level of inflammation experienced (Gunawan et al., 2019). An excessive and prolonged inflammatory response can lead to an increased injury in the tissues and worsen the healing process. Success in wound healing requires coordination between inflammation and resolution of inflammation (Eming et al., 2007).

The results of the current study reveal that the Kepok banana peel extract ointment reduced inflammatory cell counts on days 3, 6, and 9. This effect was attributed to the extract's flavonoid, tannins, and saponin content, which exhibit anti-inflammatory properties (Kim et al., 2011; Anandhi and Rajeskumar, 2023). Saponins function as anti-inflammatory by inhibiting inflammatory reactions in the early phases (Kim et al., 2011). Another anti-inflammatory mechanism that saponins have was the ability to inhibit the formation of exudate and inhibit the increase in vascular permeability (Fitriyani et al., 2011). Flavonoids can show anti-inflammatory activity by inhibiting the production of pro-inflammatory cytokines such as IL-6, IL1-1β, and TNF-α (Mayangsari et al., 2023). In addition to saponins and flavonoids, tannins also have anti-inflammatory activity but the mechanism of anti-inflammatory activity remains unclear (Fitriyani et al., 2011).

Interleukin 6

The data on interleukin 6 expression in each group on days 3, 6, and 9 of treatment with Kepok banana peel extract ointment was obtained by examining histopathological preparations of mouse skin using a microscope at 400x magnification (Figure 5). Observations were made in three different fields of view and analyzed using ImageJ 1.54g software. The average expression of interleukin 6 (IL-6) in each group on days 3, 6, and 9 of treatment with Kepok banana peel extract ointment is presented in Table 3. Statistical analysis conducted using SPSS software with one-way ANOVA revealed significant differences among groups ($p < 0.05$) concerning IL-6 expression on days 3, 6, and 9, as indicated by differing notations in the Tukey post-hoc test. The lowest IL-6 expression on day 3 was observed in group K4 (treatment with 15% banana peel extract ointment), with an average expression of 24.53 ± 1.57. On day 6, the lowest IL-6 expression was again observed in group K4, with an average expression of 15.60 ± 2.81. Finally, on day 9, group K3 (treatment with 10% banana peel extract ointment) exhibited the lowest IL-6 expression, averaging 9.49 ± 2.74.

Wound healing was a complex process involving the interaction of cellular cascades and biochemical actions that trigger structural improvements and the integrity function of injured tissues. This process involved many cell populations, extracellular matrices, and the action of dissolved mediators such as growth factors and cytokines (Ionita et al., 2022). One of the cytokines involved in the wound healing process was IL-6. Interleukin 6 was a multifunctional cytokine and was described as an inflammatory mediator (Nosenko et al., 2019). Interleukin 6 has a role in the wound healing process, but excessive expression of IL-6 can slow down the wound healing process because IL-6 provided a signal for leukocytes, thereby increasing the inflammatory process (Gulo et al., 2022). Although inflammation is an integral part of the physiological healing of wounds that aimed to clean the wound site from debris and pathogens, excessive or prolonged inflammatory reactions can impede the wound healing process, lead to incomplete recovery, and trigger chronic wounds (Geissler et al., 2022).

Based on the obtained results, Kepok banana peel extract therapy effectively reduced the expression of interleukin 6 on days 3, 6, and 9. This reduction can be attributed to the flavonoids and saponins present in Kepok banana peel extract, known to be able to inhibit or decrease pro-inflammatory cytokines, including interleukin 6. Khayri et al. (2022) report that flavonoids not only inhibit inflammation by targeting key signaling pathways, including Nuclear Factor-Kappa Beta (NF-κB), Mitogen-Activated Protein Kinase (MAPK), Extracellular Signal-Regulated Kinases (ERK), and the Akt pathway, but they also diminish the production of inflammatory cytokines. This reduction affects various cytokines, including Interleukin 6 (IL-6), Interleukin 8 (IL-8), Interleukin 1 Beta (IL-1β), Interleukin 17 (IL-17), Tumor Necrosis Factor Alpha (TNF-α), and Interferon gamma (IFN-γ). Yao et al. (2014) suggested that saponins exhibit anti-inflammatory effects by inhibiting the production of various pro-inflammatory cytokines, including Interleukin 6 (IL-6), Tumor Necrosis Factor Alpha (TNF-α), and Nitric Oxide (NO). This mechanism underscores the potential of saponins in

modulating inflammatory responses. In addition to flavonoids and saponins, banana peel extract also contains tannins, which possess anti-inflammatory properties (Fitriyani et al., 2011).

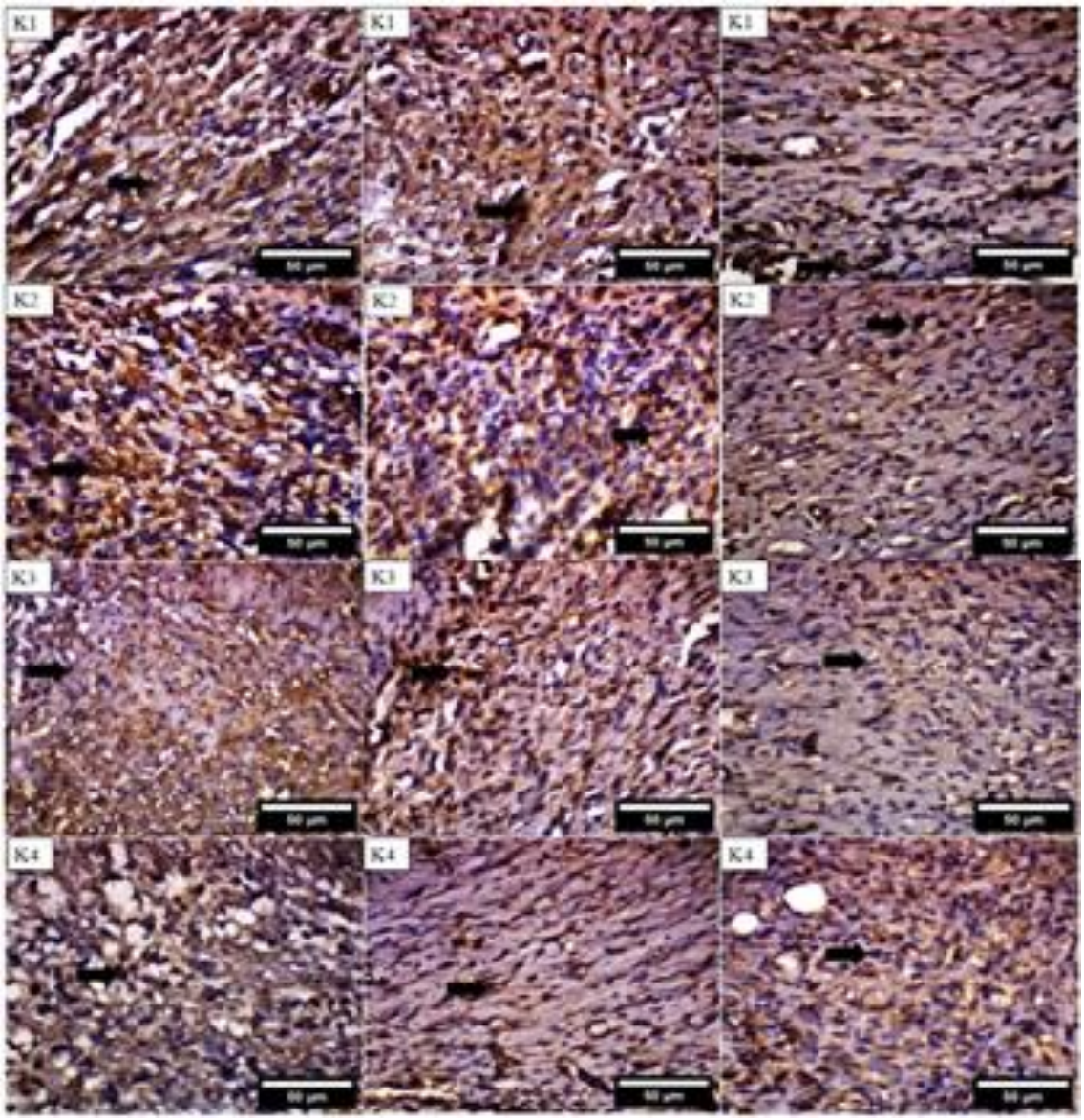

Figure 5. "Histopathological feature of Interleukin 6 (IL-6) expression during wound healing with Kepok banana peel extract (immunohistochemical staining, magnification 400x). The black arrows indicate IL-6 expression, which is stained brown in the immunohistochemical analysis. **K1**: Control, **K2**: Treatment with 5% Kepok banana peel extract ointment, **K3**: Treatment with 10% Kepok banana peel extract ointment, **K4**: Treatment with 15% Kepok banana peel extract ointment.

Table 3. Average number of Interleukin 6 (IL-6) expression in Kepok banana (*Musa paradisiaca*) peel extract ointment groups on days 3, 6, and 9 after treatment

Groups \ Subgroups (Days)	Day 3	Day 6	Day 9
K1(0%)	41.42±2.60^{a}	28.25±1.12^{e}	21.50±1.58^{i}
K2 (5%)	30.85±2.54^{b}	21.12±4.21^{f}	11.77±2.27^{j}
K3(10%)	29.05±2.73^{bc}	17.27±2.89^{f}	9.49±2.74^{j}
K4(15%)	24.53±1.57^{c}	15.60±2.81^{f}	12.81±3.84^{j}
P value	0.000	0.000	0.027

Note: The presence of different notations indicates a significant difference among treatment groups within the same column ($p < 0.05$). K1: Control, K2: 5% concentration Kepok banana peel extract ointment therapy, K3: 10% concentration Kepok banana peel extract ointment therapy, K4: 15% concentration Kepok banana peel extract ointment therapy.

Fibrocyte

Data on the number of fibrocytes in each group were obtained from observation using a microscope with 400x magnification in three fields of view (Figure 6). The average number of fibrocytes in each treatment group using Musa paradisiaca peel extract ointment on days 3, 6, and 9 is presented in Table 4. One-way ANOVA was used for statistical

analysis of fibroblast counts on days 3 and 9, while the Kruskal-Wallis test was employed for the counts on day 6. The results indicated no significant differences in fibrocyte numbers among groups on days 3, 6, and 9 after therapy with Kepok banana peel (Musa paradisiaca) extract ointment; therefore, no post-hoc tests were conducted.

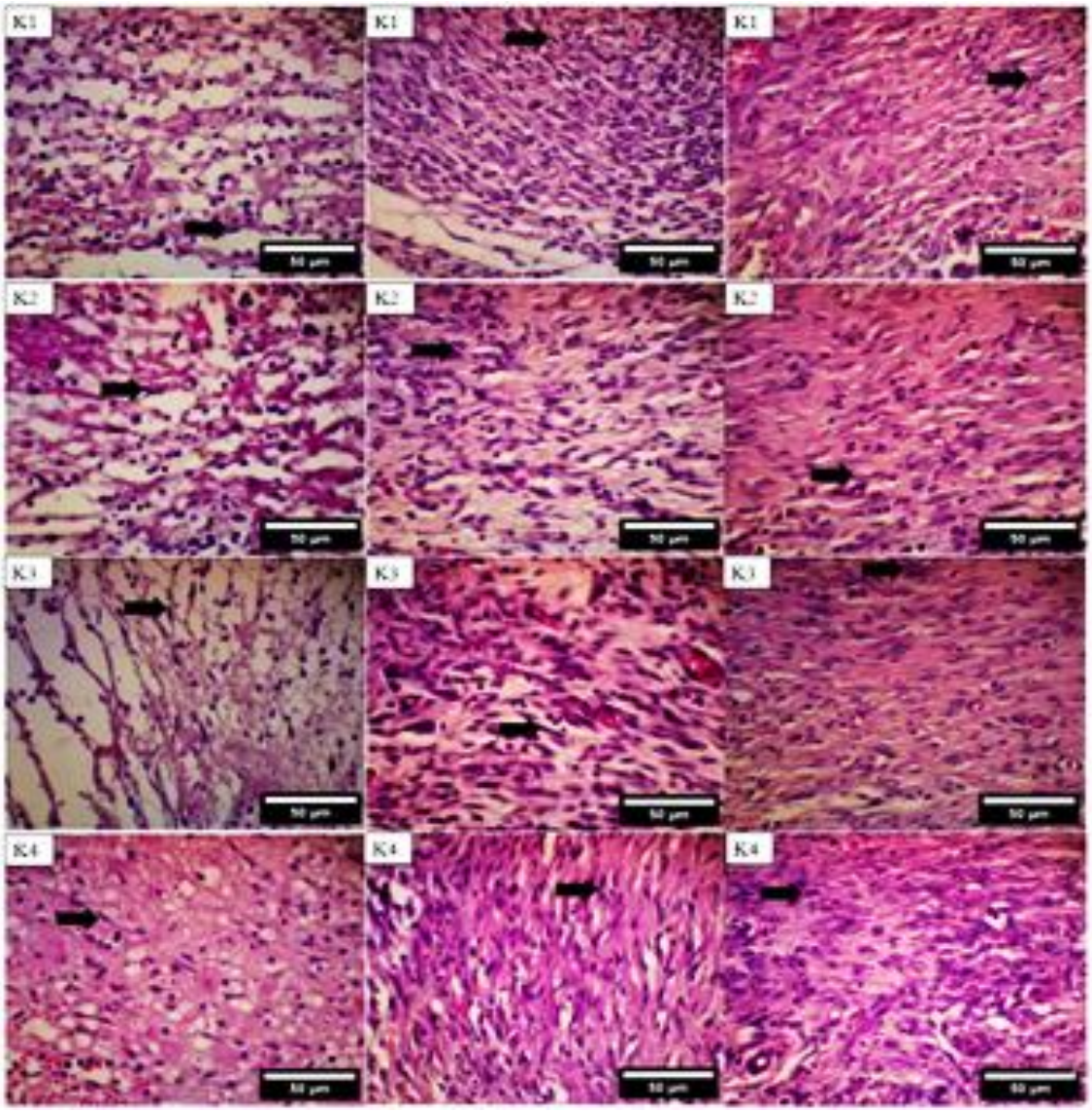

Figure 6. Histopathological examination of fibrocytes during the wound healing process with Kepok banana peel extract (hematoxylin-eosin staining, magnification 400x). Fibrocytes are indicated by black arrows. **K1**: Control, **K2**: Treatment with 5% Kepok banana peel extract ointment, **K3**: Treatment with 10% Kepok banana peel extract ointment, **K4**: Treatment with 15% Kepok banana peel extract ointment.

Table 4. The average number of fibrocyte cells in Kepok banana (*Musa paradisiaca*) peel extract ointment groups on days 3, 6, and 9 after treatment

Groups \ Subgroups (Days)	Day 3	Day 6	Day 9
K1(0%)	2.08±2.67^{a}	40.67±22.95^{e}	77.75±11.63^{i}
K2 (5%)	2.58±2.87^{a}	44.67±5.69^{e}	83.08±20.31^{i}
K3(10%)	2.67±1.65^{a}	67.17±9.34^{e}	99.17±2666^{i}
K4 (15%)	4.00±4.05^{a}	79.42±2.10^{e}	113±33.85^{i}
P value	0.817	0.431	0.219

Note: The absence of different notations indicates no significant difference between treatment groups within the same column ($p > 0.05$). K1: Control, K2: 5% concentration Kepok banana peel extract ointment therapy, K3: 10% concentration Kepok banana peel extract ointment therapy, K4: 15% concentration Kepok banana peel extract ointment therapy.

Fibrocytes were among the critical cells involved in the process of wound healing and tissue repair (Metz, 2003). These cells play an essential role in the wound healing process through several mechanisms, including their ability to function as antigen-presenting cells (APCs) during the inflammatory phase and their secretion of several proteins that trigger endothelial cell proliferation, migration, and angiogenesis (Xia and Ping, 2010). Fibrocytes play a significant role

in the proliferation phase of wound healing, participating in processes such as angiogenesis, collagen deposition, and wound contraction. Furthermore, they are also involved in tissue remodeling during the maturation phase of healing, contributing to the overall restoration of tissue integrity (de Oliveira and Wilson, 2020).

Kepok banana peel extract was known to contain flavonoids, saponins, and tannins, all of which can enhance wound healing through various mechanisms (Syakri, 2019). Flavonoids were recognized for their ability to enhance the production of growth factors essential for the wound healing process (Jaya et al., 2023). This ability can accelerate the transition from the inflammatory phase to the proliferative phase so that the wound healing process that occurs is faster, as compared to the physiological process (Jaya et al., 2023). Saponins have been shown to inhibit excessive inflammatory responses, promote re-epithelialization in wounds, and stimulate matrix synthesis during the wound healing process (Kim et al., 2011). Additionally, tannins possess both anti-inflammatory and antiseptic properties, which contribute to the acceleration of wound healing (Apriliana et al., 2021).

Collagen

Collagen density data for each group on days 3, 6, and 9 after treatment with Musa paradisiaca peel extract ointment was obtained through microscopic observation with 400x magnification (Figure 7) in three fields of view and processed with ImageJ software. Statistical analysis of the collagen density data was carried out with SPSS software. The Kruskal-Wallis test showed no significant difference in collagen density among groups on day 3. On day 6, one-way ANOVA revealed a significant difference between groups ($p < 0.05$) in collagen density, as indicated by distinct notations in the subsequent Tukey test. The highest collagen density was observed in group K4 (15% Kepok banana peel extract ointment).

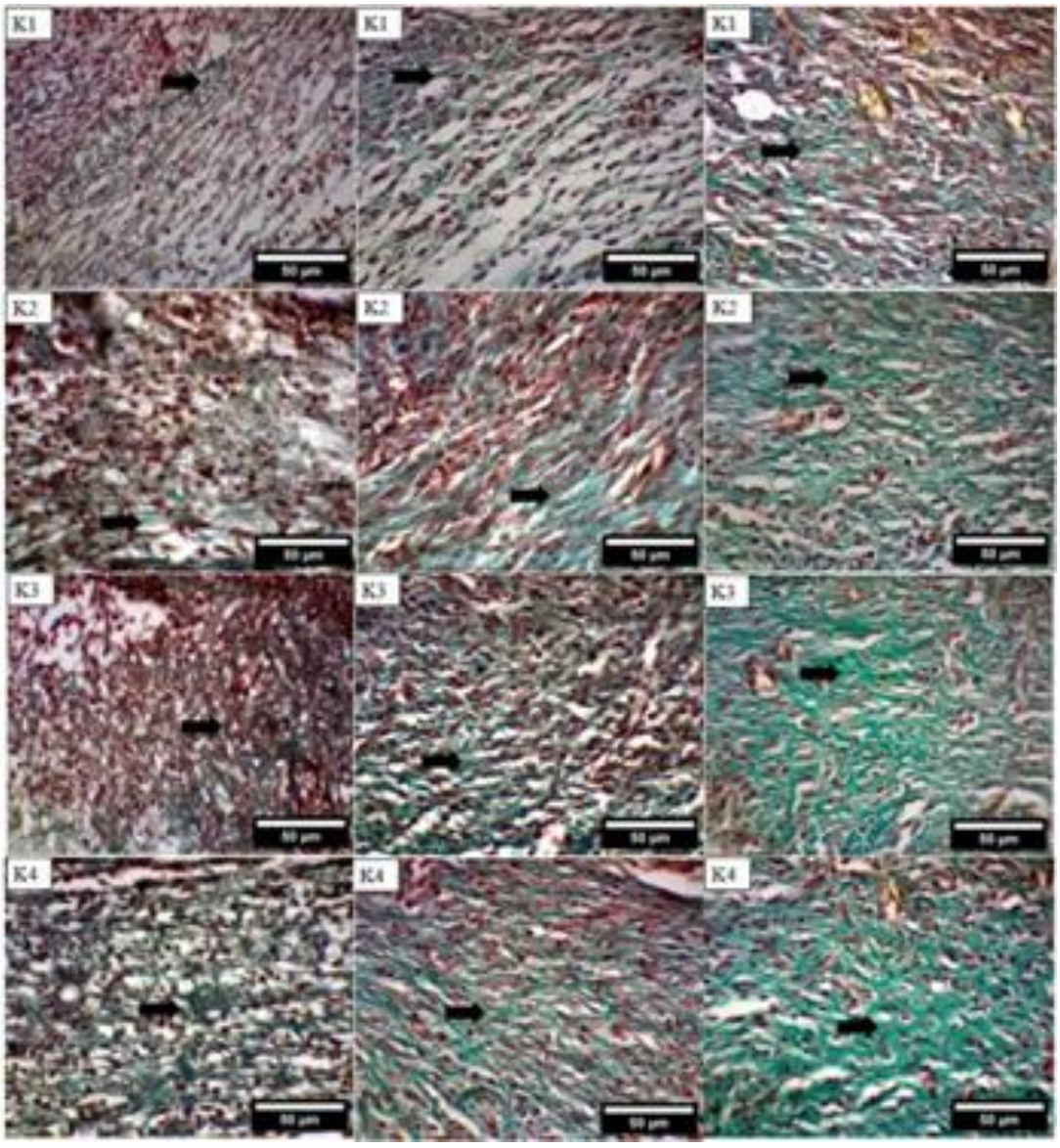

Figure 7. Histopathological feature of collagen fibers in wound healing with Kepok banana peel extract (*Masson's trichrome* staining, 400x magnification). The black arrows indicate collagen fibers stained green with *Masson's trichrome*. **K1**: Control, **K2**: 5% Kepok banana peel extract ointment therapy, **K3**: 10% Kepok banana peel extract ointment therapy, **K4**: 15% Kepok banana peel extract ointment therapy.

Table 5. Collagen density in Kepok banana (*Musa paradisiaca*) peel extract ointment groups on days 3, 6, and 9 after treatment

Groups \ Subgroups (Days)	Day 3	Day 6	Day 9
K1(0%)	19.02±8.72[a]	25.63±3.41[e]	29.55±4.77[i]
K2(5%)	22.60±1.52[a]	32.86±6.99[ef]	40.74±3.18[ij]
K3(10%)	25.40±3.11[a]	37.51±4.81[f]	44.40±6.97[j]
K4 (15%)	38.79±12.25[a]	43.36±5.19[f]	56.16±7.64[j]
P value	0.156	0.003	0.006

Note: The presence of different notations indicates a significant difference among treatment groups within the same column ($p < 0.05$). K1: Control, K2: 5% concentration Kepok banana peel extract ointment therapy, K3: 10% concentration Kepok banana peel extract ointment therapy, K4: 15% concentration Kepok banana peel extract ointment.

Additionally, statistical analysis using the Kruskal-Wallis test in SPSS demonstrated a significant difference between treatments ($p < 0.05$) in collagen density expression on day 9, as indicated by distinct notations in the Kruskal-Wallis post hoc test, with the highest collagen density found in group K4 (15% Kepok banana peel extract ointment).

Kepok banana peel extract was known to contain flavonoids, saponins, and tannins, which can accelerate wound healing (Syakri, 2019). These compounds interact with the growth factor receptors of fibroblasts, stimulating their activity and proliferation, thereby triggering collagen synthesis and accelerating granulation to accelerate wound healing. Flavonoids, saponins, and tannins can stimulate the proliferation and differentiation of fibroblasts into myofibroblasts. This stimulation leads to the synthesis of collagen and several other matrix proteins in large quantities (Marlinawati et al., 2023).

The inflammatory phase of wound healing was marked by hemostasis to stop bleeding and the activation and recruitment of immune cells. Collagen III was the first type of collagen produced during the wound healing process. Type III collagen was synthesized in the proliferative phase and was replaced with type I collagen in the remodeling/maturing phase. Fibroblasts were a major source of new collagen synthesis (Steiner et al., 2021).

By day 6, the wound healing process enters the proliferation phase (Das, 2013). This phase was characterized by the presence of fibroblast proliferation, collagen deposition, angiogenesis, formation of granulating tissue, and re-epithelialization (Steiner et al., 2021). The results of the present study demonstrate that Kepok banana peel extract therapy significantly increased collagen density on day 6 of the wound healing process. This increase in collagen density was attributed to the flavonoids, saponins, and tannins present in the banana peel extract. Flavonoid compounds inhibit Matrix Metalloproteinase (MMP), thereby increasing the amount of synthesis of collagen by fibroblasts for the formation of a new matrix and consequently, accelerating the wound healing process (Stipcevic et al., 2006). Saponins increase collagen production, thereby accelerating the wound healing process (Budiawan et al., 2023). Marlinawati et al. (2023) report that saponins can enhance fibronectin synthesis by fibroblasts and modify the expression of the Transforming Growth Factor Beta (TGF-β) receptor. Moreover, fibronectin is a versatile glycoprotein with binding sites for various macromolecules, including collagen, proteoglycans, and fibrin. Enhanced fibronectin synthesis promotes faster fibroblast migration, supporting the wound healing process by facilitating collagen production. Increased fibroblast migration to the wound site leads to higher collagen synthesis. Newly synthesized collagen combines with the existing collagen in the extracellular matrix, resulting in a denser matrix and promoting faster wound healing. Tannins can also enhance collagen synthesis by promoting the migration and proliferation of fibroblasts to the wound site (Rahati et al., 2020).

Based on the results obtained on day 9 of therapy with Musa paradisiaca peel extract ointment, the treatment with Kepok banana (Musa paradisiaca) peel extract was found to increase collagen density. This was evidenced by a higher average collagen density in the Kepok banana peel extract therapy group when compared to the control group. The higher collagen density in the therapy group with Kapok banana peel extract is due to the presence of flavonoids, tannins, and saponins. Flavonoid compounds inhibit MMP, thereby increasing enhancing collagen synthesis by fibroblasts and promoting the formation of a new extracellular matrix, which accelerates wound healing (Stipcevic et al., 2006). According to Deen et al. (2020), Falvonoids and tannins increased the viability and formation of collagen fibers, leading to an increase in the strength of the produced collagen fibers. Saponins enhanced collagen production during wound healing (Budiawan et al., 2023). They promote the migration and proliferation of fibroblasts to the wound site, which were the primary cells responsible for new collagen synthesis (Marlinawati et al., 2023). Additionally, saponins support the formation of type I collagen, a crucial element for stabilizing tissues during the remodeling phase (Meliawaty et al., 2021). Besides saponins, tannins also play a role by stimulating fibroblast migration and proliferation in the wound area, thereby boosting collagen synthesis and expediting the healing process (Rahati et al., 2023).

CONCLUSION

Topical therapy using Kepok banana peel extract ointments at purity percentages of 5, 10, and 15 formed wound healing areas on the skin of mice on days 3, 6, and 9. The treatment effectively reduced the number of inflammatory cells on days 3, 6, and 9, and decreased IL-6 expression on days 3, 6, and 9. While it did not increase fibrocyte levels on days 3, 6, and 9, it significantly enhanced collagen density on days 6 and 9. The 15% concentration of Kepok banana peel extract, applied for 9 days, exhibited the greatest potential in accelerating wound healing. Further research could utilize extracts from unripe Kepok banana peel, known to contain higher levels of bioactive tannins, and employ animal models with larger wound sizes to assess potential differences in wound closure rates among treatment groups.

DECLARATIONS

Authors' contributions

Sitarina Widyarini and Yuli Purwandari Kristianingrum supervised the study. Husnur Rukyat conceptualized, managed, and conducted data analysis and interpretation and drafted the manuscript. Husnur Rukyat and Dini Agusti Paramanandi performed all the experimental procedures. All authors read and approved the final edition of the manuscript.

Competing interests

The authors have not declared any conflicts of interest.

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Ethical considerations

The authors confirm that this manuscript is an original submission to this journal and has been screened for plagiarism.

Availability of data and materials

All original contributions from this study are provided within the article and supplementary materials. For further information, please reach out to the corresponding author.

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Effects of Adding Natural Honey to Semen Extender on Ram Epididymal Sperms Quality

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ABSTRACT

Numerous studies have indicated that male infertility is often associated with poor semen quality. The present study investigated the use of natural honey as an antioxidant and nutrient additive to semen extender to evaluate its effect on sperm parameters. A total of 16 fresh testes from sexually mature rams were collected for the investigation and immediately transported to the reproductive technology laboratory at the College of Veterinary Medicine, Baghdad University. For the first group, sperm was collected and diluted with an egg yolk extender mixed with 10% natural honey**,** while the second group consisted of epididymal sperm diluted with 0.9% normal saline. Epididymal fluid was collected and evaluated for both groups. The results showed significant differences in mean individual motility between the two groups after 48 and 72 hours, as determined by the comparison of proportions. Egg yolk plus honey diluent was significantly more effective than normal saline diluent in preserving sperm cell viability after 48 and 72 hours. The same finding applied to progressive motility; the egg yolk plus honey diluent was significantly more efficient than the normal saline diluent for the time frame after 48 and 72 hours, respectively. In conclusion, the findings demonstrated that the egg yolk extender supplemented with 10% honey was more effective in preserving ram sperm motility over time than normal saline. The addition of honey to the egg yolk extender improved the motility, the live-dead ratio, and the viability of the liquid storage of ram epididymal fluid. Furthermore, egg yolk plays a crucial role in protecting sperm from the detrimental effects of low temperatures.

Keywords: Epididymal sperm, Honey, Ram, Semen extender, Sperm motility

INTRODUCTION

Honey may greatly raise the quality of semen in various bull breeds (Chung et al., 2019). A study by El-Sheshtawy et al. (2016) on Arab stallions in Egypt has revealed that supplementation of semen with honey not only improves its motility and morphology but also protects the sperm against free radical-induced DNA breakage and cryoprotectant damage. Another study indicated that a 15% concentration of pure honey in cryoprotectant media could maintain the quality of infertile semen (El-Sheshtawy et al., 2016). In another study, combining honey with a cryoprotectant medium increased the fertility of rabbits and enhanced the quality of their semen during conservation (Chung et al., 2019). Additionally, studies on Jersey bulls suggested that Bioxcell without additives was more effective than a 1% honey concentration for cryopreserving bull semen (Chung et al., 2019; Gulov and Laskin, 2021).

Honey provides a safe, efficient, and natural alternative for enhancing semen quality and prolonging its conservation in various sheep species. Farmers and breeders choose honey due to its natural qualities (Cheepa et al., 2022). Because of its ability to support oxidative stability, protect sperm from cryoprotectant damage, and prevent DNA breakage caused by free radicals, it also presents a viable strategy for increasing the fertility of infertile semen and fostering spermatogenesis (Kotze et al., 2024). The addition of honey to semen samples, particularly in artificial insemination and cryopreservation, requires careful consideration to maintain sperm quality and viability. Research has explored various methods and additives to optimize semen handling and storage, focusing on factors such as sperm motility, concentration, and the effects of cryoprotectants. These studies provide valuable insights into the challenges and potential solutions for enhancing the reproductive success of honey (Tsvetkov et al., 2024).

The studies have shown epididymal epithelium, the lining of the epididymis, plays a significant role in the processing of sperm (Rodriguez-Martinez et al., 1990). Studies have also been conducted to understand the impact of various factors on sperm maturation in the epididymis of rams (Wegener et al., 2014; Martínez-Fresneda et al., 2019). For instance, research on the human epididymis has focused on the consequences of vasectomy on the epididymal transcriptome, which is the complete set of RNA transcripts produced by the epididymis (Martin-DeLeon, 2006).

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Additionally, there is research on how environmental elements affect sperm health, such as the impacts of biphenyl A on the antioxidant system of rat epididymis sperm (Chitra et al., 2003). In summary, epididymal sperm are crucial for sperm maturation and fertility, and their health and function can be influenced by various factors (Collins, 2003).

The epididymis is responsible for the transportation, maturation, and storage of sperm (James et al., 2020). During transit through the epididymis, spermatozoa undergo changes in the luminal environment of each epididymis region, acquiring motility and fertilization capabilities (James et al., 2020). The epididymis is comprised of four anatomical regions, including the initial segment, caput, corpus, and cauda. The epithelial cells lining the epididymis lumen play a crucial role in establishing a unique luminal environment, providing a protective barrier, and performing the sectorial transport of ions, nutrients, solutes, proteins, and water. Additionally, the epididymis prevents autoimmune responses against spermatozoa and protects against ascending and blood pathogens through interactions with immune cells (Breton et al., 2019). The epididymis also synthesizes and secretes proteins that are important for sperm maturation (Fouchécourt et al., 2000). Overall, the epididymis facilitates the maturation, protection, selection, and storage of spermatozoa in the male reproductive system (Wong et al., 2000).

Epididymal fluid is a crucial component of the male reproductive system. Produced by the epididymis, a tube-like structure that transports and matures sperm, the composition of epididymis fluid is essential for sperm survival and function (Nasreen et al., 2020). The studies have shown that the epididymis epithelium, the lining of the epididymis, plays a significant role in forming the fluid microenvironment (Wong et al., 2000).

A study on stallions aimed to expand the understanding of epididymal protein composition and its dynamic changes (Fouchécourt et al., 2000). Other studies have examined the acid-base status of epididymal fluid, such as research on boars that reported on the *in vitro* acid-base status and *in vivo* pH of epididymal fluid (Rodriguez-Martinez et al., 1990; Zuo et al., 2011). The use of natural honey as a semen extender or diluent is advantageous due to its natural acidity, which does not negatively affect sperm motility or health (Banday et al., 2017). Moreover, honey possesses unique antibacterial properties that benefit semen quality (Banday et al., 2017; Nasreen et al., 2020). The main objectives of the present study were to evaluate the physical and microscopical properties of epididymal sperm from ram testes after slaughter.

MATERIALS AND METHODS

Ram testicles were cut shortly after the animal was slaughtered, placed in a cooled box containing ice and maintained at 4°C, and transported to the laboratory within 1–2 hours as fresh semen without extenders. The study involved the collection of 16 fresh testes from sexually mature rams post-slaughter. The samples were immediately transported to the Laboratory of Reproductive Technology, Department of Surgery and Obstetrics, College of Veterinary Medicine, Baghdad University. The study was conducted from December 2023 to February 2024. The samples were divided into two groups, each consisting of 8 tests. In the first group, sperm were collected and diluted with an egg yolk extender mixed with 10% natural honey (Kanz Al-tabeaa, natural clover honey made in Iraq). The second group consisted of epididymal fluid diluted solely with 0.9% normal saline following sperm collection.

Procedure

The addition of honey to the semen extender was performed prior to sperm collection. A 15% honey solution was added slowly to the semen extender while mixing to ensure an optimum mixture of the semen extender and honey. All testes were transported from the slaughterhouse to the laboratory under cooled conditions. Each testis was cleaned with soap and water and disinfected with 0.5% Povidone Iodine. All the layers that covered the testis were removed to reach the epididymis. A total of 5 ml of diluent was injected into the head, body, and head of the epididymis to facilitate mixing with epididymal sperm. Since the epididymis lacked sufficient fluid, an injection was necessary to aid sperm evacuation and collection. A longitudinal incision was made in the epididymis using a surgical blade and scissors. The area was then rinsed with an additional 5 ml of diluent to maximize the recovery of epididymal sperm (Figures 1 and 2). The epididymal fluid was then collected and evaluated. This procedure was repeated for both groups (the egg yolk plus honey group and the normal saline group). Semen evaluation was conducted at 24, 48, and 72 hours after cooling in a refrigerator set at 8°C.

Sperm's evaluation

Individual motility

Epididymal fluid was used to measure sperm motility. A single drop of freshly collected epididymal fluid was placed on a spotlessly heated slide before being covered with a cover slide. A light microscope (Noval, China) with a 40X magnification was used to score each semen's motility individually (Vilakazi and Webb, 2004).

Sperm's concentration

Hemacytometers, made of specially built slides with two counting chambers and two dilution pipettes, were used to concentrate sperm. The process was carried out by adding 0.1 ml of epididymal fluid to 19.9 ml of coloring solution in a test tube with a dilution rate of 1:20. One drop was then placed on the hemocytometer's slide chamber, and the calculation was done using the following formula (Vilakazi and Webb, 2004):

Number sperm/ml = number of sperm in 0.1 mm^3×10×dilution rate ×1000.

Sperm morphology

Eosin and Nigrosin were the chemical staining agents used to determine the viability of the sperm (dead and living sperm). While the backdrop was dyed with Nigrosin to make the unstained sperm visible, eosin was able to penetrate through the membranes of the non-living cells (Al-Dahabi, 2010). A drop of the solution was added to a drop of fresh semen situated on a heated microscope slide after 1% eosin and 5% nigrosine had dissolved in 2.9% sodium citrate dehydrated buffer (Kushwaha, 2019).

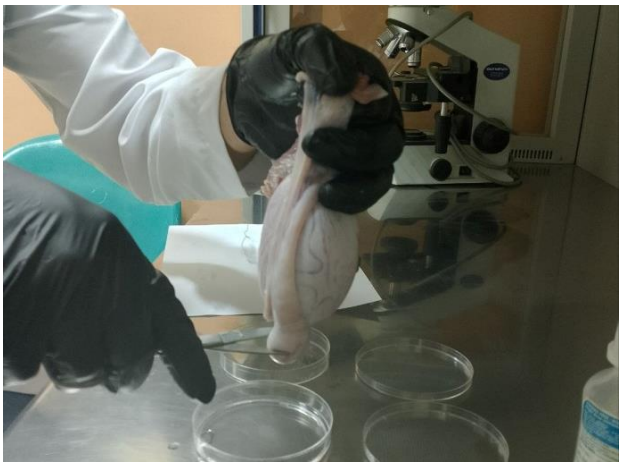

Figure 1. The longitudinal incision in the ram epididymis

Figure 2. Evacuation of ram epididymal fluid using 10 ml of extender containing 15% honey

Statistical analysis

The data were analyzed statistically using a two-way ANOVA. Significant differences ($p < 0.05$) between means were determined using the Least Significant Differences (LSD) post hoc test. Additionally, the Chi-square test was employed to identify any significant variations in proportions.

RESULTS AND DISCUSSIONS

The examination of sperm morphology was conducted to determine any primary and secondary abnormalities. After staining a drop of fresh semen with eosin and nigrosine, the semen slide field exhibited an acceptable standard for normal morphology (70%). Figures 3 and 4 illustrate the sperm morphology, indicating normal sperm structure (head and tail).

Table 1 presents the mean individual motility of sperm in the first group (egg yolk plus 10% honey). The individual motility and progressive motility were found to be 70% and 54%, respectively, at day 0. After 24 hours, these values decreased to 47% and 47%, respectively. By 48 hours, the individual and progressive motility further declined to 37% and 43%, respectively. On day three (72 hours), the individual and progressive motility reached their lowest values of 27% and 38%, respectively.

Table 2 presents the mean individual motility of sperm in the second group (normal saline). At day 0, the individual motility and progressive motility were recorded as 67% and 53%, respectively. Individual and progressive motility values started to decrease with time; after 24 hours, they were 43% and 37%, respectively, while the values of individual and progressive motility after 48 hours reached 20% and 23%, respectively. Finally, after 72 hours, the individual and progressive motility were found to be 12% and 13%, respectively.

Tables 3, 4, and 5 present the physiological characteristics of ram epididymal sperm diluted with egg yolk diluent plus 15% honey compared to normal saline. At day 0, the individual motility scores of ram sperm diluted with egg yolk plus honey and normal saline were 27% and 12%, respectively. On day three, the progressive motility percentages of ram sperm diluted with egg yolk plus honey and normal saline were 38% and 13%, respectively. The maintenance of sperm motility was considerably better with egg yolk plus honey diluent compared to the normal saline diluent.

Statistically significant differences were observed in the mean individual motility between the two groups after 48 hours and 72 hours, as demonstrated by the comparison of proportions. Egg yolk plus honey diluent at 15% was significantly ($p < 0.05$) more effective than the normal saline diluent in preserving sperm cell viability after 48 hours ($p < 0.05$) and after 72 hours ($p < 0.05$). The same trend was observed for progressive motility, where the egg yolk plus honey diluent of 15% was significantly more efficient than the normal saline diluent.

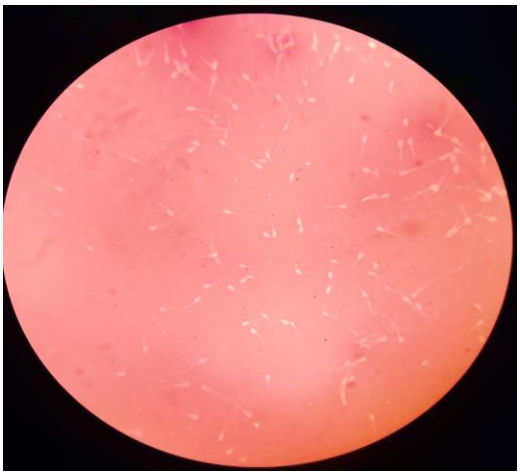

Figure 3. Sperm cells of ram epididymal fluid under the light microscope (40X). This image demonstrates ram sperm progressive motility stained with an eosin-nigrosine.

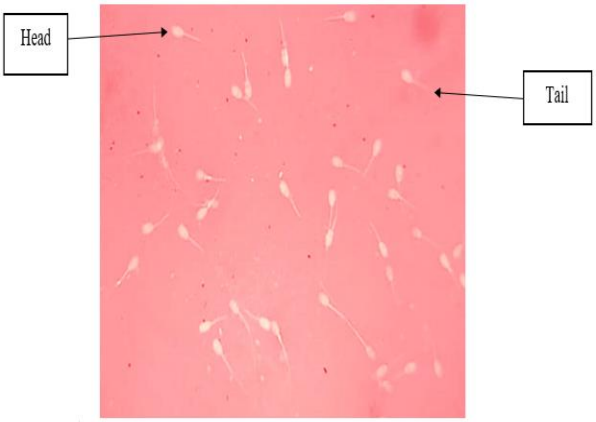

Figure 4. Sperm cells of ram epididymal fluid under the light microscope. A normal feature of ram epididymal sperms (head and tail) stained with eosin-nigrosine at (40X)

Table 1. Mean of individual motility of epididymal sperm from rams, diluted with egg yolk plus 15% honey

Period	Individual motility (%)	Progressive motility (%)
Day 0	70[a]	53[b]
24 hours	47[a]	47[a]
48 hours	37[a]	43[b]
72 hours	27[a]	38[b]

[a, b] Different superscript letters in the same row are significantly different at $p < 0.05$.

Table 2. Mean individual motility of ram epididymal sperm diluted with normal saline

Period	Individual motility (%)	Progressive motility (%)
Day 0	67[a]	53[b]
24 hours	43[a]	37[a]
48 hours	20[a]	23[a]
72 hours	12[a]	13[a]

[a, b] Different superscript letters in the same row are significantly different at $p < 0.05$.

Table 3. Sperm evaluation of ram epididymal sperm diluted with egg yolk diluent plus 15% honey in comparison with normal saline

Day 0 / Characters	Egg yolk plus honey	Normal saline
Individual motility	70%	67%
Progressive motility	53%	53%
Sperm concentration (ml)	1.36 × 106	1.36 × 106
Sperms anomalies	8%	9%

Table 4. A comparison of proportions was used to compare the two groups- egg yolk with 15% honey and normal saline- in terms of motility, individual motility, and progressive motility of ram sperm

Period	E+H In motility	NS In motility	P-value	E+H Pr motility	NS Pr motility	P-value
Day 0	70%	67%	0.648	53%	53%	0.1000
After 24 h	47%	43%	0.570	47%	37%	0.1530
After 48 h	37%	20%	0.007*	43%	23%	0.0027*
After 72 h	27%	12%	0.007*	38%	13%	0.0001*

NS: Normal saline diluent, E+H: Egg yolk + honey. In motility: Individual motility, Pr motility: Progressive motility

Table 5. Sperm evaluation of ram epididymal sperm diluted with egg yolk diluent plus 15% honey in comparison with normal saline

Characters	Day 0		P-value	After 72 h		P-value
	Egg yolk + honeybee	Normal saline		Egg yolk + honeybee	Normal saline	
Individual motility	70%	67%	0.648	27%	12%	0.0076*
Progressive motility	53%	53%	1.000	38%	13%	0.0076*
Sperm concentration (ml)	1.36 ×106	1.36 × 107	-	-	-	-
Sperm anomalies	8%	9%	0.800	-	-	-

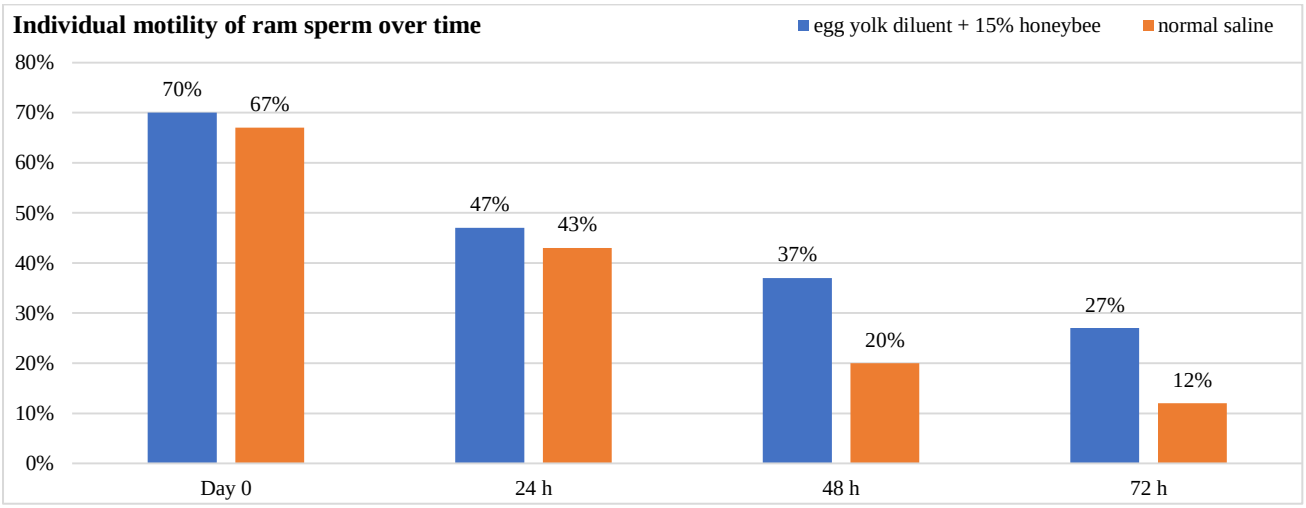

Figure 4. The individual motility of ram sperm diluted with egg yolk plus 15% honey and normal saline diluents

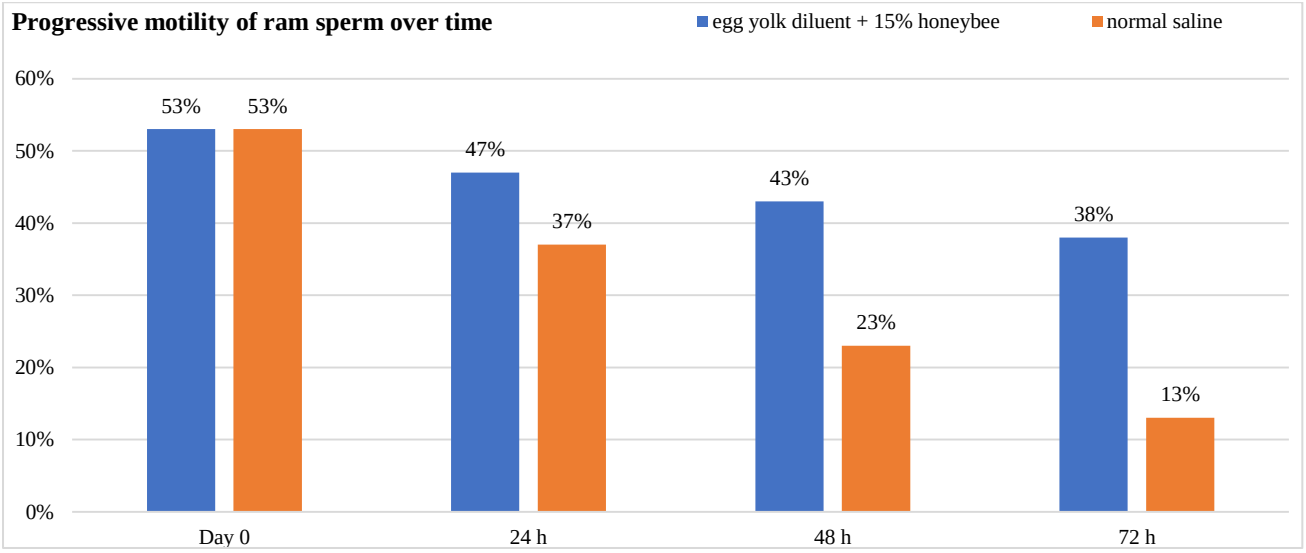

Figure 5. The progressive motility of ram sperm diluted with egg yolk plus 15% honey and normal saline diluents

It is widely recognized that several parameters, such as antibiotics, dilution components, semen storage techniques, frequency of semen collection, and dilution times influence the quality of semen (Liaqat et al., 2022). The natural habitat of sperm cells is replicated *in vitro* using semen dilution. It is feasible to decrease the velocity of sperm migration, block sperm metabolism, extend the period that spermatozoa survive, raise the amount of semen, and enhance spermatozoa consumption by adding an appropriate diluent, as supported as well by Van de Hoe et al. (2022). Honey possesses antibacterial and antioxidant properties due to the presence of flavonoids, which can scavenge free radicals and prevent consequent damage to DNA (Zhang et al., 2024). Studies have demonstrated that adding honey to an egg yolk extender enhances the viability, motility, and live-dead ratio of liquid-stored goat semen (Wong et al., 2000). Egg yolk plays a critical role in protecting sperm from the detrimental effects of low temperatures, as noted by Zhang et al. (2024). Lecithin and unsaturated fatty acids in the yolk may increase sperm metabolism, stabilize the cell membrane, promote the production of lipoproteins, and mitigate the effects of free radicals.

