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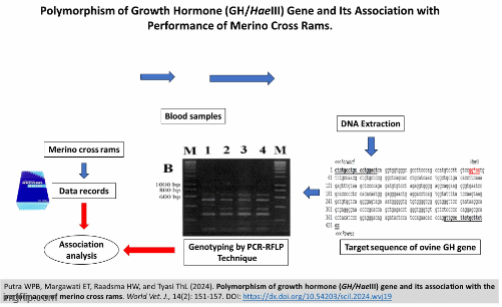
Volume 14 (2); June 25, 2024

Research Paper

Polymorphism of growth hormone (*GH/Hae*III) gene and its association with the performance of merino cross rams

Putra WPB, Margawati ET, Raadsma HW, and Tyasi ThL.

World Vet. J. 14(2): 151-157, 2024; pii:S232245682400019-14
DOI:https://dx.doi.org/10.54203/scil.2024.wvj19



ABSTRACT: Merino cross sheep (75% Merino × 25% Garut) are introduced by the government of Indonesia for meat production purposes. The present study aimed to determine the polymorphism in the exon 2 region of the growth hormone (*GH*) gene (422 bp) in Merino cross rams using the PCR-RFLP technique and to analyze its relationship with body weight and body measurements of the rams. A total of 145 rams aged one-year-old with an average body weight of 29.08 ± 7.96 kg from the breeding station in West Java, Indonesia were considered as the experimental animals. It was indicated that a missense mutation of c.55G > A (p.G19S) was detected in the target sequence of the *GH* gene in Merino cross rams. The PCR-RFLP analysis in the *GH* gene of Merino cross with HaeIII restriction enzyme (*GH*/*Hae*III) was observed in a moderate category with a polymorphic informative content (PIC) value of 0.22. Therefore, the G allele was more frequent than the A allele (0.85 versus 0.15). Furthermore, the genotype AA was not present among the sheep that were part of the study. However, the polymorphism of p.G19S was found to have no significant association with birth weight and body measurements in one-year-old Merino cross sheep. However, the *GH*/*Hae*III gene in Merino cross rams exhibited polymorphism, primarily with two genotypes: GG (wildtype) and GA (carrier). The G allele was identified as the dominant allele in the ovine *GH* gene, occurring with a frequency of 0.85. Importantly, the polymorphism of the *GH/Hae*III gene was significantly linked to birth weight and chest depth in one-year-old Merino cross rams. These findings provide preliminary insights that could potentially aid in the early stages of molecular selection for Indonesian Merino cross sheep.
Keywords: Growth hormone gene, Merino cross ram, Performance, Polymorphism

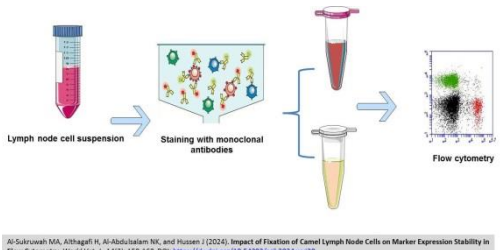
[Full text-PDF] [Crossref Metadata]

Research Paper

Impact of Fixation of Camel Lymph Node Cells on Marker Expression Stability in Flow Cytometry

Al-Sukruwah MA, Althagafi H, Al-Abdulsalam NK, and Hussen J.

World Vet. J. 14(2): 158-168, 2024; pii:S232245682400020-14
DOI: https://dx.doi.org/10.54203/scil.2024.wvj20



ABSTRACT: Single cell immunophenotyping by flow cytometry has proven a useful and high sensitive method for the analysis of immune cell composition and phenotype in different lymphatic and non-lymphatic tissues. Fixation of stained cells is usually recommended when the cells need to be preserved for later analysis by flow cytometry to avoid changes in cell morphology and expression of the level of cellular antigens. In the present study, a stain-fix approach was used in combination with flow cytometry to investigate the impact of fixation of camel lymph node cell suspension (n = 5 camels) after labeling with monoclonal antibodies to some leukocyte antigens on their cellular composition and expression density of immune cell markers. The obtained results indicated that camel lymph node cell suspension stained with fluorochrome-conjugated mAbs to leukocyte antigens and fixed with paraformaldehyde (PFA) will keep stable values for their immune cell composition for at least six days when analyzed by flow cytometry. However, if cell subsets were to be identified, fixation may result in different values that were obtained when analyzing fresh stained unfixed cells. Especially the instability in the fluorescence intensity of CD14, CD172a, and MHCII will lead to significant changes in the frequency of monocyte subsets (classical versus intermediate or non-classical) and the identification of macrophage functional subtype (M1 versus M2). Similarly, the instability in CD44 expression may affect the identified

phenotype of T cells with significantly lower frequency of activated T cells. In conclusion, flow cytometric data collected from stained and PFA-fixed cell suspension prepared from camel lymph nodes should be interpreted with care if the functional subtype of cells is to be identified based on surface molecule expression.
Keywords: Camel, Fixation, Flow cytometry, Immune cell, Lymph node

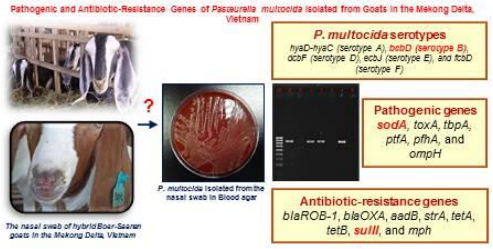
[Full text-PDF] [Crossref Metadata]

Research Paper

Pathogenic and Antibiotic-Resistance Genes of *Pasteurella multocida* Isolated from Goats in the Mekong Delta, Vietnam

Nguyen TT, Nguyen VLP, Truong TT, Nguyen CTH, and Nguyen TK.

World Vet. J. 14(2): 169-177, 2024; pii:S232245682400021-14
DOI: https://dx.doi.org/10.54203/scil.2024.wvj21



ABSTRACT: *Pasteurella multocida* (*P. multocida*) is one of the predominant pathogens that mostly cause respiratory diseases in domestic animals, such as goats. To determine *P. multocida* serotypes and the prevalence of pathogenic and antibiotic-resistance genes the PCR method was used. A total of 143 isolated *P. multocida* strains were collected from 289 healthy hybrid Boer-Saanen goats' nasal samples in the Mekong Delta, Vietnam, from March to June 2023. A total of 143 *P. multocida* strains, serotype B accounted for the highest proportion (51.05%), followed by serotype A (14.69%), and the lowest was serotype E (0.70%) while (39.86%) of strains could not be determined serotypes. Among the six virulence genes surveyed, the *sodA* gene (56.64%) had the highest presence, while the *ompH* gene (4.20%) had the lowest presence. Pathogenic genes were present mainly in serotypes A and B; *tbpA* was frequently detected in serotype A (66.67%), and *sodA* was commonly detected in serotype B (56.16%). There were 14 virulence gene combinations in 59/109 (54.13%) serotyped *P. multocida* strains, and the pattern of *sodA* + *toxA* + *tbpA* was prevalent at the highest rate (12.84%). Moreover, among the eight investigated antibiotic resistance genes, the *sulII* gene had the highest presence rate (74.13%), compared to the *tetA* gene with the lowest presence rate (13.29%). Gene *sulII* was mainly detected on strains belonging to serotypes A (80.95%), B (83.56%), and F (77.78%). A total of (77.98%) of serotyped *P. multocida* strains indicated multi-harbor from two to six antibiotic-resistance genes, and the most common pattern was *aadB* + *sulII* (10.09%). The prevalence of five pathogenic *P. multocida* serotypes harboring diverse antibiotic-resistance genes isolated from nasal samples could be a critical issue in treating and preventing the respiratory diseases caused by *P. multocida* in goats in the Mekong Delta.
Keywords: Antibiotic resistance, Goat, *Pasterella multocida*, Pathogenicity, Mekong Deltadd

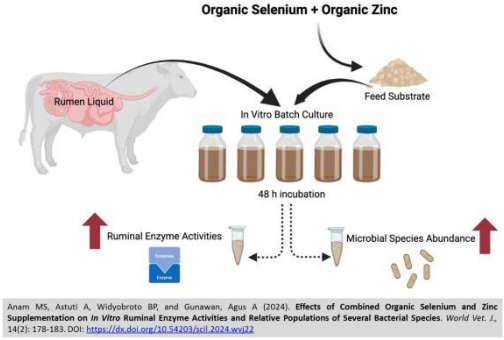
[Full text-PDF] [Crossref Metadata]

Research Paper

Effects of Combined Organic Selenium and Zinc Supplementation on *In Vitro* Ruminal Enzyme Activities and Relative Populations of Several Bacterial Species

Anam MS, Astuti A, Widyobroto BP, and Gunawan, and Agus A.

World Vet. J. 14(2): 178-183, 2024; pii:S232245682400022-14
DOI: https://dx.doi.org/10.54203/scil.2024.wvj22



ABSTRACT: Selenium (Se) and zinc (Zn) are essential animal microminerals. Combining Se and Zn (Se-Zn) as a feed additive in its influence on rumen fermentation patterns is still very limited, so further investigation is needed. The present study explored the supplementation impact of combined Se-Zn from organic sources on rumen enzyme activity and relative abundance of several bacterial species through an *in vitro* method. Five treatments, each with six replicates were used in the study. The first group treated without Se and Zn supplementation (T0, control), the second group treated with 0.3 ppm Se + 60 ppm Zn (T1), the third group treated with 0.45 ppm Se + 60 ppm Zn (T2), the fourth group treated with 0.3 ppm Se + 90 ppm Zn (T3), and the fifth group treated with 0.45 ppm Se + 90 ppm Zn (T4). The parameters observed included rumen microbial enzyme activities (carboxyl methyl cellulase, amylase, protease) and the relative abundance of rumen microbes (*Ruminococcus* sp., *Ruminococcus flavefaciens, Ruminococcus albus, Streptococcus* sp., *Prevotella ruminicola,* and *Eubacterium ruminantium*). Results indicated that carboxyl methyl cellulase (CMC-ase) and amylase activities raised in T2, T3, and T4 in comparison to T1 and T0 treatments. Protease activity and protein enzyme content increased in T2 compared to all treatments. The relative abundance of *Ruminococcus* sp. and *Ruminococcus albus* was higher in T2 and T3 compared to T0 treatment. Furthermore, an elevated *Ruminococcus flavefaciens* was indicated in T2 compared to

other treatments. The T2, T3, and T4 led to higher abundances of *Eubacterium ruminantium, Prevotella ruminicola*, and *Ruminococcus albus* compared to T0 and T1. It is concluded that organic Se and Zn enhanced the relative abundance of several bacterial species and the activity of enzymes in the rumen; optimal results are recommended when combining 0.45 ppm Se + 60 ppm Zn.
Keywords: Bacterial Species, Enzyme Activity, Rumen, Selenium, Zinc

[Full text-PDF] [Crossref Metadata]

Research Paper

The Effects of Saturated and Polyunsaturated Fatty Acids on Reproductive Performance and Reproductive Hormonal Changes in Dairy Cows at the Transition Period

Amini MR, Naserian AA, Valizadeh R, Dirandeh E, Baghshahi H, and Razavi SA.

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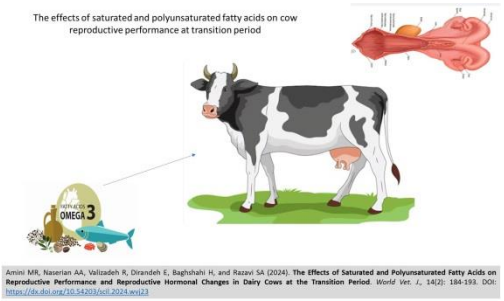
DOI: https://dx.doi.org/10.54203/scil.2024.wvj23



ABSTRACT: Reproductive success is crucial in dairy farming as it heavily relies on the consumption of a complete mixed ration for the diet. The current study investigated the effects of adding saturated (SFA) and polyunsaturated fatty acids (PUFAs) to dairy cows' diets on reproductive performance and reproductive hormones during the transition period. A total of 30 Holstein dairy cows were randomly divided into three groups (10 animals in each group), based on parity and body condition score. The cows had an initial body weight of 567.5 ± 40.3 kg (mean ± SD), a body condition score of 3.5 ± 0.26 out of 5 (mean ± SD), and a parity of 1.7 ± 0.02 (mean ± SD). The control group received a balanced ration meeting all the nutrient requirements according to the National Research Council (NRC) guidelines. The SFA group received 1.4% of dry matter (DM) as palm oil (RumiFat®), while the omega group had 5% of DM as safflower (a source of n-6 fatty acids) added from 21 days before parturition to 21 days after, and 4% of DM as flaxseed (a source of n-3 fatty acids) added from 21 to 42 days after parturition. In the Omega group, estradiol concentration significantly increased on artificial insemination (AI) day (12.54 pg/mL). Additionally, serum prostaglandin F2-alpha concentration was significantly higher in the omega group (0.732 pg/mL on day 7 and 1.68 pg/mL on day 14) compared to other groups. The control group exhibited the highest progesterone concentrations at 14 and 21 days post-calving compared to the other groups, other groups. whereas the omega group highest concentration five days after AI. The omega group also showed a significantly higher mean number of follicles >10mm and larger ovulatory follicle diameter. Moreover, a higher percentage of pregnant cows at 120 days in milk, fewer open days, and lower service per conception were observed in the omega group compared to the other groups. In conclusion, supplementing dairy cows' diets with PUFAs during the transition period positively influenced ovarian function, hormone levels, and reproductive performance.
Keywords: Flaxseed, Follicle diameter, Omega, Ovarian function, Safflower

[Full text-PDF] [Crossref Metadata]

Research Paper

Protein Concentration, Anthelmintic Activity, and Microbial Contamination of the Laboratory-Produced Chitosan-Encapsulated Bromelain Batches

Bez-bang KMR, Maina N, and Kagira J.

World Vet. J. 14(2): 194-201, 2024; pii:S232245682400024-14
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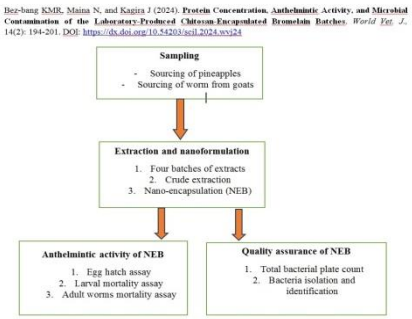


ABSTRACT: Bromelain has been shown to have potential as an anthelmintic for controlling livestock nematodes, such as *Haemonchus (H.) contortus*. The present study aimed to evaluate the *in vitro* quality of the laboratory-produced nanoencapsulated bromelain (NEB) and its activity against *H. contortus*. The acid-base extraction method was employed to extract four different batches of bromelain from the peels of fully ripened pineapples. It was encapsulated in chitosan to form the nano-encapsulated bromelain complex. Standard biochemical methods were employed to determine the bromelain concentration, protein concentration, *in vitro* anthelmintic activity against various stages of *H. contortus* (egg, larva, adult), and bacteria contamination for the four NEB batches. The mean concentration of extracted bromelain was 4.3 mg/ml in all four batches. There were no variations in the protein concentrations between the batches of NEB, which ranged from 1,090 mg/ml to 1.205 mg/ml. Although there were no significant differences in different batches, a variation in NEB inhibitory concentration (IC_{50}) was observed according to the different parasitic stages. The highest activity was for adult worms (LC_{50} =0.2454 ±

0.05 mg/ml), followed by the eggs (IC_{50} = 0.3 ± 0.07 mg/ml), and the larval stage (IC_{50} =0.9 ± 0.45 mg/ml). Despite the identification of certain bacterial species in the raw pineapple extract, the final product of all four batches of NEB remained free from any bacterial contamination. The current study indicated that NEB's concentration, protein concentrations, and anthelmintic activity did not vary significantly across the different batches of NEB. Additionally, the encapsulation process ensured that the final product was free of bacterial contamination and thus safe for use in animals.
Keywords: Anthelmintic activity, Bromelain, Chitosan, Nanoencapsulation

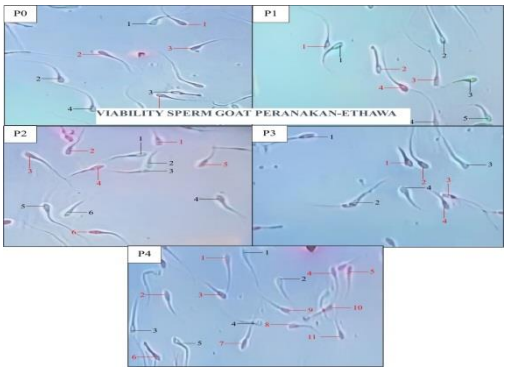
[Full text-PDF] [Crossref Metadata]

Research Paper

The Effects of Adding Coconut Water to Egg Yolk Diluent on Motility, Viability, and Abnormality of Etawa Crossbred Goat Sperm

Hoesni F, Firmansyah F, Abutani SA, and Nurhayati N.

World Vet. J. 14(2): 202-212, 2024; pii:S232245682400025-14
DOI: https://dx.doi.org/10.54203/scil.2024.wvj25



ABSTRACT: The Etawah crossbreed goat is a dual-purpose type of goat that can adapt well to tropical regions in Indonesia. The current research aimed to evaluate the effects of adding coconut water to citrate egg yolk diluent on the spermatozoa quality parameters (motility, viability, and abnormality) of the Etawah crossbred goat at the physiology and reproduction laboratory of animal husbandry, Jambi University (Indonesia). The research employed a randomized block design on Etawa crossbreed goats aged around 2-3 years with an average weight of 12 kg per head in six groups. The treatments included 100% citrate diluent of egg yolk without adding coconut water (P0) as a control, 90% citrate diluent of egg yolk + 10% coconut water (P1), 80% citrate diluent of egg yolk + 20% coconut water (P2), 70% citrate diluent of egg yolk + 30% coconut water (P3), 60% citrate diluent of egg yolk + 40% coconut water (P4). The parameters evaluated in this study included viability of spermatozoa, spermatozoa motility, and spermatozoa abnormalities. The five treatment tubes were stored in a refrigerated cabinet at 5°C for 2 days. After this period, semen quality assessment was assessed microscopically. The percentage of live spermatozoa was determined using a staining technique. The spermatozoa motility was assessed based on their ability to move. Abnormal spermatozoa were calculated based on the number of abnormal spermatozoa compared to the total number of spermatozoa. The results of the study showed that the addition of 20% coconut water to the 80% citrate diluent of egg yolk (P2 treatment) reduced the rate of decline in spermatozoa viability and did not increase the number of spermatozoa abnormalities significantly, compared to other groups. There was no decrease in the viability of Etawah crossbreed goat spermatozoa during 2 days of storage at 5°C in all groups. Therefore, it was concluded that coconut water could be added up to 20% into the egg yolk without any significant negative effects on spermatozoa quality parameters evaluated in the current study.
Keywords: Citrate diluent, Coconut water, Egg yolk, Etawah crossbred goat, Spermatozoa resistance

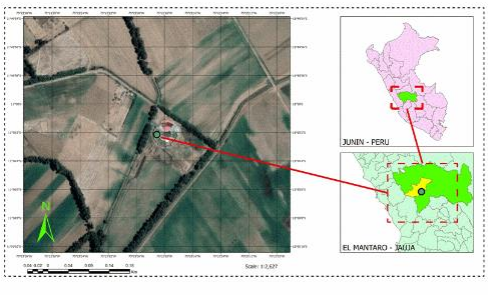
[Full text-PDF] [Crossref Metadata]

Research Paper

Effects of Different Seasons on Milk Quality: A Study on Two Cattle Breeds in Rainy and Drought Contexts

Guzman EL, Salome PH, Carhuas JN, Guzman SO, Tacza AA, Guillen MAF, and Garcia-Olarte E.

World Vet. J. 14(2): 213-219, 2024; pii:S232245682400026-14
DOI: https://dx.doi.org/10.54203/scil.2024.wvj26



ABSTRACT: The primary focus of dairy farming in the central region of Peru centers on producers. However, there is limited information on how different altitudinal zones, particularly during rainy and dry seasons, affect milk production. The present study aimed to investigate the effects of the rainy and dry seasons on the physicochemical properties of milk from Brown Swiss and Holstein cattle. A total of sixty cows were selected for the study, comprising 30 Brown Swiss and 30 Holstein. The study was conducted across two distinct seasons, including rainy and dry seasons. All animals received the same semi-intensive management and were fed ryegrass and balanced feed. Each animal provided 500 ml of milk for analysis in the morning. The milk was transported at a temperature of 2 °C, where they were analyzed with the Lactoscan equipment to evaluate protein, lactose, fat, total solids, milk density, freezing point, salts, and pH. Significant differences were observed in the interaction between

Brown Swiss and Holstein breeds across different seasons, including rainy and dry periods. Significant differences were observed in protein content, showing a positive effect in the interaction "rain: Brown" a value of 3.50 ± 0.36, while "rain: Holstein" showed 3.14 ± 0.05. Statistical differences were observed in the interactions for lactose content, with rain: Brown at 4.82% and dry: Holstein at 4.37%. Similarly, there were significant differences in fat content and total solids for rain interaction of rain: Holsten, and dry: Brown. Nevertheless, no differences were observed in terms of milk density, freezing point, salts, and pH. It is concluded that there was an influence of the interaction between breed physiology and seasonal conditions on milk composition. The results also highlight the impact of season-specific environmental factors on the quality of milk.
Keywords: Breed comparison, Milk composition, Milk quality, Physiological stability

[Full text-PDF] [Crossref Metadata]

Research Paper

Evaluation of Stored Whole Blood and Monitoring the Health of Dogs After Transfusion Using Fresh Whole Blood, Stored Whole Blood, and Packed Red Blood Cells

Nguyen TT, Nguyen HTQ, and Dinh KN.

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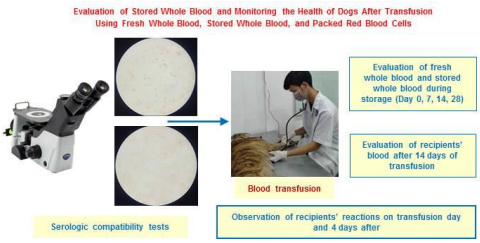
DOI: https://dx.doi.org/10.54203/scil.2024.wvj27



ABSTRACT: Blood products have been widely used in emergencies and treatment, necessitating optimal storage conditions to maintain quality. The current study aimed to evaluate the blood quality during storage, transfusion effectiveness, and reactions during and after transfusion in dogs. Five Greyhounds, including three males and two females aged 2.5 years old, and with 25-30 kg bodyweight, were selected and randomly labeled N1, N2, N3, N4, and N5. Fresh whole blood, stored whole blood, and packed red blood cells from the samples dogs were used for transfusion in the study. The investigated parameters were total protein (TP), aspartate transaminase (AST), alanine transaminase (ALT), alkaline phosphatase (ALP), lactate dehydrogenase (LDH), mean corpuscular volume (MCV), total carbon dioxide (tCO2¬), creatine kinase (CK), creatinine (CREA), blood urea nitrogen (BUN), glucose (GLU), white blood cells (WBC), red blood cells (RBC), hematocrit (HCT), plaletes (PLT), calcium (Ca), phosphorus (P), chloride (Cl), manganese (Mg), sodium (Na), and potassium (K). The results indicated that all parameters of stored blood samples were in the normal range during 28 days of storage in a refrigerator at 2-4°C. However, some parameters (TP, AST, ALT, ALP, LDH, MCV, tCO2, and K) increased, while others (CK, CREA, BUN, GLU, WBC, RBC, HCT, PLT, Ca, P, Cl, Mg, and Na) decreased during the storage period, especially Ca, P, and Na were below the normal range. All dogs indicated no reactions during and 5 hours after transfusion. However, dogs had symptoms of inappetence and mild diarrhea in 1-2 days after transfusion. Dogs received fresh whole blood recovered on day 3, while dogs of the stored blood recipient group recovered on day 4. By day 5, all dogs were healthy with no abnormal signs. The findings indicated the presence of hematological and biochemical alterations in stored blood, highlighting the importance of considering transfusion of stored blood for patients with critical medical conditions.
Keywords: Dog, Fresh whole blood, Packed red blood cell, Stored whole blood, Transfusion

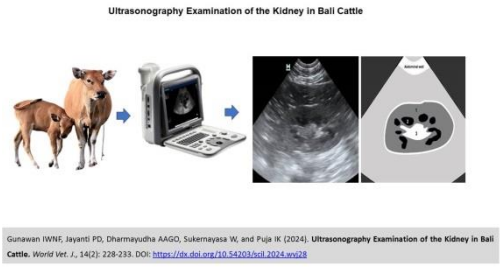
[Full text-PDF] [Crossref Metadata]

Research Paper

Ultrasonography Examination of the Kidney in Bali Cattle

Gunawan IWNF, Jayanti PD, Dharmayudha AAGO, Sukernayasa W, and Puja IK.

World Vet. J. 14(2): 228-233, 2024; pii:S232245682400028-14
DOI: https://dx.doi.org/10.54203/scil.2024.wvj28



ABSTRACT: Ultrasonography is an important technology for examining renal measurements, including length and width. The kidneys can be easily examined, and various structures in the kidneys are distinguishable with ultrasound. This research aimed to determine the normal ultrasonographic appearance of the kidneys in healthy adult Bali cattle, providing a reference for future descriptions of Bali cattle kidneys. In this research, 8 Bali cattle, aged 2-3 years with the healthy status of the urinary system were examined. The tool used was an animal ultrasound device, named Mindray DP10 Veterinary Ultrasound, with a 3-7.5 MHz convex transducer, utilizing a B-mode image mode. The transducer was placed in the right paralumbar fossae. The results indicated that the average horizontal length of the kidneys was 17.36 cm and the average vertical diameter of the kidney was 4.6 cm. The echogenicity of the renal cortex showed an echoic image, while the pyramidal part of the renal medulla indicated a relatively hypoechoic image. The results of measuring the diameter of the

left kidney in clinically healthy Bali cattle could be used as a basis for decision-making in determining the clinical status of kidney health in this breed of cattle.
Keywords: Bali cattle, Echogenicity, Kidney, Morphology, Morphometric data, Ultrasound examination

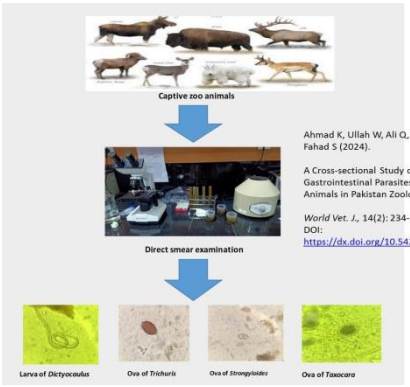
[Full text-PDF] [Crossref Metadata]

Research Paper

A Cross-sectional Study of Prevalence of Gastrointestinal Parasites in Captive Wild Animals in Pakistan Zoological Gardens

Ahmad K, Ullah W, Ali Q, Adeel M, and Fahad S.

World Vet. J. 14(2): 234-241, 2024; pii:S232245682400029-14
DOI: https://dx.doi.org/10.54203/scil.2024.wvj29



ABSTRACT: The animals held captive in zoos often face health and well-being issues. Parasitic infections can lead to health problems in wildlife animals by affecting their gastrointestinal tract. Therefore, the present study aimed to identify and evaluate the population of the various Gastrointestinal (GIT) parasites of wild animals enclosed in different zoological gardens in Pakistan. The fresh fecal samples (n = 960) of 20 captive wildlife animals were collected from Marghzar Zoo, Islamabad (n = 340), Ayub National Park, Rawalpindi (n = 221), Lohi Bher Wildlife Park, Rawalpindi (n = 296), and Bansra Galli Wildlife Park, Rawalpindi (n = 103). The samples were obtained from wildlife mammals, including urial (n = 95), blue bull (n = 106), chinkara gazelle (n = 77), zebra (n = 77), hog deer (n = 75), spotted deer (n = 43), blackbuck (n = 58), barking deer (n = 52), red deer (n = 104), yak (n = 44), grey goral (n = 40), lion (n = 37), mouflon sheep (n = 46), red fox (n = 12), bear (n = 37), grey wolf (n = 12), jackal (n = 12), vervet monkey (n = 12), rhesus monkey (n = 12), and langoor (n = 12). Various methods, such as direct smear examination, standard sedimentation, and floatation techniques were applied to detect and identify the endoparasites in the fecal sample. The detailed routine parasitological analysis identified approximately 52 endoparasites in the fecal samples, including *Haemonchus contortus*, *Eimeria bovis*, *Ostertagia curcumcincta*, *Strongyloides papillosus*, *Strogylus equinus*, *Oxyuris equi*, *Chabertia ovina*, *Protostrongylus*, and *Trichostrongylus vitrines*. The obtained results indicated that Lohi Bher Wildlife Park (46.35%) had a higher prevalence of GIT parasites, compared to Marghzar Zoo (33.23%), Bansra Galli Wildlife Park (33.02%), and Ayub National Park (19.45%). The study reports mild to moderate parasitic infection in captive wild animals and that could affect the survivability of the animals in captivity. The findings of the study can be used to formulate a proper health protocol and sanitation management in captive wild animals to control parasitic infections.
Keywords: Captive wild animal, Gastrointestinal infection, Parasite, Zoological Garden

[Full text-PDF] [Crossref Metadata]

Research Paper

Macroscopic Differences of Pig Eye after Death: A Veterinary Forensic Study

Haryo A, Widayanti R, Pangestiningsih TW, Widyatmoko AYPBC.

World Vet. J. 14(2): 242-246, 2024; pii:S232245682400030-14
DOI: https://dx.doi.org/10.54203/scil.2024.wvj30

ABSTRACT: The study of veterinary forensics is a field of science that is developing rapidly in the world of veterinary medicine. Veterinary forensics plays a crucial role in investigating and resolving cases involving animals, either as subjects or objects in incidents and ensuring the collection of all possible biological and physical evidence. Given the close relationship between humans and animals, numerous significant cases arise that are pertinent to veterinary forensics. The current research aimed to determine early post-mortem changes in pigs, providing insights into animal mortality in real-world scenarios. Observations were made on seven male Yorkshire pigs, aged 3 months old, with an average weight of 30.1 kg. Pigs were observed at four different post-mortem intervals, including 2,4,6, and 8 hours after death, with initial observations at the time of death serving as the control. Observations of changes in the eye sclera, eye lens, eyeball temperature, and eyeball pressure were carried out at each time interval. Results at the 2nd and 4th hours post-mortem showed no macroscopic changes in the eye sclera and eye lens, but there were changes in eye pressure. By the 6th and 8th hours, changes in the sclera and eye lens showed desiccation in the area of the sclera and the eye lens, which became increasingly cloudy. The eyeball temperature measurement values from the 2nd to 8th hour of the study revealed a significant decrease in eyeball pressure. The results of this study indicated observable changes in the eyes can

be used as a basic alternative method for calculating the introductory post-mortem interval in animals in the future. There was a significant decrease in eyeball temperature, and eyeball compactness, as significant differences in the eye sclera, and eye lens at 2, 4, 6, and 8 hours post-mortem, compared to the time of death. These variables offer crucial insights into early post-mortem changes in pigs, using the eyes as the primary focus of observation.
Keywords: Death, Eye, Forensic study, Pig

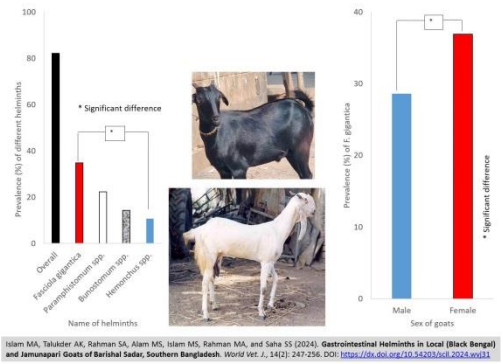
[Full text-PDF] [Crossref Metadata]

Research Paper

Gastrointestinal Helminths in Local (Black Bengal) and Jamunapari Goats of Barishal Sadar, Southern Bangladesh

Islam MA, Talukder AK, Rahman SA, Alam MS, Islam MS, Rahman MA, and Saha SS.

World Vet. J. 14(2): 247-256, 2024; pii:S232245682400031-14
DOI: https://dx.doi.org/10.54203/scil.2024.wvj31



ABSTRACT: Gastrointestinal helminths are important causes of hindering global goat production. To find the prevalence of gastrointestinal helminths of Black Bengal and Jamunapari breeds of goats, the current investigation was carried out at Barishal Sadar Upazilla of Barishal district, Bangladesh. The gastrointestinal helminths were identified through coprological examination. A total of 112 fecal samples were collected from household goats across different seasons, breeds, sexes, and ages. During the study period, four types of gastrointestinal helminths were identified based on the presence of helminth eggs in fecal samples. The overall prevalence of gastrointestinal helminths in goats was found to be 82.1%, while the prevalence rates of *Fasciola gigantica* (*F. gigantica*), *Paramphistomum* spp., *Bunostomum* spp., and *Hemonchus* spp. were 34.8% (95% CI: 1.4-2.5), 22.3% (95% CI: 0.7-1.8), 14.3% (95% CI: 0.1-1.5), and 10.7% (95% CI: 0.2-1.0), respectively. A significantly different prevalence was observed among different gastrointestinal helminths in goats. A significantly lower prevalence of *F. gigantica* and *Paramphistomum* spp. was observed in male goats, compared to females. A higher prevalence of *F. gigantica* was significantly observed during the winter, compared to the summer. The current study elucidates that *F. gigantica* was more prevalent in female goats. The current study indicated that *F. gigantica* was more prevalent in female goats. These findings underscore the importance of further research and control measures to manage gastrointestinal helminth infections in goats across southern Bangladesh and other regions with similar environmental conditions.
Keywords: *Fasciola gigantica,* Gastrointestinal helminth, Goat, Prevalence, Summer

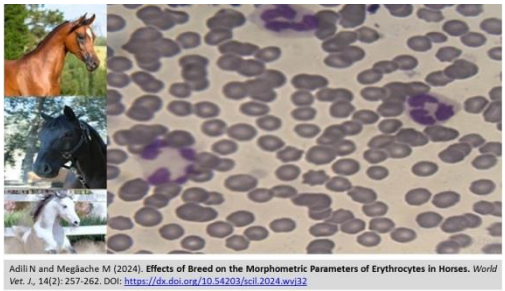
[Full text-PDF] [Crossref Metadata]

Research Paper

Effects of Breed on the Morphometric Parameters of Erythrocytes in Horses

Adili N and Megâache M.

World Vet. J. 14(2): 257-262, 2024; pii:S232245682400032-14
DOI: https://dx.doi.org/10.54203/scil.2024.wvj32



ABSTRACT: Various factors have a distinct impact on the quantity and dimensions of red blood cells across diverse animal species. These factors encompass age, sex, elevation, time of year, and lineage. The present study aimed to evaluate the influence of breed on the morphometric parameters of red blood cells in horses. To examine the impact of various horse breeds on the diameter, circumference, and surface area of red blood cells, blood samples were obtained from a total of 90 healthy horses. These horses belonged to three different breeds, including Arab Thoroughbred, English Thoroughbred, and Barbe. Each breed consisted of 30 individuals, with an equal distribution of males and females. The collected blood specimens were then divided into two separate batches for further analysis. The age range of all horses included in the study was between 5 and 12 years old. Smears were made and stained using the May-Grünwald Giemsa technique. The morphometric measurements were performed while using the OPTIKATM Vision Pro special software. The obtained results showed that there were no significant differences in the red blood cell diameters across different horse breeds. However, this factor appears to influence significantly both the circumference and surface area of erythrocytes. Specifically, the circumference and surface of Barbe red blood cells were highly smaller than both Arabian and English purebred horses. The present study demonstrated that the circumference and surface of red blood cells appear to be more indicative and representative in detecting variations in the erythrocyte morphometry between different horse

breeds.
Keywords: Breed, Circumference, Diameter, Erythrocyte, Horse, Surface

[Full text-PDF] [Crossref Metadata]

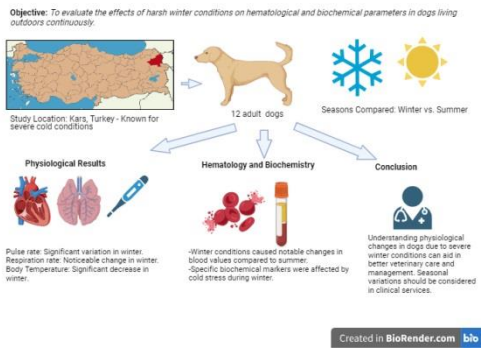
Research Paper

The Effects of Weather Conditions on Hematological and Biochemical Parameters in Dogs: A Field Study

Erkiliç EE, Merhan O, Ermutlu CŞ, Kirmizigül AH, Sezer M, Bati YU, and Özaydin İ.

World Vet. J. 14(2): 263-269, 2024; pii:S232245682400033-14

DOI: https://dx.doi.org/10.54203/scil.2024.wvj33



ABSTRACT: Although animals have adaptation abilities to different environmental conditions, various physiological changes may occur. The present research aimed to evaluate the effects of severe winter conditions on hematological and biochemical parameters in dogs kept outside all year. The research was carried out in the province of Kars, which is known for its severe cold conditions in Türkiye. Vital signs, hematological, and biochemical parameters of 12 adult dogs aged 1-8 years old (mixed breed, 8 males and 4 females) included in the study were compared in winter and summer seasons. The results indicated a significant effect of the winter season on the body temperature, respiration, and pulse rate of the dogs. In addition, it was observed that some hematological, including White blood cell (WBC), mean corpuscular hemoglobin (MCH) and mean corpuscular hemoglobin concentration (MCHC) Hemoglobin (HB), and biochemical parameters (urea, TP, albumin, cholesterol, glucose, creatinine) of dogs differed in winter from those in summer. It should be considered that the vital values of dogs living outdoors in intense winter differed from those in mild winter or summer. It is also concluded that veterinarians should consider these differences in routine clinical examinations of these animals.
Keywords: Biochemical parameter, Cold stress, Dog, Environmental factor, Hematological parameter, Winter condition

[Full text-PDF] [Crossref Metadata]

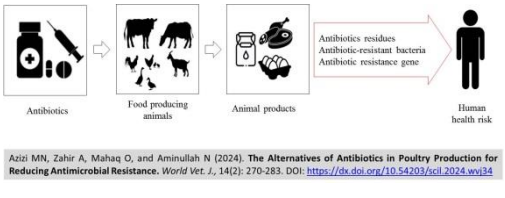
Review

The Alternatives of Antibiotics in Poultry Production for Reducing Antimicrobial Resistance

Azizi MN, Zahir A, Mahaq O, and Aminullah N.

World Vet. J. 14(2): 270-283, 2024; pii:S232245682400034-14

DOI: https://dx.doi.org/10.54203/scil.2024.wvj34



ABSTRACT: Antibiotics are natural, semi-synthetic, or chemical compounds that have anti-microbial activity and are used in livestock and poultry production for a variety of reasons, including therapeutic and growth promotion. The use of antibiotics in poultry production has been associated with the development of resistant bacteria. The present study attempted to explain the role of antibiotics as poultry growth promoters, bacterial resistance, and risks for human health, with a special focus on some selected bacterial species isolated from poultry farms and products. Furthermore, the manuscript reviewed the literature on alternative feed additives to reduce the reliance on antibiotics. Microbial resistance is a significant global health concern that has been a top global threat in the 21st century. The use of antibiotics in poultry production as non-therapeutic or growth promoters is at low doses and continuously, associated with developing resistant bacteria. Meanwhile, antibiotic-resistant genes in humans may have their roots in the diets of animals treated with antibiotics. Developing bacterial resistance has encouraged researchers to reduce the reliance on antibiotics by identifying potential feed additives, such as essential oils, bacteriophages, antimicrobial peptides, probiotics, prebiotics, organic acid, and enzymes that improve the immune system functions, reduce morbidity and mortality, improve the growth performances of poultry, and preserve consumer health.
Keywords: Antibiotic, Antimicrobial resistance, Feed additive, Human, Poultry

[Full text-PDF] [Crossref Metadata]

Review

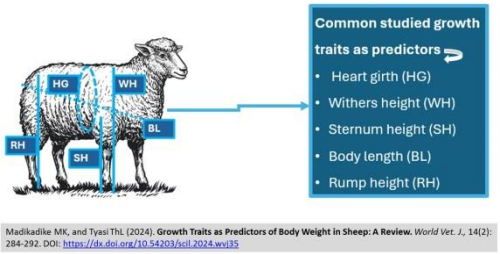
Growth Traits as Predictors of Body Weight in Sheep: A Review

Madikadike MK, and Tyasi ThL.

World Vet. J. 14(2): 284-292, 2024; pii:S232245682400035-14

DOI: https://dx.doi.org/10.54203/scil.2024.wvj35

ABSTRACT: The sheep production industry affects most rural areas and communal farm enterprises in the tropics and sub-tropics as a source of income. The motivation behind the present literature review was to provide detailed literature from various sources on the estimation of body weight from the growth traits of sheep. The review was conducted to highlight the importance of body weight and the significance of growth traits (heart girth, withers height, body length, sternum height, and rump height) as parameters to predict body weight. The main reason for livestock practice is to ensure food security. Therefore, it is important to assess economic traits and determine the carcass merit of sheep. Linear body measurement is a practical, fast, easy, and cheap method broadly utilized in national breeding programs to predict body weight and improve meat productivity in rural areas. The current review indicated that growth traits could be used to predict the body weight of sheep since they importantly provide necessary information about the morphological structure and potential development of the animals.



Keywords: Body length, Body weight, Growth trait, Heart girth, Rump height, Withers height

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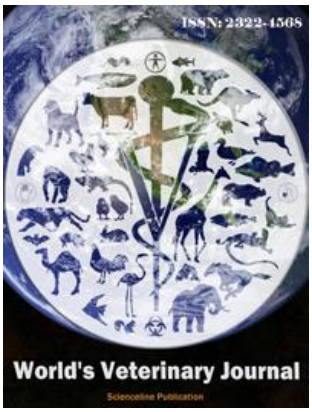

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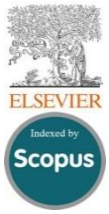

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Polymorphism of Growth Hormone (*GH*/*Hae*III) Gene and Its Association with the Performance of Merino Cross Rams

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[2]*Research Center for Applied Technologies for Animal Genetics and Reproduction, Faculty of Veterinary Science, University of Sydney, NewSouth Wales 2006, Australia*
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ORIGINAL ARTICLE
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ABSTRACT

Merino cross sheep (75% Merino × 25% Garut) are introduced by the government of Indonesia for meat production purposes. The present study aimed to determine the polymorphism in the exon 2 region of the growth hormone (*GH*) gene (422 bp) in Merino cross rams using the PCR-RFLP technique and to analyze its relationship with body weight and body measurements of the rams. A total of 145 rams aged one-year-old with an average body weight of 29.08 ± 7.96 kg from the breeding station in West Java, Indonesia were considered as the experimental animals. It was indicated that a missense mutation of c.55G > A (p.G19S) was detected in the target sequence of the *GH* gene in Merino cross rams. The PCR-RFLP analysis in the *GH* gene of Merino cross with HaeIII restriction enzyme (*GH*/*Hae*III) was observed in a moderate category with a polymorphic informative content (PIC) value of 0.22. Therefore, the G allele was more frequent than the A allele (0.85 versus 0.15). Furthermore, the genotype AA was not present among the sheep that were part of the study. However, the polymorphism of p.G19S was found to have a significant association with birth, weight, and chest depth measurement in one-year-old Merino cross sheep. However, the *GH*/*Hae*III gene in Merino cross rams exhibited polymorphism, primarily with two genotypes: GG (wildtype) and GA (carrier). The G allele was identified as the dominant allele in the ovine *GH* gene, occurring with a frequency of 0.85. Importantly, the polymorphism of the *GH/Hae*III gene was significantly linked to birth weight and chest depth in one-year-old Merino cross rams. These findings provide preliminary insights that could potentially aid in the early stages of molecular selection for Indonesian Merino cross sheep.

Keywords: Growth hormone gene, Merino cross ram, Performance, Polymorphism

INTRODUCTION

Sheep is an important livestock in Indonesia and can be developed as a potential export commodity. According to the Indonesian Ministry of Agriculture, sheep meat production in Indonesia in the year 2021 was about 50,702.06 tons (Kementan, 2022a). Meanwhile, the projection of sheep meat consumption in Indonesia in the same year was about 41,776 tons (Kementan, 2022b). Hence, there are about 8,926.06 tons of sheep meat surplus for export potency. The sheep meat production can be increased by a genetic improvement program with a selection program. Recently, a molecular selection involving functional genes has been used to obtain the genetic marker for the productivity of sheep (Bowles, 2015). In the year 2000, a grading-up program to produce Merino cross sheep (75% Merino and 25% Garut) was assessed by the government of Indonesia for meat production purposes. Therefore, previous studies recorded that sheep have 1.34±0.51 of estimated breeding value (EBV) for litter size and 0.10 ± 0.03 kg/day of post-weaned daily weight gain (Putra et al., 2023; Margawati et al., 2023).

The ovine growth hormone (*GH*) gene is one of the common candidate genes that are used for molecular selection in sheep (Gebreselassie et al., 2020). This gene is located on chromosome 11 along 1,795 bp with five exons. Previous studies reported a missense mutation (p.G19S) in exon 2 of the *ovine GH* gene that affects the production traits in sheep (Kumari et al., 2014; Susilorini et al., 2017; Rashijane et al., 2022; Muniasamy et al., 2023). Therefore, a mutation p.G19S can be detected using the PCR-RFLP technique with *Hae*III restriction enzyme (Hua et al., 2009). Unfortunately, studies aimed at detecting genetic markers for production traits in Indonesian Merino cross sheep are very limited. Margawati et al. (2023) reported a polymorphism of the BMPR1B/*Ava*II gene in Indonesian Merino cross sheep but its was not associated with the growth traits. Nonetheless, a polymorphism of *CAPN/BseSI* gene was significantly associated with birth weight of Merino cross sheep (Puruhita et al., 2023).

It is crucial to conduct research aimed at identifying genetic markers associated with growth traits of Merino cross sheep, as this will ultimately enhance their production characteristics. The purpose of the present study was to identify the mutation site or single nucleotide polymorphism (SNP) in the *GH* gene (exon 2) of Merino cross rams using PCR-

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RFLP method with *Hae*III restriction enzyme (*GH*/*Hae*III). In addition, the effect of *GH*/*Hae*III gene polymorphism on body weight and body measurements of animals in the current study was investigated.

MATERIALS AND METHODS

Ethical approval

All experimental procedures were approved by the Animal Ethics Committee of the Indonesian Institute of Science (LIPI) with the following permit number, 002/KKE/UM/VIII/2017.

Animals, research site, and DNA extraction

A total of 145 Merino crossbreed rams (75% Merino, 25% Garut) aged one year old were used in the experiment. The sheep were collected from the Cimanglid research farm at Bogor Regency, West Java, Indonesia. This farm is located at latitude 6° 38' 14" S and longitude 106° 46' 31" East with an altitude of 15-150 m asl; 20-30 °C of air temperature about 70% relative humidity and 2500-5000 mm/year of rainfall. The blood samples (± 3ml) were taken from the jugular vein of the animal using a venoject and vacutainer tube containing EDTA. The genomic DNA extraction was performed using the High Salting method according to Montgomery and Sise (1991). The DNA sample of each animal was stored at -20°C for further analysis.

PCR-RFLP

Amplification of the ovine *GH* gene (GenBank: KP120857.1) was performed using primer pairs of *GH*-F: 5'- CTC TGC CTG CCC TGG ACT -3' and GH-R: 5'- GGA GAA GCA GAA GGC AAC -3' with the target sequence of 422 bp (Hua et al., 2009, Figure 1). Amount 10 µL of PCR reaction consisted of 5 µL of PCR master mix (MyTaq Redmix, Bioline, USA), 1 µL of each primer (10 pmol/µL), 2 µL of free-nuclease water and 1 µL of DNA template (50 ng/µL) were assessed to amplify the target gene. The amplification of the ovine *GH* gene was performed in a PCR program of pre-denaturation at 94^{o}C for 5 minutes, followed by 35 cycles of denaturation at 95^{o}C for 30 seconds, annealing at 65^{o}C for 30 seconds, initial extension at 72^{o}C for 45 seconds and a final extension at 72^{o}C for 7 minutes. Therefore, the amplicons were visualized using 1% agarose gel through electrophoresis analysis at 100 Volt for 45 minutes. Subsequently. The DNA fragments were stained with EtBr and visualization under UV light. In addition, the detection of *GH* gene polymorphism was performed with RFLP analysis using *Hae*III restriction enzyme (GG*CC) at 37°C for 3 hours using the water bath. Therefore, the electrophoresis by 2% agarose gel and UV light was assessed for the visualization of RFLP results.

Management of animal

The sheep were raised in a colony stall (1 male and 30-50 females) with an intensive system at the research site. The feed ration consisted of Elephant grass (*Pennisetum purpureum*) and the commercial concentrate with the composition of 14% crude protein, 4% fat, 7% crude fiber, 8% ash, and 12% TDN with 2,700 kcal/kg of metabolizable energy (ME). The water was given *ad libitum*. The natural mating was managed in the studied farm to produce the lambs. The body weight (BW) of the animal was obtained with a hanging weight scale. Therefore, the animal weighing was performed at birth, weaning age (± 3 months of age), and continued with a regular weighing time every two weeks from weaning to yearling age (one year old).

Body weight

The Merino cross sheep in the current study were kept during the ACIAR project from 1999 to 2002. Hence, the data records of BW in sheep during that period were used in the present study for association analysis. The data correction was performed in the BW to reduce an experimental error according to the method of Hardjosubroto (1994) according to the following formulas (Formula 1-5).

$$BW_c = BW \times CTB \quad \text{(Formula 1)}$$

$$BW_{120} = \left(BW + \left(\frac{WW - BW}{T_w} \right) \times 120 \right) \times CTB \quad \text{(Formula 2)}$$

$$BW_{365} = \left(BW_{120} + \left(\frac{YW - BW_{120}}{T} \right) \times 245 \right) \times CTB \quad \text{(Formula 3)}$$

$$DG_{pre} = (BW_{120} - BW_c) / 120 \quad \text{(Formula 4)}$$

$$DG_{post} = (BW_{365} - BW_{120}) / 245 \quad \text{(Formula 5)}$$

where, BW_c is the corrected birth weight, BW_{120} is the body weight at 120 days of age, BW_{365} is the body weight at 365 days of age, BW is the actual birth weight, WW is the actual weaning weight, YW is the actual yearling weight, T_w is the weaning age, T is the period between weaning to weighing times, DG_{pre} is the pre-weaned daily gain, DG_{post} is the post-weaned daily gain, CTB is the constant for type of birth for example 1.0 (single) and 1.10 (twin).

Body measurements

Body measurements of thirteen parts, including head length (HL), head width (HW), withers height (WH), body length (BL), chest girth (CG), rump length (RL), rump width (RW), chest depth (CD), chest width (CW), front leg length (FLL), back foot length (BFL), front leg circumference (FLC), and back foot circumference (BFC) of yearling rams (365 days of age) for association analysis were recorded. All body measurements were measured according to Alderson (1999) as shown in Figure 2.

Statistical analysis

The association analysis between the genotype of the *GH*/*Hae*III gene and body weight was performed by GLM (General Linear Model) procedure in the SAS software package. The linear model was as Formula 6.

$Y_{ik} = \mu + G_i + \varepsilon_{ik}$ (Formula 6)

where, Y_{ik} was the ik traits observation value; μ was the mean; G_i was the effect of i^{th} genotype and ε_{ik} was the residual error. Therefore, the genetic diversity parameters of genotype frequency, allele frequency, observed heterozygosity (H_o), expected heterozigosity (H_e), number of effective allele (n_e), polymorphic informative content (PIC), and Chi-square (χ^2) values for *GH*/*Hae*III gene were calculated according to the studies of Nei and Tajima (1981), Weir (1990), Hildebrand et al. (1992), Nei and Kumar (2000), and Kaps and Lamberson (2004), respectively. The p value less than 0.05 considered for the significant differences.

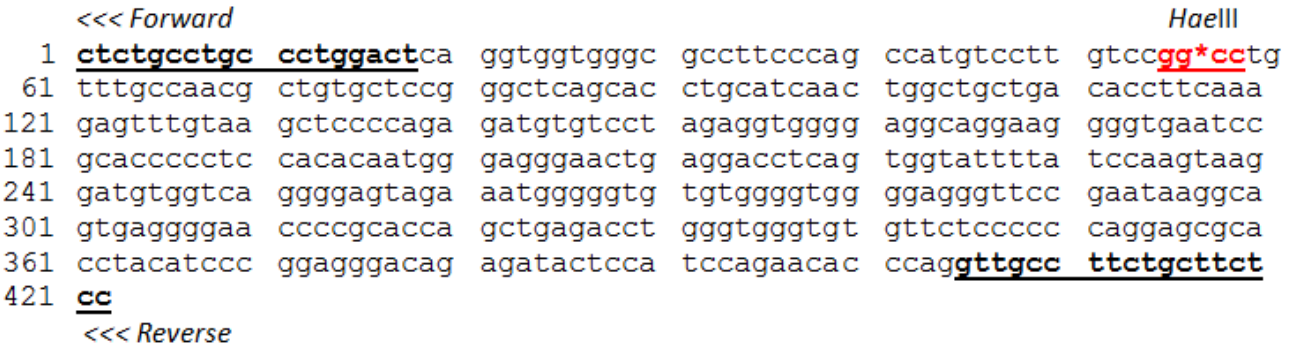

Figure 1. Primer position (black bold underline) and *Hae*III restriction site (red bold underline) in the target sequence of *ovine* Growth Hormone (*GH*) gene (GenBank: KP120857.1) along 422 bp. A transition mutation of c.55G>A (p.G19S) occurred in the exon 2 of the ovine *GH* gene

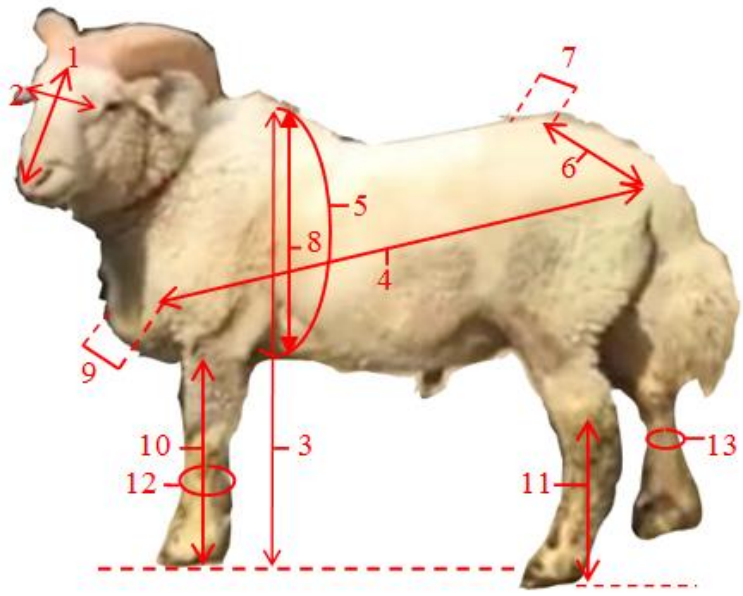

Figure 2. The sheme of body measurements in a Merino cross sheep consisted of head length (1), head width (2), withers height (3), body length (4), chest girth (5), rump length (6), rump width (7), chest depth (8), chest width (9), front leg length (10), back foot length (11), front leg circumference (12), and back foot circumference (13)

RESULTS AND DISCUSSION

The result of PCR-RFLP analysis in *GH*/*Hae*III gene of Merino cross sheep reveals two genotype patterns of GG (366 bp) and GA (422 bp and 366 bp) as illustrated in Figure 3. Nonetheless, the DNA fragment along 56 bp is not observed in the present study. Therefore, the GG genotype (0.70) and G allele (0.85) were observed superior in *GH*/*Hae*III gene of studied animals as presented in Table 1. Subsequently, a mutant AA genotype was absent in the included animals of the current study. Similar findings were observed in the Egyptian sheep breeds the presence of two genotypes of GG and GA in Barki (36% GG and 64% GA), Rahmani (19% GG and 81% GA), and Ossimi (77% GG and 23% GA) as reported by Othman et al. (2015). In Iraqi sheep breeds, two genotypes of GG and GA were observed in Awassi (70% GG and 30% GA) and Karadi (60% GG and 40% GA) as reported by Mahdi et al. (2018). Moreover, Two genotypes of GG and GA were also observed in Kenguri (42% GG and 58% GA) and Kilakarsal (29% GG and 71% GA) sheep breeds of India (Hiremath et al., 2017; Muniasamy et al., 2023). In contrast, the AA genotype was present in the Iraqi Hamdani sheep (50% GG; 10% GA; 40% AA) and Awassi sheep (47% GG; 33% GA and 20% AA) as reported by Mahdi et al. (2018) and El-Mansy et al. (2023), respectively. Moreover, the *GH*/*Hae*III gene was monomorphic in Palu sheep with 100% of the GG genotype (Malewa et al., 2019).

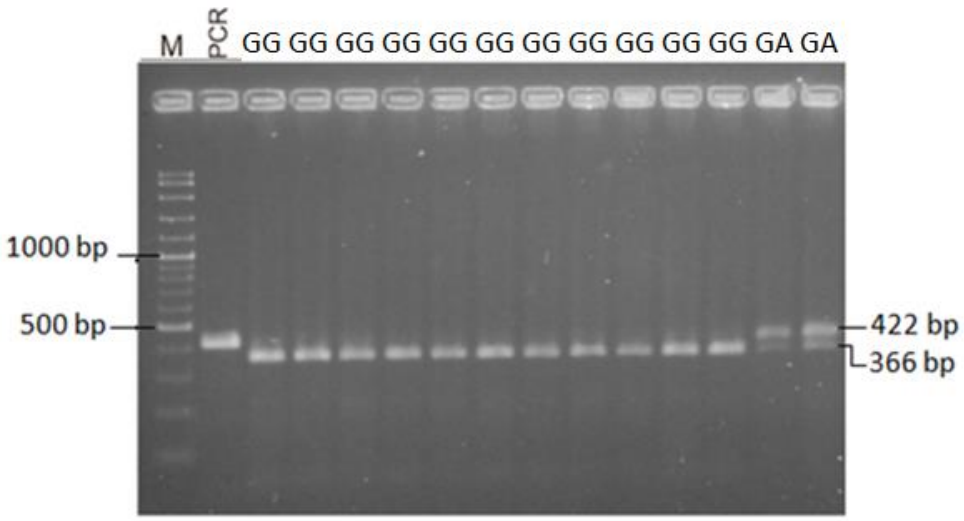

Figure 3. The PCR-RFLP results of the *GH*/*Hae*III gene in backcross rams showed two genotypes of GG (366 bp) and GA (422 bp and 366 bp). A 56 bp DNA fragment in the GA genotype is not visible. M: DNA ladder 100 bp

The observed heterozygosity (H_o) value was higher than the expected heterozygosity (H_e) and suggested that the number of heterozygous animals in the animal population was higher than the homozygous animals. The number of effective allele (n_e) values was 1.34 and suggested that this gene has a common allele of G. The genetic diversity of *GH* gene in animals included in the present study was not in Hardy-Weinberg equilibrium/HWE ($\chi^2 > 3.84$). In livestock animals, the HWE equilibrium could be attributed to selection, migration, cross-breeding, and inbreeding (Falconer and Mackay, 1989). Subsequently, the polymorphic informative content (PIC) value in the *GH* gene of the Merino cross was 0.22 and included in the moderate category. The PIC value can be classified into low (PIC < 0.10), moderate (0.10 < PIC < 0.30) and high (PIC > 0.30) categories (Nei and Kumar, 2000). The association analysis revealed that the polymorphism of *GH*/*Hae*III gene in animals under study is significantly associated with CD measurement (Table 2). Thus, the average CD in the GG genotype was higher than the GA genotype. Additionally, the polymorphism of the *GH*/*Hae*III gene in animals under study was significantly associated with birth weight (Table 3).

Hiremath et al. (2017) reported that the polymorphism of the *GH*/*Hae*III gene was not significantly associated with BW, WH, BL, and CG in the Indian Kenguri sheep which is similar to the findings of the present study. Despite this, the *GH*/*Hae*III gene polymorphism was not significantly associated with CG and BL measurements of Kacang and Boer goats (Ilham et al., 2016; Rashijane et al., 2022). Nonetheless, the polymorphism of *GH*/*Hae*III gene able to affect the CG measurement of Raini Cashmere, Sirohi and Barbari goats (Gooki et al., 2019; Singh et al., 2015). Moreover, Rashijane et al. (2022) reported that the GA genotype in the *GH*/*Hae*III gene of Boer goats had a higher adult weight than the GG genotype. In Salsk and Etawah sheep, carrying an A allele of the *GH*/*Hae*III gene influenced the body weight trait (Gorlov et al., 2017; Susilorini et al., 2017). In contrast, carrying a G allele in the *GH*/*Hae*III gene of Egyptian Awassi sheep can increase the body weight (El-Mansy et al., 2023). In Kalakasar sheep, the polymorphism of GH/*Hae*III gene was associated with yearling weight where the GA was the superior genotype (Muniasamy et al., 2023).

