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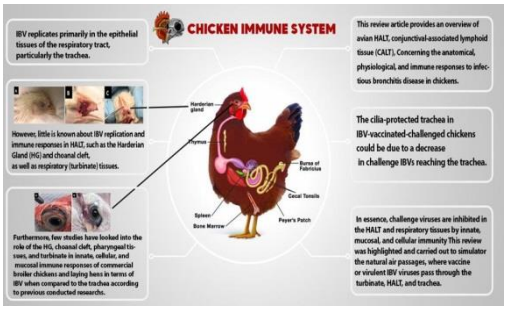
Review

The Role of Head Associated Lymphoid Tissues in Infectious Bronchitis Virus

World Vet. J. 13(1): 01-11, 2023; pii:S232245682300001-13
DOI: https://dx.doi.org/10.54203/scil.2023.wvj1



ABSTRACT: Infectious bronchitis virus (IBV) replicates primarily in the epithelial tissues of the respiratory tract, particularly the trachea. However, little is known about IBV replication and immune responses in relation to head-associated lymphoid tissue (HALT), such as the Harderian gland (HG) and choanal cleft, as well as respiratory (turbinate) tissues. Furthermore, few studies have looked into the role of the HG, choanal cleft, pharyngeal tissues, and turbinate in innate, cellular, and mucosal immune responses of commercial broiler chickens and laying hens infected with IBV, compared to the studies about the effects on the trachea. This review article overviewed the role of avian HALT, conjunctival-associated lymphoid tissue (CALT), concerning the anatomical, physiological, and immune responses to infectious bronchitis disease in chickens. The HG, choanal cleft, and turbinate in innate, mucosal, and cellular immune responses play a significant role in avian protection through virulent or attenuated vaccines of IBVs. The IBV viruses could not reach the trachea in chickens vaccinated with IBV vaccine due to the inhibition of viruses by HALT and respiratory tissues by innate, mucosal, and cellular immunity. It can be concluded that other than the trachea, the HALT and respiratory tissues play an important role in the infectivity and immune induction against IBVs due to their proximity to the upper air passages.
Keywords: Avian immunity, Chicken, Harderian gland, Infectious bronchitis, Turbinate

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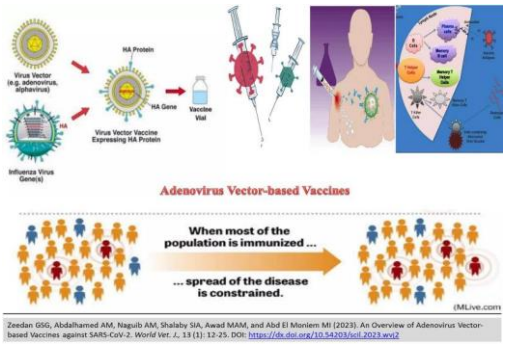
Review

An Overview of Adenovirus Vector-based Vaccines against SARS-CoV-2

World Vet. J. 13(1): 12-25, 2023; pii:S232245682300002-13
DOI: https://dx.doi.org/10.54203/scil.2023.wvj2



ABSTRACT: Adenovirus vectors have been employed to develop a vaccine against severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) for curtailing the Covid-19 pandemic spreading. Many different viral vectors have been mainly targeting the SARS-CoV-2 spike (S) protein as an antigen. Spike (S) protein is comprised of S1 and S2 subunits, in which the receptor-binding domain (RBD) of S1 is responsible for recognizing and engaging with its host cellular receptor protein angiotensin-converting enzyme 2 (ACE2), S2 accounts for membrane fusion of virus and host cell. Chimpanzee adenovirus was also used as a vector vaccine for SARS-CoV-2 (ChAdSARS-CoV-2-S) by intramuscular injection, and intranasal administration has been tested. Adenovirus vector-based vaccines are the most advanced, with several vaccines receiving Emergency Use Authorization (EUA). It was shown that rhesus macaques were protected from SARS-CoV-2 challenge after a month of being vaccinated with ChAd-SARS-CoV-2-S. A single intranasal or two intramuscular ChAd-SARSCoV-2-S vaccines could induce humoral antibodies and T cell responses to protect the upper and lower respiratory tract against SARS-CoV-2. As the effectiveness was demonstrated in non-human primates, ChAd-SARS-CoV-2-Sa potential option for preventing SARS-CoV-2 infection in humans. However, detecting novel more transmissible and pathogenic SARS-CoV-2 variants added concerns about the vaccine efficacy and needs monitoring. Moreover, the cause of recently documented rare cases of vaccine indicated immune thrombotic thrombocytopenia. This review article provided details for the adenovirus vector vaccine for SARS-CoV-2 in humans and tried to provide solutions to the adenovirus vector hemagglutinin issue.
Keywords: ACE2, Adenovirus, Immune response, SARS-CoV-2, Spike protein, Vaccine, Viral vectors

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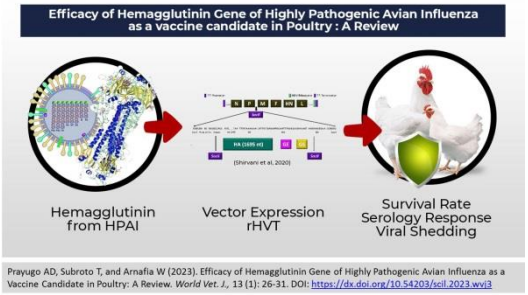
Review

Efficacy of Hemagglutinin Gene of Highly Pathogenic Avian Influenza as a Vaccine Candidate in Poultry: A Review

World Vet. J. 13(1): 26-31, 2023; pii:S232245682300003-13
DOI: https://dx.doi.org/10.54203/scil.2023.wvj3



ABSTRACT: The most prevalent fatal disease in poultry that can result in high morbidity and mortality is highly pathogenic avian influenza (HPAI), subtype H5N1. A vaccination program is the most frequent way to prevent HPAI cases in poultry, especially against the H5 subtype of HPAI. There are currently a number of avian influenza vaccines available, including recombinant and inactivated whole virus vaccines. The foundation of a recombinant vaccine is possible by the expression of an avian influenza gene of interest following insertion into a carrier vector (no pathogenic virus). A recombinant HPAI vaccine is required to further challenge avian influenza cases in poultry. As a recombinant vaccine inserted into a carrier vector, the hemagglutinin (HA) gene has proven effective. The recombinant Herpes Virus Turkey (rHVT) vector vaccine for avian influenza has been discovered and is commercially available. The rHVT vaccine was developed using a hemagglutinin insert from the HPAI virus clade 2.2. Overall, studies in this review aimed to determine the efficacy of any developed recombinant avian influenza vaccine that uses the HA gene from different clades challenged with any avian influenza virus (AIV) isolate. It was found that the efficacy of hemagglutinin as a recombinant vaccine could be promising for future HPAI vaccine development. In addition, it is possible to design a recombinant vaccine using local isolates to protect poultry farms, particularly in endemic regions.
Keywords: Avian influenza, Efficacy, Hemagglutinin, Poultry, Recombinant vaccine

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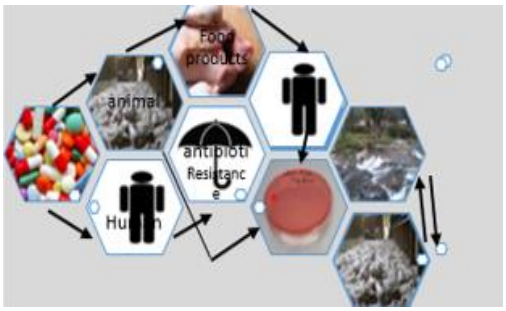
Review

Occurrence of Antibiotic Resistance in *Salmonella* Serotypes Isolated from Environment, Humans, Animals, and Animal Products in Morocco: A Systematic Review

World Vet. J. 13(1): 32-44, 2023; pii:S232245682300004-13
DOI: https://dx.doi.org/10.54203/scil.2023.wvj4



ABSTRACT: Several studies have been carried out in Morocco on *Salmonella* contamination in humans, domestic and wild animals, food products, and the environment. This bacterial genus is responsible for several infections and foodborne illnesses worldwide. The epidemiological situation of contamination by *Salmonella* is worsened by the development of antibiotic resistance to the main antibiotics used in human and veterinary medicine. The purpose of this study was to review the leading research carried out in this field, emphasizing the antibiotic resistance of this bacterium to antibiotics in humans and animals. Although some studies could not demonstrate the presence of *Salmonella* in the environments studied, the prevalence of contamination remained relatively high in humans, animals, food products, and the environment. The most critical contaminations were observed in poultry farms and poultry meat. *Salmonella* causes 42.8% of food poisoning cases in Morocco. It is the second most common cause of poisoning after pesticide poisoning. Morocco ranks first in the Middle East and North Africa for human salmonellosis, with a prevalence of 17.9% (1997-2012). Its prevalence in food products, especially those of animal origin, is very high and could reach 52.9% in turkey meat. Food products have been studied more for their contamination by *Salmonella* species. Meat products accounted for 17.35% of food poisoning cases. This study revealed that the isolation rate of *Salmonella* from food products of animal origin was dominated by isolations from meat products, with prevalence rates of 41.76 % from red meat and meat products and 25.88% from poultry meat, followed by prevalence rates of 12.44 % from fish products and 11.80 % from eggs. On the coast of Agadir, the incidence rates of *Salmonella* were 6.8% and 4.1% in sediment and seawater, respectively. This occurrence was 2.38% in the surface waters of Oued Khoumane. The development of resistance, particularly multi-resistance to antibiotics of therapeutic interest in both humans and animals, is alarming, especially with the ease of transmission of the bacterium to humans and facilitates its dissemination. Research findings indicated that 93.02% of isolates of *Salmonella* from humans, 79.37% of the strains isolated from poultry, and 46.27% of isolates from food products were resistant to at least one antibiotic.
Keywords: Animals, Environment, Food products, Foodborne disease, *Salmonella*, Resistance

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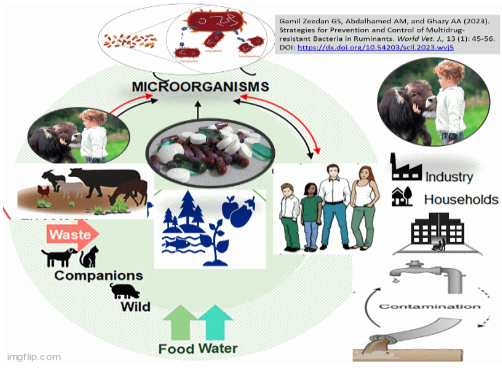
Review

Strategies for Prevention and Control of Multidrug-resistant Bacteria in Ruminants

Gamil Zeedan GS, Abdalhamed AM, and Ghazy AA.

World Vet. J. 13(1): 45-56, 2023; pii:S232245682300005-13

DOI: https://dx.doi.org/10.54203/scil.2023.wvj5



ABSTRACT: Antibiotics are no longer effective in treating bacterial infections due to antimicrobial drug resistance. Therefore, various alternative strategies have been developed to combat multidrug-resistant (MDR) bacteria. The current review article aimed to shed light on strategies to prevent and control MDR bacteria in ruminants. Due to the development of new resistant bacteria, there is a need for effective treatments and prevention protocols in livestock and humans. With growing antibiotic-resistant organisms, a few antimicrobial medicines will be available to treat the infection when no new drugs are developed. This highlights the importance of looking for other strategies for combating antibiotic-resistant bacteria. In this regard, alternative strategies have been proposed to minimize antimicrobial drug overuse in ruminants. These alternative procedures include alternatives for growth promotion (such as in-feed enzymes, probiotics, prebiotics, synbiotics, and antimicrobial peptides), alternatives for disease prevention (such as vaccines, immune modulators, chicken egg yolk antibodies, farm management, and biosecurity), and alternatives for disease treatment such as plant extracts and phage-therapy to antibiotics. These alternative methods should be safe and efficient without inducing microbial resistance.

Keywords: Antibiotic, Bacteria, Multidrug-resistant, Medicine, Ruminants

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Review

The Prevalence of Gastrointestinal Nematodes in Livestock and their Health Hazards: A Review

World Vet. J. 13(1): 57-64, 2023; pii:S232245682300006-13

DOI: https://dx.doi.org/10.54203/scil.2023.wvj6

ABSTRACT: Livestock plays an important role in the national economy and has a significant share in the gross domestic product of Pakistan. Parasitic diseases and worm infestations negatively affect their health, production, and reproductive performance. In addition, parasitic infestation in livestock reduces gross production values and renders huge economic losses globally. Among the parasites, the most important are nematodes. They are distributed worldwide and affect all kinds of livestock. This review aimed to elaborate on the main gastrointestinal nematodes, their mode of action, impacts on livestock and their control (physical, chemical or biological) strategies. Common examples of nematode worms infesting the livestock are *Ascaris, Hemonchus, Strongyloids, Trichostrongyliods, Ostertagia, Trichuris, Dictyocaulus, Trichnellia, Enterobius, Cooperia, Gunaglonema, Chabertia*, and *Oesphagostomum*. The gastrointestinal nematodes are detrimental to the animals' health. Nematodes primarily affect animals' feed consumption and efficiency, and severe ailments result in the death of the affected animals. The production and health losses primarily depend on the age of the animals, the degree of severity of worm infestation, epidemiology pattern of the parasites, management strategies of the flocks, and ecoclimatic conditions which are favorable for the worm's infestation. To minimize these issues, farmers should be educated on the importance of intensive livestock management and environmental sanitation, as well as strategic deworming of cattle using efficient broad-spectrum anthelmintics, biological control of the parasites, and breaking their life cycle and intermediate hosts.

Keywords: *Ascaris*, *Enterobius*, *Hemonchus*, Nematode, Parasitism, Roundworms, *Strongylus*

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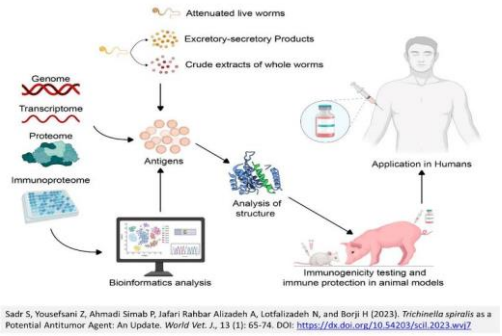
Review

Trichinella spiralis as a Potential Antitumor Agent: An Update

World Vet. J. 13(1): 65-74, 2023; pii:S232245682300007-13

DOI: https://dx.doi.org/10.54203/scil.2023.wvj7

ABSTRACT: Due to the limited success of therapeutic strategies in treating tumors, a new practical potent approach is needed. This review aimed to investigate previous literature related to tumors and *Trichinella spiralis (T. spiralis*). In recent years, there has been growing interest in utilizing biological, viral, bacterial, yeast, and parasitic agents to cure cancers. According to several studies, some parasites could interferee with the tumors' growth. There has been much discussion about some parasites' applications to cure tumors in animals and humans. In studies, *T. spiralis* was found to have antitumor properties. The active proteins in *T. spiralis,* such as Caveolin-1, Heat shock proteins, and Ribosomal proteins, are thought to inhibit the growth of cancers, such as melanoma, myeloma, sarcoma, leukemia, stomach cancer, colon cancer, breast cancer, and lung cancer. In addition, these proteins are thought to induce apoptosis in specific neoplastic cells. Accordingly, antigens derived from parasites may be helpful in cancer immunotherapy. However, there are still many unanswered questions regarding *Trichinella spiralis'* potential use as a biotherapy agent against cancer. Future studies should focus on the purification of parasite antigens and their use for wider-scale trials in animal models.



Sadr S, Yousefsani Z, Ahmadi Simab P, Jafari Rahbar Alizadeh A, Lotfalizadeh N, and Borji H (2023). *Trichinella spiralis* as a Potential Antitumor Agent: An Update. *World Vet. J.*, 13 (1): 65-74. DOI: https://dx.doi.org/10.54203/scil.2023.wvj7

Keywords: Antitumor, Apoptosis, Cancer, Immunotherapy, *Trichinella spiralis*

[Full text-PDF] [Crossref Metadata] [Scopus] [Export from ePrints]

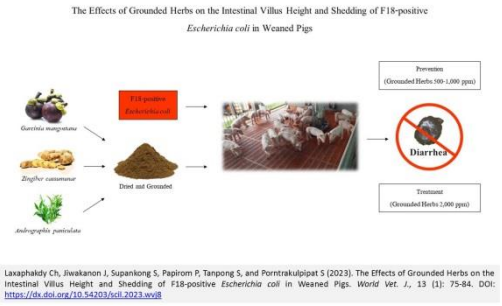
Research Paper

The Effects of Grounded Herbs on the Intestinal Villus Height and Shedding of F18-positive Escherichia coli in Weaned Pigs

Laxaphakdy Ch, Jiwakanon J, Supankong S, Papirom P, Tanpong S, and Porntrakulpipat S.

World Vet. J. 13(1): 75-84, 2023; pii:S232245682300008-13
DOI: https://dx.doi.org/10.54203/scil.2023.wvj8

ABSTRACT: Antibiotics have been widely used to control and treat infections caused by *Escherichia coli* (*E. coli*) in weaned pigs. The bacteria resistance to antibiotics can occur naturally; however, the misuse of antibiotics can accelerate this resistance. New antibiotics are developed very slowly, and only two new classes of antibiotics have been developed in the past 40 years. This makes herbal medicine a promising method for fighting against antibiotic-resistant bacteria. In the current study, 25 male crossbred (Duroc x Landrace x Large white) weaned piglets with an average weight of 6-8 kg were examined for 24 days. The pigs were randomly assigned to five groups in a completely randomized design with five replicates (1 pig/pen). All treatments included 20% crude protein corn-soybean as the basal diet. The negative control group received no supplementation, while pigs in the second experimental group received a basal diet supplemented with 150 ppm colistin sulfate. Basal diet and herbal mixture (*Andrographis paniculata*, *Zingiber cassumunar*, and *Garcinia mangostana*) were fed to three other experimental groups at 500, 1000, and 2000 ppm. The F18-positive, colistin-resistant *E. coli* were orally inoculated to all pigs for 9 days. The antibacterial and anti-diarrheal effects of this diet and its effect on the inoculated pigs' intestinal villi were evaluated. The results indicated that supplementation of this herbal mixture at levels of 500, 1000, and 2000 ppm had antibacterial effects, with no significant difference between doses. However, the positive effects of this herbal mixture on intestinal villi height and diarrhea were found only in pigs that received 1000 and 2000 ppm of the herbal mixture. From a practical point of view, supplementation of this herbal mixture at 500 and 1000 ppm could be applied for prophylaxis during the weaning period, whereas 2000 ppm of the herbal mixture could be used for the treatment of postweaning *E. coli* diarrhea.



Laxaphakdy Ch, Jiwakanon J, Supankong S, Papirom P, Tanpong S, and Porntrakulpipat S (2023). The Effects of Grounded Herbs on the Intestinal Villus Height and Shedding of F18-positive *Escherichia coli* in Weaned Pigs. *World Vet. J.*, 13 (1): 75-84. DOI: https://dx.doi.org/10.54203/scil.2023.wvj8

Keywords: *Andrographis paniculate, Escherichia coli, Garcinia mangostana*, Herbal mixture, *Zingiber cassumunar*

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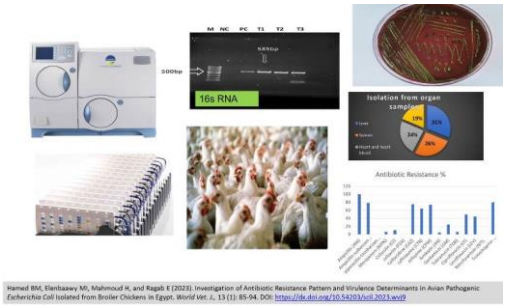
Research Paper

Investigation of Antibiotic Resistance Pattern and Virulence Determinants in Avian Pathogenic *Escherichia coli* Isolated from Broiler Chickens in Egypt

Hamed BM, Elenbaawy MI, Mahmoud H, and Ragab E.

World Vet. J. 13(1): 85-94, 2023; pii:S232245682300009-13

DOI: https://dx.doi.org/10.54203/scil.2023.wvj9



ABSTRACT: Besides its zoonotic importance, avian pathogenic Escherichia coli (APEC) causes substantial financial losses in the poultry industry globally. The progress of antimicrobial resistance in APEC is mainly associated with excessive antimicrobial use and improper sanitation. Since its beginning in the 1970s, the VITEK system has developed into the VITEK 2 system, which has used an automated system to perform all the steps required for microbial identification and antibiotic susceptibility rapidly and accurately. The present study aimed to update the available circulating data about APEC isolates by phenotypic identification, sero-grouping of APEC from broilers chickens and breeders in five governates of Egypt, investigation of their antibiotic resistance pattern by VITEK 2 system, and molecular identification of their virulence determinants. The prevalence of APEC isolated from the different internal organs (liver, lung, heart, heart blood, and spleen) was 67.5%. The most prevalent serotypes were O125, 0114, O44, O127, O142, and O78. Virulence-associated genes (iutA, fimC, and papC) were detected at rates of 84.4%, 74%, and 54.8%, respectively. The highest resistance was found against ampicillin (100%), trimethoprim-sulfamethoxazole (80%), and ampicillin-sulbactam (78.5%), which indicates that the poultry farms need a surveillance and intervention system with proper accuracy and rapidity to prevent the misuse of antibiotics and APEC outbreaks.
Keywords: *Escherichia coli*, Colibacillosis, PCR, VITEK, Virulence genes

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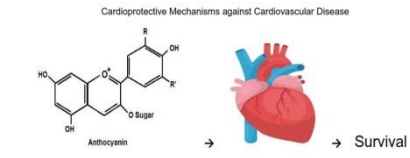
Research Paper

Effects of the Anthocyanin Compound (Cyanidin-3-glucoside) on some Histological and Physiological Parameters Related to the Heart in Male Rats Exposed to Oxidative Stress

Yasser H and Sabour AN.

World Vet. J. 13(1): 95-102, 2023; pii:S232245682300010-13

DOI: https://dx.doi.org/10.54203/scil.2023.wvj10



Yasser H and Sabour AN (2023). Effects of the Anthocyanin Compound (Cyanidin-3-glucoside) on some Histological and Physiological Parameters Related to the Heart in Male Rats Exposed to Oxidative Stress. *World Vet. J.*, 13 (1): 95-102. DOI: https://dx.doi.org/10.54203/scil.2023.wvj10

ABSTRACT: The increasing incidence of heart disease due to an unhealthy diet rich in fats has encouraged the use of plant extracts, which have shown efficiency in improving body immunity and promoting human health. The current study was designed to investigate the effect of anthocyanin cyanidin-3-glucoside on some physiological and histological parameters related to the heart in white male rats exposed to oxidative stress with hydrogen peroxide. The study included 48 adult male white rats with a weight range of 200-300 g, and an ages range of 8-12 weeks. The rats were randomly divided into six groups of eight rats per group. Group 1 was considered a negative control group supplied with water and the basal diet for 30 days. Group 2 was a positive control group in which the rats were given drinking water containing hydrogen peroxide at a concentration of 1%. The third group orally received cyanidin-3-glucoside at a concentration of 50 mg/kg. The fourth group received both cyanidin-3-glucoside compounds at a concentration of 70 mg/kg and drinking water containing hydrogen peroxide at a concentration of 1%. The fifth group was dosed orally with a cyanidin-3-glucoside only at a concentration of 50 mg/kg, and the sixth group was dosed orally with a cyanidin-3-glucoside at a concentration of 70 mg/kg. At the end of the experiment, the animals were anesthetized, then blood samples were collected from the heart directly to obtain serum for measuring the levels of troponin, lactate dehydrogenase (LDH), and creatine kinase (CK-MB). The results showed a significant increase in troponin, LDH, and CK-MB levels in the positive control group compared to the negative control group. However, there was a significant decrease in the level of these enzymes in the third and fourth groups, compared to the positive control group. The fifth and sixth groups demonstrated a significant decrease, compared to the positive control group. However, they revealed a nonsignificant difference in the levels of these parameters, compared to the negative control group. The obtained results indicated that the cyanidin-3-glucoside compound positively prevented heart muscle damage caused by oxidative stress.
Keywords: Anthocyanin compound, Heart, Hydrogen peroxide, Male rats, Oxidative stress, Physiological parameter

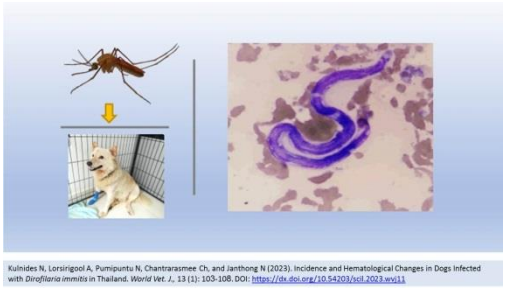
[Full text-PDF] [Crossref Metadata] [Scopus] [Export from ePrints]

Research Paper

Incidence and Hematological Changes in Dogs Infected with *Dirofilaria immitis* in Thailand

World Vet. J. 13(1): 103-108, 2023; pii:S232245682300011-13

DOI: https://dx.doi.org/10.54203/scil.2023.wvj11



ABSTRACT: *Dirofilaria immitis* is responsible for heartworm disease in dogs. Clinical signs are non-specific, ranging from asymptomatic to severe symptoms. The most common symptoms include coughing, emaciation, dyspnoea, and sudden loss of consciousness. Therefore, diagnosing heartworm infection in dogs requires a combination of methods, such as hematology and serology. This study was conducted on dogs with clinical signs, including anorexia, coughing, panting, and hind legs weakness, that was referred accidentally to a pet clinic in Thonburi district, Bangkok Province, Thailand, during 2020-2022. The examination was performed using a rapid enzyme immunoassay test and a thin blood smear. The total number of dogs admitted to a pet clinic during that period was 980. The result indicated infection of 21 (12 male and 9 female) dogs with heartworm (2.14%). The mean age of dogs was 5.62 ± 2.48 years. All infected dogs were classified under an open husbandry system that did not consistently use heartworm prevention products such as the macrocyclic lactone group. In the groups that received topical ectoparasites products, 10 dogs were detected with heartworm infection. The hematological changes in the infected dogs consisted of leucocytosis and increased levels of ALT, BUN, and creatinine. The study results can guide owners in choosing products that can prevent heartworm. Anti-mosquito nets should be deployed in areas where pets live, and always keep the environment clean.

Keywords: Dog, Heartworm, Hematology, Serum biochemistry

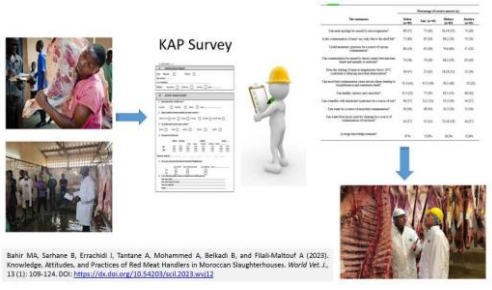
[Full text-PDF] [Crossref Metadata] [Scopus] [Export from ePrints]

Research Paper

Knowledge, Attitudes, and Practices of Red Meat Handlers in Moroccan Slaughterhouses

World Vet. J. 13(1): 109-124, 2023; pii:S232245682300012-13

DOI: https://dx.doi.org/10.54203/scil.2023.wvj12



ABSTRACT: Meat handlers are vectors of pathogens in slaughterhouses and can play a major role in the microbiological contamination of meat. The level of knowledge of meat handlers in slaughterhouses is a critical factor in food safety. Good hygienic practices in the slaughterhouse are required to reduce the risk of microbiological contamination while handling meat. This study evaluated workers' knowledge, attitudes, and practices in four municipal slaughterhouses in Morocco. A total of 267 employees were evaluated using a structured survey. The results showed that workers had acceptable knowledge and practices, and their attitudes were very satisfactory, averaging 52.87%, 50.9%, and 63.07%, respectively. A positive correlation between the workers' level of knowledge and education was found in all studied slaughterhouses. Similarly, the results indicated a positive correlation between knowledge and attitudes at Meknes and Kenitra slaughterhouses. The impact of the studied sociodemographic characteristics may vary from one slaughterhouse to another. In conclusion, the study suggested that although the knowledge, attitudes, and level of practice of food handlers were very satisfactory, some aspects related to the control of the health status of the handlers and personal protective equipment had to be underlined. Ongoing food safety training should become mandatory to enhance food safety in the slaughterhouses of study locations.

Keywords: Attitudes, Food Safety, Hygienic practices, Knowledge, Slaughterhouse

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Research Paper

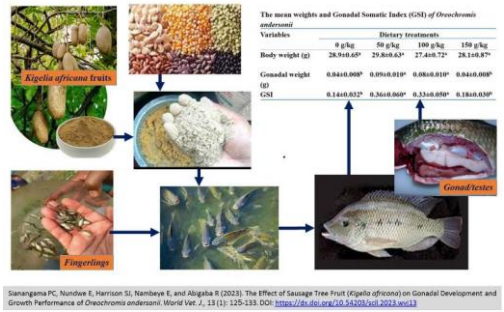
The Effect of Sausage Tree Fruit (*Kigelia africana*) on Gonadal Development and Growth Performance of *Oreochromis andersonii*

Sianangama PC, Nundwe E, Harrison SJ, Nambeye E, and Abigaba R.

World Vet. J. 13(1): 125-133, 2023; pii:S232245682300013-13

DOI: https://dx.doi.org/10.54203/scil.2023.wvj13

ABSTRACT: In Zambia fish farms, *Oreochromis andersonii* is an important common indigenous fish species. Naturally, safe phytochemicals can effectively improve fish reproduction performance and their production potential. Therefore, this study was conducted to determine the effect of *Kigelia africana* on the gonadal development and the performance of *Oreochromis andersonii.* A total of 96 male fingerlings were randomly assigned to four dietary treatments (D1-D4), and each treatment group had three replicates. The D1, D2, D3, and D4 groups were formulated to receive 0, 50, 100, and 150 g of powdered *Kigelia africana*/kg, respectively. The fish were fed the diets for 9 weeks, followed by the study parameter measurements at the end of the experiment. The highest mean body weight and gonadal weight were (29.8 ± 0.63 and 0.09 ± 0.010 g, respectively) for fish in the D2 group. There was no significant difference between the mean body weight of fish in different groups, but their mean gonadal weights differed significantly. The gonadal somatic index of fish differed significantly among treatment groups, with those in D2 having the highest mean value (0.36 ± 0.060). The highest mean standard length (103.3 ± 0.63 mm) and total length (126.0 ± 0.11 mm) of fish were observed for D1 and D2 groups, respectively. Additionally, the mean values for those parameters decreased with increasing *Kigelia africana* in the diet. The physicochemical parameters of water, including temperature and dissolved oxygen, ranged 16.8-23.1℃ and 0.6-2.2 mg/L, respectively; these were generally at low levels considering the optimum requirements for this fish species. In conclusion, *Kigelia africana* improved gonadal growth and development but did not promote overall fish growth. The best gonadal growth/development results of *Kigelia africana* powder were observed at a level of 50 g/kg, which can be used as a performance booster in the aquaculture production of *Oreochromis andersonii*.



Keywords: Aquaculture, Gonadal development, Growth, *Kigelia africana*, *Oreochromis andersonii*, Sausage tree

[Full text-PDF] [Crossref Metadata] [Scopus] [Export from ePrints]

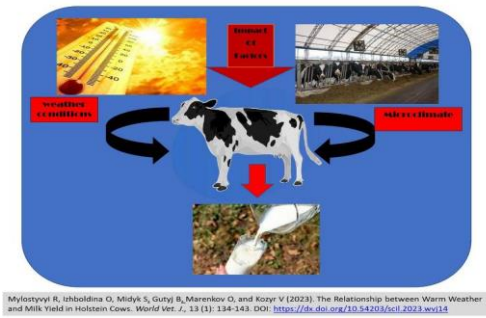
Research Paper

The Relationship between Warm Weather and Milk Yield in Holstein Cows

World Vet. J. 13(1): 134-143, 2023; pii:S232245682300014-13

DOI: https://dx.doi.org/10.54203/scil.2023.wvj14

ABSTRACT: The increasing variability of weather conditions associated with global climate change is becoming a major problem for dairy farming. The present article provided the results of studies on the relationship between the milk production of Holstein cows and environmental parameters during the warm season. The study investigated whether the relationship between weather conditions (air temperature, relative humidity, wind direction, wind strength, and insolation) and daily milk yield, as well as its components (milk fat yield and milk protein), depended on the conditions comfortable for the cows. The temperature-humidity index was calculated based on air temperature and relative humidity data, which were recorded by the nearest weather station to the farm, which is subordinate to the Ukrainian Hydrometeorological Center. It was found that the relationship between environmental parameters and milk yield was weak concerning the increase in proportion to the growth of heat load. However, the factorial analysis indicated that the total influence of weather factors on milk yield, milk fat, and protein yield was 42-46%. Moreover, weather conditions could significantly impact dairy productivity when cows are kept in naturally ventilated barns. This suggests further investigation of issues related to the microclimate improvement in cowsheds in hot seasons using sprinkler systems for cooling dairy cows.



Keywords: Components of milk, Correlation, Cows, Hot weather, Milk yield, Naturally ventilate

[Full text-PDF] [Crossref Metadata] [Scopus] [Export from ePrints]

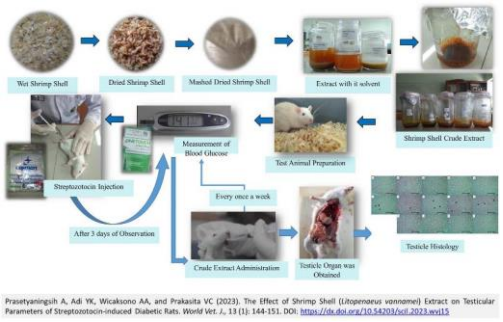
Research Paper

GA as .pdf

The Effect of Shrimp Shell (*Litopenaeus vannamei*) Extract on Testicular Parameters of Streptozotocin-induced Diabetic Rats

World Vet. J. 13(1): 144-151, 2023; pii:S232245682300015-13

DOI: https://dx.doi.org/10.54203/scil.2023.wvj15



ABSTRACT: Diabetes mellitus (DM) is a chronic metabolic disorder that has become a major health problem worldwide. Reproductive dysfunction is one of the main complications of DM, particularly in men. However, as is known, shrimp shell extract contains nutrients, such as astaxanthin, that affect reproductive traits. The present study aimed to evaluate the effect of shrimp shell extract on the volume, weight, and histological features of the testes of a DM rat model. Fifteen adult male rats were randomly divided into three groups. Group A (n = 5) was a healthy control group, group B (n = 5) was a DM control group, and group C (n = 5) was a DM group treated with shrimp shell extract. Rats in groups B and C were treated with streptozotocin to induce DM. Rats in group C were given shrimp shell extract at 25 mg/kg body weight for 30 consecutive days after DM induction. Testicles were collected and submitted to dimension, weight, and histological examinations. The testicle volume and weight of rats in group C were significantly higher and heavier, respectively, than rats in group B and did not differ from rats in group A. The seminiferous tubule diameter of rats in group C was significantly larger than rats in group B and did not differ from rats in group A. Rats in group B had a lower testicle volume and lighter testicle weight as well as a shorter seminiferous tubule diameter than rats in groups A and C. In conclusion, shrimp shell extract could improve male fertility parameters in a DM rat model. However, the mechanism of action needs to be studied further.
Keywords: Astaxanthin, Diabetes mellitus, Fertility, Seminiferous tubule, Testis

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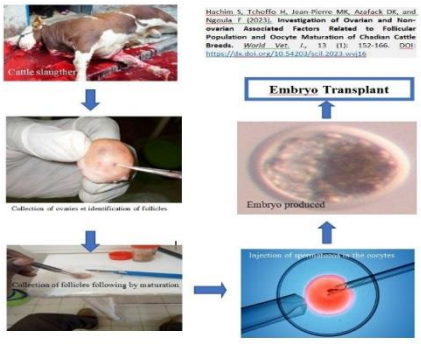
Research Paper

Investigation of Ovarian and Non-ovarian Associated Factors Related to Follicular Population and Oocyte Maturation of Chadian Cattle Breeds

World Vet. J. 13(1): 152-166, 2023; pii:S232245682300016-13

DOI: https://dx.doi.org/10.54203/scil.2023.wvj16



ABSTRACT: A cow can give birth to an average of 6-7 calves in her entire reproductive period. The remaining oocytes could be used for the in vitro production of embryos. The present study was conducted to evaluate the effects of ovarian and non-ovarian factors on the follicular population and oocyte maturation of three Chadian cattle breeds (Arab, Kouri, and Toupouri). For this purpose, the ovaries of 166 cycled cows were collected at the Farcha slaughterhouse of Chad and placed individually in labeled conical tubes containing 0.9% NaCl and 0.5 mg/ml penicillin-streptomycin. After clearing the ovaries of tissue debris, they were weighed, and the follicles were counted. The diameter of each follicle was measured and classified into three categories. A total of 2734 oocytes were collected in 28 days with a minimum of 97 per day by the slicing method using a 10X stereoscope. They were then classified into four groups according to the structure of their cumulus oophorus. Immature oocytes (class 1 and 2 [1455]) were placed in different culture media consisting of Minimum Essential Medium (MEM) alone, MEM with 10% follicular fluid, and MEM with 50% follicular fluid for oocyte maturation. The results indicated that the mean follicular population and mean oocyte yield were 24.71 ± 0.88 and 11.65 ± 0.94, respectively. The mean oocyte index and the number of cultivable oocytes for in vitro embryo production (class 1 and 2) were 1.03 ± 0.23 and 1.65 ± 0.94, respectively. The number of follicles observed in the age group of 6-9 years was higher than in other age groups. Oocyte yield was significantly higher in cows with a body condition score of 4-5 compared to average and lean cows. Among the different culture media used for oocyte maturation, the medium consisting of MEM plus 10% follicular fluid recorded a higher maturation rate than the other culture media. Cows aged 6-9 years had a higher maturation rate than other age groups. In conclusion, the good follicle (follicle that produced oocyte) and appropriate oocyte performance were observed in cows with body condition score 3-5 and an age range of 6-9 years.
Keywords: Age, Breed, Cattle, Maturation, Oocyte

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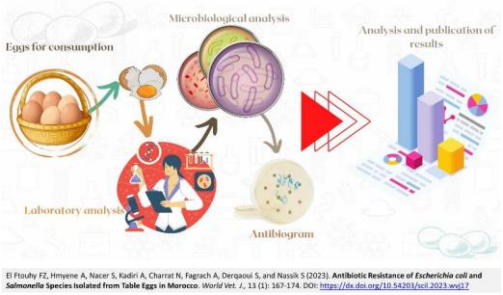
Research Paper

Antibiotic Resistance of *Escherichia coli* and *Salmonella* Species Isolated from Table Eggs in Morocco

World Vet. J. 13(1): 167-174, 2023; pii:S232245682300017-13
DOI: https://dx.doi.org/10.54203/scil.2023.wvj17



ABSTRACT: The development of antimicrobial resistance has become a severe global public health emergency. Foods of animal origin are considered possible drivers of resistant bacteria, including *Escherichia coli* (*E. coli)* and *Salmonella* spp. It is associated with the indiscriminate use of antibiotics, resulting in the inability to treat patients infected with antibiotic-resistant pathogens and a high risk of transmission of these resistant pathogens. The current study aimed to determine the prevalence and antibiotic resistance of *E. coli* and *Salmonella* spp. in raw table eggs in Morocco. A total of 870 table eggs resulting from 290 samples (3 eggs = 1 sample), were purchased from ambulatory sellers, street vendors, kiosks, and neighborhood markets from different cities in Morocco and transferred to the laboratory in the Hassan II Agronomy and Veterinary Medicine Institute of Rabat, Morocco. The egg shells and contents were tested separately then the isolation and identification of bacterial pathogens were performed according to the Moroccan Standard Norms. The bacterial isolates were tested for susceptibility to six commonly used antibiotics, namely nalidixic acid (30 µg), kanamycin (30 µg), gentamycin (15 µg), ciprofloxacin (15 µg), tetracycline (30 µg), and amoxicillin (10 µg). The findings revealed that 38 samples (13%) tested positive for *E. coli* of which 9% were on egg shells, and 4% were in egg content, while for *Salmonella enteritidis* (*S. enteritidis*), 5 samples (2%) tested positive and only in the egg contents. *Escherichia coli* showed the highest resistance to amoxicillin, followed by tetracycline and nalidixic acid with 92.10%, 84.21%, and 50%, respectively, and was sensitive to ciprofloxacin (84.21%), kanamycin (65.79%), and gentamicin (60.54%). *Salmonella enteritidis* had the highest resistance against tetracycline (80%), followed by ciprofloxacin and nalidixic acid with 40% each. The highest sensitivity rates of *S. enteritidis* were for gentamicin, amoxicillin, and kanamycin at 80%, 80%, and 40%, respectively. Given that these resistant bacteria could potentially be transferred to humans through eggs or egg products, it is necessary to employ strict hygiene measures and provide a wise and legal use of antibiotics in animal breeding.
Keywords: Antibiotic resistance, *Escherichia coli*, *Salmonella enteritidis*, Table egg

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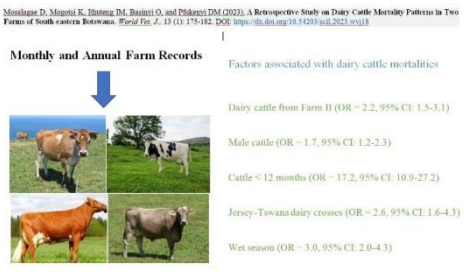
Research Paper

A Retrospective Study on Dairy Cattle Mortality Patterns in Two Farms of South-eastern Botswana

World Vet. J. 13(1): 175-182, 2023; pii:S232245682300018-13
DOI: https://dx.doi.org/10.54203/scil.2023.wvj18



ABSTRACT: Generally, high mortalities of dairy cattle due to infectious and non-infectious diseases cause huge economic losses, unprofitability, and low productivity in the dairy industry. The present study aimed at determining the mortality rates, their causes, and risk factors among 1779 cattle at two dairy farms belonging to the Department of Agricultural Research, Botswana. An 8-year retrospective study was conducted using farm records during 2005-2012. Monthly and annual records of the farms were examined regarding the total dairy cattle population, sex, breed, age, cattle deaths, and causes of death. Mortality was calculated from the total cattle population and expressed as a percentage, and it was analyzed with respect to farm, breed, age, sex, year, season, and mortality causes. The overall mortality rate was 8.5%. The semi-intensively managed Farm II, as well as young stock (<12 months old), and males recorded significantly higher mortalities than their counterparts. Dairy crosses of pure exotic and indigenous Tswana cattle had higher mortalities than the Friesians and Jerseys, and the wet season accounted for over 70% of the total deaths. Only two years (2010 and 2012) out of the 8-year study period had a mortality rate < 5%. Notably, 28.1 % of mortalities with a known cause were due to heartwater disease (n = 57), but most deaths (62.3%) were due to unknown causes. In conclusion, to improve farm herd health and husbandry practices, more efforts should be devoted to preventing heartwater and mortalities in young stock and male animals, particularly during the hot-wet season.
Keywords: Dairy cattle, Heartwater, Mortality, Risk factor

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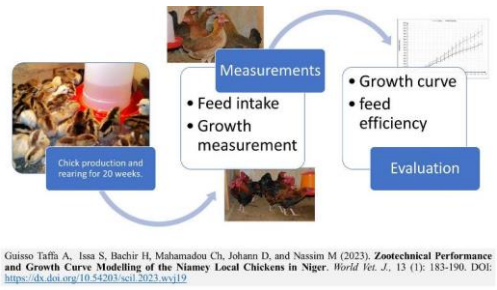
Research Paper

Zootechnical Performance and Growth Curve Modelling of the Niamey Local Chickens in Niger

World Vet. J. 13(1): 183-190, 2023; pii:S232245682300019-13

DOI: https://dx.doi.org/10.54203/scil.2023.wvj19

ABSTRACT: The Niamey region in Niger depends on imports to meet its chicken meat needs. Although consumers appreciate local poultry products, they cannot fulfill their needs. The reluctance of modern producers to use local chickens on their farms is linked to a lack of knowledge of the production characteristics of local strains, which have been little studied. Thus, this study aimed to determine the growth profile of traditional chickens from villages in the Niamey region (Niger). In doing so, 100 local chicks whose parents were collected in the surrounding villages of the Niamey region were followed from hatching until the age of 140 days. The chickens were raised in cages with 10 per compartment of 3 m length and 1.5 m width. Food consumption was recorded daily, and weights were measured weekly. The parameters of the growth curves were obtained using the Gompertz equation. Female and male chickens had a significant weight difference at the third week of age. The mean weight of chicks at hatching was 24.90 ± 0.36 g. At the end of the follow-up, males, with a mean weight of 1523.05 ± 26.22 g were significantly heavier than females (1052.73 ± 14.04 g). Over the entire period of the experiment, the average daily gain and consumption indices were 9.5 g/d and 5.12, respectively. Asymptotic weights were 2096.78 g and 1313.26 g for males and females, respectively. The maturation factor of the Gompertz equation was higher in females (0.0196 g/d) than in males (0.0181 g/d), and the inflection age averaged 75 days for both sexes. In conclusion, Niamey local chickens are slow growing and have a high feed conversion ratio compared to the modern broiler or layer strains.



Guisso Taffa A, Issa S, Bachir H, Mahamadou Ch, Johann D, and Nassim M (2023). **Zootechnical Performance and Growth Curve Modelling of the Niamey Local Chickens in Niger**. *World Vet. J.*, 13 (1): 183-190. DOI: https://dx.doi.org/10.54203/scil.2023.wvj19

Keywords: Average daily gain, Feed conversion ratio, Growth curve, Local chicken, Weight gain

[Full text-PDF] [Crossref Metadata] [Scopus] [Export from ePrints]

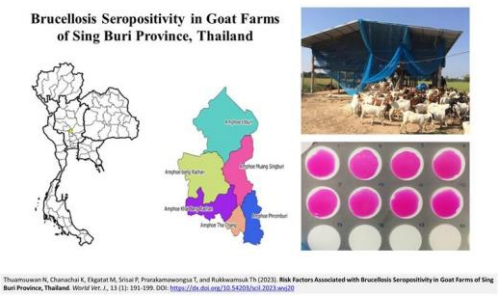
Research Paper

Risk Factors Associated with Brucellosis Seropositivity in Goat Farms of Sing Buri Province, Thailand

World Vet. J. 13(1): 191-199, 2023; pii:S232245682300020-13

DOI: https://dx.doi.org/10.54203/scil.2023.wvj20

ABSTRACT: During 2012 - 2016, goat farms in Sing Buri province were growing rapidly with support from the Thai government. In the following three years (2017-2019), the analysis of brucellosis surveillance data indicated that the seropositivity of brucellosis in goats increased. Therefore, this study attempted to identify possible risk factors associated with brucellosis seropositivity in meat goats raised in Sing Buri province of Thailand. A case-control study was conducted in a random sampling of 72 goat farms in Sing Buri province, Thailand. Questionnaires were used to collect information regarding farm production types, husbandry, goat health management, grazing management, breeding, carcass management, and goat purchasing. Bivariate and logistic regression analyses were used to determine the risk factors of *Brucella* seropositivity. Results revealed that the most frequent health complaint by the farmers was a stillbirth. *Brucella* seropositivity at the farm level was 26.4%. The two most probable risk factors for seropositivity included raising goats in a communal pasture and keeping goats with a history of clinical signs associated with brucellosis. In conclusion, approximately 25% of goat farms in Sing Buri province were infected by the bacteria genus *Brucella*. The farmers were recommended to attentively seek and cull for a brucellosis-suspected goat in their farms using clinical signs or symptoms together with active serosurveillance. Furthermore, communal pasture avoidance would also help prevent the goat from *Brucella* infection.