Furthermore, adding yolks can increase sperm's resistance to osmosis (Collins, 2003). Further research is needed to understand the mechanism of action and optimal dosage of supplementing honey products in semen extenders for different species (Hashem et al., 2021). Although studies speculate that adding egg yolk to the diluent may increase the danger of animal disease transmission, egg yolk is a frequently utilized protective ingredient in low-temperature semen preservation diluents (Vilakazi et al., 2004). The motility of individual and large sperm is seen in Figures 4 and 5. Compared to regular saline, the egg yolk plus 15% honey diluent was more effective in maintaining sperm motility over time.

Numerous stress types, including osmotic, biochemical, and thermal variables, can cause sperm destruction as a result of semen storage. Nonetheless, the normal saline solution consists of 9 grams of sodium chloride (NaCl) dissolved in water and lacks stabilizers, antioxidants, or antibacterial qualities (Vilakazi et al., 2004; Van de Hoek et al., 2022).

CONCLUSION

The findings of the current study regarding the efficiency of two dilution solutions in preserving ram sperm motility lead to the conclusion that the egg yolk plus 15% honey diluent is more efficient in preserving sperm motility over time than the normal saline diluent. The addition of honey to egg yolk extender mixed with 15% honey may improve the motility, live-dead ratio, and viability of the liquid storage of epididymal fluid in rams. The egg yolk was found to be essential in protecting sperm from low-temperature effects.

DECLARATIONS

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Authors' contributions

Baqer Jafar Hasan conducted the conceptualization, as well as writing the original draft, investigation, methodology, formal analysis, and visualization. Hayder Aabd-al-Kareem Hasan Al-Mutar contributed to the investigation, project administration, and funding acquisition. Jawed Kadhum and Taher handled the investigation, data curation, and funding acquisition. Baqer Jafar Hasan contributed to the supervision, validation, formal analysis, and writing of the review and editing. All authors read and approved the final version of the manuscript.

Competing interests

The authors declare that there is no conflict of interest.

Ethical considerations

All authors have diligently reviewed the manuscript for potential ethical issues, including plagiarism, research misconduct, data fabrication or falsification, and redundant publication.

Availability of data and materials

All data generated during this research are pertinent and have been included in the published article. For further information or inquiries, please contact the corresponding author.

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Evaluation of Immunomodulatory Properties of Fish-Protein Hydrolysate from Skipjack Tuna by-products (*Katsuwonus pelamis*, Linnaeus 1758) in Streptozotocin-Nicotinamide-Induced Diabetic Rats

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ABSTRACT

Fish protein hydrolysate (FPH) is an alternative to managing fish by-products. Protein hydrolysis by proteolytic enzymes breaks down the protein into bioactive peptides (BP). Bioactive has a high-protein content and various beneficial health properties, including antioxidants, immunoregulatory, antibacterial, anti-inflammatory, and other activities. The current study aimed to investigate the anti-diabetic and immunomodulatory activities of FPH from skipjack tuna by-products. Male *Sprague Dawley* rats (n = 25) were equally divided into five groups: healthy group, diabetic mellitus (DM) group, DM + Imunos 0.8 g/ kg BW (drug control group), DM + 0.8 g/kg BW (FPH 1), DM + 1.6 g/kg BW FPH (FPH 2). Diabetic rats were induced by being fed with a high-fat diet (HFD) for 3 months, followed by nicotinamide (NA; 120 mg/kg BW)-streptozotocin (STZ) injection (60 mg/kg BW). The initial and final body weights before and after treatment were measured. The leukocyte and lymphocyte levels were measured using a hematology analyzer. The pro-inflammatory cytokine tumor necrosis factor α (TNFα) level was measured using enzyme-linked immunosorbent assay (ELISA). The result showed that the blood glucose levels after treatment using FPH significantly decreased compared with DM rats. Leukocyte and lymphocyte numbers also decreased significantly after treatment using FPH 1 than in DM rats. The pro-inflammatory cytokine TNFα in the FPH rat groups improved significantly compared with DM rats. These study results suggested that FPH from skipjack tuna by-product administration can be used as anti-diabetic and immunomodulatory candidates.

Keywords: Diabetes mellitus, Fish protein hydrolysate, Inflammation, Skipjack tuna by-product

INTRODUCTION

Skipjack tuna (*Katsuwonus pelamis*) is a pelagic migratory fish belonging to the Scombridae family. Skipjack tuna is found in tropical and subtropical waters and has a high commercial value (Artetxe-Arrate et al., 2021; Shin et al., 2024). The catches of tuna and tuna-like species have increased in recent years, reaching their highest levels in 2018 at over 7.9 million metric tons (FAO, 2020). Tuna is mostly used as raw material for canned tuna products, resulting in a rapid increase in product demand over the past four decades (Kawamoto, 2022). The fish processing industry mostly produces 25-70% by-products or waste in the form of skin, head, fins, tail, bones, and offal (Wang et al., 2022; Abeysinghe et al., 2024). These by-products are usually used in producing pet food, animal feed, fish meal, and fertilizer, or only become waste products, resulting in the waste of biological resources and causing serious environmental problems (Kim et al., 2019; Tacias-Pascacio et al., 2021; Cai et al., 2022). Fish protein hydrolysate (FPH) is an alternate solution to decrease environmental issues that come up caused by fish by-products (Honrado et al., 2024).

Fish Protein Hydrolysate (FPH) is a processed fish waste product in liquid or powder form and consists of bioactive peptides (BP, Daroit and Brandelli, 2021). The enzymatic hydrolysis by proteolytic enzymes helps to hydrolyze the protein into short-chain peptides to produce active peptides (Caruso et al., 2020; Fadimu et al., 2022). Fish Protein Hydrolysate was reported to increase solubility, emulsifying properties, foaming, water-holding capacity, and fat-binding capacity (Dinakarkumar et al., 2022). Moreover, active peptides have many biological functions, such as antihypertensive, antioxidant, immunoregulatory, antibacterial, anti-inflammatory, anti-aging, and other activities (Fadimu et al., 2022; Ye et al., 2022; Ortizo et al., 2023). Bioactive peptides (BP) rarely accumulate in the human body, thus, it has low side effects (Nourmohammadi and Mahoonak, 2018; Akbarian et al., 2022). Meanwhile, the consumption of synthetic drugs has side effects due to high drug residue in the body (Bhardwaj and Misra, 2018).

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Diabetes mellitus, a common metabolic disorder, is linked to notable alterations in the immune system, especially regarding leukocyte and lymphocyte levels. Multiple studies indicate that inflammatory markers, including total leukocyte count, are associated with the onset and advancement of diabetes (Holt et al., 2024). The global prevalence of diabetes and obesity is rising, concerning more than 380 million and 500 million individuals, respectively (Rivero-González et al., 2017). Inflammation is now recognized as a crucial factor in the pathogenesis of both type 1 and type 2 diabetes, leading to a complex interplay between the inflammatory and immune systems (Rohm et al., 2022). Diabetic mellitus (DM) is linked to elevated reactive oxygen species and proinflammatory cytokine levels (Goycheva et al., 2023). Tumor necrosis factor-α (TNF-α) is a significant mediator in the inflammatory process and has essential roles in the development of insulin resistance and the pathogenesis of diabetes (Akash et al., 2018). Bioactive peptides represent a promising therapeutic for DM management, given their ability to affect multiple metabolic pathways and regulate immune responses (Antony and Vijayan, 2021).

Recent studies have suggested that certain peptides found in fish products may possess anti-diabetic properties (Alyari Gavaher et al., 2022) via multiple mechanisms, such as inhibition of the enzyme dipeptidyl peptidase-IV, which plays a role in the stabilization of blood glucose levels (Fakih and Dewi, 2021). Furthermore, FPH is reported to have antioxidant activity and antimicrobial activity in Gram-positive and Gram-negative bacteria (Da Rocha et al., 2018). This current study aimed to elucidate the advantages of FPH from skipjack by-products to maintain blood glucose levels and reduce pro-inflammatory cytokines in streptozotocin-induced diabetic rats.

MATERIALS AND METHODS

Ethical approval

The animal welfare and experimental procedures were approved by the Animal Ethics Committee of Brawijaya Ethics Committee, Brawijaya University with approval number 181-KEP-UB-2024 and following the principles of laboratory animal care by the National Institute of Health National Research Council (US) Committee for the Update of the Guide for the Care and Use of Laboratory Animals.

Preparation of skipjack tuna by-products protein hydrolysate

The samples in this research were framed and trimmed of skipjack tuna by-products. The samples were obtained from Cilacap Regency, Central Java, Indonesia. The procedure of protein hydrolysis from skipjack tuna followed the procedure from Prasetyo et. al. (2024). 100 g of the sample was added to distilled water with a ratio of 1:3, and then 5% papain enzyme (0.0835 ± 0.0009 U/mL) was added to the mixture. During hydrolysis, the mixture was maintained at pH 6.3 by adding 0.1 M NaOH or 0.1 M CH_3COOH. The temperature of hydrolysis was at 61 °C for 230 minutes, followed by enzyme inactivation at 80^{o}C for 30 minutes. The hydrolysis result was filtered using Whatman paper number 43, then dried using a spray dryer (manual procedure Buchi mini spray dyer B-290, BÜCHI Labortechnik AG, Switzerland) with an inlet temperature of 140 °C and an outlet of ± 95 °C. The hydrolysate powder was then kept at -20 °C for further analysis.

Animals and experimental design

Twenty-five male Sprague-Dawley rats (8±2 weeks old, 170 ± 10 g body weight) were purchased from the Animal Center of Pusat Antar Universitas (PAU), Gadjah Mada University, Indonesia. The animals were housed individually in standard cages and had free access to food and water *ad libitum*. The rats were acclimated for one week before the beginning of the experiment. Rats (n = 20) were administered a modified high-fat diet (HFD32) comprising 32% crude fat, with a caloric contribution from fat sources accounting for 60% of total energy, as formulated by Dr. Osamu Ezaki (National Institute of Health and Nutrition). The HFD32 was given for three months. The HFD32 formulation is presented in Table 1.

Following 3 months of high-fat diet feeding, blood glucose levels were assessed in the rats using blood samples from the tail vein. The rats were subsequently administered multiple doses of nicotinamide (NA, 120 mg/kg BW), followed by several low doses of streptozotocin (STZ, 60 mg/kg BW) one hour after the NA injection (Ghasemi and Jeddi, 2023; Jeong et al., 2024). The STZ stock solution was prepared by dissolving 160 mg of STZ in 16 mL of 0.1 M citrate buffer at pH 4.5. The NA stock solution was prepared by dissolving 100 mg of NA in 10 mL of 0.1 M phosphate buffer at pH 7. Fasting blood glucose levels in rats were assessed one week later through the tail vein. The fasting blood glucose ≥ 200 mg/dL was considered diabetic. The rats were then divided into five groups namely; normal as a normal control group, DM as the diabetic model control group, DM+Imunos (Lapi, Indonesia) 0.8 g/ kg BW (drug control group), DM+FPH1 at a dose of 0.8 g/kg BW, DM+FPH2 at a dose of 1.6 g/kg BW. Fish Protein Hydrolysate dosage determination is determined by dose-response considerations. The rats received the medicines orally daily for two weeks.

Weekly measurements of body weight and feed consumption tracked the experiment. Rats were fasted for twelve hours, and final blood glucose levels were examined. The rats were then dissected using Ketamine-A-Xylazine following the dosage according to the manufacturer's product (Ket-A-Cy, AgorVet, Peru). The blood was collected by cardiac puncture and then placed in a vacutainer ethylene diamine tetra acetic acid (EDTA) tube for leukocyte and lymphocyte measurement using an automatic hematology analyzer (ABX Micros 60, Horiba, Japan). Serum was obtained by collecting the blood in a vacutainer gel clot and then centrifuged at 3000 rpm, 10 ^{o}C for 10 minutes.

Table 1. High-fat diet 32 ingredients for Sprague-Dawley male rats (8 ± 2 weeks old) in 3 months

Formulation	Percent
Milk casein	20.50
Egg white	20.50
L-cystine	0.368
Powdered beef tallow (including 80% of beef fat)	13.29
Safflower oil (high oleic acid)	16.75
Crystalline cellulose	4.60
Maltodextrin	6.90
Lactose	5.80
Sucrose	5.655
AIN93 vitamin mix	1.155
AIN93G mineral mix*	4.18
Choline bitartrate	0.30
Tertiary butyl hydroquinone	0.002
Total	100.000

*A commercial mineral mix for rodents

Measurement of tumor necrosis factor α

The levels of tumor necrosis factor α (TNFα) in the serum were measured using the enzyme-linked immunosorbent assay (ELISA) method using an ELISA kit rat anti-TNFα (ER1393, FineTest, Wuhan, China) and following the manufacturer's procedures.

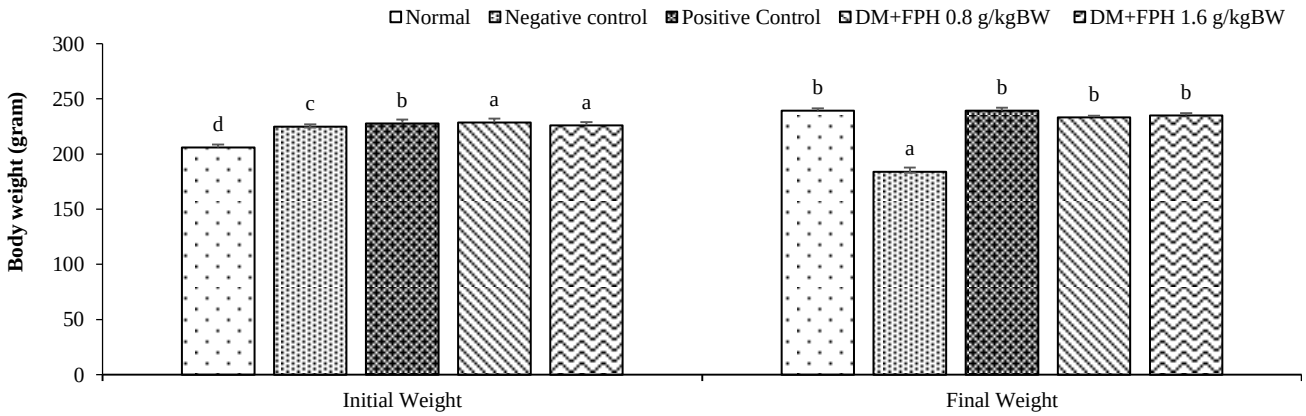
Statistical analysis

The data were analyzed using a one-way analysis of variance (ANOVA) followed by Duncan's multiple range test (DMRT) as a post hoc test using Statistical Package for the Social Sciences (SPSS) version 20 (IBM Corp, Armonk, NY). $p < 0.05$ indicated a significant difference between groups.

RESULTS

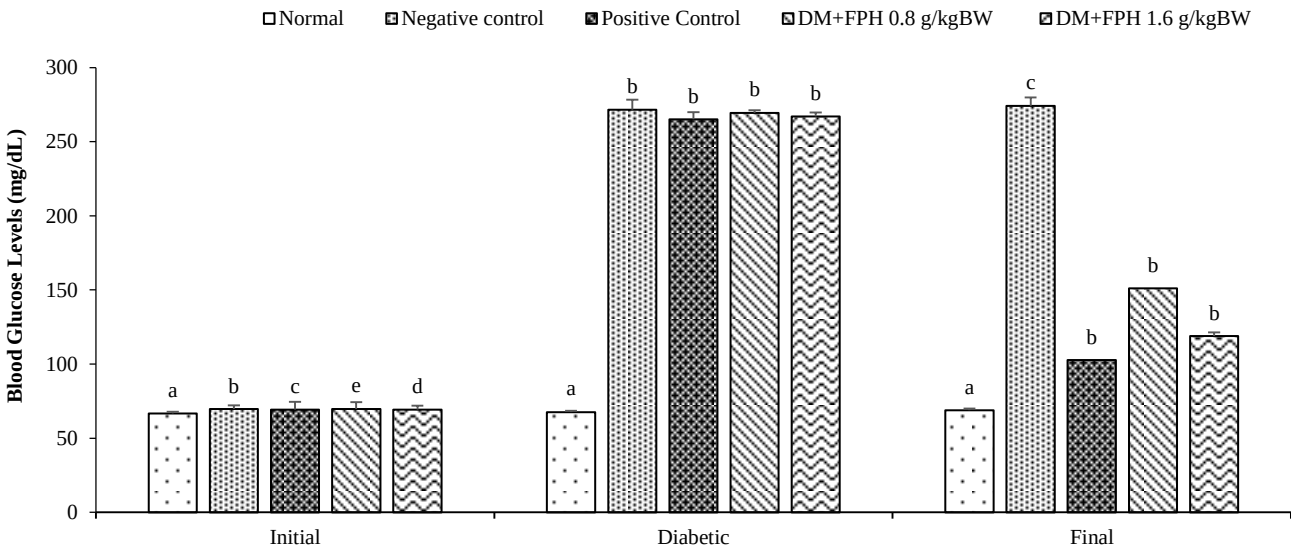
Effects of fish protein hydrolysate on body weight and glucose levels

The body weight of rats at the initial stage showed a significant difference between the groups (Graph 1, $p < 0.05$). The body weight of rats is in the range of 170 ± 10 g. High-fat diet-fed rats demonstrated a significant increase ($p < 0.05$) in body weight than normal groups that fed standard diets ($p < 0.05$). After STZ injection, the diabetic rat groups displayed a significant difference between groups ($p < 0.05$). These results indicated that the HFD fed before STZ induction affected the body weight gain of rats. The normal groups fed a standard chow diet showed a slight body weight increase, while rats fed with HFD32 increased their body weight significantly than the normal groups ($p < 0.05$).



Graph 1. Body weight change of rats at the initial stage and final stage. Normal: Normal control rats; DM: STZ-induced diabetic rats; DM+FPH1: Diabetic rats treated with fish protein hydrolysate dosage 0.8 g/kg BW; DM+FPH2: Diabetic rats treated with fish protein hydrolysate dosage 1.6 g/kg BW. ^{abcd} Different superscript letters indicated significant differences ($p < 0.05$).

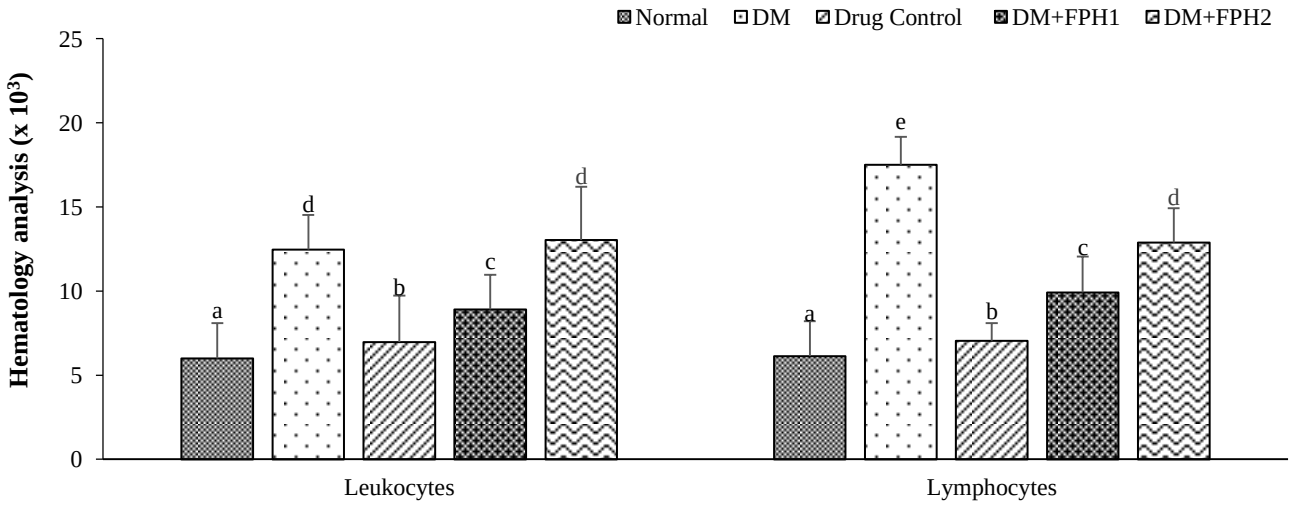
Based on the analysis as seen in Graph 2, the initial glucose levels significantly differ between groups ($p < 0.05$). After the STZ induction, the blood glucose levels in HFD-fed rats increased significantly compared to normal rats ($p < 0.05$). The blood glucose levels after STZ induction reached > 200 mg/dL. After treatment using drug control and FPH, the blood glucose levels decreased significantly compared with the DM rat group ($p < 0.05$). The blood glucose decreased by 43-55% after FPH treatment. Furthermore, decreasing blood glucose levels after treatment were also close to the normal group. This result indicated that FPH could be used to decrease blood glucose under diabetic conditions and has an ability close to drug control.



Graph 2. Blood glucose levels of rats at the initial stage, diabetic stage, and final stage. Normal: Normal control rats; DM: STZ-induced diabetic rats; DM+FPH1: Diabetic rats treated with fish protein hydrolysate dosage 0.8 g/kg BW; DM+FPH2: Diabetic rats treated with fish protein hydrolysate dosage 1.6 g/kg BW. [abcde] Different superscript letters indicated significant differences ($p < 0.05$).

Effects of fish protein hydrolysate on leukocyte and lymphocyte levels

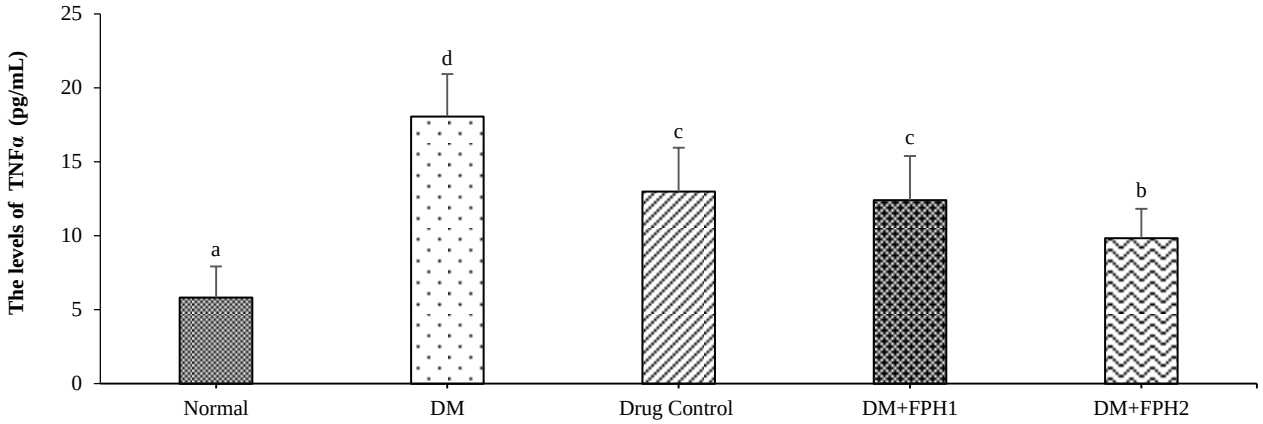
The hematology analysis on leukocytes and lymphocytes showed significant differences between groups (Graph 3, $p < 0.05$). The normal rats group showed the lowest number of both leukocytes and lymphocytes, while the DM rats group had the highest number of both leukocytes and lymphocytes. This result indicated that in diabetic conditions, the number of leukocytes and lymphocytes increased significantly ($p < 0.05$) compared to normal conditions. Furthermore, after treatment using drug control and FPH, the number of leukocytes and lymphocytes significantly decreased ($p < 0.05$). The drug control has a lower number of leukocytes and lymphocytes compared with FPH treatment, however, the treatment using FPH, especially at a dose of 0.8 g/kg BW, showed a significant difference compared with the DM rat group ($p < 0.05$).



Graph 3. Hematology analysis on the number of leukocytes and lymphocytes of normal and treatment groups in rats. Normal: Normal control rats; DM: STZ-induced diabetic rats; DM+FPH1: Diabetic rats treated with fish protein hydrolysate dosage 0.8 g/kg BW; DM+FPH2: Diabetic rats treated with fish protein hydrolysate dosage 1.6 g/kg BW. [abcde] Different superscript letters indicated significant differences ($p < 0.05$).

Effects of fish protein hydrolysate on levels of TNF-α in serum

The measurement of TNFα levels in the serum using ELISA resulted in the DM rats group having the highest TNFα levels and significantly different from other groups (Graph 4, $p < 0.05$). Treatment using drug control and FPH decreased the levels of TNFα in the serum significantly compared with DM rats ($p < 0.05$). Moreover, these treatments also improved TNFα levels almost similar to the normal group. This result suggested that treatment using FPH can reduce the pro-inflammatory cytokine TNFα in diabetic conditions.



Graph 4. The levels of TNFα in rats' serum samples. Normal: normal control rats; DM: STZ-induced diabetic rats; DM+FPH1: Diabetic rats treated with fish protein hydrolysate dosage 0.8 g/kg BW; DM+FPH2: Diabetic rats treated with fish protein hydrolysate dosage 1.6 g/kg BW. [abcd] Different superscript letters indicated significant differences ($p < 0.05$).

DISCUSSION

Diabetes mellitus is a global health concern, recognized as the seventh leading cause of mortality worldwide (Li et al., 2023). Diabetes mellitus, as a chronic metabolic disorder characterized by elevated blood glucose levels, poses significant health challenges for individuals worldwide (Shah et al., 2022). Moreover, the consumption of a high-fat diet (HFD) and changes in lifestyle lead to obesity in society. Obesity is identified as the most significant risk factor for the development of insulin resistance, which induces type 2 DM (T2DM; Ruze et al., 2023). A High-Fat Diet causes alterations in plasma membrane cholesterol and insulin binding and signaling (Sabapathy et al., 2022). Insulin facilitates glucose transport from the bloodstream into adipose cells and muscle via insulin receptors (Chadt and Al-Hasani, 2020). Accumulation of ectopic fat has an impact on insulin sensitivity, thus leading to high levels of glucose in the bloodstream (Merry et al., 2020). Obesity also contributed to impairing the antioxidant defense system, reducing the effectiveness of antioxidants like superoxide dismutase (SOD), catalase, and glutathione peroxidase leading to excessive oxidative stress (Čolak et al., 2020).

Researchers have been exploring alternative approaches to manage this menace, including the utilization of natural products (Olasehinde et al., 2021). Fish processing generates significant amounts of by-products, such as frames, bones, skins, and tails, which can be valuable sources of high-quality proteins and bioactive compounds (Caruso et al., 2020). Recent studies have highlighted the potential of bioactive hydrolysates and peptides derived from various food sources, including fish by-products (Kehinde and Sharma, 2020; Daskalaki et al., 2023; Ghalamara et al., 2024). Fish processing by-products have been reported as rich sources of bioactive material such as enzymes, polyunsaturated fatty acids (PUFA), collagen, gelatin, vitamins, minerals, and bioactive peptides (Le Gouic et al., 2018). Fish protein hydrolysate has gained attention for its potential therapeutic benefits, particularly in managing diabetes (Wan et al., 2023). Fish Protein Hydrolysate is produced by the enzymatic hydrolysis of fish proteins, resulting in a mixture of peptides and amino acids that exhibit various bioactive properties (Jafar et al., 2024). The hydrolysis process breaks down proteins into smaller peptides and amino acids, which are easier to digest and absorb (Abraha et al., 2017; Suma et al., 2023).

Skipjack tuna is a widely consumed fish species that generates significant by-products during processing, which may have untapped therapeutic potential (Ramu et al., 2022). Hydrolyzed proteins and peptides isolated from skipjack by-products have been the focus of growing scientific interest due to their reported anti-diabetic properties (Kehinde and Sharma, 2020). Based on the result of this study, the FPH from skipjack by-products decreased blood glucose levels significantly in diabetic rats. Fish protein hydrolysate might have had the ability to inhibit enzymes that are involved in carbohydrate metabolism, such as α-amylase and α-glucosidase. This result is supported by a previous study where FPH using papain enzyme resulted in the effective inhibition of α-amylase (Sarteshnizi et al., 2021). Inhibition of α-amylase

can manage the regulation of blood glucose levels (Kehinde and Sharma, 2020). By hindering the breakdown of complex carbohydrates into simpler sugars, these hydrolysates can potentially help mitigate postprandial hyperglycemia, a common challenge faced in the development of diabetes. Fish protein hydrolysate also has been reported as a function to increase insulin sensitivity (Wan et al, 2023). The bioactive peptides in FPH can enhance insulin sensitivity by modulating insulin signaling pathways, leading to improved glucose uptake by cells thus improving the blood glucose levels (Daskalaki et al., 2023). The mechanism of bioactive peptides from FPH to insulin resistance modulation by stimulating the secretion of glucagon-like peptide-1 (GLP1), which has a function to produce more glucose-dependent insulin from pancreatic β-cells (Elbira et al., 2024). On the other hand, the FPH peptides also act as inhibitors of dipeptidyl peptidase-IV (DPP4) activity, leading to increased glucose uptake and reduced blood glucose levels (Zhou et al., 2021).

On the other hand, DM significantly impacts the immune system and promotes chronic inflammation, leading to an increased risk of infections and diabetes-related complications (Gofur et al., 2024). Inflammation is associated with an increase in the production of pro-inflammatory cytokines and activation of leukocytes (Vaibhav et al., 2024). The findings revealed that the DM rats had the largest counts of leukocytes, lymphocytes, and elevated levels of the pro-inflammatory cytokine TNFα. After treatment, FPH showed a lowering in leukocytes, lymphocytes, and the levels of TNF-α (Graphs 3 and 4). Fish protein hydrolysate contains peptides with high antioxidant properties, which can decrease oxidative stress and lead to oxidative damage in the tissue and organ (Wan et al., 2023). Fish protein hydrolysate can protect pancreatic β-cells and enhance insulin production by reducing the effects of oxidative damage (Nikoo et al., 2023). Furthermore, it was reported that FPH supplementation has been shown to improve blood glucose levels, increase insulin sensitivity, and reduce oxidative stress and inflammation (Kehinde and Sharma, 2020). Oxidative stress is a major factor in the pathogenesis of diabetes, and the improvement of oxidative stress can repair the inflammation status, including leukocyte, lymphocyte, and pro-inflammatory cytokine TNF-α (Gambini and Stromsnes, 2022).

CONCLUSION

Fish protein hydrolysate (FPH) from skipjack tuna by-product ameliorates the blood glucose levels, improving the immune system activity and inflammatory markers in STZ-NA-induced diabetic rats. The hydrolysis process in fish by-products gains beneficial values, especially for anti-diabetic candidates. Further research on the benefits of FPH in various metabolic syndrome diseases is necessary to explore the function and benefits of skipjack tuna by-product protein hydrolysate.

DECLARATIONS

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Authors' contributions

Dwi Yanuar Budi Prasetyo carried out the *in vivo* animal model, performed analysis, and drafted the manuscript. Tri Winarmi Agustini participated in the study design and drafted the manuscript. Gemala Anjani participated in its design and coordination and helped draft the manuscript. Putut Har Riyadi performed fish hydrolysis and study design and drafted the manuscript. All authors read and approved the final manuscript.

Competing interests

The authors declared no conflict of interest.

Ethical considerations

This paper was originally written by the authors and has not been published elsewhere. The authors checked the text of the article for plagiarism index and confirmed that the text of the article is written based on their original scientific results.

Availability of data and materials

The original data presented in the study are included in the article. Data are available from the corresponding author upon reasonable request.

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Virulent Genes and Genetic Relationship of *Salmonella* spp. Isolated from Chickens and Husbandry Environments in Small-Scale Farms in the Mekong Delta, Vietnam

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ABSTRACT

Salmonella is one of the most severe pathogens causing diseases in poultry and humans, and several factors could become transmission vectors in the husbandry environment. This study was conducted from April to July 2024 to clarify the prevalence of common *Salmonella* serovars in chickens and the husbandry environment and their pathogenicity and genetic relationship in small-scale farms in the Mekong Delta, Vietnam. A total of 279 samples were randomly collected from fresh chickens' feces (n = 54), husbandry environment (n=81), and pests (n=144), including rats, geckos, and ants, in four small-scale farms to examine the prevalence of *Salmonella* spp. By the conventional isolation method, 75 samples were positive for *Salmonella*, accounting for 26.88%. The prevalence of *Salmonella* in chicken feces, the environment, and pests were 27.78%, 12.35%, and 34.72%, respectively. Of 75 positive *Salmonella* isolates, two common serovars were identified, including *S. Gallinarum* (13.33%) and *S. Enteritidis* (10.67%); however, *S. Pullorum* and *S. Typhimurium* were not detected using PCR. These *Salmonella* isolates were detected virulent genes by using PCR, and found that these isolates harbored several virulent genes, including *InvA* (100%), *fimA* (100%), *stn* (93.33%), *sopB* (89.33%), and *sodC1* (54.57%). The ERIC-PCR method was used to determine the genetic relationship among *Salmonella* strains carrying virulent genes present in chickens, environment, and pests in these small-scale farms. The results showed diversity in phenotype and similarity in the genetic relationship (more than 75% similarity) among *Salmonella* strains isolated from chicken feces and the livestock environment. In conclusion, the study indicated that pathogenic *Salmonella* serovars could survive and be transmitted among sources, including chickens, the husbandry environment, and pests in small-scale poultry farms in the Mekong Delta.

Keywords: Chicken, Environment, Genetic relationship, Pest, *Salmonella*, Virulent gene

INTRODUCTION

Nowadays, the demand for meat and poultry products has promoted the development of the poultry industry (Henchion et al., 2021). *Salmonella* is one of the severe infectious pathogens causing diseases that directly affect the health of livestock and result in heavy economic losses to farmers (Cortés et al., 2022). *Salmonella* is present in the environment, including soil, water, food, barns, and livestock equipment, and can cross-contaminate from one source to another intermediate species (Tabo et al., 2013). The complicated epidemiology of *Salmonella* is due to horizontal and vertical transmission routes, and animals with weak resistance or immunodeficiency will be susceptible to salmonellosis (Gast and Porter, 2020). Examining pests (shrews, mice, rats, flies, ants, cockroaches, and birds) living around the broiler chicken farms on Reunion Island validated that they were resources of *Salmonella* spp. and infected chickens in these farms (Etheves et al., 2021). Nguyen et al. (2021) previously reported that chickens, the environment, and pests were reservoirs of *Salmonella* in poultry farms and households in the Mekong Delta.

Salmonella has more than 3,500 different serovars recorded in animals and the environment. Of which, the common pathogenic *Salmonella* strains in chickens can be divided into two groups, non-motile strains, including *S. Pullorum*, causing dysentery in chicks; *S. Gallinarum*, causing chicken typhoid; and motile strains, mainly including *S. Enteritidis* and *S. Typhimurium* causing paratyphoid in animals and humans (Al-baqir et al., 2019; Wales and Lawes, 2023). The pathogenicity of *Salmonella* strains depends on the presence of several virulent genes encoded in *Salmonella* pathogenicity islands (SPIs), plasmids, and other gene cassettes (Pavon et al., 2022). Several virulence genes have

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essential roles in adhesion, invasion, intracellular survival, systemic infection, and toxin production of *Salmonella* in hosts, including *InvA*, *fimA*, *stn*, *sopB*, and *sodC1* (Kim and Lee, 2017; Tarabees et al., 2017). In research of Siddiky et al. (2021), all *Salmonella* isolated from chickens in wet markets of Bangladesh underwent polymerase chain reaction (PCR) screening for eight virulence genes—*invA*, *agfA*, *IpfA*, *hilA*, *sivH*, *sefA*, *sopE*, and *spvC*; the results revealed that *S. Enteritidis* carried all the genes while *S. Typhimurium* contained six genes, lacking *sefA* and *spvC*.

In the Mekong Delta, chickens are mainly raised in small-scale farms or households; thus, hygiene is not taken seriously because the low income of farmers affects the investment for these farms. Besides, managing risk factors, such as pests and the husbandry environment, was also limited. The transmission route of *Salmonella* in these farms was not determined. Therefore, this study elucidates the prevalence of common *Salmonella* serovars in chickens, environments, and pests and examines their genetic relationships. The findings contribute to a better understanding of *Salmonella* epidemiology in small-scale poultry farms, thereby aiding in the prevention of salmonellosis in chickens and humans in the Mekong Delta, Vietnam.

MATERIALS AND METHODS

Ethical approval

This study was conducted following the guidelines outlined in the Helsinki Declaration and the animal welfare and safety procedures of Can Tho University, Vietnam.

Isolation of *Salmonella*

From April to July 2024, 279 samples were collected from hybrid broilers (Noi chicken, a local chicken breed). These cocks were one-month-old with an average weight of 700 g, and raised in four small-scale farms in the center of the Mekong Delta, Vietnam (two farms in Vinh Long province, one farm in Can Tho City, and one farm in Hau Giang province). The Mekong Delta is located in the southern region of Vietnam, covering latitudes from 8°34' to 11°10' N and longitudes from 104°25' to 106°48' E. This region encompasses a total land area of approximately 40,547 square kilometers, with around 1.7 million hectares designated for agricultural use. The area's air temperature typically ranges between 26.5 and 29.3°C, while the average annual precipitation fluctuates between 1,287.6 and 2,832.8 mm, with 80% of this rainfall occurring during the wet season (Vu-Thanh et al., 2014; Lee and Dang, 2019; Dinh and Dang, 2022).

These farms raised about 1,000 broilers/farms. The samples were collected, including fresh chicken feces (n = 54), bedding (n = 36), feed (n = 27), drinking water (n = 18), rats (n = 18), geckos (n = 54), and ants (n = 72) in these farms. In this study, the number of samples collected was based on the number of chickens, the farm design, and the captured pests in each farm when collecting samples. The average number of samples collected on each farm includes 13 chicken feces, 9 bedding samples, 6 drinking water samples, and 36 pests.

Chicken feces (about 1 g) were collected via cloacal swabbing and put into Cary-Blair medium (Merck, Germany), while environmental samples comprising 250 g of feed, 1,000 mL of drinking water, and 250 g of bedding were obtained directly from the farms, placed in sterilized bags, and stored at 2-8°C. Pest animals, including geckos, ants, and rats, were captured in traps and housed separately in sterilized plastic boxes with ventilation. All pest' samples were transported to the laboratory on the same day as collection. Geckos and rats were dissected to collect feces in the rectum, while whole bodies of ants were used in this study. The procedures for animal dissection and feces collection followed the laboratory biosafety guidelines of Can Tho University and the guidelines of Nguyen et al. (2021).

Salmonella was isolated on Brilliant-green Phenol-red Lactose Sucrose agar (BPLS, Merck, Germany) and examined for biochemical characteristics, such as triple-sugar iron fermentation, VP test, urea test, H_2S, lysine, and idol mobility test following previously described by Tran et al. (2004) and Nguyen et al. (2021).

Identification of *Salmonella* serovars

This study used the PCR method to identify four *Salmonella* serovars, including *S. Gallinarum*, *S. Pullorum*, *S. Enteritidis*, and *S. Typhimurium*, which could commonly cause disease in chickens and humans. Firstly, the DNA of *Salmonella*-positive strains was extracted using the TopPURE Genomic DNA extraction kit (ABT, Vietnam), following the manufacturer's guidelines. Then, it was stored at -20 °C for further use. The primer sequences and PCR conditions for the detection of *Salmonella* serovars were carried out following the guidelines of Paião et al. (2013) for *S. Enteritidis* and *S. Typhimurium* and Xiong et al. (2018) for *S. Gallinarum* and *S. Pullorum*. The kit of Mastermix 2X (Bioline, Canada) was used in these PCR reactions. A total volume of 25 µl PCR reaction included 12.5 µl of Mastermix, 0.5 µl of forward primer, 0.5 µl of reverse primer, 9.5 µl of distilled water, and 2.0 µl of DNA of *Salmonella* strains. The purified water served as the negative control. *Salmonella* serovars isolated previously from chickens in the Mekong Delta served as a positive control and were maintained at the Veterinary Food Hygiene Lab, Faculty of Veterinary Medicine, College of Agriculture, Can Tho University.

Detection of virulent genes

The PCR procedure was conducted to detect virulent genes of *Salmonella* isolates, similar to the method used to identify *Salmonella* serovars in the previous experiment. This study detected five virulent genes, including *InvA, fimA, stn, sopB,* and *sodC1*. The primers and thermocycling were carried out as described by Li et al. (2021).

Genetic relationship of *Salmonella* isolated from chicken and environment

The Enterobacterial Repetitive Intergenic Consensus Polymerase Chain Reaction (ERIC-PCR) method was used to determine the genetic relationship between *Salmonella* strains isolated from chicken feces, the environment, and pests in small-scale farms. The mixture composition of each ERIC-PCR reaction was similar to that of the PCR reaction used to identify serovars and recommendations by Tawfik et al. (2022).

The ERIC-PCR primers (Forward: 5'-ATGTAAGCTCCTGGGGATTCAC-3', Reverse: 5'-AAGTAAGTGACTGGGGTG AGCG-3') and thermocycling were carried out as described by Tawfik et al. (2022). The electrophoresis figure was inserted and analyzed by GelJ software (GNU General Public License version 3.0) following the guidelines of Heras et al. (2015).

Statistical analysis

The Chi-square test was used to determine the difference in the prevalence of *Salmonella* and its virulent genes detected from chickens and the environment. The Pearson chi-square statistic was used at the significance level of 95% with $p < 0.05$ in the Minitab 17.0 software (Minitab Pty Ltd, Australia).

RESULTS

In this study, *Salmonella* was detected at a high rate (26.88%) in the collected samples of small-scale farms in the Mekong Delta (Table 1). The presence of *Salmonella* in chickens' feces (27.78%) and pests (34.72%) was higher than that in the husbandry environment (12.35%, $p > 0.05$). In environmental samples, *Salmonella* was detected from bedding (16.67%) and feed (14.81%); however, *Salmonella* was not found in drinking water. In pests, *Salmonella* was detected at the highest rate in gecko feces (70.37%).

Of 75 positive *Salmonella* samples, 75 *Salmonella* isolates were selected (one isolate/sample) to identify *Salmonella* serovars. The results indicated that *S. Typhimurium* and *S. Pullorum* were not detected in this study. In contrast, *S. Enteritidis* and *S. Gallinarum* were detected at a relatively high rate of 10.67% and 13.33%, respectively (Table 2). Among them, *S. Gallinarum* was mainly found in chickens' feces (46.67%), while *S. Enteritidis* was detected in both chicken feces (13.33%), environment (10.00%), and pests (10.00%).

Of 75 *Salmonella* isolates examined, the genes *InvA* (100%) and *fimA* (100%), followed by *stn* (93.33%), *sopB* (89.33%), and *sodC1* (54.57%), were harbored. There was no significant difference ($p > 0.05$) in the presence of each virulent gene among identified *Salmonella* serovars, and all pathogenic genes could be found in *S. Enteritidis* and *S. Gallinarum* (Table 3).

Using ERIC-PCR, the results indicated that *Salmonella* isolated from chickens, the environment, and pests showed diverse genetic relationships, with eighteen patterns obtained, which were noted from P1 to P18 in Figure 1. The results especially revealed the close genetic similarity between *S. Enteritidis* isolated from feces and bedding, geckos, and rats (Pattern 9), and *S. Gallinarum* isolated from feces, bedding, and geckos (Pattern 11), with more than 80% similarity. On the other hand, other *Salmonella* isolates showed homologous patterns (from 50% to 75%) among isolates from chickens, the environment, and pests. Moreover, most of the *Salmonella* isolates shared the same virulent gene patterns among *Salmonella* strains from chicken feces, the environment, and pests in these farms.

Table 1. Prevalence of *Salmonella* in chickens, environment, and pests in small-scale farms in the Mekong Delta, Vietnam, from April to July 2024

Samples	No. of examined samples	No. of positive samples	Percentage (%)
Feces	54	15	27.78[a]
Environment			
Bedding	36	6	16.67
Drinking water	18	0	0.00
Feed	27	4	14.81
Subtotal	81	10	12.35[b]
Pests			
Rat	18	8	44.44
Gecko	54	38	70.37
Ant	72	4	5.56
Subtotal	144	50	34.72[a]
Total	279	75	26.88

[a,b]: These letters indicate the significant statistical difference at 95% confidence; No: The number of; Subtotals is for each factor: chickens, environment, and pests; Total is for all samples collected.

Table 2. Distribution of identified *Salmonella* serovars by PCR in chickens, environment, and pests in small-scale farms in the Mekong Delta, Vietnam, from April to July 2024

Samples	No. of examined isolates	*S. Enteritidis*		*S. Gallinarum*	
		No. of positive isolates	Percentage (%)	No. of positive isolates	Percentage (%)
Feces	15	2	13.33[a]	7	46.67[a]
Environment					
Bedding	6	1	16.67	2	33.33
Feed	4	0	0.00	0	0.00
Subtotal	10	1	10.00[a]	2	20.00[a]
Pests					
Rat	8	2	25.00	0	0.00
Gecko	38	2	5.26	1	2.63
Ant	4	1	25.00	0	0.00
Subtotal	50	5	10.00[a]	1	2.00[b]
Total	75	8	10.67	10	13.33

[a,b] The letters indicate the significant statistical difference at 95% confidence in each column; No: The number of; *S.: Salmonella;* Subtotal is for each factor: chickens, environment, pests; Total is for all samples collected.