Rashijane et al. (2022) reported that the heterozygous Boer (GA) had higher body weight than wildtype (GG). Subsequently, the GA genotype was detected as the superior genotype for marketing weight and post-weaning weight gain of Egyptian Awassi sheep (El-Mansy et al., 2023).

In this study, the polymorphism in the exon 2 of the *GH* gene in studied animals was associated with birth weight and chest depth measurement at one year old. However, detection of the genetic marker in the other exonic regions of the *GH* gene is important to obtain other potential SNPs that associated with growth traits. Previously, the polymorphism of the *ovine GH* gene was observed in the exon 4 region (p.R121K) and it affected the WH in Dorper sheep (Madikadike et al., 2023) and the body weight in Harri sheep (Abdelmoneim et al., 2016). Furthermore, Cauveri et al. (2016) did not detect SNPs in all exonic regions of the Indian Nilagiri sheep *GH* gene. In addition, a missense SNP of p.G186S was observed in exon 5 of the Italian Sarda sheep *GH* gene (Vacca et al., 2013).

Table 1. The genetic diversity in *GH*/*Hae*III gene of Merino cross rams

Genotype frequency (N)			Allele frequency (%)		H_o	H_e	n_e	PIC	χ^2
GG	GA	AA	G	A					
0.70 (102)	0.30 (43)	0.00 (0)	85.00	15.00	0.30	0.25	1.34	0.22	4.395

N: Number of observation; H_o: Observed heterozygosity; H_e: Expected heterozygosity; n_e: Number of effective allele; PIC: Polymorphic informative content; χ^2: Chi-square value.

Table 2. Association results of *GH*/*Hae*III gene polymorphism with body measurements of Merino cross rams at 365 days of age

Body measurements (cm) \ Genotype	GG (N=101)	GA (N=43)
Head length	18.61 ± 4.53	18.81 ± 4.05
Head width	12.89 ± 2.43	12.99 ± 2.44
Withers height	56.47 ± 7.25	56.58 ± 6.33
Body length	55.36 ± 9.74	55.44 ± 8.42
Chest girth	67.74 ± 9.55	68.09 ± 10.73
Rump length	19.52 ± 2.81	19.14 ± 2.93
Rump width	14.64 ± 3.45	14.85 ± 3.13
Chest depth	24.46 ± 4.43^a	24.30 ± 3.18^b
Chest width	14.19 ± 2.15	14.23 ± 2.07
Front leg length	18.04 ± 1.80	18.33 ± 1.76
Back foot length	21.47 ± 2.37	21.65 ± 1.74
Front leg circumference	7.67 ± 1.24	7.83 ± 1.38

N: Number of observations. Different superscript letters in the similar row differ significantly ($p < 0.05$).

Table 3. Association results of *GH*/*Hae*III gene polymorphism with body weight of Merino cross rams

Corrected weight \ Genotype	GG (N=36)	GA (N=21)
Corrected birth weight (kg)	3.46 ± 0.88^a	3.49 ± 1.36^b
Body weight at 120 days of age (kg)	17.99 ± 4.46	18.30 ± 4.26
Body weight at 365 days of age (kg)	29.57 ± 8.57	29.59 ± 7.00
Pre-weaning weight gain (kg/day)	0.12 ± 0.04	0.12 ± 0.03
Post-weaning weight gain (kg/day)	0.05 ± 0.03	0.05 ± 0.02

N: Number of observations. Different superscript letters in the similar row differ significantly ($p < 0.05$).

CONCLUSION

The *GH*/*Hae*III gene in Merino cross rams was polymorphic with two common genotypes of GG (wildtype) and GA (carrier). Thus, the G allele was detected as the dominant allele in the *ovine GH* gene with frequency of 0.85. The polymorphism of the *GH*/*Hae*III gene in Merino cross rams was significantly associated with birth weight and chest depth at one year old. Furthermore, the obtained results can be used as early information to develop a molecular selection of Indonesian Merino cross sheep.

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

Endang Tri Margawati and Herman Willem Raadsma planned the experiment and collected the data records, Widya Pintaka Bayu Putra analyse the data, interpreted and made the write up and Thobela Louis Tyasi evaluated the paper. All authors confirmed the last content of the article before publication.

Competing interests

The authors have declared no conflict of interest.

Ethical considerations

The authors confirm that all authors have reviewed and submitted the manuscript to this journal for the first time.

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Impact of Fixation of Camel Lymph Node Cells on Marker Expression Stability in Flow Cytometry

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ABSTRACT

Single cell immunophenotyping by flow cytometry has proven a useful and high sensitive method for the analysis of immune cell composition and phenotype in different lymphatic and non-lymphatic tissues. Fixation of stained cells is usually recommended when the cells need to be preserved for later analysis by flow cytometry to avoid changes in cell morphology and expression of the level of cellular antigens. In the present study, a stain-fix approach was used in combination with flow cytometry to investigate the impact of fixation of camel lymph node cell suspension (n = 5 camels) after labeling with monoclonal antibodies to some leukocyte antigens on their cellular composition and expression density of immune cell markers. The obtained results indicated that camel lymph node cell suspension stained with fluorochrome-conjugated mAbs to leukocyte antigens and fixed with paraformaldehyde (PFA) will keep stable values for their immune cell composition for at least six days when analyzed by flow cytometry. However, if cell subsets were to be identified, fixation may result in different values that were obtained when analyzing fresh stained unfixed cells. Especially the instability in the fluorescence intensity of CD14, CD172a, and MHCII will lead to significant changes in the frequency of monocyte subsets (classical versus intermediate or non-classical) and the identification of macrophage functional subtype (M1 versus M2). Similarly, the instability in CD44 expression may affect the identified phenotype of T cells with significantly lower frequency of activated T cells. In conclusion, flow cytometric data collected from stained and PFA-fixed cell suspension prepared from camel lymph nodes should be interpreted with care if the functional subtype of cells is to be identified based on surface molecule expression.

Keywords: Camel, Fixation, Flow cytometry, Immune cell, Lymph node

INTRODUCTION

Lymph nodes are secondary organs with specialized structures that filter the lymph throughout the body (Yang et al., 2019). Lymph nodes provide sites for cross-talk between antigen-specific adaptive immune cells and antigen-presenting cells for the development of adaptive immune responses (Liao and von der Weid, 2015; Rehfeld et al., 2017). The cellular composition of the node lymphatic tissue is dominated by lymphoid cells with lower numbers of neutrophils and monocytes and their derivatives, such as macrophages and dendritic cells. Alterations in the composition and phenotype of immune cells in the lymph node reflect important events of the immune response, including migration and homing of immune cells, antigen trapping, presentation, stimulation of adaptive immunity, and generation of effector cells (Lun et al., 2007). Therefore, the analysis of these changes provides an effective method to evaluate the immune response against infection and vaccination, or for the diagnosis of immunopathology (Koets et al., 2002; Caucheteux et al., 2013).

Single-cell immunophenotyping by flow cytometry has proven a useful and highly sensitive method for the analysis of immune cell composition and phenotype in different lymphatic (Aalbers et al., 2015) and non-lymphatic tissues (Dong et al., 2016; Hagberg et al., 2018; Tighe et al., 2019). Immunophenotyping steps usually include cell separation and the preparation of cell suspension followed by labeling cellular antigens with monoclonal antibodies conjugated with fluorescent dyes (Maecker et al., 2012). One of the big challenges during immunophenotyping studies, especially in veterinary medicine, where flow cytometers are still not available in every laboratory, is extending the time between cell staining and flow cytometric analysis without affecting cell composition or phenotype (Laurin et al., 2015). Fixation of stained cells represents a way to avoid protein denaturation and loss of antigenic structure leading to extending the time between sampling and analysis (Ng et al., 2012; Qin et al., 2021). In addition, the detection of intracellular epitopes, such as cytokines or signaling molecules, requires cell fixation and permeabilization to access the target epitope by antibodies (Paavilainen et al., 2010; Cheng et al., 2019). For the selection of a fixative agent, the fixative must not impact parameters related to cell structure, including cell size and granularity, as well as the reactivity of cellular epitopes with

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the monoclonal antibodies. Cross-linking of cellular proteins and DNA using paraformaldehyde (PFA) is one of the most commonly used cell fixatives due to its ability to keep, to some extent, the cell structure, and the antigenic determinants (Cheng et al., 2019).

Fixation of human white blood cells induced a significant change in cell size and granularity as well as in the abundance of many cellular antigens (Pinto et al., 2005; Stewart et al., 2007). Similar effects of PFA on human blood leukocytes have been found in a study by Ng et al. (2012), with marked changes in cell count and reactivity to monoclonal antibodies (mAbs). Similarly, PFA fixation of pig peripheral blood mononuclear cells resulted in significant changes in the reactivity of mAbs to cell surface antigens (Schuberth et al., 1998).

In a fix-stain approach, a previous study analyzed the impact of PFA fixation of camel blood leukocytes on their reactivity to monoclonal antibodies to some cell surface antigens. The study results revealed that leukocytes fixed with PFA lost their subsequent binding to CD163 and WC1 monoclonal antibodies leading to the lack of identification of target cells. In addition, fixed cells showed reduced reactivity to CD14, CD172a, MHCII, CD11a, CD18, CD44, and CD45 monoclonal antibodies (Almohammed et al., 2022). In the present study, a stain-fix approach was used to investigate the impact of fixation of camel lymph node cell suspension after labeling with monoclonal antibodies to some leukocyte antigens on their cellular composition and expression density of immune cell markers.

MATERIALS AND METHODS

Ethical approval

The presrnt study was approved by the Ethics Committee of King Faisal University, Saudi Arabia (KFU-REC-2021- DEC -EA000326).

Animals and collection of lymph node samples

A total of five healthy camels (*Camelus dromedarius*) were selected from animals admitted for normal slaughtering at Al-Omran Slaughterhouse in Al-Ahsa Region in Saudi Arabia. The animals included in the study were examined for clinical signs of reproductive, gastrointestinal, or respiratory diseases by a veterinarian. In addition, the viscera were examined directly after euthanasia (via bleeding) to exclude animals with signs of abdominal, thoracic, or reproductive disorders. After collection, mesenteric lymph nodes were immediately placed on an ice pack in cold PBS containing 1 mM EDTA and transported to the laboratory within an hour.

Cell separation from lymph node samples

Lymph node cell suspension was prepared as previously described (Barut et al., 2022). After removing the fat and connective tissue from the capsule, the lymph nodes were placed in a sterile Petri dish filled with 10 mL of cold PBS-EDTA. The nodes were then cut into small pieces (2-3 cm) and minced using sterile scissors and forceps. The minced lymph nodes were suspended in PBS-EDTA and the cell supernatants were filtered through a cell strainer. The cells were washed in PBS-EDTA for 10 minutes at 4°C and 300 × g and the cell pellet were resuspended in PBS-EDTA and contaminating red blood cells were removed by hypotonic lysis. Finally, the cell pellet was resuspended in PBS-EDTA at 5.0 × 106 cell/mL. Cell viability (always more than 96%) was evaluated within one hour by flow cytometry after adding propidium iodide (2µg/mL) to the cells.

Antibody staining and flow cytometry

Lymph node cells were labeled with mAbs to camel leukocyte markers and analyzed on the flow cytometer (DiGiuseppe and Wood., 2019). For this, 1 x 10^6 cell/well were incubated in a 96-well plate with mAbs to CD45, CD44, CD172a, CD14, CD163, and MHCII. After incubation (15 minutes/ 4°C), cells were washed twice with PBS and were incubated with anti-mouse IgG1 and IgG2a (Invitrogen) conjugated with FITC and PE, respectively. Staining with isotype control antibodies was also done (Becton Dickinson Biosciences). Cells were then washed twice with PBS and analyzed on a Becton Dickinson Accuri C6 flow cytometer (Becton Dickinson Biosciences, San Jose, California, USA). Data from at least 100,000 cells were collected and analyzed with the flow cytometric software C-Flow (Becton Dickinson Biosciences, San Jose, California, USA).

Statistical analyses

Data analysis was performed using the flow cytometric software C-Flow (BD). The column statistic function of the Prism software (GraphPad) was used to calculate the means and standard error of the mean (SEM) of the analyzed parameters. Differences between means were tested with the t-test being considered significant with p-value < 0.05.

RESULTS AND DISCUSSION

Lymph nodes are filters of tissues and tissue fluids that trap antigens and provide sites for antigen presentation and activation of pathogen-specific immune responses (Liao and von der Weid, 2015). Their immune cell composition and phenotype may reflect pathologies in tissues they drain (Elmore, 2006; Ma et al., 2021). As there are limited data on the impact of fixation on the composition and phenotype of lymph node cells in the camel, the current study investigated the impact of fixation on the frequency of and marker expression on lymph node immune cells in camels. Antibody-labeled and paraformaldehyde-fixed lymph node cells were compared with unfixed cells by flow cytometry.

Impact of cell fixation on the cell composition of camel lymph node

Staining with the pan-leukocyte marker CD45 (Meza Guzman et al., 2024) and the myeloid marker signal regulatory protein α (SIRPα) identified total immune cells (CD45+ cells), myeloid cells (CD45+CD172a+), and lymphoid cells (CD45+CD172a-) within camel lymph node cells (Figure 1A-D). For all cell populations and all time points after fixation, no significant changes were observed between unfixed and fixed cells ($p > 0.05$) (Figure 1E-G).

Lymphocytes were identified as the major fraction of lymph node cells (86.6 %) based on their negative staining with CD14, while monocytes were identified as CD14+ cells with low granularity, as determined using the side scatter (SSC) parameter, with a mean percentage of (4.9 %) of total cells. Another minor population of CD14low cells with high SSC were identified as neutrophils with a mean percentage of (6.2 %) of total cells (Figure 2A). For all cell populations including monocytes, neutrophils, and lymphocytes, stability in their frequency was observed when comparing unfixed and fixed cells (Figure 2B). The percentages of these populations remained stable during the 6 days of measurement ($p > 0.05$). The obtained data indicated that the cellular composition of mAb-stained and PFA-fixed camel lymph node cells may be analyzed by flow cytometry with stable values for at least 6 days after fixation.

Impact of fixation on forward and side scatter properties of lymph node cell populations

The comparison between unfixed and fixed cells revealed a significant increase in the forward scatter (FSC) of fixed neutrophils (day 0) followed by normalization on day 1 and a subsequent decrease on days 3 and 6 after fixation ($p < 0.05$) (Figure 3A). For lymphocytes, a significant decrease in FSC was observed directly after fixation (day 0) and remained low on all other time points after fixation ($p < 0.05$) (Figure 3A). On the other hand, a marked increase in the SSC value was observed for monocytes and neutrophils directly after fixation and on all other time points ($p < 0.05$) (Figure 3B) when compared to unfixed cells. No Fixation-induced changes were found in the FSC value of monocytes or the SSC value of lymphocytes when comparing unfixed and fixed cells ($p> 0.05$) (Figure 3A-B).

Forward and side scatter values are important parameters that are commonly used for the identification of cells by flow cytometry (Givan, 2001; Nunez, 2001). While forward scatter is a measure that indicates cell size, side scatter reflects the structure complexity and granular content of a cell (Givan, 2001; Nunez, 2001). Increased FSC values are usually linked to stimulated phenotypes of monocytes and neutrophils (Hussen et al., 2016). Similarly, lymphocyte proliferation is associated with increased cell size with lymphoblasts being larger than lymphocytes (DiGiuseppe and Wood, 2019). In contrast, reduced cell size is an indicator of cell apoptosis and necrosis (Yurinskaya et al., 2017). On the other hand, reduced SSC, which indicates lower granularity, is usually linked to neutrophil degranulation upon stimulation (Hussen et al., 2016). In the present study, the fixation-induced changes in FSC and SSC properties of camel lymph node cells may interfere with the functional characterization of immune cells leading to false interpretation of stimulatory or inhibitory effects in functional studies or misinterpretation of cell viability studies.

Impact of cell fixation on cell markers expression on lymph node cells

The leukocyte antigens CD45 and CD44 are pan-leukocyte markers that are usually used for the identification of and gating on total immune cells for the subsequent analysis of cell subsets (Ratei et al., 2007; Gray et al., 2012). In addition, these molecules are used as markers for cell activation and migration of leukocytes (Gray et al., 2012; Senbanjo and Chellaiah, 2017). In the present study, fixed neutrophils and lymphocytes showed relative stability in the abundance (mean fluorescence intensity; MFI) of CD45 (Figure 4A) and CD44 (Figure 4B) directly after fixation followed by a continuous decrease in the MFI values starting on day 1 after fixation.

Only for monocytes, the expression stability by fixation remained until day 6 after fixation for CD45 and until day 3 after fixation for CD44. Although the observed fixation-induced change did not affect the percentage of cells stained positively with CD45 or CD44 antibodies, the reduced MFI values after 24 hours of fixation may lead to incorrect interpretation of the functional phenotype of the cells in terms of activation and migration status.

The myeloid antigens CD172a, CD14, and CD163 in addition to the class II major histocompatibility complex (MHCII) are cell markers usually used to identify monocytes, macrophages, and neutrophils (Broz and Krummel, 2015). Their detection is usually used for the identification of total myeloid cells (CD172a) (Hussen et al., 2016), for the classification of monocyte subsets (CD14 and MHCII) (Lyu et al., 2023), or for the characterization of the functional subtype of macrophages (MHCII and CD163) (Lyu et al., 2023; Feng et al., 2021). Except their CD163 expression, which indicated relative stability after fixation ($p > 0.05$), the monocyte phenotype was significantly affected by fixation in the present study. While CD14 and MHCII levels were significantly reduced on fixed monocytes, CD172a levels were significantly elevated after fixation ($p < 0.05$) (Figure 5A). In contrast, neutrophils did not show any significant fixation-induced changes in the abundance of any of the studied myeloid markers ($p > 0.05$) (Figure 5B).

Low CD14 expression has been linked to the phenotype of a non-classical subset of monocytes in camels and other species (Ziegler-Heitbrock, 2014). Similarly, heterogeneity in MHCII expression has been used for the classification of camel monocyte subsets with lower expression on classical than intermediate and non-classical monocytes (Hussen et al., 2020). The results indicated that fixation of camel lymph node cell suspension may lead to incorrect interpretation of data related to the heterogeneity of monocyte.

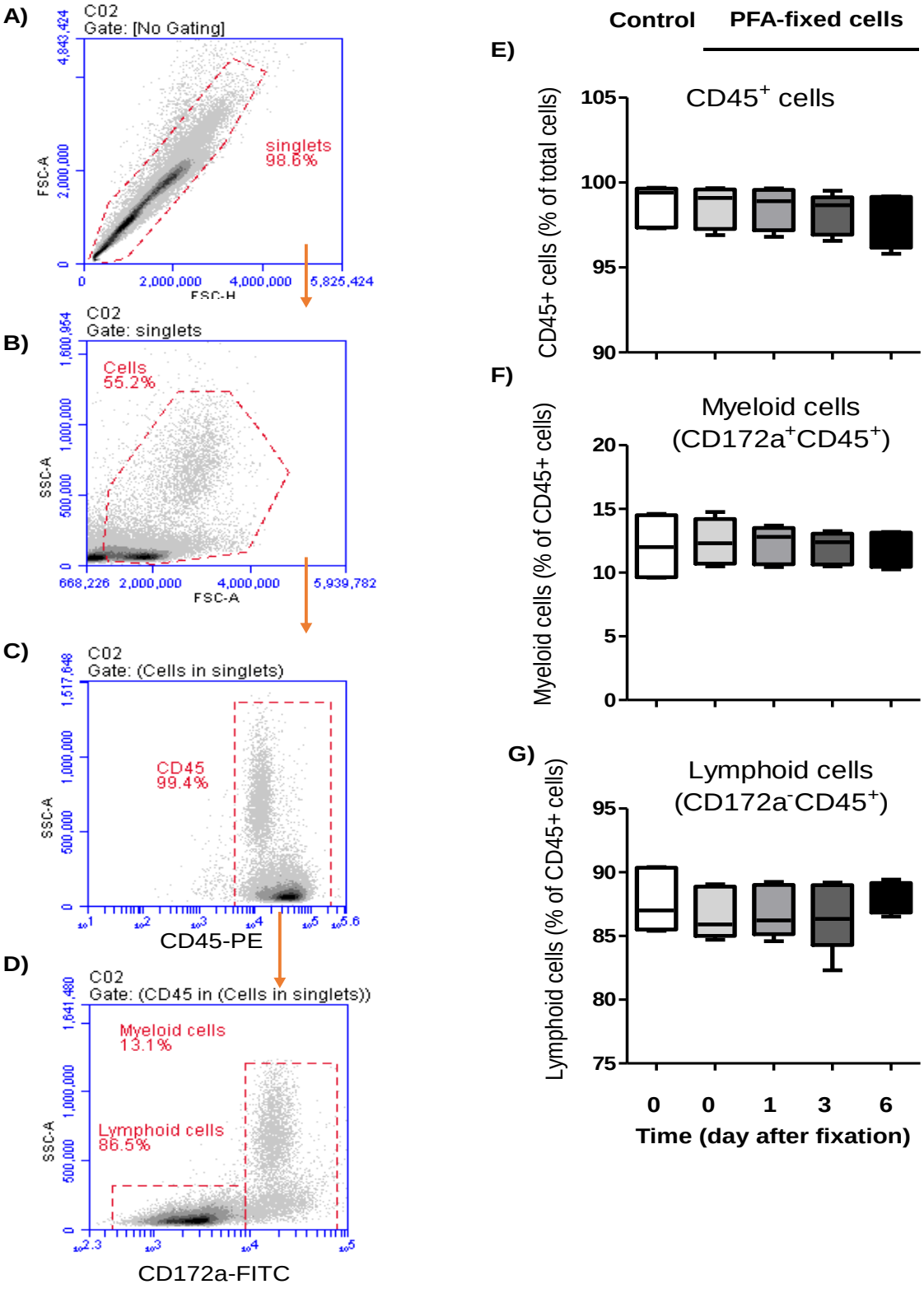

Figure 1. Gating strategy for the identification of camel lymph node cell populations using flow cytometry. After gating on single cells (**A**), lymph node cells were gated (**B**) based on their forward scatter (FSC) and side scatter (SSC) properties. Staining with CD45 (**C**) was used to identify total leukocytes and myeloid and lymphoid cells were identified based on their staining with CD172a (**D**). The percentage of total leukocytes (**E**), myeloid cells (**F**), and lymphoid cells (**G**) were calculated and presented as box plots.

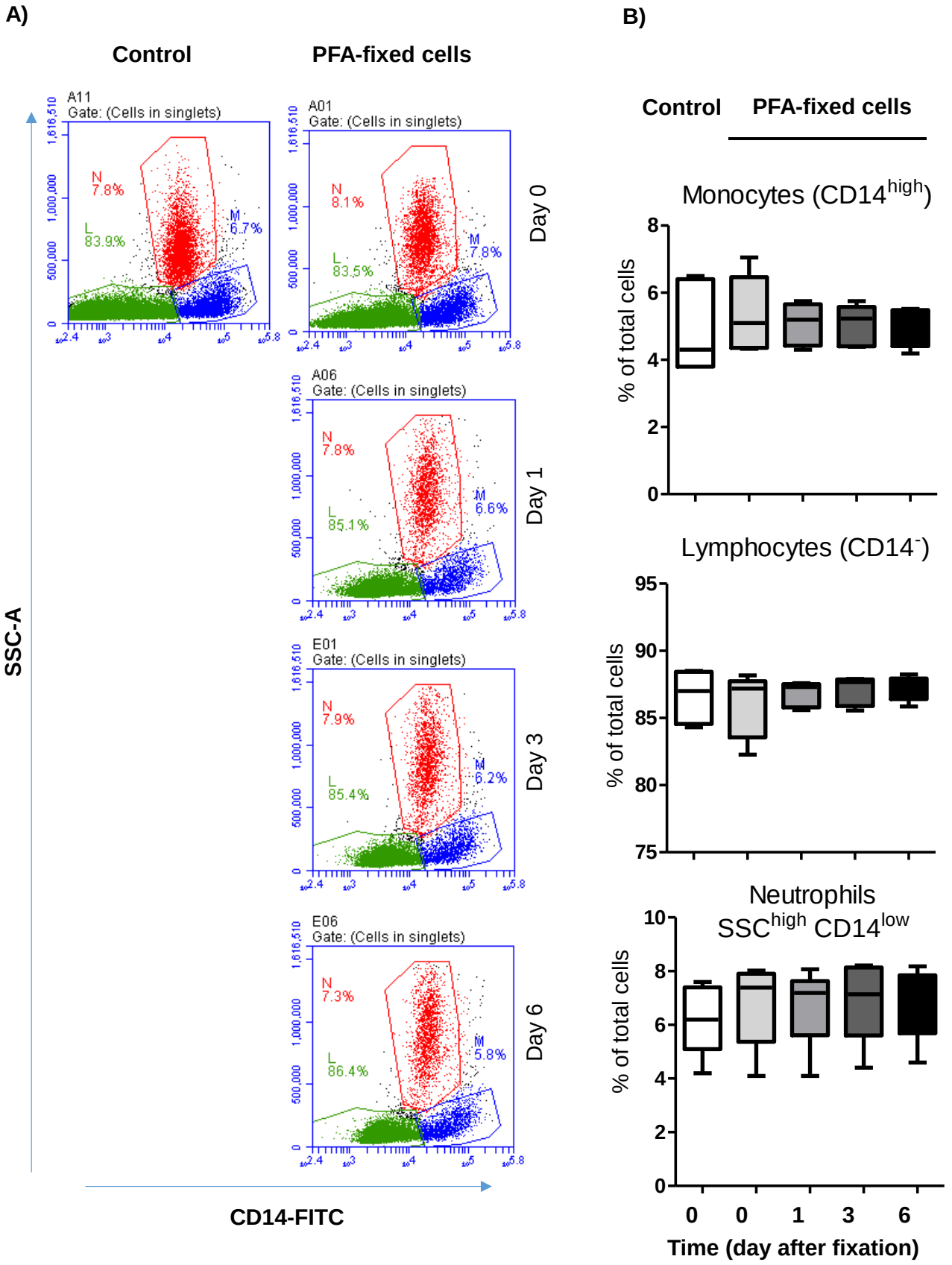

Figure 2. Identification of camel lymph node monocytes, neutrophils, lymphocytes based on their staining with monoclonal antibodies (mAbs) to CD14 (**A**) in a SSC/CD14 dot plot, and their percentages calculated for fixed and unfixed cells (**B**).

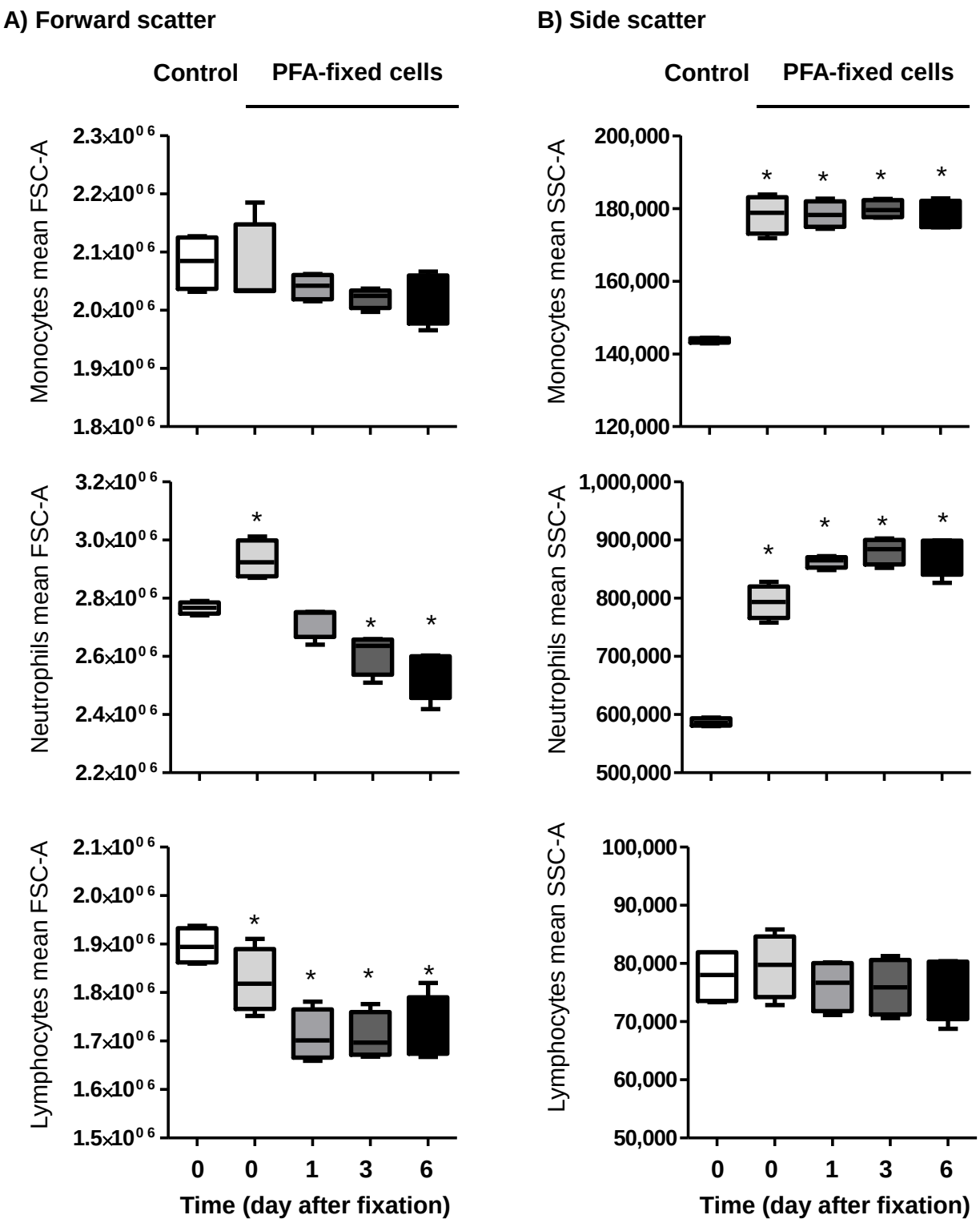

Figure 3. Changes in (**A**) forward scatter (FSC) and (**B**) side scatter (SSC) values for lymph node monocytes, neutrophils, and lymphocytes during 6 days after fixation. Lymph node cells were stained with mAbs to camel leukocyte antigens and analyzed by flow cytometry or were fixed with paraformaldehyde (PFA) and analyzed directly after fixation (d0) or on days 1, 3, and 6 after fixation. * indicated significant differences ($p < 0.05$).

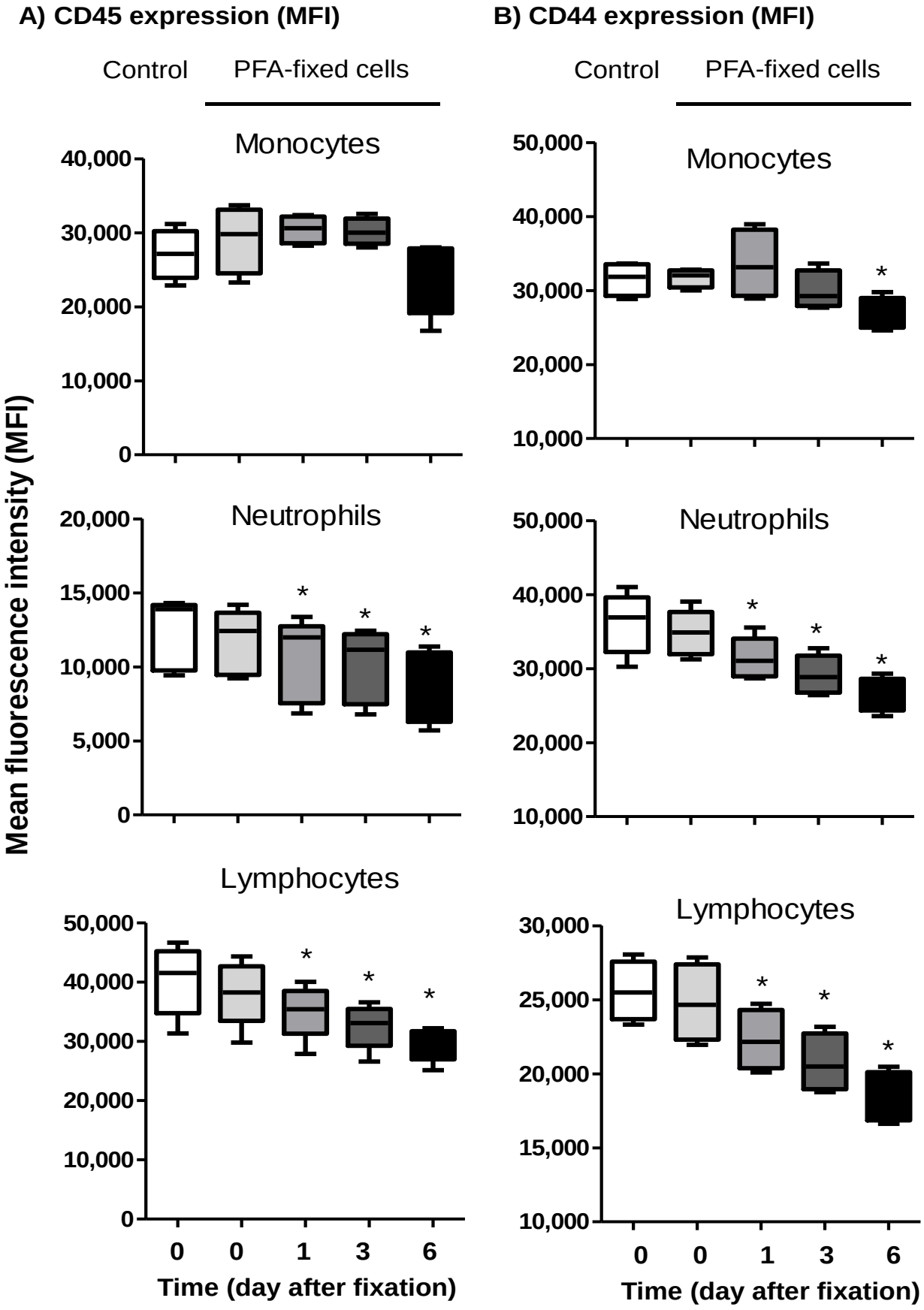

Figure 4. Changes in the abundance of CD45 (**A**) and CD44 (**B**) on lymph node monocytes, neutrophils, and lymphocytes during 6 days after fixation. Lymph node cells were stained with mAbs to CD45 and CD44 molecules and analyzed by flow cytometry or were fixed after staining with paraformaldehyde (PFA) and analyzed at day 0 (d0), d1, d3, and d6 after fixation. * indicated significant differences ($p < 0.05$).

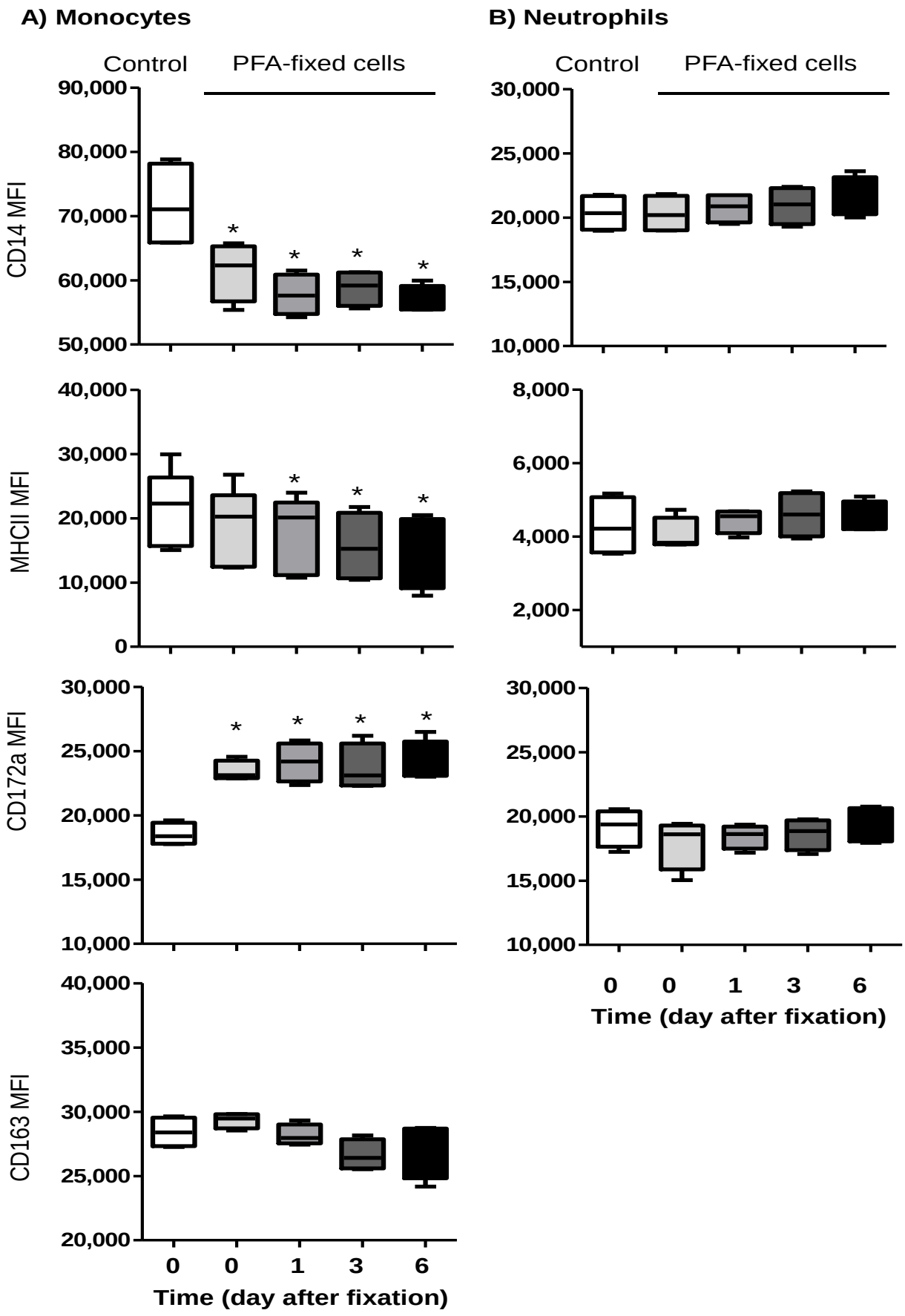

Figure 5. Fixation-induced changes in the expression levels of myeloid markers on lymph node monocytes (**A**) and neutrophils (**B**). Lymph node cells were stained with monoclonal antibodies(mAbs) to CD14, CD172a, MHCII, or CD163 molecules and analyzed by flow cytometry. Parallel set-ups were fixed after staining with paraformaldehyde (PFA) and analyzed at day 0 (d0), d1, d3, and d6 after fixation. * indicated significant differences ($p < 0.05$).

CONCLUSION

The obtained results of the study indicated that camel lymph node cell suspension stained with fluorochrome-conjugated mAbs to leukocyte antigens and fixed with PFA will keep stable values for the immune cell composition for at least six days after fixation. However, fixation may result in significant changes in the immunophenotype of monocytes, macrophages, and T cells.

DECLARATIONS

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Authors' contributions

Mohammed Ali Al-Sukruwah did sample collection and manuscript revision. Hind Althagafi did sample analysis and original draft preparation. Najla Al Abdulsalam did study design and original draft preparation. Jamal Hussen designed the study, did flow cytometry and prepared original draft. All authors have read and agreed to the published version of the manuscript.

Competing interests

The authors have no competing interests to declare

Ethical considerations

The authors confirm that all authors have reviewed and submitted the manuscript to this journal for the first time.

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Availability of data and materials

The data that support the findings of this study are available from the corresponding author, [JH], upon reasonable request.

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Pathogenic and Antibiotic-Resistance Genes of *Pasteurella multocida* Isolated from Goats in the Mekong Delta, Vietnam

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ABSTRACT

Pasteurella multocida (*P. multocida*) is one of the predominant pathogens that mostly cause respiratory diseases in domestic animals, such as goats. To determine *P. multocida* serotypes and the prevalence of pathogenic and antibiotic-resistance genes the PCR method was used. A total of 143 isolated *P. multocida* strains were collected from 289 healthy hybrid Boer-Saanen goats' nasal samples in the Mekong Delta, Vietnam, from March to June 2023. A total of 143 *P. multocida* strains, serotype B accounted for the highest proportion (51.05%), followed by serotype A (14.69%), and the lowest was serotype E (0.70%) while (39.86%) of strains could not be determined serotypes. Among the six virulence genes surveyed, the *sodA* gene (56.64%) had the highest presence, while the *ompH* gene (4.20%) had the lowest presence. Pathogenic genes were present mainly in serotypes A and B; *tbpA* was frequently detected in serotype A (66.67%), and *sodA* was commonly detected in serotype B (56.16%). There were 14 virulence gene combinations in 59/109 (54.13%) serotyped *P. multocida* strains, and the pattern of *sodA* + *toxA* + *tbpA* was prevalent at the highest rate (12.84%). Moreover, among the eight investigated antibiotic resistance genes, the *sulII* gene had the highest presence rate (74.13%), compared to the *tetA* gene with the lowest presence rate (13.29%). Gene *sulII* was mainly detected on strains belonging to serotypes A (80.95%), B (83.56%), and F (77.78%). A total of (77.98%) of serotyped *P. multocida* strains indicated multi-harbor from two to six antibiotic-resistance genes, and the most common pattern was *aadB* + *sulII* (10.09%). The prevalence of five pathogenic *P. multocida* serotypes harboring diverse antibiotic-resistance genes isolated from nasal samples could be a critical issue in treating and preventing the respiratory diseases caused by *P. multocida* in goats in the Mekong Delta.

Keywords: Antibiotic resistance, Goat, *Pasterella multocida*, Pathogenicity, Mekong Delta

INTRODUCTION

Respiratory disease caused by *Pasteurella multocida* is among the most common infections in ruminant animals. Goats and other small ruminants are at a moderate risk of contracting this pathogen due to exposure to physical stress or uncomfortable environmental conditions (Mohamed and Abdelsalam, 2008). *P. multocida* is more frequently associated with the outbreak of acute pneumonia and death of goats in all age groups than *Mannheimia haemolytica* is in previous reports (Falade, 2002). Respiratory diseases in goats cause economic losses arising from mortality and morbidity. The respiratory disease mortality rate caused by *P. multocida* is 10% or more (Smith and Sherman, 2009). Girma et al. (2023) reported 27,563 and 37,522 cases of sheep and goats with pneumonia, in Southern Ethiopia from 2016 to 2021. There were a few reports on *P. multocida* isolated from small ruminants in Vietnam. In a previous report, *P. multocida* was detected in healthy goats raised on medium-scale farms in Can Tho City, the Mekong Delta at 49.48% (Nguyen et al., 2024). These observations implied the significance of pneumonic pasteurellosis in small ruminants.

Pasteurella multocida is classified into five serotypes, A, B, D, E, and F, according to capsular antigen, and 16 serotypes according to lipopolysaccharide antigen. The capsular antigen is considered an essential form of virulence of *P. multocida*, which allows *P. multocida* to avoid innate host defense systems (Boyce et al., 2000). Each serotype has different circulation in different animals. Serotypes A and D are two serotypes that commonly appear in cases of pneumonia and pasteurellosis in goats (Rawat et al., 2009; Tabatabaei and Abdolahi, 2023). Besides, additional factors essential for the proliferation and maturation of *P. multocida* have been discovered. These encompass *P. multocida* toxin (PMT), fimbriae, adhesins, the capacity to metabolize sialic acid, outer membrane proteins, and hyaluronidases (Harper et al., 2006). Previous studies reported that the prevalence of some virulence-associated genes, including colonization

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factors (*ptfA*, *fimA*, *hsf2*), iron acquisition factors (*exbB*, *exbD*, *tonB*, *Fur*), superoxide dismutase (*sodA*, *sodC*), and outer membrane proteins (*ompA*, *ompH*, *oma87*, *plpB*), were frequently detected in the pig origin *P. multocida* isolates (May et al., 2001; Peng et al., 2016; 2018). Mombeni et al. (2021) surveyed virulence genes on *P. multocida* strains isolated from goats in Iran and showed that the *sodA* gene had the highest presence rate (100.00%), followed by *toxA*, *nanH*, and *ompH*, with the same ratio of 61.90%.

On the other hand, antibiotic resistance is becoming a global concern as more and more multidrug-resistant bacteria appear. The cause is the overuse of antibiotics when treating animal diseases or using antibiotics as growth stimulants in animals (Martin et al., 2015). Kandimalla et al. (2022) revealed that *P. multocida* strains isolated from sheep and goats were susceptible to ceftriaxone, cefoperazone/sulbactum, ceftiofur, cloxacillin, ciprofloxacin, enrofloxacin, levofloxacin and tetracycline (100.00%) but were resistant to erythromycin (41.67%), gentamycin (66.67%). Nguyen et al. (2023) reported that *P. multocida* isolated from sheep in Central Vietnam was resistant to tetracycline (51.22%), ampicillin (53.66%), and erythromycin (65.85%). The frequent impact of antibiotics leads to mutations and the formation of genetic drug-resistance factors in bacteria. Surveying the presence of genes encoding drug-resistance factors in *P. multocida* gives a more general view of antibiotic resistance issues.

Diseases of domestic animals are still a massive issue in the Mekong Delta, Vietnam. There have been a few reports on the prevalence of pathogenic and antibiotic-resistance genes of *P. multocida* isolated from small ruminants; however, there were no reports in goats. This causes challenges in controlling and preventing diseases in goats. Therefore, this study aims to clarify the characteristic prevalence of *P. multocida* serotypes originating from goats and their pathogenicity and antibiotic resistance genes in the Mekong Delta, Vietnam.

MATERIALS AND METHODS

Ethical approval

The procedure for collecting fluid swab samples on goats was performed according to NAHMS (National Animal Health Monitoring System) guidelines (USDA, 2022), and *P. multocida* strains were isolated according to Vietnamese National Standard TCVN 8400-14:2011. In the author's previous study, samples were collected according to the guidelines outlined in the Helsinki Declaration and the animal welfare and safety procedures of Can Tho University, Vietnam.

Identification of *Pasteurella multocida* serotypes

A total of 143 *P. multocida* strains were previously isolated from 289 nasal swabs of healthy hybrid Boer-Saanen meat and dairy goats at all ages in small-scale farms in the Mekong Delta, Vietnam, in 2023. *Pasteurella* spp. were isolated from nasal fluid samples in goats and isolated on blood agar with 5% of sheep blood according to Vietnamese National Standard TCVN 8400-14:2011. Then, *P. multocida* strains were identified using the PCR method to detect gene *Pm1231*. Those identified strains were kept in the Veterinary Food Hygiene Laboratory, Faculty of Veterinary Medicine, College of Agriculture, Can Tho University to conduct this study.

Pasteurella multocida strains were subcultured on trypticase soy agar (TSA, Merck. Germany) at 37^{o}C for 24 h to extract DNA. The DNA of *P. multocida* strains was extracted using the heat-shock method and stored at -20^{o}C for the following experiments (Ahmed and Dablool, 2017).

Pasteurella multocida serotypes were determined by performing PCR reactions with primers of genes encoding for each serotype, including *hyaD-hyaC* (serotype A), *bcbD* (serotype B), *dcbF* (serotype D), *ecbJ* (serotype E), and *fcbD* (serotype F). The PCR conditions and primer sequences followed the description of Townsend et al. (2001).

The PCR mixture for one reaction included Mastermix 2X (BIO25042, Bioline, Meridian Bioscience, USA, 12.5 µl), forward primer (0.5 µl), reverse primer (0.5 µL), distilled water (9.5 µL), and DNA template (2.0 µL).

Determination of pathogenic genes

This study determined six pathogenic genes encoded for capsular and lipopolysaccharide antigens, including *sodA*, *toxA*, *tbpA*, *ptfA*, *pfhA*, and *ompH*. The PCR conditions and primer sequences followed the description of Doughty et al. (2000) and Ewers et al. (2006). Among genes, the annealing temperature was 55^{o}C for *sodA*, *toxA*, *tbpA*, *ptfA*, and *ompH*, while it was 58^{o}C for *pfhA*.

The *P. multocida* strains, previously isolated from cattle in the Mekong Delta, were used as a control. The MyTaq Mix 2X (BIO25042, Bioline, Meridian Bioscience, USA) was used in those experiments as described in the above method.

Determination of antibiotic-resistance genes

The PCR assay was used to detect eight antibiotic-resistance genes representative of beta-lactam (*blaROB-1*, *blaOXA*), aminoglycoside (*aadB*, *strA*), tetracycline (*tetA*, *tetB*), sulfonamide (*sulII*), and macrolide (*mph*). The PCR conditions and primer sequences followed the description of Randall et al. (2004), Saenz et al. (2004), Carattoli et al. (2005), Momtaz et al. (2012), Klima et al. (2014), and Abo-Almagd et al. (2023). The *P. multocida* strains, previously isolated from cattle in the Mekong Delta, were used as a positive control. The PCR procedure was conducted as described in the above experiment of detection of *P. multocida* serotypes.

Statistical analysis

The difference in the prevalence of pathogenic and antibiotic-resistance genes in *P. multocida* isolated from goats was statistically analyzed at a significance rate of 95% using the Pearson Chi-square test in the Minitab 17.0 software.

RESULTS

Of 143 *P. multocida* strains, serotype B was the most predominant serotype (51.05%), followed by serotype A (14.69%), serotype F (6.29%), serotype D (3.50%), and serotype E (0.70%, $p < 0.05$). There were 39.86% of *P. multocida* strains, which could not determine serotypes in this study (Table 1).

Of the six pathogenic genes examined (Table 2), *sodA* was present at the highest rate (56.64%), followed by *toxA* (45.45%), *tbpA* (30.77%), *ptfA* (10.49%), *pfhA* (4,90%), and *ompH* (4.20%, $p < 0.05$). Most pathogenic genes were found in *P. multocida* strains belonging to serotypes A and B.

Of 109 serotyped *P. multocida* strains, gene *tbpA* was frequently detected in serotype A (66.67%), and *sodA* was commonly in serotype B (56.16%); however, only one strain belonging to serotype E harbored gene *sodA* (Table 3).

There were 59/109 (54.13%) serotyped *P. multocida* strains that harbored a combination of two to three pathogenic genes (Table 4). Among gene combinations, the *sodA* + *toxA* + *tbpA* pattern was the most common (12.84%), followed by *sodA* + *toxA* (11.93%).

Of the eight antibiotic-resistance genes examined (Table 5), gene *sulII* was detected at the highest rate (74.13%), followed by *aadB* (42.66%), *strA* (33.57%), *mph* (28.67%), *blaROB-1* (21.68%), *bloOXA* (17.48%), *tetB* (16.08%), and *tetA* (13.29%).

Moreover, *sulII* and *aadB* genes were also recorded at the highest rate in serotyped *P. multocida* strains belonging to all serotypes, followed by *mph* gene (Table 6). Besides, 85/109 (77.98%) serotyped *P. multocida* strains harbored combinations of two to six antibiotic-resistance genes (Table 7). Among gene patterns, the pattern of *aadB* + *sulII* was the most common (10.09%).

Table 1. Distribution of *Pasteurella multocida* serotypes isolated from hybrid Boer-Saanen goats in the Mekong Delta, Vietnam from March to June 2023 (n=143)

Serotype	Encoded gene	No. of positive strains	Percentage
A	*hyaD-hyaC*	21	14.69
B	*bcbD*	73	51.05
D	*dcbF*	5	3.50
E	*ecbJ*	1	0.70
F	*fcbD*	9	6.29
Untyped		57	39.86

Untyped: *P. multocida* strains were not determined in the serotypes (A, B, D, E, and F) using the specific primers in this study. No: Number

Table 2. Prevalence of pathogenic genes in *Pasteurella multocida* strains isolated from hybrid Boer-Saanen goats in the Mekong Delta, Vietnam from March to June 2023 (n=143)

Pathogenic gene	No. of positive strains	Percentage
sodA	81	56.64
toxA	65	45.45
tbpA	44	30.77
ptfA	15	10.49
pfhA	7	4.90
ompH	6	4.20

No: Number

Table 3. Distribution of pathogenic genes in serotyped *Pasteurella multocida* strains isolated from hybrid Boer-Saanen goats in the Mekong Delta, Vietnam from March to June 2023

No. of positive strains (%) / Gene	Serotype A (n = 21)	Serotype B (n = 73)	Serotype D (n = 5)	Serotype E (n = 1)	Serotype F (n = 9)	Total (n = 109)
sodA	10 (47.62)	41 (56.16)	2 (40.00)	1 (100.00)	5 (55.56)	59 (54.13)
toxA	9 (42.86)	36 (49.32)	2 (40.00)	0 (0.00)	4 (44.44)	51 (46.79)
tbpA	14 (66.67)	29 (39.73)	2 (40.00)	0 (0.00)	4 (44.44)	49 (44.95)
ptfA	1 (4.76)	9 (12.33)	1 (20.00)	0 (0.00)	1 (11.11)	12 (11.01)
pfhA	0 (0.00)	3 (4.11)	0 (0.00)	0 (0.00)	0 (0.00)	3 (2.75)
ompH	1 (4.76)	4 (5.48)	0 (0.00)	0 (0.00)	0 (0.00)	5 (4.59)

No: Number

Table 4. Pathogenic gene patterns of serotyped *Pasteurella multocida* strains from hybrid Boer-Saanen goats in the Mekong Delta, Vietnam from March to June 2023 (n=109)

No. of genes	Pattern	No. of strains	Percentage
2	*sodA + toxA*	13	11.93
	sodA + tbpA	5	4.59
	sodA + ptfA	4	3.67
	sodA + pfhA	1	0.92
	toxA + pfhA	1	0.92
	toxA + tbpA	10	9.17
	toxA + ompH	1	0.92
	ptfA + tbpA	1	0.92
	ompH + tbpA	1	0.92
3	*sodA + toxA + tbpA*	14	12.84
	sodA + toxA + ptfA	1	0.92
	sodA + ptfA + tbpA	3	2.75
	sodA + toxA + ompH	3	2.75
	toxA + ptfA + tbpA	1	0.92
Total		59	54.13

No: Number

Table 5. Prevalence of antibiotic-resistance genes in *Pasteurella multocida* strains isolated from hybrid Boer-Saanen goats in the Mekong Delta, Vietnam from March to June 2023 (n=143).

Antibiotic group	Gene	No. of positive strains	Percentage
Beta-lactam	*blaROB-1*	31	21.68
	blaOXA	25	17.48
Aminoglycoside	*aadB*	61	42.66
	strA	48	33.57
Tetracycline	*tetA*	19	13.29
	tetB	23	16.08
Sulfonamide	*sulII*	106	74.13
Macrolide	*mph*	41	28.67

No: Number

Table 6. Distribution of antibiotic-resistance genes in serotyped *Pasteurella multocida* strains from hybrid Boer-Saanen goats in the Mekong Delta, Vietnam from March to June 2023

No. of positive strains (%) / Gene	Serotype A (n = 21)	Serotype B (n = 73)	Serotype D (n = 5)	Serotype E (n = 1)	Serotype F (n = 9)	Total (n = 109)
blaROB-1	5 (23.81)	16 (21.92)	1 (20.00)	0 (0.00)	0 (0.00)	22 (20.18)
blaOXA	2 (9.52)	13 (17.81)	1 (20.00)	1 (100.00)	1 (11.11)	18 (16.51)
aadB	12 (57.14)	30 (41.10)	1 (20.00)	1 (100.00)	6 (66.67)	50 (45.87)
strA	8 (38.10)	27 (36.99)	2 (40.00)	1 (100.00)	1 (11.11)	39 (35.78)
tetA	3 (14.29)	11 (15.07)	0 (0.00)	0 (0.00)	1 (11.11)	15 (13.76)
tetB	6 (28.57)	14 (19.18)	2 (40.00)	0 (0.00)	0 (0.00)	22 (20.18)
sulII	17 (80.95)	61 (83.56)	1 (20.00)	0 (0.00)	7 (77.78)	86 (78.90)
mph	9 (42.86)	26 (35.62)	2 (40.00)	0 (0.00)	5 (55.56)	42 (38.53)

No: Number

Table 7. Multiple antibiotic-resistance gene patterns of serotyped *Pasteurella multocida* strains (on the strains harbored from two antibiotic-resistance genes) from hybrid Boer-Saanen goats in the Mekong Delta, Vietnam from March to June 2023 (n=109).

No. of genes	Gene patterns	No. of strains	Percentage
2	*blaROB-1 + sulII*	1	0.92
	blaROB-1 + strA	1	0.92
	aadB+ sulII	11	10.09
	aadB+ mph	3	2.75
	strA + sulII	2	1.83
	strA + tetB	1	0.92
	strA + mph	1	0.92
	tetA + sulII	2	1.83
	sulII + mph	1	0.92
3	*blaROB-1 + tetA + sulII*	1	0.92
	blaROB-1 + strA + sulII	3	2.75
	blaROB-1 + strA + tetB	1	0.92
	blaROB-1 + sulII + tetB	1	0.92
	blaOXA + aadB+ sulII	4	3.67
	blaOXA + strA + sulII	1	0.92
	blaOXA + aadB+ mph	3	2.75
	blaOXA + sulII + tetB	2	1.83
	blaOXA + aadB+ strA	1	0.92
	aadB+ tetA + sulII	1	0.92
	aadB+ sulII + mph	8	7.34
	strA + tetB + mph	1	0.92
	strA + sulII + mph	1	0.92
	strA + tetA + sulII	1	0.92
	strA + sulII + tetB	3	2.75
	sulII + tetB + mph	2	1.83
4	*blaROB-1 + strA + tetB + mph*	1	0.92
	blaROB-1 + blaOXA + sulII + mph	1	0.92
	blaROB-1 + aadB+ strA + sulII	2	1.83
	blaROB-1 + strA + tetA + sulII	3	2.75
	blaROB-1 + blaOXA + tetA + sulII	1	0.92
	blaOXA + aadB+ sulII + mph	1	0.92
	aadB+ strA + sulII + mph	4	3.67
	aadB+ sulII + tetB + mph	2	1.83
	aadB+ strA + sulII + tetB	1	0.92
	aadB+ tetA + sulII + mph	2	1.83
	strA + tetA + sulII + mph	1	0.92
5	*blaROB-1 + blaOXA + aadB+ sulII + mph*	1	0.92
	blaROB-1 + strA + sulII + tetB + mph	3	2.75
	blaROB-1 + blaOXA + strA + tetA + sulII	1	0.92
	aadB+ strA + tetA + sulII + tetB	1	0.92
	aadB+ strA + sulII + tetB + mph	2	1.83
6	*blaROB-1 + aadB+ strA + sulII + tetB + mph*	1	0.92
Total		85	77.98

No: Number

DISCUSSION

In this study, *P. multocida* serotypes A and B were more prevalent than the remaining serotypes. Shayegh et al. (2009) and Mombeni et al. (2021) indicated that *P. multocida* strains isolated from goats in Iran belonged mainly to two serotypes, A and D. Serotypes B and E are two serogroups reported to commonly cause hemorrhagic infections in ruminant carriers. Serogroup B was often found in the nasopharyngeal fluid of livestock in Southeast Asia, while serogroup E was more common in Africa (Markey et al., 2013). Aski and Tabatabaei (2016) recorded the prevalence of three serotypes, A, B, and D, in *P. multocida* strains isolated from healthy and clinically infected goats. *P. multocida* serotype B, specifically serotype B:2, was a common serotype detected in cases of infected cattle. However, 39.86% of *P. multocida* strains were not determined serotypes in this study. The reason could be due to the specific primers or the characteristic structure of capsular antigens in those *P. multocida* strains. Further study should be done to clarify and confirm the prevalence of diverse serotypes of *P. multocida* strains isolated from goats using other primers or serotyping methods.

Furthermore, three genes, *sodA*, *toxA*, and *tbpA*, were more commonly detected in *P. multocida* strains. The *sodA* gene was frequently detected in *P. multocida* isolated from poultry, pigs, and rabbits in previous studies (Furian et al., 2015; Li et al., 2018; Mahrous et al., 2022). Therefore, it is difficult to determine whether *sodA* is a characteristic gene in *P. multocida* strains isolated from goats or was related to serotypes A and B. According to Rimac et al. (2017), the *tbpA* gene is closely related to *P. multocida* strains isolated from ruminants with pneumonia and sepsis. Katsuda et al. (2013) also indicated a close relationship between serotype A strains and the *tbpA* gene. Research by Nguyen et al. (2023) showed that the *tbpA* gene was detected in serotypes A, B, and D strains in clinical pneumonic pasteurellosis sheep in central Vietnam at 48.78%, 7.32%, and 21.95%, respectively. The above results suggested that there might be a relationship between serotypes A and B and the *tbpA* gene, especially in diseased animals. On the other hand, Pullinger et al. (2003) reported that gene *toxA* can be transferred horizontally, and the *toxA* gene was determined to be concerning serotypes A and D strains (Furian et al., 2015). Cid et al. (2019) reported that the *toxA* gene encodes the *P. multocida* toxin (PMT), which is a dermonecrotic protein in the virulence factor of capsular type D.