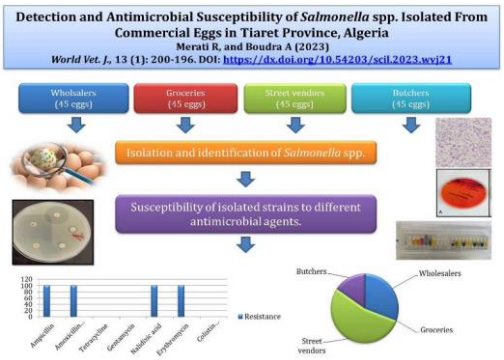
Keywords: Brucellosis, Meat goat, Risk factor

[Full text-PDF] [Crossref Metadata] [Scopus] [Export from ePrints]

Research Paper

Detection and Antimicrobial Susceptibility of *Salmonella* spp. Isolated From Commercial Eggs in Tiaret Province, Algeria

World Vet. J. 13(1): 200-204, 2023; pii:S232245682300021-13
DOI: https://dx.doi.org/10.54203/scil.2023.wvj21



ABSTRACT: Salmonellosis is a significant public health problem worldwide. The current study aimed to investigate the presence of *Salmonella* spp. in commercial eggs of Tiaret province, Algeria, and evaluate the susceptibility of isolated strains to different antimicrobial agents. A total of 180 commercial eggs collected from various retail outlets (groceries, butchers, wholesalers, street vendors) were analyzed by conventional methods, and 13 *Salmonella* spp. isolates were tested on a panel of 7 antimicrobial agents using the disc diffusion method. Of 180 chicken egg content samples examined, the findings indicated that 13 (7.22%) were positive for *Salmonella* spp. Regarding the collection site, 2 (1.11%), 4 (2.22%), and 7 (3.88%) of *Salmonella* spp. isolates were detected from butchers, wholesalers, and street vendors, respectively. Most antibiotic discs have demonstrated widespread resistance with an incidence rate of 100%, including amoxicillin + clavulanic acid, ampicillin, nalidixic acid, and erythromycin. However, colistin sulfate, gentamycin, and tetracycline were more effective against *Salmonella* isolates. It can be concluded that the highest detection rate of *Salmonella* spp. was observed for street vendors, and the highest resistance was recorded for commonly used antibiotics in poultry production.
Keywords: Antimicrobial, Chickens, Commercial eggs, *Salmonella*, Tiaret

[Full text-PDF] [Crossref Metadata] [Scopus] [Export from ePrints]

Research Paper

Effects of Phytogenic Feed Additives on Body Weight Gain and Gut Bacterial Load in Broiler Chickens

World Vet. J. 13(1): 205-213, 2023; pii:S232245682300022-13
DOI: https://dx.doi.org/10.54203/scil.2023.wvj22

ABSTRACT: Phytogenic feed additives (PFAs) have promising importance in chicken production as antibiotic alternatives to balance chicken gut microorganisms and improve productivity. The objectives of this study were to evaluate the body weight gain (BWG) and gut bacterial load of broiler chicks fed on selected herbs. For this experiment, 360 unsexed one-day-old broiler chicks of Cobb 500 with an average weight of 40.74 g were randomly allocated into six treatment groups with three replicates of 20 chicks in each pen. The treatment 1 (T1) group was fed by a basal diet alone. Chickens of T2, T3, T4, T5, and T6 were fed the basal diet containing 1% of basil, lemongrass, peppermint, rosemary, and thyme leaves powder, respectively for 49 days. Body weight (BW), BWG, and average daily weight gain (ADWG) data were recorded every week and at the end of every phase. On days 21 and 42, three chickens from each replicate were slaughtered for microbiological analysis (pathogenic and normal flora) of cecum contents aseptically. The obtained result showed that chickens kept on T3 had significantly higher BW, BWG, and ADWG during the starter and grower phases. Significantly highest final BW was recorded during the finisher phase on T3 and T6. Chickens that consumed T3 and T6 had significantly higher overall BWG and ADWG. The lowest *Escherichia coli* counts were seen in chickens fed on rosemary (T5) on both days 21 and 42 of the experimental time. Similarly, the highest *Lactobacilli* counts were recorded on chicken fed on T5 (day 21) and T3 (day 42). On the other hand, almost all treatment herbs showed a higher *Enterococcal* count, with the highest recorded for T3 (day 21) and T6 (day 42). The present findings suggest that supplementing lemongrass and thyme leaf powder improves BW performance and gut microbial composition. Likewise, rosemary leaf powder enhances the beneficial microbial composition and reduces pathogenic bacteria. However, the underlying detailed biological mechanisms and dose standardization of these herbs for inclusion in the diet of broiler chickens need to be studied further.
Keywords: Antimicrobial**,** Body weight gain, Broiler chicken, Feed additive, Gut bacteria, Phytogenic

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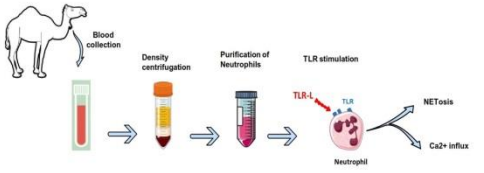
Research Paper

NETosis and Calcium influx in Dromedary Camel Neutrophils after *in vitro* Toll-like Receptor Stimulation

Albahrani Kh, Alessa J, Falemban B, Alkuwayti MA, and Hussen J.

World Vet. J. 13(1): 214-221, 2023; pii:S232245682300023-13

DOI: https://dx.doi.org/10.54203/scil.2023.wvj23



Albahrani Kh, Alessa J, Falemban B, Alkuwayti MA, and Hussen J (2023). **NETosis and Calcium influx in Dromedary Camel Neutrophils after *In Vitro* Toll-like Receptor Stimulation**. *World Vet. J.*, 13 (1): 214-221. DOI: https://dx.doi.org/10.54203/scil.2023.wvj23

ABSTRACT: Neutrophilic granulocytes are vital immune cells of the early response to pathogens. They contribute to the antimicrobial response through phagocytosis, production of reactive oxygen species, cytokine production, degranulation, and NET-formation. Neutrophil extracellular traps (NETs), also known as NETosis, are a critical antibacterial effector mechanism of cells of myeloid effector cells, including neutrophils and macrophages. Toll-like receptors (TLRs) are pattern recognition receptors (PRRs) that mediate pathogen sensing through the recognition of microbial structures known as pathogen-associated molecular patterns (PAMPs). The present study aimed to investigate the potential of several TLR ligands that mimic the sensing of bacterial and viral pathogens to stimulate NET-formation or Ca2+ influx in camel neutrophils. Neutrophils were purified from blood and were stimulated in vitro with ligands to TLR4, TLR2/1, TLR7/8, or TLR3. Net-formation was analyzed using the DNA-sensitive dye SYTOX™ Green and staining with antibodies to the neutrophil's granular enzyme myeloperoxidase. Real-time stimulation-induced Ca2+ influx was measured using the Ca2+-sensitive dye Flou-4 and flow cytometry. Only the TLR4-ligand lipopolysaccharide (LPS) could induce NET-formation in camel neutrophils, while none of the investigated TLR agonists showed a Ca2+ influx-inducing effect in camel neutrophils. The current study represents the first report on the impact of direct activation of TLR on NET-formation and Ca2+ influx in camel neutrophils with a selective effect of LPS on NET-formation induction. Future studies may investigate the molecular mechanisms behind the different responsiveness of bovine and camel neutrophils to TLR stimulation.

Keywords: Camel, Ca2+ influx, Flow cytometry, Neutrophils, NETosis, Toll-like receptor

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Research Paper

Toxic Effects of Nanographene Oxide on Testes of Rats

Abd-Alsahib EF, and Faris SA.

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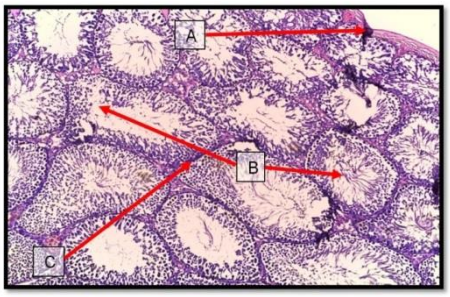

ABSTRACT: The current study aimed to examine the effects of nanographene oxide on the testes. A total of 48 male albino rats were randomly divided into 6 groups. The first, second, third, fourth, and sixth groups were treated with graphene oxide nanopowder at 20, 30, 40, 50, and 60 mg/kg concentrations, respectively. The sixth group was considered the control group. The results indicated a significant decrease in the average testis weight of rats treated with different nanographene oxide dosages, compared to the control group. There was also a significant decrease in the level of FSH and testosterone of treated rats with nanographene oxide, while there was no significant difference in the level of LH hormone when compared to the control group. The histological examination of the testes in the treated rats indicated hemorrhage, decreased sperm count, decreased thickness of the tubular epithelium, dissociation of connective tissue between the seminiferous tubules, in addition to hematological congestion, necrosis of the tubular epithelium, divergence of the seminal tubules, absence of sperm, shattering of the seminal tubule wall and degeneration sperm-forming cells and edema formation. Using the transmission electron microscope, the findings revealed a range of cellular changes, such as the presence of two-headed spermatids, the destruction of the nucleus membrane, spermatoblasts, the destruction of the cell membrane, and the denting of the nucleus membrane. It can be concluded that the nanographene oxide at 20-60 mg/kg concentrations can have harmful effects on spermatogenesis and normal function testis in rats.

Keywords: Laboratory rat, Nanographene oxide, Testes, Toxic effect

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Research Paper

Administration of *Strobilanthes crispus* in an Angora Cat with Feline Lower Urinary Tract Disease

Permadi IGWDS, Martarika R, Lienggonegoro LA, and Novita R.

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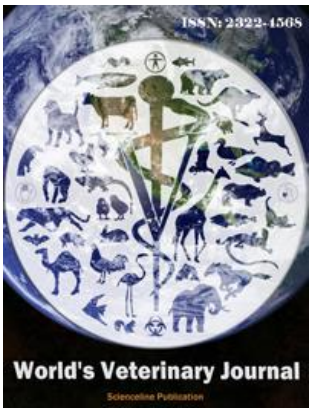
ABSTRACT: The occurrence of feline lower urinary tract disease (FLUD) in Indonesia has not been widely reported. However, the incidence of the disease has increased due to dietary cat patterns. The diet habitually consists of commercial dry food only, without wet food, such as meat. FLUD often affects certain breeds of cats. Surgical therapy is the first option to remove kidney stones; however, rural areas in Indonesia often lack animal surgical facilities. This condition requires alternative therapies to cure the disease. A one-year-old male Angora cat was brought to Rumah Satwa veterinary clinic in Tanah Datar, Indonesia, for examination, with a history of urination difficulties or dysuria, pain in the abdomen when being handled, and lack of desire to mate. A macroscopic examination of urine showed a cloudy and dense appearance. The ultrasound examination revealed a stone (struvite) and a thickening of the urinary bladder wall. The angora cat was diagnosed with obstructive FLUD caused by urolithiasis. A capsule containing 125 mg Keji Beling (*Strobilanthes crispus*, Bl) extract was administered to the cat once daily to aid the struvite stone dissolution. Keji Beling is a herbal plant easily found in Indonesia and used to treat human kidney stones. After 32 days of therapy, the clinical condition of the cat improved. The ultrasound examination did not find any stones left in the bladder. In conclusion, based on local wisdom, Keji Beling leaves can potentially be an alternative therapy for FLUD in Angora cats with certain conditions.

Keywords: Angora cats, Keji Beling, Urinary bladder, Urolithiasis



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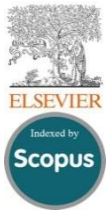

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The Role of Head Associated Lymphoid Tissues in Infectious Bronchitis Virus

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ABSTRACT

Infectious bronchitis virus (IBV) replicates primarily in the epithelial tissues of the respiratory tract, particularly the trachea. However, little is known about IBV replication and immune responses in relation to head-associated lymphoid tissue (HALT), such as the Harderian gland (HG) and choanal cleft, as well as respiratory (turbinate) tissues. Furthermore, few studies have looked into the role of the HG, choanal cleft, pharyngeal tissues, and turbinate in innate, cellular, and mucosal immune responses of commercial broiler chickens and laying hens infected with IBV, compared to the studies about the effects on the trachea. This review article overviewed the role of avian HALT, conjunctival-associated lymphoid tissue (CALT), concerning the anatomical, physiological, and immune responses to infectious bronchitis disease in chickens. The HG, choanal cleft, and turbinate in innate, mucosal, and cellular immune responses play a significant role in avian protection through virulent or attenuated vaccines of IBVs. The IBV viruses could not reach the trachea in chickens vaccinated with IBV vaccine due to the inhibition of viruses by HALT and respiratory tissues by innate, mucosal, and cellular immunity. It can be concluded that other than the trachea, the HALT and respiratory tissues play an important role in the infectivity and immune induction against IBVs due to their proximity to the upper air passages.

Keywords: Avian immunity, Chicken, Harderian gland, Infectious bronchitis, Turbinate

INTRODUCTION

Infectious bronchitis (IB) is a highly contagious and acute disease that can affect broiler, breeder, and layer chickens, resulting in economic losses for the poultry industry (Cavanagh, 2005). The IB is a respiratory infection, prevalent in chickens aged 2-3 weeks, with a high mortality rate and congested respiratory pathways at post-mortem (Schalk and Hawin, 1931). Later, a virus named infectious bronchitis virus (IBV) was identified as the causative agent of this disease (Jackwood 2012). Since the first report, many serotypes and variants have been isolated and characterized (Jackwood, 2012; Jackwood and de Wit, 2013). Many countries have reported multiple variant IBV strains circulating in their poultry farms so far (Sjaak de Wit et al., 2011; Awad et al., 2014; Alsultan et al., 2019; Sabra et al., 2020; Yehia et al., 2020; Jasim et al., 2022).

The Harderian gland (HG) produces all of the local immunoglobulins (IgM, IgA, and IgY) in the lachrymal fluids, which provide local protection in the upper respiratory tract via these immunoglobulins (Baba et al., 1988). It has also recently been reported that using both the Ma5 and 4/91 vaccine strains at the same time results in high levels of immunoglobulins (IgA and IgY) in the upper respiratory tract (URT) and high levels of CD8+ T cells by HG (Smialek et al., 2016).

Following IBV infection, immunoglobulins, such as IgM, IgA, and IgY will increase the tracheal epithelium (Nakamura et al., 1991). The IBV-specific IgA antibodies have been found in the lamina propria, tracheal washes, and epithelial cells of IBV-infected chickens' trachea (Joiner et al., 2007). IgA and IgY antibodies specific to IBV have also been found in lachrymal fluid (Al-Rasheed et al., 2021; Al-Rasheed et al., 2022). It has also been reported that IBV-specific IgA in tears correlates with resistance to IBV reinfection. IBV-specific IgA was first detected in the lachrymal fluid 10 days after vaccination with an attenuated Ark DPI-type IBV live vaccine (Gelb et al., 1998).

Recently, the role of HG, choanal cleft, and turbinate in terms of IBV M41 has been investigated in comparison with trachea. The viral load, pro-inflammatory cytokines (IL-6), and host gene mRNA expression, including Toll-like receptor 3 (TLR3), Melanoma differentiation-associated protein 5 (MDA5), IFN-α and IFN-β in the HALT and respiratory tissues were examined in 21-day-old chickens. After the virulent IBV M41 challenge, the viral RNA expression detected either by quantitative RT-PCR or immunohistochemistry peaked at 2-3 days post-challenge (dpc) in all tissues. Significant increases of lachrymal fluid anti-IBV -specific IgA and IgY levels were found at 4-5 dpc. The

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results demonstrate innate, cellular, and mucosal immune responses at 1-3 days after M41 challenge in the HALT and respiratory tissues (Al-Rasheed et al., 2022).

The host's innate, humoral, mucosal, and cellular immune responses in the HALT and respiratory tissues were also recently evaluated in 41-week-old egg-laying hens following administration of Mass (Mass) or 793B live vaccines, either by oculonasal or drinking water methods (Al-Rasheed et al., 2021).

Infectious bronchitis virus

Infectious bronchitis virus structure

Infectious bronchitis virus (IBV) is the causative agent of IB. It belongs to the genus *Gamma corona virus*, the family Corona viridae, and the order Nidovirales (Cavanagh, 2007). The IBV is a single-stranded (ss) RNA virus with a positive sense, a 120-160 nm diameter, and large surface spikes of 20 nm. The virus has a round to pleomorphic shape with heavily glycosylated spike projections. The virus genome is approximately 27.6 kilobases long. It contains structural proteins, spike (S1 and S2), envelope (E), membrane (M), nucleocapsid (N), and non-structural proteins (Nsps) that are important for virus replication or proliferation. The S1 and S2, cleaved forms of the S protein found on the surface of the viral envelope (virion), have molecular weights of 92 kDa and 84 kDa, respectively. The S1 subunit contains epitopes that induce neutralizing antibodies against IBV, whereas the S2 subunit controls virus fusion to host cells (Ignjatovic and Sapats, 2005; Belouzard et al., 2012). The replicase gene (gene 1) comprises two open reading frames, 1a and 1b, located near the 5' untranslated region (UTR) and the leader sequence. These genes encode proteins that participate in RNA replication and transcription. The *S*, *E*, *M*, and *N* genes are located near the 3' UTR and encode proteins found in virus particles. These structural protein genes are interspersed with Nsps, 3b, 5a, and 5b accessory genes that are not required for replication (Armesto et al., 2012).

Replication of infectious bronchitis virus

The virus replicates in the cytoplasm. The IBV replication begins with S1 binding to cell membrane receptors, specifically those of -2,3-sialic acid (Belouzard et al., 2012; Shahwan et al., 2013). Following that, during biosynthesis, host-cell-dependent proteolytic cleavage of the viral S protein is observed, as is viral envelope fusion with the plasma membrane. The virus enters the cell via fusion with the host plasma membrane or receptor-mediated endocytosis (Brian and Baric, 2005). Virus (+) ssRNA is used as a host polymerase template to synthesize viral RNA polymerase directly. The IBV replication begins with S1 binding to cell membrane receptors, specifically those of α2,3-sialic acid (Belouzard et al., 2012; Shahwan et al., 2013). Subsequently, host-cell-dependent proteolytic cleavage of the viral S protein during biosynthesis is observed as a viral envelope fusion with the plasma membrane. The virus enters the cell via fusion with the host plasma membrane or receptor-mediated endocytosis (Li and Cavanagh, 1992). The first step in virus assembly is the binding of N protein to viral RNA, resulting in the formation of the helical nucleocapsid (Weber and Schmidt, 2005), followed by the integration of the M and E proteins into the membrane of the host cell's endoplasmic reticulum (ER; Vennema et al., 1996). The S protein interacts with the M protein at the pre-Golgi complex, forming an S-M complex (Nguyen and Hogue, 1997).

Strains (variant) distributions

The IBVs have poor serotype cross-protection, highlighting the importance of ongoing identification and surveillance (Jackwood, 2012). The IBV exists in various antigenic or genotypic types, referred to as variants (Sjaak de Wit et al., 2011; Cook et al., 2012). It can produce new variant strains through mutation or gene recombination in the *S1* gene, and can occur due to the introduction of a current strain from another region (Jones, 2010). The mechanism underlying the emergence of new virus types and variants is mainly unknown (Jackwood, 2012). Despite being discovered in the United States, the classical M41 serotype and the Dutch H120 serotype, derived from a 1955 Dutch isolate, are the most widely used vaccine viruses (Sjaak de Wit et al., 2011). Variants have been discovered all over the world (Table 1). Arkansas is the most common type of IBV found in the United States. Connecticut and Massachusetts (Mass) viruses are also frequently detected in the United States (Jackwood et al., 2005; Jackwood et al., 2010; Jackwood, 2012).

Strain classification

Serotypes

The IBV strains are classified into different serotypes based on the antigenicity of the S protein using VN and HI testing (de Wit, 2000). Some laboratories also use enzyme-linked immunosorbent assays (ELISA) with monoclonal antibodies (mAbs) directed against specific epitopes of the S1 protein to distinguish different strains of the virus; however, cross-reactions between serotypes can occur, especially when serum is collected from field samples (Jackwood and de Wit, 2013). The ELISA method has some disadvantages, such as the limited availability of mAbs and the need to develop new mAbs for each new variant (Karaca et al., 1992). Furthermore, due to the limited availability of an increasing number of reference sera associated with various serotypes, VN and HI are not commonly used for serotyping studies.

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Genotype

In recent years, traditional serotyping methods used to demonstrate field strain identity have been replaced by DNA sequencing and genotyping based on the S1 region of the spike gene (Jackwood et al., 1992; Cavanagh et al., 1999; Lee et al., 2000; Jackwood and de Wit 2013). Strains are classified using this method based on the genetic characterization of the S1 subunit, particularly the S1 hypervariable region (Cavanagh 2005).

Protectotype

The most important system from a practical standpoint is protectotype or immunotype classification, playing an important role in the efficacy of vaccine programs in the field. Strains that induce cross-protection against each other, such as M41 (de Wit, 2000) and QX-like, belong to the same protectotype (Bru et al., 2017). However, vaccine strains that are not serologically linked (belong to a different serotype) may still provide cross-protection. For instance, the live H120 vaccine was shown to induce protection against an Australian T strain challenge or variant isolates in commercial farms (Darbyshire, 1985; Awad et al., 2015). A cross-immunization challenge study is required to determine a strain's protectotype. However, this type of research is time-consuming, costly, and necessitates a large number of birds and isolation facilities (de Wit, 2000; Sjaak de Wit et al., 2011). Alternatively, tracheal organ cultures or oviduct organ cultures from vaccinated hens were used in a cross-immunity test; these cultures were challenged using in vitro heterologous or homologous inoculation strains to assess cross-immunity (Raj and Jones, 1996).

Table 1. Infectious bronchitis virus strains types reported worldwide in broiler chickens

Country	Strains/Types	Reference
United State	Beaudette M41 Arkansas Connecticut Mass types California variant Delaware GA08 GA98	(Beaudette and Hudson, 1937) (Bracewell, 1975) (Jackwood, 2012; Jackwood et al., 2010; Jackwood et al., 2005)
Brazil	BR I BR II Mass 793B	(Villarreal et al., 2010)
Mexico	Connecticut Maxx Arkansas	(Jackwood, 2012)
United Kingdome	793B (CR88, 4/91)	(Parsons et al., 1992; Adzhar et al., 1997; Worthington et al., 2008).
Italy	Italy O2 D274 D1466 793B	(Worthington et al., 2008; Sjaak de Wit et al., 2011)
Australia	"T'' strain 793B	(Cumming 1963)
China	QX Q1	(Yudong et al., 1998) (Yu et al., 2001)
Moroccan	G strain 4/91	(El-Houadfi et al., 1986) (Jones et al., 2004)
Libya	IS/885/00 IS/1494/06 (Variant 2)	(Awad et al., 2014a)
Iran, Iraq, and Saudi Arabia (Middle East)	Variant 2 Mass 793B QX Q1	(Alsultan et al. 2019; Amin et al. 2012; Boroomand et al. 2011; Ganapathy et al., 2015)

Anatomy, physiology, and immune responses of head-associated lymphoid and respiratory tissues

The respiratory and lymphoid tissues in the head are the most likely lymphoid tissues in poultry to be initially exposed to vaccines or pathogenic respiratory agents. Since most respiratory vaccines are delivered via spray or eye/nose drops, understanding the immune responses in the head-associated lymphoid tissue (HALT) and upper respiratory tissue in domestic poultry is important. The IBV targets the mucosal surface and periocular lymphoid tissue (van Ginkel et al., 2008). The HG, conjunctiva-associated lymphoid tissue, and lymphoid follicles distributed throughout the mucosal

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surfaces are the primary inductive sites of mucosal immunity in the HALT (Maslak and Reynolds, 1995). There has been little research on the role of HALT and respiratory tissues, including the turbinate, in immune responses against vaccine or virulent strains of IBV in chickens (Al-Rasheed et al., 2021).

Harderian gland

The HG is a major periocular gland of domestic birds that is an immune-endocrine organ located in orbit behind the eye. It is located in the ventral orbit, posteromedial to the eyeball (Wight et al., 1971; Mobini, 2012; Kaiser and Balic, 2015). Since it is loosely connected to the periorbital fascia, it may remain in orbit after eye removal (Dyce et al., 2010; Olah et al., 2014, Figure 1). The HG was first introduced in 1964 (Dyce et al., 2010). Many anatomical studies on these glands, known as HGs, have been published (Davelaar and Kouwenhoven, 1976; Olcese and Wesche, 1989; Scott et al., 1993; Spalevic et al., 2012). The HG is the main orbit gland in birds (Walls 1942) and plays an important role in local eye and upper respiratory immunity, as well as lubricating and cleaning the nictitating membrane via an excretory duct (Burns 1992; Kaiser and Balic, 2015).

The gland is structurally divided into head and body sections based on differences in the surface epithelium and underlying lymphoid organization (Olah et al., 1996). The HG head resembles a typical secondary lymphoid organ, with B cell-dependent germinal centers, the follicle-associated epithelium, and T cell-dependent interfollicular regions containing isolated T cells and macrophages. Several B-lymphocytes and plasma cells are found in the HG body. The B and T lymphocytes are classified according to their developmental stage (Davison et al., 2008). Since HG contains a large population of plasma cells, it significantly affects the immunity of the eye and upper respiratory tract. Furthermore, it is thought to be a significant contributor to antibody production and protect the oculonasal mucosa from airborne viruses, such as IBV, infectious laryngotracheitis, and avian metapneumovirus (Bang and Bang, 1968; Toro et al., 1996; van Ginkel et al., 2008; Spalevic et al., 2012). Bang and Bang (1968) reported chickens raised in a germ-free environment developed lymphocytic infiltrates in the HG, showing that lymphoid tissue within the HG can be induced without microbial stimulation although the possibility of inflammatory agents in the environment cannot be completely ruled out. Van Ginkel et al. (2009) used HG mRNA analysis to confirm the expression of the polymeric immunoglobulin receptor. These findings highlighted the importance of the HG in producing mucosal and systemic immunity against a pathogen, such as avian influenza in chickens following the ocular administration of adenovirus vaccine (Ad5-H5).

From day 5 of age, the HG in young chicks contains single HIS-C7-positive leukocytes and small groups of positive leukocytes in the connective tissue glandular lobes. In addition, B cells, macrophages, and heterophils are present (Mobini, 2012). The number of plasma cells dramatically rises with age, and these leukocytes are located close to tubular ducts and inter-alveolar connective tissue (Savage et al., 1992). Furthermore, the HG is distinguished by many plasma cells capable of proliferating in the area (Scott et al., 1993). Olah et al. (1996) found many IgM and IgA-producing plasma cells in the HG, but only a few IgY plasma cells. In contrast, Jeurissen et al. (1989) found IgY+ plasma cells with IgY in the overlaying epithelium, but only in birds older than 6 weeks old. In 10-week-old chickens, Jalkanen et al., (1984) discovered more cytoplasmic plasma cells c-IgY+ than c-IgM+ and only a few c-IgA+ cells. Lymphocytes from the bursa Fabricius are seeded into the HG prior to hatching and do not appear to be involved in systemic immunity (Baba et al., 1988). Van Ginkel et al. (2008) discovered that 70-90% of HG lymphocytes come from the Fabricius bursa and 10% from the thymus. They also found that HG has many B cells with surface immunoglobulins. Although HG is thought to be the primary source of IgA in lachrymal fluid and not derived from serum IgA migration, IgY+ plasma cells have been observed in the lachrymal fluid contribution. IgA was found in the lachrymal fluid of healthy 2-week-old chicks, and levels increased with age, reaching 0.2mg/ml at 15 weeks. IgY is initially derived from maternal antibodies, decreasing over the first 3 weeks of life before gradually increasing to approximately 2-3 mg/ml at the age of 15 weeks (van Ginkel et al., 2008).

It is still unclear how environmental antigens are absorbed, processed, and presented in the lachrymal fluid, resulting in humoral antibodies. According to Survashe et al. (1979), the HG immune response starts in the lymphoid tissue near the gland duct's access point to the nictitating membrane. According to some researchers, at least in the case of the turkey, antigen uptake takes place in the lower eyelid and is processed in the lymphoid tissue connected to the conjunctiva, producing plasma cells in the HG (Fix and Arp 1989, 1991).

Using the neutralization test, the immunization of 1-day-old chicks with high neutralization index values using the H120 vaccine virus through conjunctival and intranasal routes could result in immunity after 4 weeks. This result is as significant as the induced immunity four weeks later, comparable to that attained by immunizing 20 and 15-day-old chicks with lower levels of maternal antibodies. Successful vaccination is associated with significant stimulation of the HG in the 15-day-old age groups and a rise in lymphocytes and plasma cells, implying that the HG is important in the immune response against IBV (Al-Rasheed et al., 2021).

Choanal cleft

A literature search has revealed that few publications on chicken currently focus on choanal cleft. The shape of the avian choanal cleft varies by species. The cleft in fowl and pigeons is very long, whereas it is very short in ducks and geese (Nickel et al., 1977). There are six transverse rows of caudally directed filiform papillae on either side of the choanal cleft, as in many avian species, papillae are behind the median palate ridge (Figure 2). The palates of fowl and pigeons have caudally pointing papillae arranged in several transverse rows. In contrast, the palate of the goose has a median row of papillae and 2-3 rows of blunt papillae, which are confined to the optical region (Dyce et al., 2010). Recently, some studies have focused on immune responses against IBV in this tissue, the role of HG and choanal cleft in terms of IBV M41 viral

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load were compared to those of trachea, and immune (innate, cellular and mucosal) responses were studied in 21-day-old commercial broiler chickens. Findings from these studies showed active IBV replication in the HALT (HG and choanal cleft) and turbinate tissues, and the limited subset of immunity-related genes provided further understanding of the immunobiology of IBV in naïve 21-day-old commercial broiler chickens. Such effects were dependent on the tissue type, with significant changes in TLR3, MDA5, IFN-α, and IL-6 mRNA expression in the turbinate and trachea being most notable. Meaningfully, the data highlighted the significant presence of both IgA and IgY in lachrymal fluid following the IBV M41 challenge, suggesting that early detection of both IBV-specific IgA and IgY in lachrymal fluid can be used as important indicators of mucosal immune responses of IBV M41 infection in commercial broiler chickens (Al-Rasheed, 2020; Al-Rasheed et al., 2022).

Turbinate

Conchae or turbinate projections are covered projections within the nasal cavity of extant reptiles, mammals, and birds (Geist, 2000). Birds and mammals typically have an additional nasal cavity elaboration (Hillenius 1992; Witmer, 1995). In contrast to the straightforward conchae structure of reptiles, the avian respiratory turbinate is a highly convoluted, frequently scrolled structure lined with moist mucociliary epithelium (Dyce et al., 2010). The spiral structure of the turbinate increases the surface area of the nasal mucosa, which may prevent dust and foreign matter from entering (Kang et al., 2013). The anterior and middle conchae of the respiratory turbinate of birds are similar to mammalian maxilloturbinates. They are located directly in the path of the nasal epithelial mucosa and lessen the efficiency of respiratory air from the mucosal surface (Geist, 2000). In contrast to the bony respiratory turbinate found in mammals, the avian turbinate is usually cartilaginous. The turbinates are paired and serve as intermittent counter current heat exchangers in the nasal cavity (Schmidt-Nielsen et al., 1969, Figure 3). The innate, cellular, and mucosal responses of the immune system were examined in 21-day-old commercial broiler chickens to see how the turbinate differs from the trachea in terms of IBV M41 virus load. The recent work has demonstrated increased viral loads in the turbinate, HG, and choanal cleft tissues in 21-day-old chickens at 2-3 dpc, which indicated localised infection and persistence of IBV at these tissues compared to tracheal tissues. The early innate, cellular, mucosal and humoral immune responses were also tested on a daily basis in M41-challenged chicks. Gene transcription showed a significant up-regulation of TLR3, MDA5, IL-6, IFN-α and IFN-β, where patterns and magnitude fold-change of mRNA transcription were dependent on the gene and tissue type (Al-Rasheed et al., 2022). Viral load and immune responses in the HG, turbinate and choanal cleft showed that tissues other than the trachea should be considered in IBV immunopathogenesis studies.

Trachea

The presence of lymphoid tissue in the avian trachea has not been described yet. Nonetheless, the tracheal mucosa responds vigorously to infection in Mycoplasma gallisepticum infection models, as evidenced by extensive lymphocyte infiltration following lymphoproliferation (Gaunson et al., 2000, 2006). In contrast to CD4+ cells, dispersed throughout the tracheal mucosa, CD8+ cells are found in groups or structures resembling lymphoid follicles. B cells actively proliferating are a characteristic of mycoplasma-induced tracheal lesions (Gaunson et al., 2006). Similar responses were seen in the tracheal mucosa following IBV infection. The IBV-induced lesions are associated with high heterophil and lymphocyte infiltration in the tracheal lamina propria. The formation of many lymphoid follicles and the infiltration of plasma cells heals tracheal lesions 2 weeks after IBV infection (Kotani et al., 2000a; Kotani et al., 2000b).

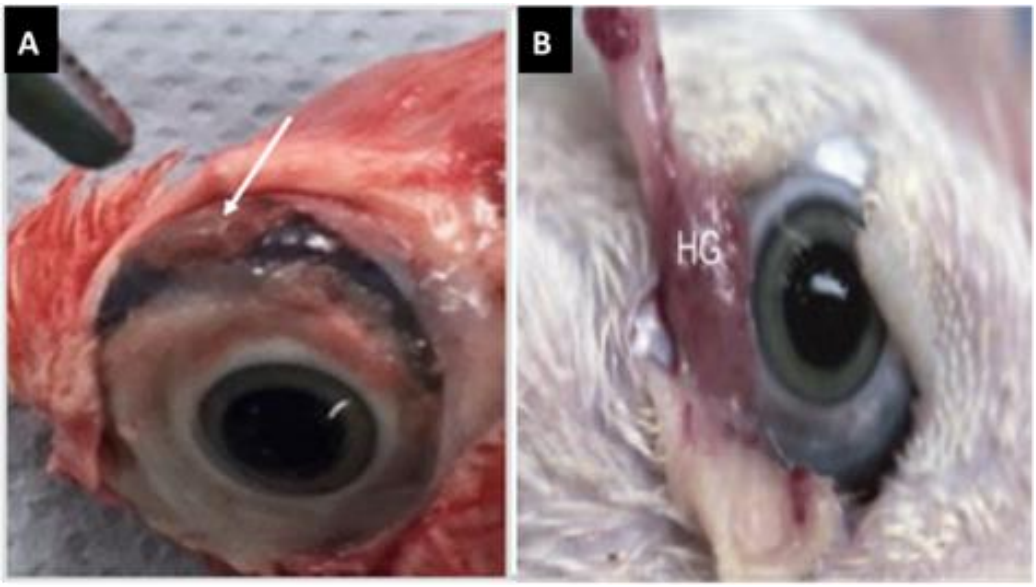

Figure 1. Anatomy of the Harderian gland (HG) in the chicken. The dorsoventral view of the chicken's skull at necropsy with removed skin and the head's rostral aspect to the right. (A) White arrow indicates the left side of HG. By pulling the nictitating membrane with forceps, the attached HG can be withdrawn from the medial surface of the orbit (B; Olah et al., 2014)

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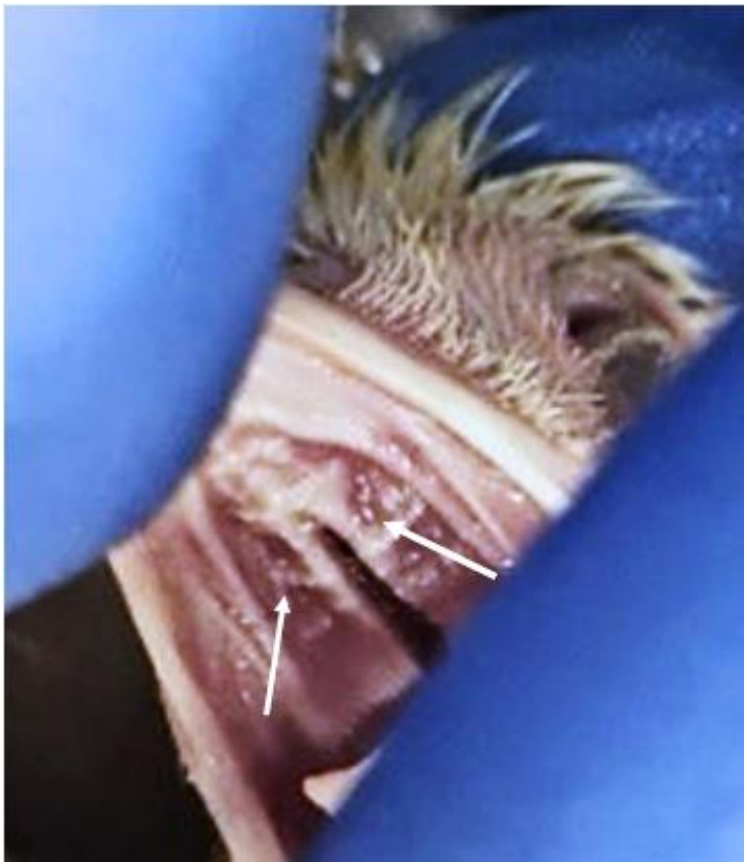

Figure 2. Interior of the upper mandible showing choanal cleft and choanal papillae inside the mouth of a healthy chicken (Al-Rasheed, 2020).

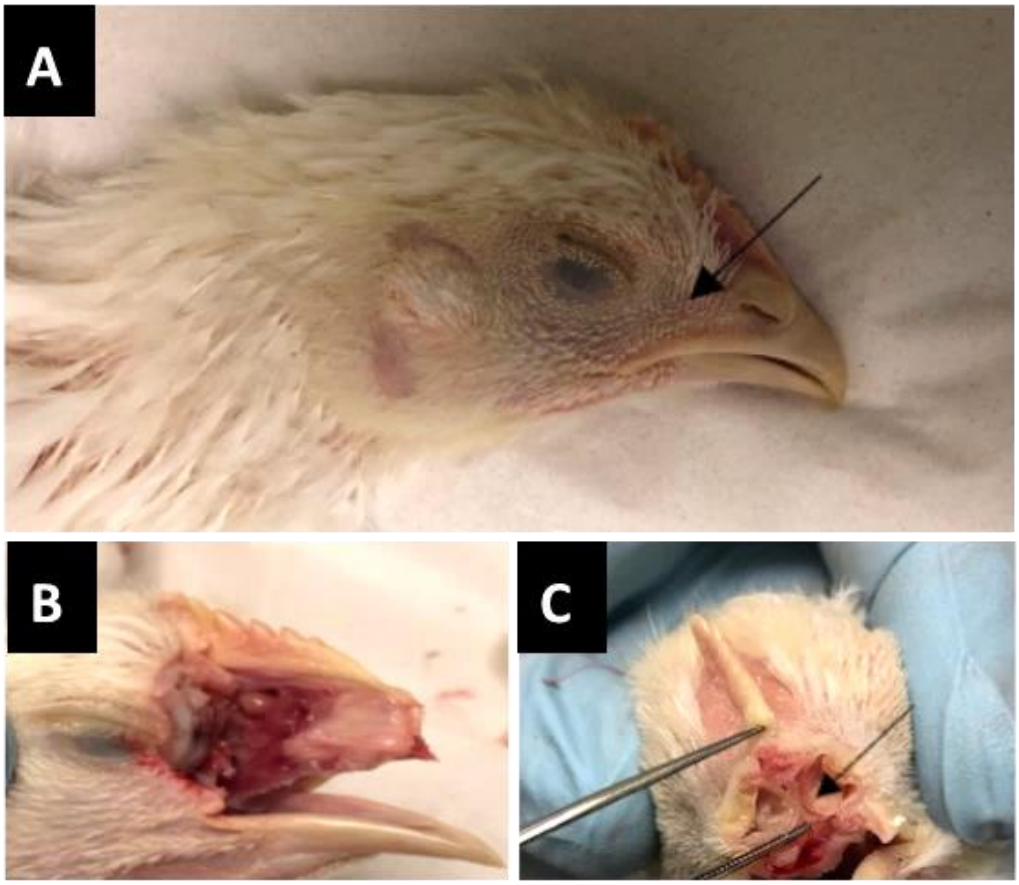

Figure 3. Lateral view of a chicken's head. Right lateral of the head showing the nasal cavity and nostril (A, Arrow). Nasal cavity through a longitudinal sectional view of a chicken's skull (B). Transverse view of the cross-sections from a chicken's nasal cavity showing the spiral structure of a chicken's turbinate, and a pair of turbinate's was located on the wall of the nasal cavity, which is termed concha nasalis media (C, Middle turbinate, arrow; Al-Rasheed, 2020).

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Pathogenesis of infectious bronchitis virus

Infectious bronchitis virus is primarily epitheliotropic, causing lesions in the kidneys, reproductive organs (testes, oviduct), lungs, and respiratory tract (nasal turbinate, trachea, HG) after replicating. The virus can also reproduce in different gastrointestinal tract cells, frequently resulting in mild lesions (Raj and Jones, 1997). It can alter the small intestine macroscopically and microscopically, which could be related to mutations that alter the S1 structure (Hauck et al., 2016). Although all IBV strains infect the respiratory system of avians, it later spreads to target tissues for replication and persistence within 18-36 hours (Jackwood and de Wit, 2013).

Harderian gland

Following experimental infection with live attenuated H120, IBV was isolated from the HG (Toro et al., 1996). This study has concentrated on the role of this lymphoid tissue in mucosal immunity. Recently, researchers investigated the role of HG, choanal cleft, and turbinate in terms of IBV M41 viral load in 21-day-old commercial broiler chickens. The antigen concentration peaked at 2-3 dpc in all head-associated lymphoid and respiratory tissues. At 4-5 dpc, there was a significant increase in lachrymal IBV-specific IgA and IgY levels, and gene transcription showed a significant up-regulation of TLR3, MDA5, IL-6, IFN-α, and IFN-β, where patterns and magnitude fold-change of mRNA transcription, demonstrating active IBV M41 replication in the HG, CC, and turbinate, compared to levels of replication found in the trachea (Al-Rasheed et al., 2021; Al-Rasheed et al., 2022).

Turbinate

In a study, nasal turbinate organ cultures inoculated with six different IBV strains (H52, H120, M41, Connecticut, Australian T strain, and British field strain HV-10) yielded maximum viral titers 48-72 hours after infection (Darbyshire et al., 1978). Following vaccination with live IBV H120, the antigen was detected in the turbinate organ culture (Darbyshire et al., 1976). Dolz et al. (2012) used in-situ hybridization to isolate and identify the presence of viral RNA of the IBV Italy 02 serotype in the nasal turbinate prior to detection in the trachea. Recently, IBV-attenuated vaccines were found in Mass and 793B-vaccinated hens after M41 challenge. In airborne infectious, the turbinate is likely the first defensive barrier (Al-Rasheed et al., 2021; Al-Rasheed et al., 2022).

Trachea

The tracheal epithelium was examined for lectin reactivity, and it was discovered that the susceptible cells have high levels of 2,3-linked sialic acid expression. According to these findings, 2,3-linked sialic acid is essential in IBV infection on the respiratory epithelium (Winter et al., 2008). The IBV replication has previously been observed in tracheal ciliated and mucus-secreting epithelial cells (Yagyu and Ohta, 1990; Nakamura et al., 1991; Owen et al., 1991; Benyeda et al., 2010), and IBV is frequently isolated from the trachea (Otsuki et al., 1990; Janse et al., 1994; Lee et al., 2000). The surveillance of the virus in the trachea depends on the virus strain. By contrast, 793B was isolated from the trachea of infected specific pathogen-free (SPF) chicks up to 7 days post-infection. In contrast, infection by strain G results in the highest viral titers at 3 days post-infection, with viral isolation observed up to 14 days post-infection (Ambali and Jones, 1990; Raj and Jones, 1996). Tracheal dysfunction has been linked to IBV strain virulence that could measure by ciliary activity (Otsuki et al., 1990; Dhinakar Raj and Jones, 1997). According to the cilia-stopping test, the QX-like and 793B strains were the least virulent, while the M41 infection was more severe (Benyeda et al., 2009).

CONCLUSION

There is a dearth of information about the role of head-associated lymphoid tissues (HALT) in chickens. The research presented in this review has provided evidence that other than the trachea, the HALT and respiratory tissues play an important role in the infectivity and immune induction against the IBVs due to their proximity to the upper air passages. Based on IBV qRT-PCR and immunohistochemistry, it is demonstrated that IBV actively replicates in HALT (HG and choanal cleft) and turbinate tissues other than the trachea, leading to infection and immunobiology. An essential finding was the role of anti-IBV IgA and IgY in the lachrymal fluid as a quantitative IBV vaccine efficacy biomarker. The findings highlighted the role of gene signatures, type 1 interferons, and cytokines following virulent or vaccine inoculation of young and adult chickens. As a result, a series of studies must be conducted to indicate the mucosal, humoral, and cellular immunity mechanisms of the HALT in chickens given either vaccine or virulent IBV viruses.

DECLARATIONS

Authors' contribution

The idea was created by Mohammed Al Rasheed, who also carried out the laboratory work. Mohamed Al Rasheed and Mohamed Shawky, who are both authors, contributed to the writing, editing, and creation of the final draft. The final submission was approved and confirmed by all authors.

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Ethical consideration

All authors check plagiarism, consent to publish, misconduct, data fabrication and/or falsification, double publication and/or submission, and redundancy already.

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Competing interests

The authors claim to have no conflicts of interest.

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An Overview of Adenovirus Vector-based Vaccines against SARS-CoV-2

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ABSTRACT

Adenovirus vectors have been employed to develop a vaccine against severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) for curtailing the Covid-19 pandemic spreading. Many different viral vectors have been mainly targeting the SARS-CoV-2 spike (S) protein as an antigen. Spike (S) protein is comprised of S1 and S2 subunits, in which the receptor-binding domain (RBD) of S1 is responsible for recognizing and engaging with its host cellular receptor protein angiotensin-converting enzyme 2 (ACE2), S2 accounts for membrane fusion of virus and host cell. Chimpanzee adenovirus was also used as a vector vaccine for SARS-CoV-2 (ChAdSARS-CoV-2-S) by intramuscular injection, and intranasal administration has been tested. Adenovirus vector-based vaccines are the most advanced, with several vaccines receiving Emergency Use Authorization (EUA). It was shown that rhesus macaques were protected from SARS-CoV-2 challenge after a month of being vaccinated with ChAd-SARS-CoV-2-S. A single intranasal or two intramuscular ChAd-SARSCoV-2-S vaccines could induce humoral antibodies and T cell responses to protect the upper and lower respiratory tract against SARS-CoV-2. As the effectiveness was demonstrated in non-human primates, ChAd-SARS-CoV-2-Sa potential option for preventing SARS-CoV-2 infection in humans. However, detecting novel more transmissible and pathogenic SARS-CoV-2 variants added concerns about the vaccine efficacy and needs monitoring. Moreover, the cause of recently documented rare cases of vaccine indicated immune thrombotic thrombocytopenia. This review article provided details for the adenovirus vector vaccine for SARS-CoV-2 in humans and tried to provide solutions to the adenovirus vector hemagglutinin issue.

Keywords: ACE2, Adenovirus, Immune response, SARS-CoV-2, Spike protein, Vaccine**,** Viral vectors.

INTRODUCTION

Coronaviruses (family Coronaviridae) are common pathogens of humans and animals. Four coronaviruses are endemic in humans (human coronavirus NL63 (HCoV-NL63), HCoV-229E, HCoV-OC43, and HCoV-HKU1) and typically infect the upper respiratory tract, causing common-cold symptoms (Drosten et al., 2003). In the past two decades, three zoonotic coronaviruses (severe acute respiratory syndrome coronavirus (SARS-CoV), Middle East respiratory syndrome coronavirus (MERS-CoV) and SARS-CoV-2) have infected humans after spilling over from animal reservoirs. Severe acute respiratory syndrome coronavirus originated in China and caused an epidemic in 2003, whereas MERS-CoV is currently causing intermittent outbreaks in the Middle East; SARS-CoV-2 emerged in late 2019 (Peiris et al., 2003; Zaki et al., 2012; Lamers and Haagmans, 2022). They cause a global pandemic of acute respiratory disease, coronavirus disease 2019 (Covid-19), which threatens human public health and safety (Redondo et al., 2021). Approximately 636 million people have been infected with SARS-CoV-2, and about 6.5 million died due to Covid-19 by September 2022 (Lamers and Haagmans, 2022). Coronaviruses enveloped, positive sense single-stranded (+ ss) RNAbelongs to the beta coronaviruses family (Wong and Perlman, 2022). This virus encodes a set of structural proteins (membrane protein, nucleocapsid protein, envelope protein, and spike glycoprotein), non-structural proteins (of which most compose the viral replication and transcription complex), and accessory proteins (Lamers and Haagmans, 2022). The structural proteins and a lipid bilayer derived from the host form an enveloped virion (or virus particle), delivering viral genomic RNA into the cell (Hoffmann et al., 2020). The spike protein is a connection with the target cell receptors (including

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angiotensin-converting enzyme 2 (ACE2), which is involved in the entrance of the SARS-CoV-2 into the host cells (Kirchdoerfer et al., 2018). The SARS-CoV-2 spike(S) protein has been the focus of vaccine research and therapeutic antibodies since its interaction with the cell surface receptor angiotensin-converting enzyme 2 (ACE2) is so essential for coronavirus entry into human cells (Sharma et al., 2020). The Spike (S) protein of SARS-CoV-2 comprises two subunits, S1 and S2, which are subsequently processed to produce a smaller S2 protein (Hassan et al., 2021). In contrast, membrane fusion is promoted by the S2 protein, which contains the receptor-binding domain (RBD) that potently neutralizes monoclonal antibodies (Chhikara et al., 2020). To combat SARS-CoV-2, several academic virology institutes and universities are working together with major pharmaceutical industrial groups to innovate and develop vaccines targeting various platforms, such as the SARS-CoV-2 inactivated virus and subunit vaccines, as well as viral-vectored (Kyriakidiset al., 2021). Emergency use authorization vaccines (Pfizer/BioNTech BNT162b2, Moderna 1273 mRNA, Johnson and Johnson Ad26.COV2, and AstraZeneca ChAdOx1 nCoV-19) were administered by intramuscular injection, which resulted in uncertain mucosal immunity, while other vaccines were in advanced clinical trials in humans to evaluate the safety and efficacy (Prakash 2022). In non-human primates, numerous intramuscular vaccinations protected against pneumonia caused by SARS-CoV-2, but they varied in their abilities to prevent upper respiratory tract infection and transmission (Bingbing et al., 2019; Sternberg and Naujokat, 2020).

An increasing number of preclinical and clinical investigations are now using adenovirus vectors to deliver vaccine antigens, including influenza, measles, hepatitis B, rabies, anthrax, Ebola, severe acute respiratory syndrome (SARS), the human immunodeficiency virus type 1 (HIV-1), malaria, and tuberculosis (Mirzaei et al., 2020). Because they do not integrate into the cells' genome and can provide large titers of recombinant viruses and high levels of gene expression when used in both dividing and non-dividing cells (Tu et al., 2020). Additionally, it was proposed to use an adjuvant to deliver DNA encoding the SARS-CoV-2 S-protein in an oral tablet vaccine based on a non-replicative recombinant adenovirus vector vaccine (Appaiahgari and Vrati, 2015). SARS-CoV-2 vaccines based on a chimpanzee adenovirus (simian Ad-36) and chimpanzee Ad-23 have been approved for use in humans as single intranasal doses and intramuscular injections in most countries, respectively (Tu et al., 2020). The ChAd-SARS-CoV-2-S intranasal dosage produced neutralizing antibodies and T cell responses, restricting or preventing upper and lower respiratory tract infection following the SARS-CoV-2 challenge. SARS-CoV-2 infection and human transmission might be minimized by administering this single intranasal dosage vaccine to non-human primates (Lee et al., 2017; Vrba et al., 2020).