Table 3. Presence of virulent genes in *Salmonella* isolated from chickens, environment, and pests in small-scale farms in the Mekong Delta, Vietnam, from April to July 2024

Genes	*S. Enteritidis* (%) (n=8)	*S. Gallinarum* (%) (n=10)	Other serovars (%) (n=57)	Total (%) (n=75)
InvA	8 (100.00)	10 (100.00)	57 (100.00)	75 (100.00)
fimA	8 (100.00)	10 (100.00)	57 (100.00)	75 (100.00)
stn	7 (87.50)	8 (80.00)	55 (96.49)	70 (93.33)
sopB	8 (100.00)	9 (90.00)	50 (87.72)	67 (89.33)
sodC1	6 (75.00)	4 (40.00)	31 (54.39)	41 (54.57)

S.: Salmonella

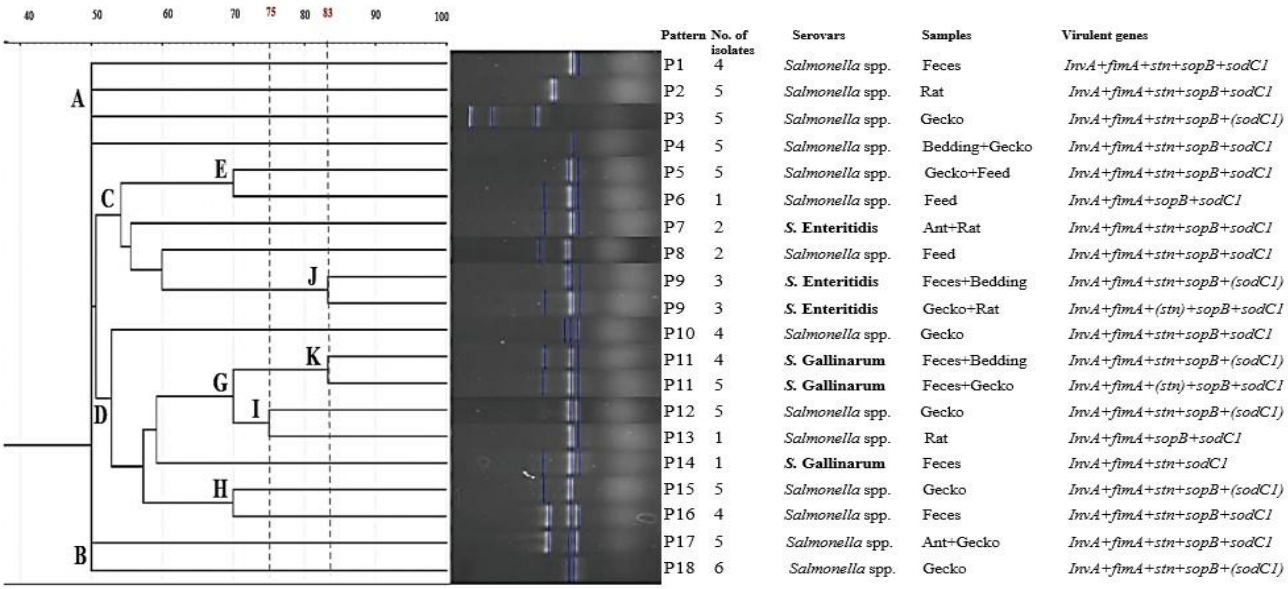

Figure 1. The dendrogram of the genetic relationship of *Salmonella* isolated from chickens, the environment, and pests in small-scale farms in the Mekong Delta, Vietnam, from April to July 2024. The dendrogram revealed diverse patterns of *Salmonella* isolated in small-scale farms (18 gene patterns); Moreover, there was a homogenous genetic characteristic among *S. Enteritidis* (P9, 83.00%) and *S. Gallinarum* (P11, 83.00%) isolated from chicken feces, environment, and pests in these farms. The gene, which was put in the bracket, is present or absent in some isolates in one group; P: Pattern

DISCUSSION

Chicken manure can contain *Salmonella* after being excreted; it can contaminate the environment and surrounding pests. In addition, farmers have raised several domestic animals, such as cattle, ducks, and dogs, on these small-scale farms, which could increase the risk of *Salmonella* contamination among animals and the environment (Lowenstein et al., 2016). There is a close relationship and interaction between livestock, the environment, and wildlife regarding hygiene; animals in the area are sources of pathogens and vectors for *Salmonella* infection (Tessier et al., 2016; Ame et al., 2022). In the study of Nguyen et al. (2021), *Salmonella* was isolated at a low rate in farms and households in the Mekong Delta, including 7.67% in chickens' feces, 4.33% in the environment, and 5.98% in wild animals. In commercial chicken farms in Nigeria, a farm-level prevalence of 47.9% and a sample-level prevalence of 15.9% for *Salmonella* were recorded (Jibril et al., 2020). Compared to the findings of this study in the Mekong Delta, Vietnam, these differences in the prevalence of *Salmonella* in chickens and the husbandry environment could be affected by the sampling location, hygiene conditions, and farm scales. Moreover, poorly preserved leftover food is a potential food source for pests, which can be contaminated with *Salmonella* through their feces and contact with their bodies (Gwenzi et al., 2021). According to research by Gosling et al. (2022), the prevalence of *Salmonella* in water, feed, and litter (husbandry waste) caused *Salmonella* contamination in chicken farms, which was harmful to human health. The bedding, where waste materials from chicken farming activities are stored, and chickens come into direct contact with feces (Dunn et al., 2022). Chickens walking and digging increase contact and diffusion of feces, increasing the risk of infection by harmful microorganisms in general and *Salmonella* in particular (Chinivasagam et al., 2010). Furthermore, this study showed that *Salmonella* was detected at a high rate in geckos in small-scale farms in the Mekong Delta, Vietnam. It was the same statement of Nguyen et al. (2021) regarding geckos and ants being a source of *Salmonella* on poultry farms. In other reports, ants living in the residential areas sporadically contain *Salmonella* at rates of 8% in Mauritius (Simothy et al., 2018), and Etheves et al. (2021) indicated that pests (shrews, mice, rats, flies, ants, cockroaches, and birds) were resources of *Salmonella* infection to chickens in the broiler farms on Reunion Island. Therefore, the prevalence of *Salmonella* in chickens and the environmental agents could become a source of *Salmonella* outbreaks in chickens and humans in these small-scale farms in the Mekong Delta.

The prevalence of the same *Salmonella* serovars in most of the samples (feces, environment, and pests) showed contamination among chickens, the environment, and pests in these small-scale farms in the Mekong Delta. Wales and Lawes (2023) stated that *S. Gallinarum* and *S. Pullorum* are limited to poultry and can be transmitted vertically and horizontally to cause fowl dysentery or typhoid. Haque et al. (2021) reported that *S. Gallinarum* was detected in 25.75% of samples in small-scale layer flocks in Bangladesh and highlighted the urgent need for effective control measures to reduce the prevalence of antibiotic-resistant *S. Gallinarum* in these farms to promote improved egg production and bolster food security and safety in resource-limited environments. Shalaby et al. (2021) reported a higher infection rate in younger broiler chicks in Egypt and identified isolates primarily as *S. Enteritidis*, *S. Shangani*, and others. In Iran, Bahramianfard et al. (2021) clarified that 2.3% of examined poultry samples and 1.3% of eggs were contaminated precisely with *S. Enteritidis*. In Singapore, Aung et al. (2020) conducted the epidemiological distribution of *Salmonella* serovars in humans, food, animals, and the environment. Their findings demonstrated that *S. Enteritidis* was the most prevalent serovar among isolates from chicken (28.5%) and egg products (61.5%). In contrast, over 80% of isolates from farms and wildlife were identified as serovars distinct from *S. Enteritidis* or *S. Typhimurium*. Thus, it underscored the importance of a coordinated one-health approach for enhanced surveillance of *Salmonella* epidemiology.

Virulent genes are essential for the survival and pathogenicity of *Salmonella* in hosts. *Salmonella* has been observed to gain virulence from other species via horizontal gene transfer, which is believed to be a primary factor in the evolution and emergence of highly pathogenic strains (Van Asten and Van Dijk, 2015; Zakaria et al., 2021). The high presence of virulence genes in *Salmonella* isolates identified in this study exhibited significant virulence, potentially leading to severe disease outcomes in susceptible humans and animals. El-Saadony et al. (2022) reported that various *Salmonella* species possess numerous virulent genes that enhance their pathogenic potential, with the *invA* gene being the most prevalent among the examined isolates. Zakaria et al. (2021) observed that the virulence genes present in *Salmonella*, particularly *S. Enteritidis*, were obtained from chickens in Malaysia. These genes predominantly included *PefD, SpvC, Spv, Rck, SseK1, T3SS, InvA*, and *Spa*. Shittu et al. (2022) identified the genes *InvA* and *sopB* (100%) in *Salmonella* strains isolated from the feces of layer chickens in Nigeria. In other research, all *Salmonella* spp. strains isolated from broiler chickens in Colombia contained virulence genes *(lpfA, csgA, sitC, sipB, sopB, sopE*, and *sivH*), which were also detected in humans within the same region. Identifying virulent genes in *Salmonella* from broilers and humans raises concerns regarding potential public health risks in Colombia (Lozano-Villegas et al., 2023). In addition, Shu et al. (2022) stated that *Salmonella* isolates from chicken in China frequently carried extended-spectrum beta-lactamases (ESBLs),

such as *blaTEM*, *blaOXA*, and *blaCTX-M*, and various virulence genes, including *invA*, *stn* (100%), *sopE* (94.87%), *spvR* (87.18%), *ssaQ* (85.47%), *avrA* (77.78%), *spvB* (71.79%), *bcfC* (69.23%), *spvC* (54.70%), *sopB* (51.28%), and *mgtC* (29.06%). These genes were horizontally transferred, significantly contributing to the spread of antimicrobial resistance and pathogenesis, thereby enhancing the pathogenic potential of *Salmonella* through the interplay of resistance and virulence factors. Kanaan et al. (2022) reported that the distribution of various virulence factors was observed, such as *phoP/Q* (40.0%), *traT* (30.0%), *stn* (22.0%), *slyA* (11.0%), and *sopB* (9.0%) in *Salmonella* isolated from chicken meat and egg samples in Iraq, especially in carbapenem-resistant *S. Enteritidis* isolates. Carbapenem-resistant *S. Enteritidis* contains various virulent and antibiotic resistance genes in chicken meat and egg samples; it poses the issue that hygienic practices are essential to prevent *Salmonella* transmission from animals to humans. In the study in the Mekong Delta, virulent genes, which were selected to clarify, were not specific for each *Salmonella* serovar and were still limited. Therefore, studies on the prevalence of virulent genes in *Salmonella* isolated from chickens and the environment should be conducted more to evaluate the pathogenicity of *Salmonella* circulating in the Mekong Delta region, Vietnam.

Gast and Porter (2020) indicated that *Salmonella* can be transmitted from chickens to the environment and vice versa. Consequently, it is crucial to comprehend the epidemiology of *Salmonella* in small-scale farms to avert disease outbreaks or further transmission. In this study, *Salmonella* isolated from chickens, the environment, and pests showed diverse genetic relationships and close genetic similarity between *S. Enteritidis* isolated from feces and bedding, geckos, and rats (Pattern 9), or *S. Gallinarum* isolated from feces, bedding, and geckos (Pattern 11). These *Salmonella* isolates also shared the same virulent patterns. These serovars were mainly detected in chickens' feces (Table 2); this demonstrated that *Salmonella* isolates could be transmitted from chickens to the environment and pests. In contrast, the environment and pests might become a source of *Salmonella* and contaminate chickens. The other *Salmonella* homologous patterns (from 50% to 75%) proved the transmission ability of *Salmonella* isolates among chickens, the environment, and pests. Zhao et al. (2016) used ERIC-PCR to analyze the genetic characteristics of *Salmonella* isolated from free-range chickens in China and found diverse gene patterns belonging to three genotypes. These genotypes were also found in humans previously; thus, free-ranging chickens could act as potential reservoirs for pathogenic *Salmonella*, representing a risk to public health. In South Africa, Ramtahal et al. (2022) reported that distinct ERIC-PCR patterns were identified across various *Salmonella* subtypes isolated from poultry, and they concluded that poultry and their environments were reservoirs for resistant and pathogenic *Salmonella* strains. Elsayed et al. (2024) reported that ERIC-PCR was effectively utilized to create biologically significant clusters of *Salmonella* strains, revealing various genetic patterns and relationships among *Salmonella* isolated from chickens and their husbandry environment in Egypt. Therefore, understanding the genetic diversity of *Salmonella* in chicken farms was essential for protecting chicken health and humans.

CONCLUSION

There was a high prevalence of *Salmonella* detected from chickens, environments, and pests in small-scale farms in the Mekong Delta, Vietnam. In addition, *S. Enteritidis* and *S. Gallinarum* were commonly detected, and these *Salmonella* isolates harbored virulent genes in the function of adhesion, invasion, intracellular survival, systemic infection, and toxin production, including *InvA, fimA, stn, sopB,* and *sodC1*. Moreover, there was a close genetic relationship between *Salmonella* isolated from chickens, the environment, and pests in these farms. It indicated that there was a transmission of *Salmonella* among these factors, especially the environment and pests, which could become a source of *Salmonella* in chickens. Therefore, working on hygiene status in small-scale farms is essential to prevent chicken salmonellosis outbreaks.

DECLARATIONS

Competing interests

The authors declare that they have no conflicts of interest.

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Authors' contributions

Thuan Khanh Nguyen, Luan Minh Huynh, Tan Van Duy Vo, and Duy Duc Tran conceptualized, designed, and supervised the research. Thuan Khanh Nguyen and Luan Minh Huynh critically reviewed the study. Tan Van Duy Vo, Duy Duc Tran, Thu Thanh Kha, and Chi Thi Hanh Nguyen collected samples and processed the data. Thu Thanh Kha, Khai Thi Lien Ly, and Chi Thi Hanh Nguyen analyzed and interpreted the data. All authors revised and approved the final 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 checked the validity of the data before writing the manuscript. This article was written originally without any copy from data from published articles and books.

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The Effect of Adding Various Concentrations of Melatonin to Beltsville Thawing Solution Diluent on Berkshire Boar Semen Quality

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ABSTRACT

Boar spermatozoa contain polyunsaturated fatty acids, rendering them susceptible to damage from free radicals. Oxidative stress in liquid semen can be prevented by modifying the diluent by adding antioxidants. Melatonin is an indoleamine compound that can be used as an antioxidant with high potential in the reproductive system. This study aimed to determine the effect of adding various concentrations of melatonin to Beltsville Thawing Solution (BTS) diluent on the quality of Berkshire boar semen. A Completely Randomized Design (CRD) method was employed, with the experiment divided into four treatment groups, each replicated six times. A total of 24 samples were used in this study. P0 was used as the control group, while groups P1, P2, and P3 were given the addition of melatonin to BTS diluent with different doses, namely 0.5 mM, 1.0 mM, and 1.5 mM. The samples were stored for 48 hours and then examined for boar semen quality. The variables examined included the percentage of motility, abnormality, viability, plasma membrane integrity, and malondialdehyde (MDA) levels. The results showed that the addition of a melatonin dose of 1.0 mM to the BTS diluent was the optimal concentration that could maintain motility, abnormalities, viability, plasma membrane integrity, and MDA levels in Berkshire boar semen compared to other treatment groups and the control group. This study indicated that melatonin functions as an effective antioxidant, neutralizing free radicals and thereby inhibiting oxidative stress in Berkshire boar semen.

Keywords: Beltsville thawing solution, Berkshire boar, Malondialdehyde, Melatonin, Semen quality

INTRODUCTION

Pigs (*Sus scrofa*) are a significant commodity in the livestock sector, having been farmed for a long time and continuing to hold potential for further development. Pigs have several advantages, such as a fast growth rate (Sarajar et al., 2019), are included in polytocus animals (giving birth to many) with the ability to farrow twice a year or even five times in two years (Armini et al., 2019), and a high feed conversion ratio, yielding a carcass percentage of 65-80% (Wijaya et al., 2019). Giarda and Nugrahini (2020) explained that pork, a major livestock product, is in high demand globally, with worldwide consumption reaching 119,845,000 tons in 2018. According to Susana et al. (2014), as much as 10% of the meat requirements are provided by pigs. In addition, the high demand for pork as a means of traditional and religious customs has also resulted in an increasing need for pork in society.

To fulfill the growing demand for pork, it is essential to increase the pig population. To achieve the goal of increasing the genetic population of pigs, artificial insemination (AI) technology can be utilized through the provision of spermatozoa sources originating from superior-quality males (Sumardani et al., 2008). In addition to other factors, such as the skills of the inseminator, the main success rate in the utilization of AI is influenced by the quality of the boar semen. The processes of dilution and storage are crucial for obtaining the best boar semen quality. At the dilution stage, it is necessary to observe the diluent use. Bebas et al. (2016) mentioned several requirements for semen diluent, including the provision of nutrients as a source of energy for spermatozoa, prevention of cold shock, containing substances that can stop or inhibit the activity of bacteria in semen, acting as a buffer to prevent changes in pH, and maintaining a balance of osmotic and electrolyte pressure. These requirements are critical because fresh semen cannot remain viable for more than 24 hours without proper treatment. Beltsville Thawing Solution (BTS) is one of the diluents currently used for boar semen. According to Foeh et al. (2016), BTS is a diluent with a short shelf life of 1 to 3 days. Nahak et al. (2022) reported that BTS can maintain motility, viability, spermatozoa abnormalities, and pH of boar semen for up to two days of storage. Similarly, Thema et al. (2022) found that BTS preserves the viability, morphology, and motility of boar spermatozoa for 48 hours at 18°C. However, during storage, spermatozoa undergo metabolic processes that not only produce energy but also generate free radicals, which can damage the spermatozoa membrane through lipid

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peroxidation reactions (Leyn et al., 2021). The produced free radicals gradually cause a decrease in semen quality. This is because the plasma membrane of boar spermatozoa has a high level of Polyunsaturated Fatty Acids (PUFA) in phospholipids, making spermatozoa vulnerable to the effect of free radicals (Gena et al., 2021). Bebas et al. (2016) explained that the formation of ROS can cause spermatozoa to experience abnormalities and death. The formation of free radicals also causes an increase in Malondialdehyde (MDA) levels, which is the end product of lipid peroxidation that serves as an indicator of oxidative stress (Gena et al., 2021).

Sperm damage during storage caused by free radicals can be prevented by modifying the diluent solution by adding antioxidant compounds. Recently, there have been many efforts to use antioxidants as additional ingredients in semen diluents to maintain spermatozoa quality (Chankitisakul, 2014). Melatonin (N-acetyl-5-methoxytryptamin) is a compound emerging as an antioxidant. According to Tarocco et al. (2019), melatonin is a molecule that is widely found in nature with several functions, showing extraordinary versatility and diversity. As an antioxidant, melatonin has good potential in improving the reproductive system, particularly in enhancing sperm quality. Its antioxidant properties are manifested either directly through the neutralization of free radicals or indirectly by inducing the activity and expression of major antioxidant enzymes, such as glutathione peroxidase, catalase, and superoxide dismutase (Lavrentiadou et al., 2023).

Studies on melatonin supplementation as an antioxidant have been conducted on spermatozoa in various species, including deer, sheep, horses, buffaloes, and cattle (Chankitisakul, 2014; Li et al., 2019). However, data regarding its effects on boar spermatozoa remains limited. Therefore, this study was conducted to determine the effect of adding melatonin compounds at various concentrations to BTS diluent on the quality of Berkshire boar semen.

MATERIALS AND METHODS

Ethical approval

The Berkshire boars used in this study were handled by workers with expertise in animal care in their fields. This study received ethical permission from the Animal Testing Ethics Committee of the Faculty of Veterinary Medicine, Udayana University, Indonesia, under the reference number B/36/UN14.2.9/PT.01.04/2025.

Study design

This study did not involve the direct use of animals as research objects. The Berkshire boar semen used was obtained from the Regional Artificial Insemination Center in Baturiti, Tabanan, Bali, Indonesia. Semen collection was performed by competent officers in their fields using the hand-glove massage technique. Observations of semen quality were carried out at the Reproduction Laboratory, Faculty of Veterinary Medicine, Udayana University, Denpasar, Indonesia. Malondialdehyde (MDA) level analyses were carried out at the Integrated Biomedical Laboratory, Faculty of Medicine, Udayana University, Denpasar, Indonesia. In this study, melatonin was added to the BTS diluent at varting doses for each treatment group. The semen was then diluted with the diluent solution to which melatonin had been added at various doses. The semen that had been treated was then stored in the refrigerator at a temperature of 15-20°C for 48 hours. Observations of semen quality consisted of motility, abnormalities, viability, plasma membrane integrity, and MDA levels.

The materials used included Berkshire boar semen, Beltsville Thawing Solution (BTS®) diluent (Minitube, Germany), crystal melatonin (Sigma-Aldrich, USA), eosin-negrosin, Hypo Osmotic Swelling (HOS) solution, aquabides, ethanol, and physiological NaCl. The equipment used included a semen container, a micropipette, a thermometer, an Erlenmeyer flask, a Microscope Binocular Olympic (Olympus Corporation, Japan), a 10 ml plain vaculab tube, a pair of object glass, a pair of cover glass, a Porcine Malondialdehyde Enzyme-Linked Immunosorbent Assay (ELISA) kit No. E0151Po (BT LAB, China), a cool box, a 2 ml eppendorf tube, ice cubes, a water bath, a pair of hand gloves, a mask, an incubator, a magnetic stirrer, a centrifuge, and an ELISA microplate reader. This experimental study employed a completely randomized design. The samples were divided into four treatment groups and six replications in each group. The treatment groups were as follows: P0 (Control group, without melatonin), P1 (BTS + Melatonin 0.5 mM), P2 (BTS + Melatonin 1.0 mM), and P3 (BTS + Melatonin 1.5 mM).

Diluent preparation

Crystalline Melatonin M5250 (Sigma-Aldrich, USA) was added to the BTS diluent. To make the diluent usable, calculations were made to determine the melatonin preparation and the volume of BTS required using the molarity formula. From the calculation results, it became clear that mixing 1 ml of melatonin and 215 ml of BTS led to a BTS diluent with a melatonin concentration of 1 mm. The preparation of other concentrations followed the above calculations. Each diluent was homogenized using a magnetic stirrer and incubated at 37°C.

Evaluation of sperm motility

Spermatozoa motility was assessed by placing one drop of semen on a glass slide and examining it under a binocular microscope (Olympus Corporation, Japan) at 400x magnification. A minimum of 5-10 fields of view were observed for evaluation (Sumardani et al., 2019).

Evaluation of sperm abnormality and viability

Sperm abnormalities and viability were evaluated by making a smear preparation using eosin-nigrosin dye. Evaluation of spermatozoa abnormalities was based on morphological abnormalities that occur in the head to tail of the spermatozoa. In the evaluation of viability, live and dead spermatozoa were distinguished based on their color absorption ability. Dead spermatozoa appeared red, while live spermatozoa remained unstained/transparent (Baku et al., 2022). The evaluation was carried out using a microscope with a magnification of 400×. Evaluation of spermatozoa abnormality was counted in a minimum of 10 fields of view with the number of all normal and abnormal spermatozoa counted as 200 cells (Butta et al., 2021).

Evaluation of plasma membrane integrity

Plasma membrane integrity was evaluated using the Hypo Osmotic Swelling Test (HOST) method. A total of 20 ml of hypoosmotic solution was added to 0.2 ml of semen, mixed until homogeneous, and then incubated at 37°C for 45 minutes (Bebas dan Agustina, 2022). After incubation, 0.2 µL of the solution was dropped onto a glass for further observation using a microscope with a magnification of 400×. The number of spermatozoa was observed in at least 200 cells in 10 fields of view (Nofa et al., 2017). Spermatozoa with intact plasma membranes exhibited circular or bulging tails, while those with damaged membranes had straight tails.

Evaluation of malondialdehyde level

A Malondialdehyde level test was conducted to measure the degree of oxidative stress in spermatozoa after treatment. The ELISA kit used in the study was Porcine Malondialdehyde ELISA kit No. E0151Po (BT LAB, China), is a special kit for detecting MDA in various tissues, serum, plasma, and other biological fluids in pigs. The ELISA test is based on the binding of antigens in the sample to antibodies in the kit wells. The research procedure was carried out in accordance with the ELISA Kit's user manual.

Statistical analysis

Data were analyzed using analysis of variance (ANOVA) in IBM SPSS Statistics version 26. If significant differences ($p < 0.05$) were detected, post-hoc analysis was performed using Duncan's Test.

RESULTS AND DISCUSSION

The macroscopic evaluation showed that the collected volume of fresh Berkshire boar semen was 280 ml and had a milky white color, watery consistency, and typical odor. Based on microscopic results, the fresh semen had 78% motility, 90% viability, and 4% abnormality. Evaluation of fresh semen quality is essential in determining the quality of spermatozoa produced by each animal (Komariah et al., 2020). Normally, fresh boar semen has a milky white color with a typical odor. Butta et al. (2021) also noted that boar semen is voluminous, with ejaculate volumes ranging from 100 to 500 ml. Changes in color, such as a pinkish hue, may indicate bleeding or infection in the urinary tract. In addition, Tamoes et al. (2014) explained that qualified fresh semen should have a motility percentage of ≥ 60%, a live spermatozoa percentage of ≥ 70%, and an abnormality rate of ≤ 20%. Based on these observations, the fresh boar semen in this study was of high quality and, therefore, suitable for further processing. The results of the evaluation of Berkshire boar spermatozoa quality after treatment are presented in Table 1.

Table 1. The evaluations of Berkshire boar sperm quality (Mean ± SD) diluted with BTS after adding various concentrations of melatonin

Result / Components	P0	P1	P2	P3
Motility (%)	40.83 ± 0.98^{a}	45.16 ± 1.32^{b}	49.33 ± 0.81^{c}	40.00 ± 0.63^{a}
Abnormality (%)	7.16 ± 0.75^{b}	$6.83 \pm 0.98^{a,b}$	6.00 ± 0.89^{a}	7.33 ± 1.03^{b}
Viability (%)	49.67 ± 1.50^{ab}	51.00 ± 0.89^{a}	61.00 ± 1.26^{c}	49.33 ± 0.81^{b}
Plasma Membrane Integrity (%)	50.33 ± 0.81^{a}	55.50 ± 1.04^{b}	62.16 ± 0.75^{c}	49.33 ± 0.81^{a}
MDA Levels (ng/ml)	1979.39 ± 108.76^{b}	1997.13 ± 134.03^{b}	1792.11 ± 106.42^{a}	2050.53 ± 147.34^{b}

[abc] Different superscript letters in the same rows indicate significantly different results ($p < 0.05$). P0: Control Group; BTS Only; P1: BTS + Melatonin 0.5 mM; P2: BTS + Melatonin 1.0 mM; P3: BTS + Melatonin 1.5 mM. MDA: Malondialdehyde.

Evaluation of sperm motility

The motility, or progressive movement, of spermatozoa is the simplest assessment in determining semen quality. Spermatozoa motility is very important because it should move forward in the female reproductive tract and fertilize the ovum (Baku et al., 2022). The movement of spermatozoa is facilitated by flagella, with the main driving force being axonema, which is formed by microtubules derived from centrioles in the cell nucleus. The progressive movement or motility of spermatozoa is caused by friction between microtubules due to the presence of oxygen derived from dynein (Gazali and Tambing, 2002). At this stage, the availability of oxygen is required to convert chemical energy into mechanical energy. In addition, Solihati et al. (2020) explained that the processing of fresh semen into liquid semen allows a lot of contact between semen and the outside air, which contains reactive oxygen species (ROS). Semen containing excessive ROS levels will cause accelerated metabolism and end in the formation of free radicals. The continuous formation of free radicals' results in lipid peroxidation reactions that are autocatalytic and very difficult to stop. In this case, antioxidant compounds can suppress or prevent oxidative stress by neutralizing free radicals (Feradis, 2009). In this study, the results showed significant differences ($p < 0.05$) across most groups. In groups P0 and P3, however, there was no significant difference ($p > 0.05$). The P2 treatment group had the highest average percentage of motility compared to the other groups, amounting to 49.33 ± 0.81%. The lowest average percentage of spermatozoa motility was obtained in the P0 control group, reaching 40.83 ± 0.98%. Nevertheless, the addition of excessive antioxidant compounds can also have adverse effects since compounds that are given in excess will cause toxicity, so compounds that were originally antioxidants turn into prooxidants. In general, high dosages of antioxidants can act as prooxidants, bind with specific molecules to generate free radicals, or do so because of other reasons. Under certain conditions, melatonin can increase singlet oxygen, peroxy radicals, and hydroxyl radicals (Sotler et al., 2019). However, no study has yet explained in detail the role of melatonin as a prooxidant in semen quality. Karepu et al. (2020) stated that a prooxidant is a compound that can encourage the oxidation of cell components involving free radical compounds. This theory is consistent with the results of the motility observation of group P3, which was treated by adding 1.5 mM melatonin to the BTS diluent. In this group, the average percentage of motility obtained was very low, which amounted to 40.00 ± 0.63%. This result was at the threshold of the Indonesian National Standard (SNI 8030: 2014), which stated that the minimum percentage of semen motility that was suitable for use in artificial insemination was 40% (Parera et al., 2018).

Evaluation of sperm abnormality

Abnormality is a state of morphological deviation in spermatozoa cells. According to Parera and Lenda (2023), spermatozoa abnormality is an indication of decreased fertility because it reduces spermatozoa capacitation at the time of fertilization and affects the development and maintenance of pregnancy. Abnormality of spermatozoa is one of the important factors in determining the quality of spermatozoa; if the percentage of abnormality is above 20% in the semen used in artificial insemination, the fertility rate will be low due to the absence of fertilization during copulation (Prastiwi et al., 2021). In this study, the highest average abnormality percentages were observed in the P0 and P3 groups ($p < 0.05$). The high percentage indicated that there was an increase in morphological deviation of spermatozoa from the percentage of abnormalities in fresh semen examination of 4%. The increase in abnormality in group P0 (Control Group) was attributed to the absence of antioxidants in the diluent, while in P3, it resulted from melatonin toxicity due to excessive concentration in the diluent. Both treatment groups caused the semen to experience oxidative stress due to increased formation of lipid peroxidation. This finding is in line with Amtiran et al. (2020), who explained that the increase in abnormality rate was not only caused during the preparations before observation but also caused by lipid peroxidation. Lipid peroxidation can cause damage to the structure and metabolism of spermatozoa which, in turn, will cause increased damage to spermatozoa morphology (Wang et al., 2025). Medrano et al. (2017) further noted that oxidative stress impairs spermatozoa morphology and function due to the formation of excessive free radicals. The results of the abnormality evaluation are presented in Figure 1A.

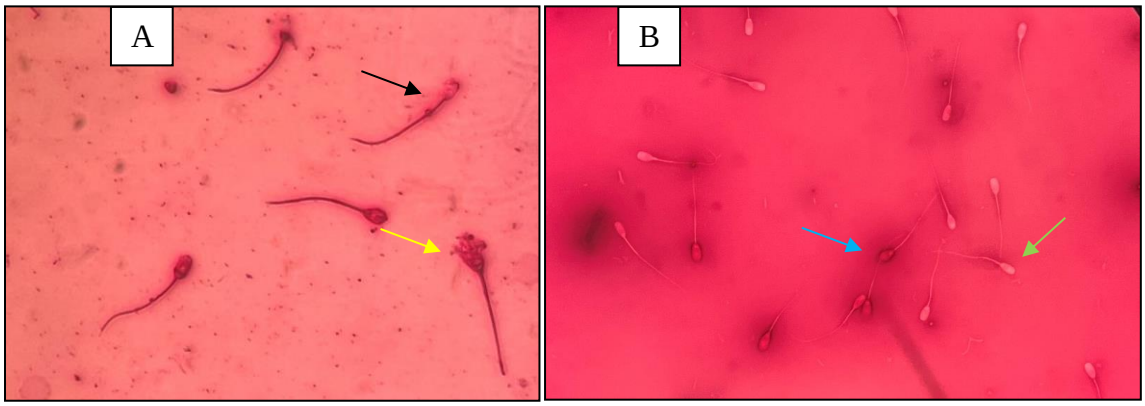

Figure 1. The abnormality (A) and viability (B) of Berkshire boar spermatozoa with eosin-negrosin staining at 400× magnification. **A**: Abnormality in spermatozoa with spermatozoa with abnormalities, such as cytoplasmic droplets (black arrow); abnormalities on the head of spermatozoa (yellow arrow). **B**: Dead spermatozoa absorb color (blue arrow); live spermatozoa do not absorb color (green arrow).

Evaluation of sperm viability

Under normal physiological conditions, stored spermatozoa, after being treated with dilution, metabolize to maintain their life. The main metabolic processes in spermatozoa consist of glycolysis and respiration. During glycolysis, spermatozoa utilize carbohydrates present in the diluent as nutrients to generate ATP. The glycolysis process can occur through two pathways, namely, metabolism with the fructose breakdown pathway and the glucose breakdown pathway. In the respiration process, spermatozoa utilize various substrates as a source of oxygen (Susilawati, 2011). Furthermore, Susilawati (2011) explained that spermatozoa respiration uses lactic acid and pyruvate derived from fructose breakdown to produce CO_2 and water. Metabolism that occurs continuously causes a reduction in available nutrients, resulting in a gradual decrease in spermatozoa viability. In addition, the metabolic process in spermatozoa cells causes a reaction between spermatozoa and oxygen, leading to the production of free radicals or ROS. Rizal and Herdis (2010) noted that while excess O_2 causes peroxidative damage, ROS compounds are formed from cell metabolism activities during semen processing during collection, dilution, and storage (Bebas et al., 2016). An imbalance between free radical formation and antioxidant levels within the cell (Sholihin and Ducha, 2024), further exacerbated by the absence of antioxidants in the diluent (Nalley et al., 2024), can induce oxidative stress, ultimately affecting the survival of spermatozoa. Lawa et al. (2021) explained that spermatozoa cell death, also known as apoptosis, can occur in the absence of antioxidant protection. This finding aligns with the results of the present study, which demonstrated a significant decline in sperm viability in the P0 group, where spermatozoa were diluted using BTS diluent without the addition of melatonin. Duncan's statistical test revealed significant differences ($p < 0.05$) in the evaluation of the viability of spermatozoa in this study. The highest viability percentage was obtained from the P2 treatment group, which amounted to 61.00 ± 1.26%. These results indicated that the addition of melatonin as an antioxidant compound in the diluent can maintain the balance of antioxidants and free radicals so that the decrease in viability percentage occurred more slowly as compared to other treatment groups. The results of the viability evaluation are presented in Figure 1B.

Evaluation of plasma membrane integrity

The plasma membrane plays an important role in spermatozoa, serving as the primary defense against external environmental damage to the cells. The functions of the plasma membrane are not limited only to protecting the organelles contained in the cell; it can function as a potential filter for the exchange of intra- and extracellular substances (Bahmid et al., 2023). Boar spermatozoa are unique in their structure, containing higher unsaturated fatty acids when compared to other mammals. Khophloiklang et al. (2024) explained that the plasma membrane of boar spermatozoa contains polyunsaturated fatty acids, such as Docosapentaenoic Acid (DPA) and Docosahexaenoic Acid (DHA). The phospholipid structure of the spermatozoa plasma membrane, which contains high unsaturated fatty acids, causes spermatozoa cells to be vulnerable to free radicals and trigger autocatalytic reactions, leading to the breakdown of their double bonds (Tamoes et al., 2014).

Figure 2. Evaluation of plasma membrane integrity of Berkshire boar spermatozoa after treatment with Hypo Osmotic Swelling Test (HOST). 400× magnification; normal plasma membrane integrity is characterized by a swelling tail (black arrow).

Duncan's statistical test showed that there were significant differences ($p < 0.05$) in the evaluation of plasma membrane integrity. The highest percentage of plasma membrane integrity was obtained in group P2, followed by group P1 with a percentage of plasma membrane integrity of 55.50 ± 1.04%. These results indicated that the addition of melatonin to BTS diluent can act as an antioxidant to maintain the integrity of the plasma membrane against the damage caused by free radicals. High levels of ROS lead to oxidative stress, which can affect cell metabolism. This is because free radicals damage the plasma membrane of spermatozoa, which will, in turn, cause damage to organelles in spermatozoa cells, where Adenosine Triphosphate (ATP) is produced during cellular respiration (Priharyanthi et al., 2021). Ultimately, decreased or damaged plasma membrane integrity results in reduced sperm motility, increased morphological abnormalities, and cell apoptosis. The results of the viability evaluation are presented in Figure 2.

The results from groups P0 and P3 showed no significant difference ($p > 0.05$), with both groups exhibiting the lowest plasma membrane integrity compared to groups P1 and P2. In group P0, the absence of antioxidant compounds in

the BTS diluent led to the absence of compounds capable of balancing free radicals. In group P3, excessive antioxidant dosage converted antioxidants into prooxidants, inducing toxicity. Plasma membrane integrity plays a critical role in spermatozoa motility. Setiadi et al. (2006) stated that if the plasma membrane of spermatozoa is damaged, the metabolism of spermatozoa will be disrupted, leading to loss of motility and, ultimately, the death of spermatozoa.

Malondialdehyde levels

Malondialdehyde (MDA) level testing is conducted to determine the level of oxidative stress in semen. This test measures the end product of free radicals, as direct measurement of free radicals is not feasible due to their autocatalytic nature and short half-life, which causes their disappearance within a few seconds (Hayati et al., 2006). MDA formation occurs through both enzymatic and nonenzymatic processes (Cordiano et al., 2023; Rizzo et al., 2024). In the formation through enzymatic processes, MDA is formed as a by-product during the formation of Thromboxan A2 (TXA2) whereas in the non-enzymatic processes, MDA is formed as a secondary product of lipid peroxidation, which is often used to measure the degree of oxidative stress (Bikulčienė et al., 2019). According to Hayati et al. (2006), MDA is a compound that is the result of lipid oxidation into peroxides through a series of processes called lipid peroxidation. The term lipid peroxidation is generally defined as a process of oxidative lipid degradation. According to Ayala et al. (2014), lipid peroxidation is the process of reaction between oxidants and lipids containing oxidant carbon-carbon double bonds, such as polyunsaturated fatty acids (PUFAs). This reaction involves the removal of hydrogen from carbon with oxygen insertion, resulting in the formation of lipid peroxyl radicals and hydroperoxides.

In the study, the result of Duncan's test showed significant differences in MDA levels among the groups ($p < 0.05$). The highest MDA levels were obtained in the P3, P1, and P0 groups, respectively, with MDA levels of 2050.53 ± 147.34 ng/ml, 1997.13 ± 134.03 ng/ml, and 1979.39 ± 108.76 ng/ml. The MDA level in group P0 was high because the diluent in this group did not contain antioxidants, while in group P1, even though melatonin had been added at a dose of 0.5 mM, it could not act as an antioxidant compound to convert reactive oxygen into neutral compounds. In group P3, high MDA levels were caused by the high dose of the administered melatonin, which caused toxicity. In this case, instead of acting as an antioxidant, melatonin transitioned into a prooxidant, accelerating oxidative stress. Munik and Ekmekçioğlu (2015) reported that under certain conditions, antioxidants can exhibit prooxidant behavior. However, the stages or processes of melatonin acting as a prooxidant have not been explained in detail, with most of the literature describing the prooxidant effects of melatonin as a hormone in the body rather than a supplement. Vega-Naredo et al. (2005) emphasized that the prooxidant effects of melatonin have only been reported *in vitro* cell culture systems, especially on cancer cells, and cannot, therefore be generalized to other cells and systems.

Oxidative stress in spermatozoa causes disturbances in the phosphorylation oxidation process, resulting in increased production of spermatozoa ROS (Hayati et al., 2006). On the other hand, the cell structure of spermatozoa in the cell membrane, which contains a lot of unsaturated polyunsaturated fatty acids, causes faster oxidative stress. Situmorang and Zulham (2020) explained that the double bonds of unsaturated fatty acids are the target of free radicals, triggering a cascade of fatty acid degradation in their vicinity. The test results showed that the lowest MDA levels were obtained in group P2, indicating that the administration of melatonin at a dose of 1.0 mM exhibited the most effective antioxidant properties in preventing oxidative stress compared to other groups. Garcia et al. (2014) highlighted the potent antioxidant properties of melatonin, emphasizing its ability to mitigate oxidative stress through multiple mechanisms. In the context of lipid peroxidation, melatonin can react with ROS, such as hydroxyl radicals. In addition, the result of the metabolism that occurs between melatonin and ROS can also neutralize free radicals. According to Zhang and Zhang (2014), melatonin detoxifies ROS through a sequential process that results in the production of cyclic 3-hydroxy melatonin (3-OHM/c3OHM), a compound that also functions as a ROS scavenger. When 3-OHM reacts with ROS, it undergoes oxidation, resulting in the formation of N^1-acetyl-N^2-formyl-methoxykynuramine (AFMK). In its stages, AFMK will then undergo deformylation, which causes the formation of N^2-acetyl-5-methoxynuramine (AMK). Reiter et al. (2014) further explained that both AFMK and AMK serve as effective peroxyl radical scavengers, reinforcing the role of melatonin in mitigating oxidative stress.

CONCLUSION

The findings of this study indicated that the administration of melatonin at a dose of 1.0 mM in Beltsville Thawing Solution (BTS) diluent is the most effective dosage for preserving the viability of Berkshire boar liquid semen. Based on these results, it is recommended that future research be conducted through *in vivo* testing in sows through the stages of artificial insemination to find out whether liquid semen supplemented with melatonin in the diluent can increase the number of piglets born or not.

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

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Authors' contributions
Putu Risma Oktaviandari wrote the manuscript and conducted the research, and Wayan Bebas and Tjok Gde Oka Pemayun supervised the research and revised the final manuscript. Ni Nyoman Werdi Susari and Desak Nyoman Dewi Indira Laksmi provided tips and feedback on the manuscript. All authors reviewed and approved the final edition of the manuscript.

Competing interests
The authors declare that they have no competing interests.

Ethical considerations
The authors confirmed that the manuscript has been submitted for the first time to this journal. All ethical issues, including plagiarism, consent to publish, misconduct, data fabrication, double publication, and redundancy, have been checked by all the authors.

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Antimicrobial Activity of *Ziziphus spina-christi* against *Staphylococcus aureus*, *Escherichia coli*, and *Shigella flexneri*

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ABSTRACT

Antibiotic resistance remains a global concern, with up to 1.91 million deaths projected to occur due to resistant Gram-negative and Gram-positive bacteria by 2050. The study hence aimed to assess the antimicrobial activity of *Ziziphus spina-christi* leaf extracts in relation to specific bacterial strains and elucidate the molecular mechanisms to validate the *in vitro* findings. *Ziziphus spina-christi* leaf extracts were tested against *Staphylococcus aureus (S. aureus), Escherichia coli (E. coli*), and *Shigella flexneri* (*S. flexneri*). The leaf powder was subjected to both aqueous and methanol-dichloromethane extraction. Phytochemical products were determined by Liquid Chromatography-Mass Spectrometry and Gas Chromatography-Mass Spectrometry for water extract and methanol dichloromethane extract, respectively. The agar well diffusion method, broth microdilution, and minimum bactericidal concentration against three bacterial species, *S. aureus*, *E. coli*, and *S. flexneri*, were used to assess the antibacterial activity of extracts. The results have shown that both plant extract has a significant level of antibacterial activity at higher concentrations (400 mg/ml) against the gram-positive bacteria. In addition, the methanol-dichloromethane extract exhibited the highest antibacterial activity against Gram-negative bacteria (*S. flexneri*, and *E. coli*), conversely, the water extract demonstrated a lower activity against *S. flexneri* and *E. coli*, with inhibition zones of 15 ± 0 mm for both bacteria. At a lower concentration (100 mg/ml), the methanol-dichloromethane extract produced inhibition zones of 19.6 ± 0.5 mm against *S. aureus*, closely followed by *S. flexneri* and *E. coli*. The water extract exhibited high antibacterial activity against Gram-positive bacteria. However, exhibited reduced antibacterial activity against *S. flexneri* and *E. coli*, indicating a concentration-dependent antibacterial effect. Extraction methods were significantly different, with products generated from non-aqueous extraction demonstrating a higher potency against both Gram-negative and Gram-positive bacteria than the aqueous extract. Docking results demonstrated that water extract had a high binding activity against penicillin-binding proteins. Moreover, it serves as a potent beta-lactamase inhibitor as it binds to their active site, rendering them inactive and inhibiting the hydrolysis of Beta lactam antibiotics. In conclusion, the methanol-dichloromethane and water *Ziziphus spina-christi* leaves could be considered a promising source of antimicrobial ingredients.

Keywords: Antimicrobial resistance, Beta lactamase, Methanol-dichloromethane Extract, Molecular docking, *Ziziphus spina-christi* leave

INTRODUCTION

Antimicrobial resistance (AMR) ranks among the foremost challenges facing public health today. It is the third greatest cause of mortality after cardiovascular diseases and cancer (Salam et al., 2023). Major research published in January 2022 found that approximately 5 million people died from drug-resistant illnesses in 2019, and nearly 1.27 million people died from infections that were resistant to antimicrobials (Salam et al., 2023). According to the 2016 Global Burden of Disease (GBD) report, cumulatively, Diarrheagenic *Escherichia coli (E. coli*), especially enteropathogenic *E. coli* (EPEC) and enterotoxigenic *E. coli* (ETEC) resulted in 63,523 deaths among all ages annually throughout the world (Kariuki et al., 2022). Whereas around 212,438 deaths, which account for roughly 13.2% of all diarrheal fatalities worldwide, were ascribed to resistant shigellosis in 2016 (Mason et al., 2023). Additionally, plant components are less expensive than chemicals and have negligible to no negative host side effects (Odhav et al., 2010). Concern over the spread of resistant bacteria and fungi, among other diseases, including healthcare-associated infections, respiratory and gastrointestinal infections, and Tuberculosis, has increased, and with it, interest in the curative power of ancient remedies (Abdulrahman et al., 2022). A variety of modern medications, nutraceuticals, dietary supplements, and medicinal products, including artemisinin, quinine, vincristine, and others, are sourced from these medicinal plants (Newman and Cragg, 2020).

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Christ thorn (Ziziphus spina-christi) is a tropically native medicinal plant that has long been used to treat many illnesses, such as fever, pharyngitis, pulmonary disease, malaria, wounds, burns, stomach pain, urinary tract infections, intestinal rheumatism, diarrhea, and bronchitis (Alhassan et al., 2019). *Ziziphus spina-christi* (*Z. spina-christi*) fruits and leaves extracts have shown antibacterial activity against *Escherichia coli, Bacillus subtilis, and Candida. albicans*, and *Staphylococcus aureus* (Ads et al., 2022). Despite the stated efficacies and the beneficial compounds found in plants, such as flavonoids, saponins, alkaloids, indole derivatives, and fatty acids, a distinct gap in understanding the antibacterial properties and molecular mechanisms of action against multidrug-resistant bacterial pathogens (Ads et al., 2022).