Pasturela multocida causes progressive atrophic rhinitis in pigs and is significantly found in ovine pneumonia isolates in Spain. Besides, the detection of the *toxA* gene could serve as a reliable indicator of the toxigenic fitness of *P. multocida* (Cid et al., 2019). Thus, the high presence of the *toxA* gene in *P. multocida* strains isolated from goats showed that those *P. multocida* strains harboring PMT toxin could cause pasteurellosis in goats in the Mekong Delta, Vietnam.

Of serotyped strains, three genotypes with high prevalence rates included *sodA* + *toxA* + *tbpA*, *sodA* + *toxA*, and *toxA* + *tbpA*, with rates of 12.84%, 11.93%, and 9.17% respectively. The *toxA* was present in most of the common patterns. Pullinger et al. (2003) showed that the *toxA* gene was encoded in the genome of a latent bacteriophage to allow the *toxA* gene to circulate easily among *P. multocida* strains of many different serotypes. Bernal et al. (2023) recorded that all *P. multocida* strains isolated from cows with respiratory disease carried more than two virulence genes. The appearance of serotypes carrying diverse virulent genes makes it difficult to prevent and control diseases in goats in the Mekong Delta.

Among antibiotic-resistance genes, gene *sulII* had the highest presence rate (74.13%). Gene *sulII* was not generally considered part of a separate genetic element; it was found on large conjugative plasmids and was associated with resistance to other antibiotics (Bean et al., 2009; Wu et al., 2010). Antibiotic-resistant genes could be silent resistance genes that are not frequently exhibited or exhibited at low levels, even when exposed to antibiotics. They are nonessential residues and do not play an essential role in the bacterial life cycle (Stasiak et al., 2021). However, antibiotic-resistance genes *blaROB-1*, *blaOXA*, *tet A*, and *tetB* had a relatively low presence rate in *P. multocida* strains in this study. In another report, Babetsa et al. (2012) indicated that *P. multocida* strains isolated from bovine, ovine, caprine, and swine pneumonic lungs were resistant to tetracycline in Greece. Among those tetracycline-resistance *P. multocida* strains, 72.22% of strains carried the *tetH* gene, and 22.22% of strains carried the *tetB* gene, while *tetA* and *tetM* were not found. The current study results showed a difference in the prevalence of antibiotic-resistance genes in *P. multocida* isolated from goats in the Mekong Delta. Thus, further research should be conducted to clarify the diverse prevalences of antibiotic-resistance genes in *P. multocida* isolated from goats in this region.

Of serotyped *P. multocida* strains, the *sulII* had the highest presence rate in serotypes A, B, and F. The gene *blaROB-1* and *tetB* genes were not found in serotype F strains. Most antibiotic-resistance genes are present in the bacterial genome through random gene transfer using mobile genetic elements (Bennett, 2008). Besides, genes *tetA* and *tetB* are two representatives of genetic factors for tetracycline resistance through the formation of ABC (ATP-binding cassette superfamily) transporters (Reynolds et al., 2016). Rendueles et al. (2018) revealed that capsular antigens were related to antibiotic resistance genes, especially genes related to antibiotic efflux pumps. Previous studies mainly focused on the relationship between serotypes and virulent genes; however, a few studies have shown the relationship between

serotypes and antibiotic-resistance genes in *P. multocida* (Harper et al., 2006; Katsuda et al., 2013; Cid et al., 2019; Nguyen et al., 2023). Thus, the current study results seemed to be the first report on the prevalence of antibiotic-resistance genes in *P. multocida* serotypes isolated from healthy goats in the Mekong Delta, Vietnam.

Moreover, 77.98% *P. multocida* strains isolated from goats in the Mekong Delta showed several antibiotic-resistance gene patterns. Among 42 antibiotic-resistance gene combinations of *P. multocida* strains, the most common patterns across serotypes were *aadB* + *sulII* (10.09%) and *aadB* + *sulII* + *mph* (7.34%) in this study. The *aadB* gene is usually associated with type 1 integron or plasmids, and the *sulII* gene is mainly located on small non-conjugative plasmids or large multiresistant plasmids that can be horizontally transferred (Antunes et al., 2005; Naderi et al., 2023). The common prevalence of *aadB* and *sulII* genes in several antibiotic-resistance gene patterns in serotypes showed that *aadB* and *sulII* might be a typical resistance gene cluster in *P. multocida* isolated from goats in the Mekong Delta, Vietnam. In addition, the diversity of antibiotic-resistance gene patterns in those *P. multocida* strains exhibited the high ability of multidrug resistance of those strains. It could cause difficulty in treating and preventing the respiratory diseases caused by *P. multocida* in goats in this region.

CONCLUSION

This study showed that five pathogenic *P. multocida* serotypes were detected in healthy hybrid Boer-Saanen goats in the Mekong Delta, Vietnam. The results indicated that *P. multocida* serotypes A and B were the dominant serotypes in goats in the Mekong Delta. Moreover, those *P. multocida* strains harbored diverse pathogenic genes and antibiotic-resistance genes with serval gene patterns. The pathogenic gene (*sodA)* and the antibiotic-resistance gene (*aadB)* were the most detected in all *P. multocida* serotypes in this study. In addition, the pathogenic gene pattern of *sodA* + *toxA* + *tbpA* and the antibiotic-resistance gene pattern of *aadB* + *sulII* were frequently prevalent in those *P. multocida* strains. It revealed that *P. multocida* strains isolated from goats were potential pathogens causing severe diseases in goats in the Mekong Delta. Therefore, the control of pathogenic and antibiotic-resistant *P. multocida* is essential to prevent and treat pasteurellosis in goats.

DECLARATION

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Conflicts of Interests
The authors declare that we do not have any conflicts of interest.

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Authors' contributions
Thuan K. Nguyen, Thuong T. Nguyen, and Trung T. Truong conceptualized, designed, and supervised the research. Thuan K. Nguyen and Thuong T. Nguyen critically reviewed the study. Thuan K. Nguyen, Thuong T. Nguyen, Chi T.H. Nguyen, Vy L.P. Nguyen, and Trung T. Truong collected samples and conducted experiments. Chi T.H. Nguyen and Vy L.P. Nguyen analyzed and interpreted the data generated. All authors revised and approved the submitted manuscript.

Competing interests
The authors declare that they have no conflict 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
The authors considered farmers' ethical concerns and consent before conducting the study. This article was written originally without any copy from data of published articles and books.

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Effects of Combined Organic Selenium and Zinc Supplementation on *In Vitro* Ruminal Enzyme Activities and Relative Populations of Several Bacterial Species

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ABSTRACT

Selenium (Se) and zinc (Zn) are essential animal microminerals. Combining Se and Zn (Se-Zn) as a feed additive in its influence on rumen fermentation patterns is still very limited, so further investigation is needed. The present study explored the supplementation impact of combined Se-Zn from organic sources on rumen enzyme activity and relative abundance of several bacterial species through an *in vitro* method. Five treatments, each with six replicates were used in the study. The first group treated without Se and Zn supplementation (T0, control), the second group treated with 0.3 ppm Se + 60 ppm Zn (T1), the third group treated with 0.45 ppm Se + 60 ppm Zn (T2), the fourth group treated with 0.3 ppm Se + 90 ppm Zn (T3), and the fifth group treated with 0.45 ppm Se + 90 ppm Zn (T4). The parameters observed included rumen microbial enzyme activities (carboxyl methyl cellulase, amylase, protease) and the relative abundance of rumen microbes (*Ruminococcus* sp., *Ruminococcus flavefaciens, Ruminococcus albus, Streptococcus* sp., *Prevotella ruminicola,* and *Eubacterium ruminantium*). Results indicated that carboxyl methyl cellulase (CMC-ase) and amylase activities raised in T2, T3, and T4 in comparison to T1 and T0 treatments. Protease activity and protein enzyme content increased in T2 compared to all treatments. The relative abundance of *Ruminococcus* sp. and *Ruminococcus albus* was higher in T2 and T3 compared to T0 treatment. Furthermore, an elevated *Ruminococcus flavefaciens* was indicated in T2 compared to other treatments. The T2, T3, and T4 led to higher abundances of *Eubacterium ruminantium, Prevotella ruminicola,* and *Ruminococcus albus* compared to T0 and T1. It is concluded that organic Se and Zn enhanced the relative abundance of several bacterial species and the activity of enzymes in the rumen; optimal results are recommended when combining 0.45 ppm Se + 60 ppm Zn.

Keywords: Bacterial Species, Enzyme Activity, Rumen, Selenium, Zinc

INTRODUCTION

Multiple microorganisms contribute to the rumen fermentation process in ruminant animals, and the feed supply significantly impacts these microbial communities in the rumen. Within the rumen, the flora is commonly comprised of diverse bacteria that undertake specific digestive roles, for instance, amylolytic bacteria break down starches, cellulolytic microbes degrade cellulose, and protein-degrading variants metabolize nitrogenous compounds (Wei et al., 2022). Bacteria alter the feed substrate, the primary source of nutrition for the host animal, by secreting several specialized enzymes (Moon et al., 2021). Due to their immediate relationship to the feed offered, rumen microbes are essential to ruminant nutrition (Puniya et al., 2015; Takizawa et al., 2020). However, the primary source of ruminant feed comes from agricultural crop wastes and forages, which have diverse nutrient contents, including minerals. In addition, many things, such as climate, plant species, soil, and farming practices, affect the mineral content of plants (Spears et al., 2022). In various regions across several countries, animal feed derived from agricultural plant residues often exhibits deficiencies in essential micronutrients, such as selenium (Se) and zinc (Zn) (Kumar et al., 2013). Nevertheless, these microminerals are essential for animal metabolism, including microorganisms present in the rumen (Zheng et al., 2022).

As a glutathione peroxidase (GSH-Px) component, Se shields cellular membranes against peroxide damage. It has been demonstrated that feeding supplements increase the antioxidant status of ruminal microbes. These circumstances improve fermentation and rumen microorganism development, which benefits animal growth and productivity (Li et al., 2023). Protease, amylase, and carboxyl methyl cellulase (CMC-ase) enzyme activities were elevated by dietary Se at concentrations of 0.3 and 0.45 ppm (Anam et al., 2023). Rumen bacterial abundance rose when Se was added to feed (Du et al., 2019; Cui et al., 2021). *Ruminococcus-1*, *Prevotella*, and *Prevotellaceae-UCG-003* might be found in higher relative abundances when hydroxy-selenomethionine (0.6 ppm) was added to dairy cattle feed (Zheng et al., 2022). Furthermore, rumen fermentation was significantly impacted by Zn in the cattle diet (Chen et al., 2019; Vigh et al., 2023). An increase in nutrient-degrading bacteria was positively connected with increased rumen microbial enzyme activity, which could be achieved with Zn supplementation. The rumen microbial protein also increased with the addition

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of 60 ppm of Zn (Chen et al., 2020). Moreover, Zn supplementation increased *Ruminococcus albus* and *Streptococcus bovis* populations (Petrič et al., 2021). Dietary supplementation of 80 ppm Zn could provide a balanced gut microbiota that promotes better growth for calves (Hou et al., 2023).

The current study focused on using organic rather than inorganic minerals. According to recent research, organic forms of minerals may prove to be more readily absorbed by the body than inorganic types due to higher bioavailability levels (Bakhshizadeh et al., 2019; Zheng et al., 2022). In addition, organic mineral supplementation can help livestock reduce environmental damage by increasing retention and absorption (Shaeffer et al., 2017). As described above, several studies have examined the effect of administering organic Se or Zn separately in ruminant feed on ruminal fermentation profiles (Chen et al., 2020; Anam et al., 2023). On the other hand, little is known regarding the organic Se-Zn combinations modifying the composition of bacteria and enzyme activity in the rumen. The present study explored the supplementation impact of combined Se and Zn from organic sources on rumen enzyme activity and relative abundance of several bacterial species through *in vitro* methods.

MATERIALS AND METHODS

Ethical approval

The Universitas Gadjah Mada, Indonesia, Animal Ethics Committee (025/EC-FKH/Eks./2023) approved all animal-handled protocols used in the current study.

Experimental design

Se and Zn were supplied as chelated-methionine containing Se and Zn at 0.4% and 15%, respectively. Five combinations of Se-Zn were evaluated, control (no Se and Zn supplementation, T0), 0.3 ppm + 60 ppm (T1), 0.45 ppm + 60 ppm (T2), 0.3 mg ppm + 90 mg ppm (T3), 0.45 ppm + 90 ppm (T4). The feeding of trace minerals into the feed was based on the dry matter (DM) of the feed used. In this case, Se and Zn were mixed with the basal premix (0.5% DM) and added to the substrate. The basal premix (per kg) contained Vitamin A 200,000 IU, Vitamin D 80,000 IU, Vitamin E 200 IU, Ca 243.4 g, P 3.2 g, K 277.9 g, Mg 1.8 g, Na 24.3 g, S 130.4 mg, Fe 12.5 mg, Mn 1.2 mg, Cu 179.4 mg, Co 5.4 mg, and I 1.2 mg. The basal diet consisted of forage to concentrate ratio of 60:40 (DM basis, percentage) based on elephant grass, wheat bran, ground corn, rice bran, dried palm kernel, and local soybean meal (Table 1).

Two Bali cattle (male and female) fitted with permanent rumen cannulas were used as rumen inoculum donors. The farm research facility at Universitas Gadjah Mada provided the cattle utilized in the present study. Healthy cattle were approximately five years old and weighed 320 ± 5 kg. During adaptation and experimentation, the animals received meals twice daily, administered at 6 a.m. and 3 p.m., alongside unrestricted access to water. Before feeding, rumen fluid was collected and filtered to eliminate any leftover feed before being transferred into a thermos flask. The rumen liquid from two cattle, each totaling 1,200 ml, was combined. Carbon dioxide (CO_2) flowed into the bottle for approximately 1 minute to remove the oxygen. Furthermore, 500mg of grounded feed substrate was transferred to a fermentation bottle, and artificial saliva and rumen inoculum were heated to 39°C and put into the fermentation bottle immediately. Each bottle was filled with 50ml of rumen fluid and artificial saliva, made in a 1:4 ratio. The artificial saliva was made anaerobically, as described by Tilley and Terry (1963). Incubation was carried out at 39°C for 48 hours with six replicates (for each treatment) and six blanks. The blanks contained only artificial saliva and rumen fluid without feed substrate. After 48 hours of incubation, the samples were centrifuged at 3,000 g for 15 minutes to separate the incubated liquid from the feed residue. The supernatant, which constitutes rumen liquid, was sampled for further analysis, including rumen enzyme activity and microbiota abundance.

Table 1. Ingredient and nutritional composition of the experimental diet

Ingredient	Value (%)	Nutrient level	Value (%)
Elephant grass	60.00	Dry matter	89.78
Wheat bran	10.00	Crude protein	14.78
Ground corn	5.00	Extract ether	3.44
Rice bran	5.00	Ash	8.21
Dried palm kernel	13.00	Acid detergent fiber	34.56
Local soybean meal	7.00	Neutral detergent fiber	52.11

Analyses

The AOAC (2005) procedure was used to determine the nutrient content of the feed substrate. Casein, amylum, and CMC substrates were used to measure protease, amylase, and CMC-ase enzyme activities, respectively (Anam et al., 2023). Ruminal genomic DNA was extracted utilizing the FavorPrep™ DNA Kit following the manufacturer's instructions. Microbial 16S rRNA was amplified, focusing on the hypervariable V3-V4 region, using primer sets V338F (5′-ACTCCTACGGGAGGCAGCAG-3′) and V806R (5′- GGACTACHVGGGTWTCTAAT-3′; Gui et al., 2021). Bioinformatics analysis followed the methodology explained by Fregulia et al. (2022), where sequencing data were identified using QIIME2 v9.2023. The relative abundance of bacterial species observed included *Ruminococcus* sp., *Ruminococcus flavefaciens*, *Ruminococcus albus*, *Streptococcus* sp., *Prevotella ruminicola*, and *Eubacterium ruminantium*.

Statistical analysis

The data were assessed using a one-way ANOVA with a completely randomized design, conducted with IBM SPSS Statistical software (version 26). The Duncan multiple range test was employed to test for differences in means. Differences in mean data were considered significant at $p < 0.05$. The association between ruminal bacterial species populations and enzyme activities was assessed using a Spearman-rank correlation analysis.

RESULTS

Table 2 presents the results of *in vitro* rumen enzyme activities. CMC-ase levels for T2, T3, and T4 were higher than T1 and T0 ($p < 0.05$). Amylase levels for T2, T3, and T4 were also higher than T1 and T0 ($p < 0.05$). In addition, enzyme protein levels for T2 were higher across all treatments ($p < 0.05$). Figure 1 depicts the bacterial species abundance across the different dietary treatments. According to the data, there was a significant improvement of *Ruminococcus* sp. in T2 and T3 compared to T0 and an equivalent rise in T4 compared to T0 ($p < 0.05$). *Ruminococcus flavefaciens* showed higher in T2 than other groups ($p < 0.05$). *Ruminococcus albus* increased in T2, T3, and T4 than in T0 and T1 ($p < 0.05$). Compared to T0, *Streptococcus* sp. increased in T2 and T4 ($p < 0.05$). Furthermore, in T2, T3, and T4, compared to other treatments, there were greater levels of *Eubacterium ruminantium* and *Prevotella ruminicola* ($p < 0.05$). As shown in Figure 2, the correlation between rumen enzyme activities and populations of bacterial species was determined using Spearman's correlation analysis under different combinations of supplemental Se-Zn. In general, it has been shown that *Ruminococcus* sp., *Ruminococcus flavefaciens*, *Ruminococcus albus*, *Streptococcus* sp., *Prevotella ruminicola*, and *Eubacterium ruminantium* had positive relationships with CMC-ase, amylase, and protease activities.

Table 2. Ruminal enzyme activities as affected by various combined organic Se and Zn supplementations in the diet of Bali cattle

Item \ Treatment	T0	T1	T2	T3	T4	SEM	*p*-value
CMC-ase (U/g)	3.40^{b}	3.85^{b}	5.01^{a}	4.79^{a}	4.46^{a}	0.18	< 0.001
Amylase (U/g)	16.90^{c}	18.11^{b}	20.42^{a}	20.42^{a}	19.89^{a}	0.31	< 0.001
Protease (U/g)	141.91^{d}	149.26^{c}	165.38^{a}	161.36^{ab}	153.64^{bc}	1.99	< 0.001
Enzyme protein (mg/ml)	0.78^{c}	0.92^{bc}	1.08^{a}	1.04^{ab}	0.89^{bc}	0.03	0.002

T0: Basal diet, no additive, T1: T0 + 0.3 ppm Se + 60 ppm Zn, T2: T0 + 0.45 ppm Se + 0.60 ppm Zn, T3: T0 + 0.30 ppm Se + 90 ppm Zn, T4: T0 + 0.45 ppm Se + 90 ppm Zn. SEM: Standard Error of the Mean. Means with different superscript letters in the same row are significantly different at $p < 0.05$.

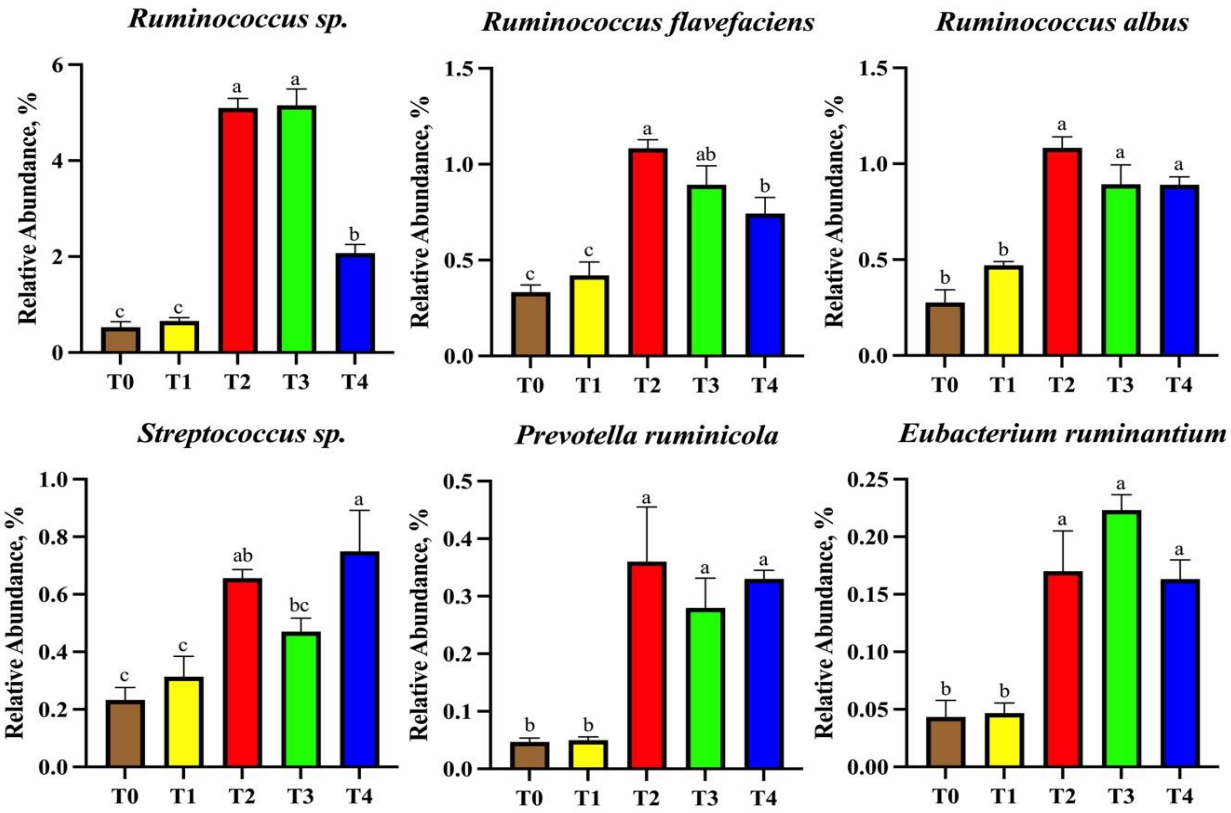

Figure 1. Ruminal bacterial abundance with different levels of combined organic selenium and zinc supplementations. T0: Basal diet, no additive, T1: T0 + 0.3 ppm Se + 60 ppm Zn, T2: T0 + 0.45 ppm Se + 0.60 ppm Zn, T3: T0 + 0.30 ppm Se + 90 ppm Zn, T4: T0 + 0.45 ppm Se + 90 ppm Zn. Means with different letters in the same figure are significantly different at $p < 0.05$.

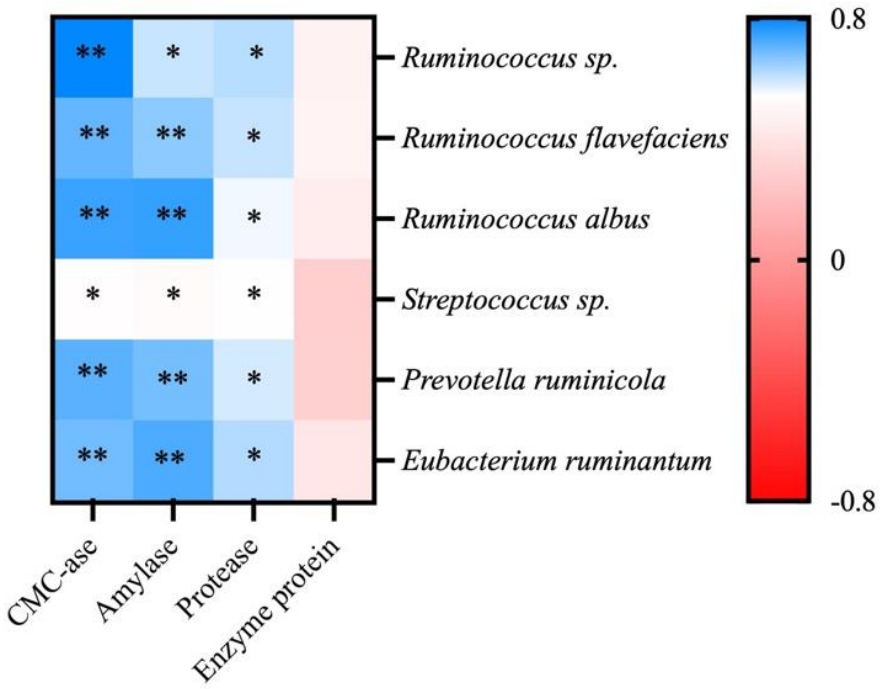

Figure 2. The heat map displays the correlation of rumen ruminal enzyme activities with bacterial abundance. Spearman's correlation coefficients were calculated and the values between -0.8 and 0.80 in the color key indicate negative (red) and positive (blue) correlations. CMC-ase: carboxymethyl cellulase. *: $p < 0.05$ and **: $p < 0.01$.

DISCUSSION

Bacteria comprise about 95% of the entire microbiota and are the most abundant microorganisms. These bacteria secreted enzymes that play an essential function in the feed degradation process (Hao et al., 2021). In the current research, supplementing some variations of Se-Zn additive in ration boosted the activity of the CMC-ase enzyme linear with the increase in fiber-degrading bacteria abundance. Se snares free radicals and guards against oxidative damage to cell membranes (Surai et al., 2019). Čobanova et al. (2016) found that after incorporating 0.4 ppm of Se into sheep diets, rumen microbes and protozoa exhibited elevated GSH-Px activity. Anam et al. (2023) reported that adding 0.45 ppm organic Se revealed CMC-ase activity. In line with Liu et al. (2019), the inclusion of inorganic Se in the diet of Holstein dairy cattle led to an increase in the numbers of *Ruminococcus albus* and *Ruminococcus flavefaciens* compared to the unsupplemented group. Additionally, Zn has antioxidant properties that might boost some enzyme activities. When Zn supplementation was applied to dairy cows, Wang et al. (2021) observed a linear rise in CMC-ase activity as well as in the number of fiber-degrading bacteria, *Ruminococcus albus* and *Ruminococcus flavefaciens*. These two bacteria belong to the two main bacteria in the *Ruminococcus* phylum category, which can produce cellulase and hemicellulase. *Ruminococcus* is known for breaking down cellulose and hemicellulose (Kim et al., 2018; Takizawa et al., 2020). The enzyme mentioned in this study, CMC-ase, breaks down β-1,4-glucan and generates free chain ends by targeting low-crystalline regions in cellulose fibers (Sun and Cheng, 2002).

Amylolytic bacteria, including *Prevotella ruminicola* and *Streptococcus bovis* can degrade nutrients, such as xylan, pectin, and starch (Wei et al., 2022). In addition, *Prevotella ruminicola* is responsible for the metabolism of peptides and proteins in the rumen (Wallace et al., 1997). The enzyme activity tests performed in the present research revealed that amylase and protease levels within the T2 group samples were markedly higher than those of the T0 or other analyzed groups. This finding may indicate that amylolytic and proteolytic flora may have better reactions in T2 treatment. Rumen microorganisms incorporate Se into proteins and their components. Similarly, the level of amylase activity was significantly increased when dairy cattle feed was supplemented with Se up to 0.5 ppm (Liu et al., 2019). In addition, Zn can help rumen fermentation patterns by directly altering microbial enzyme functions (Hilal et al., 2016). Dietary 70 ppm organic Zn caused an improvement in the relative population of *Streptococcus bovis* (Petrič et al., 2021). *Prevotella ruminicola* also developed with proteolytic bacteria as a result of the addition of inorganic Zn at 30 ppm (Wang et al., 2021). According to similar findings, rumen protease enzyme activity was raised by organic Se inclusion at a level of 0.45 ppm (Anam et al., 2023). The Se interacts closely with Zn, and both play crucial roles at the cellular level, depending on their form and dose (Yildiz et al., 2019). Giving 0.5 ppm Se plus 50 ppm Zn boosted daily body weight

gain in Awassi sheep by 31.03% as compared to Se alone (Al-Taie and Almahdawi, 2021). The improvement in sheep performance was associated with the increase in rumen enzyme activity, which degraded feed more efficiently.

CONCLUSION

In summary, the inclusion of organic Se and Zn enhanced the relative abundance of several bacterial species and the activity of enzymes in the rumen, optimal results are recommended when combining 0.45 ppm Se + 60 ppm Zn. Nevertheless, more investigation is required to find whether this result may be directly applied to live ruminant animals.

DECLARATIONS

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Authors' contributions

Moh Sofi'ul Anam and Ali Agus: conceptualization, data collection, and writing. Budi Prasetyo Widyobroto and Andriyani Astuti: data curation, validation, and editing. Gunawan: methodology, writing, and review. All authors checked and approved the analyzed data and the final edition of the manuscript for publication.

Competing interests

The authors declare that they have no competing interests.

Ethical considerations

All authors have reviewed the manuscripts for ethical issues such as plagiarism, misconduct, data fabrication, and double submission.

Availability of data and materials

The datasets produced in the present study can be obtained from the corresponding author upon a reasonable inquiry.

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The Effects of Saturated and Polyunsaturated Fatty Acids on Reproductive Performance and Reproductive Hormonal Changes in Dairy Cows at the Transition Period

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ABSTRACT

Reproductive success is crucial in dairy farming as it heavily relies on the consumption of a complete mixed ration for the diet. The current study investigated the effects of adding saturated (SFA) and polyunsaturated fatty acids (PUFAs) to dairy cows' diets on reproductive performance and reproductive hormones during the transition period. A total of 30 Holstein dairy cows were randomly divided into three groups (10 animals in each group), based on parity and body condition score. The cows had an initial body weight of 567.5 ± 40.3 kg (mean ± SD), a body condition score of 3.5 ± 0.26 out of 5 (mean ± SD), and a parity of 1.7 ± 0.02 (mean ± SD). The control group received a balanced ration meeting all the nutrient requirements according to the National Research Council (NRC) guidelines. The SFA group received 1.4% of dry matter (DM) as palm oil (RumiFat®), while the omega group had 5% of DM as safflower (a source of n-6 fatty acids) added from 21 days before parturition to 21 days after, and 4% of DM as flaxseed (a source of n-3 fatty acids) added from 21 to 42 days after parturition. In the Omega group, estradiol concentration significantly increased on artificial insemination (AI) day (12.54 pg/mL). Additionally, serum prostaglandin F2-alpha concentration was significantly higher in the omega group (0.732 pg/mL on day 7 and 1.68 pg/mL on day 14) compared to other groups. The control group exhibited the highest progesterone concentrations at 14 and 21 days post-calving compared to the other groups, other groups. whereas the omega group highest concentration five days after AI. The omega group also showed a significantly higher mean number of follicles >10mm and larger ovulatory follicle diameter. Moreover, a higher percentage of pregnant cows at 120 days in milk, fewer open days, and lower service per conception were observed in the omega group compared to the other groups. In conclusion, supplementing dairy cows' diets with PUFAs during the transition period positively influenced ovarian function, hormone levels, and reproductive performance.

Keywords: Flaxseed, Follicle diameter, Omega, Ovarian function, Safflower

INTRODUCTION

Reproductive success is a crucial aspect of dairy farming that relies on a total mixed ration-based diet. Reproductive inefficiency can be caused by negative energy balance after parturition, leading farmers to add fat to the dairy cows' diet to optimize energy status (Castro et al., 2006). The supplementation of fat aims to increase energy intake while minimizing body cows' fat mobilization during the transition period, thereby reducing the incidence of early lactation disorders, such as fatty liver (Mirzaei et al., 2020). Modifying dietary fatty acid intake has the potential to improve reproductive measures in dairy cattle (Sammad et al., 2022). Of particular interest was the supplementation of long-chain fatty acids, especially polyunsaturated fatty acids (PUFAs, Nanas et al., 2023).

Supplementation of long-chain fatty acids, particularly polyunsaturated fatty acid (PUFA) has raised considerable interest due to the possible positive effect on reproductive performance (Castro et al., 2006). Vegetable oil obtained from oilseeds (soybean oil, safflower oil, sunflower oil, or flaxseed oil) or oilseeds themselves can be added to the ration as a source of fat that contains different fatty acids (Nanas et al., 2023).

Several reviews and a meta-analyze have examined the effects of PUFAs on ruminants (Leduc et al., 2017, Roque-Jiménez et al., 2021; Angeli et al., 2021; Veshkini et al., 2023). Ruminants cannot synthesize omega 3 (n-3) and omega 6 (n-6) fatty acids, making supplementation of these fatty acids essential in their diet (Mylostyvyi et al., 2021). The N-6 fatty acids, such as linoleic acid, can alter the fatty acid profile of phospholipids in cell membranes, increasing the proportion of arachidonic acids. This change had advantages for the synthesis of prostaglandins and eicosanoids (Dyall et al., 2022). Silvestre et al. (2011) demonstrated that feeding cows a Ca salt rich in n-6 fatty acids during late and early gestation increased fertility benefits during the breeding period (Silvestre et al., 2011). Another study found that

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supplementing dairy cows' diet with 10 g of docosahexaenoic acid (n-3) enhanced pregnancy per AI and pregnancy rate (Sinedino et al., 2017). According to Dirandeh et al. (2013), supplementing the diet with polyunsaturated fatty acids (n-3 and n-6) improved fertility in dairy cows. Moreover, Leduc et al. (2017) demonstrated the positive effect of certain fatty acids (PUFA) on the pituitary, ovaries, and uterus rather than improved energy status. It is indicated that certain fatty acids (n-3, n-6) can suppress PGF2α and are known to play a role in prostaglandins (PGF2α) synthesis. Therefore, supplementation of these fatty acids can result in a decrease in embryonic mortality due to a change in the profile of fatty acids in the diet and uterine synthesis of prostaglandins during early pregnancy.

The present study aimed to evaluate the effects of adding selective saturated fatty acids (SFA) and PUFA to dairy cows' diet on ovarian function, hormone concentration (serum prostaglandin F2-alpha [PGFM], progesterone, and estradiol concentrations) during the transition period.

MATERIALS AND METHODS

Ethical approval

All animals were treated in accordance with the regulations and guidelines set by the Iranian Council of Animal Care. The experiment received approval from the Iranian Ministry of Agriculture (experimental permission no. 1828).

Animal and experimental diets

The study was done at Mashhad University Agriculture Farm, Iran (from June to August 2020). A total of 30 Holstein dairy cows (10 animals in each group), consisting of 15 primiparous and 15 multiparous cows, were included in the study. The cows had an initial body weight of 567.5 ± 40.3 kg (mean ± SD), a body condition score of 3.5 ± 0.26 out of 5 (mean ± SD), and a parity of 1.7 ± 0.02 (mean ± SD). The cows were randomly assigned to one of three experimental treatments, each group consisted of 10 cows (5 primiparous and 5 multiparous). The experimental design was completely randomized, and the testing period spanned from 21 days before the expected calving date until 42 days after calving. The cows were housed in tie stalls (3x3x3 meters) and fed individually, with free access to fresh water throughout the testing period. The animals did not receive any vaccination during the experiment.

The cows were divided into three equal experimental groups based on parity, body condition score, and expected calving date. The control group received a balanced ration that met all the nutrient requirements according to the National Research Council (NRC) guidelines (Table 1, NRC, 2001). The other two treatment groups received different dietary supplements. The SFA group included the addition of 1.4% of dry matter (DM) palm oil (RumiFat®) to the diet, while omega group included the addition of 5% of DM safflower (a source of n-6 fatty acids) to the diet from 21 days before parturition to 21 days after parturition, and 4% of DM flaxseed (a source of n-3 fatty acids) from 21 days after parturition to 42 days after parturition. Milking was done twice a day (At 8 a.m. and 5 p.m.). The diets were fed as a total mixed ration (TMR) twice daily (0700 and 1700 hour) for *ad libitum* intake, with 10% of refusals on an as-fed basis.

Experimental procedure, sampling, and feeding

The cows were housed in a tie-stall barn that was randomly designed, with each group consisting of 10 cows, starting from 21 days before parturition. They were provided with a close-up diet from the first day after parturition until 21 days after parturition, followed by a fresh cow's diet until 21 days after parturition, and final diet from 21 days after parturition to 42 days after parturition (Table 1). The total mixed rations were sampled weekly, pooled monthly, and analyzed in the laboratory of Mashhad University Agriculture for dry matter content using a drying oven method (Mylostyvyi et al., 2021). The samples (500 g) were also analyzed for crude protein (CP), neutral detergent fiber (NDF), and acid detergent fiber (ADF) according to the official methods of analysis by the Association of Official Analytical Chemists (AOAC, Mylostyvyi et al., 2021).

Blood sampling and analysis

Blood samples (10 ml) were collected from all cows 7, 14, and 21 days after parturition, on the day of AI, and 5 days after AI. The samples were collected via coccygeal venipuncture and placed in Vacutainer tubes (BD Vacutainer Systems, Plymouth, UK) without anticoagulants. After allowing the blood to coagulate for two hours, it was centrifuged for 15 minutes at 3500 rpm. The serum was then transferred to microtubes and frozen at -20°C for further laboratory analysis. The concentrations of progesterone (ng/mL), estradiol (pg/mL), and PGFM (pg/mL), in the serum were determined using ELISA kits (progesterone and estradiol: Diaplus, North York, Ontario, Canada, Intra- and intra-assay coefficients of variation were < 5% (PGFM: Cayman Chemical, Ann Arbor, MI, USA). The sensitivity of the PGFM assay was 0.02 ng/mL.

Table 1. Ingredient and chemical composition of experimental diets of Holstein dairy cows during the transition period (21 days before calving to 42 days after calving)

Item	Close up period[1]			Fresh cow period[2]			Early lactation period[3]		
	Control	SFA	Omega (Safflower seed)	Control	SFA	Omega (Safflower seed)	Control	SFA	Omega (Flaxseed)
Ingredient (Percentage of dry matter)									
Alfalfa hay	21.22	21.13	21.14	27.24	27.38	27.38	13.47	13.46	13.46
Corn silage	29.12	29	29.01	17.85	17.95	17.95	26.93	26.92	26.92
wheat straw	10.04	10	10.01	-	-	-	-	-	-
Barley, rolled	19.72	18.3	14.68	5.57	4.15	0.26	5.96	5.96	5.96
Corn	-	-	-	24.26	23.85	23.85	25.63	24.31	21.77
Soybean meal	4.25	4.23	4.23	9.54	9.59	9.59	13.12	13.42	11.15
Corn gluten meal	-	-	-	2.38	2.39	2.39	2.98	2.98	3
Canola meal	4.3	4.72	2.75	2.76	3.55	1.54	2.98	2.98	3
Meat meal	1.79	1.79	1.79	-	-	-	-	-	-
Bran	8.74	8.63	10.62	2.73	2.74	2.74	6.23	5.96	8.19
Sugar beet pulp	-	-	-	5.4	4.67	6.68	-	-	-
Calcium salt of palm fatty acid	-	1.38	-	-	1.45	-	-	1.31	-
Safflower seed	-	-	4.96	-	-	5.34	-	-	-
Flaxseed	-	-	-	-	-	-	-	-	3.85
Calcium bicarbonate	0.29	0.28	0.28	0.43	0.43	0.43	0.42	0.42	0.42
Dicalcium phosphate	-	-	-	-	-	-	0.12	0.12	0.12
Sodium bicarbonate	-	-	-	0.55	0.55	0.55	0.62	0.62	0.62
Magnesium oxide	-	-	-	0.18	0.18	0.18	0.23	0.23	0.23
Vitamins/minerals[4]	0.53	0.53	0.53	0.92	0.92	0.92	0.88	0.88	0.88
Vitamin E and Selenium Supplement[5]	0.15	0.15	0.15	0.15	0.15	0.15	0.23	0.23	0.23
Salt	-	-	-	0.18	0.18	0.18	0.19	0.19	0.19
Nutrient composition (Dry matter basis)									
NEL,[6] Mcal/kg	1.58	1.62	1.6	1.55	1.59	1.58	1.61	1.65	1.63
CP, % of DM	13.8	13.8	13.8	16.2	16.2	16.2	15.7	15.8	15.9
NFC, % of DM	36.5	35.6	33.4	46.1	45.1	44.1	45.2	44	41.8
Fatty acids, % of DM	2.8	4.1	4.1	2.9	4.1	4.1	2.7	4.1	4
NDF, % of DM	42.4	42	44	30.1	29.9	31.1	31.5	31.2	33.3
ADF, % of DM	26.4	26.3	27.8	18.3	18.3	18.6	20.2	20.2	22
Ca, % of DM	0.7	0.7	0.7	0.6	0.6	0.6	0.7	0.7	0.7
P, % of DM	0.5	0.5	0.5	0.4	0.4	0.4	0.4	0.4	0.4

[1] Control: no fat supplement; SFA: Saturated fatty acid supplement; Omega: Polyunsaturated fatty acids (PUFAs) supplement (from 21 d before predicted calving until calving).
[2] Control: no fat supplement; SFA: Saturated fatty acid supplement; Omega: PUFAs fatty acid supplement (from calving until 21 d postpartum).
[3] Control: no fat supplement; SFA: Saturated fatty acid supplement; Omega: PUFAs fatty acid supplement (from 21 to 42 d postpartum).
[4] Contained 68,679 mg/kg Ca; 30,000 mg/kg P; 40,000 mg/kg Na; 20,700 mg/kg Mg; 1,840 mg/kg of Cu; 5,000 mg/kg of Mn; 100 mg/kg of Co; 10,395 mg/kg of Zn; 100 mg/kg of Se; 2 mg/kg of organic Se; 120 mg/kg of I; 1,000 kIU/kg of vitamin A; 500 kIU/kg of vitamin D; 1 kIU/kg of vitamin E; and 1,000 mg/kg of antioxidants.
[5] Contained 300 mg/kg of Se; 11,000 mg/kg of vitamin E; and 400 mg/kg of antioxidants.
[6] Calculated based on the DMI of 11.5 kg per day and using NRC software (version 2001).

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Body weight and body condition score

The body weight of all cows in the study was measured, and their body condition score (BCS) was evaluated at the beginning of the study. The BCS was assigned to each cow on a scale of 1 to 5, with the increase of 0.25, according to the method described by Edmonson et al. (1989).

Reproductive management

The cows in the study were synchronized using two injections of $PGF_{2\alpha}$ (150 mg cloprostenol sodium, syva, Spain) administered 14 days apart, starting at 30 ± 3 and 44 ± 3 days in milk (DIM) postpartum. The day after the second $PGF_{2\alpha}$ injection, the cows' uterus and ovaries were scanned using an ultrasound linear rectal transducer (Ecogra, 7.5 MHz transrectal linear transducer, Aloca Co., Ltd., Tokyo, Japan) for three days (45 ± 3 DIM, 46 ± 3 DIM, and 47 ± 3 DIM) to evaluate ovarian function (number of follicles and diameters). The follicles were categorized into three diameter classes (Ultrasound device measurement was used to categorize the follicles) including small (3.0 to 4.9 mm), medium (5.0 to 9.9 mm), and large (≥ 10 mm). The diameter of each follicle was measured as the average length and width of the antrum (Dyall et al., 2022). Cows that exhibited estrus after the second $PGF_{2\alpha}$ injection were artificially inseminated, while cows that did not show estrus were inseminated during their subsequent estrus. Artificial insemination was performed by a technician using commercially available frozen-thawed semen (USA). Pregnancy information, open days, and insemination for pregnancy were recorded using livestock information software (Modiran software version 2.3, Iran).

Statistical analysis

Data were analyzed using the MIXED procedure of SAS software (version 9.4, 2018, SAS Institute Inc., Cary, NC, USA). The statistical model included treatment, parity, and their interaction as fixed effects. The individual cow was considered as the experimental unit. The repeated measures of blood parameters were analyzed using the MIXED procedure with repeated measures. The random residual error was also included in the model.

$Y_{ijkl} = \mu + D_i + T_j + C_k + DT_{ij} + \varepsilon_{ijkl}$

Where Y_{ijkl} = the dependent variable, μ = the overall mean, D_i =the effect of diet, T_j = the effect of time of sampling, C_k = the effect of cow, DT_{ij} = the interactions between diet (D) and time (T), and ε_{ijkl} = the random residual error.

The best fitting covariance structure for the data was chosen based on the Schwartz Bayesian Information Criterion (BIC). The compound symmetry (CS) structure was chosen as it had the lowest BIC among the considered structures, which also included first-order autoregressive (AR1), heterogeneous first-order autoregressive (ARH1), and unstructured (UN) structures.

The significance level for the analysis was set at $p < 0.05$, with $p < 0.1$ considered as a trend. The Chi-square test with PROC FREQ was used to examine the number of pregnant cows after the first AI, as well as the overall number of pregnant cows. This allowed for comparisons of all possible dietary treatment pairs. The number of AI and open days were examined using the Kruskal-Wallis test with PROC NPAR1WAY, and the Mann-Whitney W test was used to compare the medians of the three groups two by two (Wilcoxon). The Dunn-Sidák multiple comparison method was used to adjust p-values derived from all tested comparisons.

RESULTS

Serum estradiol concentration

The results of estradiol concentration were indicated in Figure 1. The Omega (when supplemented with flaxseed) group had the highest estradiol concentration (12.54 pg/mL), while the SFA group had the lowest (8.9 pg/mL) estradiol concentration on AI day ($p < 0.05$).

Serum prostaglandin concentration

The effects of SFA and PUFAs on PGFM concentration at 7 and 14 days after parturition were indicated in Figure 2. Experimental groups showed a significant effect on PGFM concentration on day 7 and day 14 ($p < 0.05$). The Omega (when supplemented with safflower) group had the highest PGFM concentration on both days (0.732 pg/mL on day 7 and 1.68 pg/mL on day 14), while the control group had the lowest concentration on both days (0.32 pg/mL on day 7 and 0.94 pg/mL on day 14).

Serum progesterone concentration

The effects of SFA and PUFAs on serum progesterone concentration at 14 and 21 days after parturition, AI day, and 5 days after AI were indicated in Figure 3. The control group had the highest progesterone concentration (1.15 pg/mL) at 14 days after parturition compared to the SFA and Omega (safflower) groups ($p < 0.05$). The Omega

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(safflower) group had the lowest concentration (0.5 pg/mL) at 14 days after parturition. At 21 days after parturition, the control group had the highest progesterone concentration (1.65 pg/mL), while the SFA group had the lowest (0.76 pg/mL, $p < 0.05$). Five days after AI, the Omega (flaxseed) group had the highest progesterone concentration (2.57 pg/mL), while the control group had the lowest concentration (0.85 pg/mL, $p < 0.05$).

Reproductive performance, number and diameter of the ovulatory follicle

Effect of SFA and PUFAs on the mean number of follicles, diameter of the ovulatory follicle, and reproductive performance during the transition period were presented in Tables 2 and 3. The Omega group had a higher mean number of follicles >10 mm and a larger diameter of the ovulatory follicle ($p < 0.05$). The percentage of pregnant cows until 120 DIM was higher in the Omega group ($p < 0.05$), and they also had fewer open days ($p = 0.004$). Additionally, the Omega group had a lower service per conception compared to the other groups ($p = 0.002$).

Table 2. Means number of follicles, the diameter of the ovulatory follicle of adults Holstein dairy cows supplemented with saturated fatty acids and polyunsaturated fatty acids during the transition period (21 days before calving to 42 days after calving)

Variable \ Treatments [1]	Control	SFA	Omega	SD	P-value
Follicle no.					
<5 mm	7.00	6.91	7.1	0.43	0.90
5-10 mm	3.30	3.49	3.47	0.28	0.81
>10 mm	0.92[b]	0.60[c]	0.96[a]	0.06	<0.01
Diameter of the ovulatory follicle (mm)	13.6[b]	12.2[c]	15.2[a]	0.44	<0.05

[1]Control: No fat supplement; SFA: Saturated fatty acid supplement; Omega: Poly unsaturated fatty acids (PUFAs) fatty acid supplement. [a,b,c] Means within a row with different lowercase superscripts differ ($p < 0.05$). SD: Standard deviation.

Table 3. Reproductive performances of adults Holstein dairy cows supplemented with saturated fatty acids and polyunsaturated fatty acids during the transition period (21 days before calving to 42 days after calving)

Variable \ Treatments [1]	Control	SFA	Omega	SD	P-value
Pregnant cows until 120 DIM (%)	42.85 [c]	66.60 [b]	85.71 [a]	0.13	0.001
Open days (day)	108.28[a]	111.5[a]	86.14[b]	3.2	0.004
Service per conception	2.28[a]	1.89[b]	1.50[c]	0.08	0.002

[1]Control: No fat supplement; SFA: Saturated fatty acid supplement; Omega: Poly unsaturated fatty acids (PUFAs) supplement. DIM: Days in milk. [a,b,c] Mean within a row with different lowercase superscripts differ $p < 0.05$. SD: Standard deviation.

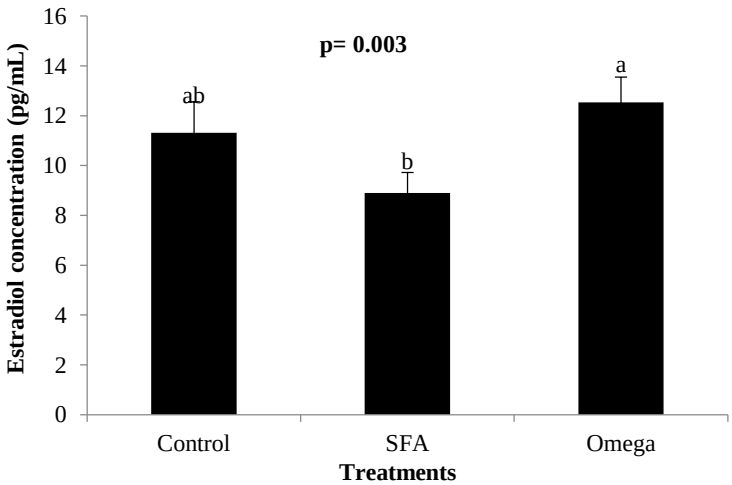

Figure 1. Mean (± SD) serum estradiol concentration (pg/mL) on artificial insemination day in Holstein dairy cow in the experimental groups from 21 days before to 42 days after calving. The groups including control, saturated fatty acids group (SFA), and polyunsaturated fatty acids group (Omega). [a, b] Mean different lowercase letters shows significant differences between the groups $p < 0.05$.

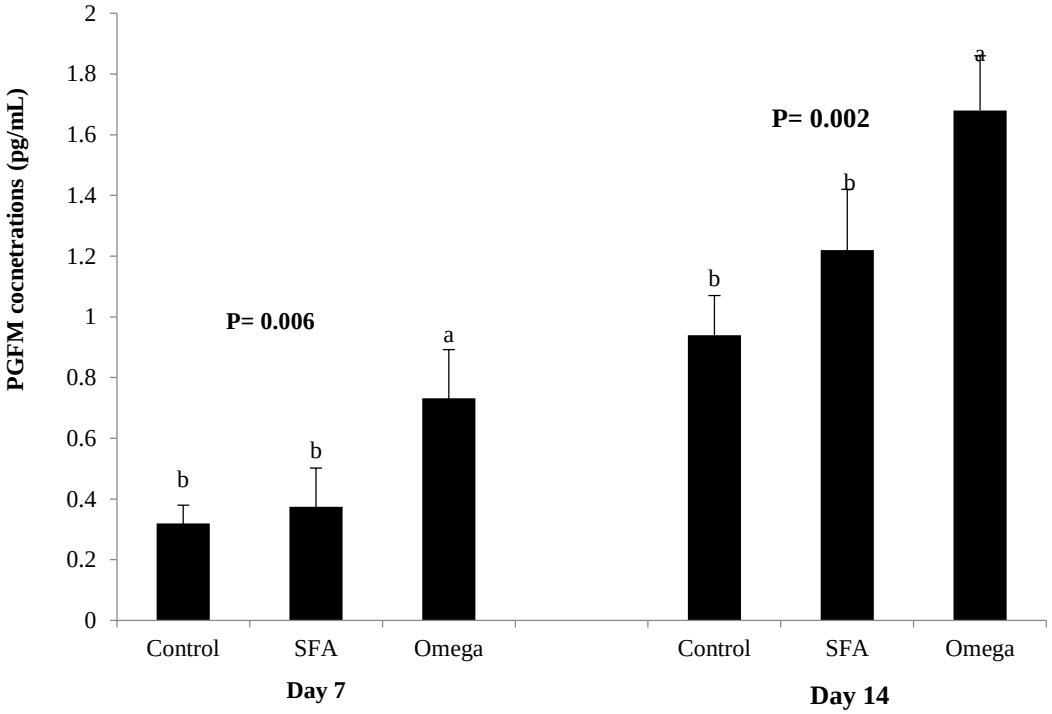

Figure 2. Mean (± SD) serum PGFM concentration (pg/mL) concentration on days 7 and 14 after parturition Holstein dairy cow in the experimental groups from 21 days before to 42 days after calving. The groups including control, saturated fatty acids group (SFA), and polyunsaturated fatty acids group (Omega). [a, b] Mean different lowercase letters shows significant differences between the groups $p < 0.05$.

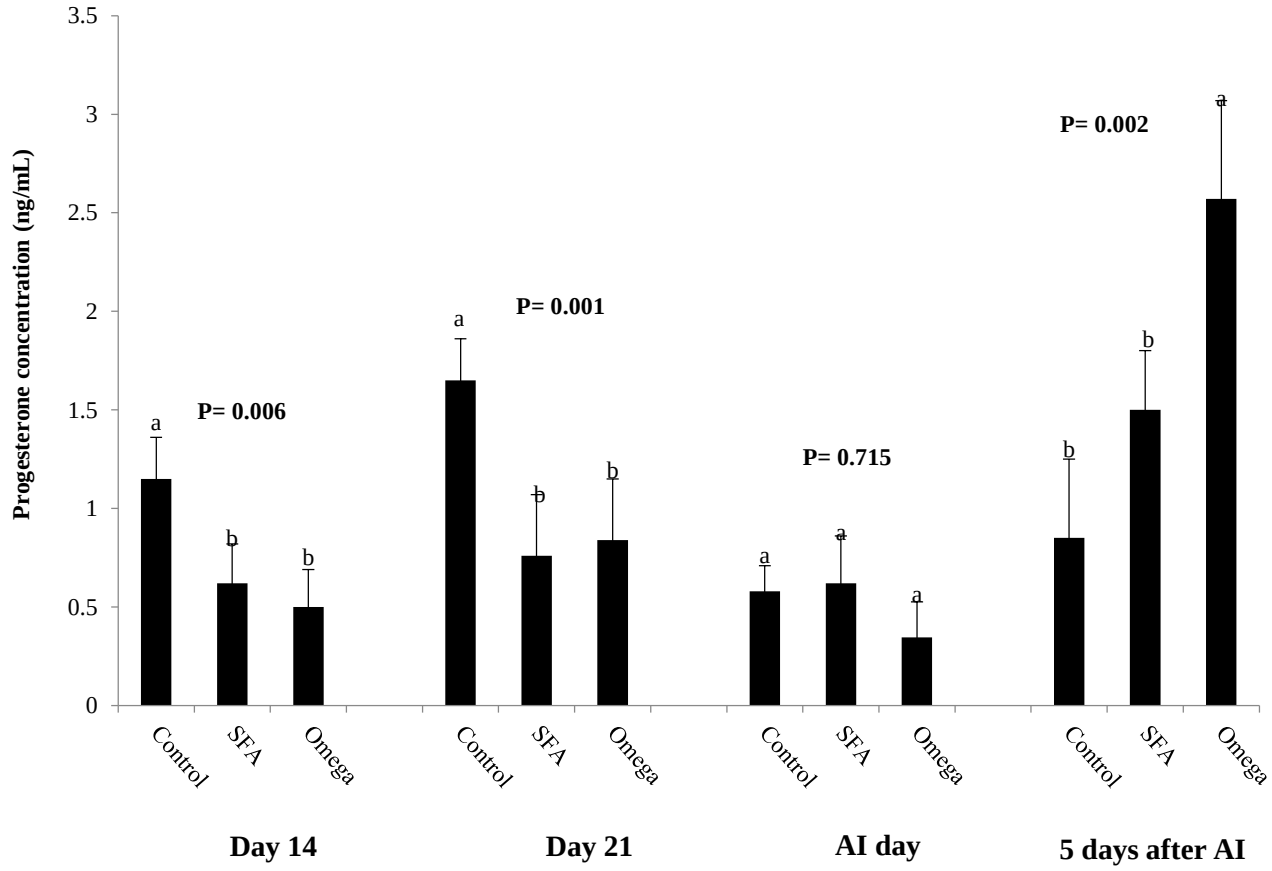

Figure 3. Mean (± SD) serum Progesterone concentration (ng/mL) on days 14 and 21 after parturition, artificial insemination day, and 5 days after artificial insemination Holstein dairy cow in the experimental group. The groups including control, saturated fatty acids group (SFA), and polyunsaturated fatty acids group (Omega). [a, b] Mean different lowercase letters shows significant differences between the groups $p < 0.05$.

DISCUSSION

An increase in estradiol concentration is associated with the initiation of estrus and can have positive effects on the process of AI (Tippenhauer et al., 2023). In this study, the group supplemented with safflower (source of omega 6) had the highest estradiol concentration on AI day, it is indicating that supplementation of PUFA to Holstein dairy cows could enhance the AI process and result in a higher conception rate.

Prostaglandins, particularly $PGF_{2\alpha}$, play a crucial role in luteolysis in ruminants (Greco et al., 2018). The precursor of PGFM is arachidonic acid, formed from the elongation and desaturation of linoleic acid (Tallima et al., 2018). PUFA, such as those found in safflower, can affect the synthesis of $PGF_{2\alpha}$ and progesterone (Gulliver et al., 2012). The safflower group in this study had higher concentrations of PGFM at 7 and 14 days after parturition compared to other groups. The current study results align with Caldari-Torres et al.'s (2006) study, which demonstrated that an increased ratio of n-6 to n-3 fatty acids in the culture media led to elevated synthesis of $PGF_{2\alpha}$ (Caldari-Torres et al., 2006). This indicated that modifying the fatty acid composition of the diet may result in the release of PG from the endometrium and potentially mitigate luteolytic signals. However, contrary to current study findings, Dirandeh et al. (2013) and Petit et al. (2004) found that increased n-3 fatty acids suppressed the release of $PGF_{2\alpha}$ following an oxytocin challenge (Petit et al., 2004; Dirandeh et al., 2013). This increase in PGFM may influence the lifespan of the corpus luteum and result in an earlier return to estrus. Silvestre et al. (2011) observed an increase in the pregnancy per AI ratio and a decrease in pregnancy loss among cows fed Ca salts enriched with PUFA during the breeding period (Silvestre et al., 2011). In the current study, the Omega group had a higher number of pregnant cows, fewer open days, and a lower service per conception compared to other groups. These results indicate that PUFA supplementation may improve reproductive outcomes in dairy cows.

Furthermore, Demetrio et al. (2007) and Lopes et al. (2009) found that dairy cows supplied with n-3 PUFA had higher plasma progesterone levels (Demetrio et al., 2007; Lopes et al., 2009). Moreover, cows treated with PUFA exhibited a higher number of pregnant cows on the first timed artificial insemination (TAI), improved conception rates, and enhanced overall reproductive performance. Stevenson et al. (2006) and Chebel et al. (2010) discovered that $PGF_{2\alpha}$ secretion may have been reduced during pregnancy detection in cows fed flaxseed as an n-3 supplement. In accordance with the findings of Castro et al. (2006), the group that received PUFA-rich vegetable oil exhibited higher plasma progesterone levels and a greater number of pregnant cows on the first AI. Additionally, these animals had a lower incidence of AI. The omega group (PUFA) had the highest progesterone levels 5 days after AI, and a higher number of cows in this group became pregnant. This aligns with previous studies that had shown higher plasma progesterone levels and improved reproductive outcomes in cows treated with PUFA (Stevenson et al., 2006; Chebel et al., 2010). It had been suggested that the production of $PGF_{2\alpha}$ may have been reduced during pregnancy recognition in cows that were fed flaxseed (a source of omega 3). Progesterone plays a crucial role in preparing the uterus for embryo implantation and supporting pregnancy by nourishing the conceptus (Castro et al., 2006).

The supplementation of PUFA in the diets of dairy cows had the potential to improve fertility. Several studies have indicated that PUFA may enhance fertility by influencing follicular growth and ovulation (Robinson et al., 2002; Libera et al., 2020). It is demonstrated that cows fed with PUFA exhibited larger follicle diameters and an increased number of follicles (Robinson et al., 2002; Kabirian Moghadam et al., 2020; 2023). In the current study, the authors observed a higher number of follicles and a larger diameter of the ovulatory follicle in the group that received PUFA.

In this study, safflower (a source of n-6) was incorporated into the cows' diets from 21 days before parturition to 21 days after parturition. This was done because safflower is known to increase $PGF_{2\alpha}$ concentration and potentially attenuate luteolytic signals, thereby influencing the lifespan of the corpus luteum and resulting in an earlier return to estrus. Additionally, the inclusion of flaxseed (a source of n-3) from 21 days after parturition to 42 days after parturition was associated with higher plasma progesterone levels. This increase in progesterone may lead to improved reproductive performance, including an increased number of pregnant cows and a higher rate of successful conception.

CONCLUSION

In this study found that supplementing a source of n-6 fatty acids before and after parturition and a source of n-3 fatty acids after parturition can have positive effects on reproductive performance in dairy cows. Safflower (source of n-6) was found to increase prostaglandin concentration and potentially mitigate luteolytic signals, while flaxseed (source of n-3) resulted in higher plasma progesterone levels and improved reproductive outcomes such as the number of pregnant cows and service per conception. The selective supplementation of n-6 and n-3 fatty acids based on the follicular cycle of dairy cows could be beneficial for their reproductive performance. As a suggestion, further studies need to evaluate the effects of PUFA in different pregnancy and lactation periods in dairy cows.