Viral vector-based vaccines for SARS-CoV-2

There are two types of viral vector-based vaccines; replicating and non-replicating. Non-replicating viral vector-based vaccines use replication-deficient viral vectors to deliver genetic material of a particular antigen to the host cell to induce immunity against the desired antigen; there are seven viral vector-based vaccines in use, two of which are Ebola vaccines and five are COVID-19 vaccines (Vanaparthy et al., 2021). Replicating vector vaccines produces new viral particles in the cells they enter, which then enter more new cells and will also make the vaccine antigen (it expresses two types of genes; early genes and late genes (Figure 1). Early genes are responsible for viral replication, while late genes are responsible for virion release (Bulcha et al., 2021). They are used as vaccine vectors against numerous infections like human immunodeficiency virus (HIV), malaria, and tumor-associated antigens (Vanaparthy et al., 2021).

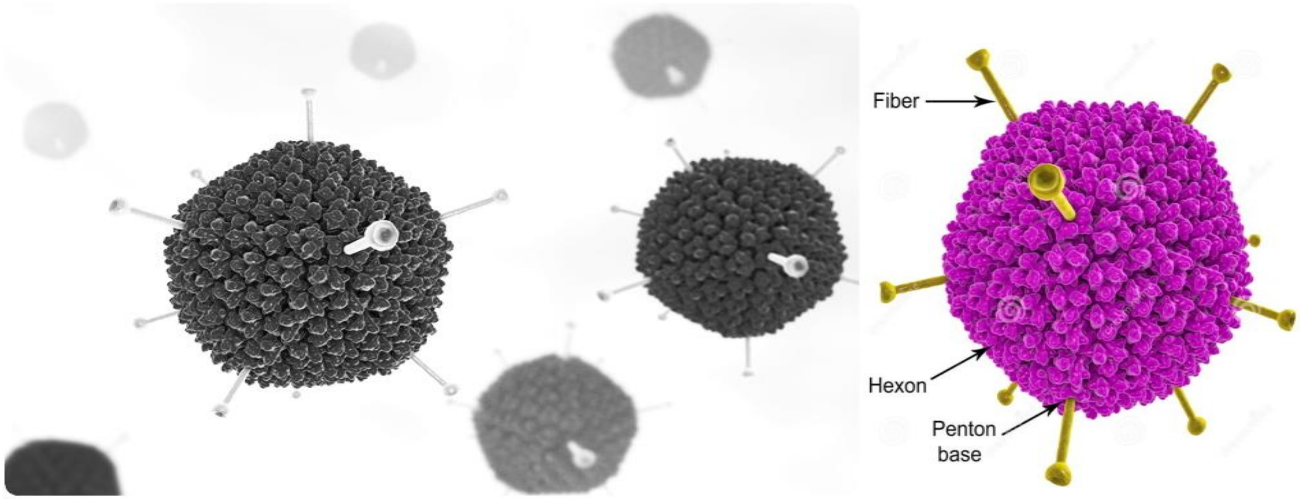

Figure 1. Molecular model of an adenovirus (Image retrieved from https://www.dreamstime.com/stock-illustration)

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Advantages and disadvantages of viral vector

Advantages of viral vectors

Viral vectors have the unique property of acting as vaccine vectors and inducing innate and adaptive immune responses in mammalian hosts (Zaiss et al., 2005). The concept of viral vector vaccines differs from that of subunit vaccines, as the latter help prevent infectious diseases by eliciting a humoral response (Sasso et al., 2020). Recombinant viral vectors are potentially therapeutic because they enable intracellular antigen expression and induce a robust cytotoxic T lymphocyte (CTL) response, eliminating virus-infected cells. Despite their efficacy, viral vectors present unavoidable problems that need to be addressed. In the near future, viral vector-based vaccines may be increasingly used to fight major diseases (Zaiss et al., 2005).

Disadvantages of viral vectors

Some viral vector integration of their genome into the host genome can lead to cancer. Another obstacle to the clinical use of viral vectors presents pre-existing immunity against the vector due to previous exposure to the virus and the production of neutralizing antibodies that reduce vaccine efficacy (Wang et al., 2019). The development of viral vectors requires a high biological safety level to gain public acceptance, non- (or low) pathogenic viruses are often selected (Verdera et al., 2020). In most cases, viruses are genetically engineered to reduce or eliminate pathogenicity. Additionally, most viral vectors are replication-defective. For example, in adenovirus-based vectors, the E1A and E1B encoding regions needed for replication in infected cells are deleted and replaced with the target gene (Custers et al., 2021). If adenovirus is used as a vaccine vector, it may be difficult or impossible to provide future booster doses because the human body develops tolerance to the vector. People with immunity to adenoviruses may find that vector distribution is ineffective in certain situations (Wang et al., 2019; Afshar et al., 2022).

Although only a few hundred cases have been reported among the more than several million vaccinated people worldwide, the problem should be solved promptly. After the first detected cases in individuals vaccinated with the ChAdOx1 nCoV-2 vaccine, persons were vaccinated with the Ad26. CoV2.S vaccine also developed vaccine-induced immune thrombotic thrombocytopenia (Chen et al., 2021). Adenovirus gene transfer was previously associated with vaccine-induced immune thrombotic thrombocytopenia and has been induced by adenovirus vaccine administration (Poland, et al., 2020). One of the common factors for all vaccines causing vaccine-induced immune thrombotic thrombocytopenia is using the SARS-CoV-2 S protein as the antigen. It was postulated that the generated soluble S protein variants are responsible for severe side effects by binding to ACE2-expressing endothelial cells in blood vessels leading to thromboembolic events (DeFrancesco, 2020; Nappi et al., 2021). The vaccine-induced immune thrombotic thrombocytopenia disease mechanism includes the interaction of the SARS-CoV-2 S protein with C-type lectin receptors, heparin sulfate proteoglycans and the CD receptor, and interaction of the adenovirus vector with the CD receptor or platelet factor antibodies (Desheva, 2018). Although some ideas and hypotheses have been presented, the reasons for causing vaccine-induced immune thrombotic thrombocytopenia are still unresolved and require further investigations (Chung et al., 2020). As initially established by Rosén in 1958, HA by human adenoviruses (A through F) exhibits various HA characteristics. Subgenus D adenoviruses may be divided into three clusters: cluster DI adenoviruses agglutinate both rat and human erythrocytes, cluster DII adenoviruses agglutinate only rat erythrocytes, and cluster DIII adenoviruses agglutinate only rat erythrocytes (Amanat et al., 2021). Erythrocyte agglutination is fiber-mediated, and particular receptors on the erythrocyte membrane appear to be involved. Intact virions can build a bridge between erythrocytes, resulting in HA, since they contain multiple fibers. Fibers alone cannot induce HA since they are just one valent. The polymers that may agglutinate erythrocytes are derived from fibers derived from tissue cultures and recombinant fibers. A study of the amino acid sequences on the fiber knob showed unique domains that may be involved in rat and human erythrocyte agglutination (Pring-Åkerblom et al., 1998). The 27 chimeric and mutant Ad9 (subgenus DI), Ad17 (subgenus DII), Ad28 (subgenus DIII), and Ad3 (subgenus B) fiber proteins produced in Escherichia coli were used to identify and describe these domains (Lee et al., 2017; Rhodes, 2021). The simian adenovirus vector ChAdOx1 was utilized in one approach to avoid any pre-existing adenovirus immunity in humans. As shown in tables 1 and 2, The ChAdOx1 nCoV-19 vaccine candidate showed protection in immunized rhesus macaques (Vrba et al., 2020; Wang et al., 2021a).

Adenovirus and adeno-associated virus vector-based vaccines

Adenovirus vectors have a long tradition as gene transfer and vaccine vectors, particularly the second and third-generation adenovirus vectors, have demonstrated high safety levels and good delivery efficacy (Dormond et al., 2009). The codon-optimized SARS-CoV-2 S protein has been utilized as the common antigen although different strategies related to vector engineering have been applied, as shown in figures 2 and 3. COVID-19 vaccines derived from viral vectors have been produced using adenoviruses (Ad5) serves as vector' for the SARS-CoV2 surface protein gene in four prospective COVID-19 vaccines that expression of the spike glycoprotein from SARS-CoV-as shown in figures 2 and 3 (Ku et al., 2021; Berndt et al., 2021). The efficacy of Ad5-nCoV was assessed in mice and ferrets, in which SARS-COV-2 replication occurs in the upper respiratory tract but not in the lungs (Wu et al., 2020). Both intranasal and

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intramuscular administration routes were tested, and IN resulted in complete protection against SARS-CoV-2 in the upper and lower respiratory tracts in mice (Table 1). However, concerns regarding issues with IN administration in people with asthma led to IM being chosen for Ad5-nCoV vaccination in the first human clinical trials (Zhu et al., 2020).

Human adenovirus serotype 26 vaccines

Janssen/Johnson and Johnson (Ad26.COV2-S)

The Ad26.COV2-S vaccine developed by Janssen Vaccines and Prevention BV (Johnson and Johnson) uses a first-generation Ad26 vector (E1/E3 deleted) to deliver the pre-fusion stabilized SARS-CoV-2 spike protein. This protein has been stabilized through a mutation in a furin cleavage site, and a proline substitution. Details of these modifications are reviewed in (Bos et al., 2020). A single-dose vector administration protected the animals against severe SARS-CoV-2 pneumonia and mortality. In a non-human primate model, the vaccine elicited strong neutralizing antibody production after a single dose intramuscular administration and conferred protection against the SARS-CoV-2 challenge. The authors noted that additional studies are needed to assess this vector's mucosal delivery and evaluate the durability of the established near-complete protection against SARS-CoV-2 infection (Berndt et al., 2021). Ad26.COV2-S was used as a single-shot vaccine in people aged 18 and ups in the United States and 40 other countries (Kurup and Schnell, 2021). However, in mid-April, US regulators temporarily paused Ad26.COV2-S vaccine administration to investigate 15 reported cases of severe thrombosis with thrombocytopenia, out of 7.98 million doses administered. Similar results have been reported in individuals receiving the ChAdOx1-nCov19 vaccine outside the US (Table 1). Following an FDA/CDC review and a risk/benefit analysis, vaccine administration was resumed - the risk of developing the rare vaccine-induced condition, termed thrombosis with thrombocytopenia syndrome (TTS, Kowarz et al., 2022).

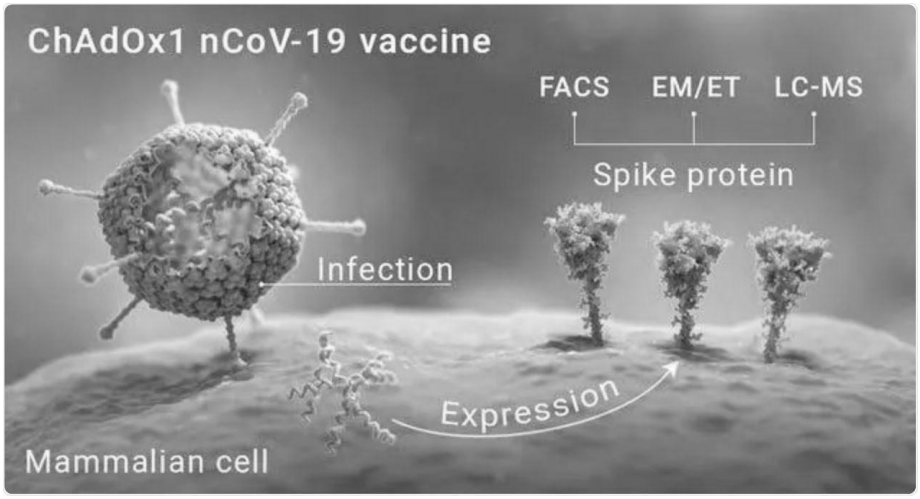

Figure 2. The protein spikes form on the surface of cells presented with the vaccine (Watanabe et al., 2021).

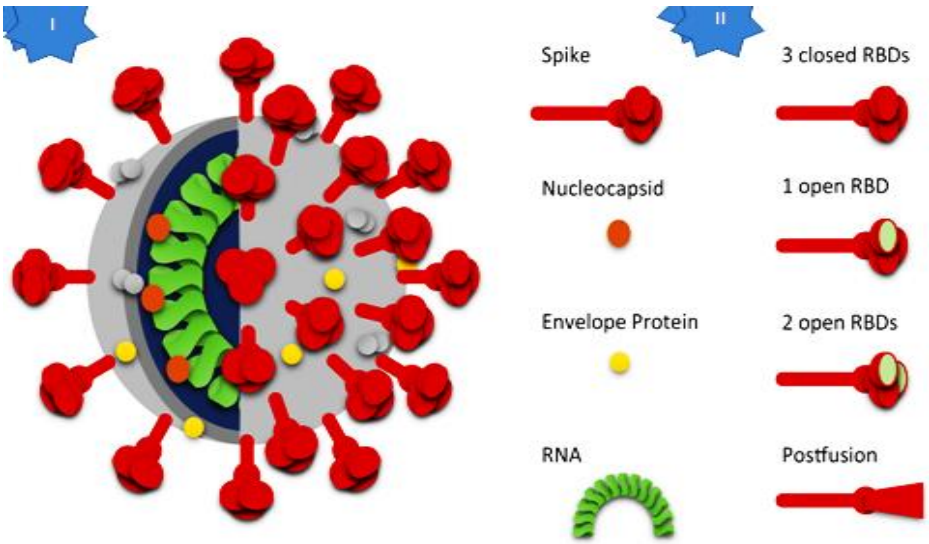

Figure 3. Coronavirus structure and relevant aspects for vaccine development. I: Current vaccines are capitalizing on epitopes in the SARS-CoV-2 proteins to elicit immune responses. The major proteins used for vaccine development are the nucleocapsid, and the spike protein, essential for cell entry. II: Spike protein can have conformation modifications protease-mediated. The stabilization of the protein in its prefusion form improves protein expression as well as immunogenicity (Mendonça et al., 2021).

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Table 1. List of SARS-CoV-2 vaccines for their characteristics, efficacy, and effectiveness against SARS-CoV-2

Type of vaccine / Manufacturer	Dose/ Injection dose interval in the phase III trial	Condition of use/storage	Composition	Reference
RNA-based BNT16b2 Pfizer/BioNtech	30 µg 5–7-dose vial 0.3 mL per dose Intramuscularly 2 doses 21 days apart	Frozen vials prior to use can be stored before dilution fom -80°C to -60°C up to the end of their expiry date or from-25°C to -15°C for up to 2 weeks	A synthetic messenger ribonucleic acid (mRNA) encoding the spike protein of SARS-CoV-2, lipids, Pbs, and sucrose	World Health Organization (2021)
mRNA-1273/ Moderna	100 µg 11 or 15-dose vial 0.5 mL per dose/ Intramuscularly 2 doses 28 days apart	Supplied as a frozen suspension stored between -50°C and-15°C Unopened vial: +2°C to +8°C for up to 30 days +8°C to +25°C for up to 24 hours After opening: +2°C to +25°C and discarded after 12 hours	A synthetic messenger ribonucleic acid (mRNA) encoding the spike protein of SARS-CoV-2. The	
RNA-based / CVnCoV	12 µg/ Intramuscularly 2 doses 28 days apart	Concentrated CVnCoV will be stored frozen at -60°C (in the clinical trial) CVnCoV must be diluted Unopened vial: 3 months at +2°C to +8°C Room temperature for 24 hours	NA	
AstraZeneca/University of Oxford / AZD ChAdOx1 nCoV-19 vaccine Non-replicating viral vector	5×10^{10} viral particles (standard dose) 8 doses or 10 doses of 0.5 mL per vial/ Intramuscularly 2 doses 4–12 weeks apart	Do not freeze Unopened vial: 6 months (+2°C to +8°C) After opening: no more than 48 hours in a refrigerator (+2°C to +8°C) Used at temperature up to +30°C for a single period of up to 6 hours	Chimpanzee Adenovirus encoding the SARS-CoV-2 spike glycoprotein (ChAdOx1-S), not less than 2.5×10^{8} infectious units (Inf.U)	
Ad26.COV2.S/ Johnson and Johnson Non-replicating viral vector	5×10^{10} viral particles 10 doses of 0.5 mL per vial/ Intramuscularly A single dose	Should be protected from light Supplied as a liquid suspension Unopened vial can be stored at +2°C to +8°C until the expiration date or at +9°C to +25°C for up to 12 hours	Replication-incompetent recombinant adenovirus type 26 vector expressing the SARS-CoV-2 spike protein in a stabilized conformation. (5×10^{10} vp)	
Gam-COVID-Vax Sputnik V/ Gamaleya Research Institute/ Non-replicating viral vector	10^{11} viral particles per dose for each recombinant adenovirus 0.5 mL/dose/ Intramuscularly 2 doses 21 days apart	Transport: two forms: lyophilized or frozen Storage: +2°C to +8°C	Two vector components, rAd26-S and rAd5-S	
NVX-CoV2373/ Novavax/ Protein-based	5 µg protein and 50 µg Matrix-M adjuvant/ Intramuscularly 2 doses 21 days apart	Liquid formulation Storage: +2°C to +8°C	SARS-CoV-2 rS with matrix-M1 adjuvant (5 µg antigen and 50 µg adjuvant)	
CoronaVac/ Sinovac Biotech/ Inactivated virus	3 µg 0.5 mL per dose/ Intramuscularly 2 doses 28 days apart	Supplied as a vial or syringe of 0.5 mL Do not freeze Protect from light Storage and transport between +2°C and +8°C Shake well before use Shelf-life: 12 months	Inactivated CN02 strain of SARS-CoV-2 created with Vero cells Aluminum hydroxide, disodium hydrogen phosphate dodecahydrate, sodium dihydrogen phosphate monohydrate, sodium chloride	
BBIBP-COrV	4 µg 0.5 mL per dose/ Intramuscularly 2 doses 21–28 days apart	Supplied as a pre-filled syringe or vial Cannot be frozen Protect from light Store and transport refrigerated (+2°C to +8°C)	Inactivated virus 19nCoV-CDC-Tan-HB02 Excipients: disodium hydrogen phosphate, sodium chloride, sodium dihydrogen phosphate, aluminum hydroxide adjuvant	
Covaxin	6 µg Single dose: 0.5 mL 10-dose or 20-dose vial/ Intramuscularly 2 doses 28 days apart	Supplied as a single dose or multidose vial Do not freeze Stored at +2°C to +8°C	6 µg whole-virion inactivated SARS-CoV-2 antigen (strain: NIV-2020-770), and other inactive ingredients such as aluminum hydroxide gel (250 µg), TLR 7/8 agonist (imidazoquinolinone) 15 µg, 2-phenoxyethanol 2.5 mg, and phosphate buffer saline® up to 0.5 m	

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Table 2. Based on observation, adverse reactions to vaccinations were observed in several cases during Coronavirus Disease 2019

Vaccine	Serious adverse events	Cases per million doses administered	Country	Age	Number of participants or doses studied	References
RNA-based BNT16b2 Pfizer/BioNtech	Anaphylaxis	4.8/million	USA	≥12 years	11.8 million doses administered (57% BNT162b2) to 6.2 million individuals	(Klein et al., 2021)
	Anaphylaxis + anaphylactoid reactions	476 cases among 40 million doses	UK	≥16 years	40 million doses (1 and 2)	
	Myocarditis Lymphadenopathy Appendicitis Herpes zoster infection	2.7/100 000 78.4/100 000 5/100 000 15.8/100 000	Palestine	≥16 years	1 736 832 participants (884 828 vaccinated)	
	Bell's palsy Myocarditis/Pericarditis Transverse myelitis	2.6/100 000 0.86/100 000 0.01/100 000	Hongkong	≥12 years	4 776 700 doses	
	Myocarditis Pericarditis	6/million 4.9/million	UK	≥16 years	40 million doses (1 and 2)	
mRNA-based / CVnCoV	Anaphylaxis	5.1/million	USA	≥12 years	11.8 million doses administered (43% mRNA-1273) to 6.2 million individuals	(Klein et al. 2021)
		2.5/million	USA	≥16 years	4 041 396 doses	(CDC, 2021)
	Myocarditis Pericarditis	20.4/million 14.8/million	UK	≥18 years	2.3 million doses (1 and 2)	
AstraZeneca/University of Oxford / AZD ChAdOx1 nCoV-19 vaccine Non-replicating viral vector	Thromboembolic events	0.61/million	India	≥18 years	Retrospective survey of 75 random subjects	
	Thrombosis with thrombocytopenia syndrome Capillary Leak Syndrome Myocarditis Pericarditis Anaphylaxis or anaphylactoid reactions	14.9/million 20.5/million 12 cases among 48,9 million doses 2.1/million 3.3/million 816 cases among 48.9 million doses	UK	≥18 years 18–49 ≥18 years	48.9 million doses (1 and 2)	
	Guillain-Barré syndrome	833 cases among 592 million doses	Worldwide	≥18 years	592 million doses	(EMA, 2021)
	Thrombosis with thrombocytopenia syndrome	1503 cases among 592 million doses	Worldwide	≥18 years	592 million doses	
Ad26.COV2.S/ Johnson and Johnson Non-replicating viral vector	Thrombosis with thrombocytopenia syndrome Guillain–Barré syndrome	45 cases for 14.3 million doses (3/million) 185 cases for 14.3 million	USA	≥18 years	14.3 million doses	
Sputnik V	Expected local and systemic reactions The most frequent symptoms were local pain, asthenia, headache, and joint pain	2.1% of participants suffered severe reactions in San Marino's population	Republic of San Marino	18–89 years	Cohort of 2558 participants	(Montalti et al., 2021)
CoronaVac	Bell's palsy Encephalopathy	3.8/100 000 0.01/100 000	Hong Kong	≥12 years	n = 2 811 500 doses	
	Anaphylaxis Thromboembolic events Bell's palsy Guillain–Barré syndrome	2/million 1.15/million 8.73/million 0.29/million	Chile	≥16 years	n = 13 862 155 doses	
BBIBP-COrV	No serious side effects were reported	—	Jordan	Mean age: 35–40 years	No date specified	
	No severe side effects were reported.	—	Iraq	≥18 years		

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Sputnik V (gam-COVID-vac)

Sputnik V is a vaccine candidate developed by the Gamaleya Research Institute in Russia. The vaccination protocol consists of a two-dose regimen utilizing two human Ads: Ad26 as prime and Ad5 as a boost. Heterologous Ad vector prime-boost immunization protocols, where a different type of virus is applied at each dose, are used to circumvent immune responses against the viral vector. To assess the efficacy of Sputnik V, two phases with three clinical trials were carried out (NCT04530396, NCT04564716). In an interim report of the NCT04530396 trial, involving 21,977 adults, the vaccine candidate with a prime-boost regimen showed 91.6% efficacy against COVID-19 (Table 1). Regarding safety, the reported adverse events were mostly graded 1, and none of the reported serious adverse events could be associated with the vaccination. As of April 2021, Sputnik V was approved for emergency use in Russia and several other countries (Mendonça et al., 2021).

Non-human adenovirus vaccines

Oxford/AstraZeneca (ChAdOX1-nCoV)

During the 2012 Middle East respiratory syndrome coronavirus (MERS-CoV) outbreak, the Oxford group developed a vaccine using their ChAd (chimpanzee Ad) technology, which circumvents pre-existing immunity to Ad5 (Ewer et al., 2017. The vector was developed from an Ad isolated from a chimpanzee fecal sample and vectorized by deletion of E1/E3 and modifications in E4 (E4Orf4, Orf6, and Orf6/7 swapped with human Ad5). The Oxford AstraZeneca COVID-19 vaccine uses a replication-deficient chimpanzee adenovirus vaccine vector (ChAdOx1) that has been authorized for usage, recombinant adenovirus type 26 (Ad26) in the Johnson and Johnson COVID-19 vaccineAd5-nCoV and UQ-CSL V451 both employ recombinant adenovirus type-5 (Ad5), Gam-COVID-Vac (also known as Sputnik-V) vaccine uses an Ad26-based vaccination as shown in figures 2 and 3 (Zhu et al., 2020). The fiber protein is responsible for the virion's attachment to specific cell surface receptors (Giacca and Zacchigna, 2012). An N-terminal tail, a variable-length shaft, and a globular C-terminal knob make up the fiber protein that protrudes from the 12 vertices of the capsid (Holterman et al., 2004). Molecular recognition and nuclear localization signals are both located at the conserved N terminus (Wang et al., 2019). Adenovirus fibers' receptor binding selectivity may be adjusted by swapping knob domains, as evidenced by studies demonstrating the Ad5 knob's ability to prevent viral infection. Adenoviruses of subgenus C and Ad9 (of subgenus D) share a fiber receptor, but subgenus C and B adenovirus serotypes recognize different receptors. A 46-kDa HeLa cell surface protein has recently been shown to function as a shared receptor for adenoviruses of subgenus C and coxsackie B viruses (Tatsis and Ertl, 2004). Furthermore, it has been found that the class I major histocompatibility complex can also act as an adenovirus receptor (Appaiahgari and Vrati, 2015). The fiber knob also carries the type-specific antigen, which defines, together with the hexon's antigen, the adenovirus's serotype specificity. The determinant consists of at least 17 amino acids that are not confined to a specific area on the fiber knob (Ricobaraza et al., 2020). The positive findings from preclinical studies in rodents and non-human primates supported the transfer to clinical trials with the ChAdOx1 nCoV-19 vaccine candidate (Lombardi et al., 2021). High safety and both humoral and cellular immune responses were obtained in phase I/II clinical trials, as shown in figures 2 and 3 (Lu et al., 2020; Lombardi et al., 2021). Additionally, phase III clinical evaluation of more than 30,000 volunteers has been conducted (Bricker et al., 2021). Interim phase III results from the UK, Brazil, and South Africa showed good vaccination safety and 62.1% vaccine efficacy after two vaccinations with 5×10^{10} ChAdOx1 nCoV-19 particles and up to 90% in individuals receiving a prime dose of 2.2×10^{10} particles and a boost of 5.5×10^{10} particles as shown in Table 1 and 2(Douglas, 2007; Basheeruddin Asdaq et al., 2022).

The ChAdOx1 nCoV-19 vaccine received a EUA in the UK in December 2020 (Cederwall and Påhlman, 2019; Kashte et al., 2021). In contrast to the ChAdOx1 nCoV-19 vaccine, the Ad26.COV2.S vaccine is based on the human Ad26 serotype expressing the prefusion-stabilized SARS-CoV-2 S protein, and requires only one immunization (Cederwall and Påhlman, 2019). This was confirmed in hamsters, where a single injection of Ad26.COV2.S elicited neutralizing antibodies and protected the animals from SARS-CoV-2-associated pneumonia and death (Biserni, et al., 2021). Moreover, a single immunization of macaques elicited strong neutralizing antibody responses and protected against SARS-CoV-2 challenges (Baron et al., 2018). In the context of clinical trials, a single administration of Ad26.COV2.S elicited rapid binding, neutralization antibody responses, and cellular immune responses in a phase I study in 25 healthy volunteers (Ricobaraza et al., 2020). Moreover, 1,045 healthy volunteers were vaccinated with a single dose of 1×10^{10} or 5×10^{10} Ad26.COV2.S particles in phase I/II study showed good safety and strong immune responses (Spunde et al., 2022).

The Ad26.COV2.S vaccine has been subjected to large phase III clinical trials with 60,000 participants (Coughlan, 2020, Bibby et al., 2022). As mentioned earlier, simian adenovirus vectors have been used for SARS-CoV-2 vaccine development to address any potential pre-existing immunity against human adenoviruses in the population (Folegatti et al., 2022).

However, the current adenovirus-based vaccines, except for Ad26.COV2.S requires a prime-boost regimen (Almuqrin et al., 2021). Neutralizing antibodies against adenoviruses might reduce the efficacy of a second or a third

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immunization with the same adenovirus serotype. For this reason, a strategy of prime vaccination with an Ad26 serotype vector expressing the SARS-CoV-2 S protein followed by a booster vaccination with another adenovirus serotype, the Ad5 expressing the SARS-CoV-2 S protein, was evaluated (Pei et al., 2019). In preclinical studies, the rAd26-S/rAd5-S vaccine candidate showed 100% protection in transgenic mice, hamsters, and primates (Punga et al., 2020). Moreover, good safety, mild adverse events, and robust immune responses were observed in phase I/II clinical trials (Soudet and Stutz, 2019). Phase III study with the rAd26-S/rAd5-S vaccine showed tolerability and 91.6% vaccine efficacy (Poland et al., 2020). The rAd26-S/rAd5-S (Sputnik V) vaccine received a EUA in Russia in July 2020, although only preliminary vaccine evaluation had been conducted in 76 volunteers (DeFrancesco, 2020).

A third-generation Ad5 serotype vector expressing the SARS-CoV-2 S protein (Ad5-S-nb2) was intramuscularly administered into mice and ferrets, which resulted in protection against challenges with SARS-CoV-2 (Desheva, 2018). Moreover, the Ad5-S-nb2 vaccine provided projection against SARS-CoV-2 in rhesus macaques (Chung et al., 2020). In the case of clinical trials, a single dose of Ad5-S-nb2 induced both binding and neutralizing antibodies in healthy volunteers (Zhu et al., 2020). However, the level of response depended on pre-existing Ad5 antibodies and the age of the vaccinated person (Giacca and Zacchigna, 2012). Interim results from a phase III trial indicated that a single dose of the Ad5-S-nb2 vaccine showed an overall efficacy of 65.3% in preventing all symptomatic COVID-19 disease 28 days post-vaccination (Wang et al., 2019). Moreover, Ad5-S-nb2 showed a 90.1% efficacy in preventing severe COVID-19 disease 28 days post-immunization. The Ad5-S-nb2 received a EUA in February 2021 in China (Holterman et al., 2004). The gorilla adenovirus GRAd has been used to express the perfusion-stabilized SARS-CoV-2 S protein (Appaiahgari and Vrati, 2015). The GRAd-COV2 vaccine candidate elicited robust immunogenicity in both mice and macaques. The functional antibodies neutralized SARS-CoV-2 infection blocked SARS-CoV-2 S protein binding to angiotensin-converting enzyme 2 (ACE2) and generated robust Thelper 1(Th1)-dominated cellular responses. The GRAd-COV2 vaccine candidate is undergoing phase I evaluation (Ricobaraza et al., 2020). In another vaccine approach, the chimpanzee adenovirus serotype 68 (ChAdV68; Rhodes, 2021) was combined in a prime-boost regimen with a SAM expressing the SARS-CoV-2 S protein and T-cell epitopes from the SARS-CoV-2 N protein. A dose-escalation phase I clinical trial with a ChAdV68 prime vaccination and SAM boost vaccination is in progress (Lu et al., 2020). The AAV vector-based vaccine candidate AAVCOVID-1 was recently introduced (Wang et al., 2021b). The SARS-CoV-2 S gene was expressed from an AAV2 inverted terminal repeat (ITR) with an AAVrh32.33 capsid, showing potent immunogenicity in mice and non-human primates. Moreover, a single immunization provided complete protection in macaques challenged with SARS-CoV-2. Neutralizing antibodies were sustained for a year. Neither pre-existing immunity against AAVCOVID-1 in humans nor cross-reactivity to common AAV vectors used in gene therapy were detected. Single-dose administration, high-yield manufacturing, and one-month stability at room temperature make the AAV-based approach attractive for potential global use once efficacy has been confirmed in clinical trials (Zabaleta et al., 2021).

Immunogenicity, safety results of current adenovirus vaccines

Evaluation of vaccine immune response

A reliable assessment of a vaccine capacity to generate T-cell responses might be made using quantitative approaches, such as intracellular cytokine testing (Zeedan et al., 2014; Zeedan et al., 2019). It is impossible to verify the effectiveness of vaccination using such quantitative approaches as titration or challenge with a virus that produces severe injury or death in experimental animals (Wang et al., 2021, Zabaleta et al., 2021). Due to their capacity to stimulate innate immune system cells, induce the maturation of immature dendritic cells into antigen-presenting cells, and express significant levels of transgene products in the majority of vectors, deleted AdHu5 vectors have a high level of immunogenicity (Zhou and Ertl, 2006; Horton et al., 2007, McLean, 2018).

ChAdOx1 nCoV-19 was safe, tolerated, and immunogenic, while reactogenicity was reduced with paracetamol. A single dose elicited both humoral and cellular responses against SARS-CoV-2, with a booster immunization augmenting neutralizing antibody titers (Das et al., 2022). The preliminary results of this first-in-human clinical trial supported clinical development progression into ongoing phase 2 and 3 trials. Older age groups with comorbidities, health-care workers, and those with higher risk for SARS-CoV-2 exposure are being recruited and assessed for efficacy, safety, and immunogenicity of ChAdOx1 nCoV-19 given as a single-dose or two-dose administration regimen in further trials conducted in the UK and overseas. Evaluation of the vaccine in children once sufficient safety data have been accumulated in adult studies. Phase 3 trials are now underway in Brazil, South Africa, and the UK and will evaluate vaccine efficacy in diverse populations, as shown in tables 1 and 2 (Folegatti et al., 2020).

SARS-CoV-2 variants and vaccine efficacy

Despite the success achieved in developing vaccines against COVID-19, the detection of novel SARS-CoV-2 lineages has raised concern about vaccine efficacy. For instance, the B.1.1.7 variant (alpha) was initially claimed to possess higher transmission rates and was found to spread rapidly in the UK (Dhawan et al., 2022). The alpha variant

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carrying the N510Y mutation and deletion of amino acids 69 and 70 in the RBD of the SARS-CoV-2 S protein was determined to be 75% more transmissible than the wild-type strain with the 501N sequence. It was recently demonstrated that individuals who tested positive for the alpha variant showed a mean log10 viral load 1.05 higher than non-alpha variant subjects (Jones et al., 2021). In addition to the alpha variant, the South African B.1.351 (beta, Mwenda et al., 2021), the Brazilian B.1.1.28.1 (gamma), and the Indian B.1.617 (Cantón et al., 2021) variants have been identified. Related to vaccine efficacy, adenovirus vector-, RNA-, and protein subunit-based vaccines have been tested. A small but significant reduction in neutralizing antibody activity against the N501A and the K417N-E484K-N501Y mutations in the SARS-CoV-2 S protein was detected for the two approved RNA-based vaccines (Wang et al., 2021). In another study, 20 volunteers vaccinated with the BNT162b2 RNA vaccine showed similar neutralizing titers to SARS-CoV-2 with either N501 or Y501 in the S protein, nanoparticle encapsulated SARS-CoV-2 S protein subunit vaccine NVX-CoV22373 the efficacy against the alpha variant was 86%, and against the beta, the variant was 60% (Xie et al., 2021). In the case of adenovirus-based vaccines, variability related to protection efficacy has been discovered. For instance, in a phase II/III trial, similar vaccine efficacy against the alpha variant and other lineages was obtained (Chi et al., 2022). However, reduced neutralization activity was measured against the alpha variant compared to non-alpha variants in vitro after ChAfdOx1 nCoV-19 vaccine administration (Mahase, 2021). Despite that, the vaccine protected against the alpha variant. However, in another study, the ChAdOx1 nCoV-19 failed to provide protection against mild-to-moderated COVID-19 caused by the beta variant (Madhi et al., 2021).

In contrast, Ad26.COV2.S vaccine showed clinical efficacy against symptomatic COVID-19 and also against the beta variant despite its partial resistance to neutralizing antibodies (Alter et al., 2021). Moreover, humoral and cellular responses against the original SARS-CoV-2 strain and the beta variant were observed. However, the median pseudovirus-neutralizing antibody titers were 5-fold lower than the original SARS-CoV-2 strain. Overall, the detected and potentially emerging new variants demand a thorough follow-up on vaccine efficacy and the readiness to re-engineer available vaccines to ensure the efficacy of vaccine protection (Alter et al., 2021).

Properties of adenoviruses

Double-stranded DNA adenoviruses have genomes between 34 and 43 kb, making them easier to manipulate for reprogramming. Figure 1 shows how alternative splicing and poly(A) sites in different polypeptide strands helped the virus adapt to its small genome. Adenoviruses were the first respiratory viruses to be identified in tissue culture. The capacity of diverse human and animal organ and tissue cells to develop in vitro on synthetic medium, as well as the ability of viruses to replicate on sensitive cells, resulting in cytopathic effects. The Adenoviridae family includes at least 120 viruses that may infect mammals, birds, reptiles, amphibians, and fish (Bricker et al., 2021; Lombardi et al., 2021). There are 51adenovirus serotypes from humans and 27 serotypes from simians, including seven chimpanzee serotypes that are isolated from other mammalian species and cause infections ranging from mild respiratory infections to life-threatening multi-organ diseases. The six most studied human serotypes (A-F; B1, and B2) were split into B1 and B2 based on sequence homology and their capacity to agglutinate red blood cells (Tatsis and Ertl, 2004; Douglas et al., 2007). Adenovirus types are presently included in Rosen's hemagglutination Group I, although they are generally considered typical members. Three types indicated three important points included hemagglutination titers which were consistently higher with a rat than with rhesus or African green monkey erythrocytes; hemagglutination inhibition titers might be readily demonstrated with rats but not with rhesus erythrocytes. Standardized hemagglutination and hemagglutination inhibition procedures were described and statistically evaluated for all (Cederwall and Påhlman, 2019; Zeedan et al., 2020).

Adenovirus infections

Adenoviruses can cause both acute and long-term symptoms. Adenovirus of the human serotypes (AdHu), including AdHu1, AdHu2, and AdHu5 (subgenus C), frequently infect people and cause various symptoms, such as mild upper respiratory infections in children. Pneumonia (AdHu4) and meningoencephalitis (AdHu7, 12, and 32) can result from adenoviruses, especially in immunocompromised persons and children (Baron et al., 2018). The signs and symptoms of adenoviruses in chimpanzees, which are being studied for vaccine development, are yet unclear. Several human adenovirus serotypes were widely distributed and infected the majority of infants and young children in the early stages of their lives (Ricobaraza et al., 2020). According to several studies, 45% to 80% of persons had antibodies that neutralized AdHu5 viruses (Spunde et al., 2022). Depending on the location where they live, 5-15% of adults had virus-neutralizing antibodies to AdHu 35. Chimpanzee viruses were typically utilized in preclinical vaccination testing (Biserni et al., 2021).

Tropism

The capacity of adenoviruses to bind to host cell receptors is known as tropism (Baron et al., 2018; Spunde et al., 2022). The distal knob domain of the fiber initially binds to the coxsackie adenovirus receptor (CAR), which is

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expressed on many cell types, including hepatocytes, the basolateral surface of epithelial cells, endothelial cells, myoblasts, and heart muscle cells. Even though lymphoid cells lack the ability to produce CAR, they serve as a reservoir for viral infections that utilize CAR (Baron et al., 2018).

Hemagglutination

Adenoviruses, rubeola, and myxoviruses have a standardized hemagglutination (HA) test reproducibility of 84 to 96 percent, whereas reoviruses have a standardized hemagglutination-inhibition test reproducibility of 78 to 93 percent (Ricobaraza et al., 2020). Since Roséninitially demonstrated HA by human adenoviruses in 1958, serotypes of six subgenera(A-F) exhibit various characteristics. Subgenus D adenoviruses may be divided into three clusters. Cluster DI adenoviruses agglutinate both rat and human erythrocytes, and cluster DII adenoviruses agglutinate only rat erythrocytes (Coughlan, 2020). The adenovirus fiber mediates the agglutination of erythrocytes. Based on differential hemagglutinating properties, subgenus D adenoviruses can be subdivided into clusters DI, DII, and DIII. While subgenus DI adenoviruses agglutinate rat and human erythrocytes, DII adenoviruses simply agglutinate rat erythrocytes and DIII adenoviruses display no or only weak rat erythrocyte agglutination (Spunde et al., 2022). Amino acid sequence comparisons revealed distinct domains on the fiber knob, which could be involved in hemagglutination. To localize and characterize the domains responsible for the interaction with rat and human erythrocytes, potential hemagglutination domains of the adenovirus type 9 (Ad9, subgenus DI) fiber knob was introduced into Ad17 (subgenus DII) and Ad28 (subgenus DIII) fiber knobs by primer-directed mutagenesis (Bibby et al., 2022). Furthermore, rat erythrocyte hemagglutination domains were also introduced into the Ad3 (subgenus B) fiber knob, which only agglutinated monkey erythrocytes (Folegatti e al., 2022). The recombinant proteins were tested in HA tests. All eight subgroups I strains were related to prototype C-1 chimpanzee adenovirus and human adenovirus type 16. Six strains of subgroup II were closely antigenically related to each other, and human adenovirus type 5 by hemagglutination inhibition (HI). Four additional strains were partially cross-reactive in HI tests with human adenovirus type 2 and highly cross-reactive with one another. The remaining two strains of subgroup II represented previously unreported serotypes that were not related to known adenoviruses or to each other, as demonstrated by HI techniques (Bibby et al., 2022). The linear genome flanked by two origins for DNA replication (ITRs) has eight units for RNA polymerase II-mediated transcription (Pei et al., 2019; Punga et al., 2020).

Chimpanzee adenoviruses

Twenty-seven serotypes from simians, including seven from chimpanzees, each containing adenovirus complement-fixing antigen, are divided into three main subgroups according to their hemagglutinating properties. Subgroup I is composed of eight strains that cause hemagglutination of rhesus or vervet monkey erythrocytes; subgroup II consists of 12 strains that agglutinate selected rat erythrocytes in the presence of heterotypic immune serum to members of the Rosen subgroup III of human adenoviruses. Finally, chimpanzee subgroup III is composed of two strains that fail to agglutinate monkey, rat, guinea pig, or human-type O erythrocytes (Soudet and Stutz, 2019; DeFrancesco 2020; Chung et al., 2020; Poland et al., 2020).

CONCLUSION

The SARS-CoV-2 pandemic has shown that Ad vectors are strong vaccine candidates. Clinical trials with Ad vaccines have demonstrated they are safe in humans, with no serious adverse events observed in most individuals. Ad vaccines produced protective humoral and cellular immune responses, even after a single dose in some cases. Adenovirus studies have allowed researchers to circumvent the problem by using blood clotting in a vaccinated person and show promise as carriers for antigen delivery of vaccines currently in development. Numerous adenovirus-based vaccines have approved the use of viral vectors in the creation of COVID-19. They have been widely utilized in mass immunizations. Despite having slightly lower vaccine efficacy than RNA-based vaccines, they have been frequently used in bulk vaccinations. However, comparing the effectiveness of several COVID-19 vaccines is difficult due to the various phases to determine their safety, dosage schedule, and level of protection to evaluate vaccines on an individual basis, not in comparison to one another and broad range of protection from 80 to 95% efficacy for Pfizer and BioNTech, to approximately 60 to 70% revealed by a vaccine made by AstraZeneca of Oxford. Although phase III clinical trials involve a significant number of people who have been vaccinated, the vaccines are often tested in different geographical locations and at different stages of the pandemic rather than being compared in the same way. In this context, the adenovirus-based ChAdOx1 nCoV-19 and mRNA-based BNT162b2 and mRNA-1273 vaccines showed prior to the emergence of the SARS-CoV-2 alpha, beta, gamma, and delta variants, which does not make the vaccines any less effective. Finally, a deep understanding of the structural features of S will facilitate the design and development of successful vaccines against coronavirus SARS-CoV-2 for large populations.

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Authors' contribution

All authors equally contributed Research ideas planned the study design and performed data. All authors checked and approved the final version of the manuscript for publication in the present journal.

Competing interests

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Ethical considerations

All ethical issues have been checked by the authors, including plagiarism, double submission and data originality.

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Efficacy of Hemagglutinin Gene of Highly Pathogenic Avian Influenza as a Vaccine Candidate in Poultry: A Review

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ABSTRACT

The most prevalent fatal disease in poultry that can result in high morbidity and mortality is highly pathogenic avian influenza (HPAI), subtype H5N1. A vaccination program is the most frequent way to prevent HPAI cases in poultry, especially against the H5 subtype of HPAI. There are currently a number of avian influenza vaccines available, including recombinant and inactivated whole virus vaccines. The foundation of a recombinant vaccine is possible by the expression of an avian influenza gene of interest following insertion into a carrier vector (no pathogenic virus). A recombinant HPAI vaccine is required to further challenge avian influenza cases in poultry. As a recombinant vaccine inserted into a carrier vector, the hemagglutinin (HA) gene has proven effective. The recombinant Herpes Virus Turkey (rHVT) vector vaccine for avian influenza has been discovered and is commercially available. The rHVT vaccine was developed using a hemagglutinin insert from the HPAI virus clade 2.2. Overall, studies in this review aimed to determine the efficacy of any developed recombinant avian influenza vaccine that uses the HA gene from different clades challenged with any avian influenza virus (AIV) isolate. It was found that the efficacy of hemagglutinin as a recombinant vaccine could be promising for future HPAI vaccine development. In addition, it is possible to design a recombinant vaccine using local isolates to protect poultry farms, particularly in endemic regions.

Keywords: Avian influenza, Efficacy, Hemagglutinin, Poultry, Recombinant vaccine

INTRODUCTION

Due to the high mortality rate from highly pathogenic strains in poultry and the possibility of zoonotic transmission made by the spread of domestic poultry species, avian influenza (AI) poses a significant threat to the entire world, especially in the poultry industry (Suttie et al., 2019; El-Shall et al., 2021). Numerous highly pathogenic avian influenza (HPAI) outbreaks have occurred since 1996, resulting in significant losses in Southeast Asia, the Middle East, Europe, and Africa (Balzli et al., 2018). The majority of HPAI viruses of subtypes H5 and H7 evolved from low pathogenic H5 and H7, resulting in significant mortality and economic losses in poultry (OIE, 2021). One of the most significant HPAI outbreaks is a subtype of H5N1. These H5N1 viruses have spread to several nations and become endemic, including China, Indonesia, Vietnam, and Egypt (FAO, 2011). Mass culling is no longer acceptable in developing countries, according to the World Organization for Animal Health (OIE) and the United Nations Food and Agricultural Organization (FAO), for ethical, ecological, and economic reasons (Peyre et al., 2008). Restricting bird migration, enhancing biosecurity, and starting a vaccination campaign are all necessary for controlling AI in endemic countries (Nassif et al., 2020).

Vaccination has been recommended as an AI eradication or control program strategy in endemic countries (Hsu et al., 2014). It is a powerful combination when combined with good biosecurity and monitoring programs (Kapczynski et al., 2015). The antigens in vaccines should be sufficient to produce a protective level of antibody titer (vaccine potency). The vaccine must protect the bird against virus infection (Vaccine efficacy) and be properly administered to a large proportion of the susceptible population (Swayne and Kapczynski, 2008). In different countries, inactivated vaccinations have been used to limit the spread of highly dangerous H5 and H7 avian influenza viruses (Qiao et al., 2009). In fact, the parental route is the only way to administer inactivated vaccines individually, which is laborious, time-consuming, and puts the vaccination crews at risk of spreading the field virus (Rauw et al., 2011).

In order to make sure that inactivated vaccines are still effective against field virus strains that are currently circulating, their efficacy should be routinely evaluated. The effectiveness of inactivated vaccines is primarily determined by the vaccine properties, passive immunity's presence or absence, and the targeted host's age (Rauw et al.,

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2011; Kapczynski et al., 2016). However, due to inadequate protection and weak flock immunity, AI vaccination had only a limited impact on domestic poultry (Peyre et al., 2009). In addition, there have been numerous reports of HPAI vaccination failures in the commercial broiler, layer, and breeder flocks (Swayne et al., 2015). This happened because the immune systems of the immunized poultry population were compromised, favoring viral mutation and antigenic drift field viruses from the vaccine strain (Kilany et al., 2015). The ideal AI vaccine should be effective, safe, only require one dose, be affordable, and make it possible to distinguish between infected and vaccinated animals (Bertran et al., 2015).

AIV vaccines can be classified into two broad technological groups in field usage; there are inactivated whole AIV vaccine and recombinant vectored AI vaccine expressed HA protein (Capua and Alexander, 2008; Swayne, 2009). To combat current threats of H5N1 infection in the poultry industry, a recombinant Herpes Virus of Turkey (rHVT) vaccine was recently created. This vaccine expresses the HA gene of an HPAI H5N1 strain (Soejoedono et al., 2012). The rHVT could be a good candidate for a recombinant vaccine-based viral vector that meets most of the criteria for an ideal AI vaccine (Reemers et al., 2021). Furthermore, the studies in this review were aimed to determine the efficacy of developed recombinant avian influenza vaccine that uses the HA gene from clade 2.2 challenged with any AIV isolate. Therefore, the present review article focused on the efficacy of the hemagglutinin gene as a vaccine candidate in a poultry clinical trial.