The acquisition of antibiotic resistance in bacterial species can occur through different mechanisms, including vertical and horizontal gene transfer. The conjugative transfer of plasmids containing resistance genes among bacterial species is regarded as a critical method for resistance transmission in bacteria (Mansour et al., 2024). Bacteria may develop many strategies to resist antibiotics, including alterations in drug targets, restriction of cellular entrance, removal via efflux pumps, or drug inactivation. To pick the most effective antibiotics for treating multidrug-resistant bacteria, it is essential to comprehend and anticipate resistance trends (Mansour et al., 2024). The rising occurrence of multidrug-resistant strains has underscored the significance of advancing antibacterial that utilize mechanisms of antibiotic action that have not been previously exploited, such as targeting the adhesion molecules or quorum-sensing pathways, reducing biofilm formation, and pathogenicity (Tondi, 2021). Recent advancements in computational methodologies have fundamentally established the basis for the creation and identification of therapeutically active natural compounds that can target specific proteins. Penicillin-binding proteins (Freischem et al., 2021), fatty acid synthesis, and DNA Gyrase (Spencer and Panda, 2023) enzymes are recognized targets within a broader class of antibiotics that feature. Thus, the present study aimed to examine the antibacterial activity of *Z. spina-christi* leaf extracts against three bacterial isolates.

MATERIALS AND METHODS

Plant extract preparation

Fresh leaves were collected from Jomo Kenyatta University of Agriculture and Technology (JKUAT) Seedlings, Juja- Kiambu. The plant material was identified, verified, and authenticated by a plant taxonomist at the JKUAT Herbarium and was assigned the identification number HMMA.JKUATBH.001/2024. The leaves were washed twice with distilled water, and stored at -80°C for 72 hours, then the water content was removed using a freeze dryer (Model FDL-10N-50-8M) under controlled conditions of sample temperature -72°C, cold trap temperature -62°C, and a vacuum pressure 0.00001pa for 48 hours. The desiccated leaves are ground into a fine powder utilizing a blender. 100 grams of dried leaves to 500 ml of distilled water was used for aqueous extract preparation. The mixture was incubated for 24 hours in a shaker incubate (GYROMAX^{TM} 727), followed by filtration using cotton and Whatman no.1 filter paper. The filtrate was stored at -40°C for 1 day and -80°C for 3 days before being freeze-dried under controlled conditions of sample temperature -68°C, cold trap temperature -62°C, and a vacuum pressure 0.00001pa for 72 hours to get the powder extract. Cold maceration of 100 g of dried leaves in 500 ml of dichloromethane and methanol mixture (1:1) was done for 72 hours regarding non-aqueous extract preparation. The solution underwent further filtration with Whatman No. 1 filter paper (Aleixandre-Tudo and du Toit, 2018). The solvents were removed from the extracts by drying them out with a rotary evaporator (Labtech DAIHAN, VP30, EV11) set at 40 rpm and 60°C. The powders were dried further in a 37°C oven and then kept at room temperature until needed.

Phytochemical compositions analysis

The study of phytochemical contents of non-aqueous extract was conducted using a Gas Chromatography-Mass Spectrometer system (Model; Shimadzu, GC-MS QP-2010SE) and a low polarity BPX5 capillary column (30 m× 0.25 mm× 0.25 µm film thickness). A liquid chromatography-mass spectrometer system (An ExionLC AC system, SCIEX Triple Quad Ascentis® Express 90 Å C18 Column [2.1×150 mm, 2.7 µm]) was used for water extract phytochemical composition analysis. C hromatography mass spectrometry was used to characterize phytochemical substances, with the identity and quantification of each compound determined by retention time and peak area percentage. A larger area indicated a higher concentration of that compound.

Bacterial isolates and media

Bacterial strains maintained in 1.5 ml Trypticase Soy Broth with 20% glycerol at -80°C were obtained from Kenya Medical Research Institute (KEMRI) Center for Global Health Research in Kisumu-Kenya. The bacterial strains included *Escherichia coli* (ATCC25922), *Staphylococcus aureus* (ATCC25923), and *Shigella flexneri* (*S. flexneri*) (ATCC12522).

Assessment of antibacterial activity of plant extracts

Well diffusion assay

The antibacterial activity of each extract was assessed against isolates of bacteria using the agar well diffusion technique. One gram of both dry methanol-dichloromethane extract and aqueous extract was dissolved in 1 ml of 98% dimethyl sulfoxide (DMSO) to generate a stock solution. Each stock culture bacterium was inoculated onto separate Trypticase Soy Agar (TSA) plates and incubated overnight. The newly established colonies were subsequently suspended in a normal saline tube to attain a 1.5×10^8 CFU/ml dilution, utilizing the 0.5 McFarland standard. Mueller Hinton agar plates (pH 7.3) were uniformly injected with bacterial suspensions by the Kirby Bauer method (Joseph et al., 2011). Five circular wells, each with a diameter of 11 mm, were aseptically formed on each plate using a sterile cork borer. One hundred microliters of extracts at concentrations of 100 mg/mL, 200 mg/mL, and 400 mg/mL were dispensed into separate wells on each plate (Abdallah et al., 2016). Similarly, 98% Dimethyl sulfoxide (DMSO) was introduced to the negative control wells, whereas the reference antibiotic Ceftriaxone (100 µg/mL, Gondane and Pawar, 2023) was administered to the positive control wells. A control plate for the three bacterial isolates with a DMSO concentration of 98% was performed to verify that DMSO demonstrates no antibacterial activity at high doses. The plates were incubated in aerobic conditions for 24 hours at 37°C. The zones of inhibition were quantified in millimeters and studied using the two extracts.

Assessment of the minimum inhibitory concentration and minimum bactericidal concentration

The minimal inhibitory concentration was determined using the broth dilution method in a sterile 96-well plate. A stock solution with a concentration of 400 mg/ml was prepared from the leaf extracts. 100 microliters of Mueller-Hinton Broth (MHB) were poured into each well of a 96-well plate. One hundred microliters of the extract stock solution were added to the first well of each test column, followed by serial dilution to a concentration of 6.25 mg/ml. Except for the negative control wells, which contained broth for quality assurance, twenty microliters of an 18-hour bacterial culture were introduced to the test and positive control wells after dilution using a 0.5 McFarland standard. Following a 24-hour aerobic incubation at 37ºC, 20 microliters of Resazurin dye (0.0025 mg/mL) were added to each well, and bacterial viability was assessed visually based on color change. The resazurin reduction by metabolically active bacteria resulted in a color shift from blue to pink, indicating growth. This color alteration was used to determine the minimum inhibitory concentration (MIC) of each extract by evaluating bacterial proliferation within the broth culture for each strain (Teh et al., 2017). A tiny volume from the wells exhibiting no discernible bacterial growth (turbidity) was moved onto fresh TSA media for an additional culture to determine the minimum concentration that kills bacteria. Following that, the dishes were incubated aerobically for 24 hours at 37°C. The bacterial colonies cultivated on each agar plate were meticulously examined, and the lowest concentration of extracts exhibiting complete bactericidal activity was identified as the minimum bactericidal concentration (MBC).

***In silico* evaluation**

Screening for drug-like compounds in *Ziziphus spina-christi* leaves extracts

The PubChem IDs and the Simplified Molecular Input Line Entry System (Canonical SMILES) of the Mass Spectrometers (GC-MS and LC-MS) identified compounds were retrieved for PubChem. The Canonical SMILES were submitted to the Swiss ADME tool and ADMETlab 3.0 to predict the physicochemical properties and drug-likeness of the candidates (Soltani Rad et al., 2023). The prediction was based on the parameters of the Blood Brain Barrier (BBB), Total Polar Surface Area (TPSA), Cytochrome P450s (CYP2D6 and CYP3A4) enzymes, and Lipinski's Rule of five (RO5), which stipulates that a drug candidate should possess a molecular weight under Five hundred Daltons, less than 10 rotatable bonds, less than 5 hydrogen bond donors, less than 10 hydrogen bond acceptors, and lipophilicity (log P) value below 5. A hypothesis was made that a molecule would not be suitable for oral administration if it failed to meet two or more RO5 criteria (Soltani Rad et al., 2023). The polar atoms within a molecule play a significant role in determining the topological polar surface area (TPSA), which is normally utilized to forecast the transport mechanism of the drug. The TPSA for an authorized medication, in the majority of instances, was below 140 Å2 (Patel et al., 2020). Compounds capable of penetrating the blood-brain barrier pose a nervous system risk because of their shifting ability from the hydrophilic environment of the blood to the lipophilic conditions of the brain. A therapeutic candidate must not inhibit CYP450 enzymes, as they are crucial for drug metabolism (Patel et al., 2020).

Molecular docking

The structural data file of chemicals was obtained from PubChem; the ligands library was created using Chimera 1.15rc software and saved in Mole2 format (Khan and Lee, 2022). The selected potential targets were three-dimensional

structures obtained from the Protein Data Bank (PDB) in PDB format and had been previously characterized through X-ray crystallography. These proteins were retrieved in their inhibitory state, with their native inhibitors bound at the active site. The XYZ coordinates of the structure's active sites were identified using BIOVIA Discovery Studio and subsequently used for molecular docking analyses. PDB formats were introduced in Chimera 1.15rc Software; existing ligands and water were eliminated from the targets. The ligand and target protein structures were produced for docking by energy minimization using the identical algorithm. The prepared targets and ligands were brought in Autodock Vina via PyRx 0.8 for docking and later transformed into PDBQT (Protein Data Bank, Partial Charge [Q], and Atom Type [T] files. The grid box was expanded using the XYZ coordinates of the active site obtained from BIOVIA Discovery Studio to ensure binding at the most suitable sites. Docking was executed at the standard exhaustiveness level of eight, ensuring precise localization of the binding pocket. Upon run completion, an output file (CSV) providing affinity scores for each ligand was generated in an Excel spreadsheet. The generated data was evaluated to create complexes in Notepad, which were subsequently saved as PDB files and viewed with BIOVIA Discovery Studio Visualizer 2021 for visualization (Khan and Lee, 2022).

Statistical analysis

The data were statistically analyzed using Origin Pro Lab 2024b (Student version) graphing and analysis software. Triplicate results for each bacterium treated with different extracts were analyzed using one-way analysis of variance (ANOVA), followed by Tukey's test to determine significant differences between the two extracts. The corresponding p-values were calculated to assess statistical significance, with values below 0.05 considered significant. Measurements were then averaged, and results were expressed as mean ± standard deviation to generate graphs illustrating the differences. In molecular docking, binding energy was utilized to forecast the ligand's affinity for the receptor, and the type of interactions was used to determine the stability of the protein-ligand complexes.

RESULTS

Identification and analysis of *Ziziphus spina-christi* bioactive compounds

A total of 34 unique compounds, together with their retention times (R. Time) and area percentages (Area%), were identified by GC-MS chromatogram. In GC-MS analysis, retention time (RT) helps identify metabolites based on their volatility and interaction with the stationary phase, while peak area reflects their relative concentration. RT aids structural identification by comparison with reference standards, and the peak area provides quantitative insights. Variations in ionization efficiency and matrix effects can affect peak area, requiring normalization techniques for accuracy. Together, RT and peak area enable precise metabolite profiling. Squalene (Peak 24) was the most predominant compound, comprising 46.12% of the area. Squalene serves as a precursor in sterol biosynthesis and exhibits antioxidant properties, contributing to skin protection and antibacterial activity by disrupting bacterial membranes, followed by Vitamin E (Peak 25) at 9.87%. Vitamin E functions as a potent antioxidant, supporting immune function and potentially enhancing antibacterial defense by affecting membrane integrity. The additional metabolite presented in a high concentration was 3,7,11,15-Tetramethyl-2-hexadecen-1-ol (Peak 21) at 8.74%, which plays a role in chlorophyll metabolism and demonstrates antimicrobial activity by disrupting bacterial lipid membranes (Figure 1).

The LC-MS analysis identified 25 metabolites along with their retention times and areas under the peak (Figure 2). The predominant metabolite was Beta-indoleacetic acid (ID 11) with an area of 5.89 \times 10^9. Beta-Indole Acetic Acid (IAA) functions as a plant hormone regulating growth and may influence bacterial quorum sensing and inhibit bacterial growth, followed by N-Isovaleroylglycine (ID 46) with an area of 3.34/times 10^9, known to have antimicrobial effects by altering bacterial membrane stability and inhibiting growth in certain microbial species and Delphinidin-3-O-(6''-O-alpha-rhamnopyranosyl-beta-glucopyranoside; IDs 95 and 96) with an area of 3.19/times 10^9 each, is a potent antioxidant anthocyanin that has antibacterial activity by rupturing bacterial membranes and preventing the formation of biofilms.

Figure 1 shows the predominant metabolite was Beta-indoleacetic acid (ID 11) with an area of (5.89 \times 10^9). Out of a total of 34 compounds, the 2-Methoxy-4-vinylphenol, 2-Formyl-9- (beta -d-ribofuranosyl) hypoxanthine, and 2,4-Di-tert-butylphenol were predominantly recognized for their potent antibacterial properties, shown in Table 1. The detailed results on all 34 compounds are shown in S1 Table.

Results of the LC-MS phytochemical analysis indicated the presence of 53 compounds (Positive mood and negative mood), including cerulenin, Quercetin-3-O-arabinoglucoside, chlorogenic acid, and Esculin, among others that have been identified for their antibacterial properties (Table 2). Further details about the rest of the compounds are presented in S2-S3 Tables.

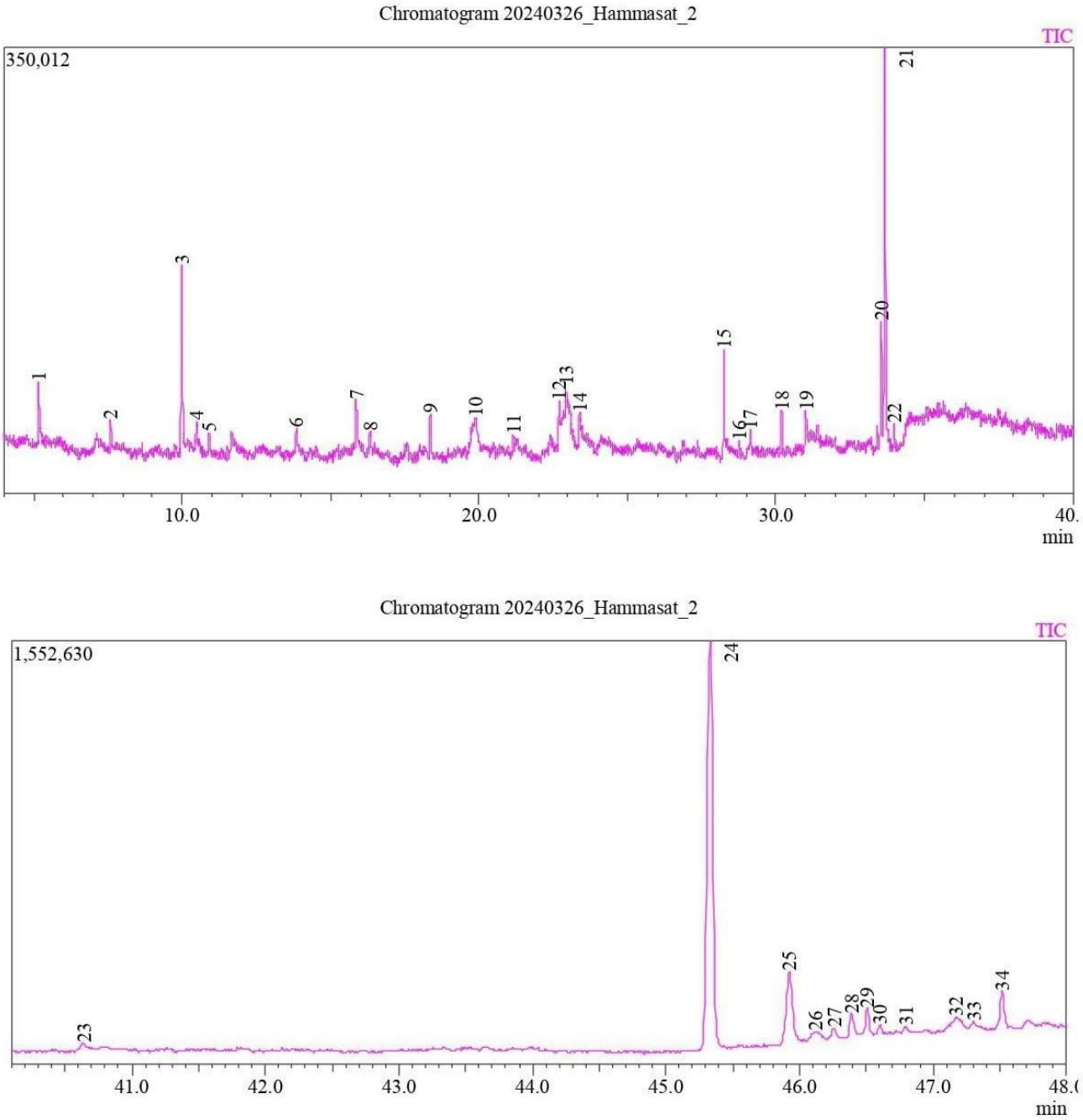

Figure 1. GC-MS chromatogram illustrating the Phyto-compounds present in the methanol-dichloromethane extract of *Ziziphus spina christi*. The peaks show that Squalene (Peak 24) was the most predominant compound, comprising 46.12% of the area.

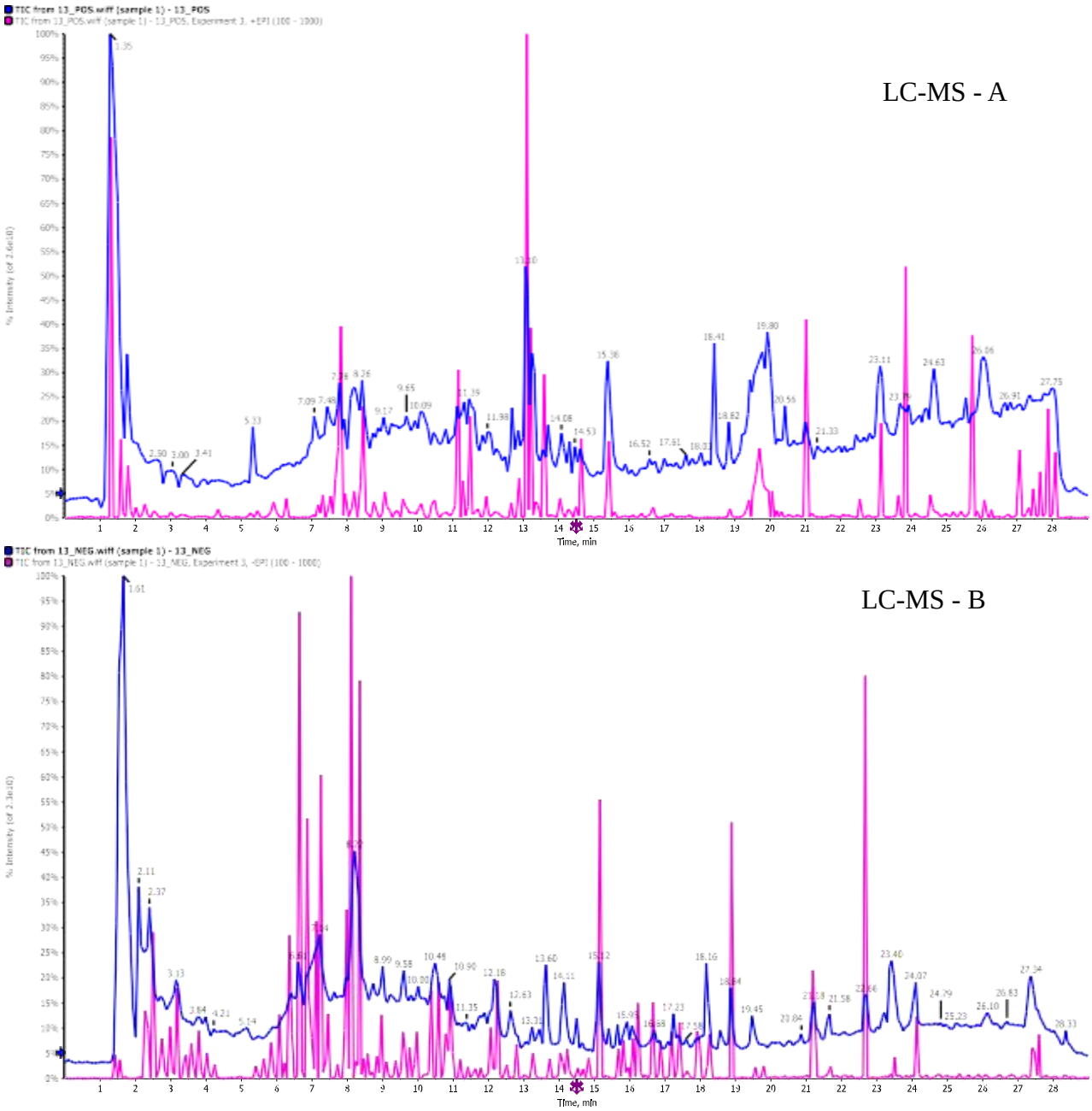

Figure 2. LC-MS chromatogram illustrating the chemical constituents found in *Ziziphus spina-christi* water extract. Presentations of both positive (LC-MS A) and negative (LC-MS B) moods of compounds from water extracts.

Table 1. The GC-MS identified compounds from *Ziziphus spina christi* methanol-dichloromethane extract

Peak Number	R. time (min)	Compound identified	MW	MF	Structure type	Function
8	16.320	2-Methoxy-4-vinylphenol	150.07	C9H10O2	Phenoxy compound	Antimicrobial and antioxidant Agent
10	19.878	2-Formyl-9-[.beta.-d-ribofuranosyl]hypoxanthine	296.08	C11H12N4O6	Tetrahydrofurans	Antiviral and Antimicrobial agent
11	21.149	2,4-Di-tert-butylphenol	206.17	C14H22O	Benzenoids	Antimicrobial agent

MW: Molecular weight; MF: Molecular formula; RT: Retention time

Table 2. The LC-MS identified compounds in *Ziziphus spina-christi* water extract

ID	RT (min)	Metabolite name	MW	MF	Structure Type	Function
56	2.328	Quercetin-3-O-arabinoglucoside	596.49	C26H28O16	Flavonoids	Antimicrobial and antioxidant agent
500	15.780	Chlorogenic acid	354.31	C16H18O9	Cyclic alcohol	Antimicrobial and antioxidant agent
549	16.767	Esculin	340.28	C15H16O9	Coumarins glycosides	Antimicrobial and antioxidant Agent

MW: Molecular weight; MF: Molecular formula; RT: Retention time

Antimicrobial activities of *Z. spina-christi* leaves extracts against *Staphylococcus aureus, Escherichia coli,* and *Shigella flexneri*

Well diffusion assay

Mean zone of inhibition in millimeters ± standard error of the mean beyond the good diameter (11 mm) generated on *E. coli* (ATCC25922), *S. aureus* (ATCC25923), and *S. flexneri* (ATCC12522). The methanol-dichloromethane extract exhibited the highest antibacterial activity against *S. aureus*, with a zone of inhibition measuring (22.3 ± 0.6) mm at a concentration of 400 mg/mL. This was followed by *S. flexneri* (21.3 ± 1 mm) and *E. coli* (19.6 ± 0.6 mm) at the same concentration. In comparison, the water extract demonstrated strong activity against *S. aureus* (21.6 ± 0.5 mm) at 400 mg/mL but showed lower activity against *S. flexneri* and *E. coli*, with mean inhibition zones of (15 ± 0) mm and (14.6 ± 0.5) mm, respectively, as shown in Figure 3. The methanol dichloromethane extracts consistently exhibited superior antibacterial activity compared to the water extract, particularly against *S. aureus.*

In the present study, Methanol-dichloromethane extracts frequently demonstrate enhanced antibacterial efficacy relative to aqueous extracts, attributable to variances in their capacity to extract bioactive components. Organic solvents like methanol and dichloromethane efficiently dissolve both polar and non-polar bioactive chemicals, including alkaloids, flavonoids, terpenoids, and phenolics, which are recognized for their potent antibacterial effects. Conversely, water, being a highly polar solvent, predominantly removes hydrophilic substances like tannins, carbohydrates, and proteins, which may lack substantial antibacterial properties (Truong et al., 2019).

The methanol-dichloromethane extract demonstrated superior antibacterial activity, particularly against Gram-negative bacteria, due to its higher concentration of flavonoids, alkaloids, and terpenoids, which are well-documented for their antimicrobial properties. In contrast, the aqueous extract, which contained coumarins, flavonoids, alkaloids, terpenoids, and cinnamic acids, exhibited lower activity against Gram-negative bacteria, possibly due to structural differences affecting their interaction with bacterial cell walls.

A key observation in this study was that the zones of inhibition for both extracts against *S. aureus* (at 400 mg/mL) were relatively close to that of ceftriaxone (25 mm). This suggests that both extracts have significant antibacterial activity against Gram-positive bacteria (P = 0.0001 However, in the case of Gram-negative bacteria, the zones of inhibition for ceftriaxone were generally larger than those of the extracts, particularly for *S. flexneri* (31 mm for ceftriaxone versus the extract's inhibition zone). Interestingly, the inhibition zones for *E. coli* were relatively closer between ceftriaxone and the methanol-dichloromethane extract, indicating a moderate yet promising activity against this strain.

The differences in activity could be attributed to the structural complexity of Gram-negative bacterial cell walls, which include an outer membrane with lipopolysaccharides that act as a barrier against many antibacterial agents. The methanol-dichloromethane extract likely contained more lipophilic compounds, facilitating better penetration through this outer membrane, whereas the aqueous extract, despite containing known antibacterial compounds, exhibited lower efficacy due to possible limited permeability or compound stability in bacterial environments. Both extracts exhibit maximal efficacy against Gram-positive *S. aureus*, whereas their activity against Gram-negative bacteria *S. flexneri* and *E. coli* is comparatively diminished. This finding corroborated the antibacterial activity observed with methanol dichloromethane extract and water extract (Figure 4). Additional detailed results are provided in Table S4.

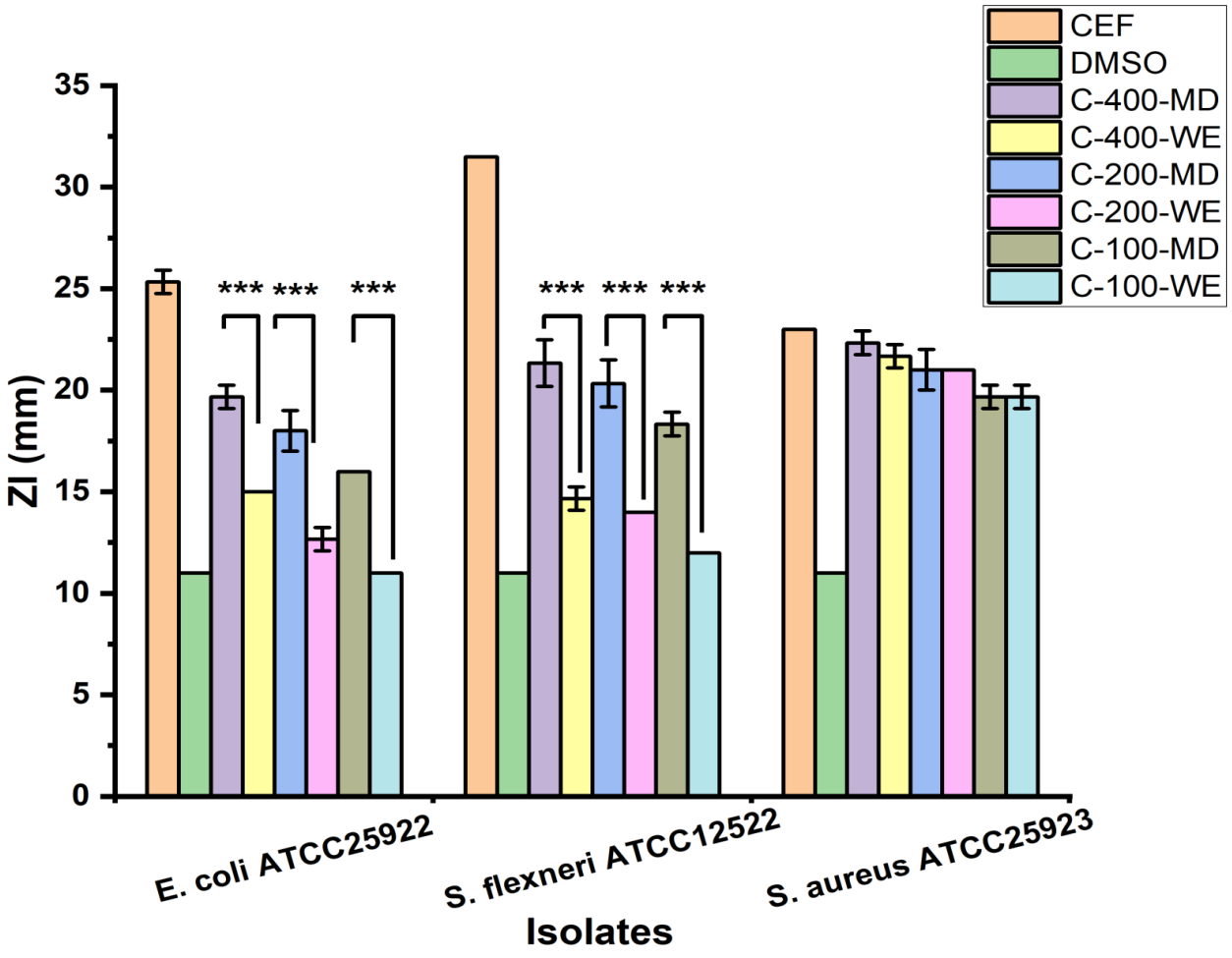

Figure 3. Antimicrobial activities (well diffusion assay) of *Ziziphus spina-christi* extracts with bacterial isolates

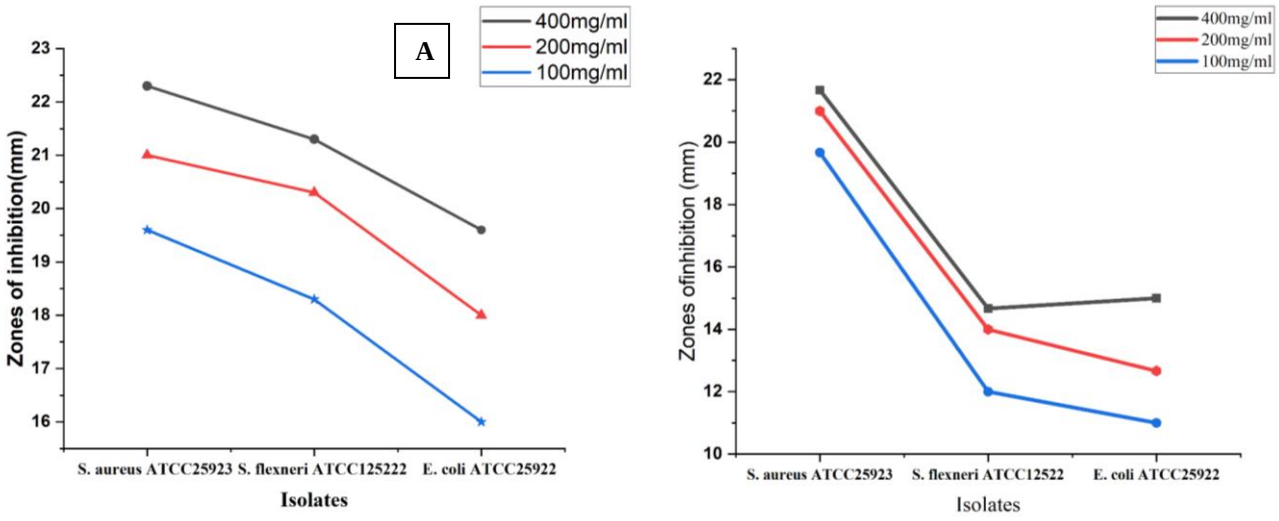

Figure 4. The performance of the *Ziziphus spina-christi* extracts against the bacterial isolates. **A**: Methanol-dichloromethane extract, **B**: Water extract

Minimum inhibitory concentration and minimum bactericidal concentration

The minimum inhibitory concentration (MIC) results indicated that both extracts demonstrated high potency against *S. aureus*, with a MIC of 12.5 mg/mL for each extract. The methanol dichloromethane extract demonstrated greater inhibitory activity of 12.5 against the tested isolates, effectively inhibiting *S. aureus* and S. *flexneri* at a concentration of 12.5 mg/mL, while *E. coli* showed inhibition at a higher concentration of 50 mg/mL (Table 3).

The methanol dichloromethane extract demonstrated killing activity against the tested isolates at 100 mg/mL concentration for *S. aureus*, *E. coli*, and *S. flexneri*. The water extract demonstrated killing activity against *S. aureus* at 100 mg/ml concentration, while both *E. coli* and *S. flexneri* showed resistance at this concentration.

Table 3. Minimum inhibitory concentrations of *Z. spina-christi* leaf extracts

Microorganism	Water Extract MIC (mg/mL)	Methanol- Dichloromethane Extract MIC (mg/mL)	Mean	Standard Deviation	CV (%)
***Staphylococcus aureus* ATCC25923**	12.5	12.5	12.5	0	0%
***Shigella flexneri* ATCC12522**	50	12.5	31.25	26.53	84.9%
***Escherichia coli* ATCC25922**	50	50	50	0	0%

CV: Coefficient of variance, MIC: Minimum inhibitory concentration

***In silico* evaluation**

Screening for pharmacologically viable chemicals in Ziziphus spina-christi leaf extract

Out of 34 chemicals extracted from *Z. spina-christi* using dichloromethane-methanol and identified using GC-MS analysis, 14 were deemed optimal drug-like candidates based on their compliance with Lipinski's Rule of Five and exhibited favourable ADMET (Absorption, Distribution, Metabolism, Excretion, and Toxicity) profiles, positioning them as viable candidates for drug development. Compounds molecular weight was under 500 Dalton, a logP value below 5, fewer than 5 hydrogen bond donors, and fewer than 10 hydrogen bond acceptors and they demonstrated favourable oral bioavailability and potential systemic distribution through Total polar surface area. In addition, the compounds were not permeable to the blood-brain barrier, and they do not inhibit Cytochrome P2D6, Lipinski's RO5, ADMET analysis offered insights into the pharmacokinetic properties of the selected compounds, confirming their optimal absorption, distribution, and metabolic stability, alongside low toxicity and minimal side effects. The favourable ADMET profiles indicate a reduced likelihood of rapid metabolism or elimination, potentially resulting in prolonged antibacterial effects. In the aqueous extract of *Z. spina-christi*, LC-MS analysis found 52 chemicals, of which 38 were deemed suitable drug-like candidates based on the same previous criteria. The presence of these bioactive compounds in both the methanol-dichloromethane and aqueous extracts aligns with the observed antibacterial effects shown by both extracts. The prioritized compounds are delineated in Tables 4 and 5.

Table 4. *Ziziphus spina-christi* dichloromethane-methanol compounds with the ideal drug candidate qualities

N	Compounds	ID	MW (g/mol)	BBB	Lipinski's Rule (Violation)	CYP2D6	HA	HD	TPSA
1	DL-Glyceraldehyde	751	93.03	No	Yes;0	No	2	3	20.23
2	2-Hydroxy-gamma-butyrolactone	545831	102.09	No	Yes;0	No	3	1	46.53
3	Coumaran	10329	120.06	No	Yes;0	No	0	1	13.14
4	8-Methyl-8-azabicyclo [3.2.1] octane-3-carbonitrile	15405279	136.1	No	Yes;0	No	2	1	35.82
5	2-Methoxy-4-vinylphenol	332	150.07	No	Yes;0	No	2	1	29.46
6	2-Formyl-9-[beta-d- ribofuranosyl] hypoxanthine	135599166	296.08	No	Yes;0	No	8	4	150.56
7	2,4-Di-tert-butylphenol	7311	206.17	No	Yes;0	No	8	4	150.56
8	cis-1,4-Cyclohexanediol	11162	116.08	No	Yes;0	No	2	2	40.46
9	Arachidic Acid	10467	312.3	No	Yes;0	No	2	1	37.3
10	Octadecanoic acid	5281	284.27	No	Yes;0	No	2	1	37.3
11	Tetraprenol	5281365	290.26	No	Yes;0	No	0	0	0
12	1-Heptacosanol	74822	396.43	No	Yes;0	No	1	1	20.23
13	1,2-Benzenediol	289	110.4	No	Yes;0	No	2	2	40.46
14	Undec-10-ynoic acid	91692467	378.35	No	Yes;0	No	2	0	26.3

ID: PubChem ID, MW: Molecular weight, BBB: Blood-brain barrier, CYP2D6: Cytochrome. P450 2D6, HA: Number of hydrogen bond acceptors, HD: Number of hydrogen bond donors; TPSA: Total polar surface area

Table 5. *Ziziphus spina-christi* water compounds with ideal drug candidate qualities

N	Compounds	ID	MW (g/mol)	BBB	Lipinski"s Rule (Violation)	HA	HD	CYP2D6	TPSA
1	Thiamine	1130	265.35	No	Yes;0	3	2	No	104.15
2	4-hydroxy-3-Methoxyphenylacetic acid (2,5,6-d3,alpha,alpha-d2)	1738	182.17	No	Yes;0	4	2	No	66.76
3	4-hydroxy-3-methoxyphenylacetic acid (2,5,6-d3,alpha,alpha-d2)	1738	182.17	No	Yes;0	4	2	No	66.76
4	N-Isovaleroylglycine	546304	159.18	No	Yes;0	3	2	No	66.4
5	3-Methylxanthine	70639	166.14	No	Yes;0	3	2	No	83.54
6	Robinetin	5281692	302.24	No	Yes;0	7	5	No	131.36
7	L-Saccharopine	160556	276.29	No	Yes;0	8	5	No	149.95
8	L-Saccharopine	160556	276.29	No	Yes;0	8	5	No	149.95
9	L-Saccharopine	160556	276.29	No	Yes;0	8	5	No	149.95
10	Chlorogenic acid	1794427	354.31	No	Yes;0	9	6	No	164.75
11	Esculin	5281417	340.28	No	Yes;1	9	5	No	149.82
12	Acadesin	17513	258.23	No	Yes;0	6	5	No	156.85
13	Acadesin	17513	258.23	No	Yes;0	6	5	No	156.85
14	Sinapoyl malate	14605050	340.28	No	Yes;0	9	3	No	139.59
15	Sinapoyl malate	14605050	340.28	No	Yes;0	9	3	No	139.59
16	Sinapoyl malate	14605050	340.28	No	Yes;0	9	3	No	139.59
17	trans-Cinnamate	444539	184.16	Yes	Yes;0	2	1	No	37.3
18	Beta-indoleacetic acid	802	175.06	No	Yes;0	2	3	No	53.09
19	Beta-indoleacetic acid	802	175.06	No	Yes;0	2	3	No	53.09
20	3,4-Dihydroxy-L-phenylalanine	6047	197.07	No	Yes;0	5	5	No	103.78
21	3,4-Dihydroxy-L- phenylalanine	6047	197.07	No	Yes;0	5	5	No	103.78
22	Scoulerin	439654	327.15	No	Yes;0	5	2	No	62.16
23	Scoulerin	439654	327.15	No	Yes;0	5	2	No	62.16
24	3-Hydroxyanthranilic acid	86	153.04	No	Yes;0	4	4	No	83.55
25	3-Hydroxyanthranilic acid	86	153.04	No	Yes;0	4	4	No	83.55
26	4-Hydroxyphenylpyruvic acid	979	180.04	No	Yes;0	4	2	No	74.6
27	4-Hydroxyphenylpyruvic acid	979	180.04	No	Yes;0	4	2	No	74.6
28	3,4-Dihydroxymandelate	85782	184.04	No	Yes;0	5	4	No	97.99
29	Genistein	5280961	270.05	No	Yes;0	5	3	No	90.9
30	Genistein	5280961	270.05	No	Yes;0	5	3	No	90.9
31	Glutamine	5961	146.07	No	Yes;0	5	5	No	106.41
32	L-(+)-Lysine	5962	146.11	No	Yes;0	4	5	No	89.34
33	Guanosine-3',5'-cyclic monophosphate	135398570	345.05	No	Yes;0	12	5	No	184.62
34	Guanosine-3',5'-cyclic monophosphate	135398570	345.05	No	Yes;0	12	5	No	184.62
35	4-aminophenol	403	109.05	No	Yes;0	2	3	No	46.25
36	Cerulenin	5282054	223.12	No	Yes;0	4	2	No	72.69
37	Cerulenin	5282054	223.12	No	Yes;0	4	2	No	72.69
38	Cerulenin	5282054	223.12	No	Yes;0	4	2	No	72.69

ID: PubChem ID, MW: Molecular weight, BBB: Blood-brain barrier, CYP2D6: Cytochrome. P450 2D6, HA: Number of hydrogen bond acceptors, HD: Number of hydrogen bond donors; TPSA: Total polar surface area

Molecular docking

Chemical formulas of the selected phytocompounds (ligands) from both extracts were retrieved from the PUB Chem database and prepared for docking using Chimera. Docking results showed that compounds from water extract such as Quercetin-3-O-arabinoglucoside with a docking score of -8.9 kcal/mol, Esculin with a docking score of -8.1 kcal/mol, and Chlorogenic acid with docking score -7.8 kcal/mol exhibited a strong interaction with the enzyme Peptidoglycan D, D-Transpeptidase (6G9F) from *E. coli*, in contrast, 2-Formyl-9-(beta-d-ribofuranosyl) hypoxanthine, 2,4-Di- tert-butylphenol and 2-Methoxy-4-vinylphenol from methanol dichloromethane demonstrated lower affinity energy with docking score -7 kcal/mol, - 6.1 kcal/mol and -5.3 kcal/mol, respectively. Within this framework, of the selected microbial enzymes considered in the docking analysis, Esculin, Quercetin-3-O-arabinoglucoside, Chlorogenic acid, and 2-Formyl-9-(beta.-d-Ribofuranosyl) hypoxanthine exhibited interactions with the TEM-1 Beta-Lactamase (8GII), demonstrating enhanced antibacterial activity with docking scores of (-8.5, -8.4, -8.1 and -7.6) kcal/mol, respectively. While, 2,4-Di-tert-butylphenol and 2-Methoxy-4-vinylphenol with docking scores -6.3 kcal/mol and -5.4 kcal/mol, respectively, demonstrated lower affinity energy compared to 2-Formyl-9- (beta-d-Ribofuranosyl) hypoxanthine from the same extract and compounds from water extract. Quercetin-3-O-arabinoglucoside and Chlorogenic acid demonstrated a binding affinity with the Beta-Lactamase (1BLH), attaining a docking score of -8.8 kcal/mol and -8.1 kcal/mol, respectively. The rest Chlorogenic acid and 2-Formyl-9- (beta -d-ribofuranosyl) hypoxanthine, 2,4-Di-tert-butylphenol and 2-Methoxy-4-vinylphenol demonstrated lower binding affinity with docking score -7.5 kcal/mol, 6.8 kcal/mol, 6.4 kcal/mol and -5.5 kcal/mol, respectively (Table 6).

Table 6. The predicted docking scores of the different ligands for inhibitor binding with the tested proteins

Extract type	Extract phytochemical	Targets binding affinity (kcal/mol)		
		Peptidoglycan D, D-Transpeptidase (6G9F)	**TEM-1 Beta-Lactamase (8GII)**	**Beta Lactamase (1BLH)**
Methanol-dichloromethane extract	2-Methoxy-4-vinylphenol	-5.3	-5.4	-5.5
	2,4-Di-tert-butylphenol	-6.1	-6.3	-6.4
	2-Formyl-9-beta-d- ribofuranosyl hypoxanthine	-7	-7.6	-6.8
Water extract	Quercetin-3-O- arabinoglucoside	-8.9	-8.4	-8.8
	Chlorogenic Acid	-7.8	-8.1	-8.1
	Esculin	-8.1	-8.5	-7.5

Quercetin-3-O-arabinoglucoside, Esculin, and Chlorogenic acid from water extract demonstrated a higher interaction with the docking enzymes than the compounds from methanol, dichloromethane extract. The 2D diagram and the ligand interactions with the distance in Angstrom and the amino acid incorporated in the interaction for the three compounds are represented in figures 5, 6, and 7, while detailed results on the methanol dichloromethane compounds are shown in S1, S2, and S3 Figures.