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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

Mahmood Reza Amini, Abasali Naserian and Reza valizadeh planned the experiment. Seyed Amin Razavi and Essa Dirandeh interpreted and analyzed the data the write the manuscripts and Hojjat Baghshahi revised the paper. All authors read and approved the final edition of the manuscript.

Ethical considerations

The authors confirm that all authors have reviewed and submitted the manuscript to this journal for the first time.

Competing interests

The authors declare no conflict of interest.

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Protein Concentration, Anthelmintic Activity, and Microbial Contamination of the Laboratory-Produced Chitosan-Encapsulated Bromelain Batches

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ABSTRACT

Bromelain has been shown to have potential as an anthelmintic for controlling livestock nematodes, such as *Haemonchus (H.) contortus*. The present study aimed to evaluate the *in vitro* quality of the laboratory-produced nanoencapsulated bromelain (NEB) and its activity against *H. contortus*. The acid-base extraction method was employed to extract four different batches of bromelain from the peels of fully ripened pineapples. It was encapsulated in chitosan to form the nano-encapsulated bromelain complex. Standard biochemical methods were employed to determine the bromelain concentration, protein concentration, *in vitro* anthelmintic activity against various stages of *H. contortus* (egg, larva, adult), and bacteria contamination for the four NEB batches. The mean concentration of extracted bromelain was 4.3 mg/ml in all four batches. There were no variations in the protein concentrations between the batches of NEB, which ranged from 1,090 mg/ml to 1.205 mg/ml. Although there were no significant differences in different batches, a variation in NEB inhibitory concentration (IC_{50}) was observed according to the different parasitic stages. The highest activity was for adult worms (LC_{50} =0.2454 ± 0.05 mg/ml), followed by the eggs (IC_{50} = 0.3 ± 0.07 mg/ml), and the larval stage (IC_{50} =0.9 ± 0.45 mg/ml). Despite the identification of certain bacterial species in the raw pineapple extract, the final product of all four batches of NEB remained free from any bacterial contamination. The current study indicated that NEB's concentration, protein concentrations, and anthelmintic activity did not vary significantly across the different batches of NEB. Additionally, the encapsulation process ensured that the final product was free of bacterial contamination and thus safe for use in animals.

Keywords: Anthelmintic activity, Bromelain, Chitosan, Nanoencapsulation

INTRODUCTION

Helminthiasis is an important tropical disease affecting livestock where it causes severe diarrhea, reduction in weight gain, impairing fertility, and extreme cases causes high mortalities (Nwoke et al., 2015). The most important nematode affecting ruminants in the tropics is *Haemonchus (H.) contortus*, which causes the livestock industry significant financial losses (Vineer et al., 2020). Proactive approaches are critical to maintaining ruminant health and productivity, and effective management tactics include rotational grazing, targeted deworming, and a focused effort to prevent drug resistance. Farmers use anthelmintics frequently to treat helminthiasis (Nwoke et al., 2015). Despite their proven benefits for animal health, many commercial treatments have some significant disadvantages. Consumers have concerns about the potential existence of synthetic drug residues in animal products and also in recent years frequent use of anthelmintics has led to the emergence of drug resistance (Wainaina Kagucia et al., 2020; Sharma et al., 2020). Therefore, there is an urgent need for innovative approaches such as the development of new drugs from plants (Wasso et al., 2020; Daiba et al., 2022).

Recent studies have demonstrated that nano-encapsulated bromelain has high anthelmintic activity (Hunduza et al., 2020; Wasso et al., 2020; Daiba et al., 2022). Bromelain is abundant in pineapple (*Ananas cosmos*, L. Merr.) fruits, stems, and peels (Eguale et al., 2007; Hunduza et al., 2020; Wasso et al., 2020). However, upon treatment, bromelain's anthelmintic action decreases due to the low pH of ruminants' abomasum and the presence of rumen microbiota (Eguale et al., 2007). Thus, studies have focused on encapsulating bromelain to enhance its activity in the gut of animals (Hunduza et al., 2020; Wasso et al., 2020; Daiba et al., 2022). All developmental phases of *H. contortus* isolated from goats can be effectively inhibited *in vitro* by nanoencapsulated bromelain (Eguale et al., 2007; Hunduza et al., 2020; Wasso et al., 2020). Studies have found that encapsulated bromelain had a greater egg hatch suppression activity than extracted (IC50 = 0.325 mg/ml) and pure bromelain (IC_{50} = 0.327 mg/ml; Hunduza et al., 2020; Mahlangu et al., 2020;

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Wasso et al., 2020; Daiba et al., 2022). In addition, *in vivo* studies have shown that nano-encapsulated bromelain has anticoccidial and antibacterial properties (Mahlangu et al., 2020; Wasso et al., 2020; Daiba et al., 2023).

In products like the nano-encapsulated bromelain, before it is registered and marketed for use in animals, rigorous quality assurance is necessary to ensure the product's final safety and effectiveness. It is important to maintain consistency in batch-to-batch variations to meet quality standards and preserve product uniformity. The use of nano-encapsulation technology introduces an additional level of complexity, necessitating meticulous monitoring to avoid variations in particle size, distribution, and encapsulation efficacy (Fan et al., 2012; Nwoke et al., 2015; Lanusse et al., 2018). Furthermore, as these nanostructures may provide ideal conditions for the development of microorganisms rigorous monitoring processes are necessary to minimize the risk of microbial contamination. To guarantee that the product is safe for consumption, robust procedures for quality control should include extensive testing for microbial contaminants, such as bacteria, yeast, and mold (Dao et al., 2018). This study aimed to evaluating the quality of the laboratory-produced bromelain encapsulated (NEB) and determine its efficacy against *H. contortus* isolated from goats.

MATERIALS AND METHODS

Ethical approval

The study was approved by the Jomo Kenyatta University of Agriculture Technology (JKUAT) Institutional Animal Ethics Committee. Sampling of worms at the Ruiru slaughterhouse was approved by the meat inspector based at the facility. The protocols used for sampling and worm isolation adhered to the Kenya's Animal Diseases Act (Act No. 35 of 1984) and the University's guidelines.

Study site

The laboratory study was conducted between June and December 2023. The study was conducted at JKUAT, which is situated at latitude 1°05 S and longitude 37°00 E in Juja Sub-County, Kiambu County, Kenya. The average temperature in the area is 18.7°C with 850 mm of annual rainfall.

Batches preparation

The four different batches of extracted bromelain were produced one week apart. For all batches, the concentration of bromelain was determined after direct extraction, dialysis, and encapsulation. The extracts were stored at -20°C pending experiments on their anthelmintic properties.

Extraction of bromelain

Twenty mature pineapples (*Ananas comosus* L. Merr.) were obtained from a farm located in Kiambu County, Kenya's Gatundu Sub-County. This sub-county is located between longitudes 0° 25 and 1° 20 south and latitudes 36° 31 and 37° 15 east. It has a tropical climate with 26°C average temperature and 1200 mm of annual rainfall on average.

Bromelain was extracted using sodium acetate from pineapple peels as earlier described by Hunduza et al. (2020). The crude extract was precipitated with 40% ammonium sulfate. The resulting pellet was dissolved in 100 mM of Tris-HCl, dialyzed, and stored at -20°C.

Bromelain nano-encapsulation using chitosan

Ionic gelation method by Hunduza et al. (2020) was used to encapsulate bromelain into chitosan and this involved use of 1% sodium tripolyphosphate (STPP, Loba Chemie, India) and 1% chitosan (Sigma Aldrich, USA). After centrifugation, the pellet was recovered, washed with distilled water, dissolved in a phosphate-buffered saline (PBS) and the solution was stored at -20°C. Commercial bromelain (Jarrow Formulas, USA) was used to prepare the standard solution. The structural properties of nanoparticles were assessed using Fourier Transform Infrared Spectroscopy (FTIR), using the method outlined by Fan et al. (2012) and the result obtained was compared to other previous studies.

In vitro anthelmintic activity

Standard drug preparation

To prepare the positive control, albendazole (Sigma Aldrich, USA) was dissolved in 1% dimethyl sulfoxide (DMSO) solution (Thermo Fisher Scientific, USA). The PBS was used as a negative control at concentrations ranging from 0.125 mg/ml to 4 mg/ml.

Isolation of eggs

Mature *H. contortus* worms were obtained directly from the abomasum of goats slaughtered at the Ruiru abattoir in Kenya (Hunduza et al., 2020). The worms were subsequently placed in 7.4 pH PBS. The procedure outlined by Coles et al. (1992) was used to isolate female worms. Thereafter, five worms were added to a test tube and five milliliters of PBS

added. The worms' eggs were then gently crushed with a glass rod to release them into the solution of PBS. The mixture was then filtered to remove worm waste, and an additional 5 mL of PBS was added for homogeneity. Subsequently, 500 µL of the egg solution was applied to a McMaster slide, and the total egg concentration was counted under a microscope (Optika Microscope, Italy) at 100x magnification.

Egg hatch assay

The egg hatch assay (EHA) was carried out using the Coles et al. (1992) method. Briefly, 10 *H. contortus* eggs were suspended in 200 µL of egg solution and added to each well of a 96-well ELISA plate. Next, each well received 200 µL of an encapsulated bromelain solution, with concentrations ranging from 0.125 mg/ml to 4 mg/ml. After that, the plate was incubated for 48 hours at 28°C in a humid atmosphere. To stop the hatching process, Lugol's iodine was added to each well. The number of hatched eggs and larvae was counted under the microscope. A formula was used to determine the percentage inhibition of egg hatch (Coles et al., 1992).

$$\%\ \text{Egg inhibition} = \frac{\text{Total number of eggs} - \text{number of hatched larvae}}{\text{Total number of eggs}} x100$$

Larval and adult worms' mortality assay

The larval mortality assay (LMA) was conducted in accordance with the methodology described by Coles et al. (1992), Amphotericin B (5 g/ml, Affy Parenterals, India) was added to the egg suspension to inhibit the growth of bacteria and fungi. In each well of a 96-well titer plate, 180 µL of egg suspension was added, along with an extra 20 µL of nutrient media. The nutrient media consisted of one gram of yeast medium in 90 mL of normal saline and 10 mL of Earle's salt solution. The plate was then incubated at a temperature of 28°C for a duration of 48 hours. After this, the hatched larvae were observed using a light microscope at a magnification of 100x. An encapsulated bromelain solution (200 µL), with concentrations ranging from 0.125 mg/ml to 4 mg/ml, was then introduced into the wells. The numbers of dead and viable larvae were subsequently determined using a microscope set at a magnification of x100.

The adult worm's mortality assay (AWMA) was conducted following the procedure described by Eguale et al. (2007) and Hunduza et al. (2020). Ten actively motile adult worms obtained from the Ruiru slaughterhouse were placed in Petri plates containing NEB solutions of varying concentrations (0.125 mg/ml to 4 mg/ml). The worms were then observed for 24 hours. They were then placed in lukewarm fresh PBS for 30 minutes. A microscope was used to count the number of live and dead worms/larvae. The mortality rate for each concentration of extract was calculated using the following formula (Hunduza et al., 2020).

$$\text{Worm/larva mortality}\ (\%) = \frac{\text{Number of dead worms/larva}}{\text{Total number ofworms/larva}} \text{x}100$$

Determination of microbial contamination

Bacterial presence in the final product was evaluated as earlier described by Omorotionmwan et al. (2019). Briefly, crude extract of bromelain and NEB samples were inoculated onto nutrient agar (HiMedia Laboratories, India) and incubated at 37°C for 48 hours. The total plate count enumeration was done. After examination of the morphology of the colonies, the isolated bacteria were then gram-stained and further identified using biochemical tests, including catalase, Simmons-Citrate, methyl red / Voges-Proskauer (MR-VP), and urease.

Statistical analysis

The obtained data was entered and analyzed using Microsoft Excel (Microsoft, USA). The FTIR graphs were prepared using OriginPro 2023 (OriginLab, Germany version 10.0.5.157), while the anthelmintic activity graphs were prepared using GraphPad Prism (Dotmatics, United Kingdom, version 9.5.1). The statistical analyses were undertaken using analysis of variance (ANOVA) with a significance level set at $p < 0.05$.

RESULTS

Bromelain concentration

The concentration of bromelain after extraction from pineapples (Figure 1) was 4.3 ± 0.04 mg/ml. However, the concentrations decreased to 3.0 ± 0.31 mg/ml after dialysis and then to 1.2 ± 0.04 mg/ml after nanoencapsulation. The concentrations of bromelain did not differ significantly between the various batches ($p > 0.05$).

Fourier Transform Infrared Spectra of Nano-Encapsulated bromelain and Nano-encapsulated commercial bromelain

The effectiveness of the ionic gelation method in creating bromelain-loaded chitosan nanoparticles and the nature of interactions between bromelain and chitosan were evaluated using Fourier Transform Infrared (FTIR) analysis. The FTIR spectra of the chitosan matrix and chitosan nanoparticles loaded with bromelain are shown in Figure 2. The

chitosan spectrum exhibited an apparent and wide peak within the range of 3500 cm-1 to 3300 cm-1, which can be attributed to the stretching vibration of hydrogen-bonded O-H groups. The primary amines and the IA-type amide's N-H stretching peaks overlapped in the same area. The A-type amine's C-N stretching vibration is represented by the peak at 1317 cm-1, whereas the asymmetric C-O-C stretching peak is situated at roughly 1150 cm-1. There was no variation between peaks NEB in the various batches. The batches had the same transmittance as the standard (commercial bromelain).

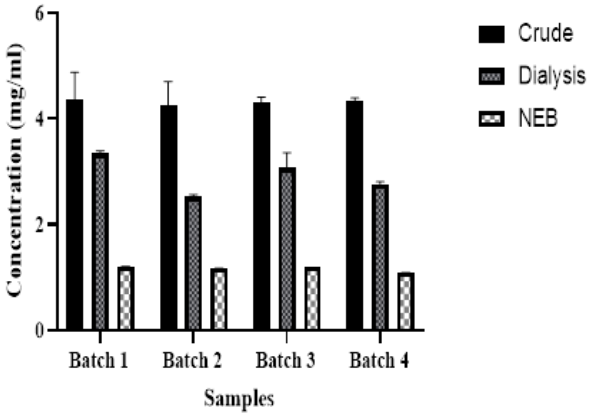

Figure 1. Concentration of bromelain from pineapple peels in the crude extract, after dialysis and nanoencapsulation of bromelain with Chitosan. NEB: Nanoencapsulated Bromelain

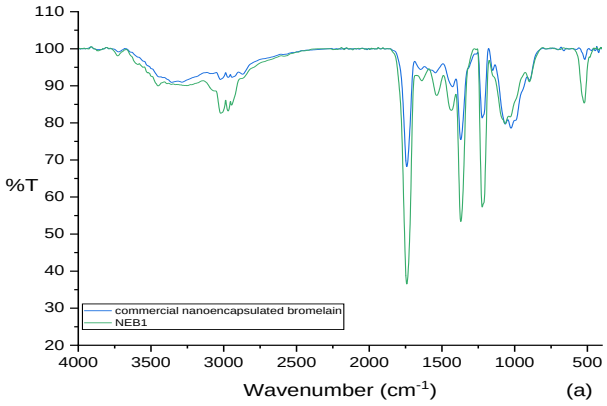

Figure 2. Fourier transform infrared spectra of the nano-encapsulated commercial bromelain and encapsulated extracted bromelain. Fourier transform infrared was prepared using OriginPro 2023. NEB: Nano-encapsulated bromelain. T: Transmittance

Anthelmintic activity of nano-encapsulated bromelain

Egg hatch assay

The results of the EHA are shown in Figure 3. The IC_{50} of the Albendazole was 0.25 ± 0.2 mg/ml, while the IC_{50} for the batches of commercial encapsulated bromelain (NCB) was 1 ± 0.5 mg/ml. The IC_{50} of the batches of NEB was 0.5 ± 0.3 mg/ml. There were no differences ($p > 0.05$) in the IC_{50} values of commercial nano-encapsulated bromelain and NEB. Further, there were no statistically significant differences ($p > 0.05$) in the IC_{50} of the various batches.

Larval mortality assay

The results of the Larval mortality assay **(**LMA) are presented in Figure 4. The IC_{50} of the Albendazole was 0.25 ± 0.3 mg/ml. The IC_{50} of NEB was 1.3 ± 0.5 mg/ml while that of NCB was 0.6 ± 0.3 mg/ml. There was no variation in the IC_{50} among NEB and NCB batches ($p > 0.05$).

Adult worm mortality

Figure 5 shows the results on the mortality of adult worms in *H. contortus*. The IC_{50} of Albendazole was 0.3 ± 0.2 mg/ml. The batches of NCB had an IC_{50} of 0.9 ± 0.05 mg/ml, while that of NEB was higher (IC_{50} = 0.245 ± 0.05 mg/ml, $p < 0.05$). The IC_{50} of the different batches did not show any statistically significant difference ($p > 0.05$).

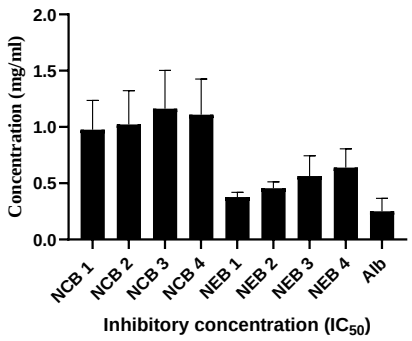

Figure 3. Inhibitory concentration (IC_{50}) in mg/ml of albendazole, nanoencapsulated bromelain (NEB), and commercial non-encapsulated bromelain (NCB) on eggs of *Haemonchus contortus.* The drugs were prepared in 4 batches (NCB 1, 2, 3, 4; NEB 1, 2, 3, 4). NEB: nano encapsulated bromelain; NCB: commercial nano encapsulated bromelain; Alb: Albendazole

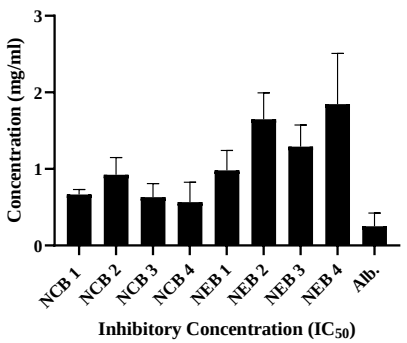

Figure 4. Inhibitory concentration (IC_{50}) in mg/ml of albendazole, nano encapsulated bromelain (NEB), and commercial nonencapsulated bromelain (NCB) on mortality of *Haemonchus contortus* larva. The drugs were prepared in 4 batches (NCB 1, 2, 3, 4; NEB 1, 2, 3, 4). NEB: nano encapsulated bromelain; NCB: commercial nano encapsulated bromelain; Alb: Albendazole \

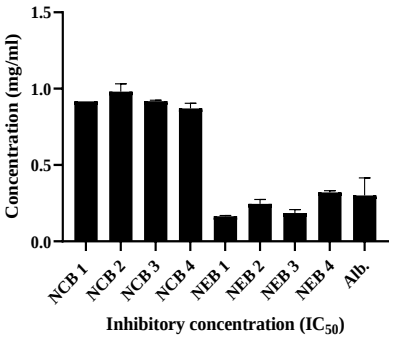

Figure 5. Inhibitory concentration (IC_{50}) in mg/ml of albendazole, nanoencapsulated bromelain (NEB), and commercial nonencapsulated bromelain (NCB) on adult *Haemonchus contortus* mortality. The drugs were prepared in 4 batches (NCB 1, 2, 3, 4; NEB 1, 2, 3, 4). NEB: nano-encapsulated bromelain; NCB: commercial nano-encapsulated bromelain; Alb: Albendazole

Microbial contamination

The total plate counts of the bacterial contamination in the crude pineapple extract batches ranged between 2000 CFU/ml and 7500 CFU/ml. The bacteria present in the crude extracts are shown in Table 1 and include *Proteus mirabilis, Klebsiella pneumoniae, Escherichia coli, Citrobacter freundii, Enterobacter cloacae Enterobacter aerogenes, Proteus vulgaris, Enterococcus faecalis, Staphylococcus aureus, Salmonella enterica, Serratia marcescens, Enterococcus faecium,* and *Klebsiella oxytoca*. The final product did not have any microbial contamination.

Table 1. Species of bacteria isolated from the crude extract of pineapples using biochemical methods in 4 batches isolated in Kenya

Isolated bacteria	Batch 1	Batch 2	Batch 3	Batch 4
Proteus mirabilis	✓			
Klebsiella pneumoniae	✓			✓
Escherichia coli	✓	✓	✓	✓
Enterobacter aerogenes	✓			
Enterococus cloaceae		✓		✓
Citrobacter freundii		✓		
Staphilococcus aureus		✓		
Enterococcus faecalis			✓	
Proteus vulgaris	✓		✓	
Enterococcus faecium			✓	
Serratia marcescens				✓
Klebsiella oxytoca		✓		✓
Salmonella enterica	✓			✓

DISCUSSION

The present study assessed the quality and efficacy of the laboratory-produced chitosan-encapsulated bromelain (NEB) compared to the nano-encapsulated commercial bromelain (NCB) against different parasitic stages of *H. contortus*. There were minimal variations in the concentrations of bromelain across the various batches which suggests some degree of reproducibility and the enzyme's relative stability during production. Similar concentration of bromelain were obtained by Daiba et al. (2022) in the crude extract and after encapsulation of bromelain. Omotoyinbo and Sanni, (2017) found that after 70% ethanol precipitation, total enzyme activity and total protein content also decreased. However, these authors showed that recovery rates for pineapple peel bromelain remained constant while specific activity and yield increased.

The FTIR spectra obtained for all batches of NEB and commercial nano-encapsulated bromelain (NCB) were similar. This additionally confirms the consistency and reliability of the observed spectral patterns. Devakate et al. (2009) reported similar FTIR spectra for extracted bromelain from the pineapple fruit. The different organic groups (O-H at 3500-3300 cm^{-1} for hydrogen bond, the amide N-H of type I'A and the amine of type A of the C-N observed at 1317 cm^{-1}, and the asymmetric groups C-O-C at 1150 cm^{-1}) obtained are similar to the results obtained by Wasso et al. (2020) and Daiba et al. (2022).

The present study showed that NEB had good activity against egg hatching, and the values obtained were close to those obtained by previous studies (Hunduza et al., 2020, Wasso et al., 2020). Anthelmintic activity has been investigated in various plant extracts. In a study conducted by Yongwa et al. (2020) and Thuo et al. (2017), extracts from *Albizia gummifera* and *Zanthoxylum usambarense* displayed significant anthelmintic activity against nematode eggs of *Haemonchus* spp., *Trichostrongylus* spp., and *Oesophagostomum* spp. For the study by Thuo et al. (2017), the inhibitory action (IC_{50}) of NEB on *H. contortus* eggs was 0.5 ± 0.3 mg/ml which is close to that reported in the present study. Further, the NEB activity obtained in the present study was comparable to those reported by Yongwa et al., (2020) in their study on *Senna italica* where they observed an IC_{50} of 0.69 mg/mL on inhibiting hatching of *H. contortus* eggs.

In the present study, the results obtained indicated that NEB has anthelmintic activity comparable to commercial bromelain in causing mortality of *H. contortus* larvae. Products that are able to limit nematode larval growth as well as cause mortality can be used to effectively control helminthiasis. Further in the current study, NEB exhibited high activity against adult worms which is similar to the results obtained by Hunduza et al. (2020) who showed that a concentration of

NEB at 0.2 mg/ml is required to kill adult worms. The activity of encapsulated bromelain in causing adult worm mortality was higher than that of inhibiting egg hatching and larval mortality. The variation in activity against adult and larval stages could be due to differences in the cuticle composition of the two stages (Hunduza et al., 2020). Previous studies have demonstrated that the cuticles of adult worms, which are fully grown, are the primary focus of bromelain activity (Daiba et al., 2022; 2023).

The adult worms' stage of *H. contortus* consume blood, leading to anemia in the host. In addition, the use of the encapsulated bromelain (NEB) can cause the death of adult worms, which in turn disrupts the parasite's life cycle by limiting egg production and decreasing environmental contamination.

The results of the current study showed that the crude extract of the pineapples had a high number of viable bacteria, whereas, in the final NEB product, there was no bacteria. This is similar to the study by Omorotionmwan et al. (2019), which isolated a variety of bacteria from pineapple peel. This could be due to contamination emanating from the production and harvesting of the pineapples. The primary bacteria observed in this study were *E. coli, S. aureus, E. faecalis, E.faecium, P. mirabilis, P. vulgaris, K. pneumoniae, P. oxytoca,* and *S. enterica*. In a study conducted by Omorotionmwan et al. (2019), it was observed that pineapple peels and pulp contained *Staphylococcus aureus, Streptococcus faecalis*, several *Bacillus* species, and *Clostridium* species. Dao et al. (2018) noted that the raw materials used in the production of pharmaceutical and cosmetic products frequently contained contamination derived from different bacteria. Therefore, the removal of these bacteria is necessary during the production process in order to guarantee the safety and quality of the product. Despite the initial high number of bacteria present in the crude extract of pineapples, the absence of viable bacterial cells in the final NEB product suggests successful bacteria elimination during the production process. The successful elimination of these bacteria in the final product illustrates that the production process effectively eliminates the bacteria.

CONCLUSION

The results of the current study indicated that the laboratory-produced NEB has anthelmintic activity against all stages of *H. contortus*, but it is more active against the adult worm stages. The laboratory production process demonstrates high levels of efficiency and consistency, particularly demonstrated by the consistent concentration levels of bromelain and the observed anthelmintic activity in the four batches of NEB. In addition, the method of encapsulating ensures the safety of the final product, thereby demonstrating high-quality assurance of laboratory-produced NEB. There is a need to undertake further development and commercialization of the product as an herbal anthelmintic drug for use in livestock.

DECLARATIONS

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Availability of data and materials

Inquiries regarding data availability should be directed to the respective authors.

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Authors' contributions

This work was completed with the assistance of all authors. The methods and design of the study were developed by authors Maanicus Bez-bang, Naomi Maina, and John Kagira. The author Maanicus Bez-bang undertook the encapsulation work, the *in vitro* analysis, and the statistical analysis. The authors Maanicus Rodolpher Bez-bang Kotangou, John Kagira, and Naomi Maina wrote the first draft of the manuscript. All the authors read and approved the final manuscript.

Competing interests

The authors have not declared any conflict of interest.

Ethical considerations

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

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The Effects of Adding Coconut Water to Egg Yolk Diluent on Motility, Viability, and Abnormality of Etawa Crossbred Goat Sperm

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ABSTRACT

The Etawah crossbreed goat is a dual-purpose type of goat that can adapt well to tropical regions in Indonesia. The current research aimed to evaluate the effects of adding coconut water to citrate egg yolk diluent on the spermatozoa quality parameters (motility, viability, and abnormality) of the Etawah crossbred goat at the physiology and reproduction laboratory of animal husbandry, Jambi University (Indonesia). The research employed a randomized block design on Etawa crossbreed goats aged around 2-3 years with an average weight of 12 kg per head in six groups. The treatments included 100% citrate diluent of egg yolk without adding coconut water (P0) as a control, 90% citrate diluent of egg yolk + 10% coconut water (P1), 80% citrate diluent of egg yolk + 20% coconut water (P2), 70% citrate diluent of egg yolk + 30% coconut water (P3), 60% citrate diluent of egg yolk + 40% coconut water (P4). The parameters evaluated in this study included viability of spermatozoa, spermatozoa motility, and spermatozoa abnormalities. The five treatment tubes were stored in a refrigerated cabinet at 5°C for 2 days. After this period, semen quality assessment was assessed microscopically. The percentage of live spermatozoa was determined using a staining technique. The spermatozoa motility was assessed based on their ability to move. Abnormal spermatozoa were calculated based on the number of abnormal spermatozoa compared to the total number of spermatozoa. The results of the study showed that the addition of 20% coconut water to the 80% citrate diluent of egg yolk (P2 treatment) reduced the rate of decline in spermatozoa viability and did not increase the number of spermatozoa abnormalities significantly, compared to other groups. There was no decrease in the viability of Etawah crossbreed goat spermatozoa during 2 days of storage at 5°C in all groups. Therefore, it was concluded that coconut water could be added up to 20% into the egg yolk without any significant negative effects on spermatozoa quality parameters evaluated in the current study.

Keywords: Citrate diluent, Coconut water, Egg yolk, Etawah crossbred goat, Spermatozoa resistance

INTRODUCTION

Etawah Crossbred goat is a dual-purpose type of goat that can adapt to tropical areas in Indonesia. This breed results from crossing the Etawah goat from India with local Indonesian goats. The purpose of growing Etawah Crossbred goats is to produce kids rather than for meat production (Pubiandara et al., 2016; Rezki et al., 2016; Barek et al., 2020; Tethool et al., 2022). Many goat mating systems are carried out naturally due to the lack of superior male goats, thereby reducing the productivity of goats (Baldaniya et al., 2020). Therefore, efforts are needed to optimize productivity through artificial insemination technology in Etawah Crossbred goats.

Increased livestock production is a crucial goal to meet the demand for animal protein. Achieving this goal relies on the farmer's capability and access to information about livestock management, especially livestock reproductive technology, which is essential for successful production (Tethool et al., 2022). Artificial insemination is one of the reproduction technology systems. Unfortunately, implementing artificial insemination in goats has not been carried out in-depth in Indonesia, compared to cows. This is due to the technical difficulties of artificial insemination in the field (da Silva Ferreira et al., 2014; Baldaniya et al., 2020; Saputro et al., 2022). Another persistent issue in the artificial insemination program is the semen preservation technique as it is necessary to maintain viability outside the body and minimize sperm mortality rates (Anakkul et al., 2014; Shafiei et al., 2015). Although there are many difficulties with artificial insemination in goats, many countries have successfully implemented this technology in goats (Abdi-Benemar et al, 2020).

Semen is the secretion of the male reproductive glands that are normally ejaculated into the female reproductive tract during copulation and can be collected for artificial insemination purposes (Saputro et al., 2022). According to Oliveira et al. (2014), the benefits of artificial insemination technology include improving the utilization of superior

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males, overcoming distance and time constraints, enhancing the genetic quality of livestock, preventing the transmission of diseases, and saving costs. Dilution is one of the methods used to reduce density and extend the survival of spermatozoa. An effective semen diluent must act as a buffer and provide nutrients as a source of energy for spermatozoa (Üstüner et al., 2015; Martínez-Fresneda et al., 2020).

Citrate egg yolk diluent has been widely used as a buffer medium to extend the survival of spermatozoa (Bustani and Baiee, 2021). One advantage of citrate buffer is that it can be mixed directly with egg yolk, serving as an energy source for spermatozoa, protecting them from cold shock, buffering to prevent pH changes due to lactic acid accumulation, and maintaining osmotic pressure and electrolyte balance (Salmani et al., 2013). Egg yolk is commonly used as a diluent since it can protect spermatozoa from cold shock with protective factors, such as lipoproteins and lecithin that act on the sperm cell membrane (Bustani and Baiee, 2021). In addition, egg yolk contains glucose, various proteins, fat-soluble vitamins, and beneficial viscosity for sperm cells (Bogdaniuk et al., 2022). Coconut water is a solution containing carbohydrates and sugars consisting of glucose, proteins, fats, and some minerals that can be used by spermatozoa as an energy source. However, coconut water does not contain cold shock factors like citrate egg yolk diluent that can anticipate a sudden drop in temperature (Baldaniya et al., 2020). The combination of coconut water and citrate egg yolk is expected to maintain the resistance of stored spermatozoa for up to 2 days. The novelty of this research lies in evaluating a combination of adding young coconut water ranging from 10% to 40% and egg yolk citrate ranging from 90% to 60%. The current research aimed to evaluate the effect of adding coconut water to citrate egg yolk diluent on the motility, viability, and abnormality of Etawah crossbred goat spermatozoa.

MATERIALS AND METHODS

Ethical approval

The Committee of Ethical Clearance of the Faculty of Animal Husbandry, Jambi University (Indonesia), has approved all of the research activities by providing a certificate of ethical clearance ref. 04/UN21.7/ECC/2023

Study design

This research was conducted in the physiology and reproduction laboratory of the faculty of animal husbandry, University of Jambi, Indonesia from June to July 2022. The diluent was made by preparing a citrate solution of 2.9 gr citrate dissolved in 100 cc of distilled water. Then, the citrate thinner and egg yolk were combined (Citric acid = 0.56 gr and egg yolk = 20 ml). After mixing, young coconut water (7-month-old coconut with green skin color) was added to the two diluents according to the specified treatment. The samples used in this study included semen from 2 to 3-year-old Etawah Crossbred male goats collected during the research period. Goat samples were collected from the Mat Beken goat farm in the Jambi City area of Indonesia during the research period with the average weight of goats being 12 kg per head. The samples for each treatment were repeated three times. All treatments appeared once in each replication, and randomization was carried out separately per group. The animals were growing in intensive goat farming. Drinking water was provided *ad libitum*.

The materials used included coconut water from the young coconut, egg yolk, a basic solution of sodium citrate as a diluent, NaCl 3%, eosin, as well as penicillin and streptomycin (1 mg/100ml). Young coconut water and egg yolk were purchased from young coconut and egg traders, and sodium citrate and eosin were obtained from the physiology and reproduction laboratory of the faculty of animal husbandry, University of Jambi. The equipment used in this study included an artificial vagina for semen collection, graduated test tubes, test tubes, a spatula, measuring glass, glass slides, cover slips, a refrigerated cabinet, an erythrocyte pipette, Neubauer counting chamber, an oven, an icebox, thermometer, aluminum foil, counter, syringe, spirit lamp, and microscope (Digital Binocular Microscope, Amscope, United States). This experimental study was designed using a randomized block design with five treatments and six groups. Sperm concentration for each treatment or each group was 3.09 ± 0.89 billion. The treatment in this study included P0 (100% egg yolk citrate + 0% young coconut water), P1 (90% egg yolk citrate + 10% young coconut water), P2 (80% egg yolk citrate + 20% young coconut water), P3 (70% egg yolk citrate + 30% young coconut water), P4 (60% egg yolk citrate + 40% young coconut water).

Research procedure

The process of mixing semen with diluent involved mixing 0.5 ccs of semen with dilution 10 times to get 5 ccs of a mixture of semen and diluent. Following this, each mixture was mixed with the respective treated diluent. Before conducting this research, the necessary equipment, including tools for semen collection (artificial vagina) and laboratory examination, was prepared. Subsequently, these tools were sterilized to ensure cleanliness and prevent contamination caused by microbes. Material sterilization through heating was performed to eliminate germs that could cause damage to

the materials (Toelihere, 1985). Before collecting semen, a diluent was prepared for semen dilution to ensure the quality of semen. Semen quality inspection was carried out by macroscopic and microscopic examination.

The semen from Etawah Crossbred goats was collected using an artificial vagina. After obtaining the semen, the volume obtained for each semen reservoir was 0.5 cc per ml, a macroscopic examination was conducted, including volume, color, odor, consistency, and pH. The semen was then stored in a temporary storage place (icebox) before being transported to the laboratory (physiology and reproduction laboratory of the faculty of animal husbandry, University of Jambi, Jambi Province, Indonesia) for initial microscopic examination (Magnification of 10 and 40 x), including spermatozoa motility and concentration. The semen was diluted according to the predetermined dilution composition, and then the five treatment tubes were stored in a refrigerated cabinet at 5°C for 2 days. After a 2-day storage period, the five tubes were stored in a refrigerator (5°C) for further examination. The observed variables included the percentage of live spermatozoa, spermatozoa motility, and spermatozoa abnormalities after storage for 2 days. The percentage of live spermatozoa was determined using a staining technique. The spermatozoa motility was measured based on the spermatozoa's ability to move. Abnormal spermatozoa were calculated based on the number of abnormal spermatozoa, compared to the total number of spermatozoa.

Statistical analysis

In this study, analysis of variance (ANOVA) was conducted using IBM SPSS Statistics version 26 to examine the significant effects of treatment on the measured parameters. The significant effects of treatment on the measured parameters ($p < 0.05$) were tested by Duncan's Multiple Range Test to compare the means of each treatment result.

RESULTS AND DISCUSSION

Table 1 shows the initial characteristics of the semen from the Etawah crossbred goats used in this study. As indicated, the initial conditions of Etawah Crossbred goat semen used in the study had an average volume of 0.55 ml, spermatozoa concentration of 3.09 billion, spermatozoa motility of 78.72%, percentage of live spermatozoa at 81.67%, and spermatozoa abnormalities at 7.40%. These parameters generally indicate that the semen meets the criteria for being considered studs. However, variations may occur depending on factors, such as the age and maturity level of the male goats, the skills of the semen collectors, and the frequency of semen collection (Wurlina et al., 2020). The declining semen volume tendency after collection may be attributed to the influence of excessively frequent collection.

According to Prastiya et al. (2021), goats suitable for breeding should produce spermatozoa with a volume ranging from 0.5 to 1.5 ml and spermatozoa concentration of 2 to 6 billion/ml. Moreover, spermatozoa motility should fall within the range of 65 to 90%. The findings of this study align with results from several other researchers (Mara et al., 2007; Musaffak et al., 2021), indicating that the parameters are within acceptable ranges. Frequent semen collection can lead to a decrease in semen volume (Bebas et al., 2018). Spermatozoa motility is influenced by the health of the breeding male, age, nutrition, collection frequency, and conditions on the collection day (Ducha et al., 2020). Influential factors on semen quality and quantity include genetics, libido, diseases, nutrition, transportation, and the environment (Susilowati et al., 2022).

Table 1. The initial characteristics of fresh semen in the Etawah crossbred goat

Description	Mean ± Standard Deviation
Volume (ml)	0.55 ± 0.07
Spermatozoa concentration (billion)	3.09 ± 0.89
Spermatozoa motility (%)	78.72 ± 0.78
Spermatozoa live percentage (%)	81.67 ± 2.87
Spermatozoa abnormality (%)	7.40 ± 2.08

The results indicated that adding young coconut water with egg yolk citrate diluent had a significant effect on the percentage of live spermatozoa ($p < 0.05$, Table 2). In this regard, treatment P2 yielded the best results (58.40%), followed by P3 (56.20%), P1 (52.80%), and P4 (54.00%). Treatment P2 achieved an optimal balance between egg yolk citrate diluent and young coconut water, effectively neutralizing metabolic waste, such as lactic acid and thereby prolonging the survival of spermatozoa. Conversely, treatments P1, P3, and P4 indicated an imbalance in neutralizing metabolic waste, potentially leading to inadequate neutralization of the remaining metabolic by-products and an elevated number of dead spermatozoa due to increased spermatozoa activity. In treatment P0, the percentage of live spermatozoa was 50.20%, which was lower than that in other treatments (P1, P2, P3, and P4). This condition indicated a lack of nutritional availability for the survival of spermatozoa in this treatment. Additionally, coconut water as a diluent contains

various sugars, such as glucose, sucrose, and fructose to meet the energy needs of spermatozoa during storage (Salmani et al., 2014; Huang et al., 2022; Zaenuri et al., 2023). Wondim et al. (2022) stated that the high activity of spermatozoa can lead to their death. The availability of sufficient nutrition for spermatozoa is very important to slow down the decline in the percentage of spermatozoa death and prevent the acceleration of metabolism. This prevention is crucial as it hinders the accumulation of lactic acid, which in turn reduces the pH of semen (Pahlevy et al., 2022). The accelerated accumulation of lactic acid can poison spermatozoa as it reduces semen pH. If a buffering solution within the medium does not neutralize the remaining metabolic by-products, it can result in poisoning and death of spermatozoa (Saputro et al., 2022; Huang et al., 2022). The analysis of variance revealed a significant effect of adding young coconut water to egg yolk citrate diluent on spermatozoa motility ($p < 0.05$, Table 3). Treatment P2 (52.60%) provided the highest spermatozoa motility, compared to other groups. This could be attributed to the optimal balance achieved in treatment P2, where the addition of 20% coconut water provided an ideal condition for maintaining spermatozoa viability during the 2-day storage period (Figure 1).

The average results for each treatment on spermatozoa motility revealed a decreasing trend in P4 and P0 (Figure 2). In treatment P4, it is likely that spermatozoa still had energy reserves to survive and undergo metabolism by utilizing glucose from egg yolk citrate and young coconut water. This observation is consistent with the findings of Michael et al. (2019), Souza et al. (2021), and Thiangthientham et al. (2023), who highlighted that spermatozoa store glucose in the form of glycogen, which is converted into ATP when energy is depleted. Then, in P0, there was also a decrease in spermatozoa motility probably due to the lack of nutrients for the survival of spermatozoa. This is consistent with studies conducted by Ranjan et al. (2020) and Nurcholis et al. (2021), indicating that sufficient nutrients for spermatozoa are crucial to maintaining the rate of decrease in spermatozoa motility.

Table 2. The effect of adding coconut water to citrate egg yolk diluent on the percentage of live spermatozoa in Etawa crossbreed goat

Coconut water addition (%)	Replication					Mean ± Standard Deviation
	1	2	3	4	5	
P0 (0)	50.00	50.00	50.00	53.00	48.00	50.20 ± 3.65^{C}
P1 (10)	52.00	50.00	55.00	57.00	50.00	52.80 ± 3.58^{BC}
P2 (20)	55.00	60.00	57.00	65.00	55.00	58.40 ± 6.05^{A}
P3 (30)	50.00	58.00	60.00	60.00	53.00	56.20 ± 4.62^{AB}
P4 (40)	60.00	55.00	50.00	55.00	50.00	54.00 ± 3.77^{B}

^{ABC}: The different capital superscript letters in the s column are significantly different at the 5% level; P0: 100% egg yolk citrate without young coconut water, P1: Combination of 90% egg yolk citrate and 10% young coconut water, P2: Combination of 80% egg yolk citrate and 20% young coconut water, P3: Combination of 70% egg yolk citrate and 30% young coconut water, and P4: Combination of 60% egg yolk citrate and 40% young coconut water.

Table 3. The effect of adding coconut water to citrate egg yolk diluent on spermatozoa motility male Etawa crossbreed

Coconut water addition (%)	Replication					Mean ± Standard Deviation
	1	2	3	4	5	
P0 (0)	36.40	37.00	42.10	44.10	45.04	40.93 ± 4.98^{B}
P1 (10)	39.70	46.00	52.60	53.40	46.19	47.58 ± 5.28^{A}
P2 (20)	48.80	53.70	52.80	55.60	48.51	52.60 ± 2.93^{A}
P3 (30)	44.40	46.70	53.60	54.50	46.91	49.14 ± 4.28^{A}
P4 (40)	53.20	41.00	45.20	46.36	44.18	45.99 ± 4.48^{AB}

^{AB}: Different capital superscript letters in the same column are significantly different at the 5% level; P0: 100% egg yolk citrate without young coconut water, P1: Combination of 90% egg yolk citrate and 10% young coconut water, P2: Combination of 80% egg yolk citrate and 20% young coconut water, P3: Combination of 70% egg yolk citrate and 30% young coconut water, and P4: Combination of 60% egg yolk citrate and 40% young coconut water.

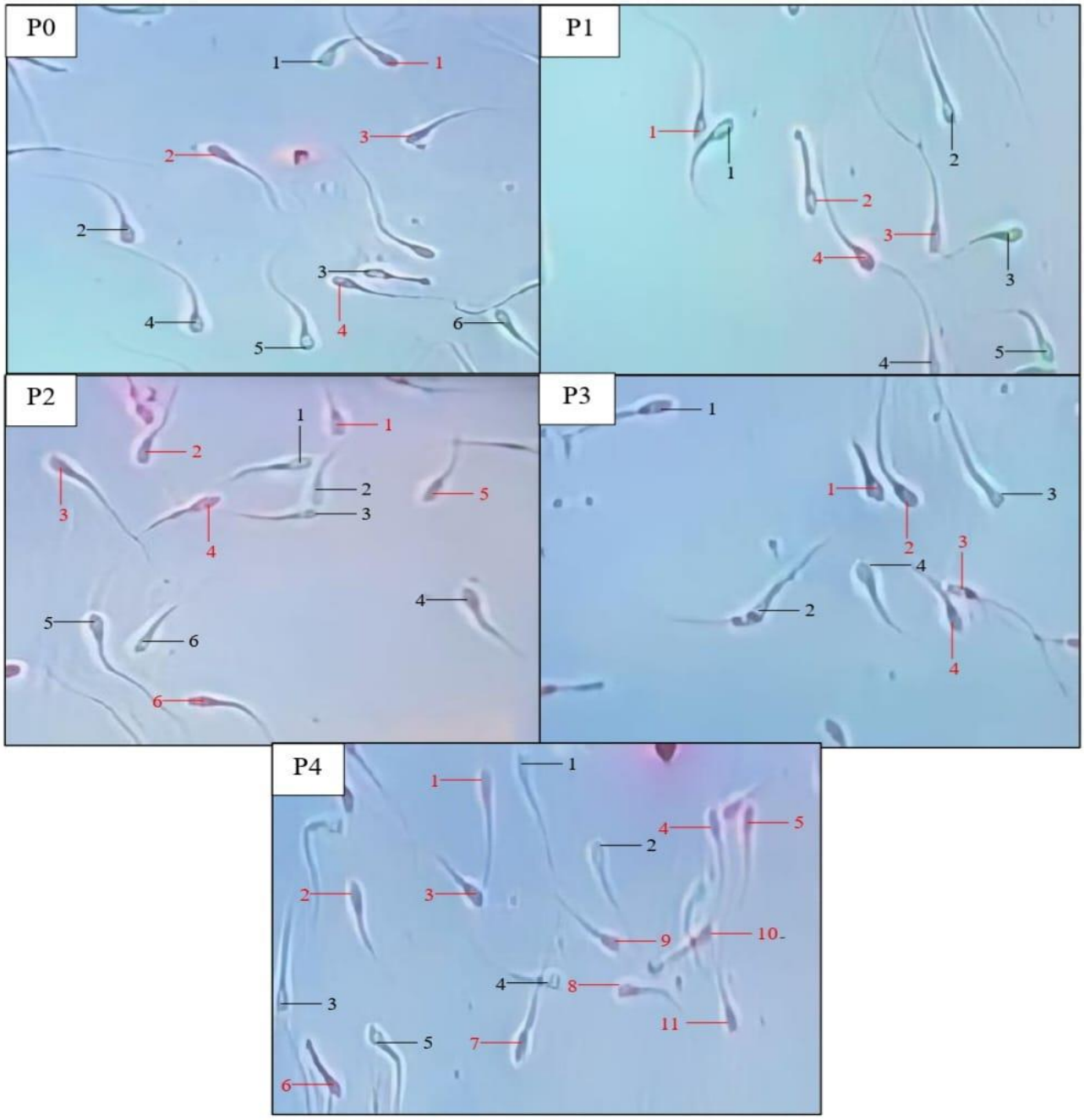

Figure 1. Viability of Etawah crossbreed goat sperm at 400x magnification. Black lines with numbers indicate live spermatozoa. Red lines with numbers indicate dead spermatozoa. P0: 100% egg yolk citrate without young coconut water, P1: Combination of 90% egg yolk citrate and 10% young coconut water, P2: Combination of 80% egg yolk citrate and 20% young coconut water, P3: Combination of 70% egg yolk citrate and 30% young coconut water, and P4: Combination of 60% egg yolk citrate and 40% young coconut water.

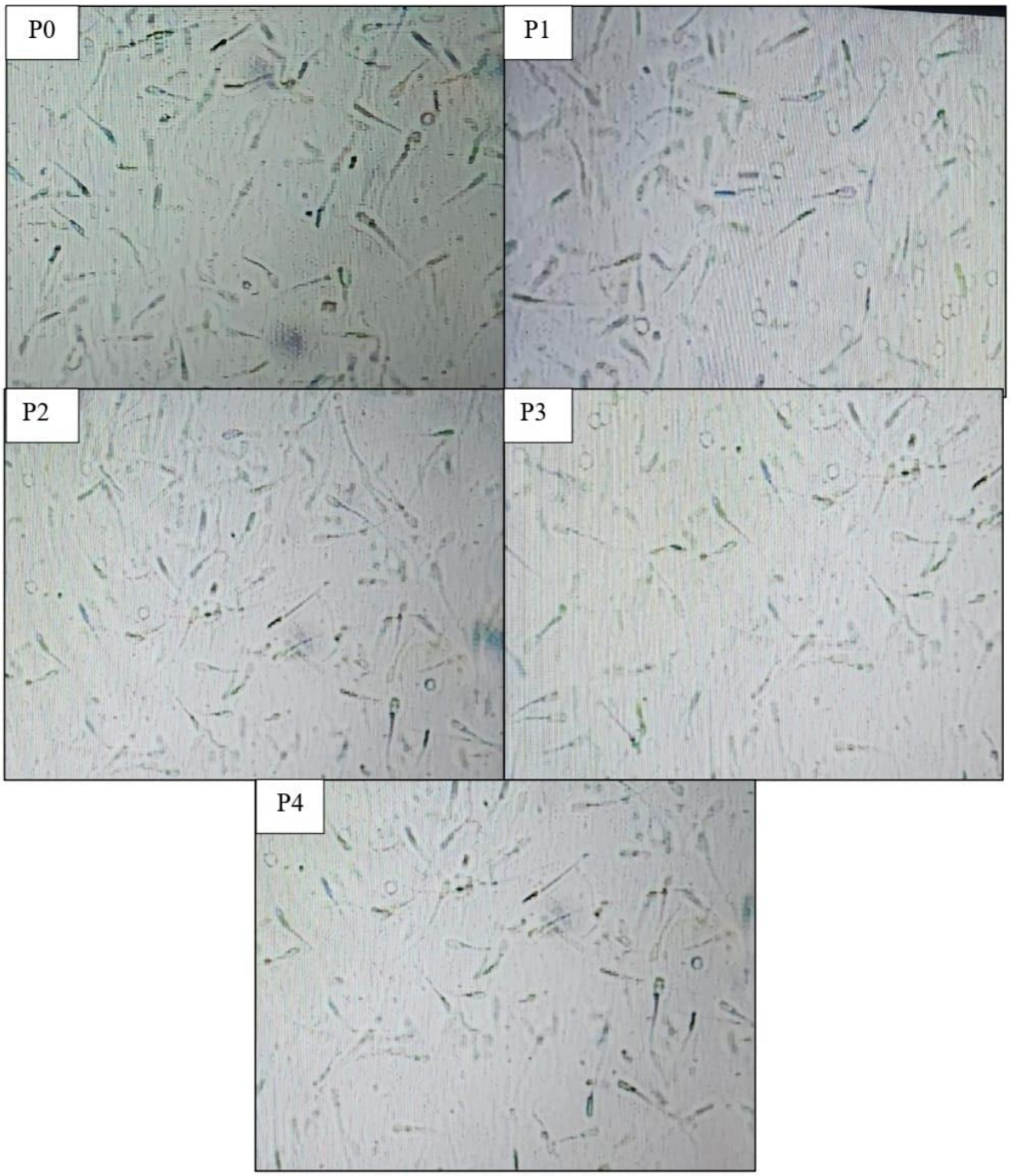

Figure 2. The sperm motility of Etawah crossbreed goats in different treatments (P0 to P4). P0: 100% egg yolk citrate without young coconut water, P1: Combination of 90% egg yolk citrate and 10% young coconut water, P2: Combination of 80% egg yolk citrate and 20% young coconut water, P3: Combination of 70% egg yolk citrate and 30% young coconut water, and P4: Combination of 60% egg yolk citrate and 40% young coconut water.

The results showed that storing semen from Etawah crossbreed goats did not significantly affect spermatozoa abnormalities between treatments ($p > 0.05$, P0, P1, P2, P3, and P4, Table 4). Moreover, the average percentage of spermatozoa abnormalities remained below 10% (Figure 3). This finding suggests that the 2-day storage period in a refrigerator can maintain the morphological condition of spermatozoa because the spermatogenesis process could affect spermatozoa abnormalities in the seminiferous tubules and during the movement of spermatozoa in the epididymis. In this study, the average percentage of spermatozoa abnormalities in Etawah Crossbred goats only involved primary abnormalities and did not vary significantly between treatments. Studies by Lukusa and Kabuba (2020) and Diansyah (2022) indicated that abnormalities or abnormal shapes of spermatozoa from failed spermatogenesis processes include coiled tails, broken necks, severed heads and necks, double heads, and double tails. This was also emphasized by Zamiri (2020), Nurcholis et al. (2021), and Macêdo et al. (2022) indicating that abnormal shapes resulting from failed spermatogenesis processes include large heads (macrocephalus) or small heads (microcephalus), short heads, broad heads, and double tails.

The preparation of smears and the storage process can lead to abnormalities in spermatozoa. The preparation of smears can result in abnormalities, such as coiled tails, broken necks, and severed heads and necks (Üstüner et al., 2015; Sutama, 2021). If the percentage of spermatozoa abnormalities exceeds 15%, it means that goats are experiencing infertility and cannot fertilize eggs (Menéndez-Blanco et al., 2019). Anand et al. (2017) and Wurlina et al. (2020) reported that spermatozoa abnormalities can be caused by the influence of semen pH, osmotic pressure, and cold-shock stress during storage.

Table 4. The effect of adding coconut water to citrate egg yolk diluent on spermatozoa abnormality in male Etawa crossbreed goats

Coconut water addition (%)	Replication					Mean ± Standard Deviation
	1	2	3	4	5	
P0 (0)	2.65	4.14	2.00	6.30	4.00	3.81 ± 1.66
P1 (10)	5.15	2.50	5.20	2.50	7.40	4.55 ± 2.08
P2 (20)	3.30	4.70	2.45	2.00	7.10	3.91 ± 2.06
P3 (30)	4.55	4.30	5.50	2.88	7.40	4.92 ± 1.67
P4 (40)	5.60	4.30	2.38	2.51	2.00	3.35 ± 1.54

P0: 100% egg yolk citrate without young coconut water, P1: Combination of 90% egg yolk citrate and 10% young coconut water, P2: Combination of 80% egg yolk citrate and 20% young coconut water, P3: Combination of 70% egg yolk citrate and 30% young coconut water, and P4: Combination of 60% egg yolk citrate and 40% young coconut water

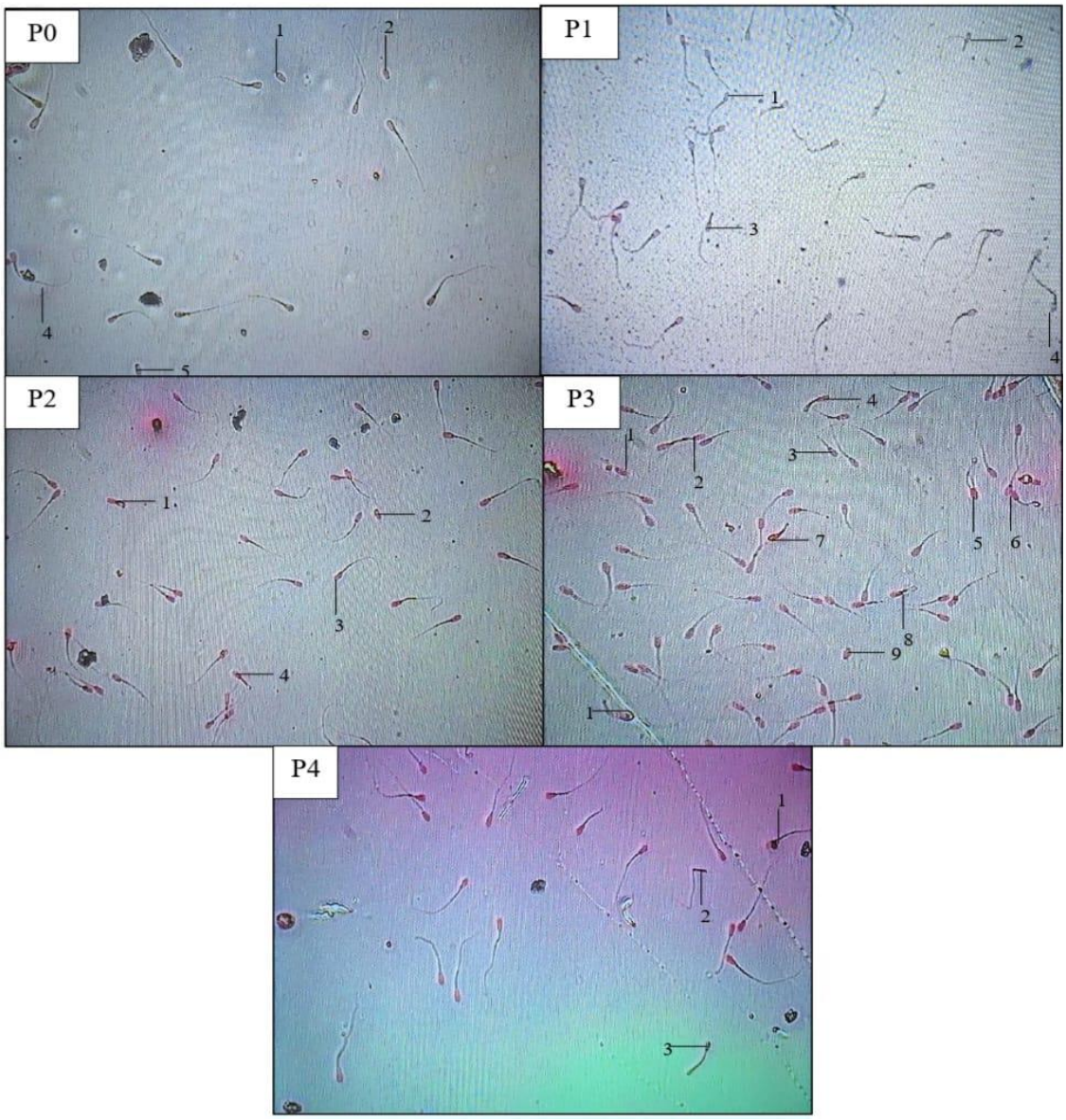

Figure 3. Etawah crossbreed goat sperm abnormalities, 400x magnification. Black lines with numbers indicate abnormalities. Red-colored spermatozoa indicate that they are classified as abnormal P0: 100% Egg Yolk Citrate without Young Coconut Water, P1: Combination of 90% Egg Yolk Citrate and 10% Young Coconut Water, P2: Combination of 80% Egg Yolk Citrate and 20% Young Coconut Water, P3: Combination of 70% Egg Yolk Citrate and 30% Young Coconut Water, and P4: Combination of 60% Egg Yolk Citrate and 40% Young Coconut Water.

CONCLUSION

It was concluded that the addition of up to 20% young coconut water to the egg yolk citrate diluent could reduce the rate of decline in spermatozoa viability in Etawah crossbreed goats and did not increase the number of spermatozoa abnormalities significantly. Further research is needed to evaluate other diluents in different temperatures.

DECLARATIONS

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Availability of data and materials
The data of the current study are available by a reasonable request.

Ethical considerations
The authors confirmed that the manuscript has been reviewed and submitted to this journal for the first time. The text of the manuscript was checked for plagiarism by authors before submission and all sentences were written by authors originally.

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Authors' contributions
Fachroerrozi Hoesni conceptualized, managed, and supervised the study. Fachroerrozi Hoesni and Firmansyah drafted the manuscript and performed all the experimental procedures. Fachroerrozi Hoesni, Firmansyah, Sri Arnita Abutani, and Nurhayati conducted data analysis and interpretation. All authors read and approved the final manuscript.

Conflict of interests
There is no conflict of interest to declare.

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Effects of Different Seasons on Milk Quality: A Study on Two Cattle Breeds in Rainy and Drought Contexts

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ABSTRACT

The primary focus of dairy farming in the central region of Peru centers on producers. However, there is limited information on how different altitudinal zones, particularly during rainy and dry seasons, affect milk production. The present study aimed to investigate the effects of the rainy and dry seasons on the physicochemical properties of milk from Brown Swiss and Holstein cattle. A total of sixty cows were selected for the study, comprising 30 Brown Swiss and 30 Holstein. The study was conducted across two distinct seasons, including rainy and dry seasons. All animals received the same semi-intensive management and were fed ryegrass and balanced feed. Each animal provided 500 ml of milk for analysis in the morning. The milk was transported at a temperature of 2 °C, where they were analyzed with the Lactoscan equipment to evaluate protein, lactose, fat, total solids, milk density, freezing point, salts, and pH. Significant differences were observed in the interaction between Brown Swiss and Holstein breeds across different seasons, including rainy and dry periods. Significant differences were observed in protein content, showing a positive effect in the interaction "rain: Brown" a value of 3.50 ± 0.36, while "rain: Holstein" showed 3.14 ± 0.05. Statistical differences were observed in the interactions for lactose content, with rain: Brown at 4.82% and dry: Holstein at 4.37%. Similarly, there were significant differences in fat content and total solids for rain interaction of rain: Holsten, and dry: Brown. Nevertheless, no differences were observed in terms of milk density, freezing point, salts, and pH. It is concluded that there was an influence of the interaction between breed physiology and seasonal conditions on milk composition. The results also highlight the impact of season-specific environmental factors on the quality of milk.