Role of hemagglutinin

Avian influenza virus is made up of eight single-stranded negative-sense RNA segments, each of which codes for one or more viral proteins. The antigenic characteristics of the hemagglutinin (HA) and neuraminidase (NA) glycoproteins determine the specificity of the AI subtype (Wibowo et al., 2015). It is currently known that there are eleven NA and 18 HA subtypes in the AI virus (N1-N11). Subtypes H1-H16 and N1-N9 are mainly found in avian species. The only viruses known to cause HPAI are viruses of the H5 and H7 subtypes although not all of these subtypes possess these characteristics (Alexander, 2000). As a prerequisite for host restriction and pathogenicity, the HA protein binds to sialic acid receptors on the surface of host cells to begin viral infection (Suttie et al., 2019). The HA is a major envelope glycoprotein with the potential for vaccine development. A subunit vaccine against H5N1 infection has been developed using recombinant HA (rHA) proteins. The rHA vaccine approach is an appealing vaccine manufacturing option. It eliminates the need for H5N1 influenza virus vaccine production based on eggs or cells (Lin et al., 2011). Protection is primarily due to a humoral immune response against HA and secondarily against NA. However, such protective responses are only subtype-specific (Swayne, 2009).

By binding to the cellular receptor sialic acid and assisting in the fusion of the viral and host membranes, the surface glycoprotein HA is in charge of identifying the target cell and facilitating viral genome entry into the target cell. A homotrimeric precursor known as HA is produced by the viral genome's fourth segment (HA0, Schrauwen et al., 2012). During the viral life cycle, the cleavage of HA0 into HA1 and HA2 subunits by host cell protease is essential for viral infection. The HA2 subunit promotes membrane fusion, while the HA1 subunit binds to the cellular receptor sialic acid (Wang et al., 2019). The nucleocapsid can be released into the cytoplasm to begin viral replication with the viral envelope and endosomal membrane fused at low pH. This is made possible by the significant conformational changes that HA experiences inside the endosome (Wu et al., 2012). The HA1 subunit can be identified by its membrane-distal globular head domain, which contains the receptor-binding site (RBS), while the HA2 subunit can be identified by its membrane-proximal stem region (Yamada et al., 2006). The majority of highly potent neutralizing antibodies induced by viral infection and vaccine immunization target the globular head domain of HA1 (Wang et al., 2019). These antibodies are generally strain or clade-specific because of the high variability of their HA1 epitope residues. Therefore, the antibodies formed against the HA protein are potent neutralizing antibodies that slow disease progression and prevent viral infection (Chiu et al., 2015).

Efficacy of rHA from clade 2.2 challenged against different isolate

During the HPAI challenge, effective AIV vaccinations have been shown to reduce virus shedding from birds' respiratory and gastrointestinal tracts and protect against morbidity and mortality (Criado et al., 2019). The Herpes Virus of Turkey (HVT) might be a good candidate for an AI vector vaccine since it meets the most optimal AI vaccine criteria. HVT may be used in the hatchery either *in ovo* or subcutaneously, and it has previously been commercially used as a vector vaccine worldwide (Reemers et al., 2021). The rHVT vaccine was created using a genetic insert derived from the HA gene of the clade 2.2 HPAI virus A/swan/Hungary/4999/2006, which is expressed for a protracted amount of time by HVT (Balzli et al., 2018). Using a live viral system that can remain in the host while expressing the targeted insert for immune modulation triggers a cellular and humoral immune response, which are the advantages of the rHVT vaccine technology over inactivated whole vaccines (Kapczynski et al., 2015). The higher homology between the H5 present in the rHVT-H5 and the inactivated H5N1 vaccines may cause the better effect seen in rHVT with the H5 insert gene (Rauw et al., 2012).

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However, some challenge studies must be conducted to determine the vaccine efficacy. It will be beneficial to conduct a clinical trial with challenges against various isolates to determine the vaccine's level of protection (Swayne et al., 2015). Considering the current review studies, the rHA vaccine uses HVT as a viral vector and has different protectivity with challenge strains from different clades described in Table 1. On specific pathogen free (SPF) Chicken, rHVT show different protectivity indicated by survival rate result. The survival rate was fully protected, explaining that no dead chickens were in the trial group vaccinated against rHVT on the first day of age and challenged with different isolates. Meanwhile, rHVT is fully effective in protecting SPF chickens from a challenged pathogenic isolate from America, Mongolia, Bangladesh, Egypt, Turkey, and Germany (Table 1). Another trial from the studies indicated that rHVT is not fully protected in SPF Chicken against challenge pathogenic isolate (Soejoedono et al., 2012; Nassif et al., 2020). As a result, many chickens died after exposure to pathogenic isolates from Asia. A few were from Egypt and Indonesia (West Java-Subang, Purwakarta-Cilingga). It demonstrates that when tested against isolates from Asian strains, the isolates used in rHVT are not entirely protective. This refers to the homology characteristics of the hemagglutinin gene of the clade 2.2 strain with isolates of other pathogenic strains and different clades. Further genomic analysis is needed regarding the gene alignment of the various isolates and whether they have significant differences.

In addition to offering excellent clinical protection against antigenically drifted H5Nx HPAI strains, the rHVT-H5 vaccine can potentially pose a significant challenge to the suppression of virus shedding (Nassif et al., 2020). Vaccine efficacy failure in the field is typically attributed to the antigenic distances between the vaccine and the circulating field strains (Swayne et al., 2015; Peeters et al., 2017). It is well known that maternal derived antibodies (MDA) prevent the development of protective immunity after vaccination (Vriese et al., 2010). The cell-associated rHVT-H5 vaccine creates a pathway inside lymphocytes that may promote cell-mediated immunity. Along with the humoral response, this cell-associated immune response is thought to be insensitive to MDA interference with the HVT virus. After using inactivated vaccines, MDA has been observed to interfere with eliciting an immune response against various antigens. On the other hand, commercial day-old chickens (DOC) have MDA against HVT. If given a sufficient dose, these antibodies do not revoke protection but may reduce the efficacy of cell-associated HVT vaccines (King et al., 1981; Poetri et al., 2011; Kilany et al., 2015). Additionally, rHVT vaccination induces long-lasting immunity because the antigen is continuously expressed (Reddy et al., 1996).

Table 1. Summary of efficacy Hemagglutinin HPAI H5N1 Clade 2.2 strain A/swan/ Hungary/4999/2006 challenged with different HPAI isolates and vaccinated using vector rHVT on the first day of chick

References	Challenge strain	Animal Test	Virus given	Virus (EID/50)	Survival rate (%)	Control (%)
(Balzli et al., 2018)	A/turkey/Minnesota/12582/2015	SPF	4 wpv	1 x $10^{7.5}$	100	0
(Kwon et al., 2021)	A/chicken/Bangladesh/NRL-AI-3237/2017	SPF	4 wpv	1 X 10^{6}	100	0
(Rauw et al., 2011)	A/Chicken/Egypt/1709-6/2008	SPF	3 wpv	1 X 10^{6}	100	0
(Reemers et al., 2021)	A/turkey/Turkey/01/2005	SPF	3 wpv	1 x 10^{6}	100	0
(Steensels et al., 2016)	A/turkey/Germany- MV/R2472/2014	SPF	4 wpv	1 x 10^{6}	100	0
(Kapczynski et al., 2015)	A/Whooper Swan/Mongolia/3/2005 A/chicken/West Java Sbg/29/2007	SPF Com	6 wpv 4 wpv	1 X 10^{6}	100 80	0
(Soejoedono et al., 2012)	A/CK/WJava-Subang/029/ 2007 A/CK/Purwakarta-Cilingga/142/2010	SPF	4 wpv	1 X 10^{6}	80 95	0
(Nassif et al., 2020)	A/chicken/Egypt/173CAL/2017 A/duck/Egypt/VG1099/2018 A/chicken/Egypt/FL6/2018	SPF	4 wpv	1 X 10^{6}	90 90 80	0
(El-Shall et al., 2021)	A/chicken/Egypt/Alex-2/2017	Com	3 wpv	1 x $10^{6.3}$	50	0
(Kilany et al., 2015)	A/Chicken/Egypt/128S/2012	Com	3 wpv	1 X 10^{6}	80	nr

*SPF: Specific pathogen-free, Com: Commercial broiler, wpv: Weeks post vaccination, EID: Egg infective dose, nr: Not reported

Serology test result and viral shedding of rHA vaccine

Measuring the humoral response to hemagglutinin, the main surface glycoprotein of the influenza virus, is the primary method for assessing the efficacy of AI vaccines. The strain-specific hemagglutination inhibition (HI) test is the gold standard for determining AI immunity response (Swayne et al., 2015). The HI antibody level thought to be the cut-off for susceptibility for the whole-virus inactivated vaccine is 4 log 2 (Qiao et al., 2009). To combat infection with particular AI strains, specific antibody titers are required. Although many vaccinated survivors also have low levels of HI antibodies, the bird that died from infection had low HI antibody titers on pre-challenged chickens. This suggests that

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the HI antibody titer to the required viral challenge is greater than 4 log 2. It might provide protection for antigenic variants and be a reliable indicator of survival (Ross et al., 2019).

The discrepancy between the achieved high protection level and the lower serologic response than a predicted protective level of HI titers observed in many studies can be explained by the rHVT-H5 vaccine's inability to induce strong specific cell-mediated immunity in the immunized chickens (Rauw et al., 2011; Criado et al., 2019; Nassif et al., 2020). According to studies, the rHVT-H5 vaccine induces a humoral and cell-mediated immune response (Kilany et al., 2014; Kapczynski et al., 2015). When antibody titers to the challenge virus strains are lower than to the vaccine virus strain, this indirectly indicates the antigenic distances between the vaccine and challenge strain (Palya et al., 2016). The summary of HI titers results from experimental vaccination with recombinant hemagglutinin (rHA) vaccine is shown in Table 2.

One-day-old chicken that had received the rHVT-H5 vaccine had significantly less viral excretion during the initial stages of infection via the oropharyngeal and cloacal routes. As a result, there was significantly less viral shedding in vaccinated chickens that were producing specific antibodies than in negative controls. Vaccinated chicks were seen to shed early after infection with high-challenge doses, especially by the respiratory tract. This was observed in both vaccinated and unvaccinated chicks. In addition to this, the effect of the dose became clear (Steensels et al., 2016). In addition, the continued development of a vaccine based on hemagglutinin has the potential to lessen the amount of virus that is shed following exposure to the virus. Vaccinated chicks were found to have significantly less viral shedding than unvaccinated chicks when exposed to high-challenge doses (Kwon et al., 2021).

Table 2. Summary of serology tests and viral shedding from rHA-based vaccine in specific pathogen-free and commercial chickens

References	HI ANTIGEN	GMT HI Pre (Log_2)	GMT HI Post (Log_2)	Swab Collected	Oral swab	Cloacal swab
(Soejoedono et al., 2012)	Vaccine	7.14	nr	2, 4, 7 dpc	7 dpc (+)	7 dpc (+)
(Nassif et al., 2020)	Vaccine	5.1	6	3, 7, 10 dpc	10 dpc (+)	10 dpc (+)
(Balzli et al., 2018)	Vaccine	6	9	2, 4 dpc	4 dpc (+)	4 dpc (+)
(Kwon et al., 2021)	Challenge	6	10	2, 4 dpc	4 dpc (+)	4 dpc (+)
(Rauw et al., 2011)	Vaccine	4	9	3, 7 dpc	7 dpc (+)	nr
(Reemers et al., 2021)	Challenge	4.6	8.6	4, 7, 14 dpc	14 dpc (+)	14 dpc (-)
(Steensels et al., 2016)	Vaccine	4.5	8.5	2, 5, 9, 14 dpc	14 dpc (+)	14 dpc (-)
(Kapczynski et al., 2015)	Challenge	5.1 5.5	6.4 8	2, 4 dpc	4 dpc (-) 4 dpc (+)	4 dpc (-) 4 dpc (+)
(El-Shall et al., 2021)	Vaccine	3	6	3, 5, 7 dpc	7 dpc (+)	7 dpc (+)
(Kilany et al., 2015)	Vaccine	Nr	4.4	3, 6, 9, 14 dpc	14 dpc (-)	14 dpc (-)

*HI: Hemagglutination inhibition, GMT: Geometric mean titer, dpc: Days post challenge, (+): Positive, (-): Negative, nr: Not reported

CONCLUSION

It has been demonstrated that the effectiveness of hemagglutinin in avian influenza as a vaccine candidate against various isolates has a high level of protective efficacy. The survival rate, the antibody titer level, and the amount of viral shedding can measure this level of efficacy. The method for developing a recombinant vaccine is a commonly used viral vector with HVT. The conclusion that can be drawn from this is that the development of an avian influenza recombinant vaccine could use any homologous isolate with the virus challenge strain in an area and give cross-protection among the various types of AIV. The vaccine will have good protectivity and inhibit viral shedding if the clade or isolate for recombinant vaccine is homologous. The developing recombinant vaccine used the HA strain identically as a vaccine and produced in vector expression to provide poultry with constant protection against virus mutation in the field. Further studies about universal clade based on ethnicity are needed to find acceptable prevention against different types of avian influenza.

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Data availability and material

All information pertaining to the review study is presented in the article.

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Authors' contribution

Armanda Dwi Prayugo initiated the manuscript drafting and production of the final draft. Both authors (Toto Subroto and Wyanda Arnafia) contributed to conceptualizing the idea, editing, and production of the final draft. All authors checked the last draft of the manuscript and confirmed it before submission to the journal.

Competing interests

No conflicts of interest are disclosed by the authors of this review.

Ethical consideration

Plagiarism, lack of consent to publish, misconduct, fabrication and/or falsification of data, duplicate publication and/or submission, and redundant information were all investigated by the authors.

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Occurrence of Antibiotic Resistance in *Salmonella* Serotypes Isolated from Environment, Humans, Animals, and Animal Products in Morocco: A Systematic Review

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ABSTRACT

Several studies have been carried out in Morocco on *Salmonella* contamination in humans, domestic and wild animals, food products, and the environment. This bacterial genus is responsible for several infections and foodborne illnesses worldwide. The epidemiological situation of contamination by *Salmonella* is worsened by the development of antibiotic resistance to the main antibiotics used in human and veterinary medicine. The purpose of this study was to review the leading research carried out in this field, emphasizing the antibiotic resistance of this bacterium to antibiotics in humans and animals. Although some studies could not demonstrate the presence of *Salmonella* in the environments studied, the prevalence of contamination remained relatively high in humans, animals, food products, and the environment. The most critical contaminations were observed in poultry farms and poultry meat. *Salmonella* causes 42.8% of food poisoning cases in Morocco. It is the second most common cause of poisoning after pesticide poisoning. Morocco ranks first in the Middle East and North Africa for human salmonellosis, with a prevalence of 17.9% (1997-2012). Its prevalence in food products, especially those of animal origin, is very high and could reach 52.9% in turkey meat. Food products have been studied more for their contamination by *Salmonella* species. Meat products accounted for 17.35% of food poisoning cases. This study revealed that the isolation rate of *Salmonella* from food products of animal origin was dominated by isolations from meat products, with prevalence rates of 41.76 % from red meat and meat products and 25.88% from poultry meat, followed by prevalence rates of 12.44 % from fish products and 11.80 % from eggs. On the coast of Agadir, the incidence rates of *Salmonella* were 6.8% and 4.1% in sediment and seawater, respectively. This occurrence was 2.38% in the surface waters of Oued Khoumane. The development of resistance, particularly multi-resistance to antibiotics of therapeutic interest in both humans and animals, is alarming, especially with the ease of transmission of the bacterium to humans and facilitates its dissemination. Research findings indicated that 93.02% of isolates of *Salmonella* from humans, 79.37% of the strains isolated from poultry, and 46.27% of isolates from food products were resistant to at least one antibiotic.

Keywords: Animals, Environment, Food products, Foodborne disease, *Salmonella*, Resistance

INTRODUCTION

The increasing emergence of salmonellosis threatens the effective control of human foodborne diseases (Ziyate et al., 2016). In European countries, salmonellosis is the second most frequently reported zoonotic infection (Schmid and Baumgartner, 2013). Non-typhoidal *Salmonella enterica* is a leading bacterial that causes acute gastroenteritis in both children and adults (Al-Rifai et al., 2020).

From 2007 to 2011, more than 6960 cases of food poisoning, including 24 deaths, were listed by the Moroccan Poison Control and Pharmacovigilance Center (Rebgui et al., 2013). *Salmonella* would be responsible for 42.8% of food poisoning cases in Morocco, knowing that the incidence of foodborne disease is often underestimated (Cohen et al., 2007). Similarly, a retrospective study conducted by the Moroccan Poison Control and Pharmacovigilance Center covering the period of 2010-2016 revealed 17,076 foodborne diseases, of which 2,963 (17.35%) were linked to the consumption of flesh foods. The majority of cases occurred in urban areas, with a rate of 67.06% (Boukili et al., 2019). The risk assessment of foodborne bacterial pathogens in axis Rabat-Casablanca Morocco revealed that the raw products, particularly poultry meat and red meat, were most contaminated by *Salmonella* with the respective prevalence of 21.05% and 5% (Oba et al., 2014)

On a local scale, the analysis of the epidemiological characteristics of foodborne diseases at the prefecture of Agadir over 3 years (2015-2017) showed 11 foodborne disease outbreaks concerning 163 poisoned individuals with 2 cases of death. *Salmonella* species and *Escherichia coli* (*E. Coli*) were identified in four of six samples subjected to microbiological analysis (Bouchriti et al., 2021). In a similar study in Kenitra, Morocco, 43 foodborne disease outbreaks were reported between 2001 and 2018, affecting 367 poisoned individuals, including 69 hospitalized and 2 death cases. In that study, 25% of the samples revealed the presence of *Salmonella* (Elkhal et al., 2021).

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As part of the salmonellosis epidemiological surveillance in some European countries, salmonellosis cases are often reported in people who have stayed in Morocco. Thus, the European Center for Disease Prevention and Control reported an increase in salmonellosis cases in six European countries due to *Salmonella* Chester in patients who would have traveled to Morocco between 2014 and 2015 (Fonteneau et al., 2017). Other countries have reported isolation in people with digestive disorders who have recently traveled to Morocco, including travelers from France during 2000-2011 (Le Hello et al., 2013), Switzerland during 2011-2012 (Schmid and Baumgartner, 2013), Finland during 1995-2009 (Hinkka, 2011) and in Norway during 2000-2016 (Siira et al., 2019).

The widespread multidrug resistance to antibiotics in human, avian, aquacultural, and environmental *Salmonella* is increasing and has been confirmed by numerous epidemiological studies in Morocco. Thus, En-nassiri et al. (2017) reported the emergence of multiresistant *Salmonella* to fluoroquinolones and third-generation cephalosporins, prescribed in the treatment of severe salmonellosis in adults and children, which is a real public health problem.

The current study aimed to review the leading research and highlight the prevalence, serotypes distribution, profiles, and importance of *Salmonella* isolated from humans, animals, food products, and the environment in Morocco.

METHODOLOGY

The current review covered the studies addressing prevalence, serotype, and antibiotic resistance tests in *Salmonella* isolates in Morocco. The study was performed using the descriptive literature review method. The diagram of the article selection process is inspired by the methodology adopted by Al-Rifaia et al. (2020). The eligibility criteria for studies included the completion of the study on animals, animals' products, the environment, and humans in Morocco in the last 25 years. The studies must be related to the serotyping of the isolated *Salmonella*, and the antibiotic resistance test must be carried out on the isolates. This review study aimed at elucidating the isolation of *Salmonella* from the environment, animals, animal products, and humans.

Since there has been a limited number of studies in Morocco, a general search of several databases was performed to collect the maximum number of studies on *Salmonella* and salmonellosis in Morocco. The search was operated on ProQuest, Cochrane Library, Web of Science, PubMed, CABDirect, Agricultural Documentation Center (CDA-IAV Hassan II), IMIST library, GeoScienceWorld, and EBSCO. The number of bibliographical resources consulted for this study consisted of 114 studies. Several articles, end-of-study dissertations, and research theses in Morocco or having a relationship with *Salmonella* carriage at the national level were derived from the studies abroad on isolates from humans who would have consumed Moroccan food products. Exclusion criteria are shown in Figure 1.

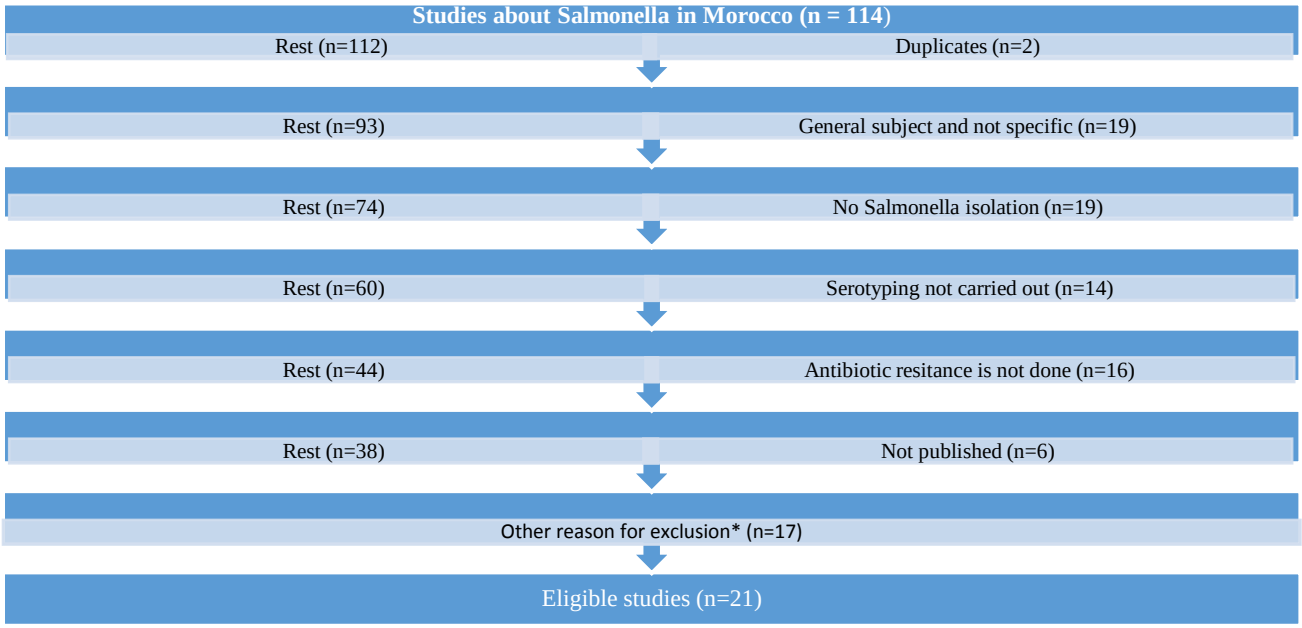

Figure 1. Flowchart of the study eligibility process. * Ancient food of vegetable origin, results not precise, and typhoid (not NTS)

The number of bibliographical resources eligible for this study consists of 21 references from scientific publications. Among these sources, the number of references for serotyping and antibiotic resistance of isolated *Salmonella* was 13 for food products, 6 for humans, 3 for the environment, and 2 for those isolated from animals (poultry). Some source studies have been concerned with more than the studied factors (animal and animal product/environment). The collected data were classified in Excel spreadsheets for their exploitation. Data were classified in terms of food product isolation (animal

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species, and food product), serotype, and antibiotic resistance profiles. Table 1 shows the list of studies selected for this study.

Table 1. List of studies eligible for this analysis

Authors	Product/species concerned by the study
Amajoud et al. (2017)	Dairy products, Red meat and meat products, Poultry meat
Ammari et al. (2009)	Food products, Humans
Ben Moussa (2014)	River water
Bouchrif et al. (2009)	Dairy products, Red meat and meat products, Poultry meat, Fish products
Boutaib et al. (2011)	Fish products (bivalve mollusk)
Dejlil et al. (2000)	Food products
Ed-Dra et al. (2018)	Food products
Ed-Dra et al. (2019)	Meat products (Chicken)
El Allaoui et al. (2013)	Turkey meat
El Allaoui et al. (2017)	Turkey
Elared et al. (2001)	Eggs
Fonteneau (2017)	Humans
Karraouan et al. (2010)	Turkey meat
Khallaf M. et al. (2014)	Chicken meat
Le Hello et al. (2013)	Humans
Siira et al. (2019)	Humans
Murgia et al. (2015)	Dairy products, red meat and meat products, Poultry meat, Fish products, Snails
Nassri et al. (2021)	Spring water
Ohmani et al. (2010)	Humans
Zahli et al. (2022)	Chicken meat
Ziyate et al. (2016)	Laying hens

RESULTS

Animals

Most of the studies carried out in Morocco on the *Salmonella* contamination and carriage of animals have addressed the poultry sector since it is the sector most concerned by the presence of *Salmonella*, particularly broiler chickens and turkeys. Several studies on the search for the genus *Salmonella* have been conducted in this sector. The data relating to the analysis of these studies are listed in Table 2, which concerns only two publications on poultry farms.

Studies revealed a highly variable prevalence of *Salmonella* contamination in poultry farms depending on the region and the sub-sector. It was found that 24% of broiler farms were infected with *Salmonella* spp. in Meknes (Chaiba and Rhazi Filali, 2016). In laying hen farms, 76.7% were contaminated by the *Salmonella* genus, with very significant regional variations ranging from 100% in farms in the Greater Casablanca region to 50% of contaminated farms in the Rabat Sale Zemmour Zaer region to 87.5% in the Sous Massa Drâa region (Ziyate et al., 2016).

However, in a recent study performed in the region of Azemmour (El Jadida-Morocco), the absence of *Salmonella* in breeder farms, broiler farms, and laying hen farms was reported due to size-reduced samples (4 farms per category), and the low quality of health supervision and the establishment of health barriers or vaccination in laying hens (Karib et al. 2021). Furthermore, the reported prevalence in broiler turkey farms in the Khémisset region was 35% (El Allaoui et al., 2014). In local free-range chicken farms, still called traditional or more commonly "Beldi" chicken, the prevalence of *Salmonella* was 6%, 10%, and 5.6% in the locality of Zemamra (El Jadida), Marrakech, and Khénifra, respectively. Seroprevalences of typhosis and pullorosis were 7.6% and 4.4%, respectively (Fagrach, 2021). It is well established that the "Beldi" chicken is a reservoir of *Salmonella* Gallinarum and *Salmonella* that continually threatens the industrial poultry sector (Bouzoubaa et al., 1992; Fagrach et al., 2021).

The serotyping of 126 strains of *Salmonella* isolated from broiler chickens and broiler turkeys is described in Table 2. Ziyate et al. (2016) and El-Allaoui et al. (2017) found that the strains belonged to 12 different serotypes. The predominance of the *S*. Kentucky serotype is notable, with a frequency of 32.54%, of which 51.2% is isolated in turkeys and 48.8% in laying hens, followed by the *S*. Enteritidis serotype representing 22.22% of the isolated serotypes, mainly in laying hens with a percentage of 85.71% of the isolates.

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Table 2. Distribution and antibiotic resistance patterns of *salmonella* species isolated from poultry farms in Morocco

Animals	Authors	Serotype	Number of Isolates		Sensitive	Amx	Na	Cip	Caz	Amc	Ctx	C	S	Te	Tmp	Cro	SxT	CEP	Gm	Am	k	SU	Spt
			By animal species	Total																			
Laying hens	Ziyate et al. (2016)	*S.* Amsterdam	2	2	2																		
Laying hens	Ziyate et al. (2016)	*S.* Enteritidis	24	28	1		23																
Turkey	El Allaoui et al. (2017)		4		2		1							2						2	2		
Turkey	El Allaoui et al. (2017)	*S.* Agona	7	10	0				3	2	3		4	4	3	3	3		1	5			
Laying hens	Ziyate et al. (2016)		3		3																		
Laying hens	Ziyate et al. (2016)	*S.* Thompson	4	4	4																		
Laying hens	Ziyate et al. (2016)	*S.* Infantis	7	7	7																		
Turkey	El Allaoui et al. (2017)	*S.* Heidelberg	4	4	1							2	2	3									
Turkey	El Allaoui et al. (2017)	*S.* Newport	3	3	0							1	2	3	3					1	1		
Turkey	El Allaoui et al. (2017)	*S.* Parkroyal	10	10	0					2		2	9	10	3		3			2	5		1
Turkey	El Allaoui et al. (2017)	*S.* Ruzizi	2	2	0								1	2	1								
Turkey	El Allaoui et al. (2017)	*S.* Saintpaul	6	6	0					1			5	5	3		5			1		3	
Laying hens	Ziyate et al. (2016)	*S.* Typhimurium	4	9	1	3							1	3									
Turkey	El Allaoui et al. (2017)		5		1		1			2		2	1		3					1			
Laying hens	Ziyate et al. (2016)	*S.* Kentucky	20	41	4	13	16	16					11	14				13	6			11	
Turkey	El Allaoui et al. (2017)		21		0		21	20		5		1	21	20	3		4	0	12	9	3		18
		Total	**126**	**126**	**26**	**16**	**62**	**36**	**3**	**12**	**3**	**8**	**57**	**66**	**19**	**3**	**15**	**13**	**19**	**21**	**11**	**14**	**19**
			Percentage		**20.63**	**12.7**	**49.21**	**28.6**	**2.38**	**9.52**	**2.38**	**6.35**	**45.2**	**52.38**	**15,08**	**2.38**	**11.9**	**10.3**	**15.1**	**16.7**	**8.73**	**11**	**15.1**

Am : Ampicilline **Caz :** Ceftazidime **Ctx :** Cefotaxime **S :** Streptomycin **Te :** Tetracycline

Amc : Amoxicilline/acide clavulanique **CEP :** Cephalothin **Gm :** Gentamycine **Spt :** Spectinomycine **Tmp :** Trimethoprim

Amx : Amoxicilline **Cip :** Ciprofloxacin **K :** Kanamycine **SU :** Sulfametoxaozal

C : Chloramphenicol **Cro :** Ceftriaxone **Na :** Nalidixic acid **Sxt :** Sulfametoxaozal/ trimethoprim

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In the same studies, the antibiotic resistance profile of strains isolated from poultry farms showed that 79.37% of the strains were resistant to at least one antibiotic (Ziyate et al., 2016; El Allaoui et al., 2017). *Salmonella* presents significant antibiotic resistance to tetracyclines, nalidixic acid, and streptomycin at 52.38 %, 49.21%, and 45.20%, respectively. All *S.* Newport, *S.* Parkroyal, *S.* Ruzizi, *S.* Saintpaul, *S.* Kentucky, and *S.* Agona strains isolated from turkey are antibiotic-resistant. However, all isolated strains, including *S.* Thompson, *S.* Infantis, and *S.* Amsterdam, as well as all *S.* Agona isolates from laying hens, indicated sensitivity to all the antibiotics studied (Ziyate et al., 2016; El Allaoui et al., 2017). The studies carried out in Morocco on the carriage of *Salmonella* by other domestic or wild animal species are old, fragmentary, and limited to certain areas of the country. The study by El Jai et al. (2003) on the causes of abortions in sheep allowed the isolation of *Salmonella* Abortusovis from the vaginal swabs of aborting ewes with a prevalence of 4.8% (n = 8820). With the same objective, a similar study on the same species in the regions of Zaer and the Middle Atlas allowed the detection of *Salmonella* antibodies with seropositivity rates in sheep herds of up to 5% (n = 604, El Idrissi et al., 1995).

Food products with animal origin

Prevalence at the product level

The *Salmonella* prevalence depends on the size of the sample studied; thus, the systematic review and meta-analysis of data on the prevalence of non-typhoid *Salmonella* in food products marketed in the countries of the MENA region (Middle East and North Africa) concluded that the prevalence in studies on less than 100 samples was 13.4%, whereas, it was only 4.1% in studies performed on more than 100 samples (Al-Rifai et al., 2020). As shown in Table 3, contamination of food products with non-typhoid *Salmonella enterica* is relatively common in food products consumed in MENA countries, with a combined global prevalence of 8.8% (Al-Rifaï et al., 2020). The variable trend in detection rates between countries could be attributed to the variability of the laboratory methods used, the types of food products analyzed, and their origins (local or imported, Habib et al., 2021). Meat products are the most frequently contaminated by *Salmonella*, predominately in poultry meat and offal. The highest prevalence reported in Morocco is 52.9% in turkey meat (Amajoud et al., 2017) and minced meat and poultry liver, with prevalence rates of 40% and 33.33%, respectively (Bennani et al., 2016). The highest prevalence (30.6%) for red meats and meat products was found in artisanal sausages (Ed-Dra et al., 2018).

Table 3. Meta-analyses of studies reporting non-typhoidal *Salmonella* in Morocco according to the tested food products

Food products	Number of studies	Analyzed samples	*Salmonella* positive	*Salmonella* prevalence		
				Range (%) Morocco	Median (%) Morocco	Pooled prevalence MENA
Animal products	18	9622	227	0.0-52.9	5.4	6.8
Fish products	6	893	46	0.0-38.4	8.9	7.7
Plant products	4	2342	6	0.0-2.0	0.1	0.0
Mixed products	4	858	11	1.7-2.0	1.6	1.1
Overall	32	13715	290	0.0-52.9	2.8	4.5

Source: Al-Rifai et al. (2020)

Distribution of isolates by serotype and by food products

Table 4 shows the results of serotypes of *Salmonella* isolated from food products of animal origin in Morocco. The findings indicated that 510 strains of *Salmonella* were isolated from food products distributed on 43 *Salmonella* serotypes, including *Salmonella* isolates that were not typable or on which serotyping was not done.

The most isolated serotypes from food products of animal origin are in descending order as *S.* Enteritidis (n = 74) at 14.51%, *S.* Infantis (n = 71) at 13.92%, *S.* Kentucky (n=54) at 10.59%, *S.* Typhimurium (n = 31) at 6.08%, *S.* Bredeney (n = 30) at 5.88%, *S.* Mbandaka (n = 28) at 5.49%, *S.* Blockley (n=23) at 4.51%, *S.* Saintpaul (n = 18) at 3.53%, *S.* Corvallis (n = 16) at 3.14%, *S.* Agona (n = 15), and *S.* Hadar (n=15) at 2.94%. Non-typable *Salmonella*, or those on which serotyping has not been carried out constituted a significant part of the isolations, including 16 isolates (3.14%).

As shown in figures 2 and 3, the genus *Salmonella* is essentially linked to meat products since 67.65% of isolates were made from these products, particularly from red meats and meat products (41.76% of isolates) and poultry meat (25.88% of isolates). However, some serotypes are much more food products bound than others. Thus, the isolation from red meat and meat products of the following serotypes was in descending order, including *S.* Montevideo (100%), *S.* Kiambu (100%), *S.* Mbandaka (96.43%), *S.* Braenderup (88.89%), *S.* Anatum (87.5%), *S.* Indiana (85.71%) and *S.* Infantis (63.38). The isolation of *S.* Saintpaul (94.44%), *S.* Agona (86.67%), and *S.* Muenster (83.33%) was made mainly from poultry meat. Similarly, the *S.* Glostrup and *S.* Newport serotypes were more closely linked to fishery and aquaculture products, with isolation rates of 100% and 69.23%, respectively. Moreover, 77.03% of *S.* Enteritidis isolations were from eggs.

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Table 4. Distribution of *Salmonella* isolates by serotype and by food products in Morocco

	FOOD PRODUCTS															
	Isolates		**Poultry meat**		**Red meat and meat products**		**Fish products**		**Dairy product**		**Eggs**		**Food products**		**Snail**	
Serotype of *Salmonella*	**No.**	**(%)****	**No.**	**(%)****	**No.**	**(%)****	**No.**	**(%)****	**No.**	**(%)****	**No.**	**(%)****	**No.**	**(%)****	**No.**	**(%)****
Enteritidis	74	14.51	2	2.70	6	8.11					**57**	77.03	9	12.16		
Infantis	71	13.92	2	2.82	45	63.38			2	2.817			22	30.99		
Kentucky	54	10.59	18	33.33	8	14.81	26	48.15	2	3.704						
Typhimurium	31	6.08	12	38.71	16	51.61	1	3.226	1	3.226					1	3.23
Bredeney	30	5.88	4	13.33	24	80.00			2	6.667						
Mbandaka	28	5.49	1	3.57	27	96.43										
Blockley	23	4.51	7	30.43	6	26.09	10	43.48								
Saintpaul	18	3.53	17	94.44	1	5.56										
ND*	16	3.14	11	68.75	3	18.75			2	12.5						
Corvallis	16	3.14	4	25.00	12	75.00										
Agona	15	2.94	13	86.67	2	13.33										
Hadar	15	2.94	8	53.33	1	6.67	6	40								
Glostrup	13	2.55					13	100								
Newport	13	2.55	4	30.77			9	69.23								
Montevideo	10	1.96			10	100										
Braenderup	9	1.76	1	11.11	8	88.89										
Anatum	8	1.57			7	87.50			1	12.5						
Indiana	7	1.37	1	14.29	6	85.71										
Muenster	6	1.18	5	83.33	1	16.67										
Kiambu	6	1.18			6	100										
Reading	6	1.18	1	16.67	5	83.33										
Bovismorbificans	4	0.78			4	100										
Chester	4	0.78	4	100												
Give	4	0.78			4	100										
Altona	3	0.59	1	33.33	2	66.67										
Heidelberg	3	0.59	3	100												
Schwarzengrund	3	0.59	3	100												
Berta	2	0.39			2	100										
Labadi	2	0.39			1	50.00	1	50								
Senftemberg	2	0.39			2	100										
Aalbert	1	0.20	1	100												
Bareilley	1	0.20			1	100										
Cerro	1	0.20			1	100										
Derby	1	0.20	1	100												
Djugu	1	0.20	1	100												
Gallinarum	1	0.20	1	100												
Hatford	1	0.20	1	100												
Israel	1	0.20			1	100										
Kiel	1	0.20	1	100												
Livingstone	1	0.20			1	100										
Othmarschen	1	0.20	1	100												
Salamae (type II)	1	0.20	1	100												
Tennessee	1	0.20	1	100												
Zerifin	1	0.20	1	100												
Overall	510	100	132		213		66		10		57		31		1	
Percentage (%)	100		25.88		41.76		12.94		1.96		11.18		6.08		0.2	

* ND: isolates not serotypable or serotyping not carried out; **: Percentage isolation of the serotype from the food product; ***: The definition of the nature of the food products was not specified by the authors of the original study; No: Number

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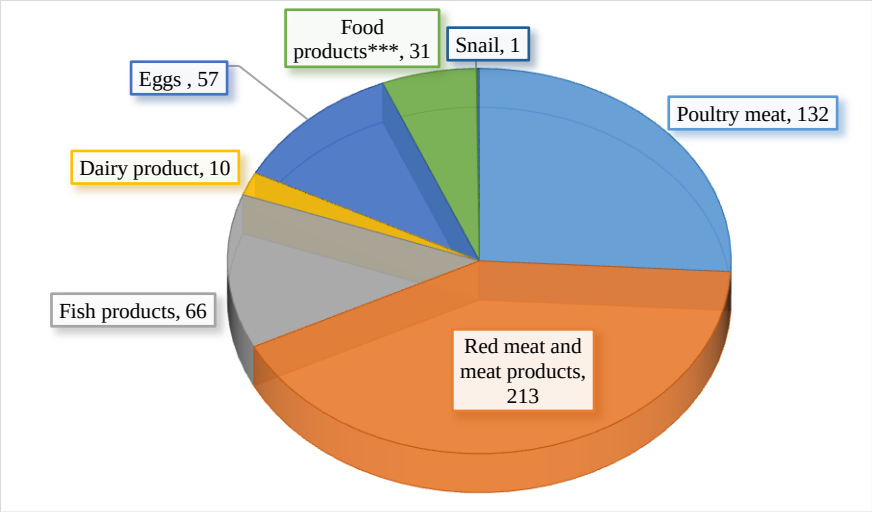

Figure 2. Number of isolates of *Salmonella* spp. from food products

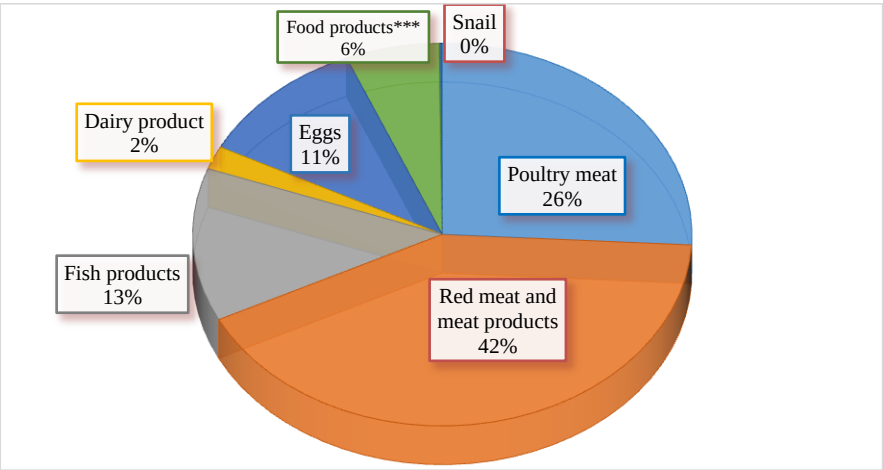

Figure 3. Percentage of isolates of *Salmonella* spp. from food products

Antibiotic resistance of isolates

Although some cases of salmonellosis can come directly from pets or contaminated water, the transmission rate through food was estimated at 95% (Korsak et al., 2004). Table 5 shows the antimicrobial resistance of *Salmonella* strains isolated from animal products in Morocco. Thus, the study of the antibiotic resistance of these isolates showed that 46.27% were resistant to at least one antibiotic. However, 53.73% of isolates were fully susceptible to the antibiotics tested. Including all isolates (n = 510), the antibiotic resistance was highest for *Salmonella* isolated from food products of animal origin against antibiotics with resistance rates of 17.88%, 16.67%, 15.29%, 11.76%, and 10.98% for amoxicillin, ampicillin, tetracyclines, nalidixic acid, and streptomycin, respectively.

The isolates showed a wide range of antibiotic resistance profiles that varied from one serotype to another or even within the same serotype. Some isolated serotypes showed a higher rate of antibiotic resistance. The *S.* Glostrup and *S.* Muenster serotypes are fully antibiotic resistant, followed by resistance rates of 92.59%, 86.67%, 83.33%, 77.42%, and 75% for *S.* Kentucky, *S.* Hadar, *S.* Saintpaul, *S.* Typhimurium, and *S.* Corvallis, respectively. On the other hand, the *S.* Bredeney, *S.* Infantis, and *S.* Anatum serotypes presented the lowest rate of antibiotic resistance at 10% and 11.27%, and 12.50%, respectively. The serotype *S.* Typhimurium isolated from food products of animal origin has antibiotic resistance to a wide range of antibiotics, including 30 different molecules with antibiotic resistance rates varying from 3.23% to 58.06%, depending on the molecules studied. Regarding *S* Typhimurium isolates from food products, it was found that 58.06% were resistant to amoxicillin, 48.39% to streptomycin, 45.16% to chloramphenicol, and 45.16% to amoxicillin-clavulanic acid. Boutaib et al. (2011) reported *S.* Glostrup serotype from aquaculture products (bivalve mollusks), with 13 isolates presenting the same multiresistant profile to tetracyclines, nalidixic acid, and ampicillin. Another most isolated serotype of the genus *Salmonella* with antibiotic resistance to antibiotics and anti-infectives among the dominant serotypes in food products was *S.* Kentucky, which showed antibiotic resistance to more than 22 molecules of different antibiotics. Furthermore, no resistance to cefquinome and imipenem has been reported; however, this finding should be taken cautiously, knowing that the range of antibiotics tested could vary from one study to another.

The antibiotic resistance profile of isolates is very diverse, ranging from resistance to one antibiotic, the most dominant profile, to multi-resistance to 18 different molecules of antibiotics. Of note, 15 different antibiotic resistance profiles have been identified in isolates from food products of animal origin; resistance to a single antibiotic constitutes was 38.30%, followed by multi-resistance to 3, 2, and 4 molecules of antibiotics at the rates of 18.72%, 17.87%, and 6.38%, respectively. Other profiles are illustrated in Table 6. Most isolates are resistant or multiresistant to less than 6 antibiotics with a rate of 91.91%.

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Table 5. Antimicrobial resistance of *Salmonella* strains isolated from animals' products in Morocco

	Isolats	Sensible	Amx	Cs	Na	Cip	Caz	Amc	Ct1	B	C	S	Te	Tmp	Cro	SSS	SxT	CEP	Gm	Am	Nor	Cn	Ipm	k	Cef	SU	Cxm	Enr	Ofx	UB	MA	Ak	CQ	F	Tic	FF	Lvx	Mec	Tim	PIP	Cfm	NN	ATM	FEP
Enteritidis	**74**	58	2	6	2								4				2		5	5														2	2									
Infantis	**71**	63								1			5							2																								
Kentucky	**54**	4	17		22	36		13			2	10	11			8		6	3	8	2					10		4	4	4	2					1	2	2	2	2				
Typhimurium	**31**	7	8	3	2			14	2	4	14	15	13	6	5	4	7	2	3	18	1	3		2		9	5			1	2	2						3	3	3	2	3	1
Bredeney	**30**	27	1							1										1						1										1			1	1				
Mbandaka	**28**	15		2							1	2	5							6				3	0	1																	
Blockley	**23**	12									1	0	7				7	1	0	0																							
Saintpaul	**18**	3	8	5	1					10		4		8		3	4			1						1										1								
Corvallis	**16**	4	4	2							1	7	1			1				8						4	1																	
ND*	**16**	9	1					3			1		4							3						2																		
Agona	**15**	8	5	1			1	1	1	6	3	2	0	4	1					1																								
Hadar	**15**	2			13	1		4			1	5	9	1			1			4	1			0	2	1											1						
Glostrup	**13**	0			13								13							13																								
Newport	**13**	7	1		3						1	0	4					1	0	1						1																	
Montevideo	**10**	6		3								3	0							3				1																				
Braenderup	**9**	4										0	1							0				3		1																		
Anatum	**8**	7		1								1	0							1						0																		
Muenster	**6**	0			1															1						4																		
Reading	**6**	5														1																												
Bovismorbifican	**4**	2										2								2																								
Chester	**4**	0												2			2				4																			1				
Give	**4**	0										4								4																								
Heidelberg	**3**	0								3				1																													
Schwarzengrund	**3**	0	1		2	2													1	2	2																		2	1	2	2			
Aalbert	**1**	0			1						1	1	1																														
Derby	**1**	0	1					1			1															1																		
Gallinarum	**1**	0									1															1																		
Israel	**1**	0												1		1	1																											
Livingstone	**1**	0																		1																								
Sérotypes sensibles	31	31																																										
Overall	**510**	**274**	**49**	**23**	**60**	**39**	**1**	**36**	**3**	**25**	**28**	**56**	**78**	**23**	**6**	**18**	**24**	**10**	**12**	**85**	**10**	**3**	**0**	**9**	**2**	**37**	**6**	**4**	**4**	**5**	**4**	**2**	**0**	**2**	**2**	**3**	**5**	**4**	**8**	**8**	**3**	**2**	**3**	**1**
Percentage (n = 510)	**100**	**53.3**	**17.88**	**4.51**	**11.76**	**7.65**	**0.20**	**7.06**	**0.59**	**4.90**	**5.49**	**10.98**	**15.29**	**4.51**	**1.18**	**3.53**	**4.71**	**1.96**	**2.35**	**16.67**	**1.96**	**0.59**	**0.00**	**1.76**	**0.39**	**7.25**	**1.18**	**0.78**	**0.78**	**0.98**	**078**	**0.39**	**0.00**	**0.39**	**0.39**	**0.59**	**0.98**	**0.78**	**1.57**	**1.57**	**0.59**	**0.39**	**0.59**	**0.20**

Ak:	Amikacin	CEP:	Cephalothin	Enr:	Enrofloxacin	MA:	Cefamandole	SSS:	Sulfonamide
Am:	Ampicilline	Cfm:	Cefixime	F:	Furane	Mec:	Mecillinam	SU:	Sulfametoxaozal
Amc:	amoxicillin-clavulanic acid	Cip:	Ciprofloxacin	FEP	Cefepime	Na:	Nalidixc Acid	SxT:	Sulfametoxaozal/ trimethoprim
Amx:	Amoxicilline	Cn:	Cefalexine	FF:	Fosfomycin	NN:	Tobramycin	Te:	Tetracycline
ATM:	Aztreonam	CQ:	Céfquinome	Fox:	Cefoxitin	Nor:	Norfloxacine	Tic:	Ticarcillin
B:	Bacitracin	Cro:	Ceftriaxone	Gm:	Gentamycine	Ofx:	Ofloxacin	Tim:	Ticarcillic-Clavulanic acid
C:	Chloramphenicol	Cs:	Colistine	Ipm:	Imipénème	PIP:	Piperacillin	Tmp:	Trimethoprim
Caz:	Ceftazidime	Ctx:	Cefotaxime	K:	Kanamycine	S:	Streptomycin	UB:	Flumequine
Cef:	Cefazoline	Cxm:	Cefuroxime sodium	Lvx:	Levofloxacin	Spt:	Spéctinomycine		

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Table 6. Resistance profile regarding the number of antibiotics resistant to *Salmonella* isolated from food products in Morocco

																Total
Number of antibiotics	1	2	3	4	5	6	7	8	9	10	11	12	13	16	18	**15**
Number of isolates	90	44	42	15	13	12	3	3	2	2	1	4	2	1	1	**235**
Percentage	38.30	18.72	17.87	6.38	5.53	5.11	1.28	1.28	0.85	0.85	0.43	1.70	0.85	0.43	0.43	**100**

Occurrence of *Salmonella* in the environment

There are a few studies concerning the presence of *Salmonella* in Morocco. When Salmonella is isolated from the environment, additional investigations such as serotyping and antibiotic resistance are rarely done. The significant contamination of poultry farms, particularly turkey and laying hen farms, by resistant *Salmonella* promotes the spread and dissemination of this bacterium in other ecosystems, such as the sea coast (Karraouan et al., 2017). Similarly, *Salmonella* contamination can be introduced into the Atlantic coast (between Essaouira and Anza) by sewage or precipitation flows (Mannas et al., 2014). The poultry farming activity developed under the impetus of private investors with sustained growth from the 1960s around the major cities of the Atlantic zone (Sraïri, 2011). The presence of clinically important *Salmonella enterica* in natural waters follows the same trends as infection in humans and wildlife in the same area, suggesting a common origin of this germ (Nassri et al., 2021).