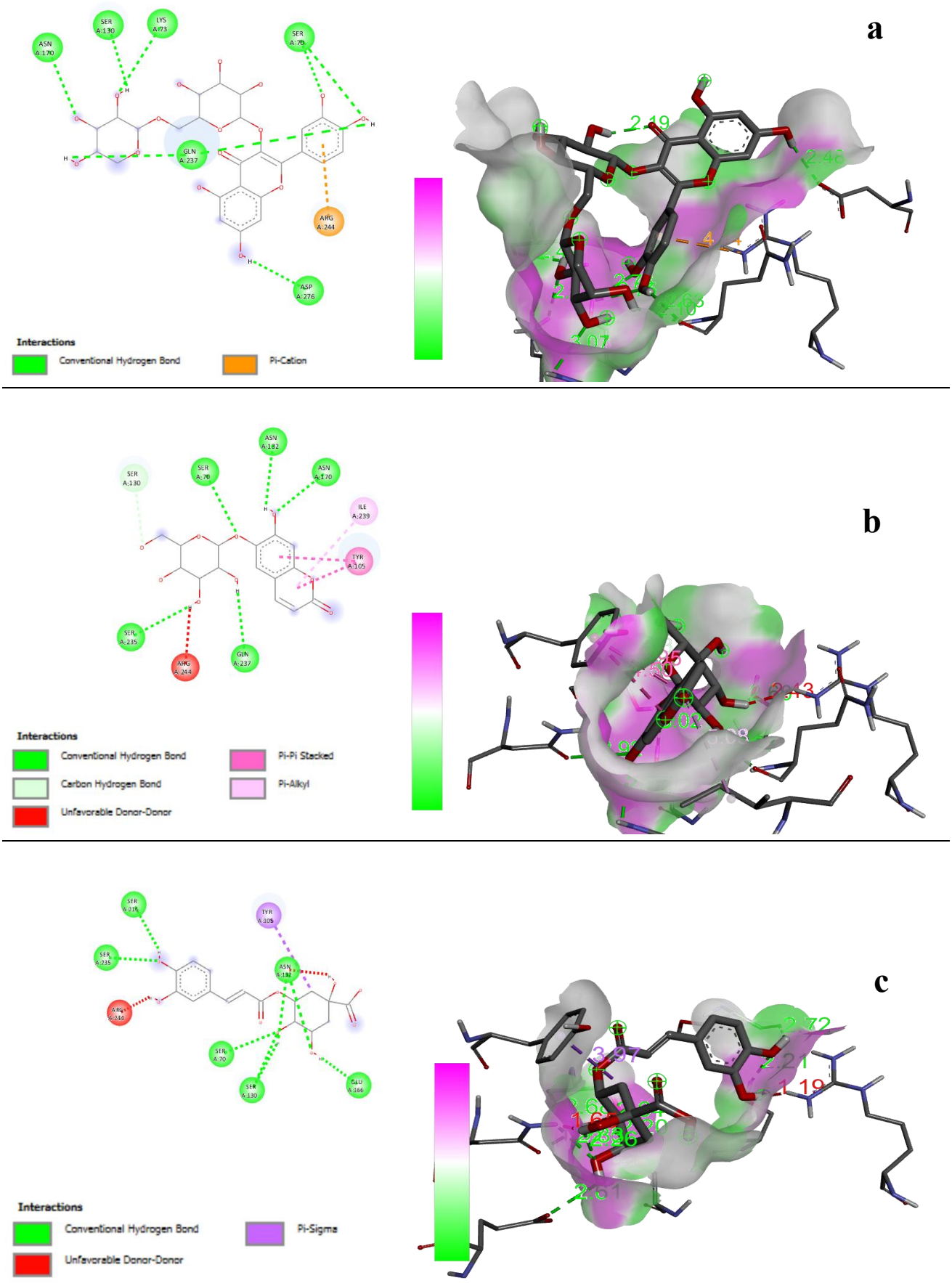

Figure 5. 2D diagrams and ligand interaction profiles of Quercetin-3-O-arabinoglucoside (a), Esculin (b), and Chlorogenic acid (c) with the 1BLH enzyme

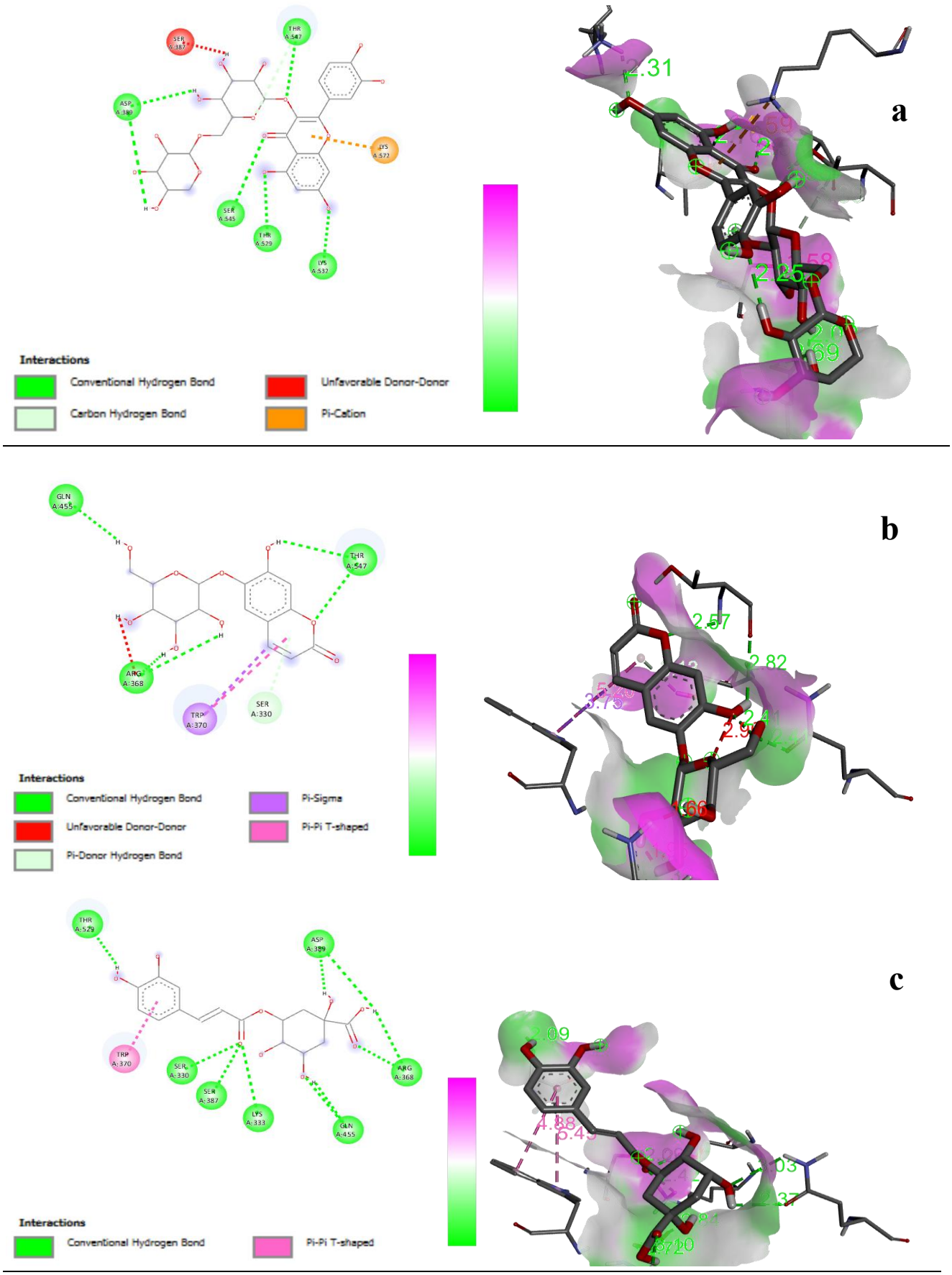

Figure 6. 2D diagrams and ligand interaction profiles of Quercetin-3-O-arabinoglucoside (a), Esculin (b), and Chlorogenic acid (c) with 6G9F enzyme

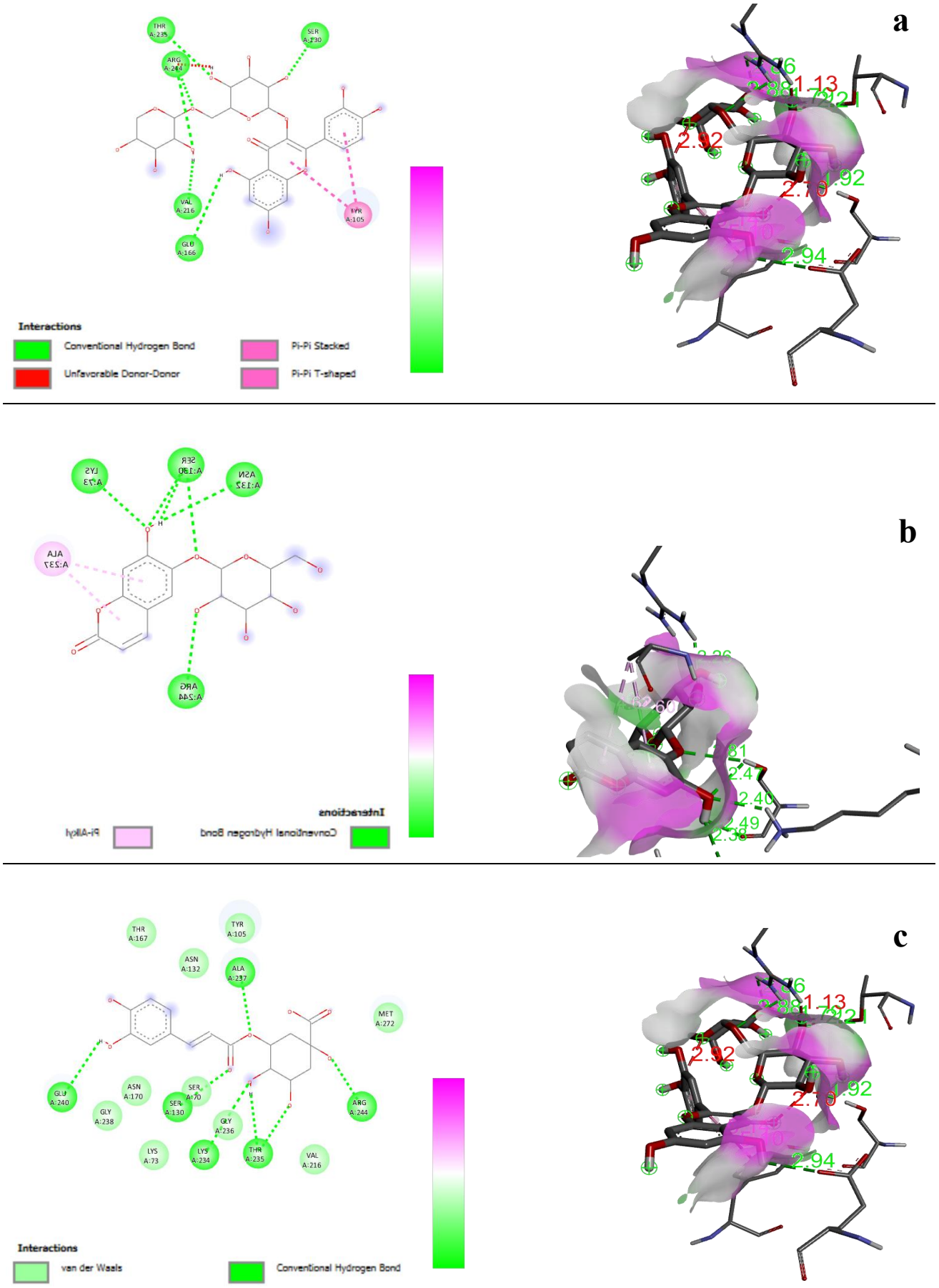

Figure 7. 2D diagrams and ligand interaction profiles of Quercetin-3-O-arabinoglucoside (a), Esculin (b), and Chlorogenic acid (c) with 1BLH enzyme

DISCUSSION

Antibiotic resistance impacts all categories of antibiotics. Multidrug-resistant bacteria (MDR) have emerged as a significant world health concern. Medicinal plants are abundant in secondary metabolites that confer antibacterial properties against many diseases while demonstrating few side effects (Mustapha et al., 2024). This study highlighted *Z. spina-christi* as a source of phytocompounds, such as flavonoids, coumarins, cinnamic acid, alkaloids, saponins, tannins, phenolic compounds, and terpenoids, which have been empirically shown to exhibit activity against both Gram-positive and Gram-negative (Kuete, 2010). Nevertheless, the safety and efficacy of Z. spina have not been fully evaluated in people, necessitating more rigorously conducted clinical trials to validate preclinical results. The mechanism of action of the leaf extract requires examination. The standard dosage and safety of the leaf must be determined (Abdulrahman et al., 2022). The phytochemical analysis results elucidated the antimicrobial activity observed in *Z. spina-christi* leaf extracts. Similar findings have been documented by El-Shahir et al. (2022), who illustrated the existence of diverse compounds in *Z. spina-christi* from Saudi Arabia, encompassing amino flavonoids, saponins, alkaloids, tannins, phenolic compounds, terpenoids, and fatty acids. Abdulrahman et al. (2022) reported that, chemically, polyphenols and flavonoids were the most reported compounds in *Z. spina-christi* leaves, with a composition of 66 compounds out of the total 193 compounds reported from different parts of the plant.

Based on the antibacterial activities observed in this study, both extracts demonstrated minimal efficacy at a concentration of 100 mg/ml while exhibiting significant effectiveness at 400 mg/ml. Results indicated the antibacterial effectiveness of both extracts in combating the studied pathogens was enhanced at higher concentrations, as demonstrated in the study of Motamedi et al. (2009).

Methanol-dichloromethane extract demonstrated antibacterial activities against the three isolates, aligning with Bukar et al. (2015) findings, indicating that *Z. spina-christi* demonstrates antibacterial activity against *Pseudomonas aeruginosa*, *E. coli*, *Shigella* species, and *S. aureus*. The extract derived from methanol and dichloromethane demonstrated remarkable antibacterial efficacy against both bacterial species, with inhibition zones measuring 19.6 ± 0.5 mm for *S. aureus*, closely followed by *S. flexneri* at 18.3 ± 0.5 mm and *E. coli* at 16.6 ± 0 mm. The water extract demonstrated significant activity solely against gram-positive bacteria, specifically *S. aureus*, which exhibited an inhibition zone of 20.3 ± 0.5 mm. In contrast, *E. coli* and *S. flexneri* displayed a diminished response to the extract 12 ± 0 and 11 ± 0 mm, respectively. The findings align with earlier research indicating that Gram-negative bacteria demonstrate heightened resistance to most plant extracts in comparison to Gram-positive bacteria, in concurrence with the findings of Suliman and Mohammed (2018) have demonstrated that in their study, water extracts derived from the *Z. spina-christi* leaves demonstrate notable antibacterial activity against *S. aureus*, with an inhibition zone measuring 14.2 mm, closely followed by 14.1 mm and 13.3 mm for *E. faecalis* and *B. subtilis*, respectively. In contrast, all tested Gram-negative bacteria, such as *S. typhi*, *P. aeruginosa*, and *E. coli*, exhibited complete resistance to the extracts, resulting in the absence of inhibition zones.

The results suggest that methanol extracts may possess more potent or higher concentrations of active antibacterial compounds than aqueous extracts, and a presence of a potential disparity in susceptibility between Gram-positive *(S. aureus*) and Gram-negative (*E. coli* and *S. flexneri*) bacteria to the extract.

The minimum inhibitory concentration for methanol-dichloromethane extract was 12.5 mg/ml for both *S. aureus* and *S. flexneri*, and 50 mg/ml for *E. coli*. While aqueous extract MIC was 12.5 mg/ml for *S. aureus* and 50 mg/ml for *S. flexneri* and *E. coli*, respectively. Conversely, Alhassan et al. (2019) indicated that the water extract MIC was 31.25 and 62.5 mg/ml for *E. coli* and *S. aureus*, respectively. Regarding those two studies, the aqueous extract of the present study has more potency towards gram-positive bacteria compared to gram-negative ones. Concerning the MBC, the methanol-dichloromethane extract had a lethal activity of 100 mg/ml against the test organisms, whereas the water extract exhibited a lethal activity of 100 mg/ml against *S. aureus*, while *E. coli* and *S. flexneri* were resistant at this concentration. There is a notable inconsistency in the Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) results between the present study and the previous findings. These inconsistencies could be attributed to differences in experimental conditions, such as extraction techniques, bacterial strains used, solvent polarity, or differences in phytochemical composition due to variations in plant origin or environmental factors as these bioactive compounds are often synthesized in response to environmental stimuli such as temperature, light intensity, soil composition, and water availability.

Penicillin-binding proteins (PBPs) function as the primary targets for β-lactams, which represent the most potent class of antibiotics developed to address bacterial infections (Freischem et al., 2021). The rapid and widespread emergence of broad-spectrum β-lactam resistance genes, including Carbapenemases, significantly undermines the effectiveness of antibiotics within this class (Sauvage and Terrak, 2016).

There is a pressing need for novel and potent PBP inhibitors, particularly those that demonstrate resistance to β-lactamase hydrolysis. Following the revolutionary finding of penicillin and its extensive application in healthcare throughout the 1940s, numerous additional classes and genera of Beta-lactam antibiotics have been formulated to effectively combat the increasing resistance of bacteria (Sauvage and Terrak, 2016). The considerable resistance to β-lactams, mostly attributed to β-lactamases, limits their effectiveness unless they are administered alongside β-lactamase inhibitors, which are predominantly non-β-lactams (Sauvage and Terrak, 2016).

Quercetin, a natural flavonoid antioxidant, possesses anti-carcinogenic, anti-inflammatory, and antibacterial characteristics (Mu et al. 2021). It may also impede biofilm formation by reducing Extracellular Polymeric Substances (EPS) synthesis and modifying the EPS composition in *Staphylococcus epidermidis* (Mu et al., 2021).

Esculin exhibits antibacterial efficacy against *S. aureus* and *E. faecalis*, as well as *E. coli*, *S. enteritidis*, *S. typhimurium*, and several multi-resistant strains of *E. coli* (Cai and Cai, 2023). It has also demonstrated antibiosis effects against certain fungi, including *T. interdigitale*, *T. mentagrophyte*, *Scopulariopsis brevicaulis*, *Microsporum canis*, and *Aspergillus fumigatus* (Cai and Cai, 2023). Conversely, chlorogenic acid significantly increased the permeability of the exterior and inner plasma membranes, resulting in impaired barrier integrity and the efflux of cytoplasmic contents, which led to cellular apoptosis (Lou et al., 2011).

To eliminate bias, TEM-1 Beta-Lactamase (8GII) from *Escherichia coli*, which is predominantly conserved among gram-negative bacteria, was chosen to represent the gram-negative source, while 1BLH Beta-Lactamase (1BLH) from *S. aureus* was selected for the gram-positive origin in the docking activity. The glycosyltransferase and transpeptidase functions of multimodular penicillin-binding proteins within complexes of multiple proteins are crucial for the formation of a functioning, stress-resistant peptidoglycan layer (Edoo et al., 2017). D, D-Transpeptidase catalyzes the synthesis of peptidoglycan cross-links in Gram-positive and Gram-negative bacteria (Edoo et al., 2017). Peptidoglycan D, D-Transpeptidase (6G9F) from *E. coli* was obtained from the PDB for docking purposes.

The docking results indicated that both extracted phytocompounds exhibited substantial interactions with the identified enzymes during docking. Quercetin-3-O-arabinoglucoside exhibits the highest binding affinity for Peptidoglycan D, D-Transpeptidase active site, with a binding score of -8.9 kcal/mol, followed by Esculin at -8.1 kcal/mol and Chlorogenic acid at -7.8 kcal/mol. The Methanol-dichloromethane phytocompounds exhibit a reduced binding affinity to PBP, with a maximum binding affinity of -7 associated with 2-Formyl-9-(beta-d-ribofuranosyl) hypoxanthine.

Within the framework of Beta lactamases (8GII and 1BLH), Esculin demonstrated the greatest degree of binding to 8GII, measured at -8.5 kcal/mol, followed by Quercetin-3-O-arabinoglucoside at -8.4 kcal/mol and Chlorogenic acid at -8.1 kcal/mol. In the methanol dichloromethane extract, 2-Formyl-9-(beta -d-ribofuranosyl) hypoxanthine exhibited the greatest binding score at -7.8 kcal/mol. Quercetin-3-O-arabinoglucoside, with a binding affinity of -8.8 kcal/mol, and Chlorogenic acid, with -8.1 kcal/mol, exhibited the best affinity for inhibiting 1BLH Beta-Lactamase.

Molecular docking analysis revealed that Quercetin, Esculin, Chlorogenic acid, and 2-Formyl-9-(β-d-ribofuranosyl) hypoxanthine exhibited significant binding affinity toward the active sites of penicillin-binding proteins (PBPs) and β-lactamases, which were identified using XYZ coordinates from the Protein Data Bank (PDB). Quercetin-3-O-arabinoglucoside exhibited the most significant affinity for peptidoglycan D, D-transpeptidase (6G9F) at -8.9 kcal/mol, presumably establishing hydrogen bonds and van der Waals interactions with essential active site residues, including Ser294, Lys386, and Thr410, which are vital for enzymatic activity. Esculin exhibited significant affinity for TEM-1 β-lactamase (8GII) (-8.5 kcal/mol), forming hydrogen bonds and π-π stacking interactions with Glu166 and Ser70, which are crucial residues in the hydrolysis of β-lactam antibiotics, thereby potentially impeding bacterial resistance. Chlorogenic acid demonstrated notable binding affinity to β-lactamases (-8.1 kcal/mol), highlighting its potential function in β-lactamase inhibition via interactions with Asp132 and Lys73.

Conversely, the compounds extracted using methanol-dichloromethane, including 2-Formyl-9-β-d-ribofuranosyl hypoxanthine, exhibited moderate binding affinities, achieving a peak score of -7.6 kcal/mol for TEM-1 β-lactamase (Bush and Bradford, 2020). This compound presumably engages in interactions via hydrogen bonds and electrostatic forces with Asn132 and Glu166; however, its affinity appears to be less robust in comparison to Quercetin-3-O-arabinoglucoside and Esculin. The comprehensive docking results correspond with the *in vitro* observations, wherein the water extract exhibited superior activity against Gram-positive bacteria, likely attributable to enhanced interactions with PBPs and β-lactamases. Conversely, the methanol-dichloromethane extract proved more efficacious against Gram-negative bacteria, potentially owing to the presence of nonpolar phytochemicals that aid in membrane penetration. The results underscore the necessity for additional *in vitro* validation and structural refinement to improve antibacterial effectiveness (Bush and Bradford, 2020). Additionally, the comparison of water versus methanol-dichloromethane extracts aligns with phytochemical profiling studies showing that flavonoids, coumarins, and polyphenols (such as

Quercetin-3-O-arabinoglucoside and Esculin) have strong β-lactamase inhibition properties (Cushnie and Lamb, 2011). Meanwhile, terpenoids and alkaloids, dominant in the methanol-dichloromethane extract, exhibit antibacterial activity through membrane disruption rather than enzyme inhibition.

The present study is unprecedented, as no prior research has examined the docking of identical compounds from *Z. spina christi* leaf extracts with these enzymes. The molecular docking results corroborate the *in vitro* antibacterial activity, establishing *Z. spina christi* leaf extract as a potent agent against bacterial infections by interfering with cellular wall synthesis through interaction with PBPs. Additionally, it may serve as a beta-lactamase inhibitor in conjunction with beta-lactam antibiotics to combat resistance to these antibiotics.

CONCLUSION

In conclusion, both methanol and aqueous extracts possess significant antibacterial properties, with methanol extracts being more potent. The results underscored the potential application of these extracts as natural antibacterial agents. *Z. spina christi* leaf extract serves as a strong treatment against bacterial infections by inhibiting cell wall synthesis through interaction with penicillin-binding proteins (PBPs). Moreover, it may function as a beta-lactamase inhibitor alongside beta-lactam antibiotics to address resistance to these agents. Future research should concentrate on isolating and characterizing active compounds in *Z. spina-christi* extracts using bioassay-guided fractionation and advanced spectroscopic techniques. Mechanistic studies, including proteomic and transcriptomic analyses, are needed to understand the mode of action. Synergistic interactions with conventional antibiotics should also be explored.

DECLARATIONS

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Authors' contributions

Hamasat Mohammed Musa Abdallah conducted the methodology and data curation and prepared the original draft. Eddy Okoth Odari and John Benjamin Ochieng supervised, reviewed, and edited the study. All authors have read and agreed to the published the last edition of the manuscript.

Competing interests

The authors acknowledge having no conflict of interest regarding this research.

Availability of data and materials

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

Ethical considerations

All authors considered and confirmed the ethical issues, including plagiarism, consent for publication, misconduct, fabrication of data, and duplicate publication or submission.

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Adoption Preferences of Greek Citizens in Stray Dogs: The Role of Morphological Characteristics

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ABSTRACT

A critical aspect of addressing the stray dog population issue in Greece involves the investigation of the likelihood of dog adoptions based on morphological traits. Recent statistics indicate that 43% of Greek households own at least one companion animal. This study aimed to explore some of the factors influencing the decision to adopt a stray and the morphological traits that are preferred by the adopters. To undertake this research, data spanning four years (2019- 2022) were used; the data comprised information on 858 adoptions from a Greek animal welfare organization on the Island of Lesvos in the Aegean Sea. The analysis revealed that younger age, smaller size, and tan or tricolor coats were associated with faster adoption rates. In addition, when comparing the data of all four years (2019-2022), the year of adoption was found to be a statistically significant variable, which confirms that Greeks also followed the international trend of increased adoptions during the Covid-19 pandemic.

Keywords: Adopter preference, Greece, Morphological trait, Shelter dog, Stray companion animal

INTRODUCTION

Greece is home to one of the largest populations of stray companion animals worldwide, with an estimated three million stray dogs and cats (Smith, 2021; MarsPetcare, 2022). According to a study at Aristotle University of Thessaloniki, there are already over 3 million stray dogs and cats, and the number is expected to rise to about 4 million in the coming years (Newsbeast, 2023). However, Greece has seen a significant change in companion animal ownership trends over the past two decades. There has been an observed gradual shift in societal attitudes toward companion animals and a growing awareness of animal welfare (Fallieros, 2024). Recent reports indicate that 43% of Greeks own at least one companion animal, with 66.1% of pet owners having at least a dog, which makes for approximately 655,000 dogs, and 42% owning at least a cat, which is calculated to approximately 606,000 cats (DiaNEOsis, 2022; Fallieros, 2024). According to the study, younger Greeks are more likely to own companion animals, while retirees are associated with the lowest percentage of companion animal ownership (DiaNEOsis, 2022; Fallieros, 2024).

Due to the COVID-19 limitations and lockdowns, people spent more time at home, and an increase in companion animal ownership was observed (Fallieros, 2024). Based on the DiaNEOsis (2022) survey, preferences for purebred animals are gradually decreasing, with 56.6% of Greeks acquiring their companion animals from the street or animal welfare organizations, compared to 16.4% who purchased their companion animal from breeders. Among those who continue to favor purebreds, popular dog breeds include Maltese, Poodles, Shih Tzus, French Bulldogs, Golden Retrievers, and Labrador Retrievers (Fallieros, 2024).

This overall increasing trend in companion animal ownership reflects a broader cultural acceptance of companion animals as an integral part of the family. Furthermore, the increasing adoption of stray animals reflects the increased awareness and concern regarding animal welfare. To better understand the Greek stray dog population problem, insights into the morphological traits of the dogs available for adoption and their probability of being adopted need to be explored. At present, and to the best of the authors' knowledge, there is no existing published study related to this issue in Greece. This study aimed to examine the relationship between the morphological traits of stray dogs and their likelihood of being adopted in Greece.

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MATERIAL AND METHODS

Ethical approval

The present study utilized publicly available data to ensure transparency and contribute to research aimed at implementing knowledge in the field of companion animals. The Greek nonprofit animal welfare organization *Kivotos* has been informed of the data usage and has granted approval. Adhering to ethical research standards, this approach facilitates data-driven insights that can enhance animal care practices and adoption outcomes.

Study design

Data was manually extracted from the official Facebook page of *Kivotos*, a stray animal welfare organization on the Island of Lesvos, Greece. The data is publicly available for Facebook and non-Facebook users as the shelter has set its page to public access. The information on 979 dog adoptions was extracted for four years (2019-2022). After excluding entries with missing values, the final dataset used in our analysis comprised 858 dog adoptions.

The collated details included the date of posting the adoption advertisement, adoption date, sex, age, coat color, and weight of the dog. For puppies, a prediction of the weight at adulthood was provided by the collaborating veterinarian of the shelter. All descriptive information was provided by the experienced animal shelter staff. The data allowed the construction of explanatory variables. Specifically, the date of adoption advertisement and the adoption date allowed the creation of a new variable, that is, the length of stay (LOS), which indicates the number of days the dog spent under the animal welfare organization's care. Age at the time of advertisement (in months) was treated as a continuous variable, while sex was coded as a binary categorical variable (0 = male, 1 = female). Coat color was created as a categorical variable and took the following values including black, black combination, white, white combination, black and white, tan, tricolor-fair, tricolor-dark, and brown/brindle/gray/other. Since stray dog breeds are often misidentified, the animal welfare organization focused on only reporting a predicted adult size in kilograms and the gender of the dog. Finally, a categorical variable depicting the year in which the dog was offered for adoption was included.

Statistical analysis

The Cox proportional hazards model (Abd El Hafeez et al., 2021) was employed to assess the influence of morphological traits on adoption speed (LOS). LOS is presented by a hazard ratio (HR), which indicates how quickly an adoption occurs, and, as such, a higher HR indicates a faster adoption. The explanatory variables included in the hazard model of this study were age, predicted size, coat color, sex, and the year the dog was offered for adoption. The analysis and the descriptive statistics were carried out on STATA (Version 16; StataCorps, College Station, Texas, USA). The Cox proportional hazards model assumes that the effect of each covariate on the hazard remains constant over time, which can be evaluated using Schoenfeld residuals. A post-estimation test was conducted using Stata command 'estat phtest' based on Schoenfeld residuals, and there was no evidence that the proportional hazards assumption had been violated. Specifically, Schoenfeld residuals were examined for each explanatory variable (age, predicted size, coat color, sex, and year of adoption) to assess whether their effects remained constant over time.

A significance threshold of $p < 0.05$ was applied, and no adjustments for multiple comparisons were necessary, as the proportional hazards assumption was tested as a model-wide diagnostic rather than a hypothesis-driven multiple comparison analysis.

RESULTS

Of the total 979 adoptions, 16.5% occurred in 2019, 26.6% in 2020, 32.3% in 2021, and 24.5% in 2022. Across all years, the average predicted weight of the dogs was consistent (approximately 11 kilograms, with the female: male dog ratio of slightly over 50%). The shortest LOS was observed in 2021 (13.3 days), and the longest in 2019 (27.9 days). Across all years, the average age of the dogs was less than 6 months old. The descriptive statistics of the data are presented in Table 1.

The results of the Cox proportional hazards regression model indicated that age, predicted size, coat color, and the year the dog was on offer had a statistically significant impact on the LOS. Table 2 provides details of the Cox proportional hazards regression model.

Specifically, the findings regarding the age at the time of advertisement ($p < 0.05$, 95% CI: 0.95-0.98) revealed that younger dogs have a shorter LOS. Dogs with a tan coat color ($p < 0.05$, 95% CI: 1.19-1.93), tricolor-fair coat color ($p < 0.05$, 95% CI: 1.06-2.29), and tricolor-dark coat color ($p < 0.05$, 95% CI: 1.05-1.83) were found to have a shorter LOS, as compared to their black-coated counterparts. The results for the variable size were found statistically significant ($p < 0.05$, 95% CI: 0.95-0.99), indicating that an increase in a dog's predicted size (measured in kilograms) was associated with a longer length of stay (LOS). This finding suggests that smaller dogs were adopted more quickly than larger dogs. Finally, the Cox proportional hazards model indicated that LOS was significantly shorter in 2020 and 2021 compared to

the reference year, 2019 ($p < 0.05$). In comparison to the 2019 baseline, dogs had a higher likelihood of faster adoption in 2020 ($p < 0.05$, 95% CI: 1.26–2.03) and 2021 ($p < 0.05$, 95% CI: 1.22-1.98). These results collectively suggest an overall increase in adoption speed during these years.

Table 1. Descriptive statistics of dog adoptions during 2019-2022 (n=979) in Lesvos, Greece

Variable	2019	2020	2021	2022
Number of Adoptions	162	261	316	240
Average LOS (days)	27.9	14.7	13.3	19.1
Average age (months)	5.74	3.05	3.76	3.2
Female	84 (52%)	143 (55%)	170 (54%)	131 (55%)
Average predicted size (kgs)	11.6	11.4	11.8	11.4
Coat colour				
Black	17 (10.5%)	46 (17.6%)	33 (10.5%)	33 (13.7%)
Black combination	5 (3.1%)	15 (5.75%)	26 (8.2%)	13 (5.4%)
White	29 (17.9%)	40 (15.33%)	29 (9.2%)	32 (13.3%)
White combination	4 (2.5%)	11 (4.2%)	43 (13.6%)	25 (10.4%)
Black/white	16 (9.9%)	20 (7.7%)	64 (20.3%)	7 (2.9%)
Tan	48 (29.6%)	43 (16.5%)	57 (18.1%)	74 (30.8%)
Tricolour - fair	14 (8.6%)	11 (4.2%)	8 (2.5%)	10 (4.2%)
Tricolor - dark	9 (5.6%)	31 (11.88%)	47 (14.9%)	21 (8.7%)
Brown/brindle/gray/other	20 (12.3%)	44 (16.9%)	8 (2.5%)	25 (10.4%)

Table 2. Cox proportional hazard model results on the Length of stay of stray dogs before being adopted in an animal welfare organization in Lesvos, Greece, during 2019-2022 (n=858)

LOS*	HR**	St. Error***	P-value	95% Confidence interval	
Age	0.97	0.01	0.001	0.95	0.98
Sex	1.00	0.07	0.95	0.87	1.15
Coat colour					
Black	1.00				
Black combo	1.06	0.18	0.73	0.75	1.48
White	1.20	0.16	0.16	0.92	1.57
White combo	1.31	0.20	0.07	0.96	1.78
Black and white	1.31	0.18	0.05	0.99	1.73
Tan	1.52	0.18	0.001	1.19	1.93
Tricolour- fair	1.56	0.30	0.02	1.06	2.29
Tricolour -dark	1.39	0.19	0.02	1.05	1.83
Brown/Brindle/Gray/other	1.22	0.17	0.16	0.92	1.63
Size	0.97	0.01	0.01	0.95	0.99
Year					
2019	1.00				
2020	1.60	0.19	0.001	1.26	2.03
2021	1.55	0.19	0.001	1.22	1.98
2022	1.22	0.14	0.09	0.96	1.54

*LOS: Length of Stay. Describes the days a dog stayed in the care of the shelter before they were adopted; **HR: Hazard Ratio. Corresponds to the relative risk of the event happening for a given unit change in the predictor variable; ***St. Error: Standard Error. Measures the accuracy with which a sample distribution represents a population.

DISCUSSION

This analysis represents the first in the literature to investigate dog adoptions in Greece. This study has obtained data from a single animal welfare organization located on the Island of Lesvos, which may be considered a limitation for the generalization of the findings to other regions or shelter environments. To tackle the potential sampling bias, the research team communicated with the organization, and it was clearly stated that the adoptive families are from throughout the country (personal communication). Thus, regardless of where they live in Greece, information on adaptive preferences is provided. The animal welfare organization publicly advertises the dogs on their social media platform and only charges adoptive families a flat fee of €50, which covers veterinary care, microchipping, and ship transport fees to other islands or mainland Greece (Personal communication). Given this, it can strongly be asserted that the findings present adoption preferences at a national level.

The regression analysis revealed that younger dogs were more appealing to adopters. This popularity of puppies is a worldwide phenomenon, with studies revealing shorter LOS in the USA (Brown et al., 2013; Cain et al, 2020) and other countries like Italy, the United Kingdom, and the Czech Republic (Normando et al, 2006; Diesel et al, 2007; Siettou et al., 2014; Žák et al, 2015). The preference for younger animals highlighted the importance of considering age when developing strategies to enhance adoption rates and address the stray dog population effectively.

Within this study's dataset, the average advertised age was less than 6 months for all years of examination, which was due to an influx of puppies (personal communication). This result provides evidence of the anecdotal knowledge that Greece has an issue of uncontrolled, unplanned breeding.

According to the literature, the size of dogs significantly impacts their LOS and likelihood of adoption, with smaller dogs being preferred by potential adopters (Siettou et al., 2014). The result of the model corroborated this trend, indicating that Greek citizens also prefer smaller dogs over larger ones. In terms of coat color, and according to international literature, black coat-colored dogs were suggested as having a higher LOS compared to tan and tricolor coat-colored dogs (Voslarova et al., 2019). Another common international trend is the preference for white coat-colored dogs over black ones (Posage et al., 1998). There is anecdotal knowledge that tan coat-colored dogs, described in Greece as cinnamon-colored, are the most popular in Greece, and this study provided significant evidence of this preference.

Finally, the year the dogs were offered for adoption was found to be statistically significant. Particularly, an adoption increase was experienced during 2020 and 2021, which was in line with the international trend observed during the Covid-19 pandemic years (2020-2021; Morgan et al., 2020; Ho et al., 2021; Siettou, 2021). This analysis indicated a shorter LOS for these two years compared to 2019. However, this study did not reveal any significant difference between the years 2019 and 2022, suggesting that LOS and adoption rates returned to pre-pandemic levels as COVID-19 restrictions were eased. This may reflect broader societal changes, such as the temporary increase in pet adoptions during lockdowns due to remote work and social isolation (Morgan et al., 2020), followed by a stabilization as routines normalized (Such as returning to office work with reduced time at home). Future research could explore the long-term impact of these shifts on adoption trends. Moreover, attention should be given to multi-center studies in examining whether adoption patterns vary across different shelters and geographic locations, particularly in other Mediterranean countries with similar socio-cultural and economic contexts as Greece. Additionally, longitudinal studies incorporating factors such as adopter preferences and policy changes could provide deeper insights into the determinants of adoption speed over time.

CONCLUSION

This analysis has provided important initial insights into Greek adoptive families' preferences regarding stray dogs' morphological traits. As mentioned in this study, Greece has a growing population of stray dogs on its streets. The results of this research contribute to defining Greek citizens' dog preferences. The trend over the country as to adopting rather than buying dogs may be a way to help with the stray population issue. Most of these preferences are aligned with international trends, although others indicate Greece's own cultural choices. Future studies should further explore the role of morphological traits in adoption preferences and even investigate how these traits may influence the likelihood of dogs becoming strays. Investigating these patterns across different regions and shelter environments could provide a deeper understanding of the factors that shape adoption trends. Additionally, research on public perceptions of stray dogs and their physical characteristics may offer valuable insights into improving welfare and rehoming strategies.

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Competing interests
The authors declare no conflicts of interest.

Authors' contributions
Anna Stefani Siettou made a major contribution to the study design, realization, data collection, analysis and interpretation, writing and preparation of the final version of the manuscript, submission of the manuscript, and coordination with the authors. Eleni Theodoropoulou made a major contribution to study design, data analysis, and interpretation, and reduction of the manuscript, participated in data acquisition, and edited and revised the final version of the manuscript. Christie Siettou made a major contribution to data analysis and interpretation and edited and revised the final version of the manuscript. Vilielmini Karagianni edited and revised the final version of the manuscript. Evangelia Sossidou edited and revised the final version of the manuscript. All authors have read and given final approval for the last edition of the article to be published.

Availability of data and materials
The datasets generated and analyzed during the current study are available from the corresponding and the first author upon reasonable request.

Ethical considerations

All authors checked and approved accuracy of ethical issues, including plagiarism, consent for publication, misconduct, fabrication of data, and duplicate publication or submission.

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Oxidative Stress Markers, Antioxidant Balance, and Protein Metabolism in Dogs with Acute Prostatitis

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ABSTRACT

The prostate gland in dogs is highly vulnerable to the action of negative pathogens due to its structure and topography. Among the numerous etiological factors in the development of prostatitis, inflammatory processes and oxidative stress play a predominant role, regardless of whether the condition is bacterial, viral, or autoimmune in origin. This study aimed to assess protein metabolism and redox balance indicators in the prostate tissue of dogs with acute prostatitis. For biochemical analyses, prostate tissue samples were taken from 24 mixed-breed dogs, including twelve animals that were considered healthy with no abnormalities of the genitourinary system (control group) and twelve animals with newly diagnosed acute prostatitis, from which samples were obtained via biopsy (experimental group). Following homogenization and sample preparation, all biochemical parameters in the prostate tissue were determined spectrophotometrically. The results of biochemical studies in dogs with acute prostatitis demonstrated a significant increase in the content of thiobarbiturate acid-reactive compounds by 102.2% and the level of lipid hydroperoxides by 35.7% compared to healthy dogs in the control group. In contrast, the total protein content was 32.9% lower than in the control group, while reduced glutathione levels decreased by 76.5%. Similar changes to the dynamics of oxidative stress markers were indicated by the activity of antioxidant enzymes, with glutathione peroxidase and catalase activities increasing by 61.3% and 21.8%, respectively, relative to the control group. These findings indicate the presence of oxidative stress in dogs with acute prostatitis. The biochemical changes observed in prostate tissue provide a foundation for future research aimed at developing therapeutic methods that incorporate anti-inflammatory, antibacterial, and antioxidant agents for the treatment of acute prostatitis in dogs.

Keywords: Biochemistry, Dog, Inflammation, Oxidative stress, Prostate

INTRODUCTION

The prostate gland is a large and unpaired organ composed of glandular tissue, smooth muscles, and connective tissue, functioning as an accessory sex gland (Krakowski et al., 2022; Zhao et al., 2023). The function of the prostate is primarily secretory, allowing it to synthesize plasma, which is a component of sperm and has a trophic and activating effect on male germ cells (Lea et al., 2022; Zhao et al., 2024). More importantly, the prostate is a gland that depends on hormone levels, such as dihydrotestosterone, and its anatomical location makes it easily exposed to pathogenic factors such as bacteria, viruses, and hypothermia, ultimately leading to prostatitis (Feng et al., 2021; Chen et al., 2024). Prostate epithelial cells are extremely vulnerable to external and internal stimuli that can induce DNA damage, cell pyroptosis, and abnormal proliferation (Mo et al., 2024; Wang et al., 2024). The etiology and pathogenesis of prostatitis are complex, involving infectious factors, autoimmune mechanisms, and endocrine imbalances. However, the manifestation of the disease is mainly accompanied by an inflammatory reaction, which contributes to the progression of the disease (Zhao et al., 2020; Ye et al., 2024). In males with prostatitis, reproductive disorders are mainly attributed to oxidative stress, which arises from the excessive formation of reactive oxygen species (ROS), resulting in damage to DNA, the lipid layer of the plasmalemma, and proteins in sperm cells (Ihsan et al., 2018; Koshevoy et al., 2021; Zhang et al., 2024a).

Experimental models of immune-mediated prostatitis and prostatitis associated with dietary or hormonal disruption in laboratory rodents (mice, rats) typically induce dorsolateral or ventral prostate inflammation (He et al., 2023). Typical changes in mice with simulated prostatitis included pronounced diffuse infiltration of leukocytes, increased levels of pro-inflammatory cytokines, and lipoperoxidation products (Chen et al., 2021). Development of oxidative stress was

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observed in mice with a model of prostatitis there was an increase in malondialdehyde (MDA) levels in prostate tissues, and a decrease in the activity of superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px) (Fu et al., 2020).

Research on proteins and their encoding genes has facilitated the development of novel therapeutic strategies for prostatitis. The response of the immune system to self-antigens is a factor that contributes to the development of chronic prostatitis in both men and rodents, making it worth studying the etiological factors, immunological mechanisms, and molecular and biochemical changes in depth to develop pathogenetic therapies (Manuel and Vezina, 2024). Metabolomic analyses allow the assessment of peripheral blood metabolites that may reflect stable changes in the internal environment affected by diseases, among which proteins, nucleic acids, and amino acids are important (Meng et al., 2022). Thus, in a mouse model, inhibition of chemokine receptor type 4 (CXCR4) was shown to effectively alleviate prostatitis by reducing inflammatory infiltration, lowering the level of markers of DNA damage as well as toxic malondialdehyde (MDA), and mitigating apoptosis of prostate epithelial cells (Zhang et al., 2024b). According to Hua et al. (2024), sodium butyrate alleviates experimental autoimmune prostatitis in mice by inhibiting oxidative stress and suppressing the activation of the family pyrin domain-containing 3 (NLRP3) inflammasome.

The effects of antioxidant therapy using a resveratrol derivative in combination with shock wave therapy be positive for nonbacterial prostatitis in rats by Song et al. (2023), who observed relief of inflammation, fibrosis, and pro-inflammatory genes. The course of nonbacterial prostatitis in rats can be alleviated by administering plant polysaccharides that regulate the level of inducible nitric oxide synthase, MDA, and SOD in the inflamed prostate, thereby enhancing the activity of antioxidant defense (Liu et al., 2020). To find new and accurate biomarkers related to prostate inflammation, including acute and chronic prostatitis, benign prostatic hyperplasia, and cancer, ongoing research seeks to improve diagnosis and evaluate the effectiveness of treatment for prostate diseases (prostatitis, hyperplasia, etc), which are characterized by common pathogenetic links and macroscopic changes (Savoca et al., 2019; Bosma et al., 2022). Therefore, the present study aimed to determine changes in redox processes and protein content in the prostate tissue of dogs with acute prostatitis.

MATERIALS AND METHODS

Ethical approval

Experimental studies to investigate the role of oxidative stress in prostatitis in dogs were reviewed and approved by the Bioethics Committee of the State Biotechnological University, Ukraine (ethical approval No. 8-10, dated October 8, 2023). All manipulations with male dogs were carried out in accordance with the provisions set out in the General Ethical Principles of Animal Experiments (National Congress on Bioethics, Kyiv, Ukraine, 2001) and the European Convention for the Protection of Vertebrate Animals Used for Experimental and Scientific Purposes (2006).

Animal groups and their characteristics

The study included 24 dogs aged 7-9 years with a live weight of 15-20 kg. The animals in both control and experimental groups included mixed-breed dogs that had been referred to the veterinary clinic of LLC *Vetexpert* (Kharkiv, Ukraine). Animals in the experimental group (n = 12) were diagnosed with acute prostatitis based on clinical examination, anamnesis, prostate ultrasound, and biochemical and hematological blood tests (Domosławska et al., 2023). The control group (n = 12) consisted of animals that, after examination by a doctor, were considered healthy with no abnormalities of the genitourinary system.

Prostate tissue sampling and homogenate preparation

Twelve samples of tissue biopsy specimens from dogs with newly diagnosed acute prostatitis were obtained surgically (Holak et al., 2010). Age-related conditions not known or reported to affect the genitourinary tract were not considered exclusion criteria in accordance with the guidelines (Weinekötter et al., 2023). Similarly, twelve prostate samples from healthy dogs were obtained (Holak et al., 2010). Animal prostates were used to prepare tissue homogenates (Aggarwal et al., 2006). The isolated glands were cooled on ice for 5-6 minutes, after which they were perfused with saline (0.9% NaCl solution; chilled). After that, the gland was crushed with scissors, and 1 g of tissue was taken for further homogenization. The tissue (1 g) was placed in a glass cylinder with chilled 5 mM Tris-HCl buffer, pH 7.4 (9 ml). The tissue was then homogenized for 30-40 seconds by moving the glass cylinder up and down relative to the Teflon rotor of the homogenizer (MRTU-421505-63). During the homogenization process, the glass beaker with the tissue under study was cooled from the outside using a bag filled with crushed ice. Afterward, the homogenized tissue was filtered into a centrifuge tube using a double-layer gauze. The homogenates were centrifuged for 10 minutes at 3000 rpm (t: 0 ± 2°C) to precipitate cellular stromata. The supernatant was then collected from the tubes and used for the determination of the parameters.

Determination of total protein content and oxidative stress markers

Total protein content in tissue homogenates was quantified using the Lowry Method via Simko LTD kits (Lviv, Ukraine), following the protocol described by Lowry et al. (1951). Absorbance measurements were taken with a spectrophotometer (Unico 1205, USA). The concentration of lipid hydroperoxides was determined via protein precipitation with trichloroacetic acid and lipid extraction with ethanol. Upon the addition of ammonium thiocyanate to the ethanol lipid extracts, a colorimetric reaction occurred. The absorbance of the resulting coloured product was measured using a spectrophotometer at a wavelength of 480 nm. The concentration of lipid hydroperoxides was calculated by subtracting the absorbance values of the control prostate homogenates from the experimental ones and expressed in units per 1 g of tissue (SU/g of tissue). Subsequently, the number of compounds that reacted with thiobarbituric acid was determined by the reaction of malondialdehyde with thiobarbituric acid in an acidic medium and at an elevated temperature of 100°C. This reaction led to a colour change. The absorbance of the resulting coloured product was measured using a spectrophotometer at the two wavelengths of 535 nm for lipid hydroperoxides content and 580 nm for thiobarbituric acid reactive compounds. The concentration of thiobarbituric acid reactive compounds was then calculated as MDA per gram of tissue (nmol/g tissue). All the methods for determining total protein content and oxidative stress markers were implemented as outlined in the manual by Vlizlo et al. (2012).

Evaluation of antioxidant protection indicators

The measurement of reduced glutathione (GSH) concentration is based on the formation of a thionitrophenyl anion (coloured product) when the 2-nitrobenzoic acid binds to the SH group of the GSH molecule. The GSH level was determined by the intensity of colour change. The absorbance of the coloured product was then determined spectrophotometrically at 412 nm. The GSH concentration was then determined, expressed in mmol per gram of tissue (mmol GSH/g tissue). Glutathione peroxidase (GSH-Px) activity was assessed by measuring the rate of GSH oxidation in the presence or absence of tert-butyl hydroperoxide. This method is based on the oxidation of SH groups of GSH tripeptide after adding a 2-nitrobenzoic acid to the medium. As a result of the corresponding oxidation reaction, a coloured compound (dinitrophenyl anion) was formed, the optical density of which was measured spectrophotometrically (λ=412 nm). Glutathione peroxidase activity was expressed in nmol GSH per minute per milligram of protein (nmol/min × mg prot.). Catalase activity was determined by the reaction of molybdenum salts with hydrogen peroxide, resulting in the formation of a coloured product. The optical density of the coloured product was measured spectrophotometrically at a wavelength of λ=410 nm. Catalase activity was calculated in mmol per minute per milligram of protein (mmol/min×mg prot.) All methods for determining the content of antioxidants were performed as described in the manual by Vlizlo et al. (2012).