Keywords: Breed comparison, Milk composition, Milk quality, Physiological stability

INTRODUCTION

Climate affects the growth and quality of forage and feed crops. Droughts, floods, and extreme weather events can reduce the availability of high-quality feed, leading to lower milk production and altered milk composition. Dairy production holds a prominent position in global agribusiness, as it plays a crucial role in supplying essential nutrients for human nutrition (Britt et al., 2018; Godfrey et al., 2019), while also contributing to employment and income generation in numerous countries (Loos and Zeller, 2014; Garcia-Olarte et al., 2024a). Milk, as a cornerstone of the economic sector, is highly valued for its nutritional richness, providing essential nutrients crucial for growth, particularly during infancy, and supporting overall health (Barłowska et al., 2011).

Ensuring food quality is paramount to guaranteeing food safety and safeguarding human health, while also maintaining the nutritional integrity of the food supply (Sachs, 2012; Hernandez et al., 2019; Kamboj et al., 2020). However, many producers lack the means to access or evaluate the nutritional or chemical composition of milk, especially in rural areas where production methods may be rudimentary and resources limited (Anstalt, 2013).

The physicochemical composition of milk represents critical variables in assessing its quality (Rodriguez et al., 2015; Guajardo et al., 2020; Garcia-Olarte et al., 2024b). Milk is a complex mixture of proteins, carbohydrates, fats, minerals, vitamins, and other constituents (Fox, 2008; Guetouache et al., 2014). Among various types of milk, cow's milk remains predominant (Vanga and Raghavan, 2018), with all milk varieties generally sharing identical constituents, albeit in different concentrations, basic nutritional elements, such as protein, fats, and minerals in all types of milk, the specific amounts of these components can differ significantly depending on the source and processing methods and especially the season (Ceballos et al., 2009). The chemical composition of specific elements and minerals commonly found in milk is of significant importance due to their absorption in the gut and subsequent biological utilization, these

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components play essential roles in maintaining health, growth, and energy supply for consumers, including transport, assimilation into cells, and conversion into biologically systemic forms (Zamberlin et al., 2012).

In Peru, while there are existing reports on the physicochemical properties of milk (Vilca and Oyarce, 2022), misinformation persists due to the diverse altitudinal zones, varied feeding practices, and differing temperature and humidity conditions across regions. It is noteworthy that the majority of producers in the central region of Peru primarily focus on cattle milk production (Wuletaw et al., 2011). However, there is lack of information on how altitudinal zones, particularly during rainy and dry seasons, impact milk production. This knowledge gap is significant given Peru's mega-diverse environment characterized by highly variable climates. Recent studies emphasize the significant impact of climate, particularly the rainy and dry seasons, on milk production and composition in dairy cows (Narmilan et al., 2021). Seasonal heat stress leads to reduced milk production, primarily due to decreased feed intake and altered metabolic processes (Wankar et al., 2021). Typically, milk production is higher during the cooler months. Additionally, genetic selection for heat-tolerant traits can enhance resistance to seasonal stress (Moore et al., 2023). Understanding the influence of seasons and breeds is crucial for optimizing milk production and improving the quality of dairy products. Therefore, the present study aimed to investigate the effects of the rainy and dry seasons on the physicochemical properties of milk from Brown Swiss and Holstein cattle in the central highlands of Peru.

MATERIALS AND METHODS

Ethical approval

Explicit approval for the research procedures was granted under the official sanction of letter Nº 006-GRJ-DRA-AAC-PERÚ-2023, dated September 25, 2023. Furthermore, the execution of all research protocols was conducted with due diligence and in full compliance with the explicit permission and authorization obtained from the pertinent overseeing authorities. The research was conducted in strict accordance with both international and national guidelines governing the ethical care and utilization of research animals.

Study area

The studies were conducted at the Mantaro Experimental Station, located in the Mantaro District, Jauja Province, in the Junín region of Peru (Figure 1). The station is located at an elevation of 3320 meters above sea level, with an average temperature ranging from 4°C to 8°C and an average precipitation of 749 mm (Senamhi, 2023). The research was carried out over two distinct periods of the year, namely the rainy season (April-June) and the dry season (July-September, 2023).

The Mantaro Experimental Station serves as a pivotal research site due to its strategic location within the Junín region, offering insights into the ecological dynamics and agricultural practices pertinent to the area. The station's altitude, coupled with its unique climatic conditions characterized by relatively low temperatures and moderate precipitation, makes it an ideal setting for studying the effects of seasonal variations on agricultural productivity and ecosystem functioning.

Animal and study design

Samples of milk (n = 60) were collected from 60 female cattle, comprising 30 Brown Swiss and 30 Holstein cows aged 6 years, all animals were from their fourth calving (Figure 2b). The animals were divided into two groups, including 15 for the rainy season and 15 for the dry season, for both breeds. Milk samples (500 ml) were collected once at 6:00 a.m. (Figure 2c). The animals were grazed on alfalfa (Figure 2a) and supplemented with commercial feed (Corina, Peru), which contained 14 % CP and 2.7 Mcal. Provided twice daily, in the morning before and evening after grazing. The feed was assessed at the Laboratory of Animal Nutrition of the Facultad de Zootecnia at the Universidad Nacional del Centro del, Perú. The physicochemical characteristics of the milk were evaluated with the Lactoscan (Figure 2e), which uses ultrasound methodology to determine temperature, fat content, density, fat solids, protein content, freezing point, salts, total solids, lactose, and pH (Figure 2f).

Data collection

For the laboratory analysis of milk samples, the following milk sample collected, an aliquot of approximately 120ml was obtained using a sample holder for its corresponding analysis in the Lactoscan Model SP of Peru (Figure 2e). Before operating and running the samples with the lactoscan equipment, calibration, and configuration were carried out using cow milk samples considered as standards (Alinezhad et al., 2024). This operation was performed in triplicate to enhance the reliability and accuracy of the results. Once the equipment was calibrated and standardized, the physical-chemical analysis of the samples was conducted, and the results and readings were visualized on a personal computer. It

is worth mentioning that the parameters and standards for milk collection were followed (Bardales et al., 2024). Sampling was carried out both in the morning and in the afternoon, depending on the milking and milk storage schedule, the distance from the livestock facilities, as well as the time of samples collection in the field. To analyze each milk sample values, such as fat, non-fat solids, density, lactose, protein, pH, total solids, freezing point, and salts were considered.

Statistical analysis

The analyzed data were recorded and organized in Microsoft Excel. Seasonal (rain and dry season) differences were performed using analysis of variance (ANOVA). A comparison test was then performed. Values of $p < 0.05$ were considered significant and all statistical analyses were performed using CRAN R software (Team et al., 2018).

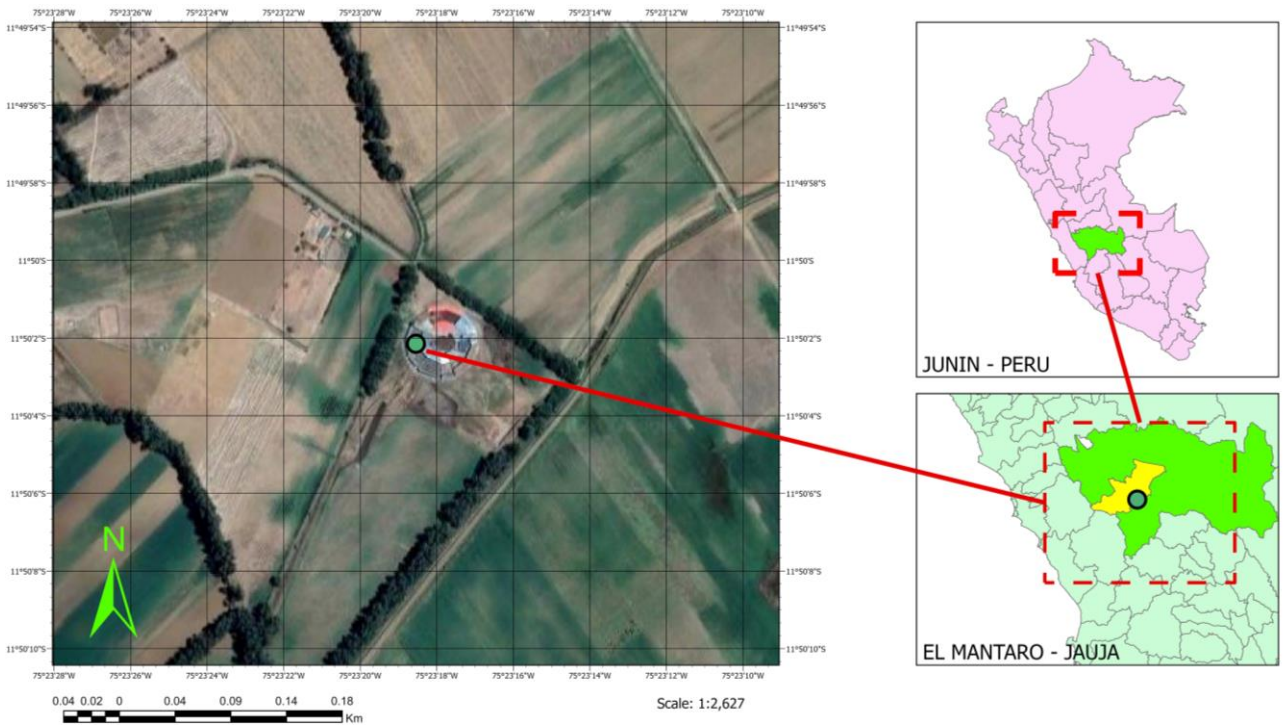


Figure 1. Location of study in Peru, specifically in the Junín region and within Junín, the research focused on El Mantaro, located in the province of Jauja

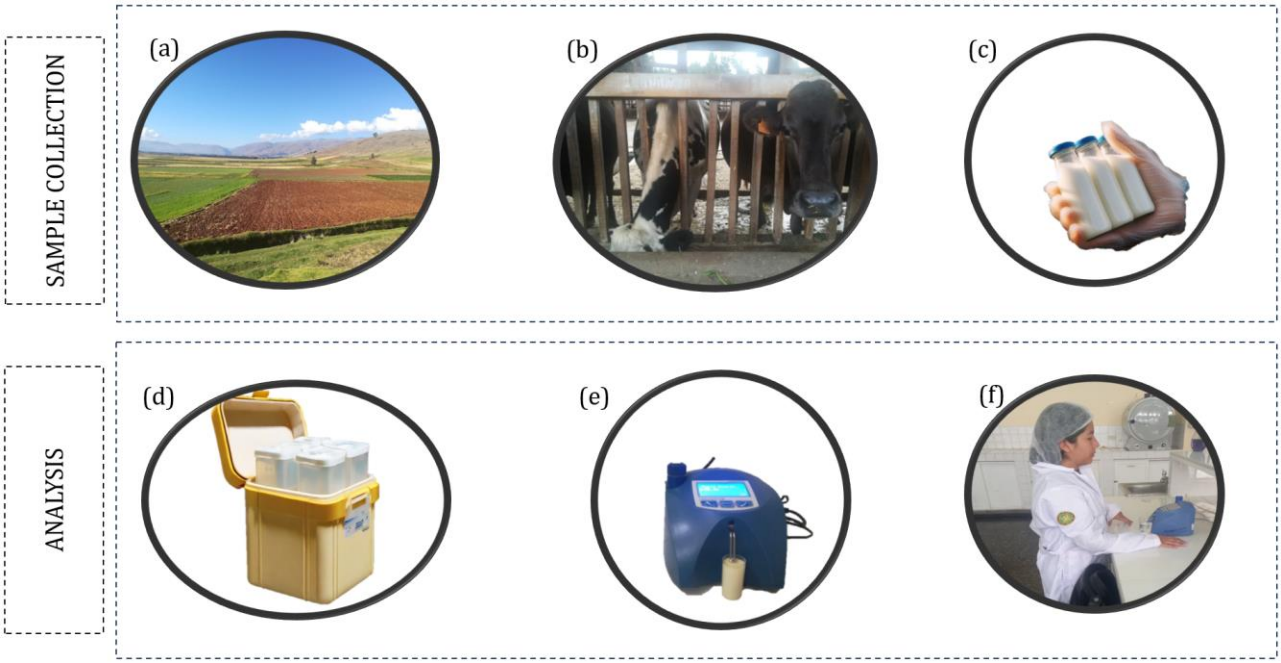
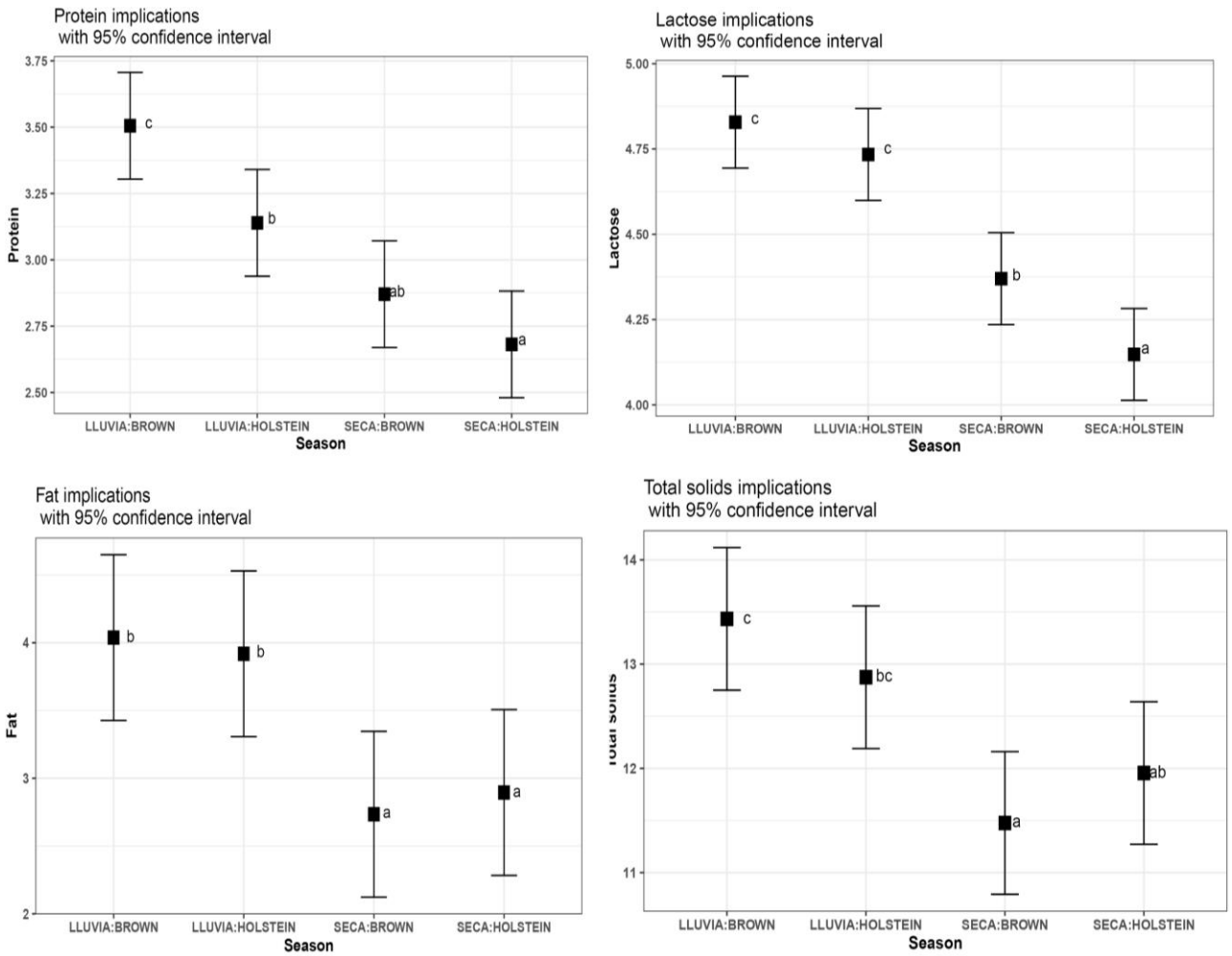


Figure 2. Methodology procedure for data collection. a: Grazing forage, b: Research animals, c: Milk samples, d: Refrigeration for sample transport, e: Lactoscan, f: Sample analysis.

RESULTS

In Graph 1, significant differences in the interaction between Brown Swiss and Holstein breeds and seasons (rainy and dry) are evident ($p < 0.05$). Variations are noticeable in the protein content (%), with the rain: Brown interaction showing a value of 3.50 ± 0.36 %, whereas rain: Holstein displayed 3.14 ± 0.05 %. Additionally, differences between the rainy and dry seasons, with values of 3.32 ± 0.31% and 2.77 ± 0.35%, respectively, were observed. In terms of lactose content (%), significant statistical differences were observed, with higher yields during the rainy season. The values were 4.83 ± 0.23 for rain: Brown and 4.73 ± 0.23 for rain: Holstein, respectively. However, there were statistical differences between dry: Brown and dry: Holstein with 4.37 ± 0.20 and 4.14 ± 0.10, respectively ($p < 0.05$). Furthermore, statistical differences were observed between the rainy and dry seasons, with values of 4.78 ± 0.23% and 4.26 ± 0.19%, respectively ($p < 0.05$). Regarding fat content (%), significant differences are also found in the rain: Brown and rain: Holstein" interactions with values of 4.04 ± 1.15 and 3.92 ± 1.13, respectively, as well as in "dry: Brown" and "dry: Holstein with 2.74 ± 0.59 and 2.89 ± 0.70, respectively ($p < 0.05$). As for total solids, slight differences are observed between the rain: Brown and rain: Holstein interactions, with values of 13.43 ± 0.87% and 12.87 ± 1.01%, respectively, and similarity between dry: Brown and dry: Holstein, with values of 12.87 ± 1.01% and 11.48 ± 0.48%, respectively. Additionally, significant differences between rainy and dry seasons are noted, with values of 13.15 ± 0.97% and 11.72 ± 1.11%, respectively ($p < 0.05$).

Regarding density percentage, as detailed in Table 1, no statistically significant differences were observed, suggesting a similar behavior for both the interaction between breeds and seasons ($p > 0.05$). Concerning non-fat solids (%), similarities were found between the rain: Brown and rain: Holstein interactions, with values of 9.07 ± 0.77% and 9.08 ± 0.97%, respectively; similarly, similarities were observed between the dry: Brown and dry: Holstein interactions, with values of 8.01 ± 0.67% and 8.29 ± 0.49%, respectively. Regarding freezing point, salts, and pH, no significant differences were detected in the interactions between breeds and seasons ($p > 0.05$).



Graph 1. Averages of protein, lactose, fat, and total solids in the interaction of cow's milk with rainy and dry seasons, and breed (Brown Swiss, and Holstein) in Perú. Equal letters do not show significant differences ($p > 0.05$)

Table 1. Mean physicochemical yields of milk in two breeds (Brown Swiss, and Holstein), and seasons (rainy and dry)

Performance	Rainy season		Dry season		P-value
	Brown Swiss	Holstein	Brown Swiss	Holstein	
Physicochemical					
Density (%)	1.0296 ± 0.0034[a]	1.0292 ± 0.0039[a]	1.0309 ± 0.0036[a]	1.0308 ± 0.0035[a]	0.5581
Non-fat solids (%)	9.07 ± 0.77[a]	9.08 ± 0.97[a]	8.01 ± 0.67[b]	8.29 ± 0.49[b]	0.0021
Freezing point	-0.56 ± 0.03[a]	-0.53 ± 0.03[a]	-0.57 ± 0.04[a]	-0.57 ± 0.05[a]	0.0923
Salts (%)	0.69 ± 0.05[a]	0.66 ± 0.04[a]	0.72 ± 0.05[a]	0.73 ± 0.08[a]	0.0832
pH	6.51 ± 0.29[a]	6.60 ± 0.19[a]	6.78 ± 0.09[a]	6.77 ± 0.41[a]	0.0721

[a,b] Equal superscript letters in the same row do not differ significantly from each other at 95%.

DISCUSSION

Regarding protein (%), superior results were observed during the rainy season for both cow breeds. According to Toni et al. (2011) and Morton et al. (2016), protein percentage values ranged from 3.0 to 3.2% in Holstein cow's milk and reached 3.5% in Brown Swiss cow's milk. These values were recorded 7 days postpartum in cows fed concentrate-based diets. Similarly, Yang et al. (2013), reported milk protein values of 3.10 %, in Holstein, and 3.51 % for Brown Swiss cows fed a concentrated diet meeting their nutritional needs and undergoing seasonal changes. These similarities stem from the use of Holstein breeds and a concentrated feeding system tailored to the animals' requirements, along with samples collected from adult cows experiencing seasonal variations between rainy and dry periods. The lactose percentages (4.78%) are similar to those reported by Costa et al. (2019), who compiled data from several countries, including New Zealand, Ireland, the United States, and Canada. The findings indicated that lactose percentages range from 4% to 4.9%, reflecting a standard expectation for lactose content in Brown Swiss and Holstein breeds. Regarding fat %, noticeable differences between seasons exist, with values of 3.98 ± 1.11 % for rainy (Brown Swiss) and 2.81 ± 0.63 % (Holstein) for dry. These values slightly exceed those reported by Toni et al. (2011), who found milk fat results of 3.91% in Holstein. The variance could be attributed to dietary intake differences, with the current research utilizing grazing with alfalfa compared to solely concentrated feed. The disparity between the rainy season's impacts on Brown and Holstein breeds is attributed to breed physiology (Golan and Assaraf, 2020), with Brown Swiss showing higher nutrient content than Holstein (Stocco et al., 2018). Holstein breed characteristics are more reflected in milk quantity (Braunschweig et al., 2000). Differences observed between rainy and dry seasons could be attributed to better quality and quantity of green forage during the rainy season, a significant factor in milk production compared to the dry season, where feed scarcity occurs.

In milk density, freezing point, salts, pH, and uniformity in milk composition no notable differences were observed between breeds and seasons, that could be attributed to various factors. Firstly, physiological stability may have influenced it, given that the studied animals were of similar ages (Pereira et al., 2008; Madhusoodan et al., 2019). Additionally, genetic adaptations could have played a significant role; being native to the study region (Casey and Van Niekerk, 1988), cows likely developed inherent resistance to abrupt changes in temperature and climate (Sutarno and Setyawan, 2016). Furthermore, consistent management and nutrition practices may have contributed to maintaining uniformity in milk composition (Zhou et al., 2022). All animals were fed and managed consistently, minimizing potential variations in milk quality (Zhu et al., 2018). Measures (similar management, feeding, milking machine, personnel, and sanitation) were implemented to control environmental factors that could have influenced the results, ensuring a controlled environment for the study. These combined factors likely contributed to maintaining stability in milk quality in terms of density, freezing point, salt content, and p/ for both Brown Swiss and Holstein cows, during the two seasons of the year.

CONCLUSION

The results reveal significant differences in protein, lactose, fat, and total solids contents between Brown Swiss and Holstein breeds, as well as between rainy and dry seasons. These disparities reflect the influence of the interaction between breed physiology and seasonal conditions on milk composition. Furthermore, they underscore the impact of environmental factors specific to each season on milk quality. Uniformity (density percentage, freezing point, salts percentage, and pH) in milk composition suggests stability influenced by factors, including physiological similarities, genetic adaptations, consistent management practices, and controlled environmental conditions. The obtained results underscore the resilience and consistency of milk quality in both breeds across the different seasons. It is recommended to conduct a study on the other breeds, such as Simmental, which has recently seen a rise in breeding in the central region of Peru.

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Availability of data and materials
All data from this study are available upon reasonable request from the authors.

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Authors' contributions
Estremadoyro Leonor Guzman supervised the investigation throughout the execution; Paucar Huaman Salome carried out the complete execution of the investigation; Jordan Ninahuanca Carhuas performed the statistical analysis; Salvador Ore Guzman edited the photographs, as well as their interpretation; Armando Aquino Tacza monitored the animals throughout the investigation; Maria Antonieta Flores Guillen was in charge of the analysis in the laboratory with the necessary equipment.; Edgar Garcia-Olarte collects samples and data for statistics. All the authors read and approved the final version of the manuscript.

Competing interests
The authors have not declared any conflict of interest.

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Evaluation of Stored Whole Blood and Monitoring the Health of Dogs After Transfusion Using Fresh Whole Blood, Stored Whole Blood, and Packed Red Blood Cells

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ABSTRACT

Blood products have been widely used in emergencies and treatment, necessitating optimal storage conditions to maintain quality. The current study aimed to evaluate the blood quality during storage, transfusion effectiveness, and reactions during and after transfusion in dogs. Five Greyhounds, including three males and two females aged 2.5 years old, and with 25-30 kg bodyweight, were selected and randomly labeled N1, N2, N3, N4, and N5. Fresh whole blood, stored whole blood, and packed red blood cells from the samples dogs were used for transfusion in the study. The investigated parameters were total protein (TP), aspartate transaminase (AST), alanine transaminase (ALT), alkaline phosphatase (ALP), lactate dehydrogenase (LDH), mean corpuscular volume (MCV), total carbon dioxide (tCO_2), creatine kinase (CK), creatinine (CREA), blood urea nitrogen (BUN), glucose (GLU), white blood cells (WBC), red blood cells (RBC), hematocrit (HCT), plaletes (PLT), calcium (Ca), phosphorus (P), chloride (Cl), manganese (Mg), sodium (Na), and potassium (K). The results indicated that all parameters of stored blood samples were in the normal range during 28 days of storage in a refrigerator at 2-4°C. However, some parameters (TP, AST, ALT, ALP, LDH, MCV, tCO_2, and K) increased, while others (CK, CREA, BUN, GLU, WBC, RBC, HCT, PLT, Ca, P, Cl, Mg, and Na) decreased during the storage period, especially Ca, P, and Na were below the normal range. All dogs indicated no reactions during and 5 hours after transfusion. However, dogs had symptoms of inappetence and mild diarrhea in 1-2 days after transfusion. Dogs received fresh whole blood recovered on day 3, while dogs of the stored blood recipient group recovered on day 4. By day 5, all dogs were healthy with no abnormal signs. The findings indicated the presence of hematological and biochemical alterations in stored blood, highlighting the importance of considering transfusion of stored blood for patients with critical medical conditions.

Keywords: Dog, Fresh whole blood, Packed red blood cell, Stored whole blood, Transfusion

INTRODUCTION

Although the first time dogs were successfully transfused with blood was in 1665, blood transfusion in dogs and cats has become commonplace in over the last 60 years (Yagi and Holowaychuk, 2016a; Khan and Sharma, 2021). Blood products are widely used in emergencies and treatment. Transfusion is an effective therapy and is considered to be life-saving, which can be used in emergencies and can save dogs in severe anemia cases with a high risk of death (Hann et al., 2014). However, transfusion of red blood cell (RBC) concentrates might cause adverse effects in the recipient, particularly when stored for more than 2 weeks (Herring et al., 2013).

To ensure the utmost quality and safety of blood and blood products, the entire process must be carried out using a standardized system. The donors must be healthy, fully vaccinated, and carrying no infectious diseases (Yagi and Holowaychuk, 2016b). The blood collecting system and blood storage must be appropriately carried out. The hematology and serum biochemistry blood profile must be periodically tested. The hematological profile includes measurement of hemoglobin (HB), hematocrit (HCT), or packed cell volume (PCV), red cell indices (mean corpuscular volume [MCV], mean corpuscular hemoglobin [MCH], and mean corpuscular hemoglobin concentration [MCHC]), total cell counts (RBC, platelets [PLT], and white blood cells [WBC]) differential white blood cells counts and comments by a veterinary hematologist on the morphology of the erythrocyte, leucocyte, and platelet populations (Day and Kohn, 2012).

Veterinarians performing the transfusion are crucial in guaranteeing the well-being of the patients. However, the limit in transfusion knowledge and experience of a practitioner could be a challenge. Furthermore, appropriate blood collection, processing, storage, and administration methods, which are usually not followed by practitioners correctly,

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can also be a problem. Any mistake in the whole transfusion process can cause serious side effects, be an infectious disease's route of transmission, or even lead to death (Moor et al., 1999; Goodnough, 2003; Buddeberg et al., 2008).

Blood storage must be carefully managed to ensure the appropriate storage duration and blood quality. Blood storage management is essential for the success and effectiveness of a treatment, and for avoiding wasting blood products (Bashir et al., 2021). Appropriate blood collection, processing, storage, administration methods, strict monitoring, and quick reaction to side effects are complicated and play an important role in blood transfusion (Dong and Vo, 2020). Veterinarians need to have enough knowledge of the transfusion process for treatment in emergency cases.

Increasing of anemia cases in dogs occurred with many causes, such as bloody diarrhea (caused by Parvovirus), blood parasites (*Ehrlichia canis, Anaplasma* spp., *Babesia canis*), endoparasites (*Toxocara canis, Ancylostoma caninum, Dipylidium caninum*), cancer, trauma (hit by a vehicle). The need for canine blood transfusion for these anemia cases is also increasing (Day and Kohn, 2012; Zakarevičiūtė et al., 2021). However, there are not many studies of blood storage and transfusion in veterinary hospitals and clinics in Vietnam. The current study was carried out to have a summary and evaluation of blood quality during storage, evaluation of transfusion effectiveness using fresh whole blood, stored whole blood and blood products, as well as the transfusion reactions during and after transfusion in dogs.

MATERIALS AND METHODS

Ethical approval

The present study was conducted by collecting blood samples and transfusion according to the procedures of the Animal Welfare Council, Nong Lam University, Ho Chi Minh City, Vietnam.

Experimental animals, blood collection, and storage

The study was conducted at Nong Lam University, Ho Chi Minh City, Vietnam. A total of five healthy Greyhounds, including 3 males and 2 females aged 2.5 years old, and weighing 25-30 kg were selected randomly and labeled N1, N2, N3, N4, and N5. All dogs were fully vaccinated within the last 12 months and received prophylactic flea treatment regularly. Dogs did not receive any raw meat diet and were completely healthy and relaxed. The N5 did not receive a blood transfusion from the others. The animals were fed with commercial dry feed (France) (900 gr). All dogs were checked physically and all clinical signs, including body temperature, heart and respiratory rate, and appetite were normal. An amount of 100 ml of blood was collected from each dog (Nong Lam Veterinary Hospital's blood bank protocol) and stored in commercially available blood bags (Ningbo Greetmed Medical Instruments Company), with anticoagulant–preservative solution (citric acid, sodium citrate, monobasic sodium sulfate, dextrose, and adenine) with 16-gauge needles. Before blood collection, the hair of the collection site was clipped. The dogs were calm and placed in lateral recumbency on a blanket, on a table. The jugular vein phlebotomy was conducted to fix the needle before collecting. The blood was collected into the blood bag containing 100 ml of blood. During the blood collection, all dogs were monitored for mucous membrane color, pulse rate, and respiratory rate. The collected blood was stored in a refrigerator at 2-4°C. The temperature of the refrigerator was checked and recorded every 12 hours with a thermometer. The whole stored blood of each dog was tested on hematological and biochemistry before performing a transfusion on days 0, 7, 14, and 28. In the present study, the IDEXX Procyte Dx® (IDEXX Laboratories Incorporation, United States) hematology analyzer was used and the IDEXX Catalyst One (IDEXX Laboratories Incorporation, United States) blood biochemistry analyzer was used as well. The hematology analyzer's work principles are laser flow cytometry, optical fluorescence, and laminar flow impedance. The commercial kits for blood biochemistry and ion tests were Chem 17 Clip and Lyte 4 CLIP, respectively (IDEXX Laboratories Incorporation, United States). The reference ranges of hematology and biochemistry parameters for adult canines were also from IDEXX Laboratories Inc., United States (IDEXX Catalyst, 2019; IDEXX ProCyte Dx, 2019).

The average of hematology and biochemistry parameters was calculated for comparison. The parameters were calculated, including white blood cells (WBC), red blood cells (RBC), hematocrit (HCT), mean corpuscular volume (MCV), platelets [PLT], total protein (TP), aspartate transaminase (AST), alanine transaminase (ALT), alkaline phosphatase (ALP), lactate dehydrogenase (LDH), creatine kinase (CK), creatinine (CREA), blood urea nitrogen (BUN), and glucose (GLU). The calculated ion parameters included (Ca), phosphate (Phos), total carbon dioxide (tCO_2), chloride (Cl), potassium (K), manganese (Mg), and sodium (Na).

Blood was transfused initially at day 0, and day 14 was the day of the second blood transfusion. The Greyhounds were divided into 2 groups, including the fresh whole-blood recipient group and the stored whole-blood recipient group. The N1 (day 0), N4 (day 0), N2 (day 14), and N3 (day 14) were in the fresh whole-blood group and the stored whole-blood group included N1 (day 14), N4 (day 14), N2 (day 0) and N3 (day 0).

Serologic compatibility tests

Before each transfusion, a compatibility test was performed to detect naturally occurring antibodies or all antibodies produced as a consequence of sensitization by glass slides under a light microscope (Model Optika IM-3MET, Italy). The compatibility test carried out in the study was crossmatching, including major crossmatch assessment and minor crossmatch assessment. The rapid slide method was applied at 37°C (Day and Kohn, 2012). These steps included collecting blood into an EDTA tube from the recipient and donor, then centrifuge tubes to settle the RBC and plasma. On each glass slide, place 1 drop of RBC and 2 drops of plasma, then mix with an applicator stick, there were four glass slides for each pair of donor and recipient dogs, labeled as donor control (donor RBC and donor plasma), major crossmatch (donor RBC and recipient plasma), minor crossmatch (recipient RBC and donor plasma) and recipient control (recipient RBC and recipient plasma). Finally, the macroscopic agglutination on the slides within 2 minutes and microscopic agglutination with oil immersion at x100 lens within 5 minutes were done. A positive result is determined by the appearance of an agglutination reaction (Day and Kohn, 2012).

Blood transfusion between dogs

Before the transfusion, each recipient was drawn 100 ml of blood. They were transfused to the same volume of blood, to ensure the total body fluid did not change. The N5 did not receive any blood from other dogs since it was used to produce pure red blood cells in the blood transfusion experiment. Before the initial transfusion, a total of 100 ml of blood was collected from each dog for storage, for 14 days. Stored whole blood was left at room temperature for approximately 30 minutes to warm up the blood and avoid hypothermia. The administrated route was intravenous. Initial blood was administered at the rate of 1 ml/kg/hour for the first 20 minutes to observe transfusion reactions, then increased to 2 ml/kg/hour (total of 130 minutes; Day and Kohn, 2012). Blood was transfused in approximately 2 hours for each recipient. The recipients were monitored during transfusion, 5 hours, and 4 days after transfusion for any reactions. To increase the viscosity of the stored whole blood and reduce the risk of embolism during transfusion, dogs could be transfused 40 ml of NaCl with blood.

Before the initial transfusion, stored whole blood was inspected for discoloration of red cells, the presence of clots, or hemolysis for safety. In the first transfusion, N1 and N4 received fresh whole blood from N2 and N3, respectively. The N2 and N3 received stored whole blood from N5 and N1, respectively (blood had been stored for 14 days). In the second transfusion on day 14, all recipients received blood from N5, including N1 and N4 received stored whole blood (the blood had been stored for 28 days), and N2 and N3 received fresh whole blood.

Monitoring

Before transfusion, both hematological and biochemistry tests were conducted on the blood to ensure its suitability. These tests were performed regardless of whether the blood was freshly collected from the donor or stored as whole blood. The physical examination (body temperature, mucosa color, heartbeat, and respiratory rate) of each recipient was recorded by veterinarians. After the transfusion, the recipients were monitored on the day they received blood (day 0 and day 14) and 4 days later. On the day of transfusion, 5 hours after transfusion the recipients were checked every 1 hour. The symptoms monitored on the transfusion day were fever, hemorrhage, cyanosis, polynea/dyspnea, urticaria/angioedema, vomiting, and diarrhea. In the next 4 days, the recipients were monitored for body temperature, gums' color, heartbeat, respiratory rate, appetite, diarrhea, and vomiting by clinical observation. The recipient dogs' blood was then checked again 14 days after transfusion.

Statistical analysis

Data were analyzed by an average mean of the values through Microsoft Excel, version 2016.

RESULTS

Evaluation of fresh whole blood and stored whole blood during storage

The average of each parameter of fresh whole blood and stored whole blood is presented in Table 1. The reference ranges are from IDEXX (IDEXX Laboratories, Incorporation, United States). All fresh whole blood parameters from 5 dogs were in the normal range. The stored blood in the present study had some changes, but all parameters were still in the normal range on day 28 of storage, which made the stored whole blood available for transfusion. The PLT significantly decreased from day 2, while WBC gradually decreased in the storage period. In the current study, there was a decrease in both RBC and HCT. The average value of WBC and PLT markedly decreased in the storage period, while the mean corpuscular volume MCV increased. Serum biochemical analysis results in the present study indicated that there was an increase in TP, AST, ALT, ALP, LDH, and a decrease in CREA, BUN, and GLU. The increase of

parameters, including TP, AST, ALT, ALP, LDH, MCV, tCO_2, K, and the decrease of the other parameters, including CK, CREA, BUN, glucose GLU, WBC, RBC, HCT, PLT, Ca, P, Cl, Mg, and Na was observed during the storage period, with Ca, P, and Na were below the normal range (Tables 2, 3).

Although changes were observed in serum biochemistry results, all parameters were still in the normal range on day 28 of storage, except for a slight increase in AST. Meanwhile, ion parameters changed during storage, and most ion parameters were out of the normal range on day 28 of storage. Only Mg and Phos results were normal. The ion results of the present study also indicated an increase in K and LDH, and a decrease in Na level. The Na level dropped specifically below the normal range on day 7 of storage (138.4 mmol/L) and kept decreasing to 114.04 mmol/L on day 28.

Table 1. Th effects of blood collection day and storage period on hematology parameters

Parameter	Unit	Reference range*	Day 0	Day 7	Day 14	Day 21	Day 28
WBC	10^9/L	5.05-16.76	13.228	10.8	9.188	7	4.624
RBC	10^{12}/L	5.65-8.87	6.574	6.374	6.056	5.744	5.384
HCT	%	37.3-61.7	45.34	44.38	43.6	44	40.6
MCV	fL	61.6-73.5	70.48	71.34	73.2	74	75.22
PLT	10^9/L	148-484	216.6	171	146	119	98.4

WBC: White blood cells; RBC: Red blood cells; HCT: Hematocrit; MCV: Mean corpuscular volume; PLT: Plaletes; *The reference range of adult canine was from IDEXX Laboratories Inc., United States

Table 2. Th effects of blood collection day and storage period on biochemistry parameters

Parameter	Unit	Reference range*	Day 0	Day 7	Day 14	Day 21	Day 28
TP	g/L	52-82	67.74	70.76	72.4	75.28	77.5
AST	U/L	0-50	28.4	31.4	38	44.6	53.8
ALT	U/L	10-125	65.4	50	56	63	65
ALP	U/L	23-212	93.6	95	102.4	111.8	119.8
LDH	U/L	40-400	65	95	156	173	212
CK	U/L	10-200	107.6	112.4	99.8	93.6	87
CREA	mmol/L	44-159	61.84	45.12	46.38	47.41	45.26
BUN	mmol/L	2.5-9.6	5.352	5.23	4.28	4.3	4.21
GLU	mmol/L	4.11-7.94	5.098	5.446	5.222	4.982	4.446

TP: Total protein; AST: Aspartate transaminase; ALT: Alanine transaminase; ALP: Alkaline phosphatase; LDH: Lactate dehydrogenase; CK: Creatin kinase; CREA: Creatinine; BUN: Blood urea nitrogen; GLU: Glucose; *The reference range of adult canine was from IDEXX Laboratories Inc., United States

Table 3. Th effects of blood collection day and storage period on ion concentrations

Parameter	Unit	Reference range*	Day 0	Day 7	Day 14	Day 21	Day 28
Ca	mmol/L	1.98-3.00	2.392	2.236	2.076	1.674	1.46
Phos	mmol/L	0.81-2.19	1.472	1.34	1.242	1.112	0.986
tCO_2	mmol/L	21-31	17	17.42	17.82	18.32	18.72
Cl	mmol/L	109-122	104.54	110.4	120.2	107.74	102.664
K	mmol/L	3.5-5.8	4.764	5.074	5.436	5.642	5.98
Mg	mmol/L	0.58-0.99	0.912	0.868	0.832	0.77	0.692
Na	mmol/L	144-160	145.3	138.4	130.88	124.18	114.04

Ca: Calcium; Phos: Phosphate; tCO2: Total carbon dioxide; Cl: Chloride; K: Potassium; Mg: Manganese; Na: Sodium; *The reference range of adult canine was from IDEXX Laboratories Inc., United States

Symptoms of recipients on transfusion day and 4 days after

There were no positive results in compatibility tests between each pair of donor and recipient. In the present study, the body temperature, mucosa color, heartbeat, and respiratory rate of recipient dogs were recorded. There were no signs of transfusion reactions in any of the dogs five hours post-transfusion. All dogs in the study had no reactions during and 5 hours after transfusion. The average body temperature showed that stored blood recipients' body temperature was slightly higher than the fresh whole blood. The average body temperature of fresh whole blood recipients was 38.8°C,

while the stored whole blood recipients were 39.2°C. During 4 days after transfusion, the average body temperature of fresh whole blood recipients and stored whole blood recipients was 38.4°C and 38.75°C, respectively. The average heartbeat was 125 beats per minute and respiratory rate was 18.5 breaths per minute (fresh whole blood recipients) and 20 breaths per minute (stored whole blood recipients). All recipients had normal mucosa color (pink). All dogs had symptoms of inappetence and mild diarrhea in 1-2 days post-transfusion. In the fresh whole blood recipient group, all recipients recovered on day 3. In the stored blood recipient group, recipient dogs slowly recovered on day 4. Eventually, on day 5, all dogs were healthy with no abnormal signs. Dogs that received stored whole blood suffered from mild diarrhea longer than ones that received fresh whole blood. All recipients in both groups did not have any abnormal body temperature, heartbeat, respiratory rate, or mucosa color 4 days after transfusion. The recipients of stored whole blood (14 days and 28 days of storage) suffered post-transfusion reactions (inappetence and diarrhea) longer than the recipients of fresh whole blood one day.

Evaluation of recipients' blood after 14 days of transfusion

The average values of hematology parameters between the fresh whole-blood recipient group and the stored whole-blood recipient group were not significantly different (Table 4). In both groups, the average values of WBC and RBC were out of the normal range, while the others were in the normal range. The maximum RBC value of stored whole blood was still below the normal range (5.60×10^{12}/L). The average values of biochemical parameters were all in the normal range in both groups (fresh whole-blood and stored whole-blood recipient group; Table 5). In the ion test, there was almost no difference between these two groups. However, the average value of total carbon dioxide and chloride were lower compared to the normal range (Table 6).

In the current study, recipients of stored whole blood suffered side effects longer than recipients of fresh whole blood. Side effects did not vary between recipients of whole blood stored for 14 days and those stored for 28 days within the first two weeks post-transfusion. Although all recipients were healthy and alive, they still had mild transfusion reactions 2-4 days after transfusion.

Table 4. Average of hematology parameters of Greyhounds dogs aged 2.5 years on day 14 after transfusion

Parameter	Unit	Reference range*	Fresh whole blood			Stored whole blood		
			Avg. value	Min. value	Max. value	Avg. value	Min. value	Max. value
WBC	10^9/L	5.05-16.76	17.87	17.21	18.46	17.59	16.46	18.46
RBC	10^{12}/L	5.65-8.87	5.35	4.90	5.90	5.32	4.90	5.60
HCT	%	37.3-61.7	40.15	38.60	43.00	40.60	38.60	42.78
MCV	fL	61.6-73.5	69.55	66.80	72.10	70.23	66.80	74.20
PLT	10^9/L	148-484	207.50	121.00	248.00	187.25	121.00	259.00

WBC: White blood cells; RBC: Red blood cells; HCT: Hematocrit; MCV: Mean corpuscular volume; PLT: Plaletes; Avg. value: Average value; Min. value: Minimum value; Max. value: Maximum value; *The reference range of adult canine was from IDEXX Laboratories Inc., United States

Table 5. Average of biochemical parameters of Greyhounds dogs aged 2.5 years on day 14 after transfusion

Parameter	Unit	Reference range*	Fresh whole blood			Stored whole blood		
			Avg. value	Min. value	Max. value	Avg. value	Min. value	Max. value
TP	g/L	52-82	74.60	64.80	81.10	74.70	63.90	91.60
AST	U/L	0-50	36.00	15.00	54.00	34.00	22.00	45.00
ALT	U/L	10-125	78.00	35.00	132.00	79.75	35.00	145.00
ALP	U/L	23-212	81.00	27.00	160.00	80.75	36.00	160.00
LDH	U/L	40-400	171.75	65.00	243.00	162.50	65.00	231.00
CK	U/L	10-200	89.00	39.00	174.00	90.50	29.00	164.00
CREA	mmol/L	44-159	112.80	56.30	169.30	116.23	68.60	157.00
BUN	mmol/L	2.5-9.6	7.88	6.40	9.70	8.18	6.82	9.70
GLU	mmol/L	4.11-7.94	5.32	4.26	6.47	5.75	4.32	6.91

TP: Total protein; AST: Aspartate transaminase; ALT: Alanine transaminase; ALP: Alkaline phosphatase; LDH: Lactate dehydrogenase; CK: Creatin kinase; CREA: Creatinine; BUN: Blood urea nitrogen; GLU: Glucose; Avg. value: Average value; Min. value: Minimum value; Max. value: Maximum value; *The reference range of adult canine was from IDEXX Laboratories Inc., United States

Table 6. Average ion concentrations of Greyhounds dogs aged 2.5 years on day 14 after transfusion

Parameter	Unit	Reference range*	Fresh whole blood			Stored whole blood		
			Avg. value	Min. value	Max. value	Avg. value	Min. value	Max. value
Ca	mmol/L	1.98-3.00	2.07	1.94	2.32	2.07	1.94	2.14
Phos	mmol/L	0.81-2.19	1.23	0.57	1.87	1.24	0.57	2.01
tCO_2	mmol/L	21-31	20.50	15.70	25.60	19.35	15.30	25.60
Cl	mmol/L	109-122	86.45	78.40	96.40	88.65	76.50	99.30
K	mmol/L	3.5-5.8	4.32	3.90	4.99	4.02	2.90	5.01
Mg	mmol/L	0.58-0.99	0.77	0.73	0.84	0.76	0.67	0.88
Na	mmol/L	144-160	129.08	108.20	144.80	132.38	112.70	144.80

Ca: Calcium; Phos: Phosphate; tCO2: Total carbon dioxide; Cl: Chloride; K: Potassium; Mg: Manganese; Na: Sodium; Avg. value: Average value; Min. value: Minimum value; Max. value: Maximum value; *The reference range of adult canine was from IDEXX Laboratories Inc., United States

DISCUSSION

Stored whole blood changes

There were no abnormal findings (discoloration of red cells, hemolysis, or blood clots) in visual inspection of the stored whole blood and inspection of the blood bags before transfusion. In human research, stored whole blood samples after 35 days had significant cellular changes, such as WBC, RBC, and PLT decrease, while MCV and HCT increased (Maruti et al., 2021). In a study by Al Nuaimy (2008), Hemoglobin (HGB) and PCV significantly decreased after ten days of storage in humans. It was unlikely that there was potassium accumulation in canine and feline, except for some Japanese and Korean dog breeds which had high RBC concentrations of K (Obrador et al., 2015). Stored whole blood in humans had increased K and LDH levels, and Na and pH levels significantly decreased (Oyet et al., 2018; Marabi et al., 2020).

Observation of recipients' reactions after transfusion

Transfusion reactions were probably under-recognized but were reported to occur in 3.3-28% of dogs and 1.2-8.7% of cats (Hohenhaus, 2010). During transfusion, monitoring plays an important role in blood transfusion. The parameters should be measured which were attitude behavior, rectal temperature, pulse rate and quality, respiratory rate and character, mucus membrane color, and capillary refill time (Day and Kohn, 2012). A transfusion reaction was most likely to occur within the first hour of the patient receiving blood (Mullineaux and Jones, 2007). Although the study was conducted with healthy individuals, dogs still had transfusion reactions for at least 3 days, which could be a major problem in patients with more severe conditions.

Evaluation of recipients' blood after 14 days of transfusion

A study of 3,095 dogs indicated that the duration of packed red blood cell (PRBC) storage did not appear to be a major contributing factor to mortality in the overall canine population, however, the longer duration of PRBC storage might negatively impact the outcome in dogs with immune-mediated hemolytic anemia (Hann et al., 2014). A mild increase in WBC, a decrease in RBC, total CO2, and chloride in both groups were observed. The selected dogs in the study were deemed to be in good health, potentially contributing to the slight alteration in blood cells, in addition to close monitoring. Accordingly, of dogs with hemolysis, 90% of which had immune-mediated hemolytic anemia, a longer duration of PRBC storage was a negative risk factor for survival (Hann et al., 2014).

Serum biochemistry changes altered the RBC and membrane structure, resulting in compromised microvascular blood flow after transfusion and increased hemolysis during storage, respectively (Barshtein et al., 2011). Some RBC changes were immediate, such as a decrease in 2.3-diphosphoglycerate (2.3-DPG), whereas alterations in lactate, pH, or adenosine triphosphate (ATP) occurred over days (Cohen and Matot, 2013). These events included alterations in the cell membrane that might lead to the formation of microparticles (MPs) which may increase from a variety of cell types under normal physiologic conditions, including RBCs, platelets, endothelial cells, and WBCs (Herring et al., 2013). The change in lactate dehydrogenase over time, which could also affect the pH of the stored blood, was also observed (Herring et al., 2013). The occurrence of platelet storage lesions was well recognized and further contributed to the short storage time. Changes in pH, LDH concentration, and morphology changes, could alter the efficacy of the platelets when transfused (Callan et al., 2009; Devine and Serrano, 2010). Not only platelets but also all hematology parameters had a

decrease below the normal range on day 28 of storage in the study (except HCT). Biochemical, biomechanical, and immunologic changes during the storage of whole blood could affect red cell viability, deformability, oxygen-carrying capacity, microcirculatory flow, and recipient response (Obrador et al., 2015).

CONCLUSION

The stored blood had some changes of composition during 28 days of storage, but all parameters of hematological and biochemistry results were in the normal range, and available for blood transfusion in adult Greyhounds. All the dogs that received the transfusion experienced a loss of appetite and mild diarrhea within 1-2 days. However, they fully recovered and displayed no abnormal symptoms after the 5-day transfusion period. The hematology parameters between the fresh whole blood recipient group and stored whole blood recipient group did not differ from each other after a 14-day transfusion, so both fresh whole blood and stored whole blood would become blood sources for transfusion in dogs. According to the obtained results, both hematological and biochemical changes in canine blood during storage, and stored blood transfusion could cause side effects for recipients with severe conditions. The study of blood transfusion needs to be conducted in dogs of different breeds and ages and evaluate the effects of blood transfusion in disease cases.

DECLARATIONS

Competing interests

The authors declare no conflict of interest.

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Authors' contributions

Thuong Thi Nguyen, Hoa Thi Quynh Nguyen conceptualized, designed, and supervised the research. Thuong Thi Nguyen, Hoa Thi Quynh Nguyen collected samples and conducted experiments. Thuong Thi Nguyen, Khanh Nguyen Dinh analyzed and interpreted the data generated. Thuong Thi Nguyen, Hoa Thi Quynh Nguyen, Khanh Nguyen Dinh critically reviewed the study. 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 from authors.

Ethical considerations

The authors considered ethical concerns and consent, animal welfare and safety procedures before conducting the study. This article was written originally without any copy from data of published articles and books.

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Ultrasonography Examination of the Kidney in Bali Cattle

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ABSTRACT

Ultrasonography is an important technology for examining renal measurements, including length and width. The kidneys can be easily examined, and various structures in the kidneys are distinguishable with ultrasound. This research aimed to determine the normal ultrasonographic appearance of the kidneys in healthy adult Bali cattle, providing a reference for future descriptions of Bali cattle kidneys. In this research, 8 Bali cattle, aged 2-3 years with the healthy status of the urinary system were examined. The tool used was an animal ultrasound device, named Mindray DP10 Veterinary Ultrasound, with a 3-7.5 MHz convex transducer, utilizing a B-mode image mode. The transducer was placed in the right paralumbar fossae. The results indicated that the average horizontal length of the kidneys was 17.36 cm and the average vertical diameter of the kidney was 4.6 cm. The echogenicity of the renal cortex showed an echoic image, while the pyramidal part of the renal medulla indicated a relatively hypoechoic image. The results of measuring the diameter of the left kidney in clinically healthy Bali cattle could be used as a basis for decision-making in determining the clinical status of kidney health in this breed of cattle.

Keywords: Bali cattle, Echogenicity**,** Kidney, Morphology, Morphometric data, Ultrasound examination

INTRODUCTION

Ultrasonography is one of the most widely used diagnostic imaging methods in clinical practice. It is a non-invasive examination tool used in both human and veterinary practices (Barreiro-Vázquez et al., 2021). Ultrasonography is cost-effective and can be carried out in the veterinary field on both small animals (Utomo et al., 2023) and large animals (Eibl and Franz, 2021). Ultrasonography has been increasingly used in large animal clinical practice and surgery. Its use has been increasing in large animal clinical practice and surgery. While most ultrasound examinations in livestock focus on pregnancy (Szenci, 2021), digestion (Khalphallah et al., 2021), and respiration (Berman et al., 2019; Cuevas-Gómez., 2021), there is a growing trend toward using ultrasonography for kidney examination (Barreiro-Vázquez et al., 2021; Tharwat, 2021).

Knowledge of the bovine kidneys' anatomy and topography relative to the body cavity is very important for veterinarians, particularly for clinical examination of the kidney, including laparotomy examination and laparoscopy/ultrasound-guided biopsy (Imran and Sharma, 2014). The use of ultrasound for assessing renal anatomy is straightforward (Hansen et al., 2016). Ultrasonography aids veterinarians in diagnosing morphologic changes in the kidney (Seif and Bark,2007), detecting kidney disorders (Debruyn et al., 2012), diagnosing infections in the upper or lower urinary tract (Floeck, 2009) and urinary system diseases in cows (Öztürk et al., 2005), measuring kidney volume, and assessing the kidney architectural appearance (Bappah et al., 2019). Additionally, ultrasound imaging is now used for the physiological examination of kidney blood flow (Barreiro-Vázquez et al., 2021).

The bovine kidneys, especially the left kidney can be mobile. This mobility is likely due to pressure exerted by the full rumen. Kidney morphology in cattle can change with the seasons, such as in summer, when heat stress affects glomerular and tubular function, as well as urine concentration (Akosman et al., 2018). Cow kidneys have a lobed shape and can be microscopically divided into three layers: the capsule, parenchyma, and renal pelvis. The kidneys are covered by a thin, light brown capsule. The parenchyma layer is divided into two parts, including the outer part, called the cortex, and the inner part called the medulla (Ishaya et al., 2021). In terms of anatomical location, cows have a right and a left kidney. The right kidney in cows is located in the abdominal cavity between the last rib and the transverse process of the 2nd to 3rd lumbar vertebrae, appearing more cranial compared to the left kidney (Braun, 1991; Barreiro-Vázquez et al.,

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2021). The left kidney, positioned slightly lower than the right kidney, can move medially depending on the ruminal filling (Braun, 1993).

Some imaging techniques for assessing kidney size and shape in humans and animals include magnetic resonance imaging, ultrasonography, contrast studies, radiography, computed tomography, and renal scintigraphy (Caroli et al., 2021). The introduction of routine abdominal ultrasound examination in large animals has resulted in various methods for estimating kidney size and volume with this modality, including using volumetric formulas based on maximum kidney length (Hussein et al., 2018).

Ultrasonography is used to assess the anatomy of the kidney, including shape and size (length, width, and thickness). Ultrasonography identifies the echogenicity of the renal parenchyma as well as details changes in the collecting system. These details help in identifying the extent of renal parenchymal damage and its possible reversibility (Ahmed et al., 2019). Additionally, ultrasonography examines the renal cortex and parenchyma (Stankiewicz et al., 2023) and complements the physical examination and clinical pathology evaluation by providing additional information about the disease.

Kidney ultrasound examination can identify primary abnormalities and differentiate between normal and abnormal conditions that often cause abdominal pain. Common urological diseases in cattle, such as urolithiasis, which result in significant economic losses for the livestock industry because they are considered the fifth most common cause of death, require ultrasonography of the kidneys for diagnostic purposes (Dangi et al., 2022). Ultrasonography can provide information on urinary system diseases, such as the size, structure, and position of the kidney and bladder parenchyma (Tharwat, 2021).

Routine ultrasound use in cattle is currently very limited, especially in Bali cattle. Bali cattle are the indigenous to Indonesia and play an important role in livestock development in Indonesia (Puja et al., 2018). They are directly domesticated from wild bulls that still live in the Baluran National Park area in East Java (Martojo, 2012). Bali cattle are known for their strong energy and the presence of horns on cows, coupled with their wild temperament, which necessitates special techniques or skills in handling. There is currently no reference available regarding the morphology of Bali cattle kidneys through ultrasound examination, using the transcutaneous method. This study aimed to describe the kidney morphology of Bali cattle through ultrasound examination. The data obtained from this study can help veterinary practitioners in the diagnosis of healthy Bali cattle kidneys.

MATERIALS AND METHODS

Ethical approval

This research has been approved by the Animal Ethic Committee Faculty of Veterinary Medicine Udayana University, Bali, Indonesia with approval number: B/98/UN14.2.9/PT.01.04/2024.

Animal samples

Eight Bali cattle, aged 2-3 years, weighed approximately 250 to 300 kg, raised at the Bali cattle breeding center, Sobangan Badung, Bali Indonesia, were selected for the study. The animals were kept under standard conditions of feeding and management. The animal is clinically healthy and has no history of pathological disorders, especially in the urinary tract based on the result of clinical examination from a veterinarian. The cows were female and not pregnant. The average body condition score was three on a five-point scale. The morphometric data was taken from post-mortem kidneys at the Pesanggaran Denpasar, Bali, Indonesia slaughterhouse in the dry season.

Ultrasound examination

The equipment utilized for this study was the Mindray DP 10 Veterinary Ultrasound (Germany), specifically designed for animal, equipped with a 3-7.5 MHz convex transducer. The image mode employed was B-mode, displayed on a 10-inch LCD. Ultrasound observations of the kidneys were carried out on the right paralumbar fossa where the hair was shaved first. Animals were positioned in a standing position without sedation. The cow is not given food or water for at least 12 hours before the examination. After adding the gel, the transducer is placed longitudinal plane on the dorsal part of the abdomen. The right and left kidneys were measured, and their internal structures, such as renal parenchyma, and echotexture of the kidney were examined (Floeck, 2009).

Data analysis

Data from ultrasound examination results were tabulated and analyzed descriptively. The results of the vertical and horizontal diameter of the kidney are presented as means and standard deviations. All statistical tests were conducted using IBM SPSS for Windows v.26.

RESULTS

Ultrasonography was performed on eight cows that were clinically healthy and not anesthetized, with imaging focused on the kidneys from the paralumbar area. While the left kidney exhibited a clear, oval appearance (Figure 1), the right kidney appeared less distinctly visible. Adjacent organs, including the large intestine and medial rumen wall, were visible alongside the left kidney. The left kidney structure image showed distinct echotexture in the ultrasound images. The renal cortex indicated an echoic image, while the pyramidal part of the renal medulla revealed a relatively hypoechoic image. In the kidney, the renal sinus section showed a hyperechoic image. A total of 15 pairs of right and left kidneys were measured to obtain kidney morphometric data for Bali cattle (Figure 2). Measurement results of the horizontal diameter of the kidneys of Bali cattle yielded an average of 17.36 ± 0.71 cm. The average diameter of Bali cattle kidney vertices was 4.6 ± 0.33 cm (Table 1). The vertical diameter of the kidney was much smaller than the horizontal diameter.