Most of the research work on *Salmonella* in the environment has been carried out in water environments, such as wells (Lotfi et al., 2020, Nassri et al., 2021), running waterways (Ben Moussa et al., 2014), water from dams (Chahboune et al., 2014), wastewaters (Ait Melloul and Hassani, 1999), and seawater (Setti et al., 2009). However, the complete study by serotyping and the study of antibiotic resistance is generally not carried out or not reported. Therefore, a comparative study addressing the contamination of the environment by *Salmonella* cannot be carried out.

Occurrence of *Salmonella* in humans

An increase in international traveling, immigration, and trade accelerates the spread of *Salmonella* pathogens (Hinkka, 2011). *Salmonella* gastroenteritis was the first disease source, with more than 39,983 cases reported in an investigation conducted between the years 1995 and 2015 in Finland on the risk of acquiring foodborne diseases and notifiable sexually transmitted infections among Finnish international travelers (Zöldia et al., 2018). Human movement facilitates the spread of resistant bacteria and antimicrobial resistance genes globally. The enteric species accounted for 65% of the 26 identified bacterial species with drug resistance (Bokhary et al., 2021). The analysis demonstrated an increase in the total number of resistant *Salmonella* spp. associated with travel from 1553 in 1990-1999 (25.75%) to 3549 in 2000-2009 (58.84%). The rates of reporting quinolone-resistant and multidrug resistance *Salmonella* spp. increased from 9.52% and 22.94% in 1990-999 (n = 283 and 329) to 84.40% and 29.08% in 2000-2009 (2510 and 417), respectively (Bokhary et al. 2021).

Morocco comes first among the MENA region countries with the highest *Salmonella* prevalence rate at 17.9%, far exceeding the general average for this region, which is estimated at 6.6% (Al- Rifai et al., 2019). The lack of widespread wastewater treatment and its use in irrigation contributes to the increase in the prevalence of salmonellosis in the exposed population. The prevalence of *Salmonella* carriage is higher in children exposed to wastewater (32.56%) than those who are not exposed with a high risk in male children younger than 10 years and sons of farmers, compared to daughters and children aged over 10 and sons of non-farmers (Ait Melloul and Hassani, 1999).

However, *Salmonella* isolated from wastewater and stool samples from hospitalized children living in the sewage-spreading field of the city of Marrakech showed a different profile from the general finding of antibiotic resistance. The percentage of antibiotic resistance is higher in isolates from children's stools, compared to isolates taken directly from wastewater. In addition, these isolates are rather very sensitive to cefotaxime, gentamicin, trimethoprim-sulfamethoxazole complex, nalidixic acid, kanamycin, trimethoprim, and chloramphenicol with the respective percentages of 100%, 99.88%, 98.04%, 98.04%, 97.30%, 97.07%, and 96.07%, respectively. In comparison, the highest levels of antibiotic resistance were observed for cephalothin, amoxicillin, sulfamethoxazole, and ampicillin, with respective percentages of 29.27%, 26.44%, 26.7%, and 25.21%, respectively (Ait Melloul and Hassani, 1999).

In terms of resistance profile based on the number of antibiotic resistance molecules, the number of isolates resistant to a single antibiotic molecule is dominant, with more than 79.17% of isolates, 90.53% of which are resistant to nalidixic acid. In comparison, resistance profiles to 2 and 4 molecules of antibiotics are 7.5% and 5%, respectively, which are of less importance. The presence of isolates multiresistant to a wide range of antibiotics in humans is worrying, although their percentage is small. Le Hello et al. (2013) reported that some *S.* Kentuchy isolates are simultaneously multiresistant to 11, 13, 14, or 15 antibiotics; this broader antibiotic resistance profile is only observed in this serotype (Le Hello et al., 2013).

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The *S.* Kentuchy serotype is fully multiresistant, while the *S.* Enteritidis and *S.* Chester serotypes showed significant antibiotic resistance of 92.54% and 92.45%, mainly to nalidixic acid with rates of 79.1% and 88.68%, respectively.

However, there is a limited number of publications and an insufficient study of antibiotic resistance to *Salmonella* isolated from humans in Morocco. Nevertheless, the data analysis shows that *S.* Enteritidis and *S.* Chester are the most frequently isolated at the rates of 51.94% and 41.09% of isolates of *Salmonella* in humans in Morocco, respectively. The *S.* Kentuchy is occasionally isolated at a rate of 4.65%, occupying the third place or among international travelers (Europeans) who have been infected following their stays in Morocco.

Regarding the conducted studies, the antibiotic resistance of *Salmonella* isolates in humans shows that 93.02% of isolates are resistant to at least one antibiotic. These isolates are resistant to a wide range of antibiotic molecules composed of 23 molecules. The majority of isolated *Salmonella* species are resistant to nalidixic acid, with a percentage of 82.3% of all isolates. In addition, less resistance was noticed to tetracyclines, sulfametoxaozal/trimethoprim, and ampicillin at the rates of 14.6%, 11.5%, and 10%, respectively.

DISCUSSION

Salmonella is among the leading causes of collective food poisoning, which can be considered one of the primary causes of infant mortality in developing countries. Control measures are established through control strategies at the level of the different countries in different ways but which remain insufficient (Nacer et al., 2021).

In Morocco, epidemiological studies show the emergence of multi-resistance in *Salmonella* of human, avian, aquaculture, and environmental origins. The main cause is the uncontrolled use of antibiotics in public health and veterinary medicine (En-Nasiri et al., 2017). Thus, the survey was conducted on the use of antibiotics in poultry farms carried out considering the leading private veterinary practices whose main activity is the health supervision of these farms. The findings revealed that 93% of flocks received at least one antibiotic treatment for a minimum of 3 days in Morocco broiler farms (Rahmatallah et al., 2018). In another study, 96.55% of practicing veterinarians surveyed in the eastern region of Morocco considered the abuse of antibiotics in poultry farms by self-medication, 93.11% of them linked it to the purchase of drugs without a prescription, and 89.66% of respondents judged that it was related to the use of contraband antibiotics (El-Youbi et al., 2016).

The emergence of poultry units and the food chain of *Salmonella* strains resistant to antibiotics is considered a cross-sectoral problem. Resistant bacteria and antibiotic-resistance genes can quickly spread through each stage of the food production chain and can cause human infections (Nacer et al., 2021).

Salmonella is ubiquitous in different environments and food products. Their prevalence can reach 52.9%, recorded in turkey meat by Amajoud et al. (2017). All the studies carried out in Morocco agree that the prevalence of *Salmonella* is high in the poultry sector, in particular, in farms intended to produce meat and eggs for consumption. The rate of antibiotic resistance and multi-resistance is alarming. In addition, Morocco comes first among Arab countries in terms of the prevalence of Non-Typhoidal *Salmonella* in humans according to a systematic review and meta-analysis in Arab countries with a prevalence of 17.9%, ranging from 5.7 to 34.8% during 1997-2012 (Habib et al., 2021). In the same geographical area, the predominant serotypes isolated from an environment or food products may not be the same reported in humans (Setti et al., 2009).

Whatever the medium of origin of *Salmonella*, it will eventually be transmitted to humans and therefore constitute a public health problem. However, studies concerning the circulation of *Salmonella* in different environments and *Salmonella* carrier food products are rare. Most studies relating to *Salmonella* are limited to an animal species, a category of products, or a circumscribed region or carried out over a limited period, and even when they exist. The analysis is often not exhaustive since it does not convert the serotyping of the *Salmonella* isolates or not studying their antibiotic resistance profiles.

Thus, the control of the expansion of antibiotic resistance should be considered a public health priority adopting an intersectoral and intermenstrual approach according to the concept “One Health” advocated by the World Health Organization (WHO) and the World Organization for Animal Health (WOAH). This is the case of the USA, which has decided to ban several antibiotics used in humans in poultry farms so that currently, 95% of the chicken produced in the USA is antibiotic-free (Nacer et al., 2021). Similarly, since non-typhoid *Salmonella* are often associated with increased morbidity and mortality, the determination of antibiotic resistance patterns should be considered an essential part of the surveillance of this *Salmonella* in food safety laboratories for public health in Arab countries (Habib et al., 2021).

CONCLUSION

The food products are the most studied in Morocco for the contamination of *Salmonella*. In animals, most studies investigated *Salmonella* contamination in poultry farms. The other animals have been rarely studied especially domestic

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animals, such as cattle, sheep, goats, and camels. Isolation of *Salmonella* from humans and the environment are seldom studied. The contamination with non-typhoid *Salmonella* is relatively common in food products consumed in Morocco, with a global prevalence of 4.5%, meat products are the most frequently contaminated by *Salmonella*, with a predominance in red meat and meat products.

The antibiotic resistance of *Salmonella* isolated in Morocco is alarming both from the nature of antibiotics and the number of multidrug resistances. The antibiotic resistance profile of strains isolated from poultry farms shows that 79.37% are resistant to at least one antibiotic; 93.02% of isolates of *Salmonella* from humans are resistant to at least one antibiotic. A rate of 46.27% of the *Salmonella* isolated from food products is resistant to at least one antibiotic. Considering the number of antibiotics to which the isolates are resistant, resistance ranges from resistance to one antibiotic, which is the most dominant profile (38.30% of resistance), to multi-resistance to 18 different molecules of antibiotics.

Although there are many studies on *Salmonella* contamination in Morocco, they remain specific, partial, sectoral, and often incomplete. This shows the importance of setting up a national observatory for surveillance and epidemiological monitoring and coordinating actions between the various departments, particularly those acting in food safety and public health. The national observatory must focus on the evolution of the antibiotic resistance character in the genus *Salmonella*, Campylobacters, *E. coli* and all pathogens transmitted by food. The study of all animal species producing food products is necessary, especially *Salmonella* contamination of meat products, while the study of contamination in ruminant breeding is rarely reported. The use of new identification methods, such as Whole Genome Sequencing is essential in the epidemiological investigations of cases of foodborne diseases for tracing the routes of transmission and origin of *Salmonella*.

DECLARATIONS

Authors' contribution

All the authors contributed to the research of the notification data, their analyses, and the writing of the final manuscript. Pr Karib Hakim and Pr Nassik Saadia participated in the collection of scientific articles and the writing of the french version. Pr Bouchriti Nourreddine and Pr Nassik participated in translating the document; Dr Motassim El Hanafi participated in data collection and analysis, and coordination. All authors participated in the advancement of the research, the development of the document, and data analysis.

Competing interests

The authors of the present study declared that there is no financial or unethical conflict related to this work, which can negatively impact its publication.

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Ethical considerations

The authors have checked for ethical issues, such as plagiarism, approval of public misconduct, data fabrication or falsification, duplicate publishing or submission, and redundancy.

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Strategies for Prevention and Control of Multidrug-resistant Bacteria in Ruminants

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ABSTRACT

Antibiotics are no longer effective in treating bacterial infections due to antimicrobial drug resistance. Therefore, various alternative strategies have been developed to combat multidrug-resistant (MDR) bacteria. The current review article aimed to shed light on strategies to prevent and control MDR bacteria in ruminants. Due to the development of new resistant bacteria, there is a need for effective treatments and prevention protocols in livestock and humans. With growing antibiotic-resistant organisms, a few antimicrobial medicines will be available to treat the infection when no new drugs are developed. This highlights the importance of looking for other strategies for combating antibiotic-resistant bacteria. In this regard, alternative strategies have been proposed to minimize antimicrobial drug overuse in ruminants. These alternative procedures include alternatives for growth promotion (such as in-feed enzymes, probiotics, prebiotics, synbiotics, and antimicrobial peptides), alternatives for disease prevention (such as vaccines, immune modulators, chicken egg yolk antibodies, farm management, and biosecurity), and alternatives for disease treatment such as plant extracts and phage-therapy to antibiotics. These alternative methods should be safe and efficient without inducing microbial resistance.

Keywords: Antibiotic, Bacteria, Multidrug-resistant, Medicine, Ruminants

INTRODUCTION

The increase in bacteria that are resistant to antibiotics increases the need for research to find alternative strategies to reduce the use of antibiotics in animals (Aizawa et al., 20016; Alfredo and Rodríguez-Hernández, 2017; Abdalhamed et al., 2018). Alternatives products with antibiotics activity should be non-toxic, easily eliminated from the body, stable through the gastrointestinal tract, easily decomposed, friendly to the environment, selectively active against pathogens with minimum effects on host gut flora, and also have a positive impact on feed efficiency and promote growth. In addition, be effective for prevention and treatment against multidrug-resistant (MDR) bacterial pathogens (Ali and Dixit, 2012)

Alternatives strategies for prevention and control of MDR bacteria in ruminants include antibiotics for growth promotion (such as in-feed enzymes, probiotics, prebiotics and synbiotics, and antimicrobial peptides), alternatives to antibiotics for disease prevention (such as vaccines, immune modulators, chicken egg yolk antibodies, farm management, and biosecurity), alternatives to antibiotics for disease treatment (such as phytochemicals plant extracts and phage-therapy), and other alternatives for disease prevention include Nanoparticles (NPs) (Alwhibi and Soliman, 2014). Alternative methods may reduce antibiotic resistance but could not be a substitution for antibiotics (Aizawa et al., 20016; Babutan et al., 2021).

Antimicrobial peptides are host defense peptides, abundantly distributed in nature, and used as alternatives to antibiotic therapy, as they have direct and indirect inhibitory effects on pathogenic bacteria (Alfredo and Rodríguez-Hernández, 2017). Using vaccines against infectious diseases as an alternative therapy has attracted much attention because no resistance occurred against vaccines (Alwhibi and Soliman, 2014; Ike et al., 2021). Hypericin, an anthraquinone, has antimicrobial activity against methicillin-resistant and methicillin-sensitive *Staphylococcus* spp. (Alwhibi and Soliman, 2014; Abdalhamed et al., 2021; Ike et al., 2021). Combinations between different plant extracts and purified plant derivative compounds could be effective against drug-resistant bacteria. Various plant compounds and antibiotic complexes could be effective against MDR bacteria (Anadón 2006, Abdalhamed et al., 2022).

Studies have revealed that about 70 species of bacteria, including methicillin-resistant *Staphylococcus aureus* and vancomycin-resistant *Enterococcus* spp., are susceptible to honey which has extreme antimicrobial activities (Atmaca, 2016; Ike et al., 2021). Bacteriophages (phages) are viral predators of bacteria and are evaluated as a potential alternative to antibiotics for treating antibiotic-resistant bacteria in human and veterinary medicine (Bai et al., 2018). Therefore, the current review article aimed to shed light on strategies to prevent and control MDR bacteria in ruminants.

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Alternatives to antibiotics for growth promotion

Probiotics, prebiotics, and synbiotic

Probiotics are live strains (yeast, fungi, and bacteria) of strictly selected microorganisms administered in adequate amounts to improve the balance of microbial activity in the gastrointestinal tract of ruminants. *Lactobacillus (L.)*spp., *Bacillus spp., Enterococcus spp., Bifidobacterium spp., Micrococcus spp., Pediococcus spp., Streptococcus spp., Propionibacterium spp., Saccharomyces spp., Aspergillus spp.* are the most common species that have been evaluated for their ability to replace with antibiotics for growth promotion and control enteric pathogens in dairy and beef cows (Baird et al., 2017; Razavi et al. 2019). An experiment that administered a probiotic mixture containing *Lactobacillus (L.) acidophilus, L. helveticus, L. bulgaricus, L. lactic, Streptococcus thermophiles,* and *Enterococcus faecium* to sheep experimentally infected with a Non-O157 Shiga toxin-producing *Escherichia coli* (*E. coli*) indicated a decreased fecal shedding of the pathogen (Baird et al., 2017). Probiotics are used as feed additives to improve animal health and productivity, stimulate intestinal microbial flora, improve digestion, enhance nutrient absorption and bioavailability, prevent enteric pathogens' colonization, stabilize pH, and increase mucosal immunity. They could act as an ideal candidate for enhancing the general health condition of ruminants. *Lactobacillus acidophilus* fecal isolate and *Bifidobacterium pseudo catenulatum* SPM1309 showed a strong growth inhibitory effect against MDR *pseudomonas aeruginosa* and MDR *acinetobacter baumannii* (Banai et al., 2002; Balakrishnan et al., 2017). The *L. fermentum* supernatant with bovine lactoferrin had synergistic inhibitory activities against methicillin-resistant *Staphylococcus aureus* (MRSA) strains (Barbu et al., 2016; Abdalhamed et al., 2021). The combinations of *L.casei* and *L. rhamnosus* with antibiotics amikacin and gentamycin (GEN) have synergistic activities on *P. aeruginosa* (Barthod et al., 2018).

Prebiotics are non-digestible dietary elements preferentially digested in gut microbes, resulting in increased immune response against antimicrobial activity and consequently improving host health. Carbohydrates, such as oligosaccharides, polysaccharides, polyols, and protein hydrolyses, are the most frequent prebiotics utilized in animal feed additives (Abd El-Moez et al., 2013). Fructo-oligosaccharide, spray-dried bovine serum, and oligosaccharides have specific health effects and decrease the incidence of enteric diseases. Manna-blocked pathogen colonization in the intestinal tract of calves that is by yeast fermentation such as yeast culture, cell walls, refined functional carbohydrates, and mannan-oligosaccharide (MOS) is used as prebiotics (Bechinger and Gorr, 2016). Mannan-oligosaccharide may act to inhibit bacterial and *cryptosporidium* attachment to the intestinal wall. Also, *Bacillus subtilis* alters the rumen microbiome and improves digestion at weaning. The administration of oligosaccharides promotes desirable intestinal microflora and improved growth performance in weaned calves. Commercial prebiotic celmanax prevents entero hemorrhagic *E. coli* colonization in the cattle gastrointestinal tract (Bhola and Bhadekar, 2019). Supplementing animal feed with probiotics increases lactate and antibody synthesis, reduces intestinal pH, inhibits intestinal microflora, and reduces pathogen bacteria in the stomach (Blair et al., 2015). Synbiotics are probiotics and prebiotics together. They are created to boost health benefits in a particular way. Continuous oral co-administration of synbiotics, such as the *Bifidobacterium breve strain Yakult* and galactooligosaccharides, enhanced survival rates and protected mice in an in-vivo investigation when they were challenged with MDR *Acinetobacter baumannii*. (Bricknell and Dalmo, 2005). Synbiotics are natural and safe, also supported by several research findings for possible application in food animals for preventing, supporting therapy, and safe alternative to current antibiotics for reducing antimicrobial usage (Chanda et al., 2011; Castillo and Gatlin 2015; Castelani et al., 2019).

Antimicrobial peptides

Antimicrobial peptides are host defense peptides, abundantly distributed in nature, and are used as alternatives to antibiotic therapy. They have direct and indirect inhibitory effects on pathogenic bacteria (Castillo and Gatlin, 2015). They are expected to be the next generation of alternatives to antibiotic therapy for preventing bacterial resistance (Chanda et al., 2011). Antimicrobial peptides (AMPs) have broad-spectrum antimicrobial activities and are amphipathic. They have hydrophobic/cationic properties which bind with the phospholipid bilayer of the bacterial cell wall, causing cell wall portion and resulting in cell death (Chusri et al., 2009; Cheng et al., 2014; Chanda et al., 2011). The AMPs enhance nutrient digestibility and benefit the growth performance of large and small ruminants (Cianciosi et al., 2018). They are formed by ribosomally synthesized bacteriocins and non-ribosomal bacitracin, gramicidin, and polymyxin (Cheng et al., 2014). The A3, P5, and cecropin AD are three synthetic AMPs that control animal growth by fostering a healthy gut microbiome (Cianciosi et al., 2018). Bacitracin zinc and methylene salicylic acid have been approved as feed additives in USA and China (Counoupas et al., 2018). SMAP-29, a cathelicidin-derived peptide isolated from sheep myeloid mRNA, induces antimicrobial activity against MRSA, vancomycin-resistant *E. faecium. faecium* and *Pseudomonas (P.) aeruginosa*. Antibacterial peptides isolated from *Enterococcus mundtii* (ST4V) have inhibitory effects against multidrug-resistant *Streptococcus* species, *P. aeruginosa, Klebsiella pneumonia, Streptococcus (S.) pneumonia* and *Staphylococcus* (Crisol-Martínez et al., 2017). Peptide AP-CECT7121 is an antimicrobial peptide produced by *Enterococcus faecalis* CECT7121, with bactericidal activity against Gram-positive bacteria, an attractive candidate for its use as a natural therapeutic tool for the treatment of infections produced by multi-resistant *Staph. aureus* and vancomycin-resistant *Ent. faecium* isolated from humans and animals (Delany et al., 2014; Delpech et al., 2017).

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Dewul (2014) suggests that AMP is used in feed additives for goats to improve rumen microbiota, and food efficiency, preventing ruminal fermentation and potentiating growth performance (Dhama et al., 2014). Dini et al. (2011) declared that the substitution and modification of peptide amino acids could enhance their antibacterial activity and be effective against all MDR bacteria spp.

Bacteriocin

Bacteriocins are ribosomal synthesized AMPs that destroy pathogenic bacteria cell walls. Bacteriocins are subdivided based on their modifications into class I (lantibiotics) and class II (heat-stable) peptides (Doolan et al., 2014; Dorneles et al., 2015). The *P. aeruginosa* is susceptible to the antibiotic actions of bacteriocins; in particular, pyocin S5 was effective at a concentration 100 times lower than tobramycin. Lactic acid (LAB) commonly secrete bacteriocins, while colicins and microcins are produced from *E. coli* (Eja et al., 2011). Probiotics using lactic acid bacteria (LAB) and bacteriocins like nisin are the most commonly explored (Giguère et al ., 2013). Founou et al. (2016) indicated that bacteriocins act on cell walls and inhibit protein expression genes. Commercially available bacteriocin products can treat superficial and systemic bacterial infections. They have several potential applications in veterinary medicine as nisin-based teat sanitizers, amrubisin, Wipe- Out® Dairy, Wipes, and Mast Out® are alternatives for antibiotics in treating mastitis. A teat dip containing lantibiotic available for therapeutic uses against staphylococcal infection. It has been demonstrated to be highly effective against *S. aureus and S. dysgalactiae* (Giguère et al., 2013). Founou et al. (2016) revealed that multidrug-resistant *Staphylococcus spp*., which causes mastitis, may be controlled by cationic nisin/dioctadecyl dimethyl ammonium bromide NPs.

In-feed enzymes

By acting on feed components in the animal's gastrointestinal tract, several enzymes are added to animal feed to improve digestion processes and nutritional bioavailability (Giguère et al., 2013). In-feed Enzymes are crucial components for minimizing drug misuse and are a stimulant for animals' immune and overall health. The most popular feed enzymes are glycanase and phytase, offered commercially as feed additives (Hamasalim, 2016). The advantages of feed enzymes are optimizing digestion and enhanced nutrient availability of high-fiber ration in the rumen of ruminants (Hambleton et al., 1988). The direct impact of feed enzymes is on animals' natural immunity; the *β-mannanase* enzyme is commercially available as β-mannanase (CTCzyme®); it could decrease the somatic cell counts in cow's milk (Hana et al., 2016). In light of these findings, in-feed enzymes could be an effective alternative to antibiotics in controlling Antimicrobial Resistance (AMR) bacteria in dairy cattle (Hazam et al., 2019).

Alternatives to antibiotics for disease prevention

Vaccines

Vaccination is an effective strategy for preventing and eradicating infectious diseases worldwide; it could be used against AMR bacteria in humans and animals. The application of vaccines as an alternative therapy to antibiotics has attracted much attention since there was no vaccination resistance (Hu et al., 2017). The common licensed veterinary vaccines include live-attenuated, inactivated, or killed vaccines and toxoids (Table 1). Veterinary vaccines are ideal candidates for preventing infections, reducing antibiotic consumption, enhancing productivity, and reducing antibiotic resistance (Jalilsood et al., 2015). Although vaccination has become a potent weapon against drug-resistant bacteria, some bacteria could evade the protection that vaccines are induced; hence, frequent vaccination updates are necessary (Jin et al., 2019). Vaccine development is crucial in the area where AMR bacteria are endemic (Jorge and Dellagostin, 2017).

Inactivated killed vaccines

The inactivated vaccine is produced *in-vitro* by inactivated bacterial cultures and adjuvant with oil-base to enhance immune responses. They are inexpensive in production and stable in storage. Oil adjuvant inactivated emulsion vaccine for Hemorrhagic septicemia (type B:6) is used for active immunization against hemorrhagic septicemia and pneumonic pasteurellosis for cattle, buffaloes, sheep, and goat vaccinations as shown in Table 1 (Jin et al., 2019). Currently, most bacterial vaccines include living attenuated and inactivated or killed microbial strains with varying degrees of efficacy; for example, *Brucella* (*B.) abortus* (RB51) is a vaccine used for brucellosis prevention in cattle (Jorge et al., 2016). Killed *S. aureus* vaccines are also developed for bovine mastitis (Jouda et al., 2016).

Live-attenuated vaccines

Live attenuated vaccines are prepared by the passage of bacteria in an unusual host or cell after several passages of the bacterial strain in different media (Kahn et al., 2019). Unlike the anti-viral vaccine, which is quite mature, the availability of antibacterial vaccines is still rare in the market (Jouda et al., 2016). However, *Brucella abortus* strain 19 and strain RB 51 vaccines, both live attenuated vaccines produced from *Brucella abortus*, are the most often utilized brucellosis vaccines in actual practice. The *Brucella abortus* strain 19 vaccination could provide longer-lasting protection for young calves than RB51. Furthermore, the RB51 vaccine aids in distinguishing between animals that have received the vaccination and those that have been infected by not interfering with serological diagnosis (Kar et al., 2016, Table 1).

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Table 1. Vaccination schedules for bacterial diseases in ruminants

Disease	Type	Time of vaccination	Immunity	Dose/ route	Indication
Black Quarter	Formal killed vaccine	Once a year, before the monsoon	One season	5 ml/ SC	Against Black Quarter in cattle and other ruminants.
`Brucellosis	*B. abortus* Strain 19 smooth (live) attenuated	About 6 months of age	3-4 calving	2 ml/ SC to female calves between 4 to 8 months old	Protection of cattle, buffaloes against Brucellosis
	RB51 Live attenuated vaccine of B. *abortus* rough strain RB51	1-2 years	animals vaccinated annually	2ml as one or 2 doses at 30-day interval	Protection cattle, buffaloes, and sheep against brucellosis by *B. abortus*
	Living attenuated *B. Melitensis* vaccine	3 to 8 months			lambs and kids
Hemorrhagic Septicemia (HS) Vaccine	Inactivated HS Oil Adjuvant Vaccine (type B:6)	Once a year, before monsoon	One season	2 ml in cattle 1 ml in sheep, IM	Protection of cattle and sheep against HS and pneumonic pasteurellosis
• Clostridial diseases • ULTRABAC® 7 • Covexin 8 • Cubolac 8 • Covaccin 10 • Polyvalient clostridia vaccine	ULTRABAC® 7 vaccine	First dose 6 Weed of age second dose 4-6 weeks later/ Annual revaccination with a single dose	One season	5 mL/ SC	Protection against clostridial diseases: • blackleg – *Clestrodium chauvoei* • malignant edema – *Clostridium septicum* • black disease – *Clostridium novyi* • gas-gangrene – *Clostridium sordellii* • enterotoxemia, enteritis – *Clostridium perfringens* types B, C, and D
	Covexin 8 Polyvalent formalized killed vaccine		6 months	2 ml - sheep 5 ml cattle IM	Protects cattle, sheep, and goat against clostridial diseases
Mixed Vaccine	Inactivated Bovine Rota, Corona Viruses, and *E. Coli* Vaccine	2 doses at least two weeks apart to pregnant cows, the second dose given 2-3 weeks before calving.	One year	4 ml in pregnant cow or buffalo	against diarrhea caused by Rota, Coronaviruses, and *E. Coli*
Tuberculosis	BCG	About 6 months of age			To be repeated every 2-3 years
Anthrax	Spore	Once a year, before monsoon	One season	1ml, SC	

B. abortus: *Brucella abortus*, RB51: *Brucella abortus* attenuated strain RB51 vaccine (RB51), BCG: Bacille Calmette-Guérin, SC: Subcutaneous, IM: Intramuscular, HS: Hemorrhagic septicemia, *E. Coli: Escherichia coli*

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Toxoids

Toxoid vaccines are bacterial toxins that physically or chemically have treated until they no longer produce disease but retain the capacity to induce immunity (Karuppiah and Rajaram, 2012). They are currently used commercially as single toxoid vaccines such as toxoid from *Corynebacterium pseudotuberculosis* causing *caseous Lymphadenitis*, and combined *Clostridium perfringens* Types C and D and tetanus (CD-T vaccine) for Sheep and goats (Karuppiah and Rajaram, 2012)

Recombinant vaccines

The DNA, subunit, and vector vaccinations are the three types of recombinant vaccines. Insertion cloning of a DNA segment into a vector is used to create recombinant vaccines. Recombinant DNA vaccines develop pathogenic agent-specific proteins, whereas subunit vaccines synthesize a recombinant protein in vitro and are injected into the host. Recombinant vector vaccines employ an attenuated bacterium to either proliferate and express the antigen within the host or multiply and express the antigen outside the host (Váradi et al., 2017). Advanced recombinant vaccines are unquestionably the future vaccines for animal disease prevention that develop safe, effective, and comprehensive protection against various infections. Before field trials, recombinant vaccinations must be safe for both the host and the environment (Kazemi et al., 2014). Recombinant *Bacillus* Calmette–Guérin (BCG) vaccine is vectored vaccine such as Ag85B antigen (a protein found on the bacterial surface) for cattle that showed protective immune responses against *Mycobacterium bovis* (Khulbe and Sati 2005). Various approaches have been investigated for vaccine development against *S. aureus* in bovine mastitis, including whole organism vaccines, live attenuated *S. aureus*, capsular-polysaccharide–protein conjugate vaccines, DNA vaccines encoding clumping factor A, and recombinant *S. aureus*-mutated enterotoxin type C (Kon and Rai 2012).

Immunostimulants

Immunostimulants are chemicals that activate phagocytes, and neutrophils and are the alternative complement system to lysozyme activity to boost the innate immune system of the hosts in a non-specific manner to promote resistance to the disease (Landers et al., 2012). By releasing cytokines and cytokine inhibitors, non-specific anti-inflammatory drugs (steroids), and changing a particular antigen-based response through interferons, immunostimulants regulate the immune response to pathogen assault (Langeveld et al., 2014). Some bacterial substances (-glucans) and different plant constituents could directly initiate innate defense mechanisms by expressing intracellular gene(s) and controlling antimicrobial compound production. Animals' natural immune protection against pathogenic bacterial assault might be improved by using immunostimulants as feed additives during stressful times (Liu et al., 2017; Lewis, 2018).

Chicken egg yolk antibodies

Chicken egg yolk antibodies (IgY) are immunoglobulin of birds, reptiles, and amphibians that transfer passive immunity to their embryos and offspring (Lubroth et al., 2007). Chicken IgY is used as an alternative to antibiotics for treating diarrhea in young calves (Figure 1). Also, IgY or hyperimmune egg products are commercially available worldwide to improve health and prevent enteric pathogens in young livestock. Now many companies are focusing on establishing and producing IgY for animal feed supplementation (Mabrouk, 2012). The oral administration of IgY to calves is commercially available against various intestinal pathogens, such as bovine enterotoxigenic *E. coli* and *Salmonella* spp. (Marquardt and Li, 2019). Data availability *in-vivo* about IgY in clinical trials indicates the possibility of using it as an alternative to current antibiotics (McCaughey et al., 2014).

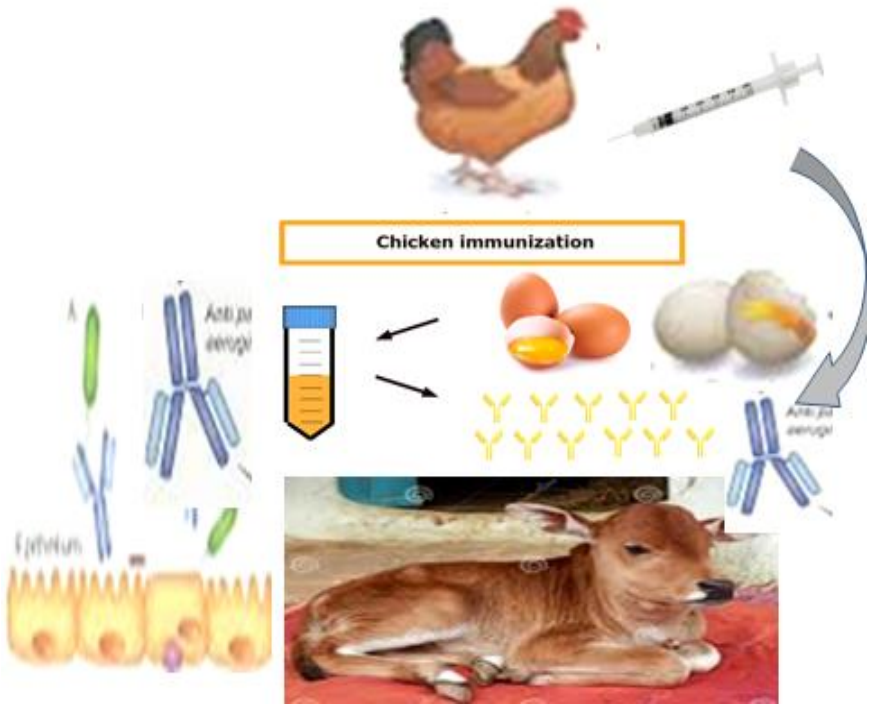

Figure 1. Hyperimmune egg-yolk antibodies as a prophylaxis and treatment of intestinal microbial diseases in livestock prepared in a dry form by spray- or freeze-drying and incorporated diet with egg yolk

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Alternatives to antibiotics for disease treatment

Plant-derived phytochemicals

Phytochemicals (phytobiotics or phytogenics) are natural bioactive compounds that originated from plants to treat MDR bacteria in ruminants or used as growth promoters (McCaughey et al., 2014; Marquardt and Li, 2018). Plant parts and extracts are mostly affordable, readily available, natural, and non-toxic. Quinones, alkaloids, flavonoids, phenols, terpenoids, essential oils, tannins, lignans, glucosinolates, and a few secondary metabolites are among the phytochemicals' bioactive constituents. Some antimicrobial agents of plants include peptides that possess antimicrobial activity against *P. aeruginosa* (Meeusen et al., 2019). Hypericin, an anthraquinone, had antimicrobial activity against methicillin-resistant and methicillin-sensitive *Staphylococcus* spp. An alkaloid called berberine has antibacterial properties against *S. Agalactiae*. Berberine, and its DNA-binding actions damage the bacterial cell membrane structure and cause cell death by making the membrane more permeable (Pliasas et al., 2022). A study showed the potent antibacterial properties of garlic against resistant bacterial strains, including *P. aeruginosa* (Molan and Rhodes, 2015). Methanol and chloroform extracts of Fenugreek (*Trigonella foenum-graecum)* inhibit *E. coli* (Mushtaq et al., 2016). *Thalictrum minus* extract showed antibacterial activities varied between the bacterial species, while 5-Hydroxy-thalidasine has good antibacterial activities in combination with ampicillin, chloramphenicol, and streptomycin against MDR. Anthraquinones and saponins in aloe vera have direct antibacterial effects (Okeke et al., 2011). Curcumin (CUR) and *Nigella sativa* have inhibitory effects against *S. cereus, and S. aureus* (Pachón-Ibanez et al., 2017). Egyptian honey, black cumin, and essential onion oils have an inhibitory effect against Gram-negative and positive AMR bacteria isolated from small ruminants with mastitis, with inhibitory zone diameter (IZD) of 13 mm to 28 mm and a minimum inhibitory concentration (MIC) of 3.25 to 25 mg/mL (Pariza and Cook, 2017). The IZD of *Comiphora (c.) molmol oils* extract against AMR *Pseudomonas spp.* isolated from dairy cows and buffaloes with mild mastitis varied from 3.25 and 6.25mg/mL (Payne, 2015). Peña-González et al. (2017) reported that the MIC of *C. molmol* methanolic extracts was 3.12 mg/mL for *E. coli, Klebsiella (K.) pneumoniae,* and *P.aeruginosa*. Meanwhile, it was 12.5 mg/mL for *A. baumannii*, and 6.25 mg/mL for *S. aureus*.

Combination therapy among herbal antimicrobials

Combinations between different plant extracts and purified plant derivative compounds synergize against drug-resistant bacteria (Purwanti and Yuwanta, 2014). The synergistic inhibitory effects of other fruits and leaves plant extracts (aqueous and ethanolic) of *Foeniculum vulgare, Priminellaanisum, Carumcarvi, Majorana hortensis, Mentha longifolia, and Salvia officinalis medicinal* plants reported on multi-drug-resistant *E. coli* O157:H7 isolated from human, cattle, and foods (Raguvaran et al., 2015). When tested individually, the aqueous extracts of *Foeniculum vulgare* had 1.4 cm (IZD) inhibition zone on E. coli O157:H7. Meanwhile, combining aqueous extracts of *Foeniculum vulgare, Priminellaanisum, and Carumcarvi* (1:1:1) had synergistic antibacterial effects against resistant *E. coli* with an inhibition zone of 4 cm . *Thymus vulgaris* combined with *Cinnamomum zeylonicum* essential oils have antibacterial activity against *E. coli* and *S. aureus*, which is indicated by the synergistic effect of the combination between *T. vulgaris, C. zeylonicum*on, and *S. aureus with* fractional inhibitory concentration (FIC) index of 0.26 (Ražná et al., 2020).

Combination between plant derivatives and antibiotics

The combination of plant compounds and antibiotics complexes synergizes against MDR bacteria. Synergistic effects were observed between tea extract and chloramphenicol against enteropathogens such as *Salmonella* (*Sa.) Typhimurium, Sa. Typhi, Sa. dysenteriae, Yersinia enterocolitica*, and *E. coli.* The synergistic inhibitory effects of chloramphenicol and tea extract against *S. Dysenteria were* 2.5 µg /mL (MIC 5 µg/mL) and 5.094 mg/ (MIC 9.089 mg/mL). The essential oil of *Helichrysum italicum* had inhibitory effects against *MDR E. coli , P. aeruginosa*, and *A. baumannii*, and when combined with beta-lactams, quinolones, and chloramphenicol increased their antimicrobial effects (Reinhardt, 2017). Mixing of ellagic and tannic acids enhanced the antibacterial activities of novobiocin, clorobiocin, rifampicin, and fusidic acid against *Acinetobacter baumannii* (Ren et al., 2019). A combination of ampicillin with *A. sativum* and, *Gongronema latifolium* extracts had additive effects and synergism against *S. aureus* (Rizzi et al., 2012). The synergistic activity between *Salvia officinalis* and *Cichorium intybus* extracts with amoxicillin and chloramphenicol against *S. aureus, E. coli, P. aeruginosa, B.subtilis, E.cloacae, K. pneumoniae, and P. mirabilis* has been noted (Rodriguez et al., 2002). The combination of *Coriandrum sativum* essential oil and gentamicin antibiotic has a synergistic effect. In contrast, the antagonistic effect was observed in the variety of *Coriandrum sativum* essential oil and erythromycin antibiotics on Gram-positive or Gram-negative bacteria (Salim et al., 2018).

Honey against multidrug-resistant bacteria

Honey has extreme antimicrobial activities. About 70 species of bacteria are susceptible to honey, including MRSA and vancomycin-resistant *Enterococcus spp.* (VRE). The antimicrobial effects of honey occur from different components, including flavonoids, phenols, high sugar concentration, acidity, and the production of hydrogen peroxide. Also, other types of honey have methylglyoxal, lysozyme, and defensin-1, which induce antibacterial activity (Ahmed and Ibrahim HM 2016). The Synergistic effect among all the bioactive components of honey is responsible for its intense antibacterial activity (Schatzschneider, 2019).

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Phage therapy

Bacteriophages (phages) are viral predators of bacteria, and they have the potential effect of being replaced with antibiotics for the treatment of antibiotic-resistant bacteria in humans and animals. Lytic phages *(Phagetherapy*) are used to treat MDR bacteria, while temperate phage is unsuitable. Bacteriophages integrate their genome into the bacterial host DNA and transfer virulence factors, including antibiotic resistance genes, to targeted pathogenic bacteria (Figures 2 and 3). However, it is challenging to discover lytic phages specific to all bacterial diseases in the cattle industry (Sharma et al., 2018). Phages can interact with bacterial-specific binding sites on specific pathogenic bacteria by tail fibers without harming beneficial microflora. Commercial phages were introduced by many companies in the USA and France (Sharma et al., 2017).

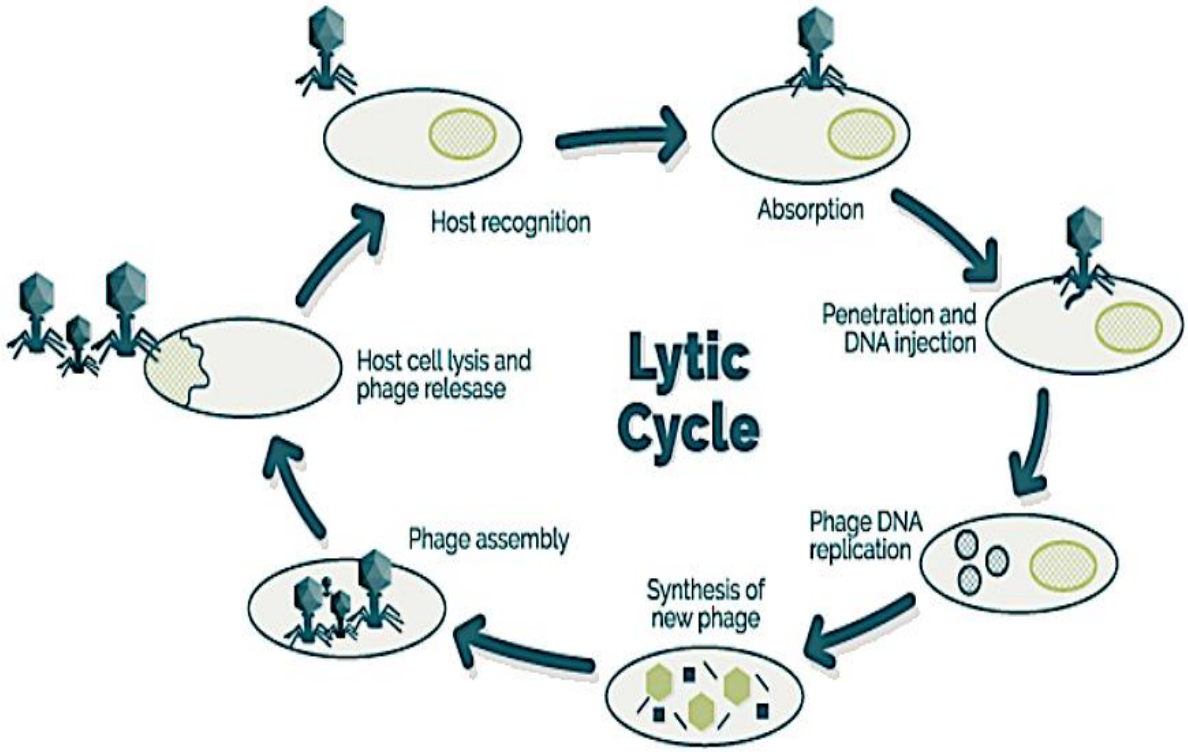

Figure 2. Differentiation between phages and lytic phages cycles (https://coliphages.com/index.php/reproduction/ with UploadWizard)

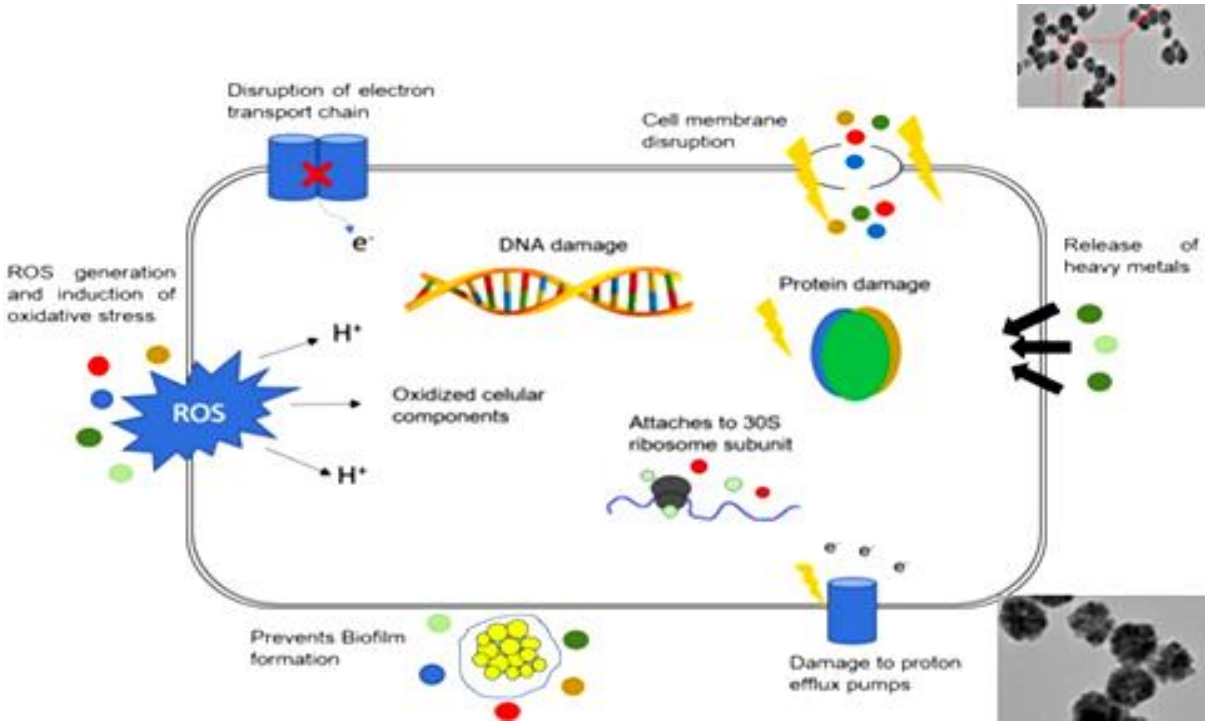

Figure 3. Different nanoparticles mechanisms in bacterial cells (Singh et al., 2014).

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Applications of nanoparticles for the prevention and treatment of multidrug-resistant bacteria

Nanotechnology has evolved into a novel, significant branch of science with broader applications in veterinary sciences, particularly against MDR bacteria (Tawfick and Gad, 2014; Suzuki et al., 2014; Siddiqi et al., 2018; Simons et al., 2020). Nanoparticles have antibacterial properties and include silver (Ag), iron oxide(Fe_3O_4), titanium oxide(TiO_2), copper oxide (CuO), gold (Au NPs), zinc oxide (ZnO), chitosan, carbon nanotubes. The antimicrobial mechanisms of NPs acted directly on the bacterial cell wall, preventing biofilm formation and triggering natural and acquired immune responses. They are produced by reactive oxygen species (ROS), which interact with DNA and proteins, as shown in the different bacterial mechanisms of NPs compared to standard antibiotics against MDR bacteria in Figure 3 (Varzakas et al., 2010; Vijayan et al., 2019).

The medicinal application of NPs for treating bovine mastitis is an important solution for treating MDR bacteria as an alternative to antibiotics against pathogenic bacteria. Wang et al. (2017) explored the synergistic activity of silver NPs and antibiotics by examining the antibacterial activity of AuNPs against *E. coli*, *Bacillus subtilis*, *S. aureus*, and *K. pneumonia* (Tiwari et al., 2005; Wittebole et al., 2014; Wang et al., 2017). They discovered that the MICs of AuNPs against the tested bacteria were 2.93 ug/ml, 7.56g/ml, 3.92 ug/ml, and 3.15 ug/ml, respectively. ZnONPs are used in animal health and production as an antibacterial, food preservative, and feed additive (Tiwari et al., 2005; Wittebole et al., 2014). Yew et al. (2016) reported that the different sizes of AuNPs successfully inhibited the growth of varying MDR bacteria, including MRSA. Zamek-Gliszczynski et al. (2018) mentioned that AgNPs and capsaicin have antibacterial characteristics and could effectively be used to inhibit the growth of MDR- extended-spectrum beta-lactamases (ESBL) producing *E. coli* of bovine origin (Thu et al., 2017; Zeedan et al., 2018). The AgNPS has a promising effect on AMR bacteria. AgNPs with an average size of 10 nm using biomolecule apigenin can inhibit cell viability and biofilm formation of MDR pathogenic bacteria such as *Prevotella Melaninogenica* and *Arcanobacterium pyogenes* isolated from uterine secretion (Zeedan et al., 2014).

Farm management and biosecurity control

Biosecurity is vital in controlling AMR bacteria and reducing antibiotic control of AMR bacteria in animals. It is defined as all actions to stop the spread of infectious diseases among people or animals on farms and in the surrounding environment. Biosecurity decreases the possibility of infectious agent spreading (Simons et al., 2020).