Statistical analysis

Mathematical and statistical analyses were conducted using the Statistical Package for Social Science (SPSS), version 22. An ANOVA test was performed to compare data from control and experimental prostate samples, with normality determined using the Shapiro-Wilk test. Significant differences between groups were confirmed by the Tukey test. Statistical significance was considered to be a P-value less than 0.05.

RESULTS AND DISCUSSION

Biochemical evaluation in prostate tissue from dogs with acute prostatitis and healthy controls revealed significant alterations in markers of oxidative stress, antioxidant enzyme activity, reduced glutathione content, and total protein ($p < 0.05$). Figure 1 shows the differences in total protein content, lipid hydroperoxides, and thiobarbiturate acid-reactive compounds between the control and experimental groups.

In dogs with acute prostatitis, a decrease in total protein content in prostate tissue was by 32.9% (4.21 ± 0.17 mg/kg, $p < 0.05$) compared to control animals (6.27 ± 0.29 mg/kg). At the same time, the intensification of free radical oxidation processes of lipids and proteins in prostate tissue was determined. Whereas the level of lipid hydroperoxides in healthy dogs was 0.42 ± 0.02 SU/g in the prostate tissue, it was significantly higher by 35.7% (0.57 ± 0.02 SU/g, $p < 0.05$) in the experimental group. The concentration of thiobarbiturate acid-reactive compounds in the prostate tissue of dogs in the experimental group was 2.71 ± 0.12 nmol/g, which was 102.2% higher than in the control group ($p < 0.05$). The presence of oxidative stress in the inflamed prostate is further confirmed by the reduced antioxidant defense, as illustrated in Figure 2.

Data in Figure 2 indicate significant alterations in reduced glutathione levels and the activity of glutathione peroxidase and catalase in dogs with acute prostatitis compared to control animals. The content of glutathione in the prostate tissue of dogs in the control group was 0.17 ± 0.007 mmol/g, while it was only 0.04 ± 0.003 mmol/g in the

animals in the experimental group, which was lower by 76.5% ($p < 0.05$). Glutathione peroxidase activity in dogs with prostatitis decreased to 24.3 ± 1.2 nmol GSH/min×mg protein, which was 61.3% lower than the control values (62.8 ± 2.7 nmol GSH/min×mg protein, $p < 0.05$). While the catalase activity in healthy dogs was 234.4 ± 11.7 mmol H_2O_2/min×mg protein, it decreased to 183.3 ± 9.4 mmol H_2O_2/min×mg protein with the development of prostatitis, which is 21.8% lower than the value for the control group ($p < 0.05$).

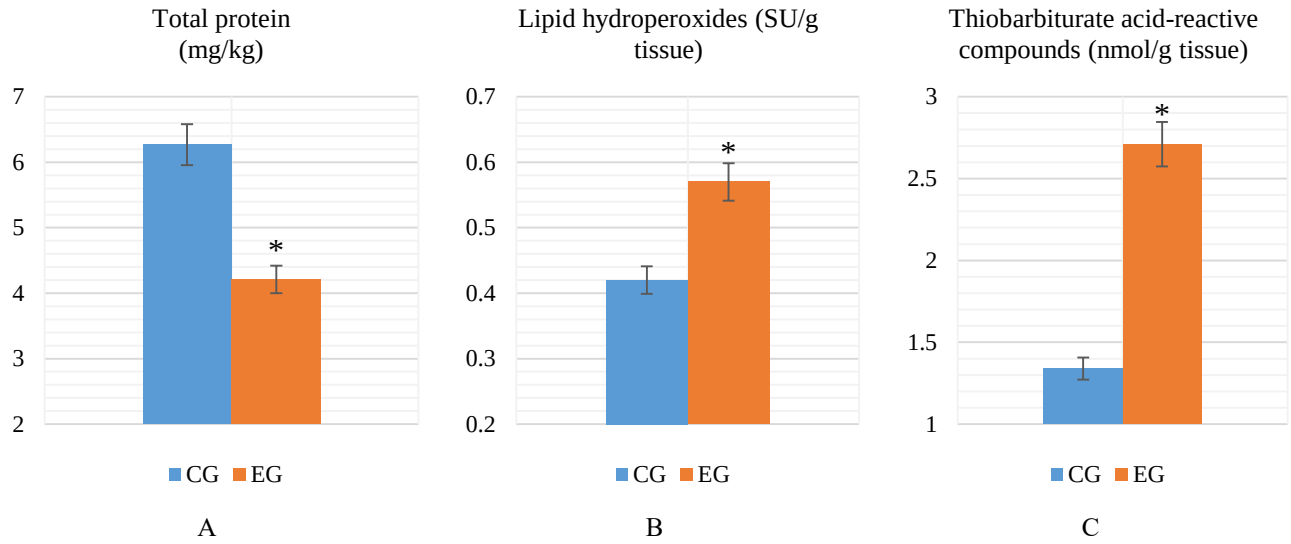

Figure 1. Total protein content (A), lipid hydroperoxide levels (B), and thiobarbiturate acid-reactive compounds (C) in prostate tissue of dogs. CG: Control group, EG: Experimental group. Significant differences ($p < 0.05$) between groups are marked with * in the figures.

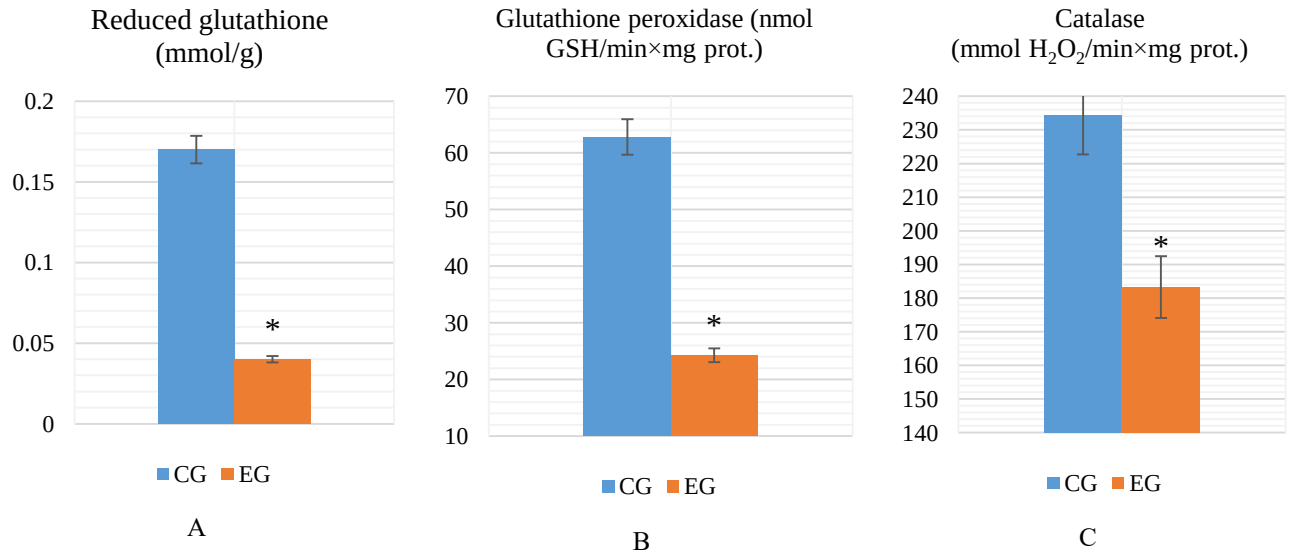

Figure 2. Reduced glutathione content (A), glutathione peroxidase activity (B), and catalase activity (C) in canine prostate tissue. CG: Control group. EG: Experimental group. Significant differences ($p < 0.05$) between groups are marked with * in the figures.

The prostate gland is the only accessory sex gland in dogs (Delaude et al., 2021; Khanbazi et al., 2021). Pathological processes in the prostate occur in older males but often remain undiagnosed, although it has been established that pathomorphological examination of the prostate in most dogs reveals morphological abnormalities with pronounced signs of inflammation (Angrimani et al., 2020; Palmieri et al., 2022). In dogs, prostatitis is a poly-etiological pathology for which there are various causes. It is accompanied by diverse clinical signs, most of which are non-specific (i.e., characteristic of several disorders of the genitourinary system) (Ryman-Tubb et al., 2022). Early diagnostic tests for disorders in the canine reproductive system, including the prostate gland, are carried out by biomarkers of reproductive function, which can be determined in the serum of male offspring, sperm, or prostate secretion, and gonadal tissues

(Koshevoy et al., 2022; Mogielnicka-Brzozowska and Cichowska, 2024). Many of these biomarkers are proteins such as albumins, clusterin, lactotransferrin, and metalloproteinases, with prostate-specific esterase being particularly important in assessing reproductive potential in male dogs (Holst et al., 2021). The present study determined a decrease in the content of total protein in prostate tissue, which confirmed the development of a pathological process. Several studies have shown that the release of proteins from the prostate is evidence of damage to this organ. These studies suggest that the presence of some proteins, particularly prostate-specific esterase, is a diagnostic tool for detecting prostate pathologies (Bucci et al., 2023; Gibson and Culp, 2024).

According to Paulis (2018), the production of ROS, mainly superoxide anion and hydroxyl radical, during acute prostatitis is aimed at destroying the bacteria that caused the infection. This is especially important for older dogs (7-10 years old) as age-related hormonal changes activate a chronic inflammatory reaction in the prostate gland, which leads to oxidative stress, DNA damage, and prostatic hyperplasia (Azadeh et al., 2022; Domosławska-Wyderska et al., 2024). The present study confirmed the development of oxidative stress in the prostate tissue of dogs during acute prostatitis. A significant increase in TBA-RC was noted in the prostate tissue of dogs in the experimental group, which is evidence of increasing free radical damage processes under acute prostate inflammation. Among the main TBA-RC metabolites, the majority consists of toxic malondialdehyde, whose accumulation in tissue indicates the destruction of prostate cell membranes and damage to lipid and protein components of the cytoskeleton (Šutulović et al., 2021; Zhang et al., 2025). The current findings revealed an increase in the content of lipid hydroperoxides in dogs with prostatitis compared to those with a healthy prostate. In general, these changes indicate the need for complex prostatitis therapy, which, along with anti-inflammatory and antibacterial effects, can neutralize toxic metabolites of oxidative stress (Park et al., 2022; Porato et al., 2023).

Aligning with the present study, Dearakhshandeh et al. (2019) reported reduced serum levels of glutathione peroxidase and superoxide dismutase (SOD) in dogs with prostatitis compared to control animals. Oxidative stress in the prostate tissue of experimental dogs developed against the background of a decrease in the activity of antioxidant defense. Thus, the content of reduced glutathione in the prostate tissue of dogs with acute prostatitis was lower than that in the control group. A substantial decrease in this compound indicates heightened oxidative stress in the prostate gland, as it represents the most active glutathione form in the animal body (Woolcock et al., 2020). Reduced glutathione content negatively affects reparative processes in the damaged prostate, as it is known to promote the induction of apoptosis and reduce ferroptosis in the hyperplastic prostate (Li et al., 2023; Zhou et al., 2024).

At the same time, a decrease in the activity of glutathione peroxidase was observed. Glutathione peroxidase is an enzyme that catalyses the reduction of lipid hydroperoxides to the corresponding alcohols and the reduction of hydrogen peroxide to water (Kendall et al., 2017). The present study also found lower catalase activity in the prostate tissue of experimental dogs compared to controls. As a result, the neutralization of hydrogen peroxide, a toxic peroxidation by-product, was significantly impaired in prostatitis-affected dogs (Aiudi et al., 2022). Current evidence suggests that catalase not only serves an antioxidant function by protecting prostate cells from damage but may also reduce apoptosis in damaged cells, thereby prolonging the recovery period, as observed in a human prostate cancer model (Giginis et al., 2023).

The findings of the present study are consistent with those of Domosławska et al. (2022), who assessed total antioxidant activity in the serum of dogs of various breeds with prostatic hyperplasia. Their study found significantly lower antioxidant levels in affected dogs than in healthy controls, with oxidative biomarkers in proteins and lipids showing a trend toward oxidative imbalance, though statistical significance was not observed. Their study also demonstrated that in prostatic hyperplasia, decreased antioxidant protection and increased oxidative modification of proteins in the prostatic fluid and semen support the pathogenetic role of oxidative stress (Domoslawska et al., 2022). The present study further confirmed the pathogenetic role of oxidative stress in acute prostatitis in dogs. Previous research by the authors of this article supported the use of redox-active nanoparticles for treating reproductive disorders in male rabbits, suggesting a potential application for dogs with prostatitis (Koshevoy et al., 2022). It is proposed that therapeutic and preventive measures for canine prostatitis should include metal nanoparticle-based treatments, which offer antibacterial, antiviral, anti-inflammatory, and antioxidant properties (Naumenko et al., 2023; Xing et al., 2024). Anti-inflammatory and antibiotic drugs, combined with phytobiotics or hormonal drugs, could enhance the effectiveness of prostate inflammation treatment and prevent reproductive complications in affected dogs (Socha et al., 2018; Clerc-Renaud et al., 2021; Tverdokhlib et al., 2024).

CONCLUSION

This study identified typical changes in biochemical processes in acute prostatitis in dogs. A significant increase in lipid hydroperoxides and thiobarbiturate acid-reactive compounds was observed in prostate tissue, alongside a decrease in the

activity of antioxidant enzymes (glutathione peroxidase and catalase), a reduction in glutathione levels, and a reduction in total protein content. These findings confirmed the key role of oxidative stress in the pathogenesis of acute canine prostatitis. The authors propose further research on changes in sperm quality in dogs with prostatitis and the development of a comprehensive treatment approach based on nanotechnology.

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.

Authors' contributions

Volodymyr Serhiienko, Vsevolod Koshevoy, and Svitlana Naumenko created the idea, developed the research design, and conducted the experimental part, and analysis of the results obtained, Bohdan Kotyk and Oksana Ilina participated in the preparation of prostate tissue samples, homogenization, and biochemical studies, Yuriy Shchepetilnikov and Diana Makhotina performed mathematical and statistical data processing, literature review on the research issues, while Ihor Marakhovskyi assisted in the selection of material for research, clinical examination of animals, etc. All authors took part in discussing the results and writing the article and agreed on the final version. The authors confirmed that the final edition of the manuscript of this article for publication in this journal has been reviewed and approved by all authors.

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Competing interests

All authors declare no conflict of interest regarding the publication of this manuscript.

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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Surgical Correction of Anal Atresia in a 4-Day-Old Brown Swiss Calf

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CASE REPORT
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ABSTRACT

A 4-day-old Brown Swiss calf was presented to a veterinary clinic in Huancayo, Peru, with congenital anal atresia. The condition was characterized by an absence of defecation, progressive abdominal distension, and a blind rectal pouch confirmed radiographically. Clinical examination revealed no anal opening, moderate tympany, and normal neurological function. Surgical correction was conducted under pre-anesthetic sedation using xylazine (0.2 mg/kg IM) and sacrococcygeal epidural anesthesia with lidocaine (0.5 mL/kg). A 12-cm vertical incision in the intertubercular ischiatic region exposed the distended rectal pouch, allowing for an end-to-cutaneous anastomosis with polyglactin 910 (Vicryl® 2-0). Subsequently, a 2-3 cm distal rectal incision was created to form a neoanus. The mucosal edges were sutured to the skin margins with interrupted simple stitches (Vicryl® 3-0), a technique aimed at preventing stenosis and maintaining a neoanal diameter of approximately 1.5-2 cm. Postoperative management included prophylactic penicillin. Within three hours of surgery, the calf passed impacted meconium, indicating the functional patency of the neoanus. Although the prognosis was favorable, the patient was closely monitored for five days to detect any signs of stricture or infection. This case illustrated the effectiveness of early surgical intervention in reestablishing gastrointestinal continuity in congenital anal atresia.

Keywords: Anal agenesis, Clinical significance, Postoperative outcome, Surgical technique

INTRODUCTION

Congenital anal atresia (atresia ani or anal agenesis) arises from abnormal embryological development of the caudal digestive tract. During normal organogenesis, the cloacal membrane (a transient embryonic structure that contributes to the formation of both the anal canal and urogenital tracts) undergoes perforation between the fifth and seventh weeks of gestation to establish the definitive anal opening. In individuals with anal atresia, incomplete perforation or persistence of this membrane leads to complete obstruction of the anal orifice, a hallmark of the condition (Ford et al., 2022; Su et al., 2024). Classified among posterior digestive tract anomalies, anal atresia involves disruption in the formation of the proctodeum between the fourth and seventh weeks of gestation in cattle (Su et al., 2024). Although its reported incidence is low (0.02-0.05% in calves), this anomaly is a significant contributor to neonatal mortality in livestock, particularly in genetically predisposed breeds, such as the Brown Swiss (Kancherla et al., 2023).

In cattle, anal atresia typically presents with acute intestinal obstruction, manifested by abdominal distension, tenesmus, and failure to pass meconium. Early surgical intervention is crucial to prevent life-threatening complications, including intestinal perforation and sepsis (Patel et al., 2024). This case report may represent one of the first documented instances of congenital anal atresia in Peruvian cattle. Worldwide, only a few similar cases have been reported recently, including those in Brazil by Fernandes et al. (2021) and Teixeira and de Araujo (2022) in Bangladesh by Rahman and Alam (2022), and in India by Patel et al. (2024), highlighting both the rarity and clinical significance of the condition. Hence, the objective of this study was to describe the successful surgical management of congenital anal atresia in a Brown Swiss calf.

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CASE PRESENTATION

The animal owner provided informed consent for the surgical procedure and submitted a certificate of animal welfare ethics (CARTA N° 001-GRJ-DRA-AAC-PERÚ-2025), which endorses compliance with health and animal welfare standards.

A 4-day-old Brown Swiss calf was brought to the Veterinaria Paulo Social clinic in Huancayo, Peru, due to an absence of defecation since birth. According to the owner, the calf had not passed meconium or feces, resulting in progressive abdominal distension. Macroscopic examination of the perineal region revealed no anal opening and no evidence of rectovaginal fistula or other urogenital anomalies. The clinical assessment confirmed moderate tympany but no systemic signs of sepsis. Both consciousness and response to stimuli were normal, ruling out any neurological involvement.

Lateral radiographs indicated a blind-ended rectal pouch located approximately 2 cm from the perineal skin, with no external communication (Figure 1). Marked distension of the rectum and colon was noted, characterized by fecal and gaseous accumulation proximal to the obstruction. Dilation of the distal intestinal loops confirmed a complete functional obstruction. No major bony malformations or additional spinal or abdominal abnormalities were identified.

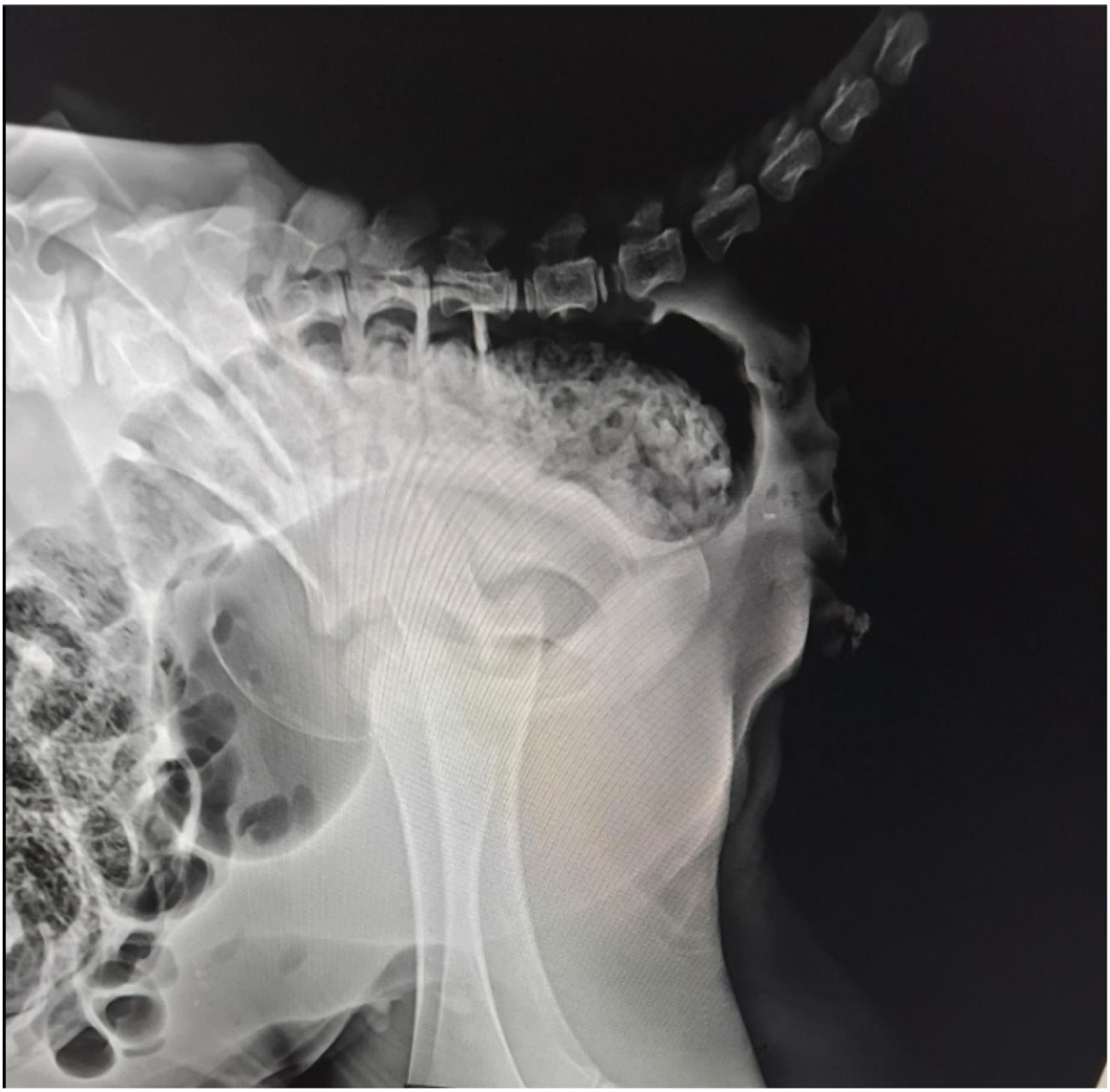

Figure 1. The caudal end of the rectum in a 4-day-old Brown Swiss calf

Prior to surgery, pre-anesthetic sedation (Xylazine, Xilagal®, 0.2 mg/kg IM) was administered, followed by sacrococcygeal epidural anesthesia (Lidocaine®, 0.5 mL/kg; Doherty et al., 2007; Alexander et al., 2022). A 12 cm vertical incision was made in the intertubercular ischiatic region corresponding to the terminal rectal pathway. Blunt dissection of the subcutaneous and fascial layers revealed pelvic floor structures, including a distended blind rectal pouch filled with fecal matter (Figure 2A). The distal rectal segment showed viable tissue (pink coloration, intact blood supply), with no signs of necrosis or previous trauma. Babcock forceps were used to secure and exteriorize the rectum. An end-to-cutaneous anastomosis was performed using a simple continuous suture pattern with absorbable polyglactin 910 (Vicryl® 2-0), approximating the rectal seromuscular layer to the perineal dermis. A 2-3 cm longitudinal incision was made in the distal rectal wall to create a functional opening. The mucosal edges were sutured to the skin margin with interrupted simple stitches (Vicryl® 3-0) (Figure 2B) to prevent stenosis and maintain a neoanus diameter of 1.5-2 cm. Patency was confirmed by digital palpation and direct visualization of healthy rectal mucosa. Meticulous hemostasis was maintained, and the surgical site was closed in layers to preserve anatomic and functional integrity.

Postoperatively, a multimodal protocol was implemented to prevent infection, control pain, and reduce inflammation. Procaine penicillin G (Pen®, 5 mL IM every 24 hours for 2 days) was given for broad-spectrum antibiotic prophylaxis, aligning with guidelines for gastrointestinal surgery in ruminants (Berge et al., 2005). Anti-inflammatory and analgesic therapy comprised Flunixin Meglumine (1.1 mg/kg IM every 24 hours for 48 hours), a COX-2-inhibiting NSAID, in combination with butorphanol (0.1 mg/kg IM every 8 hours for 2 days) for short-acting opioid analgesia (Alexander et al., 2022). The calf demonstrated early resumption of feeding and displayed only mild, localized discomfort, with no signs of acute pain (Figure 3A). Three hours post-surgery, it passed the impacted meconium, confirming the functionality of the newly created anus (Figure 3B). An Elizabethan collar was employed to prevent the calf from licking the surgical site, which could compromise wound healing or disrupt sutures. The prognosis remains favorable, with continued monitoring for potential stenosis or infection over a 5-day follow-up period.

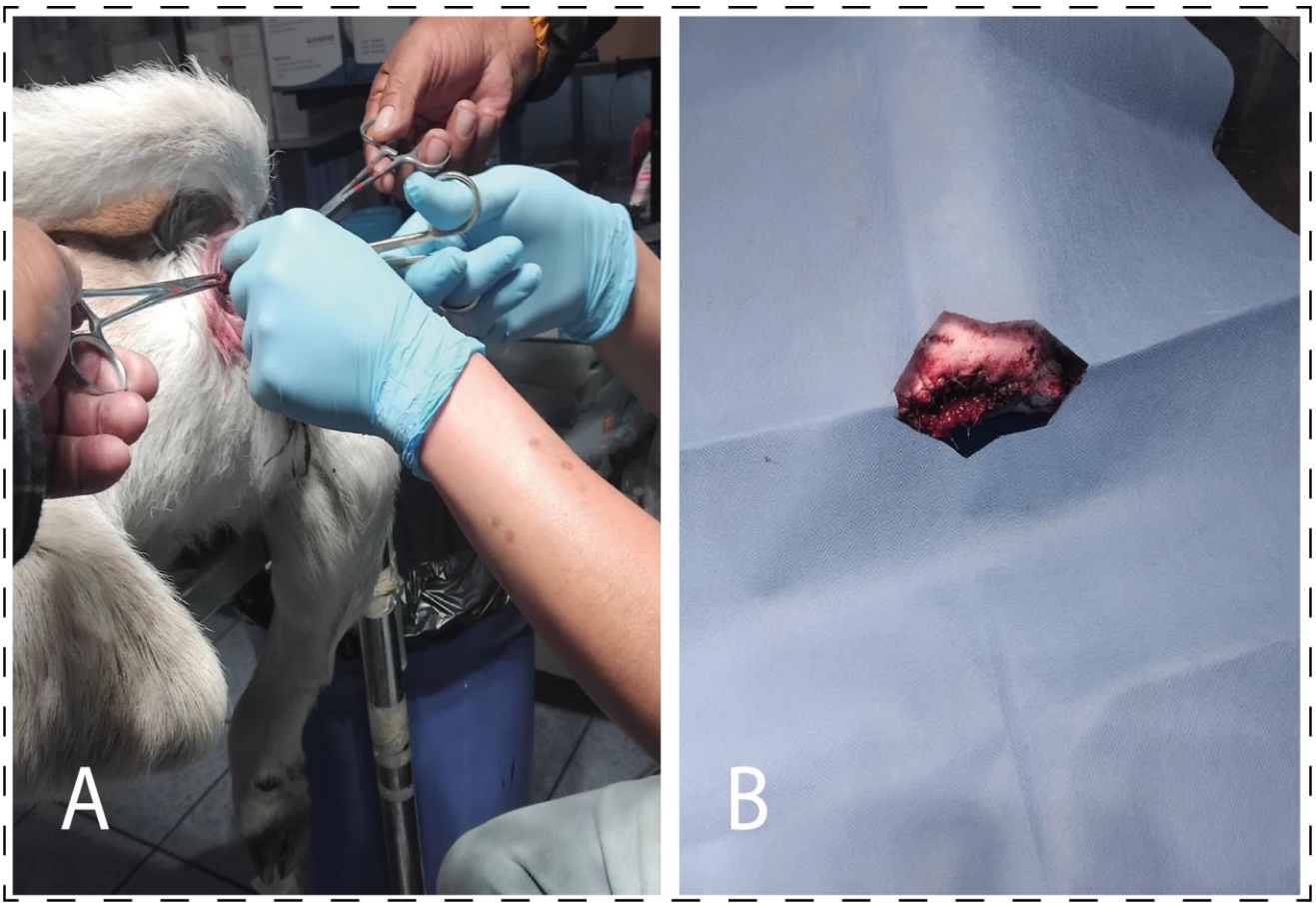

Figure 2. Surgical correction of Atresia Ani in a Brown Swiss calf. **A:** Surgical Procedure, intraoperative image showing the exteriorization and incision of the blind-ended rectal pouch. **B:** Excised Tissue, resected atretic rectal segment following dissection.

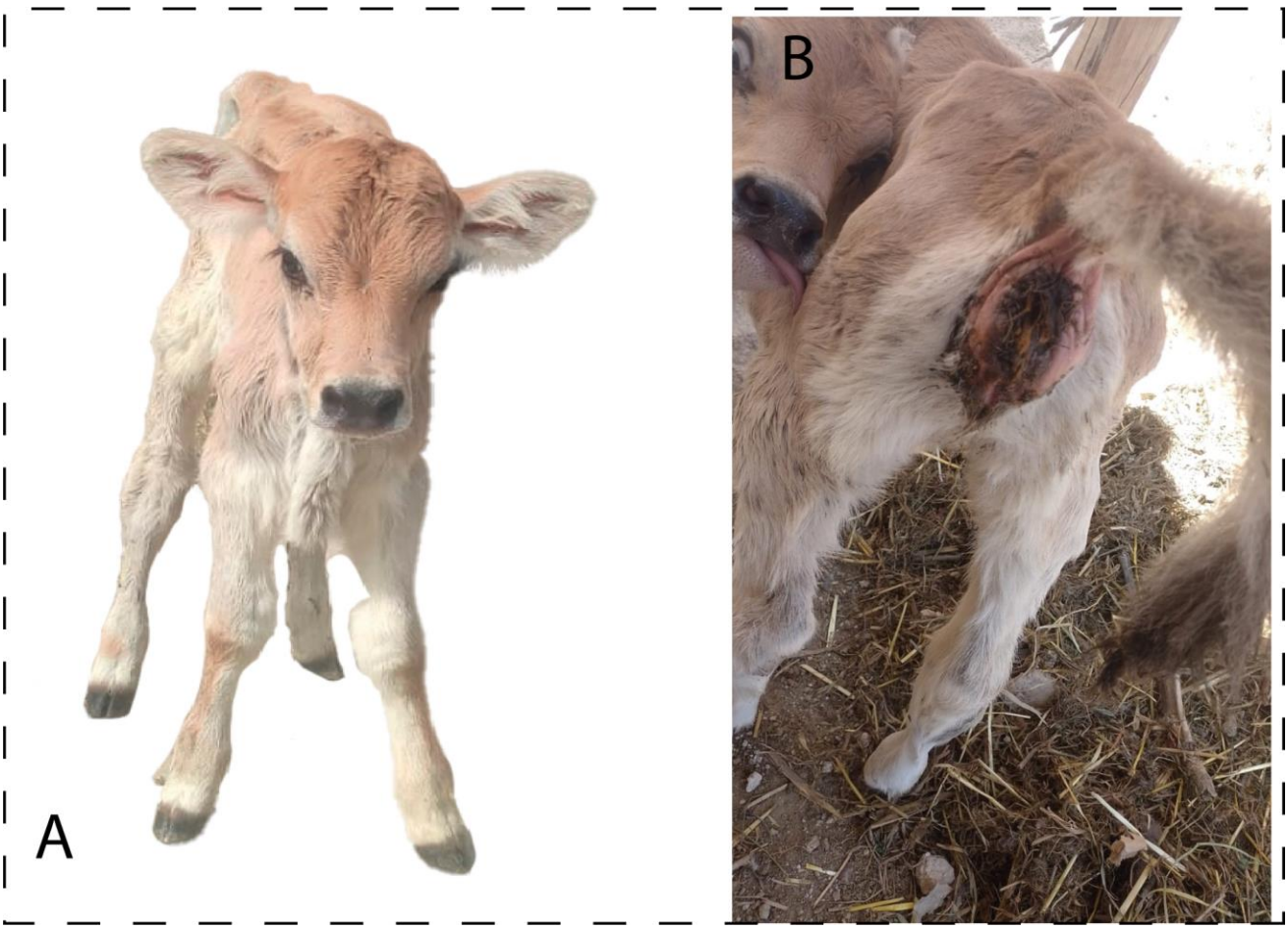

Figure 3. Postoperative condition of a brown Swiss calf with Atresia Ani. **A:** The Postoperative status, the Brown Swiss calf, shows normal behavior after surgery. **B:** The postoperative outcome, the Brown Swiss calf, shows the newly operated anal orifice after surgical correction.

DISCUSSION

Congenital anal atresia, while uncommon in cattle, presents a significant clinical and etiological challenge, as demonstrated by this first documented Peruvian case involving a 4-day-old Brown Swiss calf. Embryologically, the malformation stems from incomplete perforation of the cloacal membrane between the fourth and seventh weeks of gestation, resulting in defective proctodeal development (de Blaauw et al., 2024). The clinical presentation, characterized by acute abdominal distension, tenesmus, and failure to pass meconium, aligns with classic descriptions of the condition and highlights the need for timely intervention to prevent life-threatening sequelae, such as intestinal rupture or septicemia (Herman and Teitelbaum, 2012; Iwai and Fumino, 2013; Jacobs et al., 2022).

The perineal proctoplasty in this case successfully reestablished both anatomical and functional continuity through an end-to-cutaneous anastomosis with absorbable polyglactin 910 sutures (Vicryl®). This technique, regarded as the gold standard for low-type atresias (type I), achieved immediate postoperative patency, evidenced by the evacuation of impacted meconium within three hours. This outcome aligns with findings from Brazilian herds (Fernandes et al., 2021). The absence of fistulas simplified the surgical approach and improved prognosis since fistulas frequently require additional reparative procedures and elevate the risk of fecal contamination or postoperative infection (Rogers and Jeppson, 2016). This variation suggests the influence of genetic predisposition, epigenetic factors, or environmental triggers on phenotypic expression, warranting further research.

Prophylactic measures, including procaine penicillin administration and lumbosacral epidural analgesia, were instrumental in minimizing perioperative infection and stress, aligning with evidence-based protocols for bovine gastrointestinal surgery (Yarmuch et al., 2015). Despite these precautions, long-term complications, such as stenosis (driven by excessive collagen deposition at the anastomosis site due to surgical trauma, tension, or suboptimal mucosal apposition) and partial fecal incontinence (associated with external anal sphincter or pelvic nerve damage) may appear months after surgery (Xiong et al., 2020). To mitigate these risks, pelvic floor physiotherapy (to strengthen sphincter tone), periodic digital palpation, or endoscopic evaluations during follow-up can detect early stricture formation, allowing timely interventions, such as balloon dilation or revision surgery. This case not only demonstrates the efficacy

of early surgical correction but also addresses a critical gap in regional veterinary literature as the first reported case in Peru. It underscores the importance of documenting congenital anomalies in underrepresented livestock populations to enhance the global understanding of disease prevalence, refine breed-specific management strategies, and improve prognostic accuracy.

CONCLUSION

This case report demonstrated the effectiveness of prompt surgical correction for congenital anal atresia in calves. Timely intervention combined with precise surgical methods and preventive care successfully reestablished gastrointestinal continuity and minimized immediate postoperative complications. Long-term prognosis relies on vigilant monitoring for stenosis and adherence to breeding strategies designed to lower the incidence of congenital defects in susceptible breeds. Additionally, the study highlighted the value of recording and sharing clinical experiences in regions that are underrepresented in the literature, thereby moving toward standardized and accessible protocols for diverse livestock settings.

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Availability of data and materials

The data of the current case report are available upon reasonable request from the corresponding author.

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Authors’ contributions

Rodolfo Olivera-Calderon conceptualized the study and designed the research framework. Edgar Meza-Miguel performed the surgical procedures on the animal subjects. Jordan Ninahuanca developed and implemented the methodological approach for data collection and analysis. Ide Unchupaico Payano conducted postoperative monitoring and clinical assessments of the animals. Edgar Garcia-Olarte validated the experimental protocols and results to ensure scientific rigor. Carolina Miranda-Torpoco managed software-based image processing and analysis. Wilhelm Guerra Condor and Olivera-Acuña W supervised the operation and calibration of radiological equipment throughout the study. Vicky Sarapura coordinated the safe disposal and management of veterinary medical waste in compliance with biohazard regulations. All authors critically reviewed, edited, and approved the final version of the manuscript prior to submission for publication.

Competing interests

The authors declare that there is no conflict of interest.

Ethical considerations

The authors have checked and compiled the ethical issues, including plagiarism, consent to publish, misconduct, fabrication and/or falsification, double publication and/or presentation, and redundancy.

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Zoonotic Hepatitis E Virus: Epidemiology, Animal Reservoirs, and Control Strategies

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ABSTRACT

Hepatitis E virus (HEV) is a leading cause of acute hepatitis transmitted through the enteric route, impacting millions of individuals worldwide annually. While HEV is generally self-limiting, it can lead to considerable illness and death, particularly in gestating women, immunocompromised individuals, and those with pre-existing conditions, such as chronic liver disease. Hepatitis E virus genotypes 1 and 2 infect solely humans and are prevalent in areas with poor sanitation, whereas genotypes 3 and 4 are zoonotic, infecting both animals and humans. Hepatitis E virus genotype 7 has been reported to infect both humans and animals; however, further research is needed to clarify its zoonotic potential. Zoonotic transmission occurs primarily through the consumption of contaminated meat and close contact with infected animals, posing significant public health risks. Epidemiological studies indicated an increasing seroprevalence of HEV in humans and animals across diverse regions, highlighting the need for strengthened public health measures. While HEV infections in animal reservoirs are generally asymptomatic, they represent a critical source of human infections. The present review aimed to highlight HEV's current classification, epidemiology, modes of transmission from animals to humans, prevention, and control measures, with a special focus on HEV zoonotic genotypes and their animal reservoirs.

Keywords: Control measure, Epidemiology, Hepatitis E virus, Public health, Zoonotic genotype

INTRODUCTION

Hepatitis E Virus (HEV) is a major etiology of acute hepatitis transmitted via the enteric route, affecting millions of people worldwide annually. While HEV infections are often self-limiting (Nimgaonkar et al., 2018), they result in an estimated 3.3 million symptomatic cases and up to 44,000 mortalities each year (Rein et al., 2012). This virus is classified as a single-stranded RNA virus within the *Hepeviridae* family and the *Orthohepevirus* genus (Purdy et al., 2022). Currently, HEV includes eight classified genotypes. HEV-1 and HEV-2 solely infect humans and are generally found in areas with inadequate sanitation, including developing nations in Africa and South Asia (Nelson et al., 2019).

In contrast, HEV-3 and HEV-4 are zoonotic and can infect both humans and various animals. The global significance of HEV-3 and HEV-4 lies in their wide geographical distribution, being detected in both industrialized and low-income countries. Their distribution includes regions such as North America, Europe, and Asia, including Indonesia (Meng et al., 2010; Widasari et al., 2013). These genotypes can be transmitted through the consumption of raw or inadequately cooked meat or direct contact with infected animals (Colson et al., 2010; Primadharsini et al., 2019). Several recent hepatitis E cases have been identified that are not linked to travel in endemic areas, suggesting autochthonous or locally acquired infections. The majority of these cases are associated with genotypes 3 and 4, particularly in regions where these infections are not traditionally endemic (Samala and Ghany, 2013).

The case fatality rate of this disease is generally low, but it can rise in pregnant women, with the possibility of vertical transmission to the fetus (Bergløv et al., 2019). Fetal mortality is most likely to occur during the third trimester. Additionally, these patients may experience preterm delivery, low birth weight, or stillbirth of the fetus (Wu et al., 2020). The fatality rate is also observed to be high in individuals with immunocompromised conditions and patients with prior

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health issues, such as chronic liver (Santos-Silva et al., 2023). Although the overall number of hepatitis E cases remains low, the incidence of this disease is increasing in advanced countries (Fukuda et al., 2004; Olsen et al., 2006; Clemente-Casares et al., 2016). Wild boars (*Suis scrofa*) and domestic pigs (*Suis scrofa domesticus*) are the main reservoirs for HEV-3 and HEV-4 genotypes (Pavio et al., 2015). While these animals are identified as main reservoirs, other animal species, such as deer (*Cervidae*) and rabbit (*Oryctolagus cuniculus*), may also serve as potential reservoirs (Izopet et al., 2012; Karlsen et al., 2023). Deer, for instance, has been identified as a source of zoonotic food-borne HEV infection in humans (Tei et al., 2003). Although deer have been proven as a potential reservoir, domestic pigs have a significant impact on the spread of zoonotic HEV, particularly in regions with extensive pig farming (Kardena et al., 2024). Infected pigs usually do not show clear clinical symptoms of HEV infection, but they can release large amounts of the virus through feces, contaminating the environment and human food supplies (Salines et al., 2017). Pig farmers who have close contact with livestock are at heightened risk of HEV infection (Cossaboom et al., 2016; Hoan et al., 2019). Therefore, an in-depth understanding of HEV epidemiology in swine populations is critical for developing effective control strategies and reducing the risk of zoonotic transmission. In Indonesia, HEV is thought to be highly endemic, with several reports showing seroprevalence of the virus in humans and pigs, which serve as its main reservoirs.

This literature review aimed to comprehensively examine the general classification of HEV and animal reservoirs, with a particular emphasis on the zoonotic genotype, their animal reservoirs, associated epidemiology, transmission mechanisms, and control measures.

MATERIALS AND METHODS

This literature review was conducted using the literature study method, focusing on identifying and synthesizing existing research on HEV zoonotic genotypes, with a particular emphasis on epidemiology, transmission, and control measures. A narrative review approach was employed to collate, analyze, and summarize findings from selected sources. The review involved accessing scientific articles through ScienceDirect and PubMed. The search for relevant articles was carried out between March 2024 and September 2024. These databases were selected for their extensive coverage of biomedical and public health publications. Search terms included zoonotic HEV, HEV epidemiology, HEV transmission, HEV in Indonesia, zoonotic HEV reservoir, HEV risk factors, HEV control, and HEV prevention. The keywords were used individually and in various combinations to ensure comprehensive retrieval of relevant studies. Based on predefined inclusion and exclusion criteria, 94 articles were included in this review. Inclusion criteria required that articles were peer-reviewed, written in English, and focused on HEV zoonotic genotypes, transmission, epidemiology, and control measures. Non-English articles lacked available full text, focused exclusively on non-zoonotic HEV genotypes, or were excluded. The collected data were systematically compiled, analyzed, and synthesized to conclude.

Overview and classification of virus genotypes

The HEV is a non-enveloped, single-stranded RNA virus with an icosahedral shape and a diameter of approximately 32-34 nm (Cancela et al., 2023). The HEV virus has been successfully detected in humans and several animals (Pavio et al., 2015; Smith et al., 2020; Karlsen et al., 2023). The taxonomic classification of the virus family is based on the complete genome sequence of HEV isolates. The Hepatitis E virus belongs to the *Hepeviridae* family and the *Orthohepevirus* genus. The *Orthohepevirus* genus consists of four species, including *Orthohepevirus* A, B, C, and D. *Orthohepevirus* A includes four genotypes of HEV, namely HEV-1 to HEV-4, all of which are capable of infecting humans (Park et al., 2016).

Hepatitis E virus genotypes 1 and 2 are exclusively found in humans and are mainly found in Africa and South Asia. These genotypes are generally linked to contaminated water and inadequate hygiene and sanitation (Doceul et al., 2016; Fenaux et al., 2019). Meanwhile, HEV-3 and HEV-4 have been isolated from both human and animal hosts (Smith et al., 2020; Garbuglia et al., 2024). These genotypes are distributed across America, Europe, and Asia (Figure 1; Doceul et al., 2016), typically transmitted through eating undercooked or raw meat (Meng, 2013).

Hepatitis E virus genotype 3 is the most widely studied and well-documented in GenBank, with sequences primarily obtained from humans, pigs, and wild boars. However, HEV-3 strains have also been identified in various animal species, including mongoose (*Herpestidae*), deer, rabbits (Kenney and Meng, 2019), domestic ruminants (Wu et al., 2015; Huang et al., 2016), and white-collared peccaries (*Pecari tajacu*; Table 1; Ferreiro et al., 2020). This genotype is classified into 14 subtypes and three clades (3abchijk, 3efg, and 3ra; Smith et al. 2020). Recently, a study by Cancela et al. (2023) proposed a new subtype named 3o.

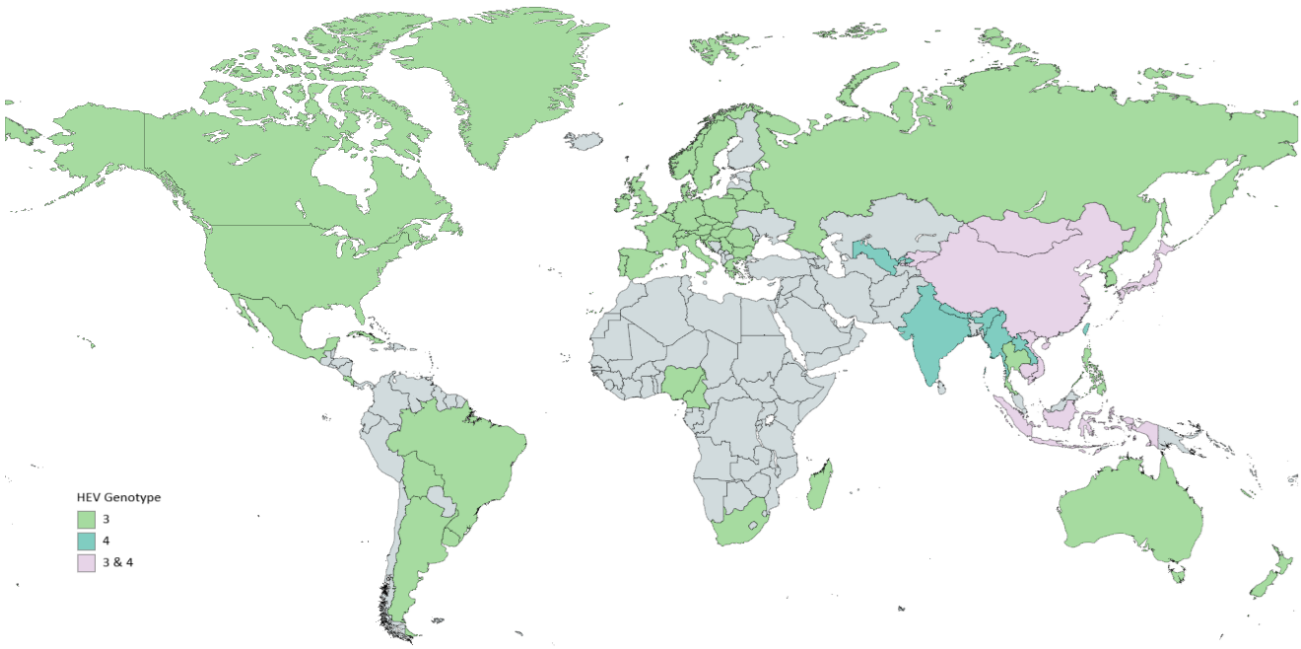

Figure 1. Geographical distribution of zoonotic HEV genotypes (3 and 4). The map was created using MapChart with the World Countries template. Source of the data: Okamoto (2007) and Treagus et al. (2021).