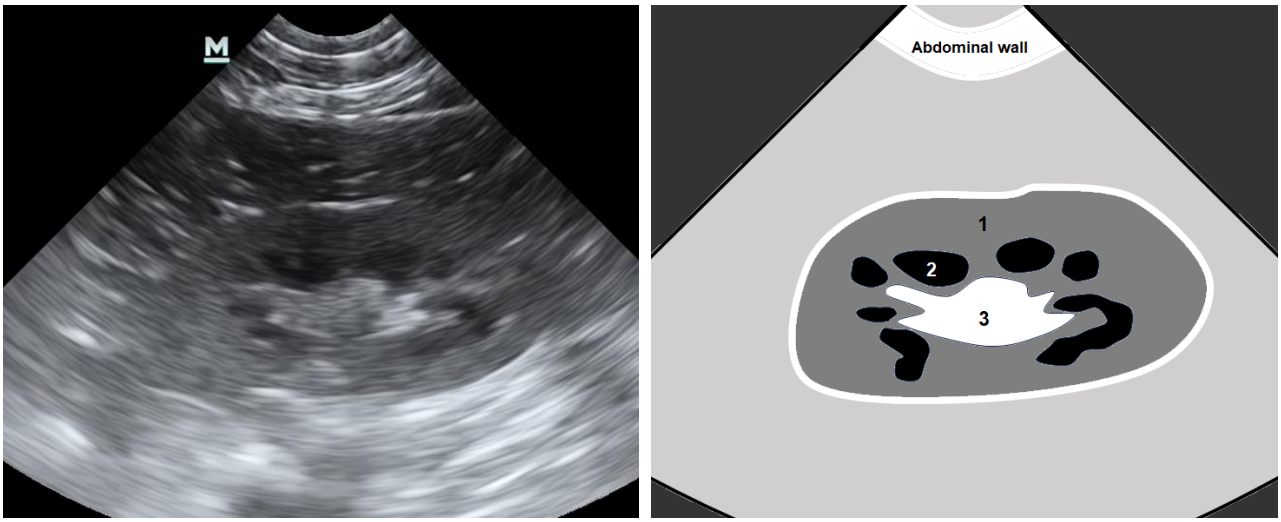

Figure 1. Longitudinal ultrasound image of left kidney in a 2-year-old female Bali cattle. **1:** Renal cortex; **2**: Medullary pyramid; **3:** Renal sinus

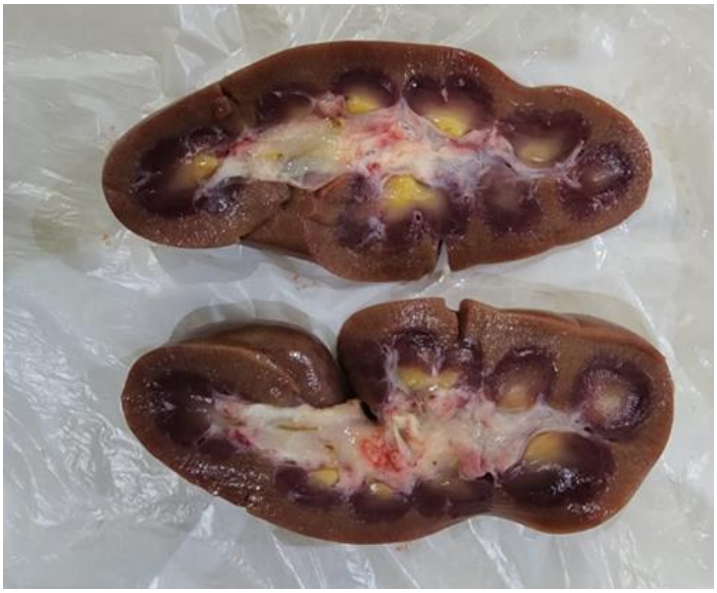

Figure 2. Morphology of three years old female Bali cattle (left kidney)

Table 1. The average size of Bali cattle kidney

Variable	N	Max	Min	Mean ± SD
Length (cm)	15	18.2	16.3	17.36±0.71
Vertical diameter (cm)	15	5.1	4.2	4.6±0.33

N: Number. SD: standard deviation.

DISCUSSION

This study represented the first report to describe the kidney morphology of healthy, non-pregnant Bali cattle using the transcutaneous ultrasonography method. These findings can be useful as a basis for further research comparing normal Bali cattle and sick Bali cattle. Transcutaneous ultrasonography images at the dorsal abdominal location succeeded in depicting the size and appearance of the kidney structure of Bali cattle. However, the ultrasound image of the right kidney cannot be clearly imaged.

The obtained results of the current study differed from the study conducted by Katsoulos et al. (2020), indicating that the image of the right kidney in Holstein cows can be recognized, compared to the left kidney. The right kidney lobes of the Holstein cow can be examined using ultrasonography, but the cortex and medulla cannot be clearly distinguished (Braun, 1991).

In this study, the echo patterns of the kidney structures, including the renal pyramids, and renal parenchyma were identified through different echogenic images. This research aligns with the report of Seif and Bakr (2007), who indicated the parenchyma and renal pyramids, could be observed with different echotexture. The results of imaging of the left kidney in Bali cattle were similar to the results of imaging carried out by Braun (1993) on Swiss Braunvieh cattle evaluated from the echogenicity of renal parenchyma and medullary pyramids. The left kidney in cattle can be imaged well using ultrasonography. Changes in the echo pattern of the structure of the left kidney may occur due to the presence of the rumen (Akosman et al., 2018). The left kidney position often varies depending on rumen distension. It can be located from the midline to the right stomach and rotated to varying positions, sometimes even in contact with the abdominal wall (Barreiro-Vázquez et al., 2021).

The results of this study showed that the average horizontal diameter of the kidney in Bali cattle ranged from 16.31 to 18.2 cm, while the width of the kidney in Bali cattle ranged from 4.2 to 5.1 cm with an average of 4.2 cm. The measurement results between individuals were similar, due to the similar age and Body Condition Score (BCS) of the examined cows. Comparatively, the average kidney size of Bali cattle fell below that of other breeds. For instance, Limousin-Cross cattle exhibited an average length of 19.63 cm in length and 10.11 cm in width (Katsoulos et al., 2020), while Holstein Friesian cattle typically ranged from 19-26 cm in length and 6.90 cm in width (Barreiro-Vázquez et al., 2020). This result indicated that differences in kidney size in other studies may be due to differences in the cattle breeds used. Breed and age are significant factors influencing cattle kidney size (Seif and Bakr, 2007; Katsoulos et al., 2020). The kidney shape of Bali cattle tended to be smoother at the margins, compared to Holstein cattle. Moreover, the lobes were less visible, compared to other cattle breeds, such as Holstein and Limousin-Cross (Katsoulos et al., 2020).

In this study, the renal cortex showed an echoic image, while the pyramidal part of the renal medulla showed a relatively hypoechoic image. The current study results are in accordance with echo patterns in healthy Jersey/Red Sindhi crossbred cows, were reported by Imran and Sharma (2014). Ultrasound regularly uses a subjective assessment of relative organ echogenicity, visual perception of echogenicity results from ultrasonic back reflection and attenuation (Stankiewicz et al., 2023).

In Bali cattle, ultrasonography images showed differences in echogenicity between the cortex and the medulla. The renal cortex showed a hypoechoic image, while the pyramidal part of the renal medulla indicated a relatively less echogenic image, compared to the cortex. These echogenicity patterns observed in the left kidney of Bali cattle mirror those documented in the left kidney of Swiss Braunvieh cattle, indicating consistent differences in the echogenicity of the kidney structure (Braun, 1993). The echogenicity pattern of renal lobulation can be visualized by ultrasonography. Various structures in the kidney, such as the renal cortex and renal medullary pyramids can show differences in echogenicity. The renal cortex is more echogenic, compared to renal medullary pyramids (Seif and Bakr, 2007). However, the thickness of the renal cortex of Bali cattle is smaller than Swiss Braunvieh. This can be the result of the difference in the size of the cows. Bali cattle is relatively smaller than most other cows, such as Holstein, Brown Swiss, and Jersy. The renal size is correlated with the body size of the cow (Seif and Bakr, 2007).

CONCLUSION

From the present results, ultrasonography images of Bali cattle's dorsal abdomen can be used to image the left kidney. The vertical diameter of the kidney is much smaller than that of the horizontal diameter. The horizontal diameter of the kidneys of Bali cattle was an average of 17.36 ± 0.71 cm and the average diameter of the kidney vertices was 4.6 ± 0.33 cm. The results of measuring kidney diameter can be used as a reference for diagnosing changes in Bali cattle's kidney morphology. Further studies involving healthy Bali cattle and Bali cattle with urinary system disease are required to establish the difference between healthy and damaged kidney ultrasound imaging.

DECLARATIONS

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Authors' contributions

I Wayan Nico Fajar Gunawan designed the research and drafted and finalized the manuscript, Putu Devi Jayanti and I Wayan Sukernayasa collected the data, Anak Agung Gde Oka Dharmayudha analyzed the data, and I Ketut Puja reviewed and finalized the manuscript. All authors reviewed and confirmed the final manuscript.

Competing interests

The authors declare there are no conflicts of interest.

Availability of data and materials

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

Ethical considerations

This article is not submitted anywhere else, and the findings are analyzed and written under the supervision of all authors. All authors wrote the article and checked the last draft of the manuscript for the similarity index.

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A Cross-sectional Study of Prevalence of Gastrointestinal Parasites in Captive Wild Animals in Pakistan Zoological Gardens

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ABSTRACT

The animals held captive in zoos often face health and well-being issues. Parasitic infections can lead to health problems in wildlife animals by affecting their gastrointestinal tract. Therefore, the present study aimed to identify and evaluate the population of the various Gastrointestinal (GIT) parasites of wild animals enclosed in different zoological gardens in Pakistan. The fresh fecal samples (n = 960) of 20 captive wildlife animals were collected from Marghzar Zoo, Islamabad (n = 340), Ayub National Park, Rawalpindi (n = 221), Lohi Bher Wildlife Park, Rawalpindi (n = 296), and Bansra Galli Wildlife Park, Rawalpindi (n = 103). The samples were obtained from wildlife mammals, including urial (n = 95), blue bull (n = 106), chinkara gazelle (n = 77), zebra (n = 77), hog deer (n = 75), spotted deer (n = 43), blackbuck (n = 58), barking deer (n = 52), red deer (n = 104), yak (n = 44), grey goral (n = 40), lion (n = 37), mouflon sheep (n = 46), red fox (n = 12), bear (n = 37), grey wolf (n = 12), jackal (n = 12), vervet monkey (n = 12), rhesus monkey (n = 12), and langoor (n = 12). Various methods, such as direct smear examination, standard sedimentation, and floatation techniques were applied to detect and identify the endoparasites in the fecal sample. The detailed routine parasitological analysis identified approximately 52 endoparasites in the fecal samples, including *Haemonchus contortus*, *Eimeria bovis*, *Ostertagia curcumcincta*, *Strongyloides papillosus*, *Strogylus equinus*, *Oxyuris equi*, *Chabertia ovina*, *Protostrongylus*, and *Trichostrongylus vitrines*. The obtained results indicated that Lohi Bher Wildlife Park (46.35%) had a higher prevalence of GIT parasites, compared to Marghzar Zoo (33.23%), Bansra Galli Wildlife Park (33.02%), and Ayub National Park (19.45%). The study reports mild to moderate parasitic infection in captive wild animals and that could affect the survivability of the animals in captivity. The findings of the study can be used to formulate a proper health protocol and sanitation management in captive wild animals to control parasitic infections.

Keywords: Captive wild animal, Gastrointestinal infection, Parasite, Zoological Garden

INTRODUCTION

Parasitic infections are a major concern of wildlife units in Pakistan and worldwide (Khan et al., 2021). In zoological gardens, animals are mainly held captive in enclosures where the environment does not resemble their natural habitat (Da Silva Barbosa et al., 2019). The physiology of animals is changed when they are kept in their enclosures, as they are suddenly exposed to unpleasant and distressing environments. This physiological alteration renders the captive animals more susceptible to numerous infectious diseases, such as viral, bacterial, fungal, and parasitic. Gastrointestinal (GI) parasitic infections are most commonly found in captive wild animals (Dev Moudgil et al., 2015; Carrera- Játiva et al., 2018). In natural habitats, animals are innately resistant to parasitic infections as there is an ecological balance between animals and their parasites. Moreover, wild animals are less exposed to parasitic infections since they freely roam in open lands with low animal density (Thawait et al., 2014; Da Silva Barbosa et al., 2019). Nevertheless, parasitic infections have negative effects on the status, behavior, reproduction, and mortality rate of wild animals (Thawait et al., 2014; Kvapil et al., 2017). The host's survival and reproduction behavior could be affected by parasitic infection through pathological effects, causing tissue damage, blood loss, spontaneous abortion, and mortality, or indirectly by declining the immune response (Thawait et al., 2014).

In recent years, A study has been conducted on wildlife pathogens to investigate the prevalence of parasitic infections with zoonotic tendencies. However, this has led researchers to overlook the ecological factors surrounding parasites while it has also damaged the efforts to manage them (Sengar et al., 2017). Extensive studies investigated identifying GIT diseases and infections among wildlife animals. Ferdous et al. (2023) noticed the occurrence of GIT infections in Bangladesh Zoo. Khan et al. (2021) identified the GIT parasitic infections among cows and buffalo in various farms located in Khyber Pakhtunkhwa province of Pakistan, and Ruhoollah et al. (2023) noted GIT parasite impacts in lower Dir region animals. However, research concerning the health of wildlife animals in Islamabad and

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Rawalpindi is still scarce and requires more data. Hence, the present study aimed to identify the various GIT parasites of wild animals in zoological gardens located in Islamabad and Rawalpindi region.

The objective of the current study was to identify the various GIT parasites of wild animals and to evaluate the percentages of prevalent GIT parasites in wildlife mammals.

MATERIALS AND METHODS

Ethical approval

Formal permission was obtained from the respective parks and zoo administration for doing research while ensuring the animals' safety. The ethical approval committee was obtained from the Department of Life Sciences Abasyn University, Islamabad Campus, and National Veterinary Laboratories Park Road Chak Shahzad Islamabad, Pakistan.

Study design

The study followed a cross-sectional study design to conduct a study on wildlife mammals in the parks and zoos in Rawalpindi and Islamabad, Pakistan.

Study method

The study chose a quantitative research method to descriptively analyze the fecal samples of the captive animals to explore GIT parasites in them.

Sample selection

To determine the sample size of the study, epitool was used to estimate 960 items as the appropriate size of the sample. Hence, 960 fresh fecal samples from 20 captive wildlife animals were collected.

Study area and time

The research was conducted from August 2020 to July 2021 in the four chosen facilities, including Marghzar Zoo, Islamabad, Lohi Bher Wildlife Park, Rawalpindi, Bansra Galli Wildlife Park, Rawalpindi, and Ayub National Park, Rawalpindi. Islamabad–Rawalpindi metropolitan area is situated in the north of Punjab, in the Potohar Plateau, against the backdrop of the Margalla Hills, and constitutes the twin cities of Islamabad and Rawalpindi, Pakistan. In Rawalpindi and Islamabad, the mean annual temperature is recorded as 21.5°C (70 °F), and June is the hottest month with an average temperature exceeding 38°C (100.4 °F). The average annual rainfall is 1,346.8 millimeters, especially during monsoon season. The average humidity is 45% whereas the average wind speed is 16 kph (10 mph).

Health and hygiene management in zoological gardens

The zoo workers followed a strict feeding and sanitary protocol to ensure the health and hygiene of the animals. The animals were well kept in warm, closed enclosures with proper access to food and water. Every six months the animals were dewormed using anthelmintic drugs, as well other measures were adopted to prevent different infections and diseases, including proper vaccination. Zoo workers most commonly used 40 grams/100 lbs dosage of Fenbendazole and Moxidectin (Symans Pharmaceuticals, Pakistan) for deworming. The enclosures were regularly cleaned and maintained in the early morning. Additionally, a weekly cleanliness routine was implemented using the necessary antibiotics, mainly phenyl prophylaxis.

Study population

Fresh fecal samples (n = 960) were collected from Marghzar Zoo, Islamabad (n = 340), Ayub National Park, Rawalpindi (n = 221), Lohi Bher Wildlife Park, Rawalpindi (n = 296), and Bansra Galli Wildlife Park (n = 103), Rawalpindi. The samples were obtained from wildlife mammals (n = 960), including urial (n = 95), blue bull (n = 106), chinkara gazelle (n = 77), zebra (n = 77), hog deer (n = 75), spotted deer (n = 43), blackbuck (n = 58), barking deer (n = 52), red deer (n = 104), yak (n = 44), grey goral (n = 40), lion (n = 37), mouflon sheep (n = 46), red fox (n = 12), bear (n = 37), grey wolf (n = 12), jackal (n = 12), vervet monkey (n = 12), rhesus monkey (n = 12), and langoor (n = 12).

Collection and preservation of fecal sample

A total of 960 samples were collected from individually caged healthy mammals with no history of any illness. The fecal samples (10 gr each) were collected in the early morning before routine cleaning and maintenance of the cages. To avoid contamination, all samples were taken from the ground with a sanitized polystyrene spatula (Rahman et al. 2023) and samples were swiftly delivered to the National Veterinary Laboratory, Islamabad. Each sample was placed in a

plastic container containing 10% formalin. The containers were kept in plastic biohazard bags to transport the samples. According to species, the samples were labeled with a marker, and the opening edge of the bag was tightly closed.

Examination of the sample

The fecal samples were analyzed through detailed routine parasitological to experiment with the presence of parasitic eggs/oocysts by direct smear examination, standard sedimentation (Rao et al., 2017), and floatation techniques (Soulsby, 1982; Qi et al., 2023). The prepared smear was placed on microscopic slides, sealed with a glass cover, and later it was inspected under a light microscope (Leica Microsystems, Germany) for parasitic eggs and larvae.

Data analysis

The data was analyzed using IBM SPSS software version 20 and descriptive statistics was used for tabulating and summarizing the data. The percentages of prevalence of different parasites in the collected samples were calculated using the below-mentioned formula (Farooq et al., 2012):

Percent prevalence = [Positive sample/Total number of samples] x 100.

RESULTS

To see the prevalence of gastrointestinal parasites, an aggregate of 340 fecal samples was collected at the Zoological Garden (Marghzar Zoo, Islamabad), and out of 340 samples, 113 cases (33.23%) were indicated positive for various kinds of endoparasites (Table 1). At Bansra Galli Wildlife Park Rawalpindi, a sum of 103 fecal samples were obtained and just 36 cases (33.02%) were infected with parasites which is presented in Table 2. At Lohi Bher Wildlife Park, Rawalpindi a total of 296 fecal samples were collected and, 140 cases (46.35%) were found tainted for different sorts of parasites (Table 3). At Ayub National Park, Rawalpindi a sum of 221 waste samples was gathered and just 43 (19.45%) fecal examples were tested for various sorts of endo-parasites (Table 4). The higher predominance of parasites was documented at Lohi Bher Wildlife Park (46.35%) compared with Marghzar Zoo (33.23%), Bansra Galli Wildlife Park (33.02%), and the lowest commonness at Ayub National Park (19.45%).

Table 1. Prevalence of oocytes/eggs, larvae of parasites isolated from zoo animals between August 2020 to July 2021 from Marghzar Zoo, Islamabad, Pakistan

Mammals	Total Sample	Number of infected animals	(%)	Parasite's encountered
Blue Bull	45	26	57.8	*Eimeria bovis, Haemonchus contortus, Trichostrongylus vistrinus, Ostertagia curcumcinta, Chabertia ovina*
Chinkara	28	16	57.1	*Haemonchus contortus, Trichostrongylus vistrinus, Ostertagia curcumcincta, Strongyloides papillosus, Nematodirus filicollis*
Urial	39	19	48.7	*Eimeria bovis, Haemonchus contortus, Trichostrongylus vistrinus, Ostertagia curcumcincta, Strongyloides papillosus, Protostrongylus, Chabertia ovina, Dictayocaulus*
Zebra	23	13	39.4	*Eimeria bovis, Strongyloides papillosus, Nematodirus filicollis, Strogylus equinus, Oxyuris equi, Protostrongylus*
Hog Deer	22	4	18.2	*Eimeria ovis, Haemonchus contortus, Chabertia ovina*
Spotted Deer	20	7	35	*Eimeria bovis, Haemonchus contortus, Trichostrongylus vitrines, Ostertagia curcumcincta, Chabertia ovina*
Red Deer	12	0	0	0
Grey Goral	18	2	11.1	*Eimeria bovis, Haemonchus contortus, Chabertia ovina*
Black Buck	13	12	92.3	*Eimeria bovis, Haemonchus contortus, Trichostrongylus vitrines, Moniezia*
Barking Deer	12	3	25	*Eimeria bovis, Trichostrongylus vitrines, Ostertagia curcumcincta*
Mouflon sheep	12	1	8.3	*Trichostrongylus vitrinus, Strongyloides papillosus*
Rhesus monkey	12	0	0	0
Vervet Monkey	12	0	0	0
Langoor	12	0	0	0
Brown Bear	12	8	66.66	*Trichuis vulpis, Toxoc1ra canis*
Grey Wolf	12	3	25	*Trichuris vulpis, Toxocara canis, Muell1rius capillaris*
Red Fox	12	2	16.66	*Toxocara canis, Muel1erius capillaris*
Jackal	12	2	16.66	*Trichuris vulpis, Toxo1ara canis*
Lion	12	2	16.66	*Toxocara canis, Dipyl1dium caninum*
Total	340	113	33.2	

As can be seen in Table 1, the maximum numbers of ungulates were found positive for gastrointestinal parasites compared to Carnivores and only one species (Brown bear) from Omnivores was found positive for gastrointestinal parasites. *Eimeria bovis* was the most prevalent (15.4%) parasite followed by *Haemonchus contortus* (10.2%), *Trichostrongylus vitrinus* (7.7%), *Strongyloides papillosus* (5.1%), *Protostrongylus* (3.7%) and *Ostertagia curcumsincta* (2.5%). A total of 45 samples were obtained from blue bull and 19 samples were infected. *Trichostrongylus vitrinus* and *Haemonchus contortus*, were most prevalent (13.3%) followed by *Eimeria bovis* (8.88%), *Haemonchus contortus* (4.4 %), and *Chabertia ovina* (2.2%). A total of 28 samples were obtained from Chinkara out of which, 15 samples were infected with endoparasites. *Ostertagia curcumsincta* was the most prevalent parasite (17.85%) along with *Trichostrongylus vitrinus* and *Fasciola hepatica* (each 7.1%), *Strongyloides papillosus* and *Nematodirus filicollis* (each 3.6%).

The obtained results indicated that 59 faecal samples of Red deer (*Cervus elaphus*) were infected with *Haemonchus contortus* (14.0%) followed by *Ostertagia curcumcincta* (12.3%), *Trichostrongylus vistrinus* (10.5%), *Eimeria bovis* (7.0%), and *Strongyloides papillosus* (1.7%). A total of 44 fecal samples from Yak (*Bos grunniens*) shown to be infected with *Ostertagia curcumcincta* (10%), *Haemonchus contortus* (7.5%), *Eimeria bovis* and *Oesophagostomum columbianum* (each 2.5%), and 12 fecal samples were obtained from Siberia tiger (*Panthera tigris altaica*) and one sample was positive for *Taxocara canis* (8.3%).

Table 2. Prevalence of oocytes/eggs, larvae of parasites isolated from zoo animals between August 2020 to July 2021 from Bansra Galli Wildlife Park, Rawalpindi, Pakistan

Mammals	Total sample	Number of infected animals	(%)	Parasite's encounter
Yak	44	9	22.5	*Eimeria bovis, Haemonchus contortus, Ostertagia curcumcincta, Oesophagostomum columbianum*
Red Deer	59	26	45.61	*Eimeria bovis, Haemonchus contortus, Trichostrongylus vitrines, Ostertagia curcumcincta, Strongyloides papillosus*
Total	103	36	36.08	

Table 3. Prevalence of oocytes/eggs, larvae of parasites isolated from zoo animals between August 2020 to July 2021 Lohi Bher Wildlife Park, Rawalpindi, Pakistan

Mammals	Total sample	Number of infected animals	(%)	Parasite's encounter
Blue Bull	43	24	55.8	*Eimeria bovis, Haemonchus contortus, Trichostrongylus vitrines, Ostertagia curcumcincta, Strongyloides papillosus, Fasciola hepatica, Chabertia ovina*
Urial	29	11	37.9	*Eimeria bovis, Haemonchus contortus, Trichostrongylus vitrines, Ostertagia curcumcincta, Strongyloides papillosus, Protostrongylus, Chabertia ovina*
Chinkara	31	11	35.4	*Trichostrongylus vitrines, Ostertagia curcumcincta, Strongyloides papillosus, Fasciola hepatica*
Black Buck	27	24	88.8	*Eimeria bovis, Trichostrongylus vitrines, Ostertagia curcumcincta, Strongyloides papillosus, Nematodirus filicollis*
Barking Deer	22	5	41.6	*Eimeria bovis, Chabertia ovina*
Mouflan Sheep	16	6	37.5	*Eimeria bovis, Ostertagia curcumcincta, Protostrongylus*
Grey Goral	22	0	0	0
Zebra	27	14	51.8	*Strongyloides papillosus, Strogylus equinus, Oxyuris equi, Protostrongylus*
Red Deer	33	1	7.6	*Ostertagia curcumcincta*
Hog Deer	26	17	65.3	*Eimeria bovis, Haemonchus contortus, Trichostrongylus vitrines, Ostertagia curcumcincta, Strongyloides papillosus, Nematodirus filicollis, Fasciola hepatica, Protostrongylus, Moniezia*
Spotted Deer	23	5	26.3	*Haemonchus contortus, Trichostrongylus vitrines, Strongyloides papillosus*
Total	296	118	46.27	

Table 3 reveals that Ungulate species had the greatest range of gastrointestinal parasites eggs/ova and hatchlings compared to Carnivores. Out of 29 samples of Urial, *Eimeria bovis* and *Chabertia ovina* eggs were more prevalent (10.34%), whereas *Trichostrongylus vitrinus* was most prevalent (19.35%) in Chinkara (*Gazella bennettii*). Other detected endoparasites included *Haemonchus contortus*, *Trichostrongylus vitrinus*, *Ostertagia curcumcincta*, *Strongyloides papillosus*, and *Protostrongylus*, however, these parasites had low prevalence.

In Zebra (*Equus quagga*), *Strongylus equines* (18.52%) had the highest prevalence rate compared to *Strongyloides papillosus*, *Oxyuris equi*, and *Protostrongylus* (14.81%, 11.11%, and 7.41%) respectively. In 26 samples of Hog deer (*Hyelaphus porcinus*) 9 different endo-parasitic species were isolated, and *Trichostrongylus vitrinus* was the most infecting parasitic species (15.38%). Other endo-parasites include *Eimeria bovis, Haemonchus contortus, Ostertagia curcumcincta, Strongyloides papillosus, Nematodirus filicollis, Fasciola hepatica, Protostrongylus*, and *Moniezia* species.

Table 4. Prevalence of oocytes/eggs, larvae of parasites isolated from zoo animals between August 2020 to July 2021 Ayub National Park, Rawalpindi, Pakistan

Mammals	Total Sample	Number of infected	(%)	Parasite's encounter
Urial	27	6	22.2	*Eimeria bovis, Haemonchus contortus, Trichostrongylus vitrines, Ostertagia curcumcincta, Chabertia ovina*
Blue Bull	18	3	16.6	*Protostrongylus, Moniezia, Dicyocaulus*
Chinkara	18	3	16.6	*Ostertagia curcumcincta, Strongyloides papillosus, Chabertia ovina*
Zebra	27	6	22.2	*Strongyloides papillosus, Strogylus equinus, Oxyuris equi*
Black Buck	18	5	27.7	*Haemonchus contortus, Ostertagia curcumcincta, Fasciola hepatica*
Mouflon Sheep	18	2	11.1	*Protostrongylus, Chabertia ovina*
Barking Deer	18	3	16.6	*Haemonchus contortus, Fasciola hepatica, Chabertia ovina*
Hog Deer	27	3	11.1	*Haemonchus contortus, Trichostrongylus vitrines, Protostrongylus*
Lion	25	3	12	*Baylisaascis procyonis, Toxocara canis, Alaria*
Bear	25	3	12	*Trichuris vulpis, Toxocara canis*
Total	221	37	16.75	

The data from blue bull (*Boselaphus tragocamelus*) obtained from 18 fecal samples, including *Protostrongylus spp.*, *Moniezia spp.*, and *Dictyocaulus spp.* were the most prevalent (each 5.5%). A total of 18 fecal samples from Chinkara gazelle (*Gazella bennettii*) were infected with *Ostertagia curcumcincta., Strongyloides, and chabertia* (each 5.5%). A total of 27 fecal samples were collected from Zebra (*Equus quagga*), *Strongylus equinis* were the most prevalent (11.1%) followed by *Oxyuris equi* (7.5%) followed by *Strongyloides* (3.7%). The most prevalent parasite isolated from black buck was *Haemonchus contortus* (16.6%) followed by *Ostertagia curcumcincta* and *Fasciola hepatica* (each 5.5%). A total of 18 fecal samples were collected from Mouflon sheep (*Antilope cervicapra*) and isolated *Protostronglus* and *Chabertia ovina* (each 5.5%). A total of 18 fecal samples of Barking deer (*Muntiacus muntjack)* were infected with *Haemonchus contortus*, *Fasciola hepatica* (each 5.5%). In 27 fecal samples of hog deer (*Hyelaphus porcinus*), *Trichostrongylus vitrinus* was the most prevalent (7.4%) parasite followed by *Haemonchus* and *Protostrongylus* species (each 3.7%). The collected 25 samples from Lion (*Panthera leo*) were infected with *Baylisascaris procyonis* and *Alaria* (each 4%), and Bear (*Ursus americanus*) was found infected with *Trichuris vulpis* and *Taxocara canis* (each 4%).

Figures 1-4 present microscopic images of endoparasites, namely *Dictyocaulus* species, *Trichuris* species, *Strongyloides* species, and *Taxocara* species. The direct smear method and Microscope image processing technique were used to get a detailed and magnified image of the parasites.

Figure 1. Larva of *Dictyocaulus* species extracted from Blue Bull in September 2020 from Ayub National Park, Rawalpindi, Pakistan. The image was obtained using 40x power of magnification.

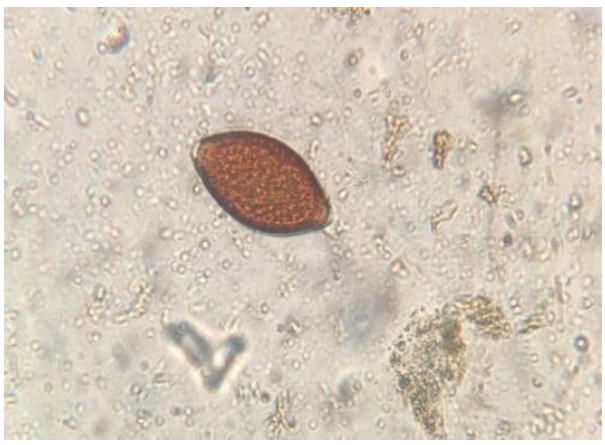

Figure 2. Ova of *Trichuris* species from Brown Bear in September 2020 from Marghzar Zoo, Islamabad, Pakistan. The image was obtained using 10x power of magnification.

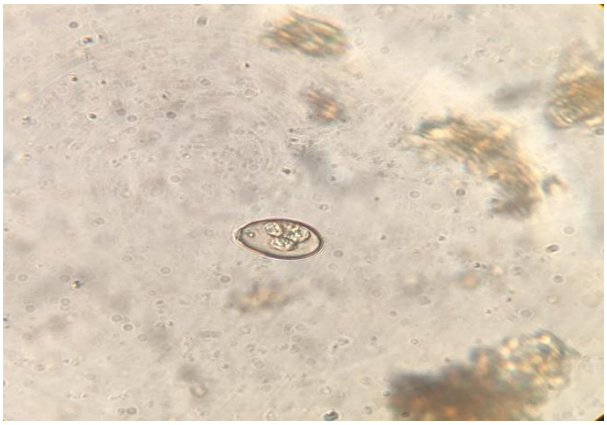

Figure 3. Ova of *Strongyloides* species from Urial in September 2020 from Lohi Bher Wildlife Park, Rawalpindi, Pakistan. The image was obtained using 10x power of magnification.

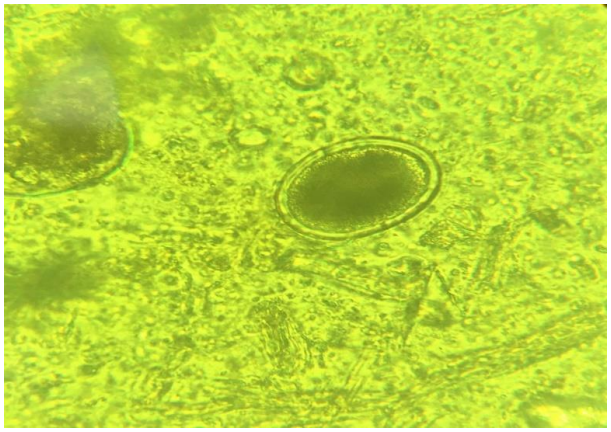

Figure 4. Ova of *Taxocara* species isolated from Siberian tiger in September 2020 from Bansra Galli Wildlife Park, Rawalpindi, Pakistan. The image was obtained using 40x power of magnification.

DISCUSSION

The present study showed that 31.14% of the wild animals were positive for nematodes, cestodes, and trematodes parasites in twin cities' parks and zoos, indicating a mild to moderate prevalence of parasitic infections. The results aligned with Khattak et al. (2023) which showed a high prevalence of GIT parasites among wild and domestic animals in Golden Life Safari Park Khyber Pakhtunkhwa and Mardan district. They attributed the high prevalence to poor settlement, body conditions, anthelminthic medications, and management systems in zoos and parks. The hygiene and cleanliness conditions in the chosen locations of the present study were also compromised, leading to poor animals' health. Moreover, the present study recognized that captive wildlife animals in Pakistan are vulnerable to GIT parasitic infections, especially ungulates including deer, chinkaras, and urials. The high prevalence of GIT infections in these animals was also depicted by Mir et al. (2016), who reported a higher prevalence of parasites from Bir Moti Bagh Mini Zoo, Patiala, Punjab, and found that Blue bull (100%), Spotted deer (50%), Hog deer, Blackbuck (75%), and Barking deer (100%) were infected with GIT parasites. Similarly, Farooq et al. (2012) isolated helminths from domesticated and wild ruminants, including cattle, goats, sheep, camels, chinkara, and blackbuck in the Cholistan desert in Pakistan. The study recorded a high prevalence of helminths in cattle (44.7%) followed by sheep (43%), goats (39%), chinkara (27%), and black buck (20%). The present study reported moderate to high prevalence of GIT parasites in Zebra (22.2%, 39.4%, and 51.8%). However, obtained results of the study from Etosha National Park, Namibia by Turner and Getz (2010) contradicted the findings of the present research, in which a low prevalence of endo-parasites in Zebra, Springbok Blue wild beast, and Gemsbok was reported. The difference in the prevalence of endo-parasites might be due to the difference in management conditions, as in Etosha National Park, animals were dewormed and vaccinated at regular intervals. The findings of the current study are supported by the majority of literature, which emphasizes the susceptibility of both wild and domestic animals to gastrointestinal and parasitic infections.

The present study sought to identify various parasites that lead to GIT infections in captive wildlife animals particularly ungulates in Pakistan. The role of various microorganisms in causing GIT infections, including *Haemonchus contortus*, *Trichostrongylus vitrinus*, *Strongyloides papillosus*, *Protostrongylus*, *Ostertagia curcumsincta*, *Trichostrongylus vitrinus*, *Haemonchus contortus*, *Eimeria bovis* (8.88%), *Haemonchus contortus*, and *Chabertia ovina* was found out. The results are also complementary to Ferdous et al. (2023) who examined wild animals at the safari parks in Bangladesh and reported a high prevalence of GIT parasitic infection, supporting the result of the present study. Beesley et al. (2018) and Frey et al. (2018) also isolated *Dictyocaulus viviperus*, *Ostertagia* spp., and *Fasciola hepatica* from wild ruminants, such as red, white-tailed, and fallow deers in New Zealand. Kvapil et al. (2017) noted that 45% of the samples were positive for endo-parasites from Ljubljana Zoo in Slovenia. They isolated various parasites, including *Eimeria* spp., *Trichuris* spp., *Ostertagia* spp., *Strongylidae* spp., *Strongyloides* spp., *Trichostrongylus* spp., *Capillaria* spp., *Eimeria* spp., and *Protostrongylidae* spp., from Ungulate species, *Ascaridida* spp., *Trichuris* spp., *Capillaria* spp. from Carnivores, and *Balantidium* spp., *Enterobius* spp., *Oxyuridae* spp., *Strongylidae* spp., and *Trichuris* spp. from primates, supporting the results of the present paper. According to Davidson et al. (2014), there was *Ostertagia leptospicularis* (83%), *Spiculopteragia spiculopter* (92%), and characteristics of *Capillaria* spp., *Moniezia* spp., *Oesophagostomum venulosum*, and *Chabertia* spp. parasites in the red deer. Hence, the obtained results of the present study are in line with the given literature on the types of parasites that result in GIT infections in captive wild animals.

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The study contributes to the literature concerning the veterinary health and well-being of wild animals and identifies a need for improved management of these animals. Thus, the findings of the study suggest inadequate deworming practices and administration at the zoos and parks in twin cities. Nehmat et al. (2015) emphasized the importance of adequate veterinary care and sanitary management of captive wildlife animals to minimize the risk and prevent the occurrence of parasitic infections. Accordingly, the present study recommends improving the health and hygiene conditions of captive wild animals. Moreover, ensuring the cleanliness of the landscape and effective administration providing high-quality food and deworming services can enhance the outcomes of the animals.

CONCLUSION

The study reported a mild to moderate level of parasitic infection in four different zoological parks across Islamabad and Rawalpindi, Pakistan, and identified that ungulates had a high prevalence of GIT parasitic infections and were mostly affected by various endoparasites. The study highlighted the adverse impacts of poor management and inadequate deworming protocols in Pakistani zoological gardens for captive wildlife animals. It warned about the worrisome situation which can endanger the lives of precious captive animals. Therefore, it is recommended that a proper deworming protocol should be developed and authorities should improve the sanitation and hygienic condition of these zoological parks. Future studies can explore the most effective strategies for managing these wild animals based on individual animal safety and health needs. Moreover, future studies can identify the type of environment and habitats to suit the health, and well-being of these animals ensure their immunity, and reduce vulnerability.

DECLARATIONS

Availability of data and materials

Due to privacy concerns of the corresponding author, the data is not openly available.

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Author's contributions

Kaleem Ahmed and Wahid Ullah collected fecal samples from premises that had captive wildlife animals. Qasim Ali and Shah Fahad analyzed the samples in the laboratory for parasitic contamination. Kaleem Ahmed and Muhammad Adeel helped in drafting the manuscript. All authors have read the final version of the manuscript for publishing in the present journal.

Competing interests

Authors have no competing interests.

Ethical considerations

All authors have checked the ethical concerns such as plagiarism, misconduct, fabricated or false data, consent to publish double publication and/or submission, and redundancy.

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Macroscopic Differences of Pig Eye after Death: A Veterinary Forensic Study

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ABSTRACT

The study of veterinary forensics is a field of science that is developing rapidly in the world of veterinary medicine. Veterinary forensics plays a crucial role in investigating and resolving cases involving animals, either as subjects or objects in incidents and ensuring the collection of all possible biological and physical evidence. Given the close relationship between humans and animals, numerous significant cases arise that are pertinent to veterinary forensics. The current research aimed to determine early post-mortem changes in pigs, providing insights into animal mortality in real-world scenarios. Observations were made on seven male Yorkshire pigs, aged 3 months old, with an average weight of 30.1 kg. Pigs were observed at four different post-mortem intervals, including 2,4,6, and 8 hours after death, with initial observations at the time of death serving as the control. Observations of changes in the eye sclera, eye lens, eyeball temperature, and eyeball pressure were carried out at each time interval. Results at the 2nd and 4th hours post-mortem showed no macroscopic changes in the eye sclera and eye lens, but there were changes in eye pressure. By the 6th and 8th hours, changes in the sclera and eye lens showed desiccation in the area of the sclera and the eye lens, which became increasingly cloudy. The eyeball temperature measurement values from the 2nd to 8th hour of the study revealed a significant decrease in eyeball pressure. The results of this study indicated observable changes in the eyes can be used as a basic alternative method for calculating the introductory post-mortem interval in animals in the future. There was a significant decrease in eyeball temperature, and eyeball compactness, as significant differences in the eye sclera, and eye lens at 2, 4, 6, and 8 hours post-mortem, compared to the time of death. These variables offer crucial insights into early post-mortem changes in pigs, using the eyes as the primary focus of observation.

Keywords: Death, Eye, Forensic study, Pig

INTRODUCTION

The study of veterinary forensics is essential in veterinary science, especially in identifying and investigating crimes against animals. The area that received special attention in this study has been the eye organ. The eyes of different animals are often the focus of veterinary forensic cases and serve as a rich source of post-mortem information (Ruiz-Ederra et al., 2005). Several factors make the eye an important subject in veterinary forensic studies, including its use in identifying animals involved in crimes through microstructure analysis and other unique signs present in the eye structure (Ang et al., 2021). In several important cases, histopathological and microscopic examination of the eye organs can provide a relatively clear picture of the animal's health condition before death, including the presence of infection, inflammation, or other pathological disorders (Sarmiento et al., 2023). The eye offers numerous advantages in forensic investigations due to its capabilities in identification, visual recording, DNA analysis, and health information retrieval (Ang et al., 2021). The current study aimed to determine the macroscopic differences in pig eyes at various post-mortem intervals. Determining the time of death of animals in the field was a critical need that must immediately receive an easy and effective solution.

MATERIALS AND METHODS

Ethical approval

All procedures conducted on animals were in accordance with the ethical standards of the institution, and the current study was approved by the Animal Care and Use Committee (Komisi Laik Etik Penelitian) Universitas Brawijaya, Indonesia (No. 153 KEP UB 2022).

Sampling

A total of seven healthy male Yorkshire pigs, aged 3 months old, and weighing 30 kg were sourced from pig farms in Kromengan, Malang, East Java, Indonesia. These pigs were euthanized using an overdose of Ketamine 10% Inj. (Dutch, Holland) 10 mg/kg and Xylazine (Interchemie, Holland) 6mg/kg following the standard AVMA Guidelines for

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the Euthanasia of Animals (AVMA, 2020). Euthanization and observation were carried out at the animal burial site in Malang City, East Java, Indonesia. Shortly after euthanasia was successfully carried out, the pig's body was laid down by manipulation with an eye retractor on both right and left eyes; observations were made at 2, 4, 6, and 8 hours post-death. The observed changes are macroscopic changes in the eyeball, eye sclera, eye lens, eyeball temperature, and eye compactness. This research was conducted at the Central Laboratory for Research and Diagnostics of Satwa Sehat Indonesia, Malang, East Java, Indonesia.

Preparation

Observations were conducted in an open-air environment in October 2023, with an environmental temperature of 25-28°C, air humidity of 80%, and a wind speed of 0 km/hour. Observation times with obvious weather conditions were carried out at 08.00 am and 04.00 pm. Macroscopic observations were carried out using a professional camera, (Canon 700D, Japan) to observe the eye sclera and eye lens. Eyeball temperature was observed using a noncontact infrared thermometer (HT 820D, China), and eye compactness was carried out using animal eye tonometry (Tonometer Schiotz, Pakistan).

Statistical analysis

The obtained data was analyzed using inferential statistical analysis to compare the different post-mortem intervals. The relationship between the time of death and macroscopic changes was carried out carefully. Multiple regression analysis ($Y = a + b1\ X1 + b2\ X2 + ... + bn\ Xn.$) was employed, starting with the coefficient of determination, F-test, and t-test. The regression equation was carried out at the end of the analysis to ensure the accuracy of the regression equation's interpretation by confirming its significance. The relationship between the time of death and macroscopic changes was analyzed carefully. The time intervals of death used in this study included 2,4,6, and 8 hours after death occurred. Observations were made by observing macroscopic changes in the eyeball, eye sclera, eye lens, eyeball temperature, and eye compactness.

RESULTS

Observations of changes in the eyes of Yorkshire pigs aged 3 months after euthanization using a combination of Ketamine and Xylazine were summarized in Table 1. Each parameter explains how the macroscopic changes in the sclera, eye lens, eyeball temperature, and eyeball pressure show precise results. Figure 1 shows the observation method carried out at the control and observation time at 8 hours after death. Data for the measured parameter, including the use of a camera, infrared thermometer, and tonometry, are presented in Table 1 and Figure 1.

The results that indicated changes in the sclera began to occur between 4 and 8 hours after death. Changes in the eye lens occurred twice 2 and hours after death. The results multiple regression test indicated significant changes in eyeball temperature ($p < 0.05$) and eyeball pressure ($p < 0.05$) using Minitab 18 software.

Table 1. The pig eyeball parameters at observation times

Parameter	Sample	Control	Time of death			
			2 Hours	4 Hours	6 Hours	8 Hours
Macroscopic change - sclera	Pig 01	White	White	Cloudy Black	Cloudy Black	Cloudy Black
	Pig 02	White	White	Cloudy Black	Cloudy Black	Cloudy Black
	Pig 03	White	White	Cloudy Black	Cloudy Black	Cloudy Black
	Pig 04	White	White	Cloudy Black	Cloudy Black	Cloudy Black
	Pig 05	White	White	Cloudy Black	Cloudy Black	Cloudy Black
	Pig 06	White	White	Cloudy Black	Cloudy Black	Cloudy Black
Macroscopic change - lens	Pig 01	Clear	Cloudy White	Cloudy White	Cloudy White	White
	Pig 02	Clear	Cloudy White	Cloudy White	Cloudy White	White
	Pig 03	Clear	Cloudy White	Cloudy White	Cloudy White	White
	Pig 04	Clear	Cloudy White	Cloudy White	Cloudy White	White
	Pig 05	Clear	Cloudy White	Cloudy White	Cloudy White	White
	Pig 06	Clear	Cloudy White	Cloudy White	Cloudy White	White
Eyeball temperature (^{0}C)	Pig 01	38.0 ^{0}C	37.5 ^{0}C	35.2 ^{0}C	33.7 ^{0}C	32.3 ^{0}C
	Pig 02	38.1 ^{0}C	37.2 ^{0}C	36.5 ^{0}C	33.5 ^{0}C	32.5 ^{0}C
	Pig 03	38.0 ^{0}C	36.9 ^{0}C	36.8 ^{0}C	33.6 ^{0}C	33.2 ^{0}C
	Pig 04	38.2 ^{0}C	37.6 ^{0}C	35.6 ^{0}C	33.5 ^{0}C	32.8 ^{0}C
	Pig 05	38.0 ^{0}C	37.0 ^{0}C	36.6 ^{0}C	34.5 ^{0}C	33.9 ^{0}C
	Pig 06	38.2 ^{0}C	37.6 ^{0}C	35.9 ^{0}C	33.8 ^{0}C	32.5 ^{0}C
Eyeball compactness (mm/Hg)	Pig 01	15.2	14.7	12.5	11.3	11.0
	Pig 02	15.3	13.9	12.1	11.5	10.9
	Pig 03	15.2	14.8	11.6	11.7	10.8
	Pig 04	15.2	14.2	12.4	10.8	10.2
	Pig 05	15.7	14.6	13.0	11.1	10.2
	Pig 06	15.4	14.8	13.2	12.1	11.1

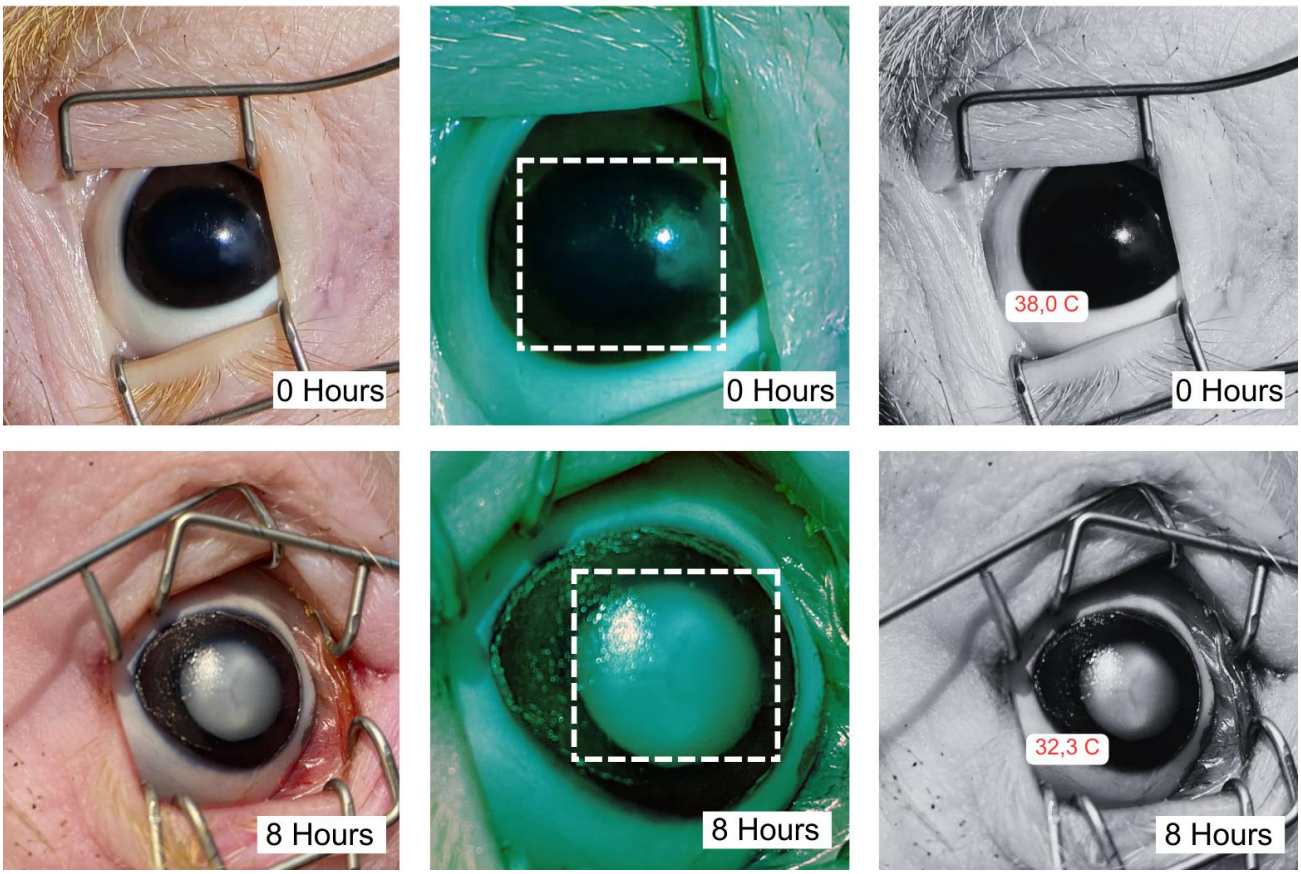

Figure 1. The observations of the sclera, lens, and eyeball temperature immediately after death and 8 hours post-mortem

DISCUSSION

Determining the time of death in animals is crucial for legal proceedings, often referred to as post-mortem interval analysis (Shrestha et al., 2023). This necessity arises due to animals being involved in various cases where evidence of death timing is essential. Reported cases of animal death encompass a range of species, including dogs, cats, cows, horses, and other wildlife (Erlandsson and Munro, 2007; Ushine et al., 2020). In the present study, homogenization of pig samples was carried out using male Yorkshire pigs aged 3 months with an average body weight of 30.1 kg. Pigs were used as experimental animals, similar to other nutritional research (Roura et al., 2016), heart failure models (Dixon and Spinale, 2009), and wound tissue healing (Kuo et al., 2022). Pigs are frequently chosen for research models due to factors such as their abundant population and their non-protected status, ensuring ethical considerations are met (Swindle et al., 2012).

The initial data from pigs used as controls were compared with observation times at 2, 4, 6, and 8 hours with immediate death time serving as a control. With four variables observed in this research, macroscopic changes in the sclera, lens, eyeball temperature, and eyeball pressure provided a practical picture. They are made easy to use as practical samples for basic data in the field. Following death, pigs undergo various phases of post-mortem changes, including algor mortis, rigor mortis, livor mortis, and decomposition, aligning with reports that natural changes occur in the body for 12 to 24 hours post-death (Brooks, 2016). These changes in death can affect all parts of the animal's body, including the eyes (Ang et al., 2021). The eye was selected as a crucial organ in the post-mortem examination process for several reasons. It stores significant data within the body and exhibits minimal developmental changes in both humans and animals Changes experienced by the eye, such as macroscopic changes in the sclera and eye lens, are associated with changes in metabolic processes in the eye, which gradually stop after death occurs (Gerometta et al., 2019). The desiccation process in the sclera layer of the eye is also associated with the cessation of the supply of fluid secretion from the lacrimal gland (Conrady et al., 2016). It is closely related to an increase in cytokines and antigen-presenting cells (APCs) in the conjunctival area (Alam et al., 2021).

The changes observed in the eye lens shortly after death are attributed to several factors. The eye lens contains specific proteins, particularly crystallin, which are crucial for maintaining lens transparency (Andley, 2007). However,

once the body's maintenance and repair mechanisms cease functioning post-mortem (Miller and Zachary, 2017), these proteins can then aggregate, leading to clumping. This aggregation disrupts the lens's transparency, resulting in a cloudy appearance. Additionally, the cells surrounding the lens cease vital functions such as cleaning or renewing lens components (Andley, 2008). With this condition, materials that are typically removed or replaced during cellular maintenance can accumulate in the lens, further contributing to cloudiness (Gerometta et al., 2019).

The decrease in eyeball temperature is associated with the cessation of metabolic processes affecting all organs, including the eyes (Kaliszan et al., 2010). This decrease in temperature is observed in pigs and other experimental animals in research settings. Furthermore, in this study, eyeball pressure demonstrated a significant decrease over time. Eyeball pressure reflects the integrity of the eye and the potential for damage. A decrease in eye pressure is observed in various animal species post-mortem, as seen in cattle (Gerometta et al, 2019), rabbits and rats (Lee et al, 2022), as well as mice (Kim et al., 2007). In this study, the changes that occur in the first 8 hours after pig death illustrate fundamental mortality changes that can be used in effective and economical examination methods in the field and serve as a tool to prove the estimated time of death of the animal.

CONCLUSION

The findings of this study confirmed a significant decrease in the compactness and temperature of the eyeball, the lens became white and cloudy, and the sclera became cloudy black at various time intervals after pig death. The findings suggest that further research should be carried out on changes over a more varied observation period from 36-72 hours.

DECLARATIONS

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Authors' contributions

Albiruni Haryo contributed to collecting samples and statistically analyzing data. Rini Widayanti, Tri Wahyu Pangestiningsih, AYPBC Widyatmoko analyzed data, and formatted and edited the manuscript. All authors discussed the results commented on the manuscript, and gave final approval for the final version of the manuscript.

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.

Conflict of interests

The authors have no conflict of interest to present.

Availability of data and materials

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

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Gastrointestinal Helminths in Local (Black Bengal) and Jamunapari Goats of Barishal Sadar, Southern Bangladesh

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ABSTRACT

Gastrointestinal helminths are important causes of hindering global goat production. To find the prevalence of gastrointestinal helminths of Black Bengal and Jamunapari breeds of goats, the current investigation was carried out at Barishal Sadar Upazilla of Barishal district, Bangladesh. The gastrointestinal helminths were identified through coprological examination. A total of 112 fecal samples were collected from household goats across different seasons, breeds, sexes, and ages. During the study period, four types of gastrointestinal helminths were identified based on the presence of helminth eggs in fecal samples. The overall prevalence of gastrointestinal helminths in goats was found to be 82.1%, while the prevalence rates of *Fasciola gigantica* (*F. gigantica*), *Paramphistomum* spp., *Bunostomum* spp., and *Hemonchus* spp. were 34.8% (95% CI: 1.4-2.5), 22.3% (95% CI: 0.7-1.8), 14.3% (95% CI: 0.1-1.5), and 10.7% (95% CI: 0.2-1.0), respectively. A significantly different prevalence was observed among different gastrointestinal helminths in goats. A significantly lower prevalence of *F. gigantica* and *Paramphistomum* spp. was observed in male goats, compared to females. A higher prevalence of *F. gigantica* was significantly observed during the winter, compared to the summer. The current study elucidates that *F. gigantica* was more prevalent in female goats. The current study indicated that *F. gigantica* was more prevalent in female goats. These findings underscore the importance of further research and control measures to manage gastrointestinal helminth infections in goats across southern Bangladesh and other regions with similar environmental conditions.

Keywords: *Fasciola gigantica*, Gastrointestinal helminth, Goat, Prevalence, Summer

INTRODUCTION

Globally livestock production is considered a crucial sub-sector of agricultural production (Islam et al., 2018; Naide et al., 2018; Sumon et al., 2018) and goat production plays a significant role in promoting human health and economy. Kamaruddin (2003) reported that by exporting skin, meats, and other by-products of goats, there was a substantial amount of foreign currency earnings by the government of developing countries. Since goats are essential production animal species for the supply of milk, they are considered as the cow for poor people. The annual production of milk, meat, and skin from goats makes a significant contribution to the gross domestic product of developing nations (FAO, 2003). The goats have a high potential to reduce poverty in emerging countries like Bangladesh (Faruque et al., 2010) and are crucial in the maintenance of nutrition and boosting of up rural economy.

Among all diseases, parasitic infection is one of the critical constraints in the worldwide production of livestock (Islam et al., 2022). The gastroenteritis caused by helminth parasites is a major problem of the health maintenance of goats. Globally helminths reduce goat production by infecting them, resulting in reduced health (morbidity) or even death (mortality) of the animals. The goat industry experiences a decrease in earnings due to the expenses for treating gastrointestinal helminths or implementing control measures (Hashemnia et al., 2013; Rahman et al., 2017). Among the diseases caused by helminths, Fasciolosis, Paramphistomiasis, Bunostomiasis, Hemonchosis, Agriostomiasis, and Moneziasis are considered economically crucial infections of goats (Win et al., 2020). The gastrointestinal helminth infection among goats in emerging countries is widespread and the financial losses due to helminth infection are significantly high in those countries (Hossain et al., 2011). Among helminths, the most important and prevalent helminth infection is *F. gigantica* and in Bangladesh only prevalent helminth fluke is *F. gigantica* in livestock (DLS, 2010-2011).

It has been reported previously that gastrointestinal helminth infection is the most prevalent parasitic disease of ruminants in Asia and Africa (Hamond and Sewell, 1990; Spithill et al., 1999; Khatun et al., 2021). Globally, there were huge economic losses in ruminants due to reduced production of meat and milk, liver condemnation, and failure of reproductive functions due to different gastrointestinal helminth infections (Fabiyi, 1986; Diaw et al., 1998; Rahman et

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al., 2012). The 7.54% condemnation of 1000 goat livers cause an economic loss of about 28.86 $ (Selim et al., 1997). Furthermore, at present, it is recognized that among gastrointestinal helminths, Fasciolosis is an emerging disease of humans. It is estimated that 2.4 million people are infected with *Fasciola hepatica* and 180 million people are at risk of *Fasciola hepatica* infection (WHO, 2006). The immature *Paramphistomum* spp. causes severe gastroenteritis and adult *Paramphistomum* spp. causes blockage of the intestinal tract of goats (Godara et al., 2014). The *Bunostomum* spp., and *Hemonchus* spp. are important helminths that suck goats' blood and ultimately cause anemia which lead to production losses in the goats (Santos et al., 2012).

Despite the wide prevalence and huge economic losses incurred by gastrointestinal helminths, no epidemiological study of the gastrointestinal helminths of goats has been undertaken so far at the Barishal Sadar, southern part of Bangladesh (Rahman et al., 2012; Talukder et al., 2015). Therefore, in the present study, an attempt was undertaken to find out the prevalence of gastrointestinal helminth infections in goats and the effects of various animal factors, including goat's sex, age, breed, and environmental factors, such as seasonal influence on the prevalence of gastrointestinal helminth infections in goats at Barishal Sadar, Southern Bangladesh.

MATERIALS AND METHODS

Ethical approval

The present study was conducted according to the guidelines of Post-graduate Studies and Research, Patuakhali Science and Technology University, Dumki, Patuakhali-8602, Bangladesh.

The area and period of study

A cross-sectional study was undertaken to find out the prevalence of gastrointestinal helminth infections in meat type goats at Barishal Sadar, Bangladesh from July 2011 to June 2012. The study was conducted during all the seasons in a year. Barishal Sadar is an Upazilla of the Barishal district (southern part of Bangladesh). An Upazilla is the smallest administrative unit of Bangladesh. The location of the Barishal Sadar Upazilla is 90°22′E 22°42′N and has a total area of 263.56 km² (Figure 1) with a riverine area of 15.54 km^2 (5.9% riverine area). The highest average annual temperature is 35.1°C, the lowest 12.1°C, and 1955 mm mean annual rainfall. The region is known as the riverine area of Bangladesh with high rainfall. Five rivers flow through the Barishal Sadar Upazilla. Goats were typically raised by the locals in that area using a semi-scavenging method (BBS, 2011).