CONCLUSION

The rising antibiotic resistance and the lack of new antimicrobials have triggered scientists to develop an alternative for antimicrobial compounds to minimize antibiotics use. Some other options have been proposed to reduce antimicrobial drug overuse to overcome the increasing rate of MDR bacteria in ruminants. These alternative procedures include alternatives to antibiotics for growth promotion (such as in-feed enzymes, probiotics, prebiotics, synbiotics, and antimicrobial peptides), alternatives to antibiotics for disease prevention (such as vaccines, immune modulators, chicken egg yolk antibodies, farm management, and biosecurity), and alternatives to antibiotics for disease treatment such as plant extracts and phage-therapy. These alternative methods should be safe and efficient without inducing microbial resistance. These strategies reduce the need for antibiotics in animals and combat antibiotic-resistant bacteria. Besides, rapid and accurate diagnostic approaches which provide sufficient sensitivity and specificity are necessary for detecting resistance in bacterial pathogens to fight and solve this problem.

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Authors' contribution

Abeer M. Abdalhamed and Gamil SG Zeedan established the research idea and drafted the manuscript. Alaa A Ghazy shared in the conception of the research idea and helped in manuscript preparation.

Ethical consideration

Not applicable.

Material and data availability

Not applicable.

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The Prevalence of Gastrointestinal Nematodes in Livestock and their Health Hazards: A Review

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ABSTRACT

Livestock plays an important role in the national economy and has a significant share in the gross domestic product of Pakistan. Parasitic diseases and worm infestations negatively affect their health, production, and reproductive performance. In addition, parasitic infestation in livestock reduces gross production values and renders huge economic losses globally. Among the parasites, the most important are nematodes. They are distributed worldwide and affect all kinds of livestock. This review aimed to elaborate on the main gastrointestinal nematodes, their mode of action, impacts on livestock and their control (physical, chemical or biological) strategies. Common examples of nematode worms infesting the livestock are *Ascaris, Hemonchus, Strongyloids, Trichostrongyliods, Ostertagia, Trichuris, Dictyocaulus, Trichnellia, Enterobius, Cooperia, Gunagylonema, Chabertia*, and *Oesphagostomum*. The gastrointestinal nematodes are detrimental to the animals' health. Nematodes primarily affect animals' feed consumption and efficiency, and severe ailments result in the death of the affected animals. The production and health losses primarily depend on the age of the animals, the degree of severity of worm infestation, epidemiology pattern of the parasites, management strategies of the flocks, and ecoclimatic conditions which are favorable for the worm's infestation. To minimize these issues, farmers should be educated on the importance of intensive livestock management and environmental sanitation, as well as strategic deworming of cattle using efficient broad-spectrum anthelmintics, biological control of the parasites, and breaking their life cycle and intermediate hosts.

Keywords: *Ascaris*, *Enterobius*, *Hemonchus*, Nematode, Parasitism, Roundworms, *Strongylus*

INTRODUCTION

Gastrointestinal (GIT) nematode infestation is the most important problem and constraint to the livestock industry in Pakistan. It negatively affects the health of small and large ruminants population and negatively influences the national economy regarding production losses at the major. The livestock share in the agriculture sector is more than 60%. Livestock plays an important role in the national economy (Government of Pakistan, Agriculture Economic Survey, 2021).

The sheep and goats possess tremendous approaches to producing meat, milk, and wool. Livestock is prone to GIT nematode infestation, and it can lead to a significant death rate. These GIT nematodes significantly affect sheep and goats' production and reproduction performance (Asmare et al., 2016).

The GIT nematodes are detrimental to the animals' health. These parasites primarily affect livestock feed consumption and the efficiency of the animals. In addition, severe ailments result in the death of the affected animals. The potential negative impact primarily depends on the animals' age, severity of worm infestation, the epidemiology pattern of the parasites, management strategies of the flock, and ecoclimatic conditions in the worm infestation (Urquhart et al., 1996).

Gastrointestinal nematodes mostly infest small ruminants. Their epidemiological patterns rely on the factors related to the parasite-host (such as inadequate host nutrition, poor hygiene, and sanitation; Tesfaye et al., 2021). Among these GIT parasites, the most common parasites infesting the livestock are *Hemonchus contortus, Ascaris, Strongyloids, Trichostrongyliods, Ostertagia, Trichuris, Dictyocaulus, Trichnellia, Enterobius, Cooperia, Gunagylonema, Chabertia, Oesphagostomum* (Mekonen, 2021).

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Therefore, strategies for the treatment and prevention of these GIT nematodes in livestock, with anthelmintic drugs, control of parasite infestations, biological control of the parasites, and breaking their life cycle and intermediate hosts should be practiced to minimize the impact of these parasites on the health status, production and performance of the animals, and safeguard the national food interest and economy (Sadr et al., 2022; Lotfalizaded et al., 2022).

Morphology of the parasites

The body of the nematodes is elongated and cylindrical, tapering at both ends and extremities. They are non-segmented creatures; however, their bodies are covered with a thick waxy cuticle layer for protection. This cuticle layer is also continuously related with the buccal cavity and digestive tract of the parasites (Jacobs et al., 1999).

General life cycle of nematodes

The nematodes are oviparous and lay a huge number of eggs per day. For example, Ascaris female lays 200,000 eggs per day and has 27 million eggs in her body at one time. The nematodes have direct and indirect life cycles, but these worms usually show a direct life cycle in livestock without involving the intermediate host. Sexes are separate and sexual dimorphism is also present in nematodes. The eggs are shed in the host feces and hatch into the first larval stage (L1), which feeds on bacteria and soil. As these worms show metamorphosis, they show ecdysis and molt into the L2 under suitable eco-environmental conditions, and subsequently, L3 larval stage is hatched. This L3 comes out of the feces, attaches itself to leaf blades of the grasses, and is transmitted to the grazing animals via the oral route. This nematode transmission can be termed oro-fecal route transmission (Mekonnen, 2021).

Overall prevalence of gastrointestinal nematodes in livestock

A study was conducted in Odisha, an eastern Indian state on the Bay of Bengal, to determine the GIT nematodes in a different breed of sheep (Kumar et al., 2021). For this purpose, about 701 fecal samples were collected from different sheep of different breeds. After fecal examination and coproculture, it was concluded that the prevalence of GIT nematodes was 61.20%. Another study was conducted in Wayu, Toka, and Diga districts, Oromia regional state, Ethiopia, to determine the prevalence of GIT nematode in sheep. The sample analysis revealed the prevalence of nematode infestation in the study was 44% (Chali and Hunde, 2021). In Sillod Tehsil, from Aurangabad district in Maharashtra, , India, the prevalence of GIT and protozoan in sheep and goats was determined. For this purpose, about 940 fecal samples were collected randomly from sheep and goats. Floatation, sedimentation, and direct smear method techniques were used for the analysis. The findings revealed a high prevalence of nematodes in sheep and goats at 72% and 61%, respectively (Shaikh and Naphade, 2021).

Another study was performed in Rajendran agar, Hyderabad, Pakistan, to determine the nematode infestation. Nematode infestation results in stunted growth, weight loss, reproduction and production loss. For this purpose, about 368 fecal samples were collected directly from the rectum of the goat. Gross, direct fecal smear, sedimentation, and flotation techniques were implied to examine and identify nematode infestation. The analysis of the samples revealed a 38% prevalence of nematodes (Shashank et al., 2019).

A total of 120 fecal samples were collected from cattle in Azare abattoir, Katagum Local Government Area, Bauchi State, North-Eastern Nigeria, to determine the GIT nematode in domestic animals. The analysis of the samples indicated the prevalence of 56% of nematodes (Umar et al., 2021). A study in Vom, Central Nigeria, determined the prevalence of GIT parasites. For this purpose, 1508 fecal samples were collected from various domestic animals, such as goats, dogs, horses, rabbits, sheep, and cattle. Formal ether concentration techniques were implied to examine worms. The analysis of the samples revealed the highest prevalence of nematodes in those species (Abraham et al., 2020).

In another study the prevalence of GIT nematodes in wild ruminants was investigated in Massachusetts, Rhode Island, Vermont, New York, Maryland, Kentucky, North Carolina, South Carolina, Arkansas, Louisiana, Iowa, Kansas, Nebraska, New Mexico, Wyoming, and Alaska of the USA. For this purpose, 548 fecal samples were collected from different wild ruminants on a large scale randomly to examine the worms. All samples were cultured and isolated DNA using PCR. The analysis of the samples revealed that Ostertagai (90%) and Trichostronylus (69%) were predominant (Barone et al., 2020).

A study was conducted in central and south-eastern regions of Ukraine to examine the parasitic infestation in sheep. For this purpose, about 710 fecal samples were collected from the rectum of sheep. After examination, it was found that 79.58% of sheep were infected, which was a great impact on the national economy (Melnychuk et al., 2020). The occurrence of GIT nematodes in goats in Karachi, Pakistan, from 42 samples was 30%, and the occurance of *Oesophagostomum rafiae* was recorded in goats for the first time (Lala et al., 2019).

In the Coastal Savannah zone of Ghana, 338 fecal samples from cattle and 502 fecal samples from small ruminants (sheep and goats) were collected. A formal ether sedimentation technique was implied to examine the worm. The analysis of the samples revealed that the prevalence of GIT helminths was 90.8% (Squire et al., 2019). A study was conducted in Oromiya Regional State of Ethiopia to determine the prevalence as well as associated risk factors of GIT

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nematodes from 384 fecal samples from sheep and goats showed 71.88% for prevalence of nematodes (with flotation and McMaster techniques), while the prevalence of sheep was 75.8%, and the goat was 61.2% (Dugassa et al., 2018).

In Ranchi, Jharkhand, India, 1506 fecal samples of sheep were collected. Modified Sheather's Sugar floatation technique and Formal ether acetic acid technique were used to examine the worms. The analysis of the samples revealed that the highest prevalence of nematodes was 39.04% (Jena et al., 2018). A study was conducted in Tandlianwala, Faisalabad, Pakistan, to determine the prevalence of GIT helminth in cattle and also the therapeutic efficacy of albendazole. For this purpose, 384 fecal samples were collected from cattle. Sedimentation, Floatation, and McMaster techniques were implied to examine the worms. The analysis of the samples revealed that the prevalence of GIT parasites was 38.02% (Sadiq et al., 2018).

A study was conducted in India on estimating the seasonal ratio of GIT helminths in goats in central Madhya Pradesh. A total of 1478 fecal samples were collected from the goats. After examinations, it was concluded that out of 1478 samples, 1194(80.78%) were positive for GIT tract helminths in which the prevalence of nematodes, trematodes and cestodes were 77.47%, 7.37%, and 14.75%, respectively. The prevalence of GIT helminths and nematode infection was highest in adults (81.04% and 96.39%) compared to kids (77.68% and 89.66%; Saiyam et al., 2018). In Suhag, Egypt, 442 fecal samples were randomly collected from small and large ruminants (171, 128, and 143 from cattle, buffaloes, and sheep, respectively). Fecal microscopy revealed a 30% prevalence of nematodes in cattle, 22.6% in buffaloes, and 31.4% in sheep of nematodes (Al-Aboody et al., 2017).

About 106 fecal samples from the rectum of sheep in the Sherpur district, Bangladesh, were collected. Direct smear and Stoll dilution egg counting techniques were implied to examine worms. The analysis of the samples revealed the highest prevalence of helminth in domestic sheep, especially nematodes (Poddar et al., 2017).

A cross-sectional study was conducted in the district of Dera Ismail Khan, Pakistan, to determine the parasitic infestation in large ruminants. For this purpose, 1920 fecal samples (960 samples from dairy calves and 960 samples from buffalo calves) were collected and examined. After fecal examination, the GIT parasitic infestation was found highest in buffalo calves (67%) as well as in cow calves (47%). The parasitic infestation was highest in summer (75.78% in buffalo calves and 70.3% in dairy calves) as compared to winter (32.91% in buffalo calves and 30.41% in cow calves (Farooq et al., 2012).

A study was conducted in Bogor, Demak, East Java, and Lombok, Indonesia, to determine GIT parasitic infestation in buffaloes. For this purpose, about 89 fecal samples were collected from buffaloes. McMaster's technique was implied to examine worms. The analysis of the samples revealed that the overall infestation of the nematode was the highest (Karim et al., 2016). In Haramaya University farms, Eastern Hararghe, Oromiya region, 383 fecal samples were randomly collected from the small ruminants (sheep 216 and goat 167). Direct and indirect techniques were used to examine and identify the worms. The analysis of the samples revealed that the highest prevalence of nematodes was 88.8% while 89.2% in sheep and 88.4% in goats (Mideksa, et al., 2016).

In Kohat, Khyber Pakhtunkhwa, Pakistan, 500 fresh slaughtered gut content of small ruminants (200 sheep and 300 goats) were collected. Direct microscopy, floatation, and sedimentation techniques were implied to examine and identify the worms. The analysis of the sample revealed a 23% prevalence of nematodes out of a 45.6% overall prevalence. The prevalence of GIT parasites in goats was 49.0% and was higher than in sheep (40.5%) (Rashid et al., 2016). A study was conducted on nomadic cattle herds in Eruwa, Oyo State, Southwestern Niger, to determine GIT parasites. For this purpose, 177 fecal samples were collected from the rectum of cattle. The analysis of the samples revealed that 62.7% was the prevalence of GIT parasites, especially *Ascaris* (Stephen et al., 2016).

A study was conducted in Meskan district, Gurage Zone, Southern Ethiopia, to determine the prevalence of GIT helminth of sheep. For this purpose, 350 sheep fecal samples were collected. The analysis of the samples revealed that the prevalence of nematodes was 60.57% *Strongyles* accounted for the majority of retrieved nematode eggs (27.1%), followed by *Strongyloides* (10.9%), *Trichuris* species (3.7%) and *Ascaris* spp. (0.6%) (Nana et al., 2016).

In Peshawar, Pakistan, fecal samples from 800 sheep were collected to determine the worm infestations. The analysis of the samples revealed a 60% parasitic infestation in sheep (Jan et al., 2015).

A study was conducted in Minna, a northcentral city in Nigeria, to determine the GIT nematodes. For this purpose, about 426 fecal samples were collected from the rectum of domestic animals (sheep, cattle, and goats). Floatation techniques were implied to examine worms. The analysis of the samples revealed a 66.19% prevalence of GIT parasites in domestic animals (Agbajelola et al., 2015).

In Udaipur, India, fecal samples were collected from 1013 buffaloes and 1012 dairy cows. After examination, the results revealed that nematode prevalence was highest in cows (79.24%) and was 28.13% in buffaloes (Swarnakar et al., 2015).

In Abeokuta, in Ogun State, southwestern Nigeria170 fecal samples from 30 goats, 40 sheep, and 100 cattle were collected. The analysis of the samples revealed that the prevalence of nematodes was 58.8% in cattle, 23.5%, and 17.6% in sheep and goats, respectively (Sylvia et al., 2015). A study was conducted in the Gechi district, Southwestern Ethiopia, to determine the occurrence of GIT helminth in domestic animals. The analysis of the samples revealed that the

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overall prevalence was 82.2%, while the prevalence of GIT parasites in sheep was 84.3% and in goats was 78.7% (Emiru et al., 2013).

A study was conducted in Jatoi district of Muzaffargarh, Pakistan, to determine the prevalence of GIT helminth in domestic animals. For this purpose, a total of 500 fecal samples were collected from the rectum of cattle. Direct, indirect, and copro-culture techniques were implied to examine the worms. The sample analysis revealed a 51% overall prevalence of helminth and a 21% prevalence of nematodes were recorded (Raza et al., 2013a).

Prevalence of haemonchus and other species in livestock

A study was conducted in Nyagatare District, Rwanda, to determine the prevalence of *Haemonchus contortus*. For this purpose, 949 fecal samples were randomly collected from sheep and goats. Parasitological techniques such as Fecal egg counts (FEC) using the Modified Wisconsin Sugar Floatation method and the Fluorescent-labeled peanut-lectin agglutination test were implied to examine the worms. The analysis of the samples revealed that the overall prevalence of *Haemonchus contortus* in sheep and goats was the same at 75.7% (Mushonga et al., 2018). Another similar study was conducted in Uttar Pradesh, India, to determine the occurrence of *Haemonchus contortus* in goats. For this purpose, about 635 goat fecal samples were collected. The analysis of the fecal samples revealed that 60.0% prevalence of *Haemonchus contortus* in goats (Rashid et al., 2018). Another study at Kamrup, India, analyzed 510 fecal samples of goats and revealed a 16.40% prevalence of *Haemonchus contortus* in goats (Dutta et al., 2017).

A study was conducted in Jabalpur, Madhya Pradesh, India, to investigate the occurrence of GIT parasites in domestic animals. For this purpose, 1675 goat fecal samples were collected from the goat population and were examined microscopically. The highest prevalence of *Strongyles* (61.43%), *Coccidia* (25.97%), *Amphistomes* (9.73%), *Monieziaexpansa* (8.66%), *Trichuris* spp. (2.03%), *Strongyloides* spp. (1.79%) and *Fasciola gigantica* (0.66%) were recorded. The prevalence of GIT parasites was highest in adults (73.83%), compared to young goats (69.1%, Lata et al., 2017).

In the Mulwa region, Madhya Pradesh, India, 200 fecal samples of goats were collected to determine the prevalence of *Strongylus* infestation. The analysis of the sample revealed that the 65% prevalence of *Strongyle* seven genera of parasites were recognized. *Haemonchus* spp. (32.33%) was the predominant GIT nematode, followed by *Oesophagostomum* spp. (22.51%), *Trichostrongylus* spp. (18.67%), *Cooperia* spp. (15.03%), *Nematodirus* spp. (12.65%), *Ostertagia* spp. (3.34%) and *Bunostomum* spp. (3.12%; Rajpoot et al., 2017).

A study was conducted in Tandojam, Sindh, Pakistan, to determine the prevalence of endoparasites in domestic animals. For this purpose, about 120 fecal samples were collected directly from the rectum of domestic animals (buffalo, cow, goat, and sheep), 30 samples for each animal, and 80 blood samples were also collected from buffalo, cow, goat, and sheep (20 samples for each animal). McMaster, floatation, sedimentation, and thin blood smear techniques were implied to determine the prevalence of endoparasites. The analysis of the samples revealed that the *Hamenochus contortus* 30% and liver fluke 40% were predominant in the fecal sample (Khaskheli et al., 2016).

An investigation of the prevalence of GIT parasites in goats from the western region of Santa Catarina, Brazil, comprised 24 farms in seventeen distinct towns. Animals (n=217) with various production goals (milk and meat) and ages were randomly selected. The feces were collected, placed in plastic bottles, and transferred in 10°C portable coolers to the laboratory. Centrifugal flotation with a saturating sugar solution was employed to assess the existence of eggs, cysts, and oocysts of GIT parasites,. In 88.9% of the examined animals, nematode eggs belonging to the Strongylida order were discovered. After cultivation and larvae identification, *Haemonchus* spp. *Trichostrongylus* spp. *Teladorsagia* spp. *Cooperia* spp. and *Oesophagostomum* spp. were identified. Moreover, eggs of the genera *Thysanosoma*, *Trichuris*, *Moniezia*, and *Neoascaris* were observed. In addition, the presence of *Eimeria* spp. and *Cryptosporidium* spp. oocysts and *Giardia* spp. and *Entamoeba* spp. cysts were confirmed. In all investigated farms, animals had a single or mixed infection, with helminths of the *Haemonchus* and *Trichostrongylus* genera and the protozoan Eimeria, being the most prevalent (Radavelli et al., 2014).

A study was conducted in Chittagong, Bangladesh, to determine the helminth infestation in goats and its relation to host, age, sex, breed, and season. For this purpose, 1600 fecal samples were collected from goats. The analysis of the samples revealed that the nematode infestation was highest than other helminths. The prevalence of different helminths in goats was *Fasciola* spp (2.62%), *Paramphistomum* spp. (2.00%), *Moniezia* spp. (2.31%), *Bunostomum* spp. (4.62%), *Strongyloides* spp. (6.93%), *Oesophagostomum* spp. (4.31%), *Haemonchus* spp. (5.87%). The age prevalence of helminths in goats was 11.41%, 28.59%, and 71.88% at ages 0-12 months, 13-24 months, and above 24 months, respectively. According to sex, the prevalence of helminths in goats was 34.50% in females and 22.87% in males (Rahman et al., 2014).

In the district of Multan, Punjab, Pakistan426 GIT tracts of large domestic ruminants (buffalo and cattle) were collected from slaughtered houses. Direct and indirect techniques were implied to examine the *Toxocara vitulorum.* The analysis of the sample revealed the 54.63% prevalence of *Toxocara vitulorum* (Raza et al., 2013b).

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A study was conducted in Southern Botswana, to determine parasitic infestation in domestic animals. For this purpose, a total of 465 fecal samples were collected from different domestic animals (131 dairy calves, 94 beef calves, 143 goat kids, and 97 lambs) to investigate the prevalence of helminth. McMaster and evaluation of enzyme immune-assay (EIA) techniques were implied to examine the worms. The results of parasitological techniques showed helminthic and *Eimeria* infections. The prevalence of helminthic and *Eimeria* species in dairy calves was 2.75 and 2.9%, while, in beef calves, the prevalence was 3.25 and 2.9%. The prevalence of helminthic and *Eimeria* infections in goat kids was 3.4 and 2.8%, while in lambs was 4.45% and 3.2% (Sharma and Busang, 2013).

In Sargodha, Pakistan about 390 GIT tracts were collected from different slaughtered houses. Direct and indirect techniques were implied to examine and identify the worms. The sample analysis revealed the 40.51% helminth prevalence (Ahmad et al., 2012).

A study was conducted in Jenin, Palestine, to determine the incidence and diversity of GIT nematodes from the intensive and extensive rearing farm system. For this purpose, about 810 fecal samples were collected from ruminants composed of both 525 extensive and 285 intensive farm systems. Of 13 genera, 11 were nematodes, 1 cestode (Moniezia), and 1 protozoan (*Eimeria*) were recovered from the GIT parasites (GIPs). Less GIP diversity was observed in intensive rearing systems. The prevalence of GIPs was significantly greater ($p < 0.01$) in animals raised using an extensive method (26.5 versus 7.9%). Several localities had significantly different GIP prevalence values ($p < 0.01$). The prevalence of infection was highest in Tarem, with a proportion of (21.1%), and lowest in Betqad, with a proportion of (5.3%). *Eimeria* spp. was the most prevalent parasite in the region. Afterward, *Dictyocaulus* spp (49.1% prevalence) and *Haemonchus* spp (23.1% prevalence) come. Animals maintained under an intensive grazing system had a lower prevalence of GIP with low diversity (*Eimeria* spp, *Dictyocaulus* spp, *Trichostrongylus* spp, *Neoscaris* spp, and *Ascaris* spp) than those maintained under an extensive grazing system (*Eimeria* spp, *Dictyocaulus* spp, *Haemonchus* spp, *Moniezia* spp. (Badran et al., 2012).

A study was conducted in the District Ganderbal, Kashmir, to determine the prevalence of GIT helminth. For this purpose, the GIT tract was collected from 284 sheep and 318 goats. The analysis of the samples revealed the 82% prevalence of *Haemonchus contortus* while the prevalence of helminth in sheep was 64.08% and in goats was 83.64% (Kuchai et al., 2012).

In Addis Ababa Municipal, Abattoir, Ethiopia, 535 slaughtered cattle were examined for worm infestation. Out of the total selected animals, 19 (3.6%) were affected by *Taenia saginata*, while 24 (4.5%) were affected by *Cysticercosis bovis* (Ibrahim and Zerihun, 2012).

A study was conducted in Iceland, to determine the prevalence of GIT helminth in sheep and rams. For this purpose, random fecal samples were collected from sheep and rams. Direct and indirect techniques were implied to examine worms. The analysis of the samples revealed that the highest prevalence of four types of helminth eggs was remarkable, such as *Moniezia expansa*, *Trichuris ovis*, *Nematodirus filicollis (N. filicollis)* and *N. apathiger* (Skirnisson et al., 2018).

A study identified the patent *strongylid* nematode infections using McMaster worm egg counts, and PCR assays of Internal transcribed spacer (ITS-2 nuclear ribosomal DNA) to screen genomic DNA extracted directly from lamb fecal samples. Lambs from four farms in southern Western Australia were sampled rectally on two separate occasions, with McMaster WECs and PCRs conducted on 858 samples. Negative controls (n = 96), worm egg count (WEC) <50 eggs per gram, and positive controls (n = 96) containing approximately equal proportions of *Teladorsagia circumcincta*, *Trichostrongylus colubriformis*, *Haemonchus contortus*, *Oesophagostomum* spp., and *Chabertia ovina*) were generated. All control samples were amplified following positive controls. The two diagnostic tests identified high levels of agreement (Kappa values ≥ 0.93). PCRs detected an additional 2.0% of samples as strongylid-positive, but there was no significant difference in the number of strongylid-positive samples identified using PCR or McMaster WEC (Sweeny et al., 2011).

A study was conducted in Peshawar, Pakistan, for which 4490 fecal samples were collected from cattle of different breeds, including males and females to investigate GIT parasites. The Direct smear method was used for the detection of parasite ova. The prevalence of parasites was higher in females 2411 (83.10%) than in males 490 (16.90%). Out of 2901 GIT parasites, 2209 (76.15%) were helminths, and 395 (13.62%) were protozoan parasites. In males, *Trichostrongylus colubriformis* had the highest prevalence (13.83%), while in females, *Trichostrongylus colubriformis* (16.24%) had the highest prevalence (Rafiullah et al., 2011).

A study was conducted at Government Research Centre for Conservation of Sahiwal Cattle (RCCSC) Jehangirabad, District Khanewal, Pakistan. For this purpose, about 333 fecal samples were collected directly from the rectum of sheep to determine the *Haemonchus contortus* prevalence. Direct microscopy and McMaster techniques were implied to determine *Haemonchus contortus* prevalence. The sample analysis revealed that the highest prevalence of *Haemonchus contortus* at 77.7% (Tasawar et al., 2010).

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In Hyderabad, Sindh, Pakistan, 1200 fecal samples were collected from sheep. McMaster, pastures larval count, and necropsy worm count techniques were implied to examine and identify the nematodes in sheep. The analysis of the sample revealed that the 24.6% prevalence of *Haemonchus contortus* recorded was symbolic (Al-Shaibani et al., 2008).

A study was conducted in Mymensingh district, Bangladesh, the determination the prevalence of GIT parasites in goats. For this purpose, 150 viscera from different slaughtered houses were collected. After examination, it was concluded that helminths were responsible for Bengal goat diseases. A total of 5 species were identified, such as *Oesophagostomum columbianum* (92%), *Trichuris ovis* (56.66%), *Schistosoma indicum* (38%), *Moniezia expansa* (10.66%) and *Moniezia benedeni* (2.66%) and these infections were highest in winter (100%) than that in summer (89.33%; Mohanta et al., 2007).

CONCLUSION

The overall high prevalence of gastrointestinal parasites in this review in some regions implies this issue is a very serious problem that reduces the productivity of livestock around the globe. The frequency of GIT parasites in cattle should be reduced by teaching farmers the value of intensive cattle management, environmental sanitation, strategic deworming of livestock using effective broad-spectrum anthelmintics, biological control of the parasites and breaking their life cycle, and intermediate hosts

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Arsalan Khan, Muhammad Jamil and Saeed Ullah wrote the manuscript, Faiqah Ramzan, Hina Khan and Naimat Ullah reviewed the manuscript, Mubarik Ali, Atta Ur Rehman collected the materials and Norina Jabeen and Rahila Amber formatted and revision of the manuscript.

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The authors declare the competing interests as none.

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Ethical considerations have been made by the authors.

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Trichinella spiralis as a Potential Antitumor Agent: An Update

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ABSTRACT

Due to the limited success of therapeutic strategies in treating tumors, a new practical potent approach is needed. This review aimed to investigate previous literature related to tumors and *Trichinella spiralis (T. spiralis*). In recent years, there has been growing interest in utilizing biological, viral, bacterial, yeast, and parasitic agents to cure cancers. According to several studies, some parasites could interferee with the tumors' growth. There has been much discussion about some parasites' applications to cure tumors in animals and humans. In studies, *T. spiralis* was found to have antitumor properties. The active proteins in *T. spiralis*, such as Caveolin-1, Heat shock proteins, and Ribosomal proteins, are thought to inhibit the growth of cancers, such as melanoma, myeloma, sarcoma, leukemia, stomach cancer, colon cancer, breast cancer, and lung cancer. In addition, these proteins are thought to induce apoptosis in specific neoplastic cells. Accordingly, antigens derived from parasites may be helpful in cancer immunotherapy. However, there are still many unanswered questions regarding *Trichinella spiralis'* potential use as a biotherapy agent against cancer. Future studies should focus on the purification of parasite antigens and their use for wider-scale trials in animal models.

Keywords: Antitumor, Apoptosis, Cancer, Immunotherapy, *Trichinella spiralis*

INTRODUCTION

Malignant tumors are one of the most threatening issues concerning people's well-being and are responsible for many human deaths (Carneiro and El-Deiry, 2020). Immunotherapy is a new approach in the field of oncology that confronts cancerous tumors by amplifying natural antitumor defenses (Schirrmacher, 2019; Wu et al., 2020). Detection of tumor antigens indicates that immunotherapy may be beneficial by stimulating the immune system's tumor suppressor mechanisms, and does not have side effects of chemotherapy or surgery (Harrington et al., 2019; O'Donnell et al., 2019). Cancer patients show early treatment improvement with autologous and allogeneic tumor cell vaccines (Pallerla et al., 2021). Many obstacles can hamper clinical success. These obstacles include inadequate antigenic natures, immune tolerance, and active immune evasion mechanisms used by progressing tumors to circumvent the immune system (Martin et al., 2020; Jhunjhunwala et al., 2021). The body's immune system must be stimulated to develop a new cancer treatment (Mulder et al., 2019). Furthermore, the immune system must be ready to attack cancer cells individually (Netea et al., 2020). This specificity will enable the immune system to overcome these obstacles (Bassiony et al., 2020).

The concept of tumor biotherapy has been developed as a clinical strategy for cancer treatment. It aims at suppressing or eradicating tumors using biological agents as therapeutic tools. Examples of these therapies include cytokines, monoclonal antibodies, growth factors, differentiation factors, cancer gene therapy, and antitumor bioactive materials (Kelley and Greten, 2021).

Several parasitic infections have been shown to induce antitumor activity in both laboratory animals and humans (Callejas et al., 2018; Daneshpour et al., 2019; Hu et al., 2019; Berriel et al., 2021). To justify this claim, a documented negative correlation has been established between the prevalence of some parasitic infections and cancer cases (Krementsov, 2009). Some cancer patients infested with certain parasites have reported a much longer lifespan than those who were not (Suresh et al., 2005). However, it is not feasible to activate the anticancer response through parasitic infections due to the morbidity and virulence of parasites. The administration of live vaccines containing non-pathogenic parasites could be an appropriate alternative (Kurup and Thomas, 2020). Acquired and innate immunity, anti-angiogenesis properties, increased cell apoptosis, and common antigen presentation may all contribute to tumor resistance induced by parasites (Albini et al., 2018). Mucins play a critical role in maintaining mucosal homeostasis and are responsible for the differential effector and regulatory responses against many microorganisms, including

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commensals and parasites (Dharmani et al., 2009). It has been found that several parasites share mucin-type O-glycan compounds, which are common antigens between cancer cells and parasites (Tarp and Clausen, 2008; Darani and Yousefi, 2012; Grondin et al., 2020).

In addition, parasites with high amounts of glycosylated antigens like *Echinococcus granulosus* may demonstrate superior anticancer effects against immune tolerance of cancer (Berois et al., 2022).

The active proteins in *T. spiralis*, such as Caveolin-1, Heat shock proteins, and Ribosomal proteins, inhibit cancers, such as melanoma, myeloma, sarcoma, leukemia, stomach cancer, colon cancer, breast cancer, and lung cancer (Kang et al., 2013; Liao et al., 2018). It is not yet known how *T. spiralis* inhibits tumor growth. The intestinal phase of *T. spiralis* is an example of a complex multicellular organism, and its potential to induce a T helper 2 (Th2) immune response is matchless (Ilic et al., 2012). It also secretes lipids, proteins, and metabolites that the immune system recognizes. So, the proliferation, differentiation, and activation of natural killer cells, cytotoxic T cells, and macrophages could be stimulated by *T. spiralis* infection (Zhang et al., 2018; Wang et al., 2020; Sun et al., 2022). Therefore, they would secrete elevated amounts of interleukins, interferons, transfer growth factor, tumor necrosis factor, and colony-stimulating factor (Fabre et al., 2009; Ilic et al., 2011). *In vivo* could activate macrophages to produce oncolytic molecules that kill tumor cells directly (Khan, 2008; Liu et al., 2015). Inhibiting the metastasis and proliferation of neoplastic tumors, Natural Killer (NK) cells serve as the primary defense against tumorigenesis (Pachynski et al., 2012; Smyth et al., 2002). When mice are infected with *T. spiralis* during the early muscle stage, NK cells trigger a cytotoxicity reaction *in vivo* (Patel et al., 2009). Interferon-Gamma (IFN-γ) and Tumour Necrosis Factor alpha (TNF-α) are two of the most potent pro-inflammatory cytokines with a wide range of biological activities (Boshtam et al., 2017; Shapouri-Moghaddam et al., 2018). The IFN-γ and TNF-α can cause tumor cells to necrosis and directly induce apoptosis within them in addition to vascular destruction around neoplasms (Chawla-Sarkar et al., 2003; Cruceriu et al., 2020; van Horssen et al., 2006). Cluster of Differentiation 8+ (CD8+) T cells can be cytotoxic when exposed to Interleukin 10 (IL-10), which has an antitumor function (Gu et al., 2017). Mouse models of tumors with CD8+T cells expressed with IL-10 have been shown to suppress tumor growth by producing higher amounts of IFN-γ (Jarnicki et al., 2006; Ruffell et al., 2014). IL-12 can also kill primary and metastatic tumors via the T helper 1 (T1) reaction and the promoted activation of CD8+T cells (Paijens et al., 2021). Consequently, cellular immune function, mediated by Th1 cells, suppresses malignant cell proliferation and angiogenesis. This review aimed to examine previous literature investigating the relations between *T. spiralis* and tumors.

General features of *Trichinella spiralis*

The *T. spiralis* is a widely distributed food-borne parasite that could trigger antitumor immunity by modulating immune system activity (Liao et al., 2018). *T. spiralis* is an obligate intracellular parasite that causes trichinosis in humans and many animals (Gottstein et al., 2009). Excretory-secretory proteins (ESPs) are complex proteins produced by *T. spiralis* during infestation (Babal et al., 2011). It is believed that polypeptide proteins, as well as ESPs, may inhibit tumor growth during the life cycle of *T. spiralis*, which includes the muscle larva (ML), the newborn larva (NBL), and the adult worm (AD, Romaris et al., 2002). The muscle larva has a more significant effect on enhancing host immunity because it lives longer, compared to the newborn and adult stages (Hewitson et al., 2009). Besides, ML is more accessible to collect than NBL or AD (Figure 1).

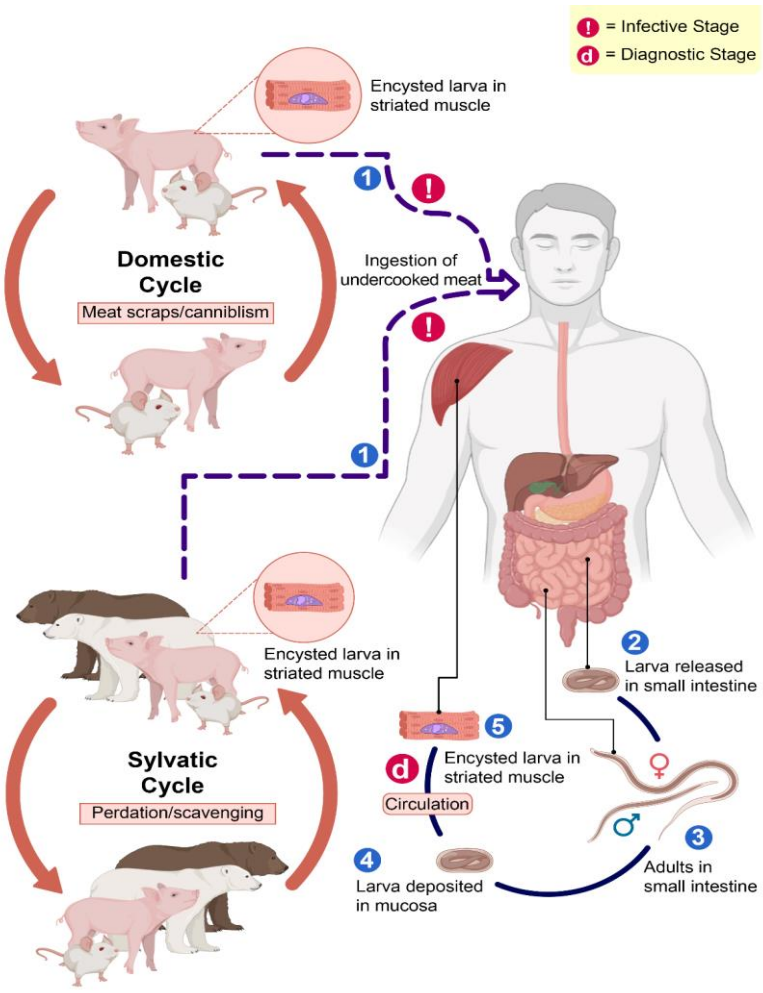

Figure 1. Life cycle of *Trichinella spirali*

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Trichinella spiralis proteins

Translationally controlled tumor protein

Recently some studies demonstrated that Translationally Controlled Tumor Protein (TCTP) has a high conservation level and is an abundant protein across various eukaryotic organisms (Bommer and Thiele, 2004). In tumor reversion, this protein is significantly downregulated (Bommer, 2017). It has been shown that factors, including cell cycle progression, histamine-releasing factors, malignant transformation, antiapoptotic, immunomodulatory functions, and calcium-binding proteins, play a crucial role in cell growth. Translationally controlled tumor protein has been found in *Plasmodium* subspecies and trematodes, and also some parasitic worm species, such as *Trichinella* (Mak et al., 2007; Nagano et al., 2009).

Caveolin-1

The caveolin-1 protein (*cav-1*) is part of the caveolae, which are introversions of the cells' plasma membrane in the form of flasks (Raja et al., 2019; Gokani and Bhatt, 2022). In some cancers, *Cav-1* causes apoptosis and cell cycle arrest at the first stages of tumorigenesis (Volonte and Galbiati, 2020; Arfin et al., 2021). Suppression Subtractive Hybridization (SSH) technique has been used to clone the *cav-1* gene from *T. spiralis* as an adult-specific antigen, which has been demonstrated to be extracted from maturing embryos and oocytes of this parasite (Wu et al., 2021).

Heat shock proteins

In addition to regulating cell growth, survival, and differentiation, heat shock proteins (HSPs) play an active role in the flexibility, intracellular arrangement, and proteolytic turnover of cells (Villesen et al., 2020; Karamanos et al., 2021; Lang et al., 2021). They are considered powerful immunoadjuvants that can lead to more substantial antitumor impacts (Banstola et al., 2020). Heat-inducible proteins, including sHSP, HSP60, HSP70, and histone H3, have been isolated from ES products and somatic extracts of *T. spiralis*, (Sun et al., 2018; Grzelak et al., 2020; Grzelak et al., 2022). Thw HSPs prevent cell death in *T. spiralis* and sustain homeostasis (Bolhassani and Agi, 2019).

Ribosomal proteins

Ribosomal proteins are essential for repairing DNA structure, cell differentiation, and development, generally overexpressed in cancers, such as esophageal, gastric, liver, and colorectal cancer (Mao-De and Jing, 2007; Abraham and Meltzer, 2017; Xie et al., 2018). The use of iRNA therapy within the past 30 years could help researchers treat many types of tumors and tumorigenic viruses with iRNA (Soudyab et al., 2016). There is still an underlying mystery regarding iRNA therapy since other novel cancer immunotherapies have emerged, and the fact that iRNA therapy has not been as practical and valuable as initially believed; therefore, it is unclear how it works (Taghikhani et al., 2020; Di Martino et al., 2021). A recent study of BALB/c mice showed that *Trichinella* iRNA significantly reduced the growth of mouse myeloma tumors (SP2/0). *Trichinella spiralis* also contains two ribosomal proteins, S24 and S24e, involved in DNA repair, cell growth regulation, and cell differentiation and are overexpressed in different types of cancer including gastric, colorectal, esophageal, and liver cancer (Duan et al., 2013).

Tropomyosins

Tropomyosins (Tms) are the core components of microfilaments (or actin filaments), which are the thinnest filaments of the cytoskeleton (Karabinos, 2019). Many eukaryotes contain Tms, which are acidic proteins found in yeasts, worms, flies, crustaceans, frogs, birds, and mammals (Gunning et al., 2008; Choi et al., 2012; Jeong and Park, 2020). A number of studies have demonstrated that Tms suppress both breast and bladder cancer as well as astrocytoma, central nervous system tumors, and colon cancers (Helfman et al., 2008; Humayun-Zakaria et al., 2019). An antitumor response was observed in SP2/0 myeloma cells with *T. spiralis* associated antigen, which could also stimulate cross-protective immunity against the tumor (Gong et al., 2011).

The prevention and treatment effects of *Trichinella spiralis* on cancers

Theantitumorr properties of *T. spiralis* have been demonstrated in numerous studies. It was first described in the 1970s that *T. spiralis* had an antitumor effect (Weatherly, 1970). Nevertheless, only limited progress has been made in this field due to inconsistent research. In this regard, it remains unclear how these inhibitory effects are acted. Furthermore, clinical trials have not provided compelling evidence linking *T. spiralis* to the prevention or treatment of tumors. There is also evidence that *T. spiralis* may trigger or contribute to tumor coinfections which mainly include viral, fungal, and bacterial infections (Hu et al., 2021). *Trichinella spiralis'* antitumor effect is not only attributed to increased innate immune function but may also be due to excretory-secretory (ES), which are complex proteins produced by *T. spiralis* during the infestation may have some antitumor effects indirectly by changing the expression of a tumor gene or directly by affecting antitumor activity including the apoptosis, immunomodulatory and anti-inflammatory effects which suppress the tumor growth (Sofronic-Milosavljevic et al., 2015; Vasilev et al., 2015; Ding et al., 2020b, Figure 2).

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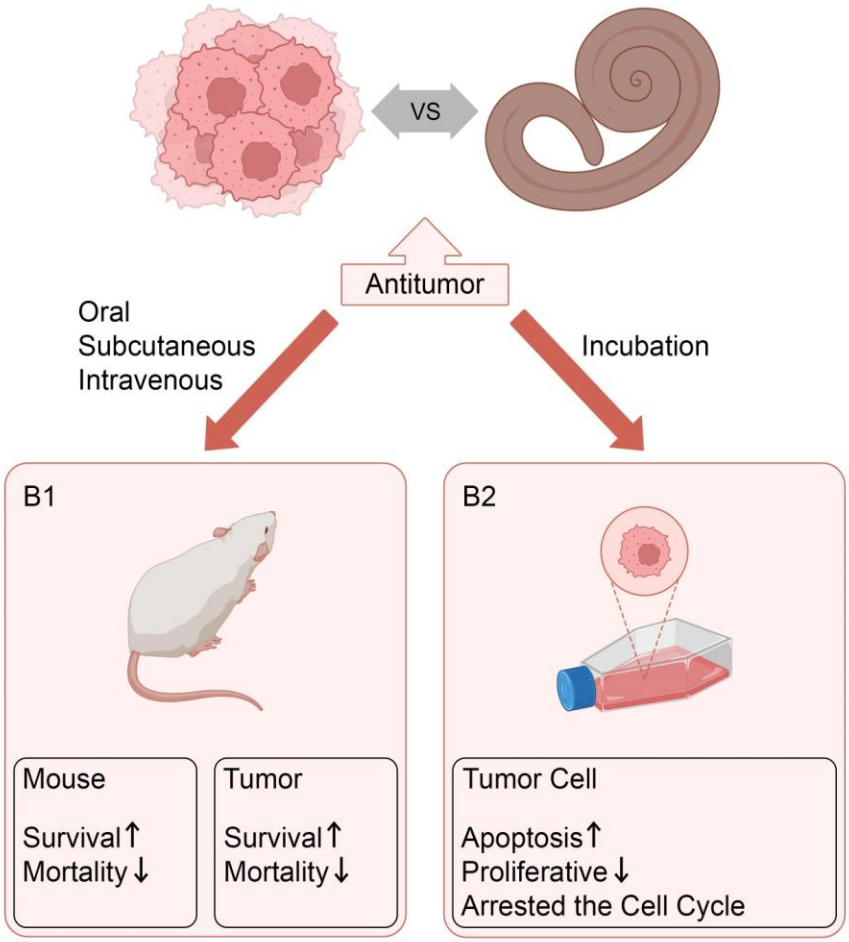

Figure 2. Relations between *T. spiralis* and tumors

Hepatocellular carcinoma

The sixth most prevalent type of cancer is hepatocellular carcinoma (HCC) in humans and animals (Balogh et al., 2016; Heimbach et al., 2018). The prognosis for HCC is driven by the tumor stage, with curative options providing a 5-year survival exceeding 70% for early-stage HCC compared with a median survival of ~1–1.5 years for symptomatic advanced-stage cases treated with systemic therapies (Villanueva, 2019). HCC is an aggressive and highly malignant tumor with a survival rate of less than 5% in 5 years (Grandhi et al., 2016). Available treatment methods have only been effective in some patients. Due to the high mortality rate and high risk of recurrence after treatment, new treatment methods are mandatory (Ringelhan et al., 2018; McGlynn et al., 2021). The regulatory mechanism of *T. spiralis* nurse cell formation is similar to tumor cell apoptosis signal regulation (Elhasawy et al., 2021). *Trichinella spiralis* nurse cell formation is a complex process and involves differentiation and cell cycle arrest of infected muscle cells. In other words, the nurse cell formation apoptotic pathways may be activated by antitumor genes that can suppress cell proliferation or induce apoptosis of the tumor cells (Wang et al., 2009). Although several parasites have been described for their ability to fight tumors, *T. spiralis* has proven particularly effective in cancer immunotherapy (Dabrowska et al., 2008). Many cytokines produced by T. spiralis are capable of inhibiting tumor growth. In addition, skeletal muscle cells are affected by *T. spiralis* infection during nurse cell formation, causing various changes (Dabrowska et al., 2016). As a result of these changes, muscle cells begin to differentiate, and apoptosis occurs, then the infected cells are stopped in the G2/M phase of the cell cycle (Wang et al., 2013). Additionally, *T. spiralis* and its extract inhibit tumor growth and induce apoptosis in tumor cells by stimulating mitochondrial pathways and death receptor pathways and apoptosis-related genes (Ding et al., 2020a; Ding et al., 2021). As part of the immune response to *T. spiralis* infection, the expression of c-Ski protein (a tumor suppressor protein) and genes associated with signaling pathways such as *p53* (apoptosis genes are expressed), *SMAD2*, and *SMAD4* are activated (Zakeri, 2017; Boros et al., 2019). These changes occur simultaneously with the increased activity of apoptosis factors involved in a mitochondrial pathway, such as caspase 9 and Bcl-2 associated protein X (BAX), as well as a death receptors pathway, such as tumor necrosis factor-alpha (TNF-α), caspase 8, and caspase 3 (Wu et al., 2005). *Trichinella spiralis* can induce apoptosis in HCC, because its infection has similar regulatory mechanisms to cancer cell apoptosis signals and it represents a promising approach to overcoming this cancer.

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Lung cancer

Among all malignant cancers worldwide, lung cancer has the highest mortality rate in humans (Bade and Cruz, 2020). Numerous factors, including population aging, smoking, and environmental pollution, have contributed to an increase in the mortality rate due to lung cancer in recent years (Rudin et al., 2021). Unfortunately, lung cancer is mostly identified at late stages, and the survival rate is less than 15% in 5 years. Due to the quick multiplication time in small cell lung cancer (SCLC), lung cancer has a poor prognosis (de Groot et al., 2018; Barta et al., 2019; Schabath and Cote, 2019; Thandra et al., 2021). Chemotherapy is the primary therapeutic option for advanced SCLCs but has significant side effects, including an increased risk of cancer recurrence (Yang et al., 2019; Oronsky et al., 2022). Contrarily, biological therapy is regarded as a secure and effective therapeutic approach. As a result, numerous experimental studies have been carried out to investigate how biological therapy can inhibit the growth of cancers, including those that examine the antineoplastic properties of parasites. According to numerous studies, *T. spiralis has two different types of antineoplastic mechanism*s. First, *T. spiralis* may cause an immunized response in the host by parasitizing tumor antigens of cancer cells. Second, *T. spiralis* may contain substances that directly start the apoptosis of cancer cells (Liao et al., 2018). *T. spiralis* could cause cell apoptosis through mitochondrial apoptosis pathways by first activating caspase-9 and then caspase-3 (Yu et al., 2014). The expression of pro-apoptosis genes like *BAX*, *Cyt-C*, *Apaf-1*, *caspase-9*, and *caspase-3* may be upregulated by ESPs, whereas the expression of anti-apoptosis genes *Bcl-2* and *Livin* may be downregulated (Bruschi et al., 2022). Therefore, it could be concluded that ESPs can activate mitochondria to release high levels of Cyt-C into the cytoplasm (Akl et al., 2014). The polymerization caused by Cytochrome C (Cyt-C) with Apoptotic protease activating factor-1 (Apaf-1) and procaspase-9 may further stimulate caspase-9 and caspase-3, which would then cut substrate proteins in the cell (Martínez-Lostao et al., 2015). The ESPs may also prevent the anti-apoptosis protein Livin from performing its apoptosis regulation functions on the cascade reaction, which would ultimately influence apoptosis in H446 cells, a small cell lung cancer cell line (Luo et al., 2017). As a result, *T. spiralis* Muscle larva (ML) ESPs trigger intrinsic mitochondrial pathways, which in turn cause apoptosis in H446 SCLC cells. In conclusion, it was found that *T. spiralis* ML ESPs may prevent human H446 SCLC cells from proliferating and trigger their apoptosis by activating mitochondrial apoptosis pathways.