Table 1. Zoonotic hepatitis E virus genotypes, subtypes, and host

Family	Genus	Genotype	Subtypes*	Host	References
Hepeviridae	*Orthohepevirus*	3	3a, 3b, 3c, 3d, 3e, 3f, 3g, 3h, 3i, 3j, 3k, 3l, 3m, 3ra	Human, domestic pig, wild boar, mongoose, deer, rabbit, domestic ruminants, and white-collared peccaries	Wu et al. (2015) Hu et al. (2016) Kenney and Meng (2019) Ferreiro et al. (2020) Castagna et al. (2024)
		4	4a, 4b, 4c, 4d, 4e, 4f, 4g, 4h, 4i	Human, domestic pig, wild boar, cattle, sheep, goat, yak	Xu et al. (2014) Wu et al. (2015) Huang et al. (2016) Garbuglia et al. (2024)
		7	7, 7a	Camel	Lee et al. (2016)

*Source of the data: Smith et al. (2020)

Hepatitis E virus genotype 4 exhibits nucleotide similarities ranging from 71.79% to 77.38% with other genotypes and is classified into nine subtypes. Similar to HEV-3, this genotype has been detected and isolated from humans, domestic pigs, and wild boars (Garbuglia et al., 2024). Research by Wu et al. (2015) and Huang et al. (2016) also reported the presence of HEV-4 in other animals, such as cattle, sheep, and goats, in China. Xu et al. (2014) reported HEV-4 infection in Yak (*Bos grunniens*). However, further studies are required to ascertain whether these animals serve as reservoirs of HEV-4 or merely as incidental hosts.

So far, HEV-5, HEV-6, and HEV-8 have only been reported to infect animals, with no confirmed cases of human infection (Doceul et al., 2016). However, these genotypes have demonstrated the ability to infect non-human primates, indicating a potential for zoonotic transmission to humans (Li et al., 2016; 2019; Wang et al., 2019). Notably, HEV-7 has been reported to infect a human liver transplantation recipient who consumed meat and milk from camel products (Lee et al., 2016). Further investigation is needed into the zoonotic potential of this genotype due to date, there has been only one reported case of human infection with HEV-7. Hepatitis E virus genotype 7 has two subtypes, but the second subtype has not been named (Smith et al., 2020).

Epidemiology

Hepatitis E is endemic in both industrial and low-income countries worldwide, with epidemic outbreaks predominantly occurring in developing regions across Mexico, Africa, and Asia (Meng, 2010). Following the discovery of HEV infection in pigs by Meng et al. (1997), concerns arose regarding the risk of zoonotic transmission, posing a significant public health concern. Worldwide, the prevalence of anti-HEV IgG among domestically raised pigs varies

widely, ranging from 20% to 100%, while the prevalence of HEV RNA is reported between 0% and 20% (Salines et al., 2017). Hepatitis E virus prevalence differs considerably between countries, regions, and even individual farms within the same country (Sooryanarain and Meng, 2020).

The prevalence of HEV-3 subtypes varies significantly between countries. In the United States, subtype 3a is the most common, while in Japan, subtypes 3a, 3b, and 3e are prevalent (Sooryanarain and Meng, 2020). In Europe, the distribution of HEV-3 subtypes varies by geographic location and country, including subtypes 3a, 3b, 3c, 3e, 3f, 3h, 3i, and 3j (Lapa et al., 2015). New subtypes continue to be identified, with 3k found in Japan (Miura et al., 2017), 3l in Switzerland (Wang et al., 2017a), and Northern Italy (De Sabato et al., 2018). In China, rabbit hepatitis E virus (rHEV) exhibits a high genetic similarity to HEV-3, leading to its classification as a genotype of HEV-3, subtype 3ra (Wang et al., 2017b; Smith et al., 2020). In South America, several studies have identified HEV-3 infections in humans and animals, including pigs, white-collared peccaries, and wild boars in Uruguay (Mirazo et al., 2018; Ferreiro et al., 2020). The anti-HEV-3 IgG seropositivity rate on Uruguayan pig farms was reported to be 46.8% (103/220), while in wild boars, it was 22.1% (31/140; Mirazo et al., 2018).

HEV-4 is predominantly endemic in Asia but has recently been detected in several European countries (Primadharsini et al., 2019). Subtypes 4c and 4i have been identified in Japan (Sato et al., 2011). According to Nakano et al. (2016), HEV-4 likely originated in Japan before spreading to China and other parts of Asia, with the global pork trade playing a significant role in its distribution. Among HEV-4 subtypes, 4a, 4b, 4d, and 4h are the most commonly isolated, while subtypes 4c, 4e, 4f, 4g, and 4i remained restricted to specific periods. All HEV-4 subtypes have been reported in China and Japan (Li et al., 2022).

Data on the seroprevalence of hepatitis E in various countries have been reported by several studies. Wu et al. (2022) conducted a study in Guangzhou, China, comparing HEV seroprevalence among swine workers, poultry workers, and the general population. The findings revealed significantly higher seroprevalence rates in swine workers (47%, 156/332) and poultry workers (40.2%, 119/296) compared to the general population (26.1%, 35/134).

In southern France, traditional liver pork sausages known as figatelli, often consumed raw or undercooked as part of local customs, have been identified as a source of HEV transmission to humans (Colson et al., 2010). A case-control study by Colson et al. (2010) reported that the seroprevalence of anti-HEV IgG among individuals consuming raw figatelli reached 54 percent, highlighting the significant risk associated with this dietary practice. The seroprevalence of HEV-3 in domestic pigs in France was 31% (1069/6565, Rose et al., 2011).

In Indonesia, zoonotic HEV genotypes, both HEV-3 and HEV-4, have been detected (Wibawa et al., 2007; Widasari et al., 2013). Wibawa et al. (2004) reported that IgG antibody testing against HEV (anti-HEV) detected a 20% (54/276) seroprevalence in the human population tested in Bali. This percentage is notably higher than the findings from Lombok, which reported 4 percent (17/446), and Surabaya, East Java, which recorded just 0.5 percent (2/393). Utsumi et al. (2011) found that the seroprevalence of anti-HEV in people over 20 years old in Bali was higher than in Java. In the pig population, Wibawa et al. (2004) reported a seroprevalence rate of 72% (51/71), while Utsumi et al. (2011) and Widasari et al. (2013) reported a prevalence of HEV antibodies in pigs from Bali at 82.4% and 81.5%, respectively. A more recent study by Kardena et al. (2024) observed a low seroprevalence of 23.5% (43/183) in pigs collected from rural areas of Karangasem and urban areas of Denpasar, Bali. The decline in prevalence is thought to be due to a pig disease outbreak in 2020-2021 that affected the pig populations and led the farmers to adopt more cautious practices and implement improved livestock systems with enhanced biosecurity measures (Kardena et al., 2024).

Widasari et al. (2013) studied the presence of anti-HEV genotype 3 antibodies among the general population and pig farm workers in Java and Bali, Indonesia, with seroprevalence rates of 5.1% (15/291) and 11.6% (23/199), respectively. This study also examined pig serum samples, showing high prevalence rates of swine HEV antibodies, including 70.3% (114/162) in Java and 81.5% (97/119) in Bali. All studies consistently show that seropositivity for anti-HEV antibodies is higher in Bali than in Java. This discrepancy is likely due to the dietary habits and cultural practices in Bali, where pork consumption is common, and many pig farms exist due to the predominantly Hindu population (Kardena et al., 2021). In contrast, other areas in Indonesia have a majority Muslim population, where pork farming is rare. Furthermore, Balinese people are closely associated with pigs in their daily lives, keeping them as domestic animals and using them in religious ceremonies (Widasari et al., 2013; Kardena et al., 2023).

Zoonotic hepatitis E virus reservoir

The primary reservoirs for zoonotic HEVs are wild boars and domestic pigs (Figure 2). Numerous studies have indicated a high prevalence of HEV among domestic pig populations globally since the first discovery of HEV infection in pigs in 1997 (Wibawa et al., 2004; Rose et al., 2011; Widasari et al., 2013; Mirazo et al., 2018; Hoan et al., 2019; Kardena et al., 2024). A rise in sporadic human cases has also been documented in developed countries (Clemente-Casares et al., 2016; Guillois et al., 2016). In pigs, infection typically occurs at an early age, following the loss of

maternal antibodies (Feng et al., 2011). Virus excretion in fecal samples peaks between 3 to 8 weeks after weaning, followed by a decline at 15-18 weeks of age (Kantala et al., 2015). However, the length of immunity gained after viral exposure is still unclear. The possibility of reinfection due to decreased immunity (postpartum or during coinfection) cannot be ruled out. Loss of protection due to decreasing antibody levels may occur in older animals, particularly in sows (Casas et al., 2011).

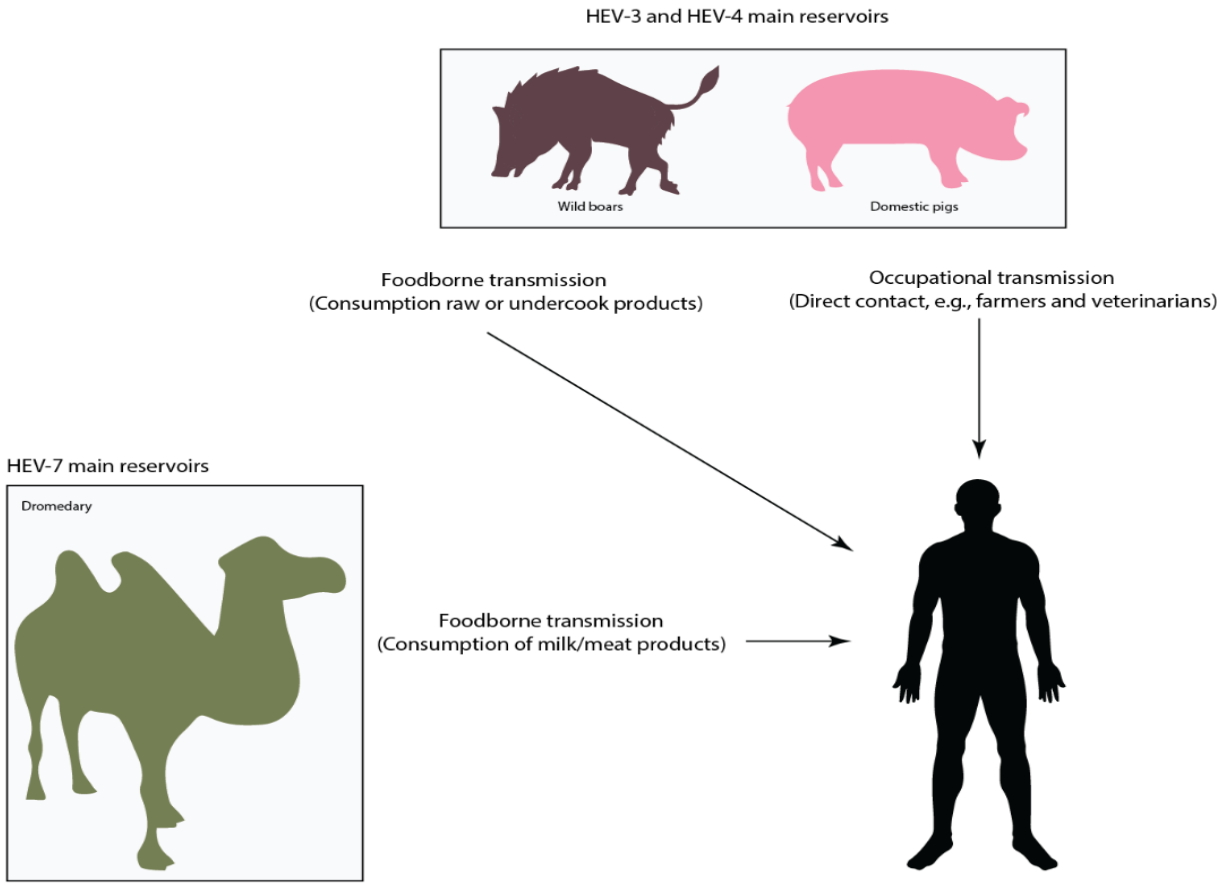

Figure 2. Transmission pathways of zoonotic hepatitis E virus genotypes (HEV-3, HEV-4, and HEV-7)

The presence of viral RNA in serum samples (viremia) is minimally documented compared to samples from the liver or feces (Grierson et al., 2015). Thus, searching for viral RNA in blood or serum samples is not an optimal method for diagnosing acute HEV infection or conducting prevalence studies in natural settings. Hepatitis E virus infection in animals does not significantly impact their health as it does not exhibit clear clinical signs (Song et al., 2013). The absence of these clinical signs complicates the surveillance and reporting of infections in livestock flocks (Pavio et al., 2017).

Several factors influence the level of viremia in pigs at slaughterhouses. Infection typically occurs before slaughter, with the spread of infection within livestock populations resulting in a high proportion of virus shedding in their feces, creating a continuous loop of new infections (Salines et al., 2017). Mirazo et al. (2018) reported that HEV-3 infection was most frequently observed in slaughter-age animals (57.3%, 70/122), which was higher than in younger animals (33.6%, 33/98). This age-related difference in infection rates is further supported by Kardena et al. (2024), who stated that pigs older than six months were four times more likely to be seropositive for HEV antibodies compared to the younger ones.

Factors contributing to the highest risk of HEV infection in pig livers include slaughtering animals at too young an age, specific pig breeds, lack of biosecurity, and using drinking water from nearby sources (Pavio et al., 2017). Kardena et al. (2024) reported several potential risk factors associated with HEV seropositivity in pig farms in Bali, Indonesia, including the type of maintenance management (extensive versus semi-intensive), type of pen floor, sewage (piled versus non-piled), cleanliness of the feed trough, the presence of domestic animals around the farm, type of drinking water, the presence of water sources around the farm, and feed processing (cooked versus not cooked). In France, identified risk factors include direct contact with wild boars, hunting, and residing in rural areas (Carpentier et al., 2012). Furthermore,

HEV exposure may result from drinking water from rivers frequented by wild boars or through contact with feces in these environments (Carpentier et al., 2012).

Rabbits are recognized as the natural hosts of HEV-3ra and are considered a significant reservoir of HEV alongside pigs (Wang et al., 2017). Experimental studies have demonstrated that HEV-3ra is capable of infecting both non-human primates and humans. However, under natural conditions, no evidence of rHEV has been detected in pig populations located near rabbit farms, and cross-species infection has never been reported (Geng et al., 2013).

In addition to wild boars and domestic pigs, recent research by Karlsen et al. (2023) identified deer as a potential true reservoir for HEV-3 and HEV-4, based on phylodynamic analyses. Supporting this, several studies have detected anti-HEV antibodies in various deer species from regions where pig farming is uncommon (Di Profio et al., 2022). Furthermore, HEV RNA has been identified in deer liver tissue, providing direct evidence of viral presence. Histopathological examinations revealed lymphocytic inflammatory cell infiltration in deer, a finding consistent with observations in other confirmed HEV hosts, further substantiating the role of deer as true hosts for HEV-3 (Fonti et al., 2022). Hepatitis E virus strains isolated from deer in Italy demonstrated a nucleotide similarity of 90%-91.5% with strains from humans (Romano et al., 2011) and pigs (Di Bartolo et al., 2017). Takahashi et al. (2004) reported that the HEV genome isolated from wild boars had a high similarity (99.7%) to HEV isolated from wild deer and patients infected with HEV after consuming raw deer meat. These findings reinforce the hypothesis that deer may contribute to the transmission of HEV-3 and HEV-4 in wildlife, potentially serving as a true reservoir for these HEV genotypes.

Zoonotic hepatitis E virus transmission

Occupational-related transmission

Hepatitis E virus infection among workers in slaughterhouses or on pig farms has been documented in several studies. Perez-Gracia et al. (2007) reported HEV-3 infection in workers at a slaughterhouse in Spain. Similarly, Acosta et al. (2022) reported that a pig farmer in Argentina suffered an acute symptomatic hepatitis E. A study conducted in Moldova found significantly higher HEV seroprevalence rates among pig farmers (51%) compared to the general population not involved in pig farming (25%). Farmers with a history of cleaning pig pens or assisting with farrowing were 2.46 times more likely to test seropositive than controls (Drobeniuc et al., 2001). Similarly, a Bayesian estimation approach in the Netherlands reported HEV seroprevalence rates of 11% among swine veterinarians, 6% among non-swine veterinarians, and 2% in the general population (Bouwknegt et al., 2008). Wu et al. (2022) conducted a multivariate analysis indicating that individuals who work in pig slaughterhouses had an elevated risk of HEV infection. In Indonesia, studies by Utsumi et al. (2011) and Widasari et al. (2013) reported several pig farmers in Java and Bali who tested seropositive for anti-HEV antibodies. The likelihood of HEV infection rises with prolonged exposure and is influenced by gender, with men generally exhibiting higher seroprevalence rates. Additionally, prevalence tends to increase with age (De Schryver et al., 2015). Although the specific transmission route in occupational exposure requires further investigation, the role of direct contact with infected animals is evident (Mrzljak et al., 2021). These findings highlight that swine workers have the highest seroprevalence, likely due to their frequent and direct contact with pigs, further emphasizing occupational risk as a significant factor in HEV exposure.

Forest workers, in addition to pig farm workers, are at risk of HEV exposure from wild animals, such as wild boars and deer. Hepatitis E virus transmission is suspected to occur through environmental contamination by wild boar feces, with forest workers becoming exposed through direct contact with contaminated environments or by consuming wild boar meat. Carpentier et al. (2012) observed a seroprevalence rate of 31% among forestry workers in France and 14% in wild boars. The study identified woodcutters as having the highest risk of HEV exposure among forestry workers (Carpentier et al., 2012).

Foodborne transmission by consuming contaminated meat

The Hepatitis E virus has been identified at the start of the food chain supply, particularly in slaughterhouses where meat processing occurs. Research has detected HEV RNA in the serum of pigs in slaughterhouses (Sooryanarain and Meng, 2020) and various commercial pork products (Cossaboom et al., 2016). For instance, HEV RNA was found in 58% (7/12) of figatelli in France (Colson et al., 2010). Similar findings have been reported in HEV RNA in raw liver samples in the United States and France (Cossaboom et al., 2016; Feurer et al., 2018), as well as in raw sausages in Italy and Germany (Di Bartolo et al., 2015; Szabo et al., 2015). In the United States slaughterhouses, approximately 40% of pigs tested positive for HEV antibodies, with around 6% exhibiting HEV-3 viremia (Sooryanarain and Meng, 2020).

The presence of viremia during the slaughter process raised significant food safety concerns, as blood containing HEV can contaminate food supplies. Foodborne transmission occurs through the consumption of raw or undercooked pork products contaminated with the virus (Meng, 2013). In Indonesia, there have been no reports regarding research on the presence of viral RNA in pork food products to date, according to the author's knowledge.

Zhang et al. (2023) investigated HEV prevalence in rabbits and rabbit meat at a slaughterhouse in Hebei Province, China. The study reported a seroprevalence of 10.9% (50/459) in slaughter-age rabbits, with HEV RNA detected in 11.7% (7/60) of rabbit livers. All identified HEV strains were classified as HEV-3ra. These findings highlighted the potential risk of HEV transmission to humans through the consumption of rabbit-derived animal products.

Control and prevention

Exposure to HEV primarily occurs through the consumption of meat from infected animals and direct contact with them. Consequently, an effective preventive strategy is to minimize human exposure, particularly among individuals who have direct contact with reservoir animals or consume them.

Food safety measures

Effective food processing techniques are essential for preventing foodborne HEV outbreaks. Several studies have demonstrated that cooking meat at 71°C for 20 minutes effectively inactivates the virus (Barnaud et al., 2012). Ensuring that animal products are thoroughly cooked before consumption can significantly reduce the risk of HEV transmission through contaminated meat (Castagna et al., 2024).

Occupational safety measures

For workers who have direct contact with reservoir animals, several measures can reduce the risk of HEV infection. These include proper handwashing after contact with animals, the use of Personal Protective Equipment (PPE), such as gloves and masks to minimize direct contact with animal fluid, and the implementation of stringent biosecurity protocols on farms and in slaughterhouses to prevent the spread of HEV among animals and from animals to humans (Mrzljak et al., 2021).

Surveillance and control

Surveillance and control of HEV infection are crucial to bridging the knowledge gap regarding its transmission and reservoirs. Regular monitoring of HEV prevalence in pig populations and pork products can help to identify and manage potential outbreaks. Enhanced surveillance can provide data for developing targeted interventions (Mrzljak et al., 2021).

In HEV surveillance, enzyme-linked immunosorbent assay (ELISA) is commonly employed to detect anti-HEV antibodies in various biological samples, including serum, body cavity transudates, and meat juices (Khudyakov and Kamili, 2011). Additionally, reverse transcription polymerase chain reaction (RT-PCR) is widely used to detect HEV RNA in diverse sample types, such as food products such as meat, sausages, semen, muscle tissue, serum, and liver tissue (Krumova-Valcheva et al., 2023).

Hepatitis E virus vaccines

Due to the widespread prevalence of HEV infection among various animal species, developing an HEV vaccine for animals is a key strategy to prevent transmission from animals to humans (Huang et al., 2024). Vaccines derived from a single genotype may achieve cross-protection across different genotypes. However, the development of a vaccine that provides cross-species immunity in pigs necessitates further studies (Park et al., 2016). In humans, vaccine development efforts include the HEV P179 vaccine, designed based on the amino acids 439-617 of the HEV-4 capsid protein. This vaccine already passed Phase I clinical trials in China (Cao et al., 2017).

Another vaccine, HEV 239, is a recombinant vaccine that has been extensively studied. The antigen in this vaccine is derived from pORF2 amino acids 368-606 of HEV-1 (Li et al., 2015). Multiple clinical trials have demonstrated its efficacy and safety, with the vaccine successfully passing Phase I through Phase IV trials (Yu et al., 2019; Zaman et al., 2024). Although developed based on HEV-1, Phase III clinical trials revealed that HEV 239 also provides cross-protection against HEV-4 infections but does not confer protection against other HEV genotypes (Huang et al., 2024).

In contrast to its performance in humans, a study by Dähnert et al. (2024) reported that the HEV 239 vaccine failed to protect pigs experimentally infected with HEV-3, highlighting limitations in its interspecies cross-protection. This underscores the need for a universal HEV vaccine capable of providing cross-protection across all genotypes and species. The successful development of such a vaccine would enable widespread immunization efforts, significantly reducing the global burden of HEV infections and improving public health outcomes.

One Health strategy

The One Health strategy, emphasizing the interconnectedness of human, animal, and environmental health, plays a vital role in HEV control and prevention. Collaborative efforts among veterinarians, public health professionals, and

environmental scientists are essential for establishing and implementing comprehensive surveillance systems, promoting research on HEV transmission dynamics and reservoirs, and designing and enforcing biosecurity measures across animal and human health sectors. Implementing these measures can substantially lower the risk of HEV transmission by reducing human exposure to the virus and limiting its spread within animal populations, thereby protecting public health (Kardena et al., 2024). Interdisciplinary collaboration is highly recommended in all disease cases (Castagna et al., 2024).

CONCLUSION

The Hepatitis E virus poses a significant global public health challenge due to its zoonotic potential and wide distribution across different regions. The Hepatitis E virus is primarily transmitted through foodborne routes, such as consuming undercooked meat and occupational exposure from direct contact with infected animals. While pigs are key reservoirs, other species contribute to transmission, emphasizing the complexity of HEV epidemiology. Preventive strategies, such as implementing biosecurity measures, promoting food safety through proper cooking practices, and enhancing surveillance systems, are essential for controlling HEV spread. The integration of the one health approach, which considers the interconnectedness of human, animal, and environmental health, is critical for the development of comprehensive control measures. Future research should focus on understanding the full spectrum of HEV reservoirs, refining diagnostic tools, and developing universal vaccines that provide cross-genotype and interspecies protection to reduce the global burden of HEV-related illnesses.

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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.

Authors' contributions

I Made Kardena and Alan P. Dargantes conducted the conceptualization and design of the study. I Made Kardena, Palagan Senopati Sewoyo, Anak Agung Gde Oka Dharmayudha, I Wayan Nico Fajar Gunawan, and Putu Devi Jayanti contributed to data collection and the drafting of the manuscript. Palagan Senopati Sewoyo contributed to the visualization/images. I Nyoman Mantik Astawa, Anak Agung Ayu Mirah Adi, I Nyoman Suarsana, I Nyoman Suartha, and Alan P. Dargantes reviewed and revised the manuscript. All authors checked and approved the final edition of the manuscript.

Competing interests

All authors declare that there are no competing interests.

Availability of data and materials

The datasets and materials analyzed in this review are derived from publicly available articles, reports, and databases, which are cited throughout the manuscript. No new data were generated or analyzed during this study.

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The Association between the Global Threat of Ocean Pollution and Climate Change on the Distribution of Antibiotic Resistance: One Health Strategy

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ABSTRACT

Antibiotic resistance represents a fundamental issue affecting public health today. Antibiotic resistance occurs when bacteria alter their response to antibiotics. Pathogenic bacteria and their genes can diffuse throughout human and natural habitats. The complicated interactions between diverse bacterial communities that affect the health of people, aquatic animals, and the aquatic environment are an illustration of ecological issues. Pollution of the oceans by antibiotic-resistant bacteria (ARB) can disturb the natural equilibrium of the oceans and may spread to humans. Also, climate change (CC) significantly affects the health of marine environments. Rising temperatures, acidification, increased sea levels, an increasing number of invasive marine animals, changed biological systems, and a decrease in biodiversity are some of the major impacts on the oceans caused by CC. However, the elevated temperatures linked to CC facilitate the higher spread of bacterial infections in aquatic environments, aquatic animals, and humans with the emergence of antibiotic resistance. The present study aimed to provide a scientific understanding of the relationship between ocean pollution and CC, as well as their impacts on ocean health. Additionally, the present study presented the current status of ARB and its associated genes in the oceans, comparing to future projections based on previous studies. One Health (OH) concept strategies for reducing antibiotic pollution in the ocean were discussed. The present paper is a foundation for further studies to determine the prevalence of antibiotic resistance in the oceans, as well as to understand the current state and key highlights of ocean pollution.

Keywords: Climate change, Drug-resistant bacteria, Heavy metal resistance gene, Ocean pollution, One Health concept

INTRODUCTION

The oceans cover 71 percent of the Earth's total surface and play a crucial role in public health, providing essential resources such as food. Recently, oceans have been vital in addressing the growing demand for animal protein (Kraemer et al., 2019). The oceans are under threat from human activities, which makes them vulnerable to pollution. However, ocean pollution is a global threat (Landrigan et al., 2020). In addition, ocean pollution is considered one of the most important challenges globally (Komijani et al., 2021). These impacts have an effect on the dissolved oxygen (DO), pH, temperature, nitrates, and other chemical and physical characteristics of seawater in oceans (Alesci et al., 2022). The major anthropogenic pollutants found in the oceans are sewage, sludge, fertilizers, heavy metals, plastic particles, pesticides, and pharmaceutical residues such as antibiotics (Buelow et al., 2021). Through rivers, runoff, and direct discharge, pollutants eventually flow into the ocean. Discharges of partial or untreated wastewater from hospitals and nursing homes into oceans are leading to the introduction of human pathogens, multi-antimicrobial resistance bacteria, and many types of resistance to the food chain of oceans (Zacharias et al., 2021). In addition, agricultural and industrial discharges and rainfall may introduce metals, fertilizers, and chemicals into the marine environment (Bukha et al., 2022).

Ocean antibiotic pollution represents a global risk that harms marine ecosystems (Kulik et al., 2023). The improper use of antimicrobial agents in animal and human treatment is considered a major factor contributing to the development of antibiotic-resistant bacteria (ARB; Cáliz et al., 2022) and antibiotic-resistant genes (ARGs) among bacteria (Kulik et al., 2023). The existence of antibiotics in marine ecosystems poses a risk, as it promotes the growth of ARB and their carriers (Sosa-Hernández et al., 2021). The ARB and ARGs are often found in contaminated marine ecosystems, but their mechanisms are not fully understood (Gothwal and Thatikonda, 2017). Globally, many studies have reported the

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presence of antibiotic agents in marine environments as emerging contaminants (Agunbiade and Moodley, 2014; Lu et al., 2019; Maghsodian et al., 2022). Residues of antibiotic agents that remain in the marine environment are considered "persistent organic contaminants" (Ben et al., 2019). Antibiotic pollution in the marine environment can reach humans via contaminated seafood or swimming in affected areas (Sosa-Hernández et al., 2021). Nevertheless, the methods of transferring ARB to humans are not yet understood.

Today, Antibiotic resistance and climate change (CC) are major public health emergencies. Nevertheless, the complex relationship between ARB and CC is now established, and it has not yet been clarified (San Lio et al., 2023). Climate change leads to a rise in ocean temperature, and it is caused by humanity's use of resultant greenhouse gasses and fossil fuels (Ford et al., 2022). Climate change significantly contributes to the increased spread of different bacterial and vector-borne illnesses in humans, plants, and animals. In addition, it may be responsible for the prevalence of novel emerging pathogens. Therefore, using a coordinated strategy such as the One Health (OH) Concept is to address antibiotic pollution at the human, animal, and associated environmental levels on an international scale (Aslam et al., 2021).

Ocean pollution has become a global problem, as it originates from many sources, and so far, polluted oceans are widespread and difficult to control. Ocean pollution threatens the health of the marine environment and humans. The oceans contaminated by antimicrobial resistance have become a major global concern. Globally, ARB and ARGs spread silently among populations. Although several studies have been conducted on ocean pollution with antibiotics, information on the extent of prevalence and occurrence of antimicrobial resistance in the oceans is still limited (Wellington et al., 2013; Victoria et al., 2022).

Therefore, the objectives of this review include: (1) discussing the role of ocean pollution and CC in resistance evaluation among bacterial communities and the pathways of antibiotic resistance spread from hotspots to oceans; (2) highlighting the impacts of antibiotic resistance pollution on people and aquatic animal health, and the health of their associated marine environment; (3) show the current global policies and OH strategies concerned with addressing the impacts of antibiotic pollution and mitigating the spread of this pollution in the oceans.

REVIEW METHODOLOGY

This study was conducted through a bibliographic review, with emphasis on literature available on the internet relevant to scientific publications. A total of 174 academic articles were examined using different databases such as Scopus, Web of Science, Google Scholar, SciELO, PubMed, and many other sources of peer-reviewed journals. The inclusion criteria for articles in the review were the presence of specific keywords related to the review title: The Association between the Global Threat of Ocean Pollution and Climate Change on the Distribution of Antibiotic Resistance: One Health Strategy. On the other hand, the exclusion criteria were the irrelevance of the content to the topic of the study, the type of publication, and the date of publication. The review article was written comprehensively to ensure the consideration of all the relevant literature.

AN OVERVIEW OF ANTIBIOTIC RESISTANCE

Antibiotics are substances naturally manufactured by bacteria, actinomycetes, or fungi (Mohr, 2016). There are common antibiotics divided into 16 families based on Modes of action and chemical properties, including fluoroquinolones, aminoglycosides, tetracycline, β-lactams, sulfonamides, macrolides, trimethoprim, and glycopeptides (Dos Santos et al., 2017). Antibacterial agents have been commonly utilized to combat bacterial infections in animals and humans since the 1950s (Carvalho and Santos, 2016). They have a significant impact on reducing the morbidity and mortality rates for many infectious bacterial infections (Carvalho and Santos, 2016). Also, they are used to prevent bacterial infections in the fish farming industry (Giguère et al., 2013) and promote the growth of livestock (Liu et al., 2021). In contrast, a part of the antibiotics is absorbed in fish products, while the rest is released into the aquatic ecosystem via sewage effluents (Liu et al., 2021).

Recently, the use of antimicrobial drugs has increased around the world (Tiseo et al., 2020; Klein et al., 2024). Further, misuse of antimicrobials can lead to increased distribution of ARB in the environment with bacteria harboring ARGs (Kraemer et al., 2019). The ARGs are considered "emerging micropollutants" due to their increasing presence in marine environments (Kulik et al., 2023). Bacteria can gain resistance to antibiotic drugs and develop intrinsic resistance (Sun et al., 2022). Antibiotic resistance and ARGs have existed for billions of years and occur naturally in the environment (Zhuang et al., 2021). Antibiotic resistance can lead to treatment failure of bacterial diseases due to the inability of antibiotics to inhibit growth or destroy the bacteria due to the transfer of resistance genes from one bacterium to another, leading to a wider spread of resistance, or bacteria produce enzymes that breakdown the antibiotic (Authority

et al., 2021). In addition, it reduces their effectiveness against many species of bacteria (Singh et al., 2022). Antibiotic pollution is a major global threat to humans and health in the 21st century. The World Health Organization (WHO) has ranked it as the most critical among six environmental challenges (Hazra et al., 2022). There is a list of 12 ARB families published by WHO (Amarasiri et al., 2020). The WHO has identified high (ARBs) such as *Escherichia coli*, *Staphylococcus aureus,* and *Klebsiella pneumoniae* (Huijbers et al., 2015).

A study was conducted in 76 countries in Europe over 5 years from 2000 to 2015, and the study concluded that antibiotic use has risen by 65% (Klein et al., 2018). Annually, antibiotic-resistant infections cause more than 30,000 deaths in countries in Europe, especially in Italy (Cassini et al., 2019). Aquatic environments are considered reservoirs and vectors for the spread of antibiotic resistance (ARB) (Amarasiri et al., 2020). The ARB and ARGs may accumulate in marine ecosystems through discharge from hospitals, farms, cities, and wastewater treatment plants (WWTPs). Increased spread of ARB and ARGs in the aquatic environment induced by human activities (Duan et al., 2020). Globally, numerous studies have documented seven families of antibiotics and their associated ARG subtypes (Zhuang et al., 2021).

The antibiotic resistance resistome is a group of all ARGs and their precursors in specific bacteria. In 2006, the antibiotic resistome term was named by Gerry Wright's group (Kim and Cha, 2021). Antibiotic resistance is categorized into intrinsic and acquired resistance, while ARGs are categorized into intrinsic resistance. Intrinsic resistance originates from the producers themselves, while acquired resistance results from another bacteria through horizontal gene transfer HGT resistance (Hu et al., 2017; Eshrati et al., 2025). The antibiotic resistome is categorized into mobile and intrinsic resistome (Hu et al., 2017).

ANTIBIOTIC-RESISTANT BACTERIA AND ANTIBIOTIC-RESISTANT GENES IN AQUATIC ECOSYSTEMS

Several research investigations have been performed that confirmed the possibility of the presence of ARB and ARGs in marine ecosystems. These studies concluded that antibiotic-resistant consequences of excessive antibiotics in hospitals and farms lead to it reaching the aquatic environments through wastewater (Stoll et al., 2012). A study by Gambino et al. (2022) detected antibiotic-resistant Gram-negative bacteria and genes that confer resistance to sulfonamide and β-lactamase antibiotics in the collected seawater samples from Sicily Coasts, Italy. In contrast, in Sishili Bay of Yantai, China detected a high prevalence of 10 ARG subtypes that confer resistance to quinolones, tetracyclines, macrolides, and sulfonamides (Zhang et al., 2020). Whereas it was monitored the prevalence of twenty-four ARGs against (aminoglycoside, glycopeptides, β-lactamase, tetracycline, macrolides, chloramphenicol, trimethoprim, and sulfonamides) in collected water samples from Australia and Germany (Stoll et al., 2012). In addition, another study by Habibi et al. (2022) detected the presence of 402 ARGs on the shores of Kuwait, which showed resistance against β-lactamase. Several studies have reported that resistance genes of β-lactamase, sulfonamide, macrolide, and tetracycline are the most widespread types of ARGs in aquatic environments (Berglund, 2015; Huang et al., 2019; Grenni, 2022). Whereas, it was noted that the β-lactamase resistance genes are abundant in marine water, fish, and sea turtles (Stoll et al., 2012).

The collected water specimens from the Coast of Southeast Louisiana, USA, were tested to detect the presence of ARB (Belding and Boopathy, 2018). The result of this study revealed the presence of Enterobacter spp., *Escherichia coli*, and *Klebsiella*, which were resistant to tetracycline, carbapenem, penicillin, monobactams, cephalosporin, and sulfonamide antibiotics (Belding and Boopathy, 2018). Other studies by Leonard et al. (2025) documented the occurrence of antibiotic-resistant *E. coli* in coastal waters of the United Kingdom UK. In contrast, in the Veraval Coast of India, the prevalence of Enterobacteriaceae family resistant to multiple antibiotics, in both water and sediment samples, having 12 ARGs (Huang et al., 2019). Also, in coastal waters of Indonesia, Malaysia, China, and Vietnam, the presence of *E. coli*, *Enterobacter* spp., and *Klebsiella* spp. that were resistant to cephalosporin, sulfonamide, tetracycline, monobactam, penicillin, and carbapenem (Boopathy et al., 2015). Guzman-Otazo et al. (2019) who reported of multi-resistant *E. coli* in the river of Choqueyapu in Bolivia. In addition, multidrug-resistant bacteria have been isolated from the coastal waters of Tunisia (Ghozzi et al., 2023).

AN OVERVIEW OF OCEAN POLLUTION

The oceans provide more than 99% of the water resources on Earth. The global ocean splits into five ocean basins: the Atlantic, Indian, Arctic, Pacific, and Antarctic (Thushari and Senevirathna, 2020). On the other hand, the oceans are threatened by human activities, including domestic pollutants, industries, hospitals, and agriculture activities, which reach the marine environments through many different pathways, such as river and aquatic drainages, the atmosphere,

and wastewater effluents (Dahms, 2014). Discharges of wastewater from hospitals and nursing homes into aquatic ecosystems are leading to the introduction of human pathogens, multi-antimicrobial resistance bacteria, and many types of resistance to the aquatic trophic chain (Zacharias et al., 2021). Furthermore, industrial discharges and rainfall may introduce metals, fertilizers, and chemicals into the marine environment. These compounds influence the health of aquatic ecosystems and accumulate in the biota (Hama Aziz et al., 2023). Marine pollution has negative impacts on aquatic life. However, these impacts affect the chemical and physical properties of seawater, such as pH, temperature, nitrates, and dissolved oxygen (DO) (Alesci et al., 2022). Pollutants can build up in the tissues of marine organisms and be transmitted through the marine food chain (Oudi et al., 2019) (Figure 1).

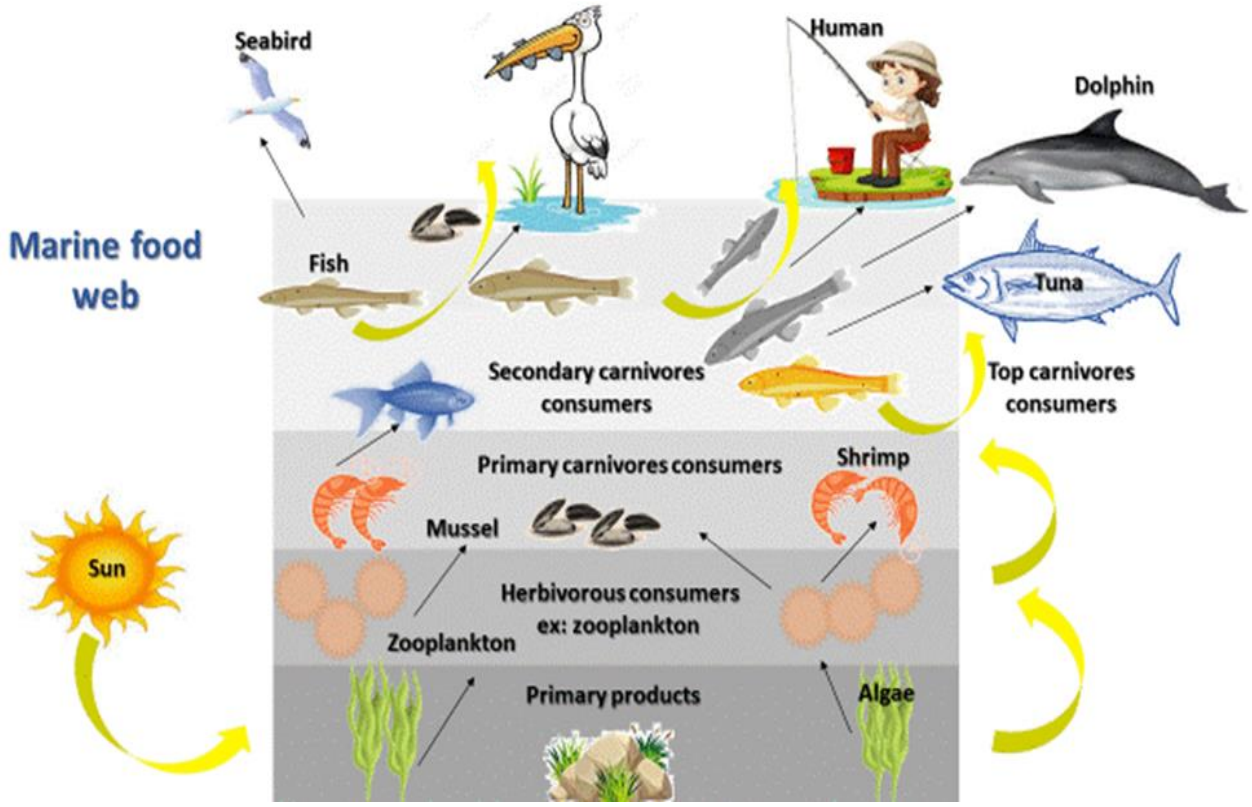

Figure 1. The marine food cycle shows the pollutants that can build up in the tissues of marine organisms and be transmitted through the marine food chain

Inadequate treatment of domestic, industrial, livestock industries, aquacultures, and hospital wastewater leads to antibiotic release into the oceans (Kraemer et al., 2019). Antibiotic pollution has become a serious threat to aquatic environments. The presence of antimicrobial agents in aquatic ecosystems may be affected by pH, temperature, bacterial populations, and dilution factors, which may lead to disturbances in their concentrations (Liu et al., 2021). However, antimicrobial agents are inadequately broken down and may find their way into aquatic environments through wastewater effluents (Chow et al., 2021). Antibiotic residues lead to increased rates of mutation and horizontal transfer among bacterial populations, even at sub-lethal or sub-inhibitory concentrations (Chow et al., 2021).

There is a difference in the pathways of ARB and ARG presence in marine environments compared with freshwater and sewage (Sucato et al., 2021). Antibiotic-resistant bacteria (ARB) and ARGs induce a negative impact on microbial populations by reducing their biodiversity. In addition, they induce changes in the ecological functions of marine and freshwater (Grenni et al., 2018). The influence of ARB and ARGs on the marine environment is related to their biodiversity, concentrations, and exposure time (Huijbers et al., 2015). In addition, they increase the risk to public health because antibiotics lose their effectiveness in treating bacterial diseases (WHO, 2023). The presence and persistence of ARB and ARGs in aquatic environments remain incomprehensible.

Currently, global oceans are threatened by plastic pollution. Plastic pollutants have become a dangerous concern for all parts of the global oceans. Moreover, the accumulated plastic debris in the oceans is divided into four forms based on their sizes, which include microplastic, megaplastic, macroplastic, and mesoplastic (Thushari and Senevirathna, 2020). Plastic debris is ubiquitous in anthropogenic aquatic ecosystems and serves as a reservoir for marine bacteria, ARB, and ARGs (Liang et al., 2023). Currently, ocean pollution is estimated to contribute to approximately 9 million deaths annually due to its impact on human health and marine environments (Landrigan et al., 2020).

EFFECTS OF CLIMATE CHANGE ON MARINE ECOSYSTEMS

Climate change (CC) correlates with shifts in rainfall and temperature patterns. Globally, over the past 50 years, an increase of 0.11°C in sea surface temperature has been recorded (Venegas et al., 2023). Similarly, in the last 130 years, sea surface temperature levels have climbed by 0.85°C (Scharsack et al., 2021). These observations highlight the ongoing trend of planetary heating and its impact on seawater temperatures.

Since the onset of the industrial revolution, there has been a sharp increase in greenhouse gas emissions, leading to a rise in atmospheric temperature at an average of 0.14^0 Fahrenheit per decade (Venegas et al., 2023). Between 1990 and 2010, there was a noticeable rise in levels of carbon dioxide CO_2 in the atmosphere, from 350 to 380 ppm, which correlated with changes in the climate (Doney et al., 2012; Coelho et al., 2013). If greenhouse gas emissions persist, we can anticipate a projected increase in the average temperature of 1.5°C by the middle of the 20^{th} century, and by 2020, levels of atmospheric carbon dioxide CO_2 will have increased by 280 ppm to 413 ppm (Scharsack et al., 2021). In February 2023, it was observed that the atmospheric carbon dioxide CO_2 concentration reached 422.9 ppm (Venegas et al., 2023). The increase in atmospheric carbon dioxide CO_2 concentrations is absorbed by the surface of the oceans, leading to an increase in ocean acidity (Wei et al., 2023). However, the high levels of atmospheric carbon dioxide $CO_{2)}$ dissolve in the oceans and then react with seawater, leading to the formation of carbonic acid, which decomposes into hydrogen ions and bicarbonate. Therefore, a decrease in ocean pH is referred to as ocean acidification. Acidification is regarded as a significant environmental issue that affects marine environments (Doney et al., 2020). The ocean acidification changed from 8.13 to 8.08 PH (Coelho et al., 2013).

The rising release of greenhouse gases into the atmosphere results in increased accumulation of heat, with apportion being absorbed by the seawater surface, leading to an increase in internal temperature "ocean heat content" known as "ocean warming" through melting ice and thermal expansion (Bindoff et al., 2019). Global ocean warming has been occurring since the 1970s (Arias et al., 2021). Around the world, CC represents a significant risk to environments, animals, and humans that plays an important role in sea acidification, sea warming, and the rise of sea levels (Ford et al., 2022). Globally, from 1901 to 2010, the level of the sea rose by 0.11 meters (11 centimeters), while in the 1980s, the level of the sea increased at a rate of 3.2 millimeters per year (Muruganandam et al., 2023). The World Health Organization WHO considers CC the greatest risk to public health between 2030 and 2050; human mortality rates are expected to rise to 250000 annually (San Lio et al., 2023).

Historically, the Mediterranean Sea has been susceptible to significant CC, making it a notable hotspot for CC (Pepi and Focardi, 2021). At present, the Mediterranean Sea experiences an average temperature is seeing an average temperature rise of 1.4 compared to late 19^{th}-century levels, with the increase being particularly noticeable during summer (Cramer et al., 2018). Lately, a substantial rise in CC levels has been detected in the ocean (Doney et al., 2012). Climate change (CC) has impacts on the physical and chemical properties of marine ecosystems, resulting in serious consequences to marine life, including dissolved oxygen concentration, pH, temperature, ice cover, and salinity of seawater (Cabral et al., 2019). Climate change affects the balance of marine ecosystems, leading to changes in the structure and biodiversity of marine organism populations (Assan et al., 2020). On the other hand, numerous research studies have reported that changes in ocean temperature may lead to alterations in the physiology of aquatic animals, resulting in changes in seasonal abundance, growth, fecundity, and feeding behavior (Doney et al., 2012) and invasive aquatic species (Neelmani et al., 2019).