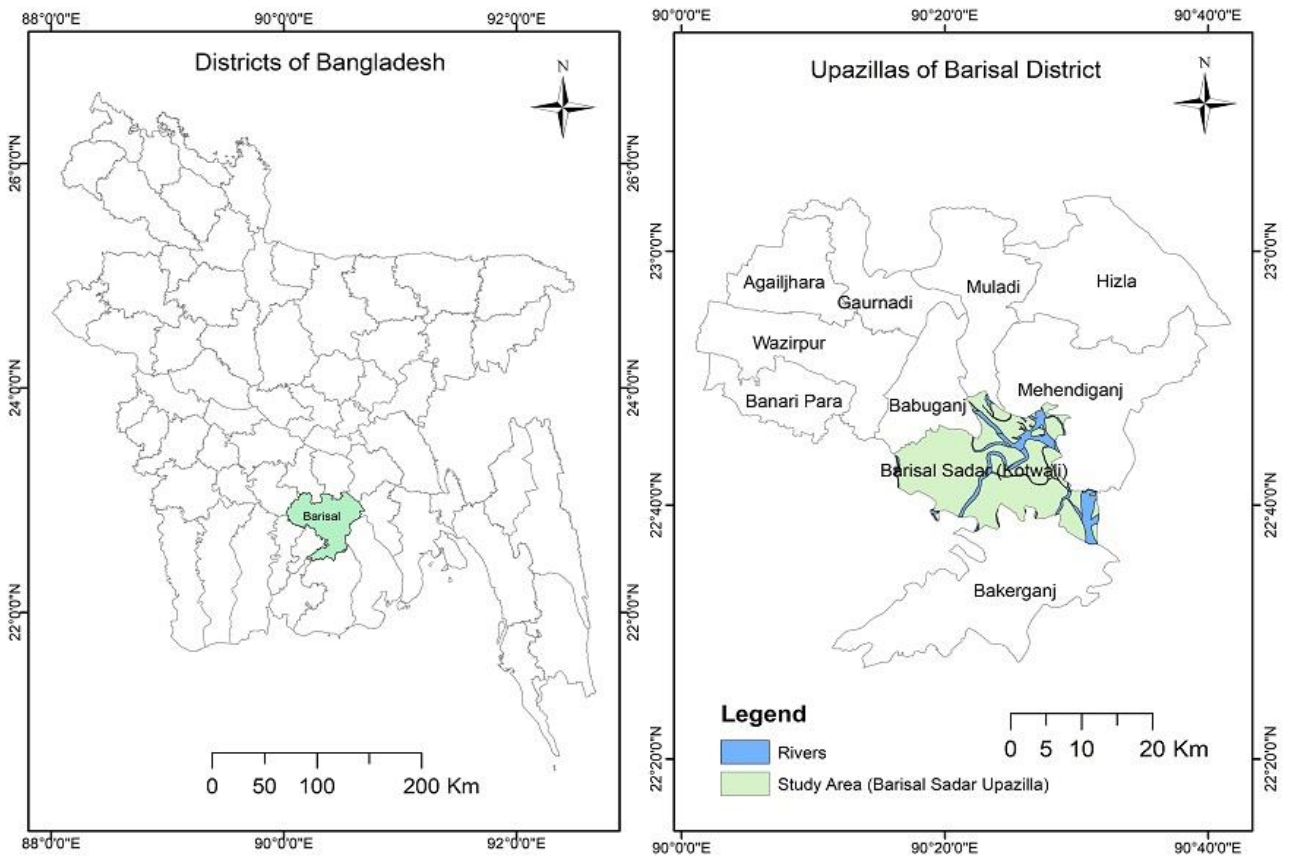

Figure 1. Study area located in Barishal Sadar Upazilla of Barishal district, Bangladesh

Study design and sample size determination

There are about 4037 households in the Barishal Sadar Upazilla. Among 4037 households, at least 200 households had goat farms commonly known as family farms (BBS, 2011). For the determination of the sample size, Epi-Info™ software Version 7 (2008) was used (CDC, Atlanta). In a one-stage cluster sampling using the StatCalc of the Epi-Info™, a total of 132 samples were required from Barishal Sadar for the study (at the level of 95% confidence interval) with at least one representative sample from each family farm. However, due to the limitations (e.g., too poor transport and communication systems), 112 fecal samples from 112 farms were collected using on-site sampling. A study questionnaire was developed to record the information of each goat, including age, sex, breed, season, and management practices, such as grazing habits and the information of the goat owner. The questionnaire was in English therefore, it was translated into the common language of the locality during the face-to-face interview with the owner of the goats. The collected information was saved in Microsoft Excel for data analysis.

Sample collection and examination

A total of 112 fecal samples were randomly collected from 112 apparently healthy goats with a history of no anthelmintic treatment in Barishal Sadar Upazilla. The samplings were performed early in the morning between 8:00-9:00 a.m. before moving the goats to the pasture areas. About 5-10g of faeces were directly collected by hand from the rectum of the goats and put into a plastic container with 10% formalin. The particulars of the goats, such as the name of the goat owner and the body weight of goats were also recorded. The label depicting the particulars of the goat, the address of the owner, and the date of the collection were taped to the plastic container's wall. The samples were immediately transferred to the Medicine laboratory of Patuakhali Science and Technology University, Babuganj, Barishal-8210 by using an ice box to examine subsequently.

The faecal samples were examined by direct smear, sedimentation, and flotation methods as described previously by Soulsby (1986). The economical binocular light microscope XSZ-107BN (2010) from Labtex Bangladesh was used for examining the fecal samples. From each faecal sample and each test, at least, two smears were prepared for the identification of eggs of different gastrointestinal helminths (Soulsby, 1986; Hendrix, 2006). The prevalence was confirmed based on the morphological characters of helminth eggs as described by Soulsby (1986) and Valero et al. (2009).

Statistical analyses

The data were entered into an Excel spreadsheet of MS Office. Determination of the prevalence was performed by employing the overall frequency of positive and negative sample test results using Equation 1 (Islam et al., 2020). For examining the statistical associations between the prevalence of gastrointestinal helminths infection and various risk factors of goats (e.g. sex, breed, and seasons), the one-way analysis of variance (ANOVA) followed by post tests and descriptive statistics were utilized. Test results were considered significant at ($p < 0.05$).

Prevalence (%) = $^{n}/_{N} \times 100$ (Equation 1).

Where n is the number of samples positive for different types of gastrointestinal helminths and N is the number of the total samples examined.

RESULTS

A total of 112 faecal samples from goats were examined in different sexes and ages during all the seasons. Four different types of gastrointestinal helminths were detected in the present study. The overall prevalence of gastrointestinal helminths in goats at Barishal Sadar is 82.1%. The prevalence of *F. gigantica*, *Paramphistomum* spp., *Bunostomum* spp., and *Hemonchus* spp. were 34.8%, 22.3%, 14.3%, and 10.7%, respectively. The prevalence of different gastrointestinal helminths was significantly different ($p < 0.05$) as shown in Table 1.

The results indicated *F. gigantica* prevalence was significantly higher in *Hemonchus* spp. prevalence ($p < 0.05$) as shown in Table 2. The highest prevalence of *F. gigantica* (36.9%) was observed in female goats, which was significantly different from the prevalence of male goats ($p < 0.05$; Table 3). As can be seen in Table 3 the highest prevalence of *F. gigantica* was observed during the winter season (52.6%) which was significantly different than the prevalence in rainy and summer seasons ($p < 0.05$). The prevalence of *F. gigantica* in winter was significantly higher than summer prevalence as shown in Table 4 ($p < 0.05$). Table 5 indicates the prevalence of *Paramphistomum* spp. was significantly higher in female goats than male goats ($p < 0.05$).

A significantly different prevalence of *Bunostomum* spp., and *Hemonchus* spp. did not observe between breed and sexes and among different seasons (Tables 6 and 7). A comparatively higher prevalence of *F. gigantica*, *Paramphistomum* spp., *Bunostomum* spp., and *Hemonchus* spp. were observed in younger goats compared to older goats

(Tables 3, 5, 6, and 7). A total of 17.85% mixed gastrointestinal helminthiasis (20 mixed infections out of 112 samples) were also observed. The mixed infections of *F. gigantica* and *Paramphistomum* spp. (8.03%), *F. gigantica* and *Hemonchus* spp. (2.68%), *F. gigantica* and *Bunostomum* spp. (2.68%), *Paramphistomum* spp. and *Hemonchus* spp. (0.90%), and *Hemonchus* spp. and *Bunostomum* spp. (3.57%) were recorded.

Table 1. Prevalence of different gastrointestinal helminths in goats of Barishal Sadar, Bangladesh

Name of helminth	Examined Samples (Mean ± SD)	Positive samples (Mean ± SD)	Prevalence percentage (95 % CI)	p-value
Fasciola gigantica (*F. gigantica*)	112 (5.6 ± 1.76)	39 (1.95 ± 1.23)	34.8 (1.4–2.5)	< 0.05*
Paramphistomum spp.	112 (5.6 ± 1.76)	25 (1.25 ± 1.25)	22.3 (0.7–1.8)	
Bunostomum spp.	112 (5.6 ± 1.76)	16 (0.8 ± 1.58)	14.3 (0.1–1.5)	
Hemonchus spp.	112 (5.6 ± 1.76)	12 (0.6 ± 0.94)	10.7 (0.2–1.0)	

CI: Confidence interval, *Significant difference ($p < 0.05$)

Table 2. The prevalence of different helminths in goats of Barishal Sadar, Bangladesh

Sl. no	Variables	Name of helminths	p-value
01	*F. gigantica*	*Paramphistomum* spp.	> 0.05
02	*F. gigantica*	*Bunostomum* spp.	> 0.05
03	*F. gigantica*	*Hemonchus* spp.	< 0.05*
04	*Paramphistomum* spp.	*Bunostomum* spp.	> 0.05
05	*Paramphistomum* spp.	*Hemonchus* spp.	> 0.05
06	*Bunostomum* spp.	*Hemonchus* spp.	> 0.05

*Significant difference ($p < 0.05$).

Table 3. Prevalence of *F. gigantica* in goats of Barishal Sadar, Bangladesh

Variables		Examined samples (Mean ± SD)	Positive samples (Mean ± SD)	Prevalence percentage (95 % CI)	p-value
Breed	Black Bengal	74 (3.70 ± 1.59)	25 (1.25 ± 1.02)	33.8 (0.8-1.7)	> 0.05
	Jamunapari	38 (1.90 ± 0.79)	14 (0.70 ± 0.66)	36.8 (0.4-1.0)	
Sex	Male	28 (1.40 ± 1.05)	8 (0.4 ± 0.68)	28.6 (0.1-0.7)	< 0.05*
	Female	84 (4.2 ± 1.58)	31 (1.55 ± 0.83)	36.9 (1.2-1.9)	
Age	0–6 months	11 (0.55 ± 1.05)	3 (0.15 ± 0.37)	27.3 (-0.02-0.3)	> 0.05
	6 months-1 year	35 (1.75 ± 1.94)	12 (0.60 ± 0.82)	34.3 (0.2-1.0)	
	1 year-1.5 years	14 (0.70 ± 1.13)	7 (0.75 ± 0.99)	50 (-0.1-0.8)	
	1.5 years-2 years	17 (0.85 ± 1.31)	7 (0.35 ± 0.75)	41.2 (0.001-0.7)	
	2 years-2.5 years	12 (0.60 ± 1.19)	4 (0.20 ± 0.52)	33.3 (-0.04-0.4)	
	2.5 years-3 years	13 (0.65 ± 1.53)	4 (0.20 ± 0.70)	30.8 (-0.1-0.5)	
	> 3 years	10 (0.50 ± 1.10)	2 (0.10 ± 0.31)	20 (-0.04-0.2)	
Seasons	Rainy	35 (5.83 ± 1.47)	11 (1.83 ± 0.75)	31.4 (1.0-2.6)	< 0.05*
	Winter	38 (5.50 ± 2.74)	20 (2.83 ± 1.47)	52.6 (1.6-4.1)	
	Summer	39 (5.50 ± 1.38)	8 (1.00 ± 0.89)	20.5 (0.3-2.0)	

Season: Rainy; July-October, Winter; November-February, Summer; March-June, CI: Confidence Interval, *Significant difference ($p < 0.05$)

Table 4. Prevalence of *F. gigantica* during different seasons in goats of Barishal Sadar, Bangladesh

Sl. no	Variables	Season	p-value
01	Rainy	Winter	> 0.05
02	Rainy	Summer	> 0.05
03	Winter	Summer	< 0.05*

*Significant difference ($p < 0.05$)

Table 5. Prevalence of *Paramphistomum* spp. in goats of Barishal Sadar, Bangladesh

Variables		Examined samples (Mean ± SD)	Positive samples (Mean ± SD)	Prevalence percentage (95 % CI)	p-value
Breed	Black Bengal	74 (3.70 ± 1.59)	16 (0.80 ± 0.77)	21.6 (0.4-1.6)	> 0.05
	Jamunapari	38 (1.90 ± 0.79)	9 (0.45 ± 0.83)	23.7 (0.06-0.8)	
Sex	Male	28 (1.40 ± 1.05)	6 (0.30 ± 0.66)	21.4 (-0.007-0.6)	< 0.05*
	Female	84 (4.20 ± 1.58)	19 (0.95 ± 1.15)	22.6 (0.4-1.5)	
Age	0-6 months	11 (0.55 ± 1.05)	1 (0.05 ± 0.22)	9.1 (-0.05-0.15)	> 0.05
	6 months-1 year	35 (1.75 ± 1.94)	9 (0.45 ± 0.89)	25.7 (0.03-0.9)	
	1 year-1.5 years	14 (0.70 ± 1.13)	2 (0.10 ± 0.45)	14.3 (-0.1-0.3)	
	1.5 years-2 years	17 (0.85 ± 1.31)	5 (0.25 ± 0.55)	29.4 (-0.008-0.5)	
	2 years-2.5 years	12 (0.60 ± 1.19)	2 (0.10. ± 0.31)	16.7 (-0.04-0.2)	
	2.5 years-3 years	13 (0.65 ± 1.53)	4 (0.20 ± 0.70)	30.7 (-0.1-0.5)	
	> 3 years	10 (0.50 ± 1.10)	2 (0.10 ± 0.31)	20 (-0.04-0.2)	
Seasons	Rainy	35 (5.83 ± 1.47)	7 (1.17 ± 1.17)	20 (-0.06-2.4)	> 0.05
	Winter	38 (5.50 ± 2.74)	6 (0.50 ± 0.84)	15.8 (-0.3-2.0)	
	Summer	39 (5.50 ± 1.38)	12 (1.83 ± 1.47)	30.8 (0.4-3.0)	

CI: Confidence interval, *Significant difference ($p < 0.05$), Rainy: July-October, Winter: November-February, Summer: March-June

Table 6. Prevalence of *Bunostomum* spp. in goats of Barishal Sadar, Bangladesh

Variables		Examined samples (Mean ± SD)	Positive samples (Mean ± SD)	Prevalence percentage (95 % CI)	p-value
Breed	Black Bengal	74 (3.70 ± 1.59)	9 (0.45 ± 1.10)	12.1 (-0.06-1.0)	> 0.05
	Jamunapari	38 (1.90 ± 0.79)	7 (0.35 ± 0.67)	18.4 (0.04-0.7)	
Sex	Male	28 (1.40 ± 1.05)	4 (0.20 ± 0.52)	14.3 (-0.04-0.4)	> 0.05
	Female	84 (4.20 ± 1.58)	12 (0.60 ± 1.39)	14.3 (-0.05-1.3)	
Age	0-6 months	11 (0.55 ± 1.05)	1 (0.05 ± 0.22)	9.1 (-0.05-0.2)	> 0.05
	6 months-1 year	35 (1.75 ± 1.94)	6 (0.30 ± 0.80)	20 (-0.08-0.7)	
	1 year-1.5 years	14 (0.70 ± 1.13)	4 (0.20 ± 0.70)	28.6 (-0.1-0.5)	
	1.5 years-2 years	17 (0.85 ± 1.31)	2 (0.10 ± 0.31)	11.8 (-0.04-0.2)	
	2 years-2.5 years	12 (0.60 ± 1.19)	1 (0.05 ± 0.22)	8.3 (-0.05-0.2)	
	2.5 years-3 years	13 (0.65 ± 1.53)	1 (0.05 ± 0.22)	7.7 (-0.05-0.2)	
	> 3 years	10 (0.50 ± 1.10)	1 (0.05 ± 0.22)	10 (-0.05-0.2)	
Seasons	Rainy	35 (5.83 ± 1.47)	5 (0.83 ± 1.60)	14.3 (-0.8-2.5)	> 0.05
	Winter	38 (5.50 ± 2.74)	7 (1.17 ± 2.40)	18.4 (-1.1-3.1)	
	Summer	39 (5.50 ± 1.38)	4 (0.67 ± 0.82)	10.3 (-0.2-1.3)	

CI: Confidence interval, Rainy: July-October, Winter: November-February, Summer: March-June

Table 7. Prevalence of *Hemonchus* spp. in goats of Barishal Sadar, Bangladesh

Variables		Examined samples (Mean ± SD)	Positive samples (Mean ± SD)	Prevalence percentage (95 % CI)	p-value
Breed	Black Bengal	74 (3.70 ± 1.59)	7 (0.35 ± 0.67)	9.5 (0.04-0.7)	> 0.05
	Jamunapari	38 (1.90 ± 0.79)	5 (0.25 ± 0.55)	13.2 (-0.008-0.5)	
Sex	Male	28 (1.40 ± 1.05)	3 (0.15 ± 0.37)	10.7 (-0.02-0.3)	> 0.05
	Female	84 (4.20 ± 1.58)	9 (0.45 ± 0.83)	10.7 (0.06-0.8)	
Age	0-6 months	11 (0.55 ± 1.05)	0 (0.00 ± 0.00)	0 (0-0)	> 0.05
	6 months-1 year	35 (1.75 ± 1.94)	4 (0.02 ± 0.62)	11.4 (-0.09-0.5)	
	1 year-1.5 years	14 (0.70 ± 1.13)	3 (0.15 ± 0.49)	21.4 (-0.08-0.4)	
	1.5 years-2 years	17 (0.85 ± 1.31)	1 (0.05 ± 0.22)	5.9 (-0.05-0.2)	
	2 years-2.5 years	12 (0.60 ± 1.19)	3 (0.15 ± 0.49)	25 (-0.08-0.4)	
	2.5 years-3 years	13 (0.65 ± 1.53)	0 (0.00 ± 0.00)	0 (0-0)	
	> 3 years	10 (0.50 ± 1.10)	1 (0.05 ± 0.22)	10 (-0.05-0.2)	
Seasons	Rainy	35 (5.83 ± 1.47)	2 (0.33 ± 0.52)	5.7 (-0.2-0.9)	> 0.05
	Winter	38 (5.50 ± 2.74)	5 (0.83 ± 1.33)	13.1 (-0.4-1.9)	
	Summer	39 (5.50 ± 1.38)	5 (0.50 ± 0.84)	12.8 (-0.2-1.6)	

CI: Confidence interval, Rainy: July-October, Winter: November-February, Summer: March-June

DISCUSSION

It is essential to maintain the optimum health of livestock to get the maximum production of farming livestock (Hendawy et al., 2022; Haque et al., 2020; 2021). Among infectious diseases, gastrointestinal helminth infections are crucial agents affecting the production of livestock as gastrointestinal helminth infections and sometimes get unnoticed during subclinical infections (Rahman et al., 2012; Rahman et al., 2017). Therefore, accurate surveillance of infections caused by gastrointestinal helminth in livestock is essential to undertake control measures against gastrointestinal helminthiasis.

The overall prevalence of gastrointestinal helminth infection in goats in Barishal Sadar was 82.1% in the present study which could be considered as high. In prior studies, a higher prevalence of gastrointestinal helminth infection in goats was reported (Islam et al., 2017; Chikweto et al., 2018; Squire et al., 2019; Win et al., 2020). The most likely reason for this high prevalence of gastrointestinal helminth infections in goats in Barishal Sadar, Bangladesh might be due to contaminated pasture where both young and adult goats were grazed together. This high prevalence of gastrointestinal helminths will cause severe losses of production in goats (Rahman et al., 2012). Therefore, immediate actions need to be undertaken to control the higher prevalence of gastrointestinal helminths in goats in southern Bangladesh.

In the present study, the overall prevalence of *F. gigantica* infection was 34.82%. Spithill et al. (1999) reported a similar rate of *F. gigantica* infection. Selim et al. (1997), Islam and Taimur (2008), and Hossain et al. (2011) reported a relatively lower infection rate of *F. gigantica* in goats which were 8.70%, 14.28%, and 20.75%, respectively. The present findings denote that *F. gigantica* infection rate increased with the progression of time. It is possible that the current samples were collected from goats that have not received any anthelmintic treatment in the past, or they could have been obtained from low-lying areas with high levels of rainfall.

The prevalence of *Paramphistomum* spp. infection in goats was 22.3%, which could also be considered as high. Previously it was reported by Godara et al. (2014) that 13.6% goats were infected with *Paramphistmum* spp. in Jammu, India. The high prevalence of *F. gigantica* and *Paramphistomum* spp. in goats in Barishal Sadar was due to low-lying areas associated with the presence of intermediate snail hosts.

A comparatively low prevalence of *Bunostomum* spp., and *Hemonchus* spp. were observed in the present study which was 14.3% and 10.7%, respectively. In the current study the most probable reason for the low prevalence of *Bunostomum* spp., and *Hemonchus* spp. infection in goats might be due to the irregular passage of eggs in faeces or low ingestion of infective stage larvae (L3) of *Bunostomum* spp., and *Hemonchus* spp. (Waruiru et al., 2001) by goats. The irregular passage of eggs through feces causes variations of gastrointestinal helminth eggs during coprological examination (Robert, 2011).

A similar rate of infection of all gastrointestinal helminths was detected both in the Jamunapari breed (a local breed of the neighboring country India) and the Black Bengal breed (local breed) in Barishal Sadar, Bangladesh. It might be due to the adjustment of the Jamunapari breed to the climate of Bangladesh. The exotic Jamunapari breed had a long habitat in Bangladesh, although the Jamunapari breed was not considered as a local breed of goat in Bangladesh. It is concluded from the present study, that gastrointestinal helminth can infect both the Black Bengal and Jamunapari breeds at a similar rate and a similar trend of endoparasitic diseases in the Black Bengal breed and Jamunapari breed has been reported previously (Parvez et al., 2014). The present findings indicated that adaptation of an exotic breed of goats in a certain climate causes a similar rate of helminth infection to the exotic breed of goats.

A significantly high prevalence of *F. gigantica* and *Paramphistomum* spp. in female goats compared to male goats was similar to the findings of other studies by Hossain et al. (2011) and Parvez et al. (2014). Change of physiological conditions during milking and/or undernutrition during production in female goats was a reason for a higher rate of infection. The other factors that might affect the rate of infection were long-time exposure of the female goats to the *F. gigantica* and *Paramphistomum* spp. as their heavy grazing habit in the submerged areas, which were characteristics of the Barisal region. A significant difference in prevalence of *Bunostomum* spp., and *Hemonchus* spp. between males and females were not observed. The most probable reason was due to the lower infection of *Bunostomum* spp., and *Hemonchus* spp. in goats in Barishal Sadar, Bangladesh.

Infection of goats at their early stages of life could have serious consequences with stunting growth and will make goats susceptible to other parasitic, bacterial, and viral infectious diseases and ultimately will cause great economic losses. Besides causing serious loss by the parasite itself, immature *F. gigantica* caused liver damage, and immature *Paramphistomum* spp. caused damage to the intestinal wall and ultimately predisposed goats to *Clostridium novyi* infection (black disease) and there may be the ultimate death of the infected goat kids (Smith, 2014).

The highest prevalence of *F. gigantica* in the younger age group (50% in 1 year-1.5-year age groups) was not in agreement with studies of Tasawar et al. (2007) and Hossain et al. (2011) and in agreement with a study of Parvez et al.

(2014). Tasawar et al. (2007) and Hossain et al. (2011) found a 35.71% and 14% prevalence, respectively in 1 year-1.5-year age groups. The probable reason for the different findings might be different geographic locations and individual immunity of goats. The lowest gastrointestinal helminth infection in the older age group was in agreement with studies of Hossain et al. (2011) and Tasawar et al. (2007). The most probable reason for the low rate of infection in adult goats than young goats could be due to the impacts of the self-cure phenomenon (Fryod, 1975; Assanji, 1988) and/or higher immunity that was acquired by goats due to aging (Singh et al., 2015). Previously it was reported that the host recovers from the infection of helminths with aging and ultimately becomes resistant to helminth diseases (Soulsby, 1986). In order to improve our understanding of the age immunity of gastrointestinal helminths in goats, it is essential to conduct studies that involve goats aged up to 10 or 12 years (as the present study includes goats up to 4 years).

The higher prevalence of Fasciolosis in winter and rainy seasons in goats was in line with the findings of Keyyu et al. (2005). The probable reason for higher findings (52%) of Fasciolosis during the winter season in the present study was due to the infection of goats in summer with *F. gigantica* and passing of *F. gigantica* ova/eggs with feces starting from the mid-rainy season and continued to whole winter season (as the prepatent period of *F. gigantica* ~120 days). Keyyu et al. (2005) reported that the passing of fluke eggs through feces gradually increased when the dry season started and egg passing reached its peak at the end of the dry season (winter) and again egg passing with feces decreased when the summer season starts which supports the present findings.

Specifically rainy weather, played a significant role in the varying prevalence of *F. gigantica* and *Paramphistomum* spp. This is due to the fact that rainy weather is essential for the reproduction of the intermediate snail hosts. Furthermore, the snail can survive a longer period under conditions that are moist and rainy (Ahmed et al., 2007). Moreover, summer in Bangladesh is rainy up to October (end of the rainy season), which facilitates the survival of *F. gigantica* and *Paramphistomum* spp. in such an environment and causes massive infection in susceptible goats during the rainy and winter season. A relatively lower prevalence of *Bunostomum* spp., and *Hemonchus* spp. were found throughout the year. The most probable reason for this lower prevalence of these two helminths were due to lower ingestion of *Bunostomum* spp., and *Hemonchus* spp. larvae by goats as evidenced by previous studies (Nyingi et al., 2001; Waruiru et al., 2001).

The mixed infections of different types of gastrointestinal helminth infections in goats were considered high (17.85%). The mixed parasitic infection in goats was also reported in other published studies by Chikweto et al. (2018) and Win et al. (2020). Single helminth infection predisposes goats to get infections with other parasites (mixed infections) and these mixed infections might severely reduce goat production compared with a single helminthic infection (Campos et al., 2008; Win et al., 2020). The high percentage of mixed infections of *F. gigantica* and *Paramphistomum* spp. (8.03%) in goats might be due to both helminths requiring snail intermediate hosts for the completion of their life cycle. A relatively lower rate of mixed infections of *Bunostomum* spp., and *Hemonchus* spp. might be due to the adverse effects of the environment on the infective stage of larvae as evidenced by previous studies (van Dijk et al., 2009; Santos et al., 2012).

A limitation of the present study was the few numbers of samples comprising only one Upazilla. Future studies should be directed at finding the prevalence of gastrointestinal helminth infections in goats using more samples including entire southern Bangladesh. Only qualitative methods (direct smear, sedimentation, and floatation) for the determination of gastrointestinal helminth infection in animals were problematic and might cause a lower detection rate. To facilitate a more precise and captivating discussion, it is imperative to consider employing serological and molecular identification techniques for the detection of gastrointestinal helminths in the future.

CONCLUSION

Gastrointestinal helminth infections in goats were 82.1% in Barishal Sadar which could be considered as endemic in Barisal Sadar, Bangladesh. Female goats were infected more with *F. gigantica* and *Paramphistomum* spp. compared to male goats. Therefore, specific and accurate diagnosis of *F. gigantica* and *Paramphistomum* spp. need to be undertaken to prevent gastrointestinal helminth infections in the female goat population. A higher prevalence (52.6%) of *F. gigantica* was observed in goats during the winter. Thus, the administration of a specific anthelmintic against *F. gigantica* during the winter will be crucial to preventing *F. gigantica* infection in goats. Control of the snail population (intermediate host), avoiding low-lying areas of grazing, and avoiding mixed grazing of adults and young in the grazing area would be other alternate options. The findings of the present study reveal the determination of other parasitic infections in goats of Barishal Sadar, Bangladesh. In the present study, due to the limitation, the species of the *Paramphistomum* spp., *Bunostomum* spp., and *Hemonchus* spp. were not determined. Future studies should be conducted to determine the species of gastrointestinal helminths in goats in Southern Bangladesh.

DECLARATIONS

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Competing interests

The authors have not declared any conflict of interest.

Authors' contributions

Md. Aminul Islam conceptualized the study idea, managed funding, developed the methodology, analyzed the data, and wrote the original draft manuscript. Anup Kumar Talukder, Sheikh Arafatur Rahman, and Mohammad Shah Alam reviewed and edited the original draft of the manuscript. Md. Sodrul Islam, Mohammad Anisur Rahman, and Shib Shankar Saha viewed and edited the manuscript. All authors checked the analyzed data and the last edition of the manuscript before submission and confirmed the previous revisions of the article before publication.

Availability of data and materials

All data related to this manuscript is included in the prepared manuscript. For any additional information, please contact to corresponding author.

Ethical considerations

The authors declared that the submitted manuscript was written originally and the data of this study is not submitted or published in any other journals.

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Effects of Breed on the Morphometric Parameters of Erythrocytes in Horses

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ABSTRACT

Various factors have a distinct impact on the quantity and dimensions of red blood cells across diverse animal species. These factors encompass age, sex, elevation, time of year, and lineage. The present study aimed to evaluate the influence of breed on the morphometric parameters of red blood cells in horses. To examine the impact of various horse breeds on the diameter, circumference, and surface area of red blood cells, blood samples were obtained from a total of 90 healthy horses. These horses belonged to three different breeds, including Arab Thoroughbred, English Thoroughbred, and Barbe. Each breed consisted of 30 individuals, with an equal distribution of males and females. The collected blood specimens were then divided into two separate batches for further analysis. The age range of all horses included in the study was between 5 and 12 years old. Smears were made and stained using the May-Grünwald Giemsa technique. The morphometric measurements were performed while using the OPTIKA^{TM} Vision Pro special software. The obtained results showed that there were no significant differences in the red blood cell diameters across different horse breeds. However, this factor appears to influence significantly both the circumference and surface area of erythrocytes. Specifically, the circumference and surface of Barbe red blood cells were highly smaller than both Arabian and English purebred horses. The present study demonstrated that the circumference and surface of red blood cells appear to be more indicative and representative in detecting variations in the erythrocyte morphometry between different horse breeds.

Keywords: Breed, Circumference, Diameter, Erythrocyte, Horse, Surface

INTRODUCTION

In horses, just like in every other mammalian species, mature red blood cells (RBCs) typically exhibit a rounded, elastic, biconcave shape with central pallor, measuring approximately 5-6 µm in width (Grondin and Dewitt, 2010; Freeman et al., 2022; Erdeljan et al., 2024). Rouleaux formation, where RBCs stack like coins, is a common occurrence in horses (Kramer, 2000; Reagan et al., 2008; Barger, 2022). Additionally, Howell-Jolly bodies, which are small, round remnants of nuclear material, may occasionally be observed in equine peripheral blood films even in healthy animals (Kramer, 2000; Grondin and Dewitt, 2010; Freeman et al., 2022). Polychromatophilic RBCs are rarely observed in the peripheral blood of horses, as equine reticulocytes tend to complete their maturation process primarily within the bone marrow, even during periods of heightened erythropoiesis (Latimer and Rakich, 1992; Malikides et al., 2000; Lording, 2008).

The total erythrocyte counts in horses range between 6×10^6/µl and 12×10^6/µl (Kramer, 2000; Grondin and Dewitt, 2010; Freeman et al., 2022). However, it is crucial to take the horse's breed into account when establishing reference values. The erythrocyte count is higher among Arab thoroughbred and English thoroughbred horses than in riding horses, ponies, and draft-heavy horses (Harvey et al., 1984; Mcgowan, 2008; Taylor et al., 2010). Racing horses typically exhibit higher erythrocytes count, compared to non-trained horses, particularly those primarily in the pasture (Rubio et al., 1996; Kästner et al., 1999; Piccione et al., 2008; Zobba et al., 2011). However, there are few studies regarding the effect of breed on erythrocyte morphometry in horses.

For many years, studies on erythrocyte morphometry have predominantly relied on linear measurements of erythrocyte size. The use of a lens micrometer (micrometric slide) and an ocular micrometer has been the established and recognized method for measuring erythrocyte size. Erythrocyte diameter is typically estimated using an optical microscope equipped with immersion magnification (x100) (Price-Jones, 1933; Adams, 1954; Todd, 1979). The present study focused on horses and aimed to examine the effect of breed on the diameter, circumference, and surface of erythrocytes through a digital method using OPTIKA^{TM} Pro Vision software.

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MATERIALS AND METHODS

Ethical approval

The Institutional Animal Care Committee has recommended that the authority be issued and under whose supervision the individual is authorized to carry out animal research, Veterinary Sciences and Agricultural Sciences Institute, University of Batna 1 - Hadj Lakhdar - Algeria. The University of Batna 1 - Hadj Lakhder authorities formed a committee to oversee the research, and their approval was obtained for the study. All specimens were obtained through standard blood collection techniques, prioritizing the animals' welfare and minimizing any stress or harm. The ethical considerations by the Institute Animal Ethics Committee related to animal handling were observed to ensure no pain to animals during those different manipulations. Throughout the study visits, researchers greeted the participants introduced themselves, and briefed animal breeders on the study's objectives and methodology.

Study area and animal habitat

The study was carried out in the Bazer-Sakra area within the Setif governorate, located in Northeastern Algeria. This area is distinguished by its highlands, approximately 300 kilometers away from Algiers, the capital of Algeria, and it rests at an altitude of 933 meters. The study was carried out on horses belonging to the Arabian thoroughbred, English thoroughbred, and Barbe breeds. An amount of 30 clinically healthy horses aged between 5 and 12 years old were chosen, from each breed, categorizing them based on sex into two groups, including 15 adult males and 15 adult females who were not pregnant. The horses and breeds were chosen after consulting their identity documents and considering identification documents, including name, registration number, breed, age, sex, and description.

Blood samples and smears

A total of 90 specimens of blood were gathered from the jugular vein, the samples were taken with standard sterile 5 ml syringes, fitted with 18-gauge needles (1.2 x 40mm). As reported by Mills (1998), Bacha and Bacha (2000), Niinistö et al. (2008), and Taylor et al. (2010), samples were promptly prepared on microscope slides post-venipuncture, without using anticoagulants to prevent any potential interference and avoid, inducing cytoplasmic and morphometric cell alterations, as reported by Mills (1998), Bacha and Bacha (2000), Niinistö et al. (2008), and Taylor et al. (2010). Acceptable blood smear must occupy two-thirds of the slide, and it must have three parts, namely a head, a body, and a tail. It is fixed just after drying by adding a few drops of methanol, this fixation aims to keep the blood cells in a state as close as possible to the living state. Each slide was meticulously labeled with the order number, breed, and sex of the animal and organized in slide racks. Using the storage boxes (slide holders) for the classification, protection, and sending of smears to the laboratory in favorable and better conditions is essential.

Blood smears staining

Blood smears were stained using the classical mixed Romanowsky staining method, particularly with the May-Gründwald Giemsa (M.G.G) dye, which is considered the most suitable for marking mammalian erythrocytes. The staining process adhered strictly to the protocol outlined by Houwen (2002).

Morphometric study of red blood cells

In the present study, an advanced optical microscope (OPTIKA B-350, Ver.4.0.0, Italy) was employed. This contemporary binocular microscope features a high-resolution digital camera, OPTIKA^{TM} (Ver.4.1.0), allowing for live visualization of microscopic images of smears on a computer screen. The morphometric examination of erythrocytes was conducted using the specialized OPTIKA^{TM} Pro Vision software provided by the OPTIKA microscope. Digital versions of traditional morphometry techniques used with optical microscopes are offered by this software.

Before the morphometric study of the red blood cells, the microscopic images of the red blood cells were digitized. Taking photos of all horses of each breed made it possible to fix the microscopic images observed and save them. As a result, they can be manipulated using the software. A correctly entered and well-calibrated scale is necessary for the accuracy of red blood cell morphometric data, in this regard the scale engraved on the micrometric slide was scanned using an immersion objective (x100). To explore how the breed of horses could influence morphological measurements of erythrocytes, the present study focused on measuring the diameter of the cells, typically estimated based on the cell's shape. Additionally, the erythrocyte circumference and surface area were two novel parameters that were introduced in horses, including the erythrocyte circumference and surface area. The morphometric examination of erythrocytes followed the guidelines and directives provided by the software developer. For each horse, the diameter, circumference, and surface area of 50 erythrocytes were measured. Subsequently, the average value for each parameter was calculated across all animals.

Statistical analysis

To ensure the clarity of the findings and evaluate rigorously the impact of breed on the diameter, circumference, and surface area, a Student's t-test was employed using MedCalc statistical software (version 12.7). A significance level of $p < 0.05$ was utilized to determine statistical significance.

RESULTS

Diameter of erythrocytes

As can be seen in Table 1, the breed factor had no significant effect on the size of red blood cells ($p > 0.05$).

In adult males, the diameter of red blood cells was slightly higher among Barbe horses, compared to Arabian and English horses. However, erythrocytes were slightly larger in English adult female mares, compared to Arab and Barbe mares. No clear impact of the horse breed on the diameter of erythrocytes was evident in these observations.

Circumference of erythrocytes

Table 2 demonstrates the effect of horse breed on the circumference of erythrocytes. The comparison of purebred Arabian horse erythrocytes with those of English thoroughbreds indicated no significant difference between these two breeds ($p > 0.05$). However, the observed values in Barbe horses are significantly lower ($p < 0.05$), compared to those of Arab and English horses.

Surface of erythrocytes

The findings displayed in Table 3 illustrate the breed effects on the surface of erythrocytes. Accordingly, they were significantly larger in Thoroughbred horses (Arabian and English) compared to those of the Barbe horse ($p < 0.05$).

Table 1. Effect of different horse breeds on the diameter of erythrocytes (µm)

Groups	Arabian thoroughbred (M ± SD)	English thoroughbred (M ± SD)	Barbe (M ± SD)
Global (n=30)	5.56 ± 0.27^{a}	5.76 ± 0.18^{a}	5.65 ± 0.32^{a}
Adult males (n=15)	5.52 ± 0.32^{a}	5.76 ± 0.19^{a}	5.83 ± 0.21^{a}
Adult females (n=15)	5.59 ± 0.21^{a}	5.75 ± 0.18^{a}	5.46 ± 0.31^{a}

M ± SD: Mean ± standard deviation. ^{a,b} data in the same line followed by different letters differ significantly at $p < 0.05$

Table 2. Effect of different horse breeds on erythrocytes circumference (µm)

Groups	Arabian thoroughbred (M ± SD)	English thoroughbred (M ± SD)	Barbe (M ± SD)
Global (n = 30)	22.77 ± 1.73^{a}	22.14 ± 0.84^{a}	20.64 ± 0.83^{b}
Adult males (n = 15)	23.41 ± 1.97^{a}	22.54 ± 0.72^{a}	21.00 ± 0.56^{b}
Adult females (n = 15)	22.13 ± 1.19^{a}	21.74 ± 0.78^{a}	20.28 ± 0.92^{b}

M ± SD: Mean ± standard deviation. ^{a,b} data in the same line followed by different letters differ significantly at $p < 0.05$

Table 3. Effect of different horse breeds on the surface area of erythrocytes (μm^2)

Groups	Arabian thoroughbred (M ± SD)	English thoroughbred (M ± SD)	Barbe (M ± SD)
Global (n = 30)	27.93 ± 3.24^{a}	27.47 ± 2.05^{a}	24.51 ± 2.07^{b}
Adult males (n = 15)	28.39 ± 3.71^{a}	28.29 ± 1.81^{a}	24.99 ± 1.65^{b}
Adult females (n = 15)	27.47 ± 2.74^{a}	26.65 ± 2.00^{a}	24.03 ± 2.37^{b}

M ± SD: Mean ± standard deviation. ^{a,b} data in the same line followed by different letters differ significantly at $p < 0.05$

Table 4. Summary of morphometric parameters of erythrocytes in different horse breeds

Parameters	Means	Ranges
Diameter (µm)	5.65	4.72-6.13
Circumference (µm)	21.85	19.01-26.99
Surface (μm^2)	26.64	19.38-34.32

DISCUSSION

The data regarding the influence of breed on the morphometric parameters of red blood cells in horses indicates no significant difference between the three breeds. The recorded values are comparable and consistently align with internationally recognized benchmarks, particularly those documented by Grondin and Dewitt (2010), Adili et al. (2016), and Erdeljan et al. (2024).

No notable distinctions were observed in the erythrocytes of Arabic and English thoroughbred horses in the present investigation. In contrast to the findings of Knill et al. (1969), who reported that the red blood cells of Arabian thoroughbreds are smaller, compared to those of English thoroughbreds. Additionally, Malikides et al. (2000), McGowan (2008), and Taylor et al. (2010) found Arabian and English thoroughbred horses have smaller erythrocytes compared to those of typical breeds. However, the breed factor seems to show a clear influence on the circumference and the surface of erythrocytes, notably, these two parameters are significantly smaller for the Barbe horses than in Arabian thoroughbreds and English thoroughbreds.

Regarding the circumference and surface area of red blood cells, reference values in the form of intervals were proposed (Table 4), considering the limited availability of studies and published standards offering comprehensive data on these parameters. The morphometric study of erythrocytes using the OPTIKA^{TM} Vison Pro Software is deemed more representative and reliable, offering significant assistance in considering morphometric changes in red blood cells. It should be noted that to establish reference values, the ideal number of animals must be between 100 and 120, to be more representative and better characteristic. The small number of horses used in the current study (30 for each breed) is essentially linked to the reduced number of purebred horses in the study region. In addition, doing the research in a specific locality allows too well control of the sample and avoids the influence of other factors, such as climate, altitude, and diet.

Utilizing the OPTIKA^{TM} Vision Pro software enables significantly more precise morphometric analyses compared to the traditional approach of measuring red blood cell diameter (Parveen et al., 2023). This software effectively reduces the impact of human error in studies conducted with an ocular micrometer by automatically determining the most suitable measurement location.

The digital type of morphometric study could serve as a basis for the diagnosis and the interpretation of several pathological phenomena in veterinary medicine, including normocytic, macrocytic, and microcytic anemias, development of inflammatory lesions, and healing of fractures.

Measurements of surface area and circumference using the evoked software are suited and more precise for comparative studies, especially when dealing with cells of varying sizes. Furthermore, It is crucial to emphasize that the morphometric analysis of erythrocytes becomes more simplified and convenient when utilizing the OPTIKATM Vision Pro software. Additionally, this new measurement approach is simple to execute, rapid, and highly cost-effective.

CONCLUSION

The findings indicated that breed did not impact the diameter of erythrocytes. However, there was a significant breed-related effect observed in the circumference and surface of erythrocytes, with those from Barbe horses being smaller compared to thoroughbred horses. No differences were found in these parameters between Arabian and English horses. Based on these results, conducting similar morphometric studies in various equine breeds, such as saddle horses, draft horses, and ponies would be beneficial to deepen the understanding of how breed influences red blood cell morphometry.

In light of the obtained results in this study, it would be more interesting to carry out new morphometric studies of red blood cells focusing on updating data relating to the size of erythrocytes and determining reference values for the circumference and surface area of red blood cells in different animal species. Additionally, comparative studies should explore the differences between males and females within different breeds of the same species, specifically examining the influence of sex on erythrocyte morphometry.

DECLARATIONS

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Authors' contributions

Nezar Adili and Mounia Megâache contributed equally to the establishment of the study plan, analysis of data, and the writing of the article. All authors read and approved the final manuscript.

Competinginterests

The authors declare that they have no competing interest regarding the publication of this research.

Ethical considerations

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

Availability of data and materials

The author confirms that the data supporting the findings of this study are available, upon a reasonable request.

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The Effects of Weather Conditions on Hematological and Biochemical Parameters in Dogs: A Field Study

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ABSTRACT

Although animals have adaptation abilities to different environmental conditions, various physiological changes may occur. The present research aimed to evaluate the effects of severe winter conditions on hematological and biochemical parameters in dogs kept outside all year. The research was carried out in the province of Kars, which is known for its severe cold conditions in Türkiye. Vital signs, hematological, and biochemical parameters of 12 adult dogs aged 1-8 years old (mixed breed, 8 males and 4 females) included in the study were compared in winter and summer seasons. The results indicated a significant effect of the winter season on the body temperature, respiration, and pulse rate of the dogs. In addition, it was observed that some hematological, including White blood cell (WBC), mean corpuscular hemoglobin (MCH) and mean corpuscular hemoglobin concentration (MCHC) Hemoglobin (HB), and biochemical parameters (urea, TP, albumin, cholesterol, glucose, creatinine) of dogs differed in winter from those in summer. It should be considered that the vital values of dogs living outdoors in intense winter differed from those in mild winter or summer. It is also concluded that veterinarians should consider these differences in routine clinical examinations of these animals.

Keywords: Biochemical parameter, Cold stress, Dog, Environmental factor, Hematological parameter, Winter condition

INTRODUCTION

Dogs have been utilized in various fields due to the close relationship with humans that began thousands of years ago, being preferred for purposes ranging from search and rescue operations to hunting and protection duties (Al-Shammari et al., 2019). These days, dogs are meticulously monitored by their owners or breeders in matters, such as health check-ups, routine vaccination tracking, and treatment management. It's crucial to combine medical history, physical examinations, vital signs, and laboratory test outcomes when assessing the health status of animals (Erkılıç, 2023). Blood analyses, particularly, play a critical role in assessing the health status of animals (Arıyıbı et al., 2002), monitoring response to treatment, and detecting potential diseases (Erkılıç, 2023). Valuable information about the general health status of the animals could be reached through blood parameters (Çınar et al., 2010). It can change under the influence of various factors (Mohammed et al., 2017), including environmental factors, such as age, gender, pregnancy, nutrition, habitat, and climate (Arıyıbı et al., 2002; Erkılıç, 2023).

Climate change is a major global concern that presents itself in a variety of ways. These manifestations include fluctuations in weather patterns, such as variations in annual precipitation and shifts in temperature (Serrano et al., 2021). Although animals have adaptive capabilities to cope with these climate changes, it's acknowledged that fluctuations in environmental temperature can impact their physiological responses (Ji et al., 2021). Severe winter weather conditions, characterized by low temperatures, can induce a range of physiological alterations in animals (Ji et al., 2021). Exceeding the lower or upper limit of the thermo-neutrality zone due to cold or heat can lead to thermal stress (Hagiu and Codreanu, 2022). Dogs are susceptible to the potential dangers of thermal stress as well (Al-Shammari et al., 2019).

Cold or thermal stress primarily increases the risk of hypothermia or freezing in dogs. Ji et al. (2021) reported it can also lead to a weakened immune system and a loss of resistance to infections and other diseases, which can be a direct cause or trigger, in combination with other factors, of many clinical problems. In areas characterized by severe winter, dogs serve as invaluable assistants to cattle and sheep farmers. These animals are typically kept outside, such as in a yard, and used for protective responsibilities. However, it is known that environmental factors, especially climate and temperature changes, can affect the health status of dogs.

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The present study aimed to determine the impact of severe winter conditions on dogs living continuously outdoors. To achieve this goal, it was planned to analyze the hematological and some routine biochemical parameters of the winter season and compare them with the values of the summer season.

MATERIALS AND METHODS

Ethical approval

The study was conducted after obtaining approval from the Kafkas University Animal Experiments Local Ethics Committee, Türkiye (KAU-HADYEK/2021-017).

Study location

Kars is located in the geographical coordinates of 42°10' and 44°49' East meridians, and 39°22' and 41°37' North parallels in the Northeast Region of Türkiye. The Kars region experiences significant variations in temperature between the summer and winter seasons (Şeker, 2001).

Meteorological data

Five-year meteorological data, including relative humidity, temperature, and rainfall amount for the region where the study was conducted, between the years 2017-2021, were obtained from the Kars Meteorology Directorate.

Animals

A total of 12 healthy adult dogs aged between 1 and 8 years (mix breed, 8 males and 4 females) were chosen randomly. The samples were kept continuously outdoors in the central district of Kars province, with regular antiparasitic and vaccination applications, under similar care and feeding conditions. The vital values and blood samples of the animals subjected to the experiment were obtained by going to the environment where the animals were living. According to the information from the animal owners, the animals were fed mostly home-made meals.

Blood sample

Blood samples were taken from the dogs twice, including in January, the coldest month, and in July, in the summer. An amount of ten mL blood samples was drawn from the *v. cephalica antebrachii* and placed into vacuum gel serum tubes (BD Vacutainer®, BD, UK) and vacuum EDTA blood tubes (BD Vacutainer®, BD, UK). Vacuum serum tubes were centrifuged at 3000 rpm for 10 minutes Hettich Rotina 380R®, Hettich, Germany, to obtain serum.

Vital parameters and hematological-biochemical analyses

Initially, clinical examinations were conducted, while the animals were at rest. The rectal body temperature (°C), pulse rate, and respiratory rate per minute were recorded. Hematological analyses were performed using a blood count analyzer (VG-MS4e®, Melet Schloesing, France) with samples collected in EDTA tubes. Alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), and creatine kinase (CK) enzyme activities from the serum samples obtained were measured using the Mindray BS120® fully automatic biochemistry device (Mindray Medikal Technology). Cholesterol, triglyceride, glucose, creatinine, urea, total protein, and albumin levels were determined by the colorimetric method (Epoch, Biotek, USA) using commercial test kits (Biolabo, France). Globulin levels were obtained by subtracting albumin levels from total protein levels.

Statistical analysis

The statistical analysis of the obtained data was conducted using the SPSS version 25 software. The Shapiro-Wilk test was applied to determine whether the data in the groups were normally distributed. Based on the obtained results, the Paired Sample T-test was performed for normally distributed data, and the Wilcoxon test was used for non-normally distributed data. P-value < 0.05 was considered statistically significant.

RESULTS

The average meteorological data between 2017 and 2021 for the Central District of Kars Province is presented in Table 1. Upon examining the temperature averages spanning five years, it is observed that January, the month of sampling, had the lowest temperature, while July had the highest temperature. Accordingly, during January in which the study was conducted, the average temperature was recorded as -8.4°C, while the average temperature for July was 19.5°C.

Significant differences were found between vital signs evaluated in winter and summer ($p < 0.05$, Table 1). The average vital signs in the winter were as follows, body temperature was recorded as 38.21 ± 0.17°C, respiratory rate was 29.83 ± 1.99 breaths/minute, and heart rate was 92.67 ± 3.22 beats/minute (Table 2). In the summer, the average of the same parameters was determined as follows, body temperature was 38.8 ± 0.21°C, respiratory rate was 44.17 ± 5.70 breaths/minute, and heart rate was 111.00 ± 6.70 beats/minute.

As can be seen in Table 3, the hematological values obtained after the whole blood count conducted in January and July are presented. It has been observed that the total leukocyte count ($p = 0.034$) along with the mean corpuscular hemoglobin (MCH, $p = 0.005$), mean corpuscular hemoglobin concentration (MCHC, $p = 0.002$), and hemoglobin (Hb) concentration ($p = 0.006$) were statistically higher in the winter compared to the summer (Table 3).

Some biochemical parameters were analyzed in the serum obtained from blood samples taken in winter and summer and are presented in Table 4. Among the evaluated biochemical parameters, blood urea ($p = 0.027$), total protein ($p = 0.03$), albumin ($p = 0.022$), and creatinine levels were determined to be statistically significantly lower in winter than in summer ($p < 0.001$, Table 4). However, it was noted that the levels of glucose and cholesterol ($p = 0.021$) were significantly higher in the winter compared to the summer ($p < 0.001$, Table 4).

Table 1. Average of meteorological data for Kars Province, Central District, Türkiye, during 2017-2021

Months	Average relative humidity (%)	Average temperature (°C)	Rainfall (mm)
January	73.14	-8.98	11.52
February	69.38	-5.76	10.16
March	65.02	0.84	34.00
April	56.28	6.24	35.76
May	56.06	11.92	76.48
June	53.80	16.18	60.20
July	53.28	19.24	58.64
August	48.88	19.00	36.52
September	49.54	15.12	29.48
October	63.06	8.30	43.60
November	70.12	1.16	26.04

SD: Standard deviation

Table 2. Seasonal variation of vital signs of dogs in Kars Province

Parameter	Winter (Mean ± SD)	Summer (Mean ± SD)	P value
Body Temperature (°C)	38.21 ± 0.59	38.8 ± 0.71	0.014
Respiratory (breath/min)	29.83 ± 6.89	44.17 ± 19.75	0.025
Pulse (beats/min)	92.67 ± 11.16	111.00 ± 23.22	0.016

SD: Standard deviation

Table 3. Seasonal changes of hematological values of mixed breed dogs

Parameter	Winter (Mean ± SD)	Summer (Mean ± SD)	p-value
WBC (10^9/L)	17.88 ± 6.32	14.34 ± 4.15	0.034
MON (10^9/L)	0.50 ± 0.17	0.45 ± 0.11	0.290
GRA (10^9/L)	12.13 ± 6.08	10.13 ± 3.32	0.480
RBC (10^{12}/L)	7.30 ± 0.82	7.38 ± 0.71	0.806
HCT (%)	53.38 ± 5.88	57.20 ± 5.84	0.123
MCH (pg)	24.93 ± 1.16	21.80 ± 2.12	0.005
MCHC (g/dL)	34.13 ± 1.27	28.13 ± 2.22	0.002
HB (g/dL)	18.24 ± 2.04	16.05 ± 1.44	0.006
THR (10^9/L)	199.42 ± 107.33	206.83 ± 99.60	0.583

SD: Standard deviation, WBC: White blood cell, MON: Monocyte, GRA: Granulocyte, RBC: Red blood cell, HCT: Hematocrit, MCH: Mean corpuscular hemoglobin, MCHC: Mean corpuscular hemoglobin concentration, HB: Hemoglobin, THR: Thrombocyte

Table 4. Seasonal changes of biochemical values of mixed breed dogs

Parameter	Winter (Mean ± SD)	Summer (Mean ± SD)	p-value
ALT (U/L)	70.72 ± 33.85	87.69 ± 11.40	0.117
AST (U/L)	28.10 ± 14.73	35.33 ± 9.96	0.117
ALP (U/L)	41.99 ± 24.02	71.13 ± 49.80	0.060
CK (U/L)	212.91 ± 81.19	312.81 ± 95.02	0.050
Urea (mg/dL)	31.38 ± 7.28	37.09 ± 5.77	0.027
TP (g/dL)	6.31 ± 0.47	6.77 ± 0.51	0.030
Albumın (g/dL)	2.98 ± 0.13	3.17 ± 0.19	0.022
Globulın (g/dL)	3.33 ± 0.49	3.60 ± 0.54	0.121
Cholesterol (mg/dL)	173.35 ± 13.95	157.69 ± 14.52	0.021
TG (mg/dL)	63.20 ± 7.81	56.33 ± 7.77	0.055
Glucose (mg/dL)	125.11 ± 20.80	79.42 ± 10.55	< 0.001
Creatınıne (mg/dL)	0.67 ± 0.12	0.88 ± 0.15	< 0.001

SD: Standard deviation, ALT: Alanine Aminotransferase, AST: Aspartate Aminotransferase, ALP: Alkaline phosphatase, CK: Creatine kinase, TP: Total protein, TG: Triglyceride

DISCUSSION

The findings reveal the differences in dogs between the winter and summer. Examining the effect of the seasons on dogs, indicated that body temperature, respiratory rate, and heart rates are significantly lower during the winter. The findings reveal that dogs reduce their metabolic activities in cold weather and activate adaptation mechanisms to ensure thermal regulation. Similarly, the changes in hematological and biochemical parameters are also noteworthy.

Evaluation of hematological, biochemical, and vital parameters provides useful information in the diagnosis of various diseases. Additionally, these parameters are important for the evaluation of some metabolic and physiological processes (Azeez et al., 2022). Heatstroke (Drobatz, 2015) and hypothermia (Todd, 2015) have numerous physiological effects, and low environmental temperatures can lead to various physiological changes (Ji et al., 2021).

The high temperature and relative humidity of the environment have been stated as the primary cause of heat stress (Azeez et al., 2022). In a study conducted on dogs, it was determined that different environmental temperatures did not affect vital parameters up to a certain level, but at very high temperatures (45°C), significant increases in body temperature, pulse, and respiratory rate were observed (Al-Shammari et al., 2019). In the present study, it was determined that vital values were significantly lower in the assessments conducted during the winter compared to those conducted in the summer. The primary factor contributing to this change is believed to be influenced by ambient temperature through a mechanism mediated by receptors in the hypothalamus and skin.

Azeez et al. (2022) noted that the total leukocyte count in dogs is lower in environments with high ambient temperature and high relative humidity compared to environments with high ambient temperature and low relative humidity. Studies conducted on sheep (Khalil et al., 2022) and horses (Fernando de Souza et al., 2018) have reported that the total leukocyte count in winter is higher compared to summer. Mohammed et al. (2017) conducted a study on dogs and reported that the total leukocyte count in the winter is statistically insignificant compared to the summer. Accordingly, the total leukocyte count was determined to be significantly higher in winter compared to summer. The larger size of lymphatic organs during the winter season (Nelson and Demas, 1996), or the stress conditions resulting from lower ambient temperatures during the winter months, have been cited as the reasons for this phenomenon.

The temperature changes occurring throughout the year have been reported as a physiological stress factor. The biological systems of animals are affected by this condition (Khalil et al., 2022). Season, ambient temperature, rainfall amount, and humidity changes are important factors affecting hematological parameters (Mohammed et al., 2017). It has been reported that heat stress may cause precipitation in Hb in erythrocytes and therefore a decrease in Hb concentration (Karthik et al., 2021). Additionally, the absence of Hb's essential components in the diet and the body's ability to absorb these components directly influence Hb levels (Šimák-Líbalová et al., 2013). Khalil et al. (2022) conducted a study on sheep that indicated the Hb levels were found to be lower in the winter compared to the summer. However, Hb levels were shown to be greater in the winter in donkeys (Longodor et al., 2020). Accordingly, winter Hb levels were higher than summertime levels. It has been proposed that the potential cause for this phenomenon could be the physiological strain induced by temperature changes (extreme cold or heat) experienced during different seasons and daily temperature fluctuations. This strain may result in insufficient absorption of the essential elements required for Hb at the intended

level. In a study conducted on sheep by Khalil et al. (2022) and dogs (Mohammed et al., 2017), MCH and MCHC values were found to be high in the winter, while in horses (Fernando de Souza et al, 2018) they were found to be low in the winter. In the present study, higher MCH and MCHC values were recorded during the winter analysis compared to the summer. The decrease observed during the summer season was associated with a decrease in Hb levels.

It is crucial to analyze the biochemical parameters in serum and plasma samples collected post-blood sampling for the clinical and metabolic assessment of animals (Çınar et al., 2010).

Mohammed et al. (2017) have reported that the serum urea level in dogs is higher in summer compared to winter, while Longodor et al. (2020) noted that it is higher in donkeys in the winter. In the present study, high serum urea levels were recorded in the summer season. It has been suggested that this change is attributed to fluid loss, which is shaped by the increasing ambient temperature during the summer season.

Creatinine moves into the blood as the end product of the nonenzymatic hydrolysis of creatine phosphate in the muscles, is distributed homogeneously into body fluids, and is filtered freely through the glomerulus (Turgut, 2000). Donkeys' serum creatinine levels were shown to be greater in the winter than in the summer, according to Longodor et al. (2020). Sheep's summertime creatinine levels were shown to be high in another investigation, and this was associated with decreased blood flow to the kidneys due to heat stress (Rathwa et al., 2017). In the current study, serum creatinine level was found to be higher in summer than in winter. It was determined that the values obtained during the winter and summer fall within the reference ranges (Turgut, 2000; Erkılıç, 2023). The transition between seasons is linked to dehydration caused by rising temperatures during the summer months. Based on biochemical parameters, the lower levels of blood urea, total protein, albumin, and creatinine during the winter are likely associated with adaptation mechanisms in metabolic activities occurring in response to cold stress.

Albumin, which constitutes approximately 50% of plasma proteins, contributes to plasma oncotic pressure. Changes in oncotic pressure are thought to control its hepatic synthesis (Turgut, 2000). Increased plasma albumin concentration due to dehydration decreases as a result of decreased hepatic synthesis, increased breakdown, or excessive loss through urine or intestines (Turgut, 2000). It has been reported that the serum total protein level in dogs decreases in cases of severe liver damage and long-term protein malnutrition, and its levels increase in cases of shock and dehydration (Kalaycıoğlu et al., 1995). According to Mohammed et al. (2017), the stress of the hot weather caused an increase in total protein levels in the summer compared to the winter in dogs, while albumin levels were recorded to be at similar levels. In the presented study, serum total protein and albumin levels were found to be higher during the summer compared to the winter. The seasonal variation of these parameters, which fall within the reference ranges (Turgut, 2000; Erkılıç, 2023), was associated with fluid loss occurring during the summer season.

There are studies in which glucose levels are determined in different animal species at different ambient temperatures, humidity levels, and seasons (Azeez et al., 2002; El-Shahat Attia, 2016; Longodor et al., 2020). Glucose analyses during the winter have noted that glucose levels are higher compared to the summer in dairy cows (Yıldız and Kızıl, 2011), sheep (Rathwa et al., 2017), and donkeys (Longodor et al., 2020). In the current study, glucose levels in dogs were also higher during the winter. Accordingly, ambient temperature may adversely affect blood glucose levels, however, the increase in temperature during the summer negatively affects food consumption in dogs. Intensive feeding by animal owners during the winter to mitigate the effects of cold conditions is effective in influencing changes in glucose levels.

Previous studies on different animal species (donkey, sheep, bull, dog) indicated that cholesterol levels differ in summer and winter, and cholesterol levels were found higher in winter than in summer (Farooq et al., 2017; Mohammed et al., 2017; Rathwa et al., 2017; Longodor et al., 2020). In the presented study, the cholesterol level in dogs was recorded to be higher in winter compared to the summer. The cholesterol levels, falling within the reference range (Turgut, 2000; Erkılıç, 2023), reveal an elevation during the winter season. This phenomenon has been linked to heightened feeding activities in winter months, a partial decrease in food intake during summer months due to rising ambient temperature, or as an adaptation to seasonal conditions. Among studies that investigated the physiological effects of thermal changes, such as heat stress, summer-winter conditions, and seasonal differences in various animals, including dog, sheep, and horse species, the lowest atmospheric temperature reported was not below zero degrees, although the study regions reflected winter conditions (Mohammed et al., 2017; Rathwa et al., 2017; Fernando de Souza et al., 2018; Al-Shammari et al., 2019). The most notable feature of the present study is that the temperature during the winter was cold adequate (-8.4°C) to affect the physiological resilience of the animals.