Melanoma

The most aggressive type of skin cancer is melanoma in humans and animals (Miller and Mihm Jr, 2006). Because this tumor is largely resistant to conventional chemotherapy, patients with advanced disease have a poor prognosis (Garbe and Leiter, 2009; Schadendorf et al., 2018). Finding new therapeutic strategies for the treatment of melanoma could be a valuable subject for research to find substances that can affect the apoptotic process of the disease (Schadendorf et al., 2015; Domingues et al., 2018; O'Neill and Scoggins, 2019). All three stages of the T. *spiralis* life cycle appear to contain elements that can control malignancy based on a few studies currently available in this field. *Trichinella spiralis* can inhibit the growth of B16 melanoma by the action of ES L1 antigens (a component unique to the chronic phase of this infection, Kang et al., 2013). Studies conducted *in vitro* showed that ES L1 antigens affect B16 melanoma cells that are both anti-survival and pro-apoptotic (Vasilev et al., 2015).

CONCLUSION

Today, the knowledge of *T. spirals'* role in antitumor therapy has greatly improved due to advancements in research on the relationships between the organism and tumors. Antigens derived from parasites may be helpful in cancer immunotherapy in humans and animals. Studies conducted *in vitro* showed that *T. spiralis* antigens affect different cancer cells in hepatocellular carcinoma, lung cancer, and melanoma by activating mitochondrial apoptosis pathways. Cellular immune function, mediated by Th1 cells, suppresses malignant cell proliferation and angiogenesis. Clinical trials have not provided compelling evidence linking *T. spiralis* to the prevention or treatment of tumors. Future studies should focus on the purification of parasite antigens and their use for wider-scale trials in animal models.

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Authors' contribution

Soheil Sadr was the principal author who directed and prepared the review paper. Zahra Yousefsani, Pouria Ahmadi Simab, Ahad Jafari Rahbar Alizadeh, and Narges Lotfalizadeh participated in the preparation of the final version of the manuscript. Hassan Borji participated as a supervisor and assisted in preparing and proofreading of the manuscript. All authors have read and approved the final version of the manuscript for publication in the present journal.

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Competing interests
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Ethical consideration
The ethical considerations including plagiarism, consent to publish, misconduct, fabrication and/or falsification of data, dual publication and/or submission, and redundancy checked by authors.

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The Effects of Grounded Herbs on the Intestinal Villus Height and Shedding of F18-positive *Escherichia coli* in Weaned Pigs

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ABSTRACT

Antibiotics have been widely used to control and treat infections caused by *Escherichia coli* (*E. coli*) in weaned pigs. The bacteria resistance to antibiotics can occur naturally; however, the misuse of antibiotics can accelerate this resistance. New antibiotics are developed very slowly, and only two new classes of antibiotics have been developed in the past 40 years. This makes herbal medicine a promising method for fighting against antibiotic-resistant bacteria. In the current study, 25 male crossbred (Duroc x Landrace x Large white) weaned piglets with an average weight of 6-8 kg were examined for 24 days. The pigs were randomly assigned to five groups in a completely randomized design with five replicates (1 pig/pen). All treatments included 20% crude protein corn-soybean as the basal diet. The negative control group received no supplementation, while pigs in the second experimental group received a basal diet supplemented with 150 ppm colistin sulfate. Basal diet and herbal mixture (*Andrographis paniculata*, *Zingiber cassumunar*, and *Garcinia mangostana*) were fed to three other experimental groups at 500, 1000, and 2000 ppm. The F18-positive, colistin-resistant *E. coli* were orally inoculated to all pigs for 9 days. The antibacterial and anti-diarrheal effects of this diet and its effect on the inoculated pigs' intestinal villi were evaluated. The results indicated that supplementation of this herbal mixture at levels of 500, 1000, and 2000 ppm had antibacterial effects, with no significant difference between doses. However, the positive effects of this herbal mixture on intestinal villi height and diarrhea were found only in pigs that received 1000 and 2000 ppm of the herbal mixture. From a practical point of view, supplementation of this herbal mixture at 500 and 1000 ppm could be applied for prophylaxis during the weaning period, whereas 2000 ppm of the herbal mixture could be used for the treatment of postweaning *E. coli* diarrhea.

Keywords: *Andrographis paniculate*, *Escherichia coli*, *Garcinia mangostana*, Herbal mixture, *Zingiber cassumunar*

INTRODUCTION

Antibiotic resistance has been among the greatest threats to global health and food security for over four decades (Swann Committee, 1969). One of the accelerating factors of antibiotic resistance is its misuse in humans and animals (WHO, 2020).

Pork is the second most popular kind of meat in terms of global consumption (Shahbandeh, 2022). The most common cause of illness and death in weaners is a gastrointestinal infection resulting from *Escherichia coli* (*E. coli*), which causes diarrhea in neonatal and weaned piglets (Aarestrup et al., 2008). Postweaning *E. coli* diarrhea (PWECD) and edema disease (ED) are common in weaned pigs. The PWECD and ED occur mainly in the first week after weaning (Bertschinger et al., 2000). The death rates of affected pigs may be as high as 25% and 90% in PWECD and ED, respectively (Fairbrother and Nadeau, 2019). The profitability of pig farms affected by PWECD and ED will decrease due to high mortality rates, decreased weight gain, and the high cost of treatments, vaccinations, and feed supplements (Wang et al., 2019). The response to the outbreak of diarrhea caused by *E. coli* needs instant action, such as antibiotics treatment. However, considerable evidence shows that antibiotic therapy in swine induces the selection of resistant bacteria (Österberg et al., 2016; Nguyet et al., 2022). Despite the growing number of antimicrobial-resistant *E. coli* strains found worldwide in pig farms (Peng et al., 2022), the pace of developing new antibiotics has been sluggish. Only two new classes of antibiotics have been developed in the last 40 years. This makes herbal medicine a promising means of fighting antibiotic-resistant bacteria (Adzitey et al., 2019). Nevertheless, the cautionary reminder to use herbs with care is highlighted by the reports of bacterial resistance to herbal antimicrobials reviewed by Vadhana et al. (2015).

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Resistance may develop if only one active principle is involved. Resistance is less likely to occur if multiple active principles are involved (Gupta and Birdi, 2017).

MuPlus® is produced in Thailand from the powder of three grounded herbs, namely *Andrographis paniculata* (*A. paniculata*), *Zingiber cassumunar* (*Z. cassumunar*), and *Garcinia mangostana* (*G. mangostana*). Each powder has shown a wide range of biological activities. For instance, *A. paniculata* has anti-inflammatory (Shen et al., 2002), anti-allergic (Xia et al., 2004), anti-platelet aggregation (Amroyan et al., 1999; Lu et al., 2011), hepatoprotective (Trivedi and Rawal, 2001), anti-HIV (Reddy et al., 2005), antibacterial (Mishra et al., 2013), and anti-diarrheal (Gupta et al., 1990) properties. The *Z. cassumunar* has pharmacological properties, such as antimicrobial, antioxidant, insecticidal, anti-cancer, anti-cholinesterase, and anti-inflammatory activities (Singh et al., 2015.(Finally, the pharmacological activities of *G. mangostana* are antioxidant activities, antitumoral, anti-inflammatory, antiallergy, antibacterial, antifungal, and antiviral properties (Pedraza-Chaverri et al., 2008; Obolskiy et al., 2009). However, these effects have been determined based on single bioactive phytoconstituents of the herbs, and most reports were derived from *in vitro* tests. Studies regarding these herbs in animals mostly involve growth performance (Herawati et al., 2020; Shi et al., 2020) and treatment of some diseases, such as Influenza (Chen et al., 2009) and Mycoplasma gallisepticum (Luo et al., 2022).

In the present study, the antibacterial and anti-diarrheal effects of a combination of these herbs and their effects on intestinal villi height were demonstrated in weaned pigs hosting hemolytic *E. coli* F18.

MATERIALS AND METHODS

Ethical approval

The Institutional Animal Care and Use Committee of Khon Kaen University, Thailand, reviewed and approved the experimental protocol based on the Ethic of Animal Experimentation of the National Research Council of Thailand (IACUC-KKU-60/63).

Escherichia coli strain

Weaned pigs with a sign of convulsion and edema of eyelids from a backyard farm in Khon Kaen province was submitted to the Faculty of Veterinary Medicine, Khon Kaen University, Thailand, for disease diagnosis. Hemolytic *E. coli* was isolated from the intestinal contents of the pigs. At necropsy, edema of mesocolon was also observed. A pure colony of isolated hemolytic *E. coli* strain selected for the experiment was detected for genes encoding F18ac+fimbrial adhesin, heat-stable toxin a (Sta), and verotoxin (STx2e). In addition, the antibiotic-resistant profile was also seen. Gene encoding of the major fimbrial subunit of F18 (fedA) was detected using fedA-specific primers (forward primer FedA 1: 5'-GTGAAAAGACTAGTGTTTATTTC-3' and reward primer FedA 2: 5'-CTTGTAAGTAACCGCGTAAGC-3', size of the amplified product 510 bp) in accordance with Imberecht et al. (1992). The PCR products (fedA) were digested with restriction enzyme *Ngo*MIV (New England Biolabs, USA) following the supplier's instructions, and the size of fedA was observed after electrophoresis on 1.5% agarose gel as described by Imberecht et al. (1994). The presence of Sta was confirmed using Sta primers (forward Sta1: 5'-tctttcccctctttagtcag-3', and reward Sta2: 5'-acaggcaggattacaacaaag-3', size of the amplified product 166 bp) according to Osek et al., (1999) whereas verotoxin-producing gene was confirmed using STx2e primers (forward STx2e -a: 5'-ccttaactaaaaggaatata-3' and reward STx2e -b: 5'-ctggtggtgtatgattaata-3', size of the amplified product 230 bp) according to Johnson et al., (1990). The isolated *E. coli* strain designated as F18ac+StaSTx2e virotype is resistant to colistin sulfate (CS) and was kept in 25% glycerol at 80°C until used.

Herbal mixture

The herbal mixture used (MuPlus®, Thailand) was supplied by Lily FoodAnSci Ltd, based in Thailand. The powder blend consisted of three herbs, namely *A. paniculata*, *Z. cassumunar*, and *G. mangostana*. The *A. paniculata* and *Z. cassumunar* were standardized to include 6% andrographolide and 0.8% volatile oil, while *G. mangostana* contained tannin and xanthones.

Pigs

A total of 25 male crossbred (Duroc × Landrace × Large white) weaned piglets weighing 6-8 kg from the Faculty of Agriculture demonstration farm at Khon Kaen University, Thailand, were used. The piglets arrived at the experimental animal building of the Faculty of Veterinary Medicine at Khon Kean University in Thailand at the beginning of February 2020. The facility was disinfected with a quaternary ammonium compound three days before the start of the experiment to acclimate the animals. Each pig was housed on a concrete floor in an individual stall measuring 1.5 x 1 meters. The piglets were randomly assigned to 5 groups in a completely randomized design with 5 replicates (1 pigs/pen) for 24 days. All treatments used 20% crude protein corn-soybean diet without antimicrobials, zinc oxide, or organic acids as the basal diet. The negative control group received no supplementation, while pigs in the second

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experimental group received a basal diet supplemented with 150 ppm colistin sulfate. Basal diet and herbal mixture (*Andrographis paniculata*, *Zingiber cassumunar*, and *Garcinia mangostana*) were fed to three other experimental groups at 500, 1000, and 2000 ppm. The F18-positive, colistin-resistant *E. coli* were orally inoculated to all pigs for 9 days. Feed mixed with different supplements was fed to the pigs from the beginning of the study, the day pigs were inoculated with hemolytic *E. coli* until day 24.

Inoculum and inoculation

The *E. coli* was prepared following Frydendahl et al. (2003). For each inoculation, *E. coli* was grown at 37°C overnight on blood agar plates. A loopful (10 µl) of colony material was taken from the blood agar and suspended in 50 ml luria-bertani broth. The bottle was incubated overnight at 37°C with shaking. The bacterial culture was centrifuged, and the pellet was suspended in 900 ml of sterile phosphate buffer solution (PBS). This suspension was adjusted to 10^8 CFU/ml adding PBS until the optical density reached 0.1 by the measure at the wavelength of 625 nanometers. The pigs were orally challenged with 1 ml of this suspension daily for up to 9 days.

Clinical sign and fecal scoring

The pigs were checked twice daily for 24 days for clinical signs such as diarrhea, edema, dehydration, anorexia, depression, vomiting, and death. The fecal scoring was performed by the same animal caregiver throughout the experiment. To determine the severity of postweaning diarrhea, the feces were scored by determining the moisture content as hard feces (0), firm feces (1), soft and formed feces (2), diarrhea with unformed and fluid feces (3), and severe diarrhea with watery and frothy feces (4; Siriwathananukul et al., 2010). Piglets with fecal scores higher than 2 were determined to have diarrhea. The pig that died during the experiment was sent to the department of Pathobiology, Faculty of Veterinary Medicine, Khon Kaen University, Thailand, for a postmortem examination. The identification of hemolytic *E. coli* was confirmed using a standard microbiological test (Quinn et al., 2004).

Quantification of *Escherichia coli* in feces

Fecal samples were collected daily from each pig in a clean plastic bag and sealed with rubber rings. The *E. coli* in the feces was measured on the day the feces were collected. One gram of feces was suspended in 9 ml of PBS. A 10-fold serial dilution was prepared. The Miles–Misra technique (for example, the drop count method) was applied to quantify *E. coli* (Miles et al., 1938). Eight drops of 0.02 ml samples were placed on blood agar. The blood agar plates were incubated at 37°C for 24 hours after the drops were dry. Hemolytic bacterial colonies with less than 40 colonies per drop were counted. The CFU number of *E. coli* in each gram of feces was calculated according to Formula 1 (Petersen and McLaughlin, 2016).

$$X = N \times 10^n \times 50 \text{ CFU / gram} \qquad \text{(Formula 1)}$$

Where, X denotes bacteria counted per gram of feces, N is the average bacterial count per 0.02 ml, and n signifies dilution factor of bacteria counted

Sampling of the small intestine

At the end of the experiment, two pigs per group were intramuscularly sedated with 2 mg/kg of azaperone following Hendrikson et al. (1995), and euthanized with an overdose of intravenous barbiturates. Two cross-sectional pieces of 1 cm length from the duodenum (6 inches distal to the pylorus), jejunum (24 inches distal to the pylorus), and ileum (12 inches proximal to the ileocecal valve) were collected from each euthanized pig immediately after death. The first piece was immediately dipped in 10% formaldehyde, whereas the second piece was longitudinally cut and pinned to a piece of styrofoam with the serosal side down. The longitudinally cut piece was then dipped in 10% formaldehyde. The intestine samples were gradually dehydrated, sectioned at 4 µm, and stained with hematoxylin-eosin, according to Nabuurs et al. (1993). One transverse sample from the cross-sectional cut and one from the longitudinal cut from each part of the intestine were mounted per slide. The intestinal morphology was recorded based on a study by Pluske et al. (1996). The height of eight intact villi (10 x objective) was measured, and the results were recorded as mean villous height in µm. The intestinal morphology was captured with the EVOSTM Core Imaging System (Invitrogen) at three megapixels, and the images were analyzed using IMAGEJ software (NIH, USA; Schneider et al., 2012).

Statistical analysis

The parameters in the current study, including the height of the villi, average fecal score, and average colony number of pathogenic *E. coli* score in 6 periods (1-3, 4-9, 10-15, 16-20, 21-24 and 1-24 days) were individually analyzed by one-way analysis of variance (one-way ANOVA) using R version 4.2.0, Vienna, Austria (R Project, 2022) and the R packages of tidyverse version 1.3.1 (Wickham et al., 2019), ggpubr version 0.4.0, ggplot2 version 3.3.6 (Kassambara, 2020) and cowplot version 1.1.1 (Wilke, 2020) were used to manipulate the data and graphic visualization. Tukey HSD

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was used to compare least-squares means between groups when overall significance for that effect was found. A p-value of ≤ 0.05 determined statistical significance.

RESULTS

Clinical results

One pig that received 150 ppm of CS as a supplement died on day 14 of the experiment. The pig was in good condition until being found dead in the morning without any signs of prior sickness. Its eyelids were swollen, its mesocolon was edematous, and its lymph nodes were enlarged. There was a pure culture of a beta-hemolytic colony derived from the mesenteric lymph nodes. Further biochemical tests, such as indole, methyl red citrate, and urease, indicated that this colony was *E. coli*, resistant to oxytetracycline, penicillin, CS, sulfamethoxazole/trimethoprim, and amoxicillin. No fimbria F18 was detected by fedA-specific primer (Imberecht et al., 1992), but a verotoxin gene was found using STx2e primers (Johnson et al., 1990).

Fecal shedding of hemolytic *Escherichia coli*

The number of hemolytic *E. coli* colonies shed was significantly affected by the supplement (antibiotic and herbal mixture) added to the pig feed ($p < 0.05$). The average number of hemolytic *E. coli* colonies in pigs that received no supplementation in their basal diet (negative control) was significantly higher than that of pigs receiving CS, 500 ppm, 1000 ppm, and 2000 ppm of the herbal mixture ($p < 0.05$). No significant difference was observed between pigs that received CS and those that received all three doses of the herbal mixture ($p > 0.05$). Moreover, different doses of the herbal mixture led to no significant difference between the groups ($p > 0.05$; Figure 1a). During the first three days of the experiment, no significant difference was observed in the feces of pigs receiving different treatments regarding hemolytic *E. coli* ($p > 0.05$, Figure 1b). Significant differences in the number of hemolytic *E. coli* shed were observed between pigs receiving no supplementation and those receiving other treatments from days 4-9 of the experiment ($p < 0.05$, Figure 1c). In this period, pigs that received 2000 ppm of the herbal mixture shed significantly higher amounts of hemolytic *E. coli*, compared to pigs that received CS. After hemolytic *E. coli* challenges stopped at day 9, differences in the number of hemolytic *E. coli* shed between treatment groups were significantly observed from days 10-15 ($p < 0.05$), 16-20 ($p < 0.05$), and 21-24 ($p < 0.05$) of the experiment (Figure 1 d, e, f). Pigs without supplements shed the highest number of hemolytic *E. coli* from days 10-15, 16-20, and 21-24. A higher level of hemolytic *E. coli* shedding was observed in pigs that received CS, compared to pigs that received all doses of the herbal mixture from days 10-15. However, differences in shedding resulting from different doses of the herbal mixture were not observed.

The excreted number of colonies in the negative control group increased over time and peaked on day 17 of the experiment (8 days after the challenges stopped). In contrast, when the challenges were stopped, the pigs that received the herbal mixture indicated decreasing hemolytic *E. coli* shedding levels. The excreted number of hemolytic *E. coli* shed by pigs that received CS continued to increase until 3 days after the challenges were stopped (Figure 1g).

Average fecal score

The result showed that pigs fed no supplementation and 500 ppm of the herbal mixture had fecal scores higher than 2, indicating diarrhea (Figure 2a). The overall average fecal scores of pigs in the negative control group were significantly higher than the average fecal scores of pigs that received CS, 500, 1000, and 2000 ppm of the herbal mixture ($p < 0.05$). Pigs administered CS had significantly lower fecal scores (less diarrhea) than pigs receiving 500 ppm of herbal mixture and significantly higher fecal scores than pigs receiving 2000 ppm of the herbal mixture ($p < 0.05$). Looking at pigs that received the herbal mixture at different doses, pigs that received 500 ppm had significantly higher fecal scores than pigs fed 1000 and 2000 ppm ($p < 0.05$). During days 1-3 of the experiment, only pigs that received 2000 ppm of the herbal mixture had significantly lower fecal scores (less than 2), compared to those that received other treatments ($p < 0.05$, Figure 2b). From days 4 to 9 of the experiment, fecal scores higher than 2, which indicated diarrhea, were observed in pigs that received no supplementation and 500 ppm of the herbal mixture (Figure 2c). Pigs that received no supplementation showed significantly higher fecal scores than other treatments. The fecal scores of pigs receiving 500 ppm of the herbal mixture were significantly higher than those receiving 1000 and 2000 ppm ($p < 0.05$). During days 10-15 of the experiment, the fecal scores of pigs that received no supplementation, CS, and 500 ppm of the herbal mixture were higher than 2, and no difference in fecal scores was observed. However, pigs receiving 1000 and 2000 ppm of the herbal mixture had fecal scores equal to or less than 2 and significantly lower fecal scores than other groups ($p < 0.05$, Figure 2d). From days 16 to 20 of the experiment, pigs that received supplements showed fecal scores equal to or less than 2, whereas pigs that received no supplementation had fecal scores higher than 2. Significantly lower fecal scores were observed in pigs that received CS and 1000 or 2000 ppm of the herbal mixture, compared to pigs that received no supplementation ($p < 0.05$, Figure 2e). From days 21 to 24 of the experiment, the pigs in all treatment groups

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indicated fecal scores lower than 2. However, pigs that received no supplementation still had significantly higher fecal scores compared to pigs that received CS and 1000 or 2000 ppm of the herbal mixture ($p < 0.05$, Figure 2f). The daily pattern of fecal score changes in Figure 2f shows that fecal scores decreased over time. The diarrheal periods of pigs that received no supplementation, CS, and 500, 1000, and 2000 ppm of herbal mixture were 21, 14, 17, 7, and 0 days, respectively (Figure 2g).

Intestinal villous height

The average villus heights of the pigs are presented in Figure 3. Pigs that received CS, 1000, or 2000 ppm of the herbal mixture revealed significantly higher villi, compared to pigs that received no feed supplementation or 500 ppm of the herbal mixture ($p < 0.05$; Figure 3a). Regarding the duodenum and jejunum, pigs in the negative control group or those receiving 500 ppm of the herbal mixture showed significantly lower villus heights ($p < 0.05$), compared to pigs that received CS and 1000 or 2000 ppm of the herbal mixture (Figure 3b and c). In the ileum, significantly lower villus heights were found in pigs that received no feed supplementation, compared to pigs that received CS and 1000 or 2000 ppm of the herbal mixture ($p < 0.05$; Figure 3d).

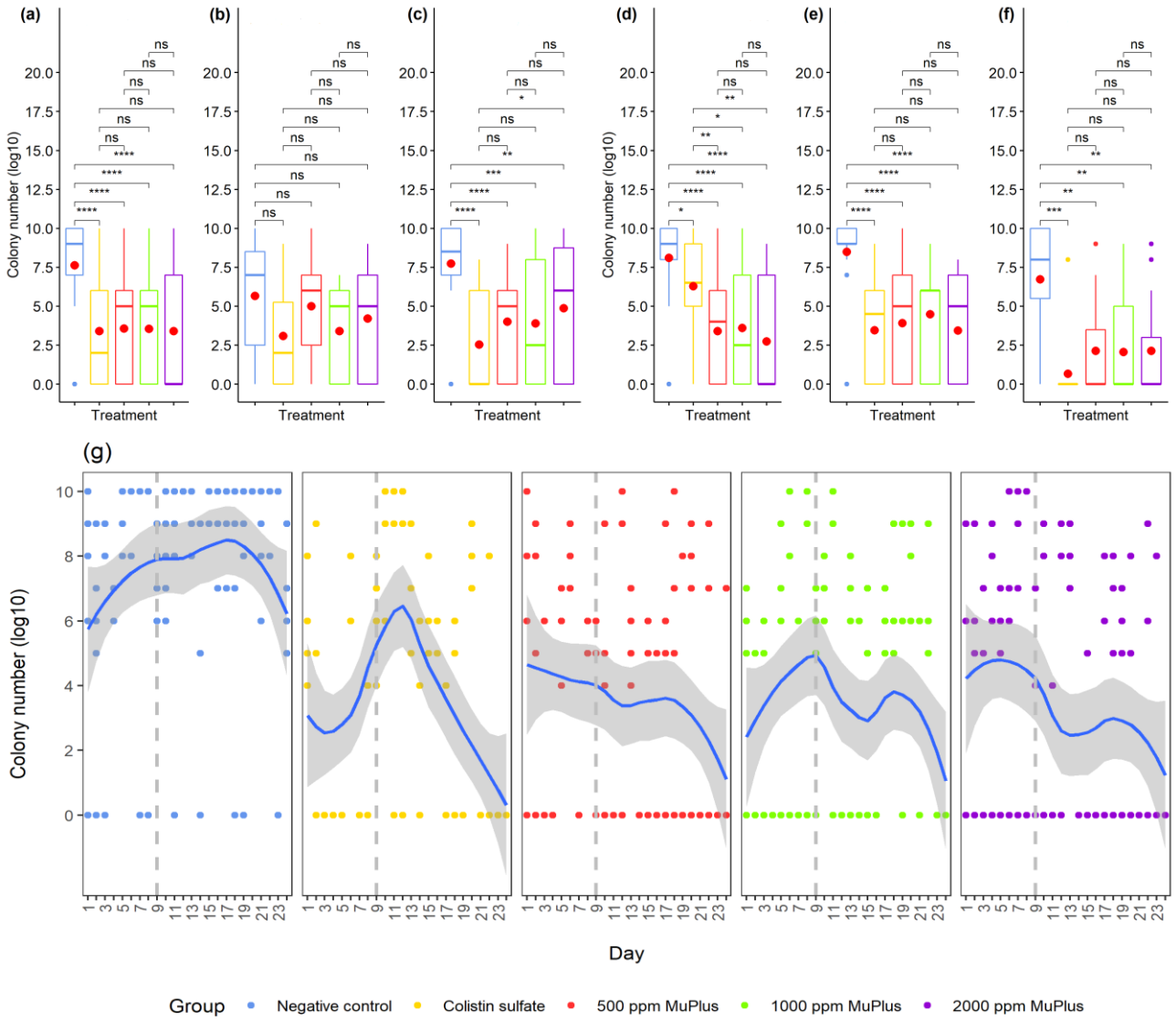

Figure 1. The number of colonies per gram feces of male crossbred (Duroc × Landrace × Large white) pigs challenged with hemolytic *E. coli*. The pigs were randomly assigned to 5 groups which were no supplementation (negative control), 150 ppm CS, 500, 1000 and 2000 ppm of herbal mixture beginning on day one of the experiment. **a:** Overall average colony number in feces of pigs (days 1-24). **b:** Average colony number in feces of pigs from days 1-3. **c:** Average colony number in feces of pigs from days 4-9. **d:** Average colony number in feces of pigs from days 10-15. **e:** Average colony number in feces of pigs from days 16-20. **f:** Average colony number in feces of pigs from days 20-24. The red circles indicate the mean of each group. ****: $p < 0.0001$, ***: $p < 0.001$, **: $p < 0.01$, *: $p < 0.05$, and ns: $p \geq 0.05$. **g:** Log10 colony number change from the beginning to the end of the experiment (day 24). The dots are the data points. The solid curve is the estimated local regression curve. Gray shading indicates the 95% confidence interval. The grey dashed line running vertically illustrates the final day the pigs received hemolytic *E.coli* treatment. Graph were plotted with ggplot2 package and arranged into complex compound figures by cowplot package in R program.

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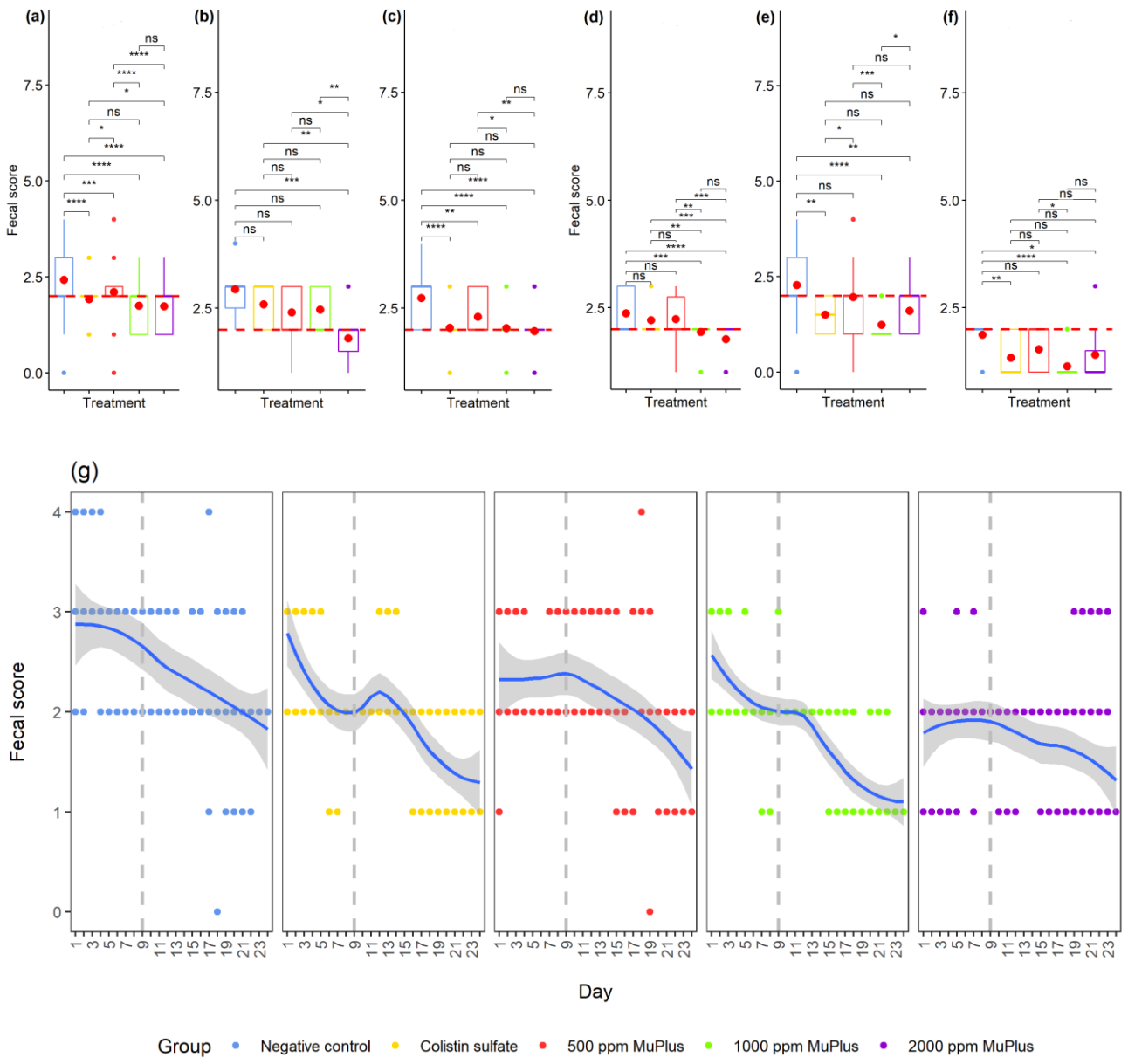

Figure 2. Change in fecal scores of male crossbred (Duroc × Landrace × Large white) pigs challenged with hemolytic *E. coli*. The pigs were randomly assigned to 5 groups which were no supplementation (negative control), 150 ppm CS, 500, 1000 and 2000 ppm of herbal mixture beginning from day one to the end of the experiment (day 24). **a:** Overall average fecal score of pigs. **b:** Fecal score from days 1 - 3. **c:** Fecal score from days 4 - 9. **d:** Fecal score from days 10 - 15. **e:** Fecal score from days 16 - 20. **f:** Fecal score from days 21 - 24. The red circles show the mean of each group. ****: $p < 0.0001$, ***: $p < 0.001$, **: $p < 0.01$, *: $p < 0.05$, and ns: $p \geq 0.05$. **g:** Daily pattern of fecal score changes from the beginning to the end of the experiment (day 24). The dots are data points. The solid curve is the estimated local regression curve. Grey shading indicates the 95% confidence interval. The grey dashed line running vertically illustrates the final day the pigs received hemolytic *E.coli* treatment. Graph were plotted with ggplot2 package and arranged into complex compound figures by cowplot package in R program.

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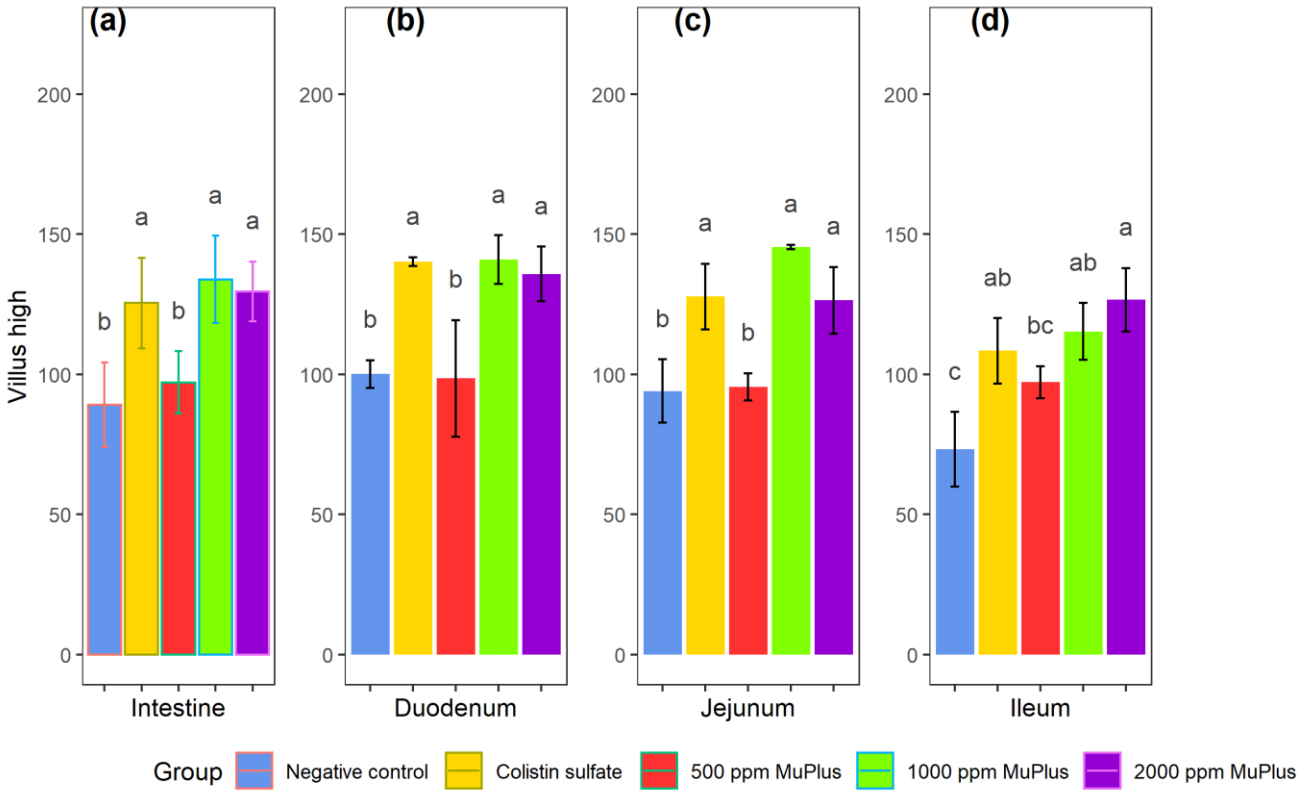

Figure 3. Villus heights at the duodenum, jejunum, and ileum of male crossbred (Duroc × Landrace × Large white) pigs challenged with hemolytic *E. coli*. The pigs were randomly assigned to 5 groups: no supplementation (negative control), 150 ppm CS, 500, 1000, and 2000 ppm of herbal mixture from day one to the end of the experiment (day 24). [abc]Different letters above the error bars indicate significant differences in villus heights ($p < 0.05$)

DISCUSSION

Pigs infected with STx2e-producing *E.coli* may die suddenly without signs of sickness. On the other hand, some affected pigs exhibit symptoms, such as edema of eyelids and forehead, incoordination, and respiratory distress (Fairbrother and Nadeau, 2019). A pig that received 150 mg/kg of CS died 5 days after the last challenge with hemolytic *E. coli*. Postmortem lesions and bacterial identification suggested that the pig died from ED. The hemolytic *E. coli* isolated differed from the one inoculated to weaned pigs because there was no fimbria F18, while STx2e was detected. The detection of other fimbria antigens, such as F4, F5, F6, and F41, was not performed. The study by Baldo et al. (2020) showed that most of the STx2e-producing isolates had the F18 adhesin factor, while only 6.01% had F6 fimbriae, and F4, F5, and F41 were not present. This bacterium could be F6-STx2e virotype, which has become habituated to the swine intestine, where it can survive and persist despite the presence of CS. The bacterium can migrate from the intestinal region to the mesenteric lymph nodes, producing the STx2e toxin that triggers ED (Fairbrother and Nadeau, 2019). Poor absorbance of CS from the gastrointestinal tract to plasma (Rhouma et al. 2016a) and the resistance to CS of this *E. coli* strain could be reinforcing factors that contribute to the development of ED.

This experiment simulated the common real-life situation in which pigs on farms become sick from *E. coli* and shed the bacteria at about 10^8 CFU per gram of feces into the environment (Frydendahl et al., 2003; Boeckman et al., 2022). Pigs in close contact inevitably consume this amount of *E. coli*, colonizing the swine's gastrointestinal tracts and proliferating. The current study revealed that in pigs in the negative control that received no supplementation and were inoculated the same amount of the real-life situation (10^8 CFU), the number of hemolytic *E. coli* shedding increased progressively over time, peaking on day 17. Even after the *E. coli* challenge was stopped, hemolytic *E. coli* shedding continued to grow. This scenario indicated the colonization and proliferation of inoculated *E. coli* in the gastrointestinal tracts of swine (Boeckman et al., 2022). In addition, inoculated *E. coli* induced diarrhea after the first day of inoculation. Despite differences in the strain of the inoculum, the results of this experiment are consistent with those reported by Jensen et al. (2006) and Rhouma et al. (2016b).

In the current study, CS-resistant *E. coli* and 150 mg/kg CS were used as the standard recommended therapeutic dose in pigs (Rhouma et al., 2016a) to simulate a realistic portrait of CS on current pig farms, where pigs might encounter CS-resistant *E. coli*. The supplementation of CS at 150 ppm did not reduce the number of *E. coli* or prevent diarrhea in pigs given CS-resistant hemolytic *E. coli* during the first 3 days or during days 10-15 of the experiment.

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Treatment of secretory diarrhea caused by *E. coli* is based on reducing or eliminating *E. coli*, controlling the motility of the intestines, and controlling secretion of the intestines (Thiagarajah et al., 2015). The Current study revealed that herbal mixture could treat secretory diarrhea caused by *E. coli.* The reduction or elimination of *E. coli* was demonstrated in pigs that received the herbal mixture at 500, 1000, and 2000 ppm. Although there was no direct evidence that motility control was affected by the herbal mixture in the current study, the authors can infer from Nwinyi et al. (2012) that *A. paniculata*, one of the herbal mixture's ingredients, has an anti-motility effect on the gastrointestinal smooth muscle. In addition, secretory diarrhea could be treated with the herbal mixture at 2000 ppm. Gupta et al. (1990) found that *A. paniculata* could control secretory diarrhea caused by the heat-labile and heat-stable enterotoxins of *E. coli*, supporting the result of the present study. The present study indicated that the anti-diarrheal property of *A. paniculata* was dose-dependent. An immediate anti-diarrheal effect was observed at 2000 ppm of herbal mixture. The anti-diarrheal effects at 1000 ppm of the herbal mixture were observed three days after ingestion. In addition to the anti-diarrheal property of the herbal mixture, pigs that received 1000 and 2000 ppm also indicated higher villi in all parts of the small intestine compared to pigs that received no supplementation or lower doses of the herbal mixture.

Regarding the practical applications and economy, each dose of the herbal mixture is appropriate for pig farms with different infection statuses. In farms where diarrhea is not observed, prophylaxis dose of 500 ppm of the herbal mixture can be used. The results from the current study confirmed that this dose of herbal mixture significantly reduced the number of *E. coli* in feces when compared to pigs that received no supplementation. Therefore, a higher dose of the herbal mixture has to be considered in farms where diarrhea is already observed, for example, 1000 ppm for low to moderate diarrhea or 2000 ppm for moderate to high diarrhea.

The herbal mixture used in this study is identical to the antimicrobial synergy concept, which combines two or more antimicrobial agents to achieve a more significant overall effect than the sum of their individual effects (Van Vuuren and Viljoen, 2011). However, combining herbs can result in complex effects due to the potential interactions among their components, leading to undesirable effects (Che et al., 2013). Although the biological activities of the herbs (whether synergistic, additive, or antagonistic) could not be determined in this study, the benefits of the herbal mixture as antibacterial and anti-diarrhea were demonstrated. Furthermore, no antagonistic antibacterial or anti-diarrheal effects or antagonistic effects on intestinal villus height were observed. Regarding the development of bacteria resistance to the herbal mixture, the possibility is slight due to the multiple sources of the compound (Caesar and Cech, 2019).

CONCLUSION

The supplementation of the herbal mixture at 500, 1000, and 2000 ppm had antibacterial effects, with no significant difference between doses. However, the positive effects of this herbal mixture on intestinal villi height and diarrhea were found only in pigs that received 1000 and 2000 ppm of the herbal mixture. From a practical point of view, supplementation of this herbal mixture at 500 to 1000 ppm could be applied for prophylaxis during the weaning period, whereas 2000 ppm of the herbal mixture could be applied for the treatment of postweaning *E. coli* diarrhea.

DECLARATIONS

Data availability

The authors can provide all necessary data to the editor upon request without delay.

Acknowledgments

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Authors' contribution

Chanthala Laxaphakdy conceptualized the idea and conducted the experimental animal work. Jatedada Jiwakanon and Sirisak Tanpong contribute to data analysis. Sansanee Supankong contributes laboratory work involving *E. coli*. Pitaya Papirom contributed to intestinal morphology work. Sarthorn Porntrakulpipat supervised all the experiments. Chanthala Laxaphakdy and Sarthorn Porntrakulpipat wrote the original manuscript. All authors have read and agreed to publish the final version of the manuscript.

Competing interests

The authors have not declared any conflict of interest.

Ethical consideration

The authors take steps to abide by all ethical standards related to plagiarism, publication approval, inaccuracies in data, multiple submissions, and double publication.

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Investigation of Antibiotic Resistance Pattern and Virulence Determinants in Avian Pathogenic *Escherichia coli* Isolated from Broiler Chickens in Egypt

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ABSTRACT

Besides its zoonotic importance, avian pathogenic *Escherichia coli* (APEC) causes substantial financial losses in the poultry industry globally. The progress of antimicrobial resistance in APEC is mainly associated with excessive antimicrobial use and improper sanitation. Since its beginning in the 1970s, the VITEK system has developed into the VITEK 2 system, which has used an automated system to perform all the steps required for microbial identification and antibiotic susceptibility rapidly and accurately. The present study aimed to update the available circulating data about APEC isolates by phenotypic identification, sero-grouping of APEC from broilers chickens and breeders in five governates of Egypt, investigation of their antibiotic resistance pattern by VITEK 2 system, and molecular identification of their virulence determinants. The prevalence of APEC isolated from the different internal organs (liver, lung, heart, heart blood, and spleen) was 67.5%. The most prevalent serotypes were O125, 0114, O44, O127, O142, and O78. Virulence-associated genes (*iutA, fimC, and papC*) were detected at rates of 84.4%, 74%, and 54.8%, respectively. The highest resistance was found against ampicillin (100%), trimethoprim-sulfamethoxazole (80%), and ampicillin-sulbactam (78.5%), which indicates that the poultry farms need a surveillance and intervention system with proper accuracy and rapidity to prevent the misuse of antibiotics and APEC outbreaks.

Keywords: *Escherichia coli*, Colibacillosis, PCR, VITEK, Virulence genes

INTRODUCTION

Escherichia coli is considered one of the normal inhabitants of the digestive tract of both humans and animals (Sarowska et al., 2019). It is considered a normal element of intestinal flora in poultry (De Carli et al., 2015). The avian pathogenic *Escherichia coli* (*E. coli*), is a subclass of extraintestinal pathogenic *E. coli*, which has many routes for entry, including the vaginal and respiratory systems, leading to various extraintestinal infections in poultry, known as colibacillosis (Matthijs et al., 2009). Perihepatitis, egg peritonitis, airsacculitis, pericarditis, omphalitis, cellulitis, and osteomyelitis/arthritis are the most major symptoms of colibacillosis in poultry (Dziva and Stevens, 2008). It is one of the leading causes of mortality (up to 20%) and morbidity in poultry, as well as a significant reduction in meat (2% decrease in live weight), 2.7% rapid decline in feed conversion ratio, and egg production (up to 20%), decreased hatching rates, and increased carcass condemnation (up to 43%) at slaughter (Guabiraba and Schouler, 2015)**.** Avian colibacillosis triggered by APEC strains is mostly associated with various virulence genes and serogroups (Wang et al., 2010). The virulence of *E. coli* is assisted by many virulence factors expressed by virulence-associated genes, including *iutA, iss, papC, iucD, tsh,ompT, hlyF, iron, and astA* (De Carli et al., 2015).

According to reports, antibiotics are utilized massively in disease prevention strategies and commonly as a growth stimulant in broilers (Osti et al., 2017). However, the therapeutic use of antibiotics in chicken has led to the emergence of multidrug-resistant bacteria, gut microflora changes, antibiotic residue in meat, and environmental effects (Subedi et al., 2018). Since these *E. coli* strains could be transmitted to people through the food chain or direct contact with infected broilers, they pose a major threat to public health. Additionally, Guerra et al. (2018) speculated that resistant *E. coli* might be a carrier of genes that makes other microorganisms resistant to antibiotics.

Avian fecal *E. coli* (AFEC), which have recently been isolated from normal broiler chicken, may also hold several virulence components that indicate their capability for virulence (Hiki et al., 2014). The putative avian hemolysin (*hly*F), the episomal increased serum survival (*iss*), the salmochelin siderophore receptor (*iro*N), the episomal outer membrane protease T (*omp*T), and the ferric aerobactin receptor (*iut*A), which are all located on the large virulence plasmid ColV, have all been linked to highly pathogenic APEC strains and are more frequently isolated from them De Oliveira et al. (2015). Type 1 fimbriae C (*FimC*) is an additional virulence factor that is necessary for fimbrial assembly and anchoring as well as for *E. coli* adhesion to host respiratory epithelial cells for colonization (Jeong et al., 2012).

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In comparison to manual biochemical testing, automation of biochemical assays has reduced the identification time to 2-10 hours (3 hours for Gram-negative rods), enhanced reliability, and increased efficiency with little manual sample preparation (Funke et al., 1998). One of the most popular integrated and automated systems for identifying bacteria based on the biochemical profiles of tested strains is the VITEK 2 system. Bacterial identification using this fluorescence and/or colorimetry-based technique is quick when used in conjunction with modest amounts of selective or differentiated media or reagents contained on compact plastic cards (Barry et al., 2003).

Through accurate microbiological identification and antibiotic susceptibility testing, the effectiveness of the VITEK 2 system equipment and VITEK® 2 PC software offers the potential to increase therapeutic success and result outcomes, according to Barenfanger et al. (1999) with a cost-effective, space-saving design, it also increases laboratory efficiency by reducing work labor and enabling quick reporting (Sanders et al., 2001).

The management of APEC infections in poultry relies on antibiotic prescription and immunization instead of reducing the environmental stressors, employing biosecurity procedures, and vaccination programs for the broiler chicken against the majority of virus-related and immunosuppressor diseases. Therefore, the current study was conducted to isolate and detect the antibiotic resistance pattern of APEC from different farms in Egypt using the VITEK 2 system, determine their serotype, and detect some virulence-related genes to improve applicable preventative measures to control colibacillosis in broilers.

MATERIALS AND METHODS

Ethical approval

The study was approved by the Institutional Animal Care and Use Committee at the Faculty of Veterinary Medicine, Cairo University, Giza, Egypt, with certificate reference Vet CU 01122022561.

Samples collection

In this study, 370 clinical cases of broiler chicken (7-21 days old) and broiler breeders (25-30 weeks old) in different breeds were examined for gross lesions of colibacillosis by postmortem examination in the Microbiology Department, Faculty of Veterinary medicine, Cairo University, Giza, Egypt during the period from January 2020 to January 2021. Samples were collected from five Egyptian governorates (Sharkia, Kafr-el-sheikh, Fayoum, Giza, and Qalubia). All investigated farms suffered clinical signs of colibacillosis, including high mortalities, respiratory signs, reduced appetite, and declined growth rate. The samples were collected from different organs (liver, lung, heart, heart blood, and spleen) aseptically and kept individually in sterile, zipped plastic bags for bacterial isolation.

Bacteriological identification

The collected organs were minced and inoculated into the nutrient broth (Oxoid®) aerobically at 37°C for 18-24 hours, before being sub-cultured on MacConkey agar (Oxoid®) and Eosin methylene blue agar (EMB, Oxoid®) aerobically at 37°C for 18-24 hours (Collee et al., 1996). All the recovered isolates were identified morphologically and biochemically as *E. coli* by observing their culture characteristics, morphology by Gram's stain, oxidase test, biochemical reactions using indole, methyl-red, Voges-Preuskuar, citrate tests (IMViC), and TSI as described by Quinn et al. (2002). The suspected isolates were maintained in cryostat tubes containing 20% glycerol with LB Luria Bertani broth at -70°C for further identification.