CLIMATE CHANGE AND OCEAN POLLUTION

Pollution of the oceans and CC are some of the most pressing global challenges (Lu et al., 2018). Additionally, CC affects pollutants present in the oceans (Scharsack et al., 2021). These are two of the top health crises and should be prioritized by the public health system because they are interconnected (San Lio et al., 2023). Lately, many researchers have proposed that the combination of CC and ocean pollution is altering the biological structure and dynamics of marine environments (Jing et al., 2015). Limited data is available on the environmental hazards resulting from this mixture.

Heavy metals and petroleum accumulate in seawater and disrupt chlorophyll function in algae, reducing their photosynthetic capacity. This decline in photosynthesis decreases dissolved oxygen levels, negatively impacting marine animals. Furthermore, the accumulation of heavy metals in algae renders them toxic to marine animals that consume them, facilitating the transfer of these contaminants through the marine food chain (Lu et al., 2018). Ocean acidification resulting from increased CO_2 may increase the toxicity of some heavy metals and organic contaminants threatening the safety of coral reefs (Landrigan et al., 2020). In addition, heavy metals affect dissolved organic matter transport and the transformation of biotic and abiotic matter (Tang et al., 2022). Heavy metals have been used as bioindicators of CC in the environment (Tang et al., 2022).

Over time, these changes can devastate aquatic environments, and mitigating their adverse impacts can prove challenging (McCrink-Goode, 2014). The interaction between pollutants and CC may exacerbate ocean pollution. The presence of heavy metals, organic pollutants, excess nutrients, and plastics in the marine ecosystem raises challenges for reducing ocean pollution (Ford et al., 2022). Overall, the stressors of CC and the toxicity of contaminants can alter the biodiversity of aquatic organisms, such as fish, mollusks, and algae (Kibria et al., 2021) (Figure 2).

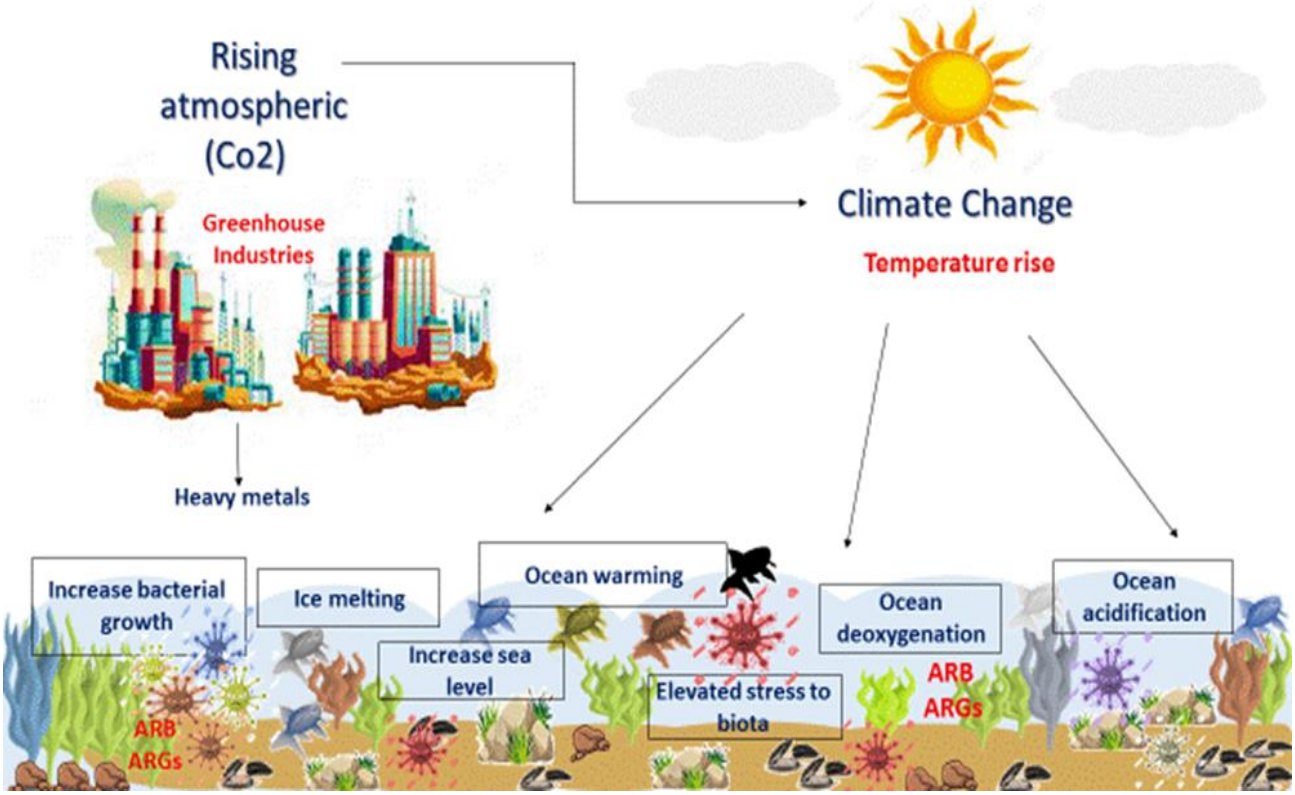

Figure 2. Negative impacts of climate change on coastal and ocean ecosystems.

CLIMATE CHANGE AND ITS ROLE IN AMPLIFYING OCEAN POLLUTION THROUGH ANTIBIOTIC-RESISTANT BACTERIA AND ANTIBIOTIC-RESISTANT GENES

Global CC is regarded as the primary driver of emerging infectious diseases (Li et al., 2022). Human activities have contributed to CC and ARB; hence, steps must be taken to mitigate their impacts. Temperature is closely related to bacterial growth, infections, the global distribution of bacteria, community composition, and survival in the presence of antibiotic agents (Philipsborn et al., 2016). Temperature rise speeds up HGT, which is a key factor in the development of antibiotic resistance. In addition, bacterial growth rates are often accelerated by temperature increases (Pietikäinen et al., 2005). Acquired ARGs are located on plasmids and can be transported to other aquatic bacteria through HGT, which is increased by increasing environmental temperatures (Zacharias et al., 2021). Many diseases are sensitive to climate, and temperature may increase the spread of bacterial infections in environments, animals, and humans (Harvell et al., 2002).

Mutations can occur in the presence of antibiotic resistance, which plays an important role in saving the life of the bacterial community (Li et al., 2022). Moreover, mutations that confer resistance to temperature stress can stimulate resistance to antibiotics and vice versa, a phenomenon known as cross-protection or collateral resistance (Okeke et al., 2022). Collateral resistance refers to the acquisition of resistance to a specific stress after prior contact with other stressor factors (Dragosits et al., 2013). The increased temperature may aid biofilm formation, as in the *Roseobacter* genus of *Rhodobacteraceae* growing at 33°C, resulting in the formation of a biofilm (Kent et al., 2018). Increased temperature induces a higher tolerance to antibiotics for marine bacteria (Kent et al., 2018). Within the United States, rising temperatures have been correlated with an increased prevalence of ARB among human bacteria (MacFadden et al., 2018; Li et al., 2022).

Numerous research studies have highlighted that the temperature increase forecasted by CC will drive an increase in antibiotic resistance in bacteria (MacFadden et al., 2018). A previous study was conducted in the USA from 1980 to 2010, collecting data on temperature and percentages of ARB from hospitals and laboratories across 41 states. The results of this study showed an association between temperature and antibiotic resistance (Pepi and Focardi, 2021).

Another study performed in 28 European countries from 2000 to 2006 reported the impact of temperature on antibiotic resistance (McGough et al., 2020). A recent review provided objective evidence that CC affects pathogenic bacteria (Cavicchioli et al., 2019). So far, there is limited research on CC and its impacts on infectious diseases. Likewise, numerous studies highlight CC impacts on the vectors of diseases more than on pathogenic bacteria. In addition, CC affects the spread of parasites, species of parasites, distribution changes, and abundance of hosts in marine environments (Assan et al., 2020). Currently, the impact of CC on the evolution of antibiotic resistance in ocean-based bacteria is not yet fully understood.

HOTSPOTS FOR THE TRANSMISSION OF ANTIBIOTIC-RESISTANT BACTERIA AND ANTIBIOTIC-RESISTANT GENES IN OCEANS

Domestic houses, healthcare settings, industrial plants, WWTPs, agriculture, aquaculture, and animal husbandry are considered "hotspots" for the spread of ARB and ARGs in the oceans (Kunhikannan et al., 2021; Gambino et al., 2022) (Figure 3). Therefore, pathogenic bacteria released from hotspots exposed to higher concentrations of antibiotics may increase their growth rates because of nutrient abundance in the ocean and may be selected before reaching marine environments (Azam and Malfatti, 2007).

Antimicrobial agents are lipophilic (Shokoohi et al., 2020) and incompletely metabolized in the human body, where they are released via the wastewater of hotspots with feces and urine (Sun et al., 2022). However, up to 70% of antibiotic agents cross the digestive tract of humans and are released via feces and urine (Shah et al., 2021). Several researchers have reported ARB and ARGs at high concentrations in hotspots. Marine environments have high levels of fecal matter, which arises from hotspots (Zieliński et al., 2021). Fecal pollution can introduce bacteriophages that harbor ARGs and pathogenic bacteria into marine environments. However, various substances in the marine environment, such as feces, soil, and plastics, act as potential reservoirs of antibiotic resistance (Amarasiri et al., 2020).

Figure 3. Impact of wastewater discharges on the marine food web.

HOSPITAL

Globally, hospitals are considered to be the most contaminated sector (Henriot et al., 2024). Antibiotics have been given extensively in hospitals for the treatment of bacterial infections in patients (Cai et al., 2021). Hospital wastewater contains the urine and feces of patients. In particular, some of these patients have taken antibiotic drugs for the treatment of their bacterial infections, which are included in the hospital wastewater as ARB and ARGs (Cai et al., 2021). The

antibiotics given to the patients in the hospital are partially metabolized in their bodies, while the rest are added to the hospital wastewater through excretion (Sosa-Hernández et al., 2021). Hospital wastewater (HWW) treatment plants are considered host sites for the spread of ARB and ARGs in aquatic environments (Yuan and Pian, 2023). Effluents of hospital wastewater discharged from infections operating rooms and diagnostic laboratories contain antibiotics, hormones, organic pollutants, heavy metals, pathogenic bacteria, viruses, and parasites (Yuan and Pian, 2023). However, the term "antibiotic-resistant hospitals" is used when hospitals acquire pollutants associated with resistant bacteria (Donker et al., 2012) (Figure 4).

Some ARB can survive chlorine disinfection and can spread in the effluent after chlorination. On the other hand, ARB may be developed via propagation, and ARGs are propagated among the bacterial community through HGT by plasmids, integrons, and transposons (Kümmerer and Henninger, 2003; Jin et al., 2018). However, the spread of ARB and ARGs in aquatic environments has negative impacts and greater challenges for public health (Rowe et al., 2017). Indeed, hospital wastewater treatment plants (HWWs) are more likely than urban wastewater systems to spread ARB and ARGs (Zheng et al., 2018). Urban wastewater contains simple pollutants, whereas HWW contains pharmaceuticals, chemicals, and pathogens that require advanced treatment (Petrovich et al., 2020).

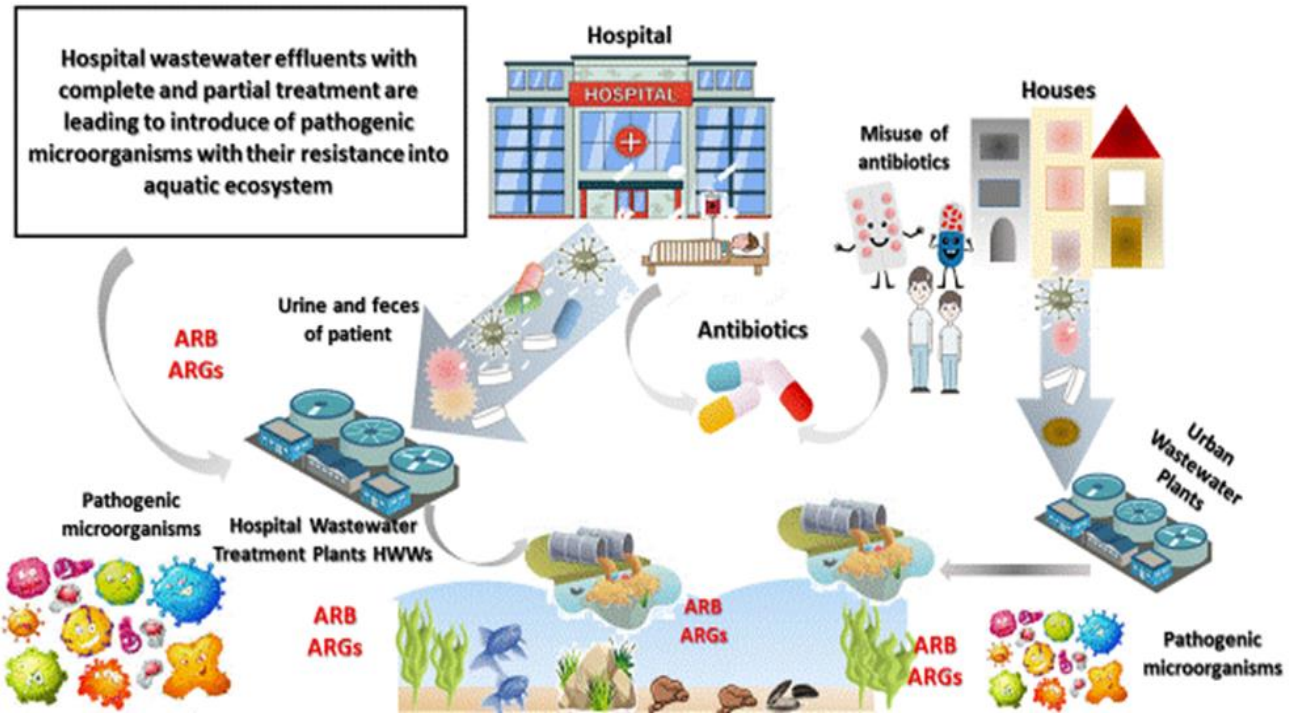

Figure 4. Hospital wastewater and urban wastewater as reservoirs for Antibiotic Resistance bacteria (ARB) and Antibiotic resistance genes (ARGs) in marine environments

In hospitals, antibiotics such as glycopeptides, vancomycin, carbapenems, piperacillin, and Cefotiam are used more frequently than in other sectors (Kümmerer and Henninger, 2003). The bacteria species commonly detected in HWWs include Vibrio spp., Salmonella, Shigella, Clostridium, Yersinia, Campylobacter, Leptospira, *Escherichia coli*, Klebsiella, Serratia, Enterobacter, *Staphylococcus aureus*, and *Acinetobacter baumannii* (Lien et al., 2017; Pulami et al., 2023). Another study has shown an increase in the presence of Gram-negative bacteria carrying *bla* genes such as bla_{NDM}, bla_{KPC}, bla_{CTX-M}, and bla_{SHV} in HWWs, together with other drug-resistant ARGs (Le et al., 2016). Studies have also identified various bacterial species in HWWs, including *Salmonella* spp., *Aeromonas* spp., *Vibrio* spp., *Escherichia* spp., *Mycobacterium* spp., *Leptospira* spp., *Pseudomonas* spp., and *Legionella pneumoniae* (Yuan and Pian, 2023). Further, another study reported the persistent presence of a complex mixture of pathogenic bacteria, ARGs, antibiotics, and viruses in HWWs (Petrovich et al., 2020). The diversity and fate of the viral community in HWWs are not yet fully understood.

Globally, 10% of hospital infection outbreaks have been associated with antimicrobial resistance. The healthcare system in poor countries is more vulnerable to the effects of antibiotic resistance pollution in the environment compared to developed nations (Manaia et al., 2024). Currently, Extensively Drug-Resistant bacteria (XDR) are on the rise among strains. They pose a major concern in healthcare settings (Siri et al., 2024), as they can result in infections that are very

challenging to manage and treat with antibiotics. Furthermore, XDR bacteria refer specifically to bacteria that are resistant to almost all available antimicrobial agents (Basak et al., 2016).

Currently, concerns about antibiotic pollution in hospitals have increased, therefore, many research studies have intensified in investigating the presence of ARB and ARGs in HWWs (Wang et al., 2018; Mehanni et al., 2023). Much research has been undertaken to detect antibiotics and residual bacteria in HWWs (Table 1).

Table 1. Isolated of Antibiotic Resistant Bacteria (ARB) and Antibiotic Resistant Genes (ARGs) from different hospitals around the world between 2023-2025

Location of study	**Type of isolates**	Year
Cambridge University Hospitals, Cambridge, United Kingdom	Tow $^{\beta}$- Lactam resistance genes (*bla*$_{GES}$ and *bla*$_{OXA}$)	2017
Beni- Suef University Hospital, Egypt	Three Multi-drug resistant bacterial isolates	2023
Three public hospitals in Xinxiang City, Central China	Escherichia and Acinetobacter were predominant in the Cultivable Multiple-antibiotic- bacteria (CMARB)	2018
Two hospitals in Singapore	The highest levels of ARGs are linked to resistance to $^{\beta}$- Lactam (*bla*$_{KPC}$), co-trimoxazole (*sull, sul2, dfrA*), amikacin, ciprofloxacin (*qnrA*), and intI1	2016
Hospital of Girona, Spain	Highest levels of ARGs linked to resistance to ($^{\beta}$- Lactam, macrolide, sulfonamide, and tetracycline)	2015
Ohashi Medical Center in Toho University, in Tokyo, Japan	Many ARB species, ARGs, and residual antibiotics	2021
Rural and urban hospitals in Vietnam	Antibiotic-resistant E coli isolated combined with ARGs	2017
Ayder Referral Hospital in North Ethiopia	Klebsiella spp, *Staphylococcus aureus*, and *Pseudomonas aeruginosa*, respectively, were frequently isolated from untreated wastewater, while Klebsiella spp, Pseudomonas aeruginosa, and Staphylococcus aureus, respectively, were frequently isolated from treated wastewater. These isolates were multi-drug resistant.	2017
Many hospitals in Benin	The highest levels of ARB are linked to resistance to Chloramphenicol and sulfamethoxazole.	2022
Regional High Specially Hospital of Ixtapaluca and the National Institute of Oncology in, Mexico	The presence of carbapenem resistance genes (CRGs) in Klebsiella spp	2022
Three hospitals in Cluj Country in, Romania	14 genes of ARGs confer resistance to $^{\beta}$- Lactam, aminoglycoside, chloramphenicol, macrolide-lincosamide-streptogramin B (MLSB) antibiotics, sulfonamide, and tetracycline	2017
Urban hospital in Japan	Proteobacteria, Bacteriodetes, Firmicute, Acinobacteria, and TM7, respectively were found in HWW before treatment.	2021

LIVESTOCK INDUSTRIES AND AGRICULTURE

Excessive antibiotic use by people and livestock may result in the discharge of partially metabolized antibiotics into the marine ecosystem through wastewater discharges. Because they are broken down or partially metabolized, most consumed antibiotics eventually enter sewer systems, either directly or indirectly (Faleye et al., 2018). The indirect release of consumed antibiotics into the marine environment through excretion is known as indirect antibiotic discharge. Unconsumed antibiotics are occasionally flushed directly into the sewer system (Aiken et al., 2014). Because of the high concentration of antibiotics, they are largely destroyed, but their active components still have an effect on the marine environment, which will eventually increase resistance in bacteria or may have ecotoxicological impacts (Aiken et al., 2014).

The rate of antimicrobial consumption in livestock reached 63.151 tons in 2010, with an expected increase of 67% by 2030 (Tiseo et al., 2020). Approximately 80% of antibiotics are distributed for veterinary use in the United States of America (Odoi et al., 2021). States in India, Canada, and Europe have strengthened their monitoring systems to spot emerging dangers or altering trends in the use of antibiotics in agriculture, particularly in settings where animals are produced (Nahrgang et al., 2018). Concerns over the rise of antimicrobial resistance have prompted many nations to implement stricter regulations on antibiotic use in veterinary practices, with the European Union notably banning the use of antibiotics as growth promoters in livestock since 2006 (European Union, 2006).

Nowadays, there is a lot of evidence suggesting that antibiotic use in livestock significantly contributes to the development of antibiotic resistance in some human infections (Collignon and McEwen, 2019). Antibiotic-resistant bacteria (ARB) and ARGs are widely distributed throughout most industrial wastes associated with animal husbandry-related sectors, and the antibiotic residue concentration in these enterprises is far higher than that in soil, hospitals, surface water, and groundwater. The continuous use of antimicrobials to enhance animal growth and prevent infections may lead to higher levels of antibiotic resistance in livestock farm wastes compared to human waste (Sim et al., 2011).

Fecal pollution creates physical contact, which increases the chances of gene transfer between environmental bacteria and bacteria that have been adopted into the intestinal tracts of people or domestic animals (Larsson and Flach, 2022). Numerous intestinal bacteria are also known to carry genetic elements, such as integrative conjugative elements, plasmids, insertion sequences, integrons, or transposons, that can help pathogens acquire genes and transmit them to other organisms (Larsson and Flach, 2022). The potential threat to human health from ARB and ARGs passing down the aquatic food chain has escalated in recent years due to the emergence of ARB and ARGs in livestock, aquatic products, and poultry meats (Rehaiem et al., 2016).

Soils have a natural abundance of ARB and ARGs. Reusing treated sewage effluents may put human health at risk if ARB and ARGs build up in agricultural soils. In addition, ARB and ARGs are mostly distributed in the soil by the use of manure in agriculture; these substances may travel to the ocean through wastewater effluents (Li et al., 2023). However, a major source of antibiotic resistance transmission to water-resistant genomes and soil is agricultural activities such as irrigation, soil fertilization, and animal rearing. Antibiotic-resistant bacteria (ARB), ARGs, and run-off chemicals are more likely to be present in intensive agricultural activities (Flores-Vargas et al., 2021). The discharge of ARB and ARGs from wastewater treatment plant effluents (WWTPs) into water sources contributes to the spread of ARB and ARGs in marine environments (Ondon et al., 2021). Antibiotic-resistant genes (ARGs) and heavy metal co-selection have been observed in agriculture-impacted resistome (Flores-Vargas et al., 2021).

INDUSTRIAL ACTIVITIES

Industrial activities such as aquaculture, medicine, and industry that cause ocean pollution may include chemical, pharmaceutical, food, and oil (Blanco-Picazo et al., 2020). The release of pollutants from industrial, wastewater treatment plants (WWTPs), and aquaculture operations (Jia et al., 2022) contribute to the prevalence of ARB and ARGs in marine environments (Baena-Nogueras et al., 2021). Nevertheless, industries that spread waste with heavy metals contaminate the soil and water sources, which causes bacteria to acquire heavy metal tolerance mechanisms. These bacteria may develop higher levels of heavy metal tolerance. The development of antibiotics and environmental studies are all significantly affected by the emergence of antibiotic and heavy metal resistance in aquatic bacteria (Tahmourespour, 2021).

There is rising global concern about antibiotic resistance and heavy metal pollution in oceans because heavy metal tolerance in pathogenic bacteria is responsible for the spread of antibiotic resistance. Studies have discovered a relationship between bacteria's resistance to heavy metals and antibiotics; comparable mechanisms exist in both situations to help bacterial growth in adverse conditions (Oves and Hussain, 2016). However, bacteria in marine environments can co-occur with ARGs and heavy metal resistance genes (HMRGs) (Håkonsholm et al., 2023). ARGs tend to be linked to gene cassettes that contain the class 1 integrons, a potentially mobile genetic element that is responsible for HMRGs and conjugative-mediated gene transfer (Lin et al., 2021). Therefore, the selection of multi-resistant bacteria and the spread of resistance into nature may be encouraged by the simultaneous existence of heavy metals and ARGs (Gambino et al., 2022). Bacteria often develop heavy metal resistance via a combination of passive and active mechanisms, such as sequestration, efflux, or alteration of metals within the microbial cell (Fardami et al., 2023).

Lead, cadmium, mercury, copper, chromium, and zinc are among the many examples of heavy metals that are frequently found in industrial operations and may constitute an important risk to both human health and the marine environment (Alghamdi et al., 2021). Copper and other heavy metals are used in fish farming as antifouling agents and feed additives. As a result, copper may contaminate the marine environment through feces, spilled feed, and nets from fish farms that have been metal-impregnated (Håkonsholm et al., 2023). Furthermore, heavy metals such as copper, arsenic, mercury, and silver have been used in different types of antimicrobials for many years and as antimicrobials in veterinary medicine and humans (Gufe et al., 2022). In addition, metals such as mercury, lead, chromium, and arsenic have carcinogenic and neurotoxic effects on both humans and animals (Morais et al., 2012). Overall, successfully mitigating the environmental effects of heavy metal pollution requires an in-depth understanding of the mechanisms via which heavy metal affects aquatic bacteria.

Over half of the seafood consumed worldwide is produced by fish farming. Fish farming uses antibiotic agents for growth and treats infections (Brunton et al., 2019). Aquatic environments can be exposed to antibiotic residues from various sources, including fish farming (Schar et al., 2021). There has been a rise in ARB and ARGs in aquaculture systems, according to several studies. The prevalence of ARB and ARGs in fish farming may have harmful effects on these industries (Kim and Cha, 2021). Many studies have shown that resistance can spread among human pathogens, aquatic bacteria, and fish pathogens (Shen et al., 2018; Pepi and Focardi, 2021). Antibiotic-resistant bacteria (ARB) and ARGs, including zoonotic pathogens, have been isolated from fish farming around the world as well as from water and products (Santos and Ramos, 2018).

WASTEWATER

Wastewater treatment plants (WWTPs) collect wastewater originating from healthcare settings, houses, companies, and other sources, making them inadvertent collection locations for ARB (Wengenroth et al., 2021). The transfer of resistance genes and storage of antibiotic resistance can occur within sewage and wastewater treatment plants (WWTPs). In addition, it has been suggested that they act as hotspots for HGT, which would allow the spread of ARGs within other bacterial species (Karkman et al., 2018). While the average bacterial density in sewage is between 10^5 and 10^8 cells per milliliter (Uluseker et al., 2021). The enhancement of biomass in contemporary biological WWTPs causes a threefold rise in bacterial density in the bioreactors, and selection by sedimentation produces dense bacterial aggregates. Microbial diversity and interactions are pervasive and frequent in WWTP bioreactors (Nielsen and McMahon, 2014).

Wastewater microbial populations are difficult to cultivate and mainly comprised of members from different phyla such as Bacteroidetes, Actinobacteria, Proteobacteria, and Firmicutes (Novo et al., 2013). Doxycycline, ofloxacin, ciprofloxacin, and norfloxacin concentrations in Swedish WWTP sludge were measured. Likewise, high levels of norfloxacin and ciprofloxacin were also found in samples of Swiss sewage sludge (Barancheshme and Munir, 2019). Moreover, β-lactamases can be easily broken down, whereas fluoroquinolones and tetracyclines are more persistent, allowing them to remain in the environment for longer and build up to higher concentrations (Hanna et al., 2023). A study by Mthiyane et al. (2024) reported that fifteen chosen antibiotics were monitored within the primary and secondary stages of WWTP.

However, the activated sludge process has been one of the most frequently used sewage treatment technologies for the removal of major contaminants from municipal sewage for more than a century (Uluseker et al., 2021). Through human and animal stool and urine, improper medicine disposal, and direct environmental contamination by wastewater from antibiotic production facilities, antibiotics are discharged into the environment (Singh et al., 2022). A study by Ng et al. (2023) reported azithromycin, erythromycin, clarithromycin, ofloxacin, and ciprofloxacin wastewater effluent samples from wastewater treatment facilities in 11 European countries, showing their presence throughout the continent's aquatic ecosystems. According to a study by Wang et al. (2019), macrolides and fluoroquinolones are the most prevalent antibiotics in the effluents of 18 designated WWTPs in Harbin City, China.

Because wastewater treatment processes are not intended to eliminate ARB and ARGs, WWTPs frequently include antimicrobial agents and other co-selective agents (Rodríguez-Molina et al., 2019). The potency of conventional treatment methods varies significantly among WWTPs, and it is still unclear how some treatment technologies work to eliminate antimicrobials, ARB, and ARGs (Pallares-Vega et al., 2019). The United Nations (UN) World Water Development Report 2020 reveals that 80% of wastewater globally is discharged into marine ecosystems without treatment (UNESCO World Water Assessment Program, 2020). In low-income countries, just 8% of wastewater undergoes treatment, while treatment rates reach 30% in lower-middle-income countries and 70% in higher-income countries (Buelow et al., 2021).

ROLE OF HORIZONTAL GENE TRANSFER IN THE DISSEMINATION OF ANTIBIOTIC-RESISTANT BACTERIA AND ANTIBIOTIC-RESISTANT GENES IN OCEANS

Bacteria can adapt to and live in settings contaminated with antibiotics according to their plastic genomes, which are supported through various genetic pathways such as conjugation, transformation, and transduction. Due to selection pressure, mutation, and gene transfer, ARB develops in bacteria (Kunhikannan et al., 2021). Horizontal gene transfer (HGT) in aquatic bacteria is the exchange of genetic material between various bacterial species in the marine environment (Eskova et al., 2022). Horizontal gene transfer (HGT) mechanisms include conjugation, transduction, and transformation. Horizontal gene transfer (HGT) is critical for assisting bacteria in sharing ARGs between species.

Horizontal gene transfer (HGT) is mediated by mobile genetic elements (MGEs) that include transposons, plasmids, bacteriophages, and integrons (Zhou et al., 2022). Although chromosomal mutations can result in antibiotic resistance in bacteria, HGT is a more prevalent method of ARGs in most cases of antimicrobial resistance (Michaelis and Grohmann, 2023). Bacteriophage transduction is a crucial method for the spread of ARGs throughout the populations of bacteria (Arnold et al., 2022). However, bacteriophages play an important role in spreading virulence genes and ARGs (Chiang et al., 2019).

Bacteria can develop resistance by degrading or modifying antibiotics, as well as by blocking their entry into cells or changing themselves. Bacteria can deactivate antibiotics by hydrolyzing them. The basic mechanism of antibiotic resistance involves antibiotic modification catalyzed by enzymes. Thousands of enzymes that can degrade and modify different antibiotics, including β-lactams, macrolides, aminoglycosides, and phenicol, have been discovered (Nordmann et al., 2011). Bacterial resistance is induced by the presence of ARGs. Pathogenic bacteria acquire ARGs via plasmid exchange at the gene level and develop antibiotic resistance. Horizontal gene transfer (HGT) of ARG-carrying plasmids, transposons, and integrons in bacteria can occur across strains of the same and distinct species (Jian et al., 2021). Different bacterial ARGs may develop resistance to various antibiotics.

About 70% of hospital-acquired infections are resistant to at least one antibiotic (Chow et al., 2021). Antimicrobials that dissolve in surface waters reach sub-inhibitory concentrations, influencing microbial ecology by raising mutation rates, generating HGT, and promoting ARB selection. Long-term exposure to sub-inhibitory antibiotic doses in the marine environment may be the primary cause of antibiotic resistance and ARG transmission (Atterby et al., 2021).

IMPACT OF ANTIBIOTIC-RESISTANT BACTERIA AND ANTIBIOTIC-RESISTANT GENES ON THE ONE HEALTH SCALE

Humans, animals, and the natural environment are interdependent, according to the OH concept (Robinson et al., 2016). A conceptual framework for creating interventions that maximize results for human, animal, and environmental health is provided by OH Concept (Gudipati et al., 2020). Despite the OH approach's propensity to emphasize zoonosis, it is crucial to consider the environmental impacts of capital-based development (Bukha et al., 2022). Because of the irresponsible and excessive use of antibiotics in many industries (human healthcare settings, livestock raising, and agriculture), antibiotic resistance is linked to each of these three factors (Velazquez-Meza et al., 2022). The improper use of antibiotics, contributing to the spread of resistance, is exacerbated by inadequate infection control, agricultural waste, environmental degradation, and the movement of humans and animals carrying resistant bacteria (Bürgmann et al., 2018). However, the OH concept underscores the interconnected health of humans, wild animals, domestic animals, and the environment. This concept emphasizes the necessity of interdisciplinary collaboration, particularly given the pressures of the rapid growth of the human population that is driven by CC, rising levels of contaminants, and the depletion of natural resources (McEwen and Collignon, 2018).

The marine ecosystems act as a significant reservoir of ARB and ARGs. The aquatic animals may consume ARB from the marine environment. On the other hand, their intestines provide an ideal environment for the horizontal transfer of ARGs via conjugation (Jia et al., 2022). Some studies have reported a high abundance of ARB and ARGs in the guts of zebrafish, this can be attributed to HGT that facilitates the ingest of ARB in water by aquatic animals (Fu et al., 2017). Resistant zoonotic bacteria can be detected in soil, where they can then infect plants, vegetables, and fruits. Antibiotic-resistant bacteria (ARB) are easily spread between and among many environments and humans (WHO, 2019). Antibiotic resistance can increase animal disease and mortality rates while also lowering productivity and causing economic losses (McEwen and Collignon, 2018).

According to current studies, the existence of ARGs and/or bacteria in wild animals is more a result of anthropogenic pollution than natural selection for resistance (Dolejska, 2020). Antibiotic-resistant bacteria (ARB) selection primarily occurs in environments with a human connection and areas with wildlife. Therefore, detecting the presence of clinically relevant ARB and ARGs in wild animals not undergoing antibiotics highlights the impact of environmental antibiotic resistance pollution (Ahasan et al., 2017). The rise of the ARB threatens both the aquatic ecosystems' health and human health, highlighting antibiotic pollution in the marine ecosystem as a critical issue (Zhuang et al., 2021) (Figure 5).

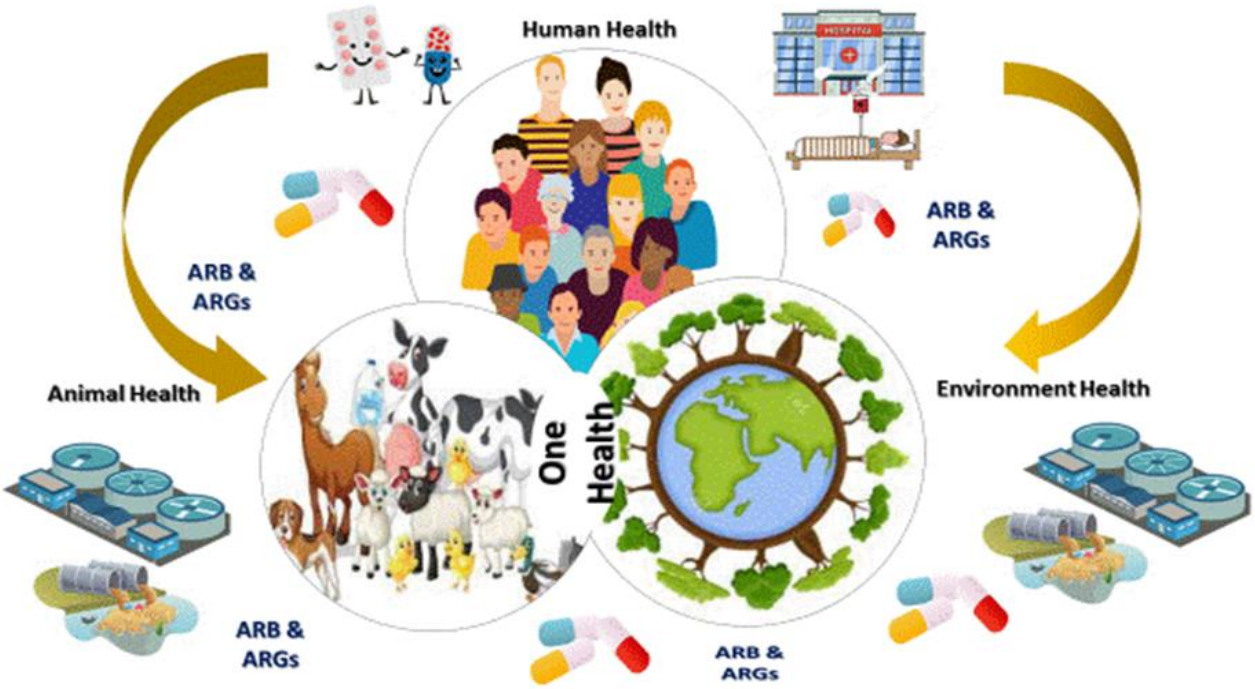

Figure 1. Spread of Antibiotic-Resistant Bacteria (ARB) and Antibiotic-Rsistant Genes (ARGs) at an On Health scale

RISKS OF ANTIBIOTIC RESISTANCE TO PUBLIC HEALTH

The rise in hospitalizations for infections caused by ARB is linked to clinically important antibiotic resistance. This situation can lead to unsuccessful treatments and increased mortality rates among patients (Ben et al., 2019). Particularly, bacteria that may colonize the human body (vectors) harbor ARGs simultaneously. To clone humans, vector bacteria must have the opportunity to come into contact with the human body, which necessitates that these bacteria occasionally or often share an environment with humans (Manaia, 2017). Antibiotic use impacts the intestinal flora, encourages resistance, and boosts the population of opportunistic bacteria, which leads to digestive issues and raises the risk of ARB (Li et al., 2021).

However, several ways may expose people to being infected with ARB, such as via ARB in medical settings, whereas it can be spread via human-to-human contact (Endale et al., 2023). Antibiotic-resistant bacteria can spread to human pathogenic bacteria when they infect the human body. Preventing and treating bacterial diseases becomes more challenging once harmful bacteria acquire resistance (Sanganyado and Gwenzi, 2019). Indirect transmission of waterborne enteric bacteria among residents in a community can also be made easier by poor sanitation (Collignon and Kennedy, 2015). Antibiotics can reach aquatic products mostly by ingestion, although they can also be ingested from sediments or surrounding water in relatively lower amounts (Maghsodian et al., 2022). Aquatic products gathered in Vietnam, China, Korea, and India contain antibiotic residues (He et al., 2016).

One of the most serious global health issues is antibiotic resistance, which causes over 33000 deaths and 67000 diseases annually in the European Union (ECDC, 2025). Globally, the threats associated with “super bacteria” or methicillin-resistant S. aureus (MRSA) are increasing due to its resistance to most antibiotics, making it one of the leading causes of death (Lin et al., 2021). The annual cost of treating infections caused by antibiotic resistance is projected to reach $35 billion in the USA, creating a considerable financial burden on the global economy (CDC, 2025). Surveillance research from 2000 to 2015 in 76 countries found a 65% growth rate in antibiotic usage, increasing it to 34.8 billion (Klein et al., 2018). According to estimates by the WHO, by the year 2050, ARB infections will cause the deaths of 10 million people annually (Chang et al., 2022; Sun et al., 2022).

GLOBAL ONE HEALTH STRATEGIES TO ADDRESS ANTIBIOTIC-RESISTANT BACTERIA AND ANTIBIOTIC-RESISTANT GENES IN THE OCEANS

To address the challenge of the antibiotic resistance crisis in the oceans, the WHO, FAO, and OIE have created a global action plan that incorporates the OH idea. The main objectives of OH are:

Awareness and knowledge of antibiotic resistance will be enhanced via education, productive communication, and training (McEwen and Collignon, 2018; Collignon and McEwen, 2019). Everyone should be aware of the basic principles of good hygiene, such as regular handwashing, disinfecting tools, and properly cleaning food. These practices help reduce the spread of diseases (WHO, 2015; Collignon and McEwen, 2019). Surveillance and research contribute to reinforcing the body of knowledge and evidence. Samples of the significant bacteria found in various animals, humans, and environmental settings, such as farms, veterinary offices, long-term care facilities, fish farming, hospitals, and community settings, should be taken as part of the OH surveillance of antibiotic resistance (WHO, 2015, 2017; Lozano-Munoz et al., 2021). Improve the administration of antibiotics to improve the health of humans and animals. While everybody can benefit from having a deeper understanding of the health aspects of antibiotic resistance, those in special need include farmers, people who keep pets, veterinarians, and those who work in the larger food industry (WHO, 2015).

Promote investment in new pharmaceuticals, vaccines, diagnostic tools, and other innovations by developing an economic case for sustainable investment that addresses the needs of all nations (Canada et al., 2015). Bacteriophages, immunomodulators, monoclonal antibodies, and enzymes encoded by phages are examples of novel treatment methods that have been developed as an outcome of increasing antibiotic resistance and a lack of new drug development. Novel therapeutic approaches may be important for treating serious infections and extending the useful life of antibiotics (Chang et al., 2022). The majority of action policies and laws fail to address the problem and do not expressly reduce the pollution of natural ecosystems by ARGs and antimicrobial agents. All national initiatives lack current regulations restricting the environmental release of antibiotics by pharmaceutical production facilities (Ifedinezi et al., 2024). When it comes to environmental preservation and conservation, global policies must be unified and unavoidable, requiring considerable investments in improving sanitary systems and wastewater management (Buelow et al., 2021).

One Health OH and global health are two interrelated approaches that must be used to fully understand the ARB problem and, more particularly, ARB transmission. The health of these systems is interconnected. Through this approach, it is possible to understand how ARB impacts not only humans but can also be transmitted from marine animals or the marine environment to humans (Hernando-Amado et al., 2020). A major approach to preventing antibiotic resistance is the prudent use of antibiotics in the aquaculture sector. This entails taking action to reduce needless antibiotic use and encouraging prudent consumption (WHO, 2015; Lozano-Munoz et al., 2021). It is crucial to implement national strategies following international norms. Restricted antibiotic use, monitoring of antibiotic resistance, and encouraging the adoption of alternative disease management techniques should be the main objectives of these programs, and to reduce ARB in marine ecosystems, the One Health approach is essential for developing integrated strategies and solutions. This approach emphasizes the importance of coordination and collaboration across various industries, such as aquaculture, public health, and environmental agencies (Lozano-Munoz et al., 2021). Sentinel species such as sea turtles can be used to track and maintain an eye on ARB in oceans. Effective measures require in-depth knowledge of the sources and factors that affect ARB in the oceans, such as WWTPs and human activities (Drane et al., 2021). Antibiotic resistance in aquatic bacteria can be monitored and noticed, which can be used to detect emerging resistance trends and guide intervention plans (Pereira et al., 2020). Despite CC, a health strategy that emphasizes simultaneously preserving the health of humans, animals, and the environment can help reduce the incidence of infections (Gudipati et al., 2020). Promoting awareness and educating the public on how antibiotic resistance is being impacted by CC in the oceans, with an emphasis on the importance of responsible antibiotic use and environmental conservation. Encouraging collaboration across disciplines among scientists working in various fields to better understand the complex connections between CC, antibiotic resistance, and ocean ecosystems (Gudipati et al., 2020).

CONCLUSION

Antibiotics are considered "pseudo-persistent" pollutants. They continually enter environments and their rate of entrance is higher than their rate of disposal. Despite this, the latest study suggests that the widespread administration of antibiotics over the past century has resulted in a selective pressure that has accelerated the acquisition and dissemination of ARGs among environmental bacteria. Moreover, this increases the possibility that these bacteria could be dangerous to human health, given their remarkable ability to swap genes**.** The unclear overlap of ARB and ARGs in the human microbiome and environment does not mean that there is no risk. Furthermore, the increase in infection rates and severity, coupled with the reduced effectiveness of antibiotics in healthcare settings, highlights the effects of this overlap on public health. Comprehending the transmission routes of ARB is crucial in tackling the issue of antibiotic resistance, which impacts not only public health but also the health of aquatic animals and marine environments to ensure the health of aquatic animals, humans, and marine environments. It is imperative to enhance cooperation among various health disciplines, engaging relevant institutions at local, national, and global levels. Applying an OH approach to understanding antibiotic pollution in oceans could lead to increased societal involvement and, ultimately, more effective

policy.

DECLARATIONS

Authors' contributions

Khwla Khirallah Bukha collected the literature and wrote the first draft of the review article. Ibrahim Mohamed Eldaghayes contributed to the critical review, editing, and overall supervision of the manuscript. All authors have read, reviewed, edited, and approved the final version of the manuscript.

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Competing interests

The authors declare that there is no conflict of interest.

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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.

Availability of data and materials

All data generated during the research are relevant and included in this published article and will be available from the corresponding author upon reasonable request.

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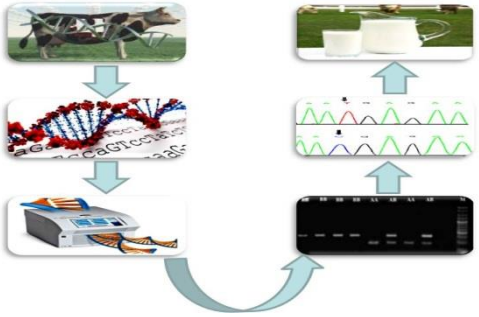

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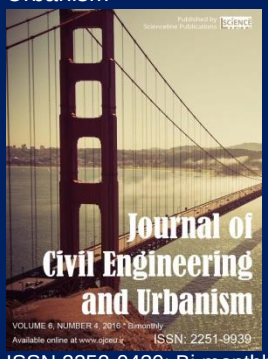

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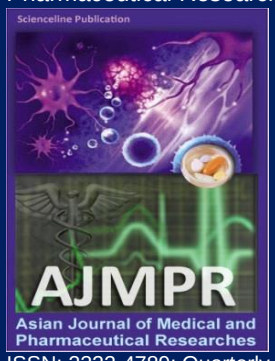

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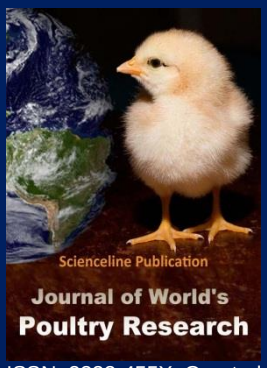

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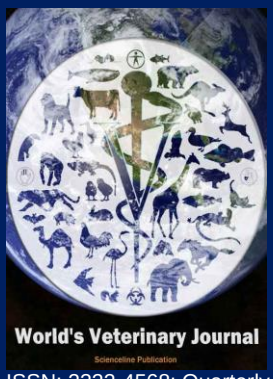

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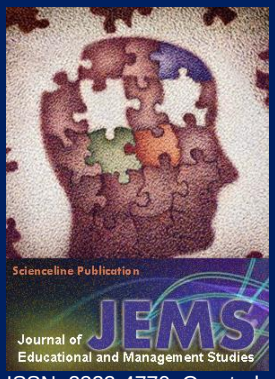

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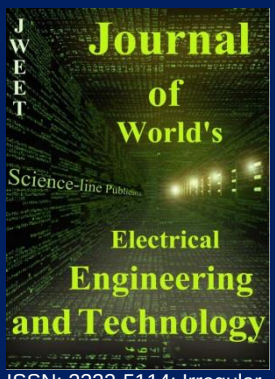

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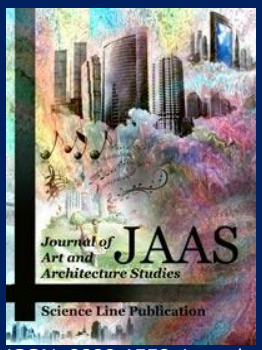

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