CONCLUSION

The winter has a significant impact on the body temperature, respiratory rate, heart rate, some hematological (WBC, MCH, MCHC, HB), and biochemical parameters (urea, TP, albumin, cholesterol, glucose, creatinine) of dogs. The

obtained results may help to understand the physiological (heart rate, respiratory rate, and body temperature), hematological, and biochemical changes that dogs are exposed to during intense winter conditions and may contribute to the adaptation of veterinary health care to these conditions. It was concluded that hematological and biochemical parameters may vary under the influence of seasonal conditions and these factors should be considered in clinical examinations. The present study emphasizes the importance of seasonal variations in assessing the health status of dogs. Future studies focusing on genetics, breed, nutrition, and environmental factors will contribute to this issue in more detail.

DECLARATIONS

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Ethical considerations

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

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author upon reasonable request.

Competing interests

The authors declare no competing interests.

Consent to publish

All authors agree to the publication of this manuscript.

Authors' contributions

Ekin Emre Erkılıç took part in the design, idea, collection of samples, and writing of the article. İsa Özaydın took part in the design, idea, and writing of the article. Oğuz Merhan took part in sample anayalsis. Mert Sezer, Yusuf Umut Batı, Celal Şahin Ermutlu, and Ali Haydar Kırmızıgul took part in the sample collection. All authors read and approved the final version of the manuscript.

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The Alternatives of Antibiotics in Poultry Production for Reducing Antimicrobial Resistance

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ABSTRACT

Antibiotics are natural, semi-synthetic, or chemical compounds that have anti-microbial activity and are used in livestock and poultry production for a variety of reasons, including therapeutic and growth promotion. The use of antibiotics in poultry production has been associated with the development of resistant bacteria. The present study attempted to explain the role of antibiotics as poultry growth promoters, bacterial resistance, and risks for human health, with a special focus on some selected bacterial species isolated from poultry farms and products. Furthermore, the manuscript reviewed the literature on alternative feed additives to reduce the reliance on antibiotics. Microbial resistance is a significant global health concern that has been a top global threat in the 21st century. The use of antibiotics in poultry production as non-therapeutic or growth promoters is at low doses and continuously, associated with developing resistant bacteria. Meanwhile, antibiotic-resistant genes in humans may have their roots in the diets of animals treated with antibiotics. Developing bacterial resistance has encouraged researchers to reduce the reliance on antibiotics by identifying potential feed additives, such as essential oils, bacteriophages, antimicrobial peptides, probiotics, prebiotics, organic acid, and enzymes that improve the immune system functions, reduce morbidity and mortality, improve the growth performances of poultry, and preserve consumer health.

Keywords: Antibiotic, Antimicrobial resistance, Feed additive, Human, Poultry

INTRODUCTION

Poultry production is an important source of the human diet globally providing essential animal protein with a suitable nutritional composition for humans. However, it also poses potential health concerns in some cases. Antibiotics, which are created in laboratories or produced by a wide range of microorganisms, including fungi and bacteria (Sahu and Saxena, 2014; Abreu et al., 2023), vary in their antibacterial effects, mode of action, and physical, chemical, and pharmacological characteristics (Dutta et al., 2019). The bactericidal or bacteriostatic mechanisms of antibiotics are inhibition of protein synthesis, cell wall synthesis, cytoplasmic membrane synthesis, and DNA synthesis (Sahu and Saxena, 2014; Diaz-Sanchez et al., 2015; Abreu et al., 2023).

Antibiotics have been used for a wide range of purposes in livestock and poultry for the past few decades. As therapeutic agents, antibiotics treat infectious diseases with high doses applied for short periods against specific diseases. As prophylactic agents, antibiotics prevent certain infections at the subclinical stage, using low (sub-therapeutic) doses periodically for several days. In addition, antibiotics are used as growth promoters, administered at a very low dose regularly in livestock feed. According to the literature, global antibiotic use increased by 39% between 2000 and 2015 (Klein et al., 2018). This surge in antibiotic demand, especially in low- and middle-income countries, is driven by economic growth and increased animal consumption. In 2013, food animals consumed approximately 131,000 tons of antibiotics, a figure expected to rise to 200,000 tons by 2030 (Van Boeckel et al., 2017).

While utilizing antibiotics significantly improves poultry performance and farm economics, it also poses potential risks. The spread of antibiotic-resistant (ABR) strains into the environment and human transmission through the food chain, compounded by inadequate drug withdrawal protocols, can pose significant public health risks (Klein et al., 2018; Abreu et al., 2023). The current review aimed to provide an up-to-date overview of antibiotic use as a poultry growth promoter, as well as bacterial resistance, and human health risks. The present study also discussed alternatives to antibiotics in poultry production.

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ANTIBIOTICS AND POULTRY PRODUCTION

The antibiotic as a growth promoter was first discovered in 1940 when aureomycin-containing pharmaceutical wastes were fed to poultry (Castanon, 2007). Adding antibiotics to animal feed as growth promoters at a concentration of 200 gm per ton for more than 14 days is a common experiment in animal production (Diaz-Sanchez et al., 2015). Adding antibiotics to the poultry diet can positively affect poultry growth performance by preventing enteritis, reducing growth-depressing metabolites produced by gram-positive bacteria, improving intestinal microbiota balance, enhancing nutrient utilization efficiency, and increasing energy harvesting from the intestine (converting feed to products; Allen and Stanton, 2014; Agyare et al., 2019; Haque et al., 2023).

The antibiotics apply their effect on bacteria in a few well-defined ways called the mode of action (Table 1). DNA replication is the process used to generate two new daughter DNA molecules, which result in the division of a bacterial cell into two daughter cells (Nagaraja et al., 2017). Antibiotics, such as fluoroquinolones (Ciprofloxacin, Levofloxacin, and Moxifloxacin) inhibit the DNA gyrase and topoisomerase IV, preventing the synthesis of bacterial DNA (Shree et al., 2023). Protein biosynthesis inhibition includes essential bacteria protein synthesis which has DNA to encode mRNA, rRNA, and tRNA. The 50s and 30s ribosomal subunits make up the bacterial ribosome and play a role in bacterial protein synthesis. Antibiotics, such as aminoglycosides (Tetracycline) target the 30s ribosomal (Halawa et al., 2024), macrolides (Chloramphenicol and Oxazolidinone) target the 50s (Syroegin et al., 2022) and inhibit protein synthesis. Cell wall synthesis inhibition means the peptidoglycan is the most important part of the cell wall, and several steps are included to synthesize the same and form the bacterial cell wall. The distinctive structure of antibiotics, including beta-lactams (penicillins and cephalosporins) and glycopeptides allows them to bind to peptidoglycan cross-linking enzymes (transpeptidase and carboxypeptidase), inhibiting bacterial peptidoglycan synthesis and preventing cell wall formation. In folic acid metabolism inhibition some antibiotics, such as sulfonamide, inhibit the specific enzymes involved in folic acid metabolism (Capasso and Supuran, 2014).

Various health and food organizations developed guidelines for using antibiotics in livestock to address and prevent antibiotic resistance. The key guidelines for using veterinary antibiotics include the recommendation of antibiotics after diagnosis of diseases with bacterial etiology, administration of antibiotics under the supervision of a veterinarian, priority being given to the health status of an infected animal, improvement in the understanding and awareness of antibiotics resistance, implementation of effective sanitation, and infection prevention, and encouragement of sustainable investment to discover new effective medicines, diagnostic tools, and vaccines (Haag, 2015; Diaz-Sanchez et al., 2015; Salam et al., 2023).

ANTIMICROBIAL RESISTANCE

Antibiotic resistance (ABR), which is also known as antimicrobial resistance (AMR), and emerging infectious diseases are severe global health concerns. Antimicrobial agents used in livestock and poultry production and their AMR in contributing sources and are connected to serious illness and a heavy financial burden in individuals and different countries. Achieving sustainable development objectives, livelihood security, food safety, and nutrition security can all be impacted. Since humans, animals, and the environment are all interconnected as a cause and a cure, ABR is truly a one health issue (Wang et al., 2021a; Salam et al., 2023).

Both direct and indirect contact between the various actors and environments can spread drug resistance up the food chain, serving as pathways for the transmission of zoonotic diseases. Humans come into direct touch with resistant microorganisms found in animals or their products. Resistance strains are more likely to colonize or infect occupational workers, including farmers, veterinary professionals, abattoir workers, food handlers, and others with whom they come into contact (Salam et al., 2023). Antibiotic residues are the byproducts of antibiotic degradation or related metabolites that accumulate in manure, wastewater, and soils and have a significant negative impact. Therefore, the environment becomes a significant reservoir of antimicrobial drug resistance due to the spread of antibiotic-resistant bacteria and antibiotic residues via food and animal waste (Abreu et al., 2023).

Antimicrobial resistance arises when bacteria lose their susceptibility and acquire resistance to the medications employed for their treatment (Vikesland et al., 2019). Infections caused by these microbes are harder to treat due to the resistance they develop, leading to increased morbidity, mortality, and healthcare costs (Almansour et al., 2023). Bacteria utilize several approaches to resist the antibiotics, such as enzymatic degradation or modification of bacteria (e.g., chloramphenicol acetyltransferases), modification of the antibiotic target (e.g., vancomycin-resistant *enterococci*, which enzymatically modify peptidoglycan), and keeping the antibiotic out of the bacterial cell through either efflux pumps or alteration of the permeability of the cell membrane (Baker et al., 2023; Salam et al., 2023).

Table 1. Selected classes of commonly used antibiotics in poultry and humans, their action mechanisms, and activity spectrum

Antibiotic Class	Synthesis	Drug name	Action mechanism	Activity spectrum	Use in human and poultry	Reference
Tetracyclines	*Streptomyces* spp.	Oxytetracycline, Chlortetracycline, Doxycycline	Inhibition of protein synthesis	Gram-positive and gram-negative	In humans: Urinary tract, respiratory tract, and sexually transmitted infections In poultry: Respiratory infection and sinusitis, and growth promoter	(Chopra and Roberts, 2001)
Sulfonamides	Synthesized from non-natural compounds (Sulfanilamide) containing a sulfonamide group.	Sulfamethoxazole	Inhibiting folic acid-producing enzyme	Gram-positive and gram-negative	In humans: Urinary tract, respiratory tract, and skin infections In poultry: Restricted for infection treatment and banned as a growth promoter due to resistance concern	(Yoneyama and Katsumata, 2006)
Beta-lactams (Cephalosporins)	Synthesized from non-natural compounds (beta-lactam ring)	Amoxicillin, Efazolin, Meropenem	Inhibiting of penicillin-binding proteins	Gram-positive and gram-negative	In humans as well in poultry: For respiratory, urinary, sepsis, and sexually transmitted diseases, and as growth promoters in poultry	(Yoneyama and Katsumata, 2006)
Fluoroquinolones	Quinolone compounds	Ciprofloxacin, Levofloxacin, Moxifloxacin	Inhibiting bacterial DNA synthesis	Gram-positive and gram-negative	In humans: Respiratory, urinary tract, skin, and gastrointestinal infections In poultry: Colibacillosis, salmonellosis, respiratory infections, and as a growth promoter	(Gouvêa et al., 2015)
Macrolides	Derived from the macrolide ring	Azithromycin, Erythromycin	Inhibition of protein synthesis	Gram-positive and gram-negative	Use in humans: Pharyngitis, sinusitis, and bronchitis treatment In poultry: Respiratory infection, and as a growth promoter	(Yoneyama and Katsumata, 2006)
Aminoglycosides	*Streptomyces* spp.	Tobramycin, Gentamicin, Amikacin	Inhibition of protein synthesis	Gram-negative	In humans: Urinary, respiratory, and abdomen infections. In poultry: Has no use in poultry due to its potential residue, special care due to resistance and residue concern is required	(Tolmasky, 2000)
Monobactams	6-aminopenicillanic acid	Aztreonam	Inhibition of protein synthesis	Gram-negative	In humans: Urinary, respiratory, skin, and soft tissue infections. In poultry: Enteritis due to *Escherichia coli* (*E. coli*) and *Salmonella*, and has no use as a growth promoter	(Li et al., 2023)

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ZOONOTIC MICROORGANISMS

Escherichia species

Escherichia coli (*E. coli*) is a pathogenic and commensal bacterium that causes infections, such as septicemia, cystitis, peritonitis, meningitis, and gastroenteritis in humans and animals (Zhang et al., 2020). The most imperative reservoirs for pathogenic *E. coli* are poultry and livestock (Yassin et al., 2017). Antimicrobials play a crucial role in animal farming by promoting the spread, emergence, and selection of AMR microbes (Abdalla et al., 2022). *E. coli* strains, which are part of human, animal, and environmental microbiotas, act as key indicators of AMR due to their resistance to antimicrobial agents and resistance gene accumulation (Poirel et al., 2018). Avian pathogenic *E. coli* is a major zoonotic disease that leads to significant financial losses for the poultry sector globally due to antibiotic resistance, primarily due to overuse and poor sanitation (Hamed et al., 2023). The world health organization (WHO) identifies *Salmonella* spp. and *E. coli* as the primary microorganisms to transmit AMR from poultry meats and products (Hamed et al., 2023).

E. coli isolates from poultry and animal farms showed resistance to at least three antimicrobial classes, while 94% showed resistance to at least one medication (Wang et al., 2021b). However, in a different investigation, the pathogenic *E. coli* which was isolated from chicken species showed a high level of resistance to widely used antimicrobials, such as colistin (82.88%), trimethoprim (89.04%), tetracycline (95.89%), and nalidixic acid (95.89%) (Bhave et al., 2019). The study revealed that 37% of turkey, 20% of chicken, 13% of duck, and 8% of game poultry *E. coli* isolates were multidrug-resistant fecal *E. coli* (Varga et al., 2019). A study by Ngai et al. (2021) revealed that 62% of *E. coli* isolates from chicken feed were resistant to ampicillin Benklaouz et al. (2020), looked at first-line antibiotics used on a chicken farm in Western Algeria to treat *E. coli*. The analysis indicated that of all the antibiotics employed in this study, nalidixic acid had the highest level of resistance (90.34%), followed by tetracycline (86.89%), ampicillin (82.75%), and other antibiotics. However, *E. Coli* isolates from the same investigation demonstrated ABR to colistin (84.64%), enrofloxacin (34.64%), neomycin (80.62%), norfloxacin, spectinomycin (0.89%), trimethoprim with sulfamethoxazole (53.47%), amoxicillin (24.38%), and amoxicillin with clavulanic acid (73.05%) (Benklaouz et al., 2020). Majewski et al. (2021) reported that the *E. coli* species frequently exhibit resistance to antimicrobials commonly employed for treating bacterial infections in poultry.

Salmonella species

Annually, 93.8 million cases of salmonellosis and 155,000 deaths are reported globally due to *Salmonella*, one of the most crucial zoonotic agents of *Salmonella* (Gong et al., 2023). The ABR bacteria list of the WHO now encompasses antimicrobial resistance against *Salmonella* as one of its top priorities (Tillotson, 2018). *Salmonella*, as a potential risk in poultry, is a common vector for the distribution of AMR to humans (Hoque et al., 2020). Food-borne zoonotic *enterobacterium* spp. can transmit ABR from animals' microbiomes to humans (Ali and Alsayeqh, 2022). However, *Salmonella* spp. infections are the most frequently reported bacterial diseases in poultry, which can potentially lead to human food-borne illnesses (El-Sharkawy et al., 2017). The development of multidrug resistance (MDR) in *Salmonella* strains may cause complications in treating humans and animals (Marin et al., 2022). Standard serological and microbiological techniques including polymerase chain reaction (PCR), conventional culture methods, immunology-based assays, miniaturized biochemical assays, and biosensors are usually used to isolate and identify *Salmonella* spp. (Kadry et al., 2019). The invasion gene (*invA*), often linked to bacterial virulence, is frequently used to accurately identify *Salmonella* spp. in clinical samples (Kadry et al., 2019).

Salmonella spp. is being considered as a potential AMR pathogen, originating from livestock, humans, and the environment (Pornsukarom et al., 2018). Non-typhoid *Salmonella* spp. is a major food-borne pathogen that globally infects humans and is linked to livestock and food (Mthembu et al., 2021). *Salmonella enterica*, comprising over 2600 serovars, is the most pathogenic species and is frequently linked to the contamination of poultry products (Jajere, 2019). Accordingly, inside the poultry production chain, the highest *Salmonella* isolates ABR levels were reported for nalidixic acid (80.3%) and ampicillin (64.8%, Castro-Vargas et al., 2020). The majority of the *Salmonella* isolates from chickens analyzed in another investigation were found to be resistant to trimethoprim/sulfamethoxazole, ciprofloxacin (73.17%), colistin (92.68%), and tigecycline (62.20%, Uddin et al., 2021). Poultry-related products, such as eggs can be exposed to pathogenic bacteria like *Salmonella*, either horizontally or vertically through transovarian transmission, which are crucial sources of pathogens (Borges et al., 2017). Adesiyun et al. (2020) revealed a 7.7% prevalence of resistant *Salmonella* spp. in eggs from layer farms in Gauteng Province, South Africa. *Salmonella* resistance in raw retail table eggs was found to be high, with 80% resistance against tetracycline and 60% resistance against ampicillin, indicating the presence of bacteria inside and outside the eggs (Kapena et al., 2020).

Staphylococcus species

Staphylococcus is a widely spread bacterium in the environments (air, dust, and household items), and are commensal colonizer of the mucous membranes and skin of humans and various animals including cats, cattle, and poultry (Lee et al., 2020). Ajoke et al. (2018) reported that 51 species and 27 subspecies of the genus *Staphylococcus*

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have demonstrated resistance to all antibiotic classes utilized in their treatment through different mechanisms. S*taphylococci* are usually classified as either coagulase-negative (CoNS) or coagulase-positive (CoPS). The coagulase-positive *Staphylococcus aureus* (*S. aureus*) causes illnesses in animals and humans and is the most vital species in this genus which causes food intoxication (Lee et al., 2020). In addition, there are examples of resistant CoNS in poultry products, such as meat, eggs, and litter (Amoako et al., 2019). The major class of antibiotics used against *S. aureus* is beta-lactam, against which the *S. aureus* develops resistance often owing to a plasmid-encoded penicillinase/beta-lactamase (Pugazhendhi et al., 2020). However, methicillin-resistant *S. aureus* (MRSA) of livestock origin especially in poultry meat has been increasingly reported in recent years (Bortolaia et al., 2016). Another study by Ali et al. (2017) investigated the presence of MRSA in poultry samples and determined the highest resistance against penicillin-G (93.33%) and the lowest resistance was detected against neomycin (23.33%) against five antibiotics.

Non-*aureus Staphylococci* (NAS), which includes CoNS, have been identified as potential sources of food poisoning and significant contributors to opportunistic infections in humans and animals in recent times (Lee et al., 2020). Multidrug resistance in NAS, particularly *S. agnetis* (19.4%) and *S. chromogenes* (14.5%) with high rates against tetracycline and fluoroquinolones, was confirmed. Tetracycline resistance was linked to mutations in *gyrA* and *parC*, while fluoroquinolone resistance was linked to *QRDR* mutations (Lee et al., 2020). Ogundipe et al. (2020) explored the AMR against MRSA in chicken meat, chickens, live poultry markets, and environmental samples within poultry farms in southwestern Nigeria. The study found that 56 MRSA isolates were detected in tested samples which demonstrated 100%, 60.7%, 33.9%, 28.6%, 32.1%, and 10.7% resistance to beta-lactams, tetracycline, ciprofloxacin, erythromycin, gentamicin, and trimethoprim/sulfamethoxazole, respectively. Accordingly, live poultry markets may be a major source of MRSA infections among the general public and that chicken meat is tainted with the disease. Moreover, every isolate of *S. aureus* and *Streptococcus* spp. tested was 100% resistant to the majority of the antibiotics that were evaluated for poultry (Sharma et al., 2017). According to Rao et al. (2022), the highest AMR of *Streptococcus* spp. against clindamycin was found, followed by erythromycin and penicillin. Furthermore, a study on CNS isolated from Polish poultry revealed that fewer CNS strains exhibited genes resistant to macrolides, chloramphenicol/florfenicol, and lincosamides (Pyzik et al., 2019).

***Campylobacter* species**

Campylobacter spp. are emerging infections responsible for 95% of diarrhea cases in humans (Kirk et al., 2015). Campylobacteriosis is a human disease caused by contaminated foods and drinks, with broilers being the primary source of *Campylobacter* and meat in many countries (Tang et al., 2020; Gao et al., 2023). The use of antibacterial drugs like macrolides, fluoroquinolones, and tetracyclines to treat *Campylobacter* infections has been criticized for causing global fluoroquinolone resistance and macrolide-resistant strains (Gahamanyi et al., 2021). *Campylobacter* from food-producing animals shows high tetracycline resistance, with strains resistant to erythromycin, tetracycline, and ciprofloxacin becoming more prevalent (Gao et al., 2023). Viswanathan et al. (2017) found that cattle *Campylobacter* isolates showed higher ABR compared to wildlife isolates, with *Campylobacter jejuni* being more common but showing multidrug resistance. A study in Casablanca, Morocco, found that tetracycline (100% resistance against *Campylobacter jejuni* isolates) and gentamicin (12.0% resistance) were the most effective antibiotics (Es-Soucratti et al., 2020). *Campylobacter jejuni* AMR profiles from a Moroccan poultry farm revealed the highest resistance to co-trimoxazole (84.1%), cephalothin (81.1%), and tetracycline (59.4%) in poultry meat and associated samples (Khan et al., 2018). *Campylobacter jejuni* indicated no resistance to gentamicin, erythromycin, or kanamycin, but resistance was observed to tetracycline (78.6%), ciprofloxacin (87.8%), and nalidixic acid (81.6%, Adiguzel et al., 2018). Using the microdilution method, 93 *Campylobacter* spp. (45 *Campylobacter jejuni* and 25 *Campylobacter coli* from chickens; 23 *Campylobacter coli* from pigs) were examined for resistance to antibiotics to nine antimicrobial agents. There were lower resistance rates to florfenicol (8.6%), but higher resistance rates to nalidixic acid (79.6%), erythromycin (75.3%), tetracycline (68.8%), azithromycin (66.7%), ciprofloxacin (64.5%), and gentamicin (35.5%, Tang et al., 2020). Excessive antibiotic use in humans and animals has increased ABR infections, particularly resistant to fluoroquinolones. Understanding AMR mechanisms in *Campylobacter* spp. is crucial for improved ABR programs.

PUBLIC HEALTH

It is well known that ABR bacteria develop and propagate in animals, humans, and the environment, posing a cross-boundary concern that impacts ecosystems and public health. Poultry, their products, carcasses, litter, and bird feces have been reported to have MDR bacteria, which can be a threat to handlers, consumers, and in general to public health (Agyare et al., 2019). The ABR bacteria and resistance genes developed in livestock transfer to humans through various channels, particularly through the food chain (Figure 1). Many of these bacteria are serious human pathogens. *Campylobacter* spp. is the primary culprit behind cases of food-borne diarrhea in humans. For instance, *Campylobacter*

spp. is a leading cause of food-borne diarrhea worldwide, responsible for 4-5 hundred million cases annually (Chibwe et al., 2023). In immunocompromised or elderly persons, as well as in extremely young children, infections caused by *Campylobacter* can be severe or even fatal. *E. coli* bacteria are another widespread cause of sickness. Furthermore, Salmonellosis is one of the world's most widespread and common food-borne infections that result in mild gastroenteritis (Mehdi et al., 2018).

Based on the literature, people from impoverished and developing countries are the most vulnerable to ABR bacteria threats. It is estimated that at least 700,000 people die each year as a result of resistant bacterial infections, a figure that is expected to rise to 10 million globally by 2050, in case the current trends continue. Furthermore, the worldwide cost of ABR is predicted to increase to $100 trillion in the coming decades (Crofts et al., 2017).

The risk of ABR bacteria in poultry production can be reduced through several approaches, including the utilization of antibiotics alternatives, prevention of environmental contaminations, and improvements in all stages of the poultry production system, such as poultry health, biosecurity, cleaning procedures, and implementing hazard analysis (Abreu et al., 2023; Salam et al., 2023).

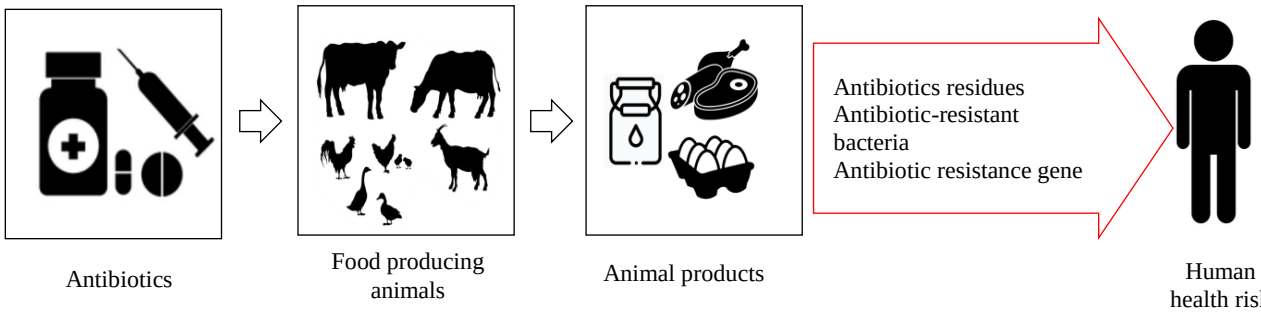

Figure 1. Human health concerns about antibiotic resistance bacteria in animal sources

ALTERNATIVES OF ANTIBIOTICS IN POULTRY PRODUCTION

Feed additives aim to reduce the reliance on antibiotics by identifying feed additives that stimulate the immune system, decrease morbidity and mortality, improve the growth performances of poultry, and preserve consumer health (Rahman et al., 2022). The modern chicken industry needs high levels of output and effective feed conversion, which can be partially attained by articular feed additives (Alagawany et al., 2016; Khan and Iqbal, 2016; Azizi et al., 2021a; 2021b, Danladi et al., 2022). Researchers have investigated several groups of feed additives and their prospective application as alternatives to antibiotics in poultry production (Rahman et al., 2022). Studying the overall alternatives for antibiotics used in poultry feeding as growth promoters is a controversial topic. Therefore, in the present review, the most frequently used feed additives, such as essential oils, phytochemicals, bacteriophages, antimicrobial peptides, probiotics, prebiotics, organic acids, and enzymes are discussed briefly.

Essential oils

Essential oils and nano-emulsions can be cutting-edge alternatives for antibiotics that reduce bacterial infections, and improve gut health intestinal environment, and gastrointestinal tract enzyme activities in chickens (Abd El-Hack et al., 2022). Nanoencapsulation herbal essential oils increase the growth performance of broiler chickens and efficiently work against antibiotic-resistant pathogens (Meimandipour et al., 2017). Similarly, ginger, garlic, limes, and lemongrass as sources of essential oil used in poultry nutrition increase the health performance of broiler chickens (Amiri et al., 2021). In addition, the application of herbal essential oils in poultry nutrition has positive effects on the antioxidant capacity, immunity, and growth performance of chickens (Linh et al., 2022). In another study, essential oils from garlic and cumin in nano-encapsulated form improved some structural features of the digestive tract including villi width and length (Amiri et al., 2021). Additionally, it has been identified that herb extracts, such as oregano, effectively inhibit the proliferation of pathogenic coliform bacteria in broiler chickens without affecting the proliferation of beneficial microbes (Mohebodini et al., 2019). The mechanism underlying the effectiveness of essential oils in poultry nutrition may involve bioactive components that enhance the production of the mucin-2 gene in the digestive tract. Mucin-2 plays a crucial role in protecting the gastrointestinal tract from infections, aiding in the secretion of digestive enzymes, and maintaining acidic conditions (Amiri et al., 2020). Furthermore, the chemical components present in the essential oils stimulate the secretion of digestive enzymes from the mucosal layer of the intestine (Jemaa et al., 2018). Thus, essential oil could be a

potential alternative to antibiotics in poultry nutrition for increasing growth performance, as well as producing low-cholesterol meat with high quality and durability (Namdeo et al., 2022).

Phytochemicals

Phytochemicals, which are secondary metabolites derived from natural plant sources, are utilized in poultry nutrition as feed additives due to their potential antimicrobial properties and ability to enhance chicken growth performance (Hashemi et al., 2008). The inclusion of phytochemicals in poultry diets has been shown to effectively replace antibiotics, improving growth performance and overall poultry production (Valenzuela-Grijalva et al., 2017; Azizi et al., 2023). These compounds possess antimicrobial, antioxidant, and anti-stress properties, contributing to enhanced immune responses, growth performance, and modulation of gut microbiota in broiler chickens (Chowdhury et al., 2018; Al-Mnaser et al., 2022; Azizi et al., 2023). Moreover, phytochemicals promote the growth of beneficial bacteria while reducing the population of pathogenic bacteria (Cencic and Chingwaru, 2010). This reshaping of the gut microbial community, alongside increased activity of digestive enzymes like amylase and maltase, likely underlies their mechanism of action (Jang et al., 2007; Al-Mnaser et al., 2022). Overall, incorporating phytochemicals as feed additives represents a promising alternative to antibiotics in poultry nutrition, enhancing metabolism, growth, antioxidant capacity, and immune function in chickens (Hassan et al., 2022).

Bacteriophages

According to Żbikowska et al. (2020), bacteriophages are a unique type of viruses that exclusively infect bacteria and are considered non-pathogenic to humans. In the poultry industry, bacteriophages are gaining attention as a promising alternative to antibiotics due to their high specificity (Lin et al., 2017). Research has demonstrated that bacteriophages can effectively control various pathogenic bacteria in chickens (Hong et al., 2013; Lee et al., 2016). Studies have shown that adding a 0.05% bacteriophage cocktail can enhance the immune system and promote the growth of beneficial gut microorganisms (Upadhaya et al., 2021). Dietary supplementation with bacteriophages has also been found to improve growth performance in broiler chickens and reduce the populations of specific pathogenic bacteria in their gastrointestinal tract (Kim et al., 2014). Similarly, feeding bacteriophages has been shown to enhance production efficiency in both broilers and layers and reduce overall excreta microflora levels (Noor et al., 2020). The improved microbiological environment in the gastrointestinal tract of broilers and layers appears to be the underlying mechanism (Lee et al., 2016). Thus, the findings from several studies suggest that bacteriophage dietary supplementation would be a safe alternative to antibiotics for raising broiler chickens.

Antimicrobial peptides

Antimicrobial peptides (AMPs), also known as host defence peptides, offer a promising alternative to antibiotics when used as feed additives in poultry nutrition (Kurt et al., 2019). The AMPs have been shown to positively impact gut microbiota and enhance overall health and performance in chickens. Research indicates that incorporating AMPs into poultry diets improves intestinal microbiota balance, intestinal morphology, nutritional digestibility, and growth rates (Wang et al., 2016). By promoting a healthy and immune-competent gut microbiota, AMPs contribute to enhanced growth performance metrics such as feed conversion efficiency, daily weight gain, feed intake, and reduced mortality (Nazeer et al., 2021). Bacteriocins, which are ribosomally synthesized antibacterial peptides, are another form of AMPs that show potential for controlling bacterial diseases and serving as alternatives to antibiotics (Ben Lagha et al., 2017). Studies have demonstrated that synthetic AMPs can increase feed intake and growth performance while protecting against intestinal damage in broiler chickens (Choi et al., 2013). Additionally, AMPs from various sources have been found to modulate the expression of pro-inflammatory and anti-inflammatory molecules in the intestine, improve intestinal morphology, enhance digestion processes, and regulate the immune system in broilers (Abreu et al., 2023). As immune modulators, AMPs also help reduce bacterial infection rates in broilers (Choi et al., 2013). The AMPs perform several antibacterial activities through several mechanisms that have been previously reviewed (Wang et al., 2016). These mechanisms include the suppression of nucleic acid and protein synthesis, the inhibition of enzymatic activities, and cell membrane synthesis (Brogden, 2005).

Probiotic

Due to the detrimental effects associated with antibiotics used in poultry nutrition, including dysbiosis, ABR, and elimination of beneficial microbial communities in the intestine (Yang et al., 2021), alternative approaches like probiotics are increasingly considered (Alagawany et al., 2016). Probiotics consist of living microorganisms that confer health benefits to the host when administered in adequate amounts (Rahman et al., 2022). Various probiotic microbe families are utilized as feed additives in the nutrition of poultry (Floch, 2014). Probiotics have been shown to have

beneficial and protective effects in several areas, such as improving growth and productive performances as well as egg quality. They also have applications as beneficial bacteria to reduce harmful bacteria, improve nutrient absorption and digestion, increase production, and protect the health of chickens (Ritzi et al., 2014; Alagawany et al., 2016; Popova, 2017). In Japanese quails, the lower dose of kefir administered as a probiotic reduced stress effect, liver weight, and alkaline phosphatase activity (Vahdatpour and Babazadeh, 2016). The use of synbiotics combined with the vaccine for infectious bursal disease promoted both the growth performance and related antibody titers (Babazadeh and Asasi, 2021).

Due to their negative proportional relationship, recent research showed that probiotics affect antimicrobial peptide modulation in chickens (Ma et al., 2020; Rahman et al., 2022). The antimicrobial peptide gene expression was reduced as probiotic concentration increased, suggesting that antimicrobial peptides might not always be required when probiotics are present (Akbari et al., 2008; Schlee et al., 2008). Furthermore, research indicates that adding probiotics to the diet during the later stages of reproduction can enhance the laying ducks' productivity and egg quality (Cao et al., 2022).

Prebiotic

The term prebiotics was first coined to describe non-digestible food components that provide benefits to the host by selectively promoting the growth or activity of specific bacteria in the colon (Floch, 2014). Chemically, prebiotics are carbohydrates with a short carbon chain that selectively increase the activity of certain types of beneficial bacteria in the gut (Kolida and Gibson, 2011; Al-Sheraji et al., 2013). Prebiotics are broken down by beneficial bacteria in the gut to produce short-chain fatty acids (Mountzouris and Tsirtsikos, 2009). Other health benefits of prebiotics in the large intestine include reduced cancer risk and enhanced gastrointestinal absorption of calcium and magnesium (Karakan et al., 2021). Different oligosaccharides have been evaluated in poultry feed as prebiotics, and their effects on the gastrointestinal microbiome of the chickens and the inhibition of pathogenic bacteria have been described by Clavijo and Flórez (2018).

Prebiotics have been associated with multiple mechanisms and roles that affect the avian gastrointestinal microbiota, including immune system interaction, alterations in intestinal morphology, and competitive elimination of pathogenic microorganisms (Pourabedin and Zhao, 2015). Prebiotics may be metabolized by a wide variety of gastrointestinal bacteria, which makes it more difficult to comprehend how they benefit the host and/or prevent the growth of pathogens (Alloui et al., 2013). However, further research is required to explore the exact mechanism behind the health and growth-promoting effects of prebiotics on livestock.

Organic acid

It has been established that short-chain organic acids (C1-C7) have antibacterial effects. Based on chemical structure, organic acids are divided into two groups, namely simple mono-carboxylic acids like butyric, propionic, fumaric, and sorbic acids, and short-chain carboxylic acids with double bonds like fumaric and sorbic acids (Wang et al., 2009; Scicutella et al., 2021). Another category of organic acids is carboxylic acids with an extra hydroxyl group including lactic, malic, tartaric, and citric acids (Shahidi et al., 2014). Due to their widespread safety, the European Union approved the use of organic acids and their salts in poultry production (Adil et al., 2010). The majority of organic acids with antimicrobial action have a pH of 3 to 5, and variable chemical and physical characteristics (Scicutella et al., 2021). Many of these acids are utilized in poultry nutrition as feed additives or as supplements to drinking water (acidifiers; Khan and Iqbal, 2016). It was found that organic acid mixtures (calcium format, calcium propionate, potassium sorbate, calcium butyrate, calcium lactate, and hydrogenated oil of vegetable) used in poultry nutrition were more effective at reducing intestinal *Salmonella* spp. and *E. coli* than the antibiotic growth promoter such as Enramycin (Hassan et al., 2010). The total bacterial count in ceca was significantly lower in the groups that received drinking water treatments with acetic acid (3 mL/L) and an organic acid mixture (3 mL/L; acetic acid, phosphoric acid, lactic acid, fumaric acid, and tartaric acid) 7 days after infection than in the non-treated group (Hamed and Hassan, 2013). Some types of organic acids perform effectively against organisms that cannot tolerate acidity (Cao et al., 2022). Organic acids improve nutrient digestibility by reducing endogenous nitrogen losses and microbial competition with the host for nutrients, as well as by lowering the incidence of subclinical infections and the secretion of immune mediators and by reducing the production of ammonia and other growth-depressing microbial metabolites (Khan and Iqbal, 2016).

Enzymes

Enzymes have gained prominence in poultry nutrition as effective feed additives, addressing challenges posed by anti-nutritional factors and optimizing nutrient digestion and utilization (Son and Ravindran, 2012; Pirgozliev et al., 2019). Enzymes that are commonly utilized in poultry nutrition include non-starch polysaccharides, which break down

the non-starch polysaccharides in viscous cereals, and microbial phytases, which target phytate-complexes in plant components (Amerah et al., 2011; Cross et al., 2011).

Particularly in young chickens, proteases play a significant role in the digestion of protein and amino acids (Pirgozliev et al., 2015). In addition to acting as antioxidants, dietary phytogenic may impact immunological function, nutritional availability, endogenous secretions, and daily feed consumption (Amerah et al., 2011; Cross et al., 2011). Enhancing animal performance through increased feed intake, weight gain, and feed efficiency is the ultimate goal of applying feed enzymes (Rahman et al., 2022). The underlying mechanism may be the breakdown of certain bonds in feed components that are not usually digested by endogenous digestive enzymes (Selle and Ravindran, 2007; Son and Ravindran, 2012), degradation of ant-nutritional elements that limit the digestion of nutrients and cause modifications to the intestinal tract's morphology (Bedford, 2000; Wu et al., 2004). Moreover, the positive effects of the enzyme may be due to the decreases in intestinal protein wastes and endogenous secretions, which reduces the amount of protein required for maintenance, and alterations to the small and large intestine's microbiota profile (Apajalathi et al., 2004; Cowieson and Ravindran, 2007).

CONCLUSION

The use of antibiotics in poultry production has been associated with the development of resistant bacteria. The spread of ABR bacteria in the environment and their transmission to humans could have serious consequences for public health. *Salmonella* spp., *E. coli*, *Campylobacter* spp., and *Staphylococcus* spp. are considered very common ABR bacteria isolated from poultry products. A ban on the use of non-therapeutic antibiotics would help to minimize additional harm to food safety, contamination of the environment, and overall health risks. Further studies are required to create substitute approaches, such as the utilization of alternative bioactive compounds as feed additives for instance essential oils, bacteriophages, antimicrobial peptides, probiotics, prebiotics, organic acid, and enzymes, and discovering their exact action mechanisms to maintain poultry health and productivity, environmental contaminations as well as preserve consumer health.

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Authors' contribution

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Competing interests

The authors disclose no conflict of interest.

Ethical consideration

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 related to the present review are presented in this article.

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Growth Traits as Predictors of Body Weight in Sheep: A Review

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REVIEW ARTICLE
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ABSTRACT

The sheep production industry affects most rural areas and communal farm enterprises in the tropics and sub-tropics as a source of income. The motivation behind the present literature review was to provide detailed literature from various sources on the estimation of body weight from the growth traits of sheep. The review was conducted to highlight the importance of body weight and the significance of growth traits (heart girth, withers height, body length, sternum height, and rump height) as parameters to predict body weight. The main reason for livestock practice is to ensure food security. Therefore, it is important to assess economic traits and determine the carcass merit of sheep. Linear body measurement is a practical, fast, easy, and cheap method broadly utilized in national breeding programs to predict body weight and improve meat productivity in rural areas. The current review indicated that growth traits could be used to predict the body weight of sheep since they importantly provide necessary information about the morphological structure and potential development of the animals.

Keywords: Body length, Body weight, Growth trait, Heart girth, Rump height, Withers height

INTRODUCTION

Sheep production in South Africa is a wide-growing livestock industry in the husbandry systems (Meissner et al., 2013). Some major economic factors related to this industry include ensuring efficient economic production through management practices focusing on shortening breeding production. Cannas et al. (2019) believe that the sheep production industry affects the economic resources in most rural areas and communal farm enterprises in the tropics and sub-tropics worldwide (Gökçe and Atakışİ, 2019). According to Ayichew (2019), sheep are primarily raised for consumption (meat and milk) and their skin for clothing and belts. Additionally, these animals are used in traditional rituals or given as gifts during ceremonies such as weddings.

The livestock industry aims to ensure food security, which can be achieved by assessing important economic traits and determining their carcass merit and value (Agamy et al., 2015). Body weight is the foremost consideration in the animal industry for marketing purposes. Additionally, understanding an animal's body weight is crucial for appropriate feeding, medication, and breeding practices (Abbas et al., 2021). In the animal industry, growth traits, such as body weight and linear body measurements, are the primary concerns during breeding for the economy, especially meat production (Moradian et al., 2013).

The sheep production industry should try to maximize production since customers have a high demand for heavier sheep (Kumari et al., 2014). Due to the lack of measuring scales, such as weighing scales in rural areas, rural farmers rely only on animal physical appearance and body weight estimates, leading to poor decision-making on medical dosing, selling, feeding, and selection criteria (Abdel-Mageed and Ghanem, 2013). Compared to visual observations and breeding value, Kumar et al. (2017) state that accurate techniques are used daily to improve growth traits, and the use of linear body measurements to predict body weight is a practical, fast, easy, and cheap method widely utilized in national breeding programs to improve the productivity of meat mostly by rural area farmers.

The motivation behind the present study was to provide a detailed review of literature from various sources on the estimation of body weight regarding sheep body parameters to enrich the literature and provide a deep understanding of this concept. In this regard, the significance of body weight is explained. Then, the importance of growth traits and body parameters, such as heart girth, withers height, body length, sternum height, and rump height, in the prediction of body weight as the primary purpose of the study is clearly explained.

Significance of body weight

According to Akpa et al. (2011), growth is determined as the total sum of structural body components that could be measured by body parameters and live weight and is mainly considered in the livestock industry animal husbandry.

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Body weight is an important parameter for selecting animals for various purposes, such as breeding and marketing (Lakew et al., 2017).

Animal breeding practices aimed to improve the economy. Body weight is an economically important trait in the quantitative selection of animals for breeding and maximizing production (Kumar et al. 2017). Dekhili and Aggoun (2013) indicated that body weight is the most significant trait in meat industries, where breeders select Ettawa sheep of high body carcass weight to breed for the next generation. According to Shirzeyli et al. (2013), body weight could be used to monitor animal growth and make good decisions for choosing male and female replacements by determining the value of animals and the rearing efficiency. Moreover, knowledge of animal body weight assists breeders in knowing breeding regarding carcass production per animal (Iqbal et al., 2013; Yilmaz et al., 2013).

Kumar et al. (2017) reported body weight as the most important feature of economic growth. Some rural farmers are still disadvantaged when selling their sheep due to a lack of weighing scales. Tsegaye et al. (2013) found that knowledge of animal weight helps the estimation of the market price to avoid selling the animals or their product at a price they do not deserve. Chitra et al. (2012) also agree and mention that sheep body weight helps in determining the correct drug dosage, while Asefa et al. (2017) indicate that knowledge about the body weight of small ruminants plays a role in ensuring proper management of feeding and avoiding obesity and anorexia. Recent studies showed that body parameters serve either to supplement body weight as an estimate of production or as a predictor of some less visible traits (Dekhili and Aggoun, 2013). According to Babale et al. (2018), the most reliable traits to estimate live body weight are wither height, body length, heart girth, rump height, and width. Moreover, they can also be used as indicators of breed origin and relationships within species.

As reported by Prasad (2010), knowledge about the body weight of goats, sheep, and pigs is important for the computation of balanced ratio, evaluation of growth, and health management. Birteebi and Ozoje (2012) documented that precise body weight could guarantee that farmers or producers were well repaid for their hard work and investments when raising the West African long-legged and West African dwarf sheep. Animal breeding practices aim to improve traits of economic importance, such as body weight, for better selection and production improvement (Shirzeyli et al., 2013).

Significance of growth traits as parameters to predict body weight

Prediction of body weight from linear body measurements is the simple, cheap, and best method, especially in communal areas where they lack resources, such as weighing scales, to determine the body weight of their animals (Sandeep et al., 2017). According to Younas et al. (2013), the livestock and meat industry aims to have an accurate and objective measurement method for improving traits and predicting the weight, values, and merit of the carcass of the live animal at a low cost. Moreover, Asefa et al. (2017) added that linear body measurements could be used indirectly to determine body weight as they are easy and simple to measure. Body linear measurements are the quantitative traits of carcass or meat and assist in developing selection criteria based on farmers' objectives (Kumar et al., 2017).

The primary aim of the animal industry is to have a technique that can accurately measure, access traits of economic importance, and establish animal merit and value of the carcass when the animal is alive (Agamy et al., 2015). One of the most popular methods for determining the relationship between two traits is a correlation between traits (Shirzeyli et al., 2013). Knowledge about the relationship between live body weight and body parameters helps the selection of superior animals in body weight in the Balochi male sheep for breeding purposes (Jahan et al., 2013).

Based on reports by Shirzeyli et al. (2013), linear body measurements differ in terms of breed, gender, and age. Many studies have been performed where body parameters are used to predict the body weight of different livestock (Babale et al. 2018). Kumar et al. (2017) concluded that body measurements serve as valuable growth indicators for sheep, particularly in rural areas where scales are scarce, aiding in the estimation of body weight and carcass characteristics. Birteeb et al. (2012) added that they have been used by other authors to predict body weight in exotic sheep breeds. As stated by Kumar et al. (2017), linear body measurement in livestock is a tool for farmers to recognize the morphological genetic strengths and weaknesses of sheep. Furthermore, body measurements can indirectly give an accurate estimate of body weight, especially when measured in the morning before the time sheep are released to graze or given feeds. According to Lakew et al. (2017), body measurements are key data sources for reflecting the breed standards and can also be used as qualitative growth indicators that reflect animal body changes during the animal's lifetime.

Knowledge of the correlation between body weight and linear body parameters is vital for better improvement and management in terms of adequate medicine administration and feed supply (Mohammad et al., 2012). Body traits are important when selecting superior indigenous Mengali sheep to gain more genetic progress in reproductive yield (Tariq et al., 2012).

According to Verma et al. (2016), linear body measurements detail the skeletal structure, growth, and development

capability of the Harnali sheep. Body parameters have been used to predict body weight in many sheep breeds (Younas et al., 2013). A study by Shirzeyli et al. (2013) indicated that linear body parameters provide information about the morphological structure and potential for development in Iranian sheep. Additionally, linear body parameters, such as rump height, withers height, body length, and heart girth, could be considered to predict body weight (Olawumi and Farinnako, 2017).

The capacity and skill of producers and purchasers to understand linear body measurements to growth traits are important in increasing production (Babale et al., 2018). As reported by Kumar et al. (2017), body measurements in sheep are used to evaluate the quantitative traits of meat. Eghahi et al. (2011) added that body measurements had been used in predicting body weight and helping in setting appropriate meat prices. Berhe (2017) highlighted that linear body measurements, mostly in smallholder farmers, could sustain the improvement of indigenous sheep. Common traits used for breed improvement are body length, heart girth, and withers height. As confirmed by Weldeyesus and Yayneshet (2016), the aforementioned body measurements are critical traits used for selecting and improving commercial goats. Many researchers have studied these traits as predictors of body weight in small livestock, including sheep and goats (Eyduran et al., 2017).

Heart girth

Heart girth is measured as the body circumference around the chest just behind the front legs and withers (Kumar et al., 2017). Most studies have revealed that heart girth could be used to predict body weight in different livestock and give reasonably accurate results. Body weight was predicted from linear body measurements in Awassi Crossbred sheep in a study conducted by Lakew et al. (2017). The findings indicated a highly significant relationship between body weight and heart girth, implying that heart girth impacts body weight and that improving heart girth might result in improved body weight. Kumar et al. (2017) reports agree with the above results. Their results showed that body weight could be predicted by heart girth. Therefore, heart girth could be used as the independent trait to estimate body weight in rural areas, where they lack weighing scales, and can also be used as a trait for the development and improvement of Harnali sheep.

In West African Dwarf goats, the correlation between body weight and linear body measurements was evaluated, and heart girth was revealed to have a strong relationship with body weight despite gender and location (Olawumi and Farinnako, 2017). A study by Younas et al. (2013) on Hissardale sheep showed that when body length, height at withers, and heart girth increase in the early stages of an animal's life, body weight increases, as well. Body weight in three Egyptian Fat-tailed sheep was estimated using linear body measurements, and a significant relationship between heart girth and body weight was found in Barki sheep Agamy et al. (2015). Furthermore, Abd-Alla (2014) considers heart girth a reliable predictor of body weight.

In their study on Jamuna basin sheep of Bangladesh across various age groups, Sun et al. (2020) found that heart girth was statistically significant in the 1-9 months group and 1-2 years group, correlating strongly with body weight. This positive relationship suggests that heart girth can be used effectively to estimate and potentially enhance body weight. Similar findings were reported in studies involving animals aged 2-6 years (Yilmaz et al., 2013), indicating the reliability of heart girth in estimating body weight across different age ranges. Moreover, a study focusing on sheep aged 13-24 months identified heart girth as the most effective trait for estimating body weight. This correlation implies that Nigerian sheep breeds with larger heart girth tend to have higher body weights (Mahmud et al., 2014).

Linear body parameters have been used to estimate body weight by some workers in exotic sheep breeds (Birteeb et al., 2012). Research by Kumar et al. (2017) described heart girth as the most important trait, and it was observed to be the trait that can be used to estimate body weight, especially in rural areas where weighing scales are absent. Similar suggestions were reported by Petrovic et al. (2012) and Jafari and Hashemi (2014) using different sheep breeds. According to Kumar et al. (2017), farmers who lack a weighing scale can use heart girth as a predictor of body weight. Prasad (2010) conducted a study and stated that body parameters, such as heart girth, are commonly used in estimating body weight in small ruminants, such as goats, sheep, and pigs.

Body length

According to Babale et al. (2018), body length is measured from the occipital protuberance to the base of the tail. Body parameters, such as body length, can be used to evaluate quantitative traits of meat, and it is easy to measure. It can be used as an indirect way to predict live weight and carcass traits (Agamy et al., 2015). Shirzeyli et al. (2013) conducted a study involving four sheep breeds and found that certain linear body measurements, such as body length, could predict body weight specifically in the Shaal breed. The relationship between animal body measurements and body weight varies depending on factors like breed, sex, and age, offering insights into their growth and development (Tariq et al., 2012). In another study focusing on sheep, Shirzeyli et al. (2013) highlighted a strong correlation between

body length and body weight. Similarly, Kumar et al. (2017) reported a positive statistical relationship between body weight and body length from a study conducted on Harnali sheep.

In indigenous sheep populations of Southern Ethiopia study by Melesse et al. (2013), the body length of Sidama-Gedeo, Wolaita, KmabataTembaro-Hadiya, and Gamogofa ewe had a high positive statistical correlation with body weight. A study on highland sheep in Tigray in Ethiopia by Berhe (2017) showed that body length in areas with no weighing scales could predict body weight due to a high positive correlation with body weight.

Wither height

As stated by Agamy et al. (2015), the wither height of an animal is the distance from the bottom of the front foot (phalanges) to the highest point of withers between the shoulders and is measured when the animal is standing. A study on indigenous sheep breeds showed a positive relationship between wither height and body weight (Mohammad et al., 2012).

Jahan et al. (2013) documented that withers height is a statistically significant trait related to body weight, suggesting that an increase in withers height could lead to an increase in body weight. Shirzeyli et al. (2013) studied four different breeds, namely Mehrabani, Macoei, Zandi, and Shaal. The results showed that withers height could be used to predict body weight in the Shaal breed; however, it was not significant in other breeds. In Ossimi and Rahmani ram-lambs, Agamy et al. (2015) outlined that withers height was correlated to body weight. The results are in line with Shehata (2013), who stated that body weight and withers height had a highly significant relationship.

Linear body measurements of Kashmir Merino Sheep were used as predictors of body weight by Rather et al. (2021), and a positive phenotypic correlation was observed between body weight and all the traits in the study. Wither height was the best predictor of body weight in that study. Genetic and phenotypic relationships between various body measurements were found by Petrovic et al. (2012) in Merino lands chaf sheep, Jafari and Hashemi (2014) in the Makuie sheep breed, and Kumar et al. (2017) in Harnali sheep. Additionally, wither height correlated to body weight in a study conducted by Mahmud et al. (2014) in 37 months and in different sheep age groups.

Different linear body measurements and prediction equations for the live weight of indigenous sheep populations in southern Ethiopia were studied, and withers height had a positive statistical relationship with body weight in Wolaita and Sidama-Gedeo ewes aged 1-2 years (Melesse et al., 2013). In rams, a significant relationship was also found between body weight and body length

Sternum height

Tyasi et al. (2020) reported sternum height as the vertical distance from the lower tip of the sternum to the ground in standing animals in the Nguni cattle breed. Prediction of body weight from body parameters was reported by Sun et al. (2020). The study revealed a correlation coefficient between body weight and sternum height among sheep aged 1-9 months, indicating that these sheep tended to have higher body weights compared to other breeds like Dorper. Furthermore, Temoso et al. (2017) conducted a study in Botswana on both sheep and goats, disregarding sex. Sternum height was also found to have the highest correlation with body weight, and the results are consistent with those of Norris et al. (2015) on indigenous goats.

Correlation and path analysis of body weight and biometric traits of Nguni cattle breed study by Tyasi et al. (2020) confirmed sternum height as the best predictor of body weight. Yilmaz et al. (2011) described sternum height as the distance between the height point of the wither and the ground. Their results on Kangal Type Akkaraman sheep revealed sternum height could be a candidate trait for estimating body weight. Patbandha et al. (2018) findings also state that sternum height could be used to predict body weight.

In a study on Female Etawah Grade Goats, Dakhlan et al. (2020) found that body weight correlated with sternum height. Similarly, Baleseng et al. (2016) conducted research on goats and sheep, indicating that shoulder height is statistically significantly correlated with animal weight. These findings suggest that shoulder height can potentially serve as a predictor of weight in both goats and sheep. These reports suggest that shoulder height can potentially be a predictor of weight in both goats and sheep. The study by Musa et al. (2012) showed heart girth is a better proxy measure of weight than shoulder height; however, both can be used to estimate the weight of goats and sheep under communal grazing.

Rump height

Based on Olawumi and Farinnako (2017), rump height is measured as the distance from the surface of a platform to the rump in a standing West African Dwarf Goat. A study by Sun et al. (2020) indicated that rump height had a high statistical relationship with body weight in 1 to 2 years of age Jamuna sheep. Lavvaf et al. (2012) and Melesse et al. (2013) findings are in agreement with results obtained from Jamuna sheep that body weight has a strong positive

relationship with rump height (Berhe, 2017).

Deribe et al. (2018) used correlational analysis to estimate the relationship between two variables. Linear body measurements of Begait, Gumz, and Rutana sheep were used to estimate body weight. Results showed a positive correlation between body weight and some linear body measurements, with rump height as one of the traits in Begait, Gumz, and Begait sheep, rump height was found to be the trait statistically correlated to body weight. Asefa et al. (2017) report on indigenous sheep of the Bale zone agrees with the above findings by Deribe et al. (2018).

Mahmud et al. (2014) documented a study on the estimation of body weight using cannon bone length and other body measurements in Nigerian breeds of sheep. Body weight was correlated to rump height as one of the linear body measurements in sheep under 12 months of age. Lavvaf et al. (2012) found similar results in a study conducted on sheep. Özen et al. (2019) report on Awassi sheep showed rump height was the most accurate trait to predict body weight. Ambacıoğlu et al. (2017) added that linear body measurements could help farmers estimate their livestock's body weight for management purposes, and rump height appeared to be the best candidate to be used to estimate body weight.

Prediction methods for weight measurement

The regression analysis method is the most widely used in multivariate statistical analysis to estimate the association between dependent and independent variables (Mohammad et al., 2012). A study by Mokoena et al. (2022) reveals that classification and regression trees are the optimum models with body length as the essential linear body measurement for estimating the body weight of Kalahari Red goats. Multivariate adaptive regression splines showed that age and wither height influenced the body weight in Nguni cows. In contrast, the results obtained from the regression tree data mining algorithm indicated that body length played a significant role in the body weight estimation of Nguni cows in the study conducted by Hlokoe et al. (2022). Additionally, it was noted that marker-assisted selection is the best-fit model for estimating body weight in Nguni cows.

Mathapo (2022) utilized data mining algorithms to analyze South African non-descript indigenous goats, concluding that body length alone could predict body weight based on a chi-square automatic interaction detector and classification and regression tree methods.

Avijit et al. (2022) employed recursive partitioning and regression trees (RPART) and stepwise regression model tools to predict body weight using linear body measurements, RPART, and stepwise regression models indicated that heart girth influenced live weight. Mohammad et al. (2012) estimated body weight from withers height, body length, and chest girth measurements using the Regression Tree (RT) Method. Statistically significant correlation coefficients were detected between body weight and wither height.

CONCLUSION

The present review indicated the use of growth traits as the most convenient method for estimating body weight, especially for farmers who lack weighing scales to determine the weight of their animals for breeding, nutrition, marketing purposes, and health management. Body length, heart girth, and wither height are popular traits for sheep breeds. However, more studies need to be conducted to predict body weight using more linear body measurements and other growth traits on different breeds to provide more information on improving the growth of the small stock.

DECLARATIONS

Availability of data and materials

Datasets and materials generated during the current study are available at the University of Limpopo and are accessible from the corresponding author upon reasonable request.

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Ethical considerations

Consent to publication and misconduct, plagiarism, data fabrication and double submission of the manuscript, and redundancy and other ethical issues were checked by the authors.

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Authors' contributions

Molabe Kagisho Madikadike and Tyasi Thobela Louis designed the study, Molabe Kagisho Madikadike wrote the review, and Tyasi Thobela Louis proofread the review. All authors confirmed the last content of the article before publication.

Competing interests

There is no competing interest to declare.

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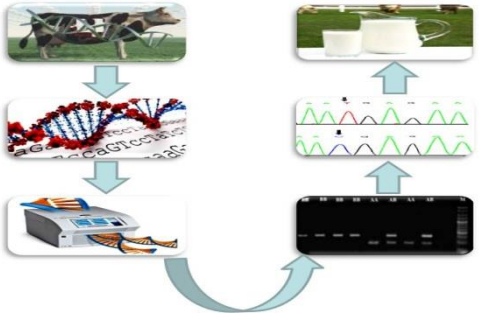

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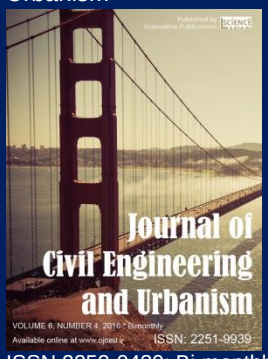

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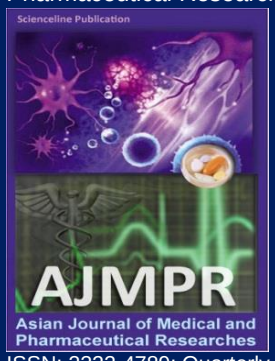

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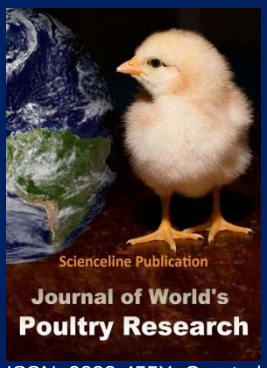

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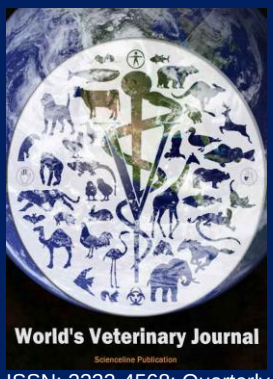

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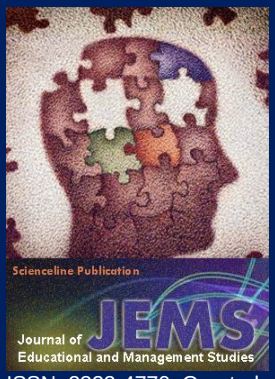

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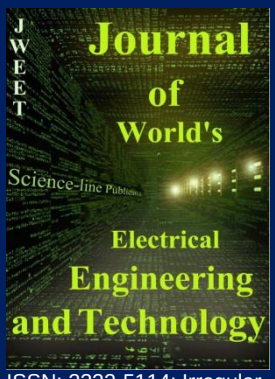

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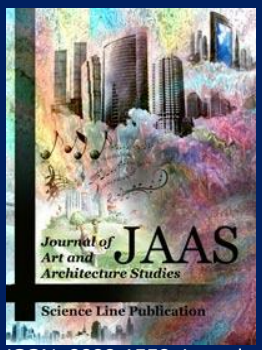

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