Identification of isolates using the VITEK 2 system

Each isolate was identified by the Gram-negative test kit VITEK 2 system (bioMérieux, France) according to the manufacturer's instructions. Using VITEK 2 DensiChek equipment (bioMe'rieux, France), a bacterial suspension was adjusted to the McFarland standard of 0.5 in 2.5 ml of a 0.45% sodium chloride solution. There were never more than 30 minutes between making the inoculum and filling out the card. The card was placed on the cassette made specifically for the VITEK 2 device, put inside, automatically filled with vacuum, sealed, incubated at 35.5°C, and subjected to an automatic colorimetric measurement using a new optical reading head every 15 minutes for a maximum incubation period of 10 hours. Version 9.02 of the VITEK 2 database was used to examine the data. The 64-well plastic GN card contains 41 tests, including 18 tests for sugar incorporation, 18 tests for sugar fermentation, 2 tests for decarboxylase, and 3 tests for miscellaneous (Urease, tryptophan deaminase, and utilization of malonate).

Serotyping of *Escherichia coli* isolates

The identified *E. coli* isolates were subjected to serotyping as described by Edward (1972). Polyvalent and monovalent diagnostic *E. coli* antisera were used for the sero-grouping of *E. coli* isolates according to somatic (O) and capsular (K) antigens. Suspected isolates of *E. coli* were subcultured on semisolid or slop agar and incubated for 24 hours at 37°C, then subcultured on MaConkey agar medium and incubated for 24 hours at 37°C. Three to five colonies were suspended in 3 ml saline and kept in the water bath at 100°C for one hour, then centrifuged at 2000 rpm for 10 minutes. The supernatant was discarded, and the precipitate was maintained, to which 0.5 ml saline was added. A drop

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from this suspension was placed on a glass slide and mixed with one drop from the O polyvalent anti-serum using a wooden strike.

Antimicrobial susceptibility testing using VITEK 2 system

Escherichia coli isolates were subjected to antimicrobial susceptibility testing using AST-GN73 TEST KIT (bioMérieux, Incorporation., Durham, NC) using VITEK 2 system version 9.02 software version according to manufacturer's instructions. Minimal Inhibitory Concentration (MIC) interpretation guideline was done according to (CLSI, 2020). The AST-GN73 card contains multiple cards of antimicrobial agents as dehydrated substances at the indicated concentrations. The cards overflowed with an inoculum ready by transferring 200 µL of culture suspension from the 0.5 McFarland culture suspension. The VITEK-2 system automatically processes the antimicrobial susceptibility cards until MICs are obtained (Joyanes et al., 2001).

Molecular identification of *Escherichia coli 16sr RNA* and their virulence determinants

Genomic DNA extraction was accomplished as described by Blanco et al. (2004). Bacterial isolates were first subcultured overnight at 37°C in Trypticase Soya broth before being suspended in 200 µL of sterile water. Bacteria were boiled for 10 minutes to disrupt the cells to release the DNA, followed by centrifugation at 10,000 rpm for five minutes. The supernatant containing DNA was stored at -20°C until used for polymerase chain reaction (PCR).

Molecular identification was made for the analysis of *16sr*RNA, *pap*C, *fim*C, and *iut*A genes in *E. coli* isolates by simple PCR using 200 ng of DNA, 12.5 µL of DreamTaq PCR Master Mix (2X, Thermo Scientific™), 10.5 µL nuclease-free water (Thermo Scientific™) and 0.5 µL of each primer (10 µM, Table 1). The cycling conditions consisted of five minutes activation step at 95°C, followed by 35 cycles of 95°C for 30 seconds, annealing temperature for 30 seconds, 72°C for one minute, and followed by the final extension step at 72°C for ten minutes. Amplicons were separated by 1% ethidium bromide-stained agarose gel electrophoresis along with a 100-bp ladder (BIOWEST, Hong Kong, China) and visualized under UV light (Sambrook et al., 1989).

Table 1. The sequence of used primers and the used annealing temperature in PCR for the different used genes

Target gene		Primers' sequences (5'- 3')	Product size	Annealing Temperature	Reference
16srRNA	Eco-1	GACCTCGGTTTAGTTCACAGA	585 bp	50°C	(Candrian et al., 1991)
	Eco-2	CACACGCTGACGCTGACCA			
fimC	F	GGAAATAACATTCTGCTTGC		51°C	(Jeong et al., 2012)
	R	TTTGTTGCATCAAGAATACG	288 bp		
papC	F	TGATATCACGCAGTCAGTAGC	501 bp		(Ewers et al., 2005)
	R	CCGGCCATATTCACATAA		59.2°C	
iutA	F	ATGAGCATATCTCCGGACG	587 bp	55°C	(Moulin et al., 2006)
	R	CAGGTCGAAGAACATCTGG			

RESULTS

Visceral organs were collected aseptically for bacteriological investigation. According to the findings of the bacteriological study, *E. coli* was detected and confirmed in 251 (67.8 %) out of 370 samples by standard biochemical tests and VITEK 2 system Id cards from diseased and freshly dead broiler chickens. They showed distinctive green metallic sheen with a black center colony on EMB agar, while they indicated medium-sized rounded pink colonies on MacConkey's agar media. The prevalence of *E. coli* isolates varied among the internal organs as the liver samples were the highest (78.6%), followed by spleen samples (67%), heart and heart blood samples (60%), and lung samples (47%, Table 2).

The serological examination of 135 *E. coli* isolates resulted in the detection of different serogroups using specific eight polyvalent, then 43 monovalent group O somatic antisera. Different serogroups were detected contained O142 K 86, O91 K -, O125 K 70, 0114 k 90, O44 k 74, O127 K 63, O1 K -, O166 K -, O158 K -, O144 K -, O103 K -, O86 K 64, O27 K -, O103 K -, O151 K -, O78 K -. O55 K 59 while 19 strains were un-typed (Table 3).

The results of the antibiotic resistance pattern of *E. coli* isolates are presented in Table 4. All *E. coli* isolates showed a high rate of resistance against ampicillin (100%), followed by trimethoprim-sulfamethoxazole (78.5%), ampicillin-sulbactam (78.4%), Ceftazidime (75.5%), cefepime (74%), ceftriaxone (65.3%), ciprofloxacin (47.8%), gentamicin (29%), and levofloxacin (28.8%). Low levels of resistance were indicated against Cefazolin 17.5%, Meropenem 9.5%, Tobramycin 9%, and Amikacin 5%, respectively.

The molecular identification of *E. coli* isolates by PCR revealed that all isolates were confirmed as *E. coli* by the universal primer for *16srRNA* with 585 bp band size. *Escherichia coli* ATCC 25922 was used as a positive control, as

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demonstrated in figures 1- 4. In this study, the prevalence of three virulence-associated genes was investigated. Out of 135 APEC isolates, 114/135 (84.4%) were positive for the *iutA* gene, 100/ 135 (74%) for the *fimC* gene, and 74 /135 (54.8%) for the *papC* gene.

Table 2. Prevalence of *Escherichia coli* isolated from internal organs of broiler chickens in five Egyptian governorates

Examined organs	Number of examined organs	Positive samples	Percentage
Liver	140	110	78.6
Spleen	97	65	67
Heart and heart blood	84	47	60
Lung	49	29	59.1
Total	370	251	67.8

Table 3. Serogrouping of *Escherichia coli* isolates from broiler chickens of five different Egyptian governorates from January 2020 to January 2021

Isolated *E. coli** serotypes	Number of positive samples	Percentage
O125 K 70	27	20
0114 k 90	16	12
O44 k 74	10	7.4
O127 K 63	10	7.4
O142 K 86	9	6.7
O78 K -	8	6
O91 K -	7	5.2
O1 K -	6	4.4
O166 K -	2	1.4
O158 K -	4	3
O144 K -	4	3
O103 K -	3	2.2
O86 K 64	3	2.2
O27 K -	2	1.4
O103 K -	2	1.5
O151 K -	2	1.5
O55 K 59	1	0.7
Untyped	19	14
Total	135	100

*****E. coli*: *Escherichia coli*

Table 4. Antibiotic resistance patterns of *Escherichia coli* isolates from broiler chickens of five different Egyptian governorates from January 2020 to January 2021.

Classes of antibiotics		N	S (%)	N	I (%)	N	R (%)
ß-lactam	1. ESBL producers	63	25	0	0	188	74.9
	2. Ampicillin	0	0	0	0	251	100
	3. Ampicillin-Sulbactam	0	0	54	21.5	197	78.4
	4. Piperacillin-Tazobactam	251	100	0	0	0	0
	5. Meropenem	217	86.5	10	4	24	9.5
Cephalosporins	6. Cefazolin	188	75	48	19	44	17.5
	7. Cefoxitin	198	78.8	53	21	0	0
	8. Ceftazidime	49	19.5	13	5	189	75.5
	9. Ceftriaxone	77	31	10	4	164	65.3
	10. Cefepime	60	24	6	2.4	185	74
Aminoglycosides	11. Amikacin	217	86.5	21	8.3	13	5
	12. Gentamicin	128	51	50	20	73	29
	13. Tobramycin	176	70	52	20.7	23	9
Fluoroquinolones	14. Ciprofloxacin	131	52	0	0	120	47.8
	15. Levofloxacin	122	48.6	57	23	72	28.7
Nitrofurans	16. Nitrofurantoin	234	93.2	17	6.7	0	0
Sulfonamides and potentiated sulfonamides	17. Trimethoprim-Sulfamethoxazole	54	21.5	0	0	197	78.5

N: Number of the isolates, S: Susceptible, I: Intermediate, and R: Resistant to antimicrobial agents by VITEK GN AST37 card

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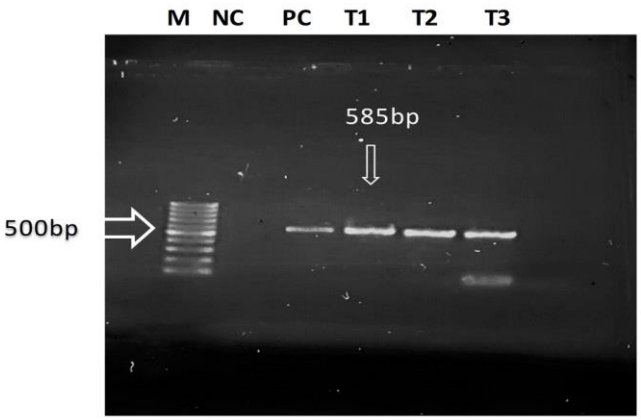

Figure 1. Agarose Gel electrophoresis of the amplified 16sRNA gene of *Escherichia coli* isolates. Lane M: 100 bp ladder; Lane NC: Negative control; Lane PC: Positive control *Escherichia coli* ATCC 25922; Lane T1-T3: Tested samples showing a positive result for 16srRNA gene with band size 585 bp

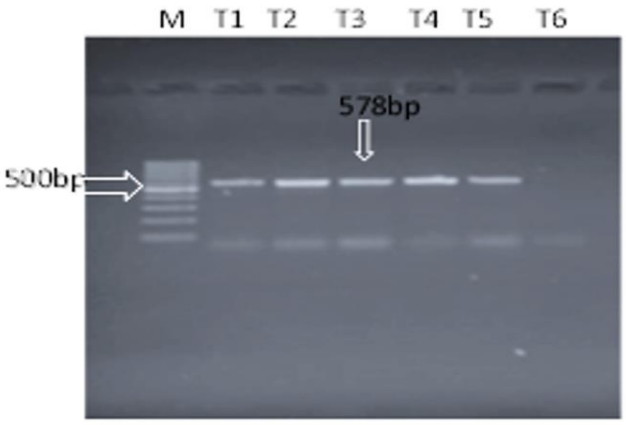

Figure 2. Agarose Gel electrophoresis of the amplified *iut*A gene of *Escherichia coli* isolates. Lane M: 100 bp ladder; Lane T1-T5: Tested samples showing a positive result for *iut*A gene with band size 578 bp; Lane T6 indicating a negative result.

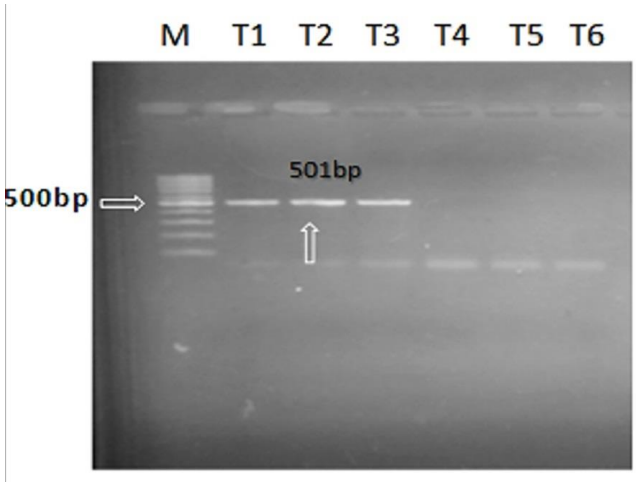

Figure 3. Agarose Gel electrophoresis of the amplified *pap*C gene of *Escherichia coli* isolates. Lane M: 100 bp ladder; Lane T1-T3: Tested samples showing a positive result for *pap*C gene with band size 501bp; Lane T4-T6 showing a negative result.

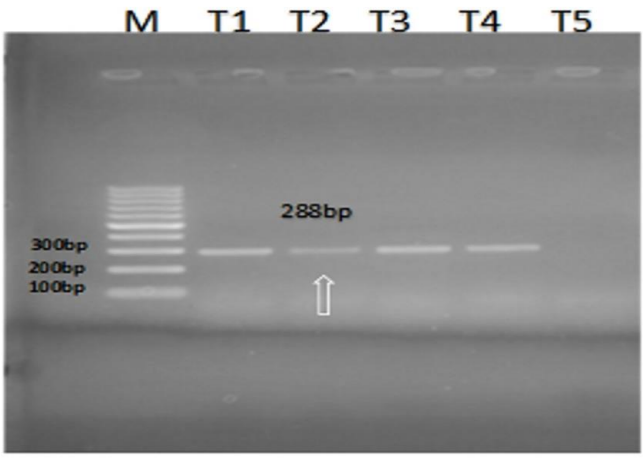

Figure 4. Agarose Gel electrophoresis of the amplified *fim*C gene of *Escherichia coli* isolates. Lane M: 100-bp ladder; Lane T1-T4: Tested samples showing a positive result for *fim*C gene with band size 288bp; Lane T5 indicating a negative result.

DISCUSSION

Avian pathogenic *Escherichia coli* (APEC) is a widespread and significant cause of economic loss in the poultry industry owing to morbidity, mortality, and loss of production (Barnes et al., 2008). Additionally, it is reported as a danger to the most affordable sources of high-quality protein in the world (Hussein et al., 2013). Strains of APEC are categorized as the extraintestinal pathogen, which is characterized by the presence of virulence genes that permit their accommodation in various organs other than the intestine (Johnson et al., 2006). Besides, evidence suggests that some APEC strains are zoonotic, allowing them to spread widely (Tivendale et al., 2010).

In the present study, broiler chickens and breeders were examined for gross lesions of colibacillosis from five different Egyptian governorates. The prevalence of *E. coli* isolates among the internal organs varies, but the highest was in liver samples, followed by spleen, heart, heart blood, and lung samples. Similar results were obtained by Abd El Tawab et al. (2016), who recorded a high isolation rate from the liver followed by heart, blood, and lung. In contrast, this result agreed with Eid and Erfan (2013), who recorded a high isolation rate from the liver (57.14%), followed by the lung (54.29%) and heart blood (37.14%). In addition, Yousef et al. (2015) reported that out of 95 liver samples, 88 were positive for *E. coli* with a percentage of 92.6%, while this result disagreed with Ola (2017), who found that 14% of *E. coli* isolates (28/200) were isolated from liver. The higher rates of *E. coli* isolation from the liver and lung assume the extraintestinal *E. coli* invasion into other organs and tissues, including lung, heart, and liver tissues (Awad et al., 2020).

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The serological examination revealed the detection of different serogroups including O142 K 86, O91 K -, O125 K 70, 0114 k 90, O44 k 74, O127 K 63, O1 K -, O166 K -, O158 K -, O144 K -, O103 K -, O86 K 64, O27 K -, O103 K -, O151 K -, O78 K -. O55 K 59 while 19 strains were untyped. Early studies on avian *E. coli* strains showed that O1, O2 O15, O35, and O78 serotypes, were mostly associated with colibacillosis outbreaks (Dziva and Stevens, 2008). A later study by Nolan et al. (2013) revealed the presence of O18, O81, O115, O116, and O132, serotypes, was a signal for the emergence of new pathogenic serotypes. Recently El-Sawah et al. (2018) showed that a wide antigenic diversity is existed among avian pathogenic *E. coli* strains in Egypt, and worldwide. Thus, the involvement of a particular O serotype in the infection process appeared to vary according to the geographical region. Some of the studies conducted in Egypt, nearly isolated the same serotypes with a predominance of the O78 serotype (Awawdeh et al., 2019; Ramadan et al., 2016).

In the present study, the highest rate of antibiotic resistance was shown against ampicillin and trimethoprim-sulfamethoxazole, while low levels of resistance were against cefazolin, meropenem, tobramycin, amikacin, and cefoxitin. These antibiotic resistivity patterns of *E. coli* strains were in agreement with other previous studies (Matin et al., 2017).

The resistance of APEC isolates to the cephalosporins, ceftazidime, ceftriaxone, and cefepime was the lowest resistance level among the tested panel of antimicrobials because they are not used in the poultry industry (Johar et al., 2021). Antimicrobials used in broiler chicken feed, water, and as growth promoters in suboptimal levels need to be monitored. The widespread usage of these antibiotic families in poultry is reflected in the significant levels of resistance that have been found (Ibrahim et al., 2019) The widespread use of these antibiotics for treatment and prevention of the disease without veterinary advice in Egypt is responsible for the high frequencies of antimicrobial resistance of *E. coli* isolates in broiler chickens (Ibrahim et al., 2022). The tremendous rise of multi-drug-resistant bacteria still poses a serious concern despite the effectiveness of modern antibacterial medications, necessitating the introduction of certain alternatives like nanoparticles, herbal extracts, and probiotics (Khalil et al., 2020; Ragab et al., 2020; Syed et al., 2020; Hassanen and Ragab., 2021; Prentza et al., 2022). Virulence genes accompanied by antimicrobial resistance are considered the main factors that increase the pathogenicity of bacteria and lead to an increase in infection severity leading to a therapeutic failure (Abd El-Baky et al., 2020). Although many techniques can be used to identify virulence factors (some phenotypic characters on chromogenic medium), PCR is still a powerful technique for detecting pathogens because of its rapidity, specificity, and sensitivity. It is an effective procedure for generating large quantities of a specific DNA sequence *in vitro* (Holland et al., 2000)

It was stated that the main virulence markers for APEC are *iro*N, *omp*T, *hly*F, iss, and *iut*A genes (Johnson et al., 2006). These genes were verified to be essentially present in APEC. In addition to that, it was suggested that the presence of two of these genes in an *E. coli* avian isolate could mean that this isolate is an APEC, and the absence or presence of a gene could reveal non-pathogenic *E. coli* (Schouler et al., 2012). The *iut*A gene encodes an outer membrane protein implicated in the high-affinity binding of Fe^{+3} aerobactin and can be plasmid-located (De Carli et al., 2015) or encoded on chromosomes in some *E. coli* strains (Unno et al., 2011).

The present study found that the prevalence rate of the *iut*A gene was 84.4% of the tested *E. coli* isolates. This finding is in accordance with a study that reported a high prevalence of about 80% (Eftekharian et al., 2016). However, this result disagrees with previous studies showing low levels of prevalences of 64% and 70% (Kwon et al., 2008; Subedi et al., 2018)

Adherence of bacteria to tissue surfaces is an important initial step in bacterial infections. In *E. coli*, P-fimbriae, which mediates bacterial colonization in the respiratory epithelium, is coded by the pyelonephritis-associated pili (*pap*C) gene. In addition to tissue adhesion, P-fimbriae protects *E. coli* from the antibacterial activity of neutrophils (Varga et al., 2018). The obtained results revealed that the prevalence rate of *pap*C gene in *E. coli* isolates was 54.8%. The same result was detected by Subed et al. (2018) with a rate of 55.6%, while lower rates were obtained by Kown et al. (2008), Varga et al. 2018), and Tidiane et al. (2019) who reported papC gene prevalence 11%, 10.27% and 12.9% in *E. coli* isolates, respectively. In the same line, a low prevalence rate was recorded by Oliveira et al. (2019), who detected papC gene in 1.5% of *E. coli* isolates.

Fimbrial adhesins are hypothesized to start host tissue colonization. The most typical type of fimbria found in APEC is type 1. According to Huja et al. (2015), fimbriae type 1 bind to mannose-containing glycoproteins on epithelial host cells. The fim cluster gene, which includes nine related genes (*A*, *B*, *C*, *D*, *E*, *F*, *G*, *H*, and *I*) necessary for its production, encodes type 1 fimbriae (Pusz et al., 2014). A chaperone protein that aids in the translocation of fimbria proteins through the periplasm is often produced by the type 1 fimbriae C (*fim*C) gene (Kostakioti et al., 2004). The present study found that 74% of the tested isolates were positive for *fim*C, which is comparable to the research done by Dou et al. (2016), who found that APEC isolates had a high prevalence of this gene, 95.88%.

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CONCLUSION

The spread of APEC is global, which emphasizes the importance of studying APEC from diverse geographical angles. The results of the present study revealed that *E. coli* is one of the main factors contributing to various disease conditions in chickens that create financial losses for the poultry business. Almost all isolated *E. coli* serotypes have been determined to be harmful to all broilers breed; however, no single illness condition or age group could be associated with a specific serotype. The present study revealed a significant prevalence of multidrug-resistant *E. coli* strains accompanied by a high frequency of virulence genes. Implementing an intervention program to reduce the risk of colibacillosis requires regular screening and monitoring of the virulence genes linked to the antibiotic-resistant APEC strains. Future studies must be established to monitor the expression of virulence genes and find suitable antibiotic alternatives.

DECLARATIONS

Availability of data and materials

All data generated or analyzed during this study are included in this published article.

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Authors' contribution

Mona I. Elenbaawy, Eman Ragab, and Basma M. Hamed hypothesized the idea and conducted laboratory work. Both authors (Hossam Mahmoud and Eman Ragab) contributed to the drafting, editing, and production of the final draft. Eman Ragab is the corresponding author. All authors confirmed and consented to the final submission.

Competing interests

The authors declare that they have no competing interests.

Ethical consideration

All authors have confirmed ethical issues, including plagiarism, double publication and/or submission, and redundancy, data fabrication and/or falsification, consent to publish.

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Effects of the Anthocyanin Compound (Cyanidin-3-Glucoside) on Some Histological and Physiological Parameters Related to the Heart in Male Rats Exposed to Oxidative Stress

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ABSTRACT

The increasing incidence of heart disease due to an unhealthy diet rich in fats has encouraged the use of plant extracts, which have shown efficiency in improving body immunity and promoting human health. The current study was designed to investigate the effect of anthocyanin cyanidin-3-glucoside on some physiological and histological parameters related to the heart in white male rats exposed to oxidative stress with hydrogen peroxide. The study included 48 adult male white rats with a weight range of 200-300 g, and an ages range of 8-12 weeks. The rats were randomly divided into six groups of eight rats per group. Group 1 was considered a negative control group supplied with water and the basal diet for 30 days. Group 2 was a positive control group in which the rats were given drinking water containing hydrogen peroxide at a concentration of 1%. The third group orally received cyanidin-3-glucoside at a concentration of 50 mg/kg. The fourth group received both cyanidin-3-glucoside compounds at a concentration of 70 mg/kg and drinking water containing hydrogen peroxide at a concentration of 1%. The fifth group was dosed orally with a cyanidin-3-glucoside only at a concentration of 50 mg/kg, and the sixth group was dosed orally with a cyanidin-3-glucoside at a concentration of 70 mg/kg. At the end of the experiment, the animals were anesthetized, then blood samples were collected from the heart directly to obtain serum for measuring the levels of troponin, lactate dehydrogenase (LDH), and creatine kinase (CK-MB). The results showed a significant increase in troponin, LDH, and CK-MB levels in the positive control group compared to the negative control group. However, there was a significant decrease in the level of these enzymes in the third and fourth groups, compared to the positive control group. The fifth and sixth groups demonstrated a significant decrease, compared to the positive control group. However, they revealed a nonsignificant difference in the levels of these parameters, compared to the negative control group. The obtained results indicated that the cyanidin-3-glucoside compound positively prevented heart muscle damage caused by oxidative stress.

Keywords: Anthocyanin compound, Heart, Hydrogen peroxide, Male rats, Oxidative stress, Physiological parameter

INTRODUCTION

The composition of diet plays a key role in the initiation and development of cardiovascular diseases and also acts as an important factor in lifestyle to prevent these and other diseases. Cardiovascular diseases (CVD) are disorders that affect the heart and blood vessels and represent the main cause of disease and mortality worldwide (Libby et al., 2011; Bokov et al., 2022; Hafsan et al., 2022). Data from epidemiological and clinical studies have shown a negative relationship between the development of CVD and diets rich in fruits and vegetables (Tang et al., 2017; Zhao et al., 2017; Ansari et al., 2022).

Numerous reports have indicated that fruits and vegetables rich in flavonoids contribute to cardiovascular health as these compounds can exhibit anti-inflammatory, anticoagulant, and antioxidant activities through complex mechanisms (Cassidy et al., 2011; Tang et al., 2017; Zhao et al., 2017). Flavonoids can interact with cell membranes, which leads to changes in their structure and physical and chemical properties (Oteiza et al., 2005). This can alter cell function, interfere with and modulate the activities of enzymes and transcription factors, and affect gene expression (Krga et al., 2016; Krga et al., 2018; Huldani et al., 2022).

Epidemiological evidence suggests that dietary intake of flavonoid-rich foods is associated with a lower incidence of CVD (Hooper et al., 2008; Dohadwala and Vita, 2009; Zadeh et al., 2022). An imbalance of oxidative stress and cellular reduction is believed to cause endothelial dysfunction (Paravicin and Touyz, 2006). Therefore, the protective properties of flavonoids for the cardiovascular system are mainly related to their antioxidant activities (Perez-Vizcaino et

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al., 2006; Perez-Vizcaino et al., 2009) directly by scavenging free radicals or indirectly as inducers of antioxidant enzymes (Schewe et al., 2008).

Flavonoids include most of the biologically active molecules found in fruits and vegetables, such as anthocyanins, which are water-soluble pigments responsible for giving the red, blue, and purple color to fruits, flowers, seeds, and vegetables (Khaki et al., 2010; Khoo et al., 2017; Zainab and Qasim, 2021). Anthocyanin is one of the natural and effective colors in reducing the danger of free radicals. It is a natural alternative to industrial antioxidants, which raised many doubts about its health safety (Yang et al., 2011).

Anthocyanins have been widely used in food manufacturing. Studies have focused on biological activities and their health effects through medical applications since they are important sources of antioxidants, besides having a high inhibitory ability against microorganisms, which increase the duration of food preservation (Pazmino-Duran et al., 2001; Kuntz et al., 2014; Martin et al., 2017). Numerous studies have also been conducted on its health effects on humans in reducing cardiovascular diseases and anti-carcinogen factors and inflammation (Cassidy et al., 2011). In addition to having good color ability due to its high stability in storage conditions, it has been used as a safe and effective food coloring (Strack and Wray, 1994).

Due to an unhealthy diet rich in fats, there has been an increase in the incidence of CVD among individuals. On the other hand, there has been a growing interest in using plant extracts to improve the body immunity and human health since they contain phytochemical compounds in high concentrations, especially anthocyanin pigment. With this in mind, the current study aimed to evaluate the ability of an anthocyanin type (cyanidin-3-glucoside) to reduce the oxidative stress induced by hydrogen peroxide at a concentration of 1% in adult male albino rats.

MATERIALS AND METHODS

Experimental animals

Ethical approval

This study was confirmed by the ethical committee of the University of Al-Qadisiyah, Iraq. The authors followed the rules related to the rights of animals during the study. In the present experiment, 48 male Albino rats of the *Rattus norvegicus* strain were used. The rats were within the age range of 8-12 weeks and had a weight range of 200-300 g. They were placed in plastic cages; each cage had a metal cover, a clamp fitted with a water bottle, and a place to put food. The cage floor was covered with sawdust, which was replaced periodically to maintain the cleanliness of the rats while cleaning the litter of cages was done three times a day. The high-protein ration was used to feed the rats freely. The animals were subjected to controlled laboratory conditions for water, ventilation, and lighting for 12 hours of light and 12 hours of dark under temperature (26±2) for 30 days.

Study design

In the current study, 48 male adult white rats were used, randomly divided into six groups and two replicates for each group (Four rats in each replicate). The first group (G1) was a negative control group that received water and food *ad libitum* for 30 days. The rats in the second group (G2), the positive control group, received the rations but drinking water containing hydrogen peroxide at the concentration of 1% using special drinking bottles. The third group (G3) received drinking water containing hydrogen peroxide at a concentration of 1% and Cyanidin-3-glucoside at a concentration of 50 mg/kg (Chayati et al., 2019). The fourth group (G4) took orally drinking water containing hydrogen peroxide at a concentration of 1% and Cyanidin-3- glucoside at a concentration of 70 mg/kg (Chayati et al., 2019). The fifth group (G5) was given Cyanidin-3-glucoside orally at a concentration of 50 mg/kg. Finally, the rats in the sixth group (G6) received Cyanidin-3-glucoside compound at the concentration of 70 mg/kg.

At the end of the experiment, the animals were anesthetized using chloroform, and then blood samples were taken immediately from the heart directly through a sterile 2-ml syringe; then it was placed in clean test tubes free of anticoagulant and left for 15-20 minutes at laboratory temperature. Therefore, the samples were centrifuged at 3000 rpm for 15 minutes the serum. The serum was kept at -20°C to measure enzyme levels.

RESULTS

Troponin level

Regarding troponin changes, the results of the current study indicated a significant increase in the G2-positive group treated with 1% hydrogen peroxide compared to the G1-negative control group ($p < 0.05$; Table 1). Moreover, there was a significant decrease in G3, compared to the G2-positive group, G1-negative control, and other experimental groups ($p < 0.05$). On the other hand, G4 showed a significant decrease, compared to G2, G1, and G5 ($p < 0.05$). A significant decrease was also observed in the G5 in comparison with the positive control group ($p < 0.05$), while no difference was observed when compared to the negative control ($p > 0.05$). In G6, there was a significant decrease in

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troponin level compared to the positive control group ($p < 0.05$), and there was no significant difference when compared to G1 and G5 ($p > 0.05$).

Table 1. The effect of Cyanidin-3-glucoside on the levels of troponin, creatine phosphokinase, and lactate dehydrogenase enzymes in male rats exposed to oxidative stress

Parameters / Groups	LDH (U / I)	CK-MB (ng / ML)	Troponin (ng / ML)
G1	209.4 ± 21.2^{bc}	2.76 ± 0.73^{b}	3.67 ± 0.73^{b}
G2	277.7 ± 18.5^{a}	4.43 ± 1.55^{a}	4.1 ± 0.18^{a}
G3	211.4 ± 45.1^{bc}	3.07 ± 0.74^{b}	2.34 ± 1.02^{d}
G4	234.2 ± 54.2^{b}	1.72 ± 0.60^{c}	2.73 ± 0.56^{c}
G5	198.0 ± 22.5^{bc}	3.05 ± 0.51^{b}	3.12 ± 0.45^{b}
G6	183.7 ± 41.3^{c}	3.17 ± 0.70^{b}	3.01 ± 0.77^{bc}
L.S.D	42.51	0.835	0.31

The values represent the mean ± standard error. LDH: Lactate dehydrogenase, CK-MB: Creatine kinase, G1: A negative control group in which they received water and food *ad libitum*, G2: A positive control group that received the drinking water containing hydrogen peroxide at the concentration of 1%. G3: Received drinking water containing hydrogen peroxide at a concentration of 1% and Cyanidin-3-glucoside at a concentration of 50 mg/kg, G4: Received drinking water containing hydrogen peroxide at a concentration of 1% and Cyanidin-3- glucoside at a concentration of 70 mg/kg, G5: Received Cyanidin-3-glucoside at the concentration of 50 mg/kg, G6: Received Cyanidin-3-glucoside compound at the concentration of 70 mg/kg. Different letters within the same column indicate significant differences at the probability level $p < 0.05$.

Creatine kinase level

As can be seen in Table 1, there was a significant increase in the level of creatine kinase (CK-MB) in G2, compared to G1 and other groups ($p < 0.05$). The rats in G3 did not differ significantly from those in G1, G5, and G6 ($p > 0.05$) and recorded a significant decrease when compared to G2 ($p < 0.05$). There was a significant decrease in the level of creatine kinase (CK-MB) in G4 when compared to other groups ($p < 0.05$). Regarding G5, no significant was observed compared to G1, G3, and G6 ($p < 0.05$), while a significant decrease was recorded when compared to G2 ($p < 0.05$), and there was a significant increase ($p < 0.05$) in the level of CK-MB, compared to the fourth group treated with hydrogen peroxide and C3G dye at a concentration of 70 mg/kg. The rats in G6 did not differ from those in G1 and G3 in terms of CK-MB level ($p > 0.05$), while there was a significant decrease in the level of CK-MB level in compared to G2 ($p < 0.05$).

Lactate dehydrogenase level

Table 1 shows the results of the lactate dehydrogenase (LDH) enzyme in the six groups of this experiment. Accordingly, there was a significant increase in G2, compared to all groups ($p < 0.05$), while no significant difference was observed in G3 when compared with G1, G4, and G5 ($p > 0.05$). A significant decrease in LDH level was recorded in G3 when compared to G2 ($p < 0.05$). Moreover, G4 recorded a significant decrease in the level of LDH enzyme compared to G2 ($p < 0.05$); however, no significant difference was recorded when compared to G1, G3 and G5 ($p > 0.05$). As for G5 there was a significant decrease, compared to G2 ($p < 0.05$), while no significant difference was indicated compared to G1, G3, and G4 ($p > 0.05$). The results indicated a significant decrease when comparing G6 with G2 G3, and ($p < 0.05$), although no significant difference was shown in the level of the enzyme when comparing G1 with G5 ($p > 0.05$).

Histological changes in heart

The tissue sections of G1 taken from the heart showed normal cardiac muscle fibers with elongated nuclei and regular transverse layout Figure 1. As G2 was treated with 1% hydrogen peroxide, the histological sections of the heart showed clear pathological changes. The expanded space between the muscle cells, bleeding, and congestion are illustrated in Figure 2. The cardiac tissue section of G3 and G4 treated with C3G compound at a concentration of 50 mg/kg and 70 mg/kg and hydrogen peroxide 1%, respectively, showed a significant improvement in tissue with the spaces between the muscle cells figures 3 and 4. The tissue sections of the heart taken from G5 and G6 treated with C3G compound at a concentration of 50 mg/kg, and 70 mg/kg indicated the normal cardiac tissue in figures 5 and 6.

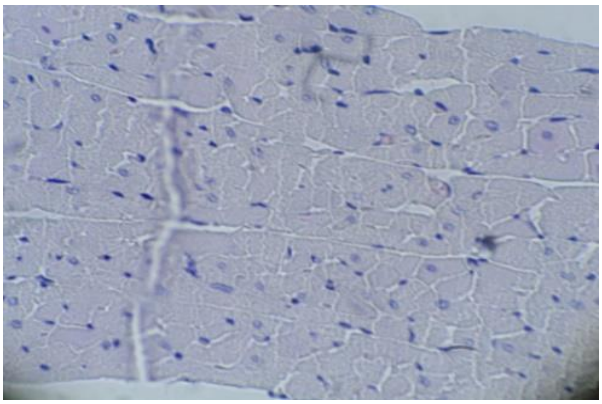

Figure 1. Normal heart tissue of a healthy rat. 40X (H&E)

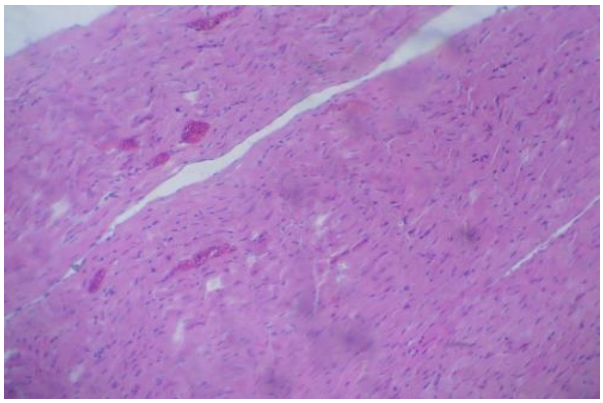

Figure 2. The heart tissue of a rat treated with 1% hydrogen peroxide. The congestion hemorrhage, and an expansion of the space between muscle cells are shown. 40X

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(H&E)

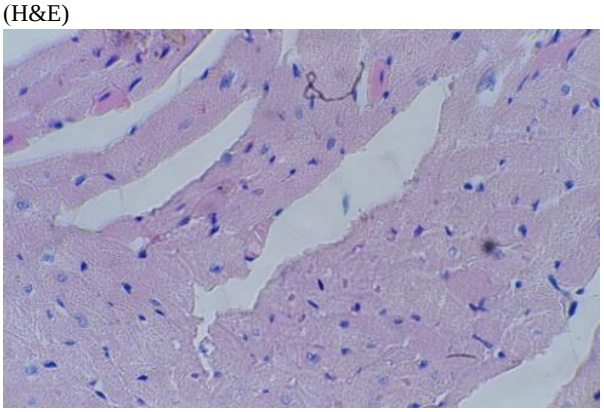

Figure 3. The heart tissue of a rat treated with Cyanidin-3-glucoside at a concentration of 50 mg/kg and hydrogen peroxide. The expansion of the space between muscle cells is obvious. 40X (H&E)

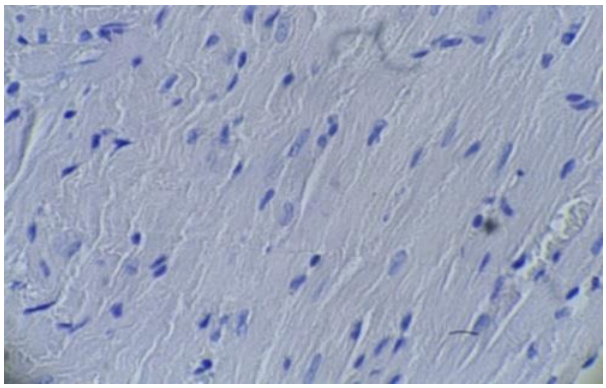

Figure 4. The heart tissue of a rat treated with Cyanidin-3-glucoside at a concentration of 70% mg/kg and hydrogen peroxide. The expansion of the space between the muscle cells is obvious. 40X (H&E)

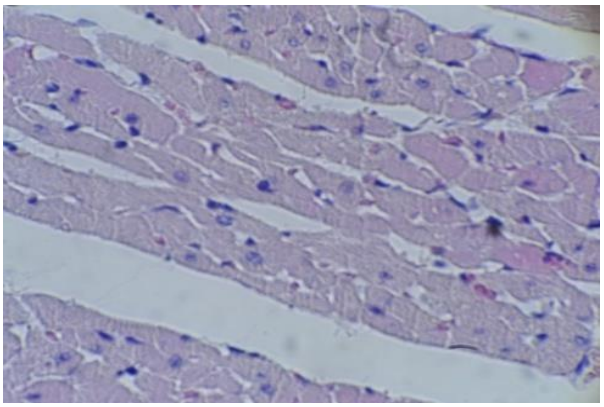

Figure 5. The heart tissue of a rat treated with Cyanidin-3-glucoside at a concentration of 50% mg/kg. 40X (H&E)

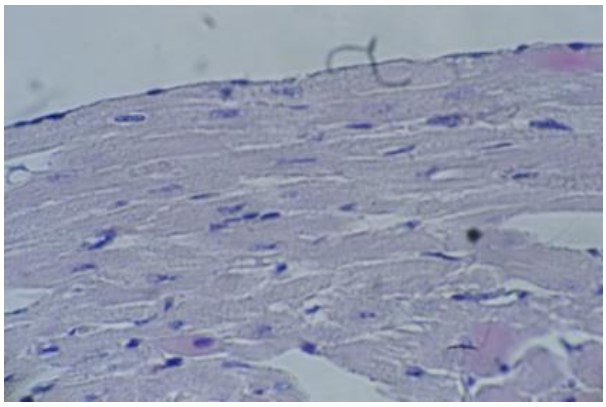

Figure 6. The heart tissue of a rat treated with Cyanidin-3-glucoside at a concentration of 70% mg/kg. 40X (H&E)

DISCUSSION

Troponin, CK-MB, and LDH are the enzymes related to cardiac activity. The increased level of each enzyme in the positive control group treated with hydrogen peroxide at a concentration of 1% could be evidence of damage to the heart muscle due to oxidative stress caused by free radicals. This risk may increase or develop into cardiomyopathy and heart failure later (Nimse and Pal, 2015). Membrane damage and leakage of Troponin, CK-MB, and LDH into cardiac tissue induced by hydrogen peroxide are prominent signs of experimentally induced myocardial infarction (Padmanabhan and Prince, 2006).

There was a significant decrease in the levels of CK-MB, Troponin, and LDH in the G3 and G4 groups compared to the positive control group. This could support reflect the positive role of Cyanidin-3-glucoside in terms of the ability to protect the heart and resist stress. Treatment with cyanidin extracted from red cabbage protected the heart through what was observed in the percentage of heart weight, a decrease in the level of CK-MB, troponin, and LDH an improvement in the levels of the antioxidant enzymes Superoxide Dismutase Catalase. In a study conducted by (Li et al., 2018), rats treated with cyanidin at a concentration of 5 mg/kg for five days before treatment with (LPS) for 18 hours showed a significant reduction of 30.4% and 30.6% in the levels of CK-MB and LDH, respectively.

Another study showed an improvement in cardiac function by treatment with cyanidin in rats that induced heart failure with doxorubicin (Petroni et al., 2017). Several reports have also indicated a protective role of cyanide against oxidative stress that induces myocardial and endothelial cell damage (Serraino et al., 2003; Qian et al., 2018).

Previous studies reported that the natural food component (cyanidin) plays an important role in the pre-death effect of oxidative cells in the heart muscle (Akhlaghi and Bandy, 2012), and they also reported that flavonoids reduced heart damage (Elberry et al., 2010; Hao et al., 2013). In another study by Mahmmoud (2013) on the profile of lipids and oxidative stress in animals treated with a high-calorie diet and with different concentrations (2.5%, 5%, 10%) of two types of blackberries, namely *Morus albal* and *Morus nigra* 4four weeks, they noticed a decrease in the levels of Nitric Oxide (NO) and Malondialdehyde (MDA) in *Morus albal* (10%, 5%) and *Morus nigra* group (2.5%, 5%, 10%). These fruits also increased the total antioxidant capacity at all used concentrations. The same results were obtained for fats.

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Compared to the control group, there was a significant decrease in total cholesterol, triglycerides, LDL, and VLDL and an increase in HDL in plasma. Consuming blackberries, which are rich in natural antioxidants, can prevent the risk of developing vascular diseases and reduce lipids and oxidative stress.

In a study by Sankhari et al. (2012), it was found that animals using an atherosclerosis-inducing diet plus red cabbage extract (rich in anthocyanins) showed a decrease in GSH, an increase in HDL-C and a decrease in liver enzymes (alanine transaminase and aspartate aminotransferase), compared to the group of animals in which atherosclerosis was induced arteries.

As for the G5 and G6 groups, there was a significant decrease in the levels of CK-MB, LDH, and troponin enzymes, compared with the positive control group. As an antioxidant, cyanidin, which can remove destructive molecules generated in the body (free radicals), destroys cell membranes and causes DNA changes and cell death. Cyanidin acts as an anti-inflammatory agent and helps protect the heart against disease (Ischizawa et al., 2011). Therefore, flavonoid cyanidin reduces damage to cardiac muscle cells and maintains the function of mitochondria, thus preventing heart muscle failure. Moreover, this substance can strengthen the immune system by supporting the main enzymes in the metabolic pathways in cells and removing free radicals that affect them (Faddah et al., 2013).

A study by Cara et al. (2017) demonstrated the high antioxidant capacity of gooseberry and gon-berry, which is consistent with studies showing that gooseberry contains high levels of anthocyanins and has antioxidant activity (Zheng and Wang, 2003; Grace et al., 2014; Isaak et al., 2015). Numerous reports have shown that antioxidant compounds, including anthocyanins, protect cells from apoptosis caused by oxidative stress through several mechanisms, including induction of autoinflammatory, inhibition of mitochondrial dysfunction, and activation of antioxidant enzymes. (Angeloni et al., 2007; Lv and Zhou, 2012; Kim et al., 2014; Lei et al., 2015).

Histological changes in heart

The tissue sections in G2 (positive control) showed an expansion in the space between muscle cells, blood bleeding, and clear congestion. Results reflect the negative cardiac tissue changes due to oxidative stress and species generation (Argun et al., 2015). Studies have indicated that free radicals of active oxygen species such as hydroxyl, hydrogen peroxide, and superoxide radicals lead to nucleolytic and programmed death (Kalivendi et al., 2001; Wang et al., 2004).

The generation of oxidative stress by hydrogen peroxide through drinking water leads to a significant increase in the activity of AST, ALT, and CK enzymes, in addition to damaging body tissues, including endothelial cells of vessels and cardiac muscle (Tao et al., 2006). The pathological changes in the tissue of the heart may be due to the fact that hydrogen peroxide can break down cell walls, which leads to the release of many chemicals that work to attract inflammatory cells to the area of injury (Zhu et al., 2018).

The tissue sections of the heart, which were taken from the third and fourth groups, showed significant improvement and residual negative effects in the tissues. This could be evidence of the recovery state after the tissue was exposed to hydrogen peroxide, as it is known that flavonoid intake is associated with a reduction of damage and muscle recovery (Hollinger et al., 2015). Rahman et al. (2007) indicated that flavonoids, such as anthocyanins and quercetin could activate the defense mechanism in cardiac cells. Flavonoids are effective in scavenging free radicals, reactive oxygen species (ROS), and reducing oxidative stress as well as their role in preventing the biosynthesis of enzyme proteins that contribute to reactions of oxidation–reduction where gene expression activates the synthesis of active proteins of cardiac muscle fibers to replace damaged proteins, are indicated.

A study by Li et al. (2018) showed the role of cyanidin in inhibiting oxidative stress resulting from treatment with the endotoxin LPS, where histological analyzes of myocardial tissues of the treated groups showed a significant increase in protein nitration. The quantitative estimation of the mentioned study showed an increase of 5.6 times, and treatment with cyanidin affected the decrease in LPS-induced protein nitrate by 26.6%. They also examined the state of intracellular oxidation and reduction by determining the amount of oxidized glutathione and reduced glutathione in myocardial tissues, where the endotoxin caused an increase in the level of oxidized glutathione up to 47.3% and also reduced the level of reduced glutathione up to 36.9%, the oxidized glutathione increased by 17.3% and reduced glutathione increased by 22.2%.

CONCLUSION

The treatment with H2o2 has led to oxidative stress in the heart tissue through indicators of cardiac parameters and histological changes. The treatment with C3G could positively affect the biochemical parameters and heart tissues induced by oxidative stress with hydrogen peroxide. C3G did not negatively affect the general health or the heart of the animals used in the study. This can be inferred in particular by the results in the two groups subjected to compound treatment. The results showed the effectiveness of C3G as an antioxidant through its ability to scavenge free radicals and protect cells from oxidative stress.

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DECLARATIONS

Competing interests

The authors confirm that they do not have any conflicts of interest.

Authors' contribution

Aseel Najah Sabour designed the study and critically revised the manuscript. Huda Yasser Aliwi brought the experimental animals and dosed them with the materials throughout the experiment. Aseel Najah and Huda Yasser performed the process of drawing blood from animals, as well as the process of dissection. Huda participated in the biochemical tests for blood serum and writing. Both authors checked and approved the final version of the manuscript for publishing in the present journal.

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 the authors.

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Incidence and Hematological Changes in Dogs Infected with *Dirofilaria immitis* in Thailand

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ABSTRACT

Dirofilaria immitis is responsible for heartworm disease in dogs. Clinical signs are non-specific, ranging from asymptomatic to severe symptoms. The most common symptoms include coughing, emaciation, dyspnoea, and sudden loss of consciousness. Therefore, diagnosing heartworm infection in dogs requires a combination of methods, such as hematology and serology. This study was conducted on dogs with clinical signs, including anorexia, coughing, panting, and hind legs weakness, that was referred accidentally to a pet clinic in Thonburi district, Bangkok Province, Thailand, during 2020-2022. The examination was performed using a rapid enzyme immunoassay test and a thin blood smear. The total number of dogs admitted to a pet clinic during that period was 980. The result indicated infection of 21 (12 male and 9 female) dogs with heartworm (2.14%). The mean age of dogs was 5.62 ± 2.48 years. All infected dogs were classified under an open husbandry system that did not consistently use heartworm prevention products such as the macrocyclic lactone group. In the groups that received topical ectoparasites products, 10 dogs were detected with heartworm infection. The hematological changes in the infected dogs consisted of leucocytosis and increased levels of ALT, BUN, and creatinine. The study results can guide owners in choosing products that can prevent heartworm. Anti-mosquito nets should be deployed in areas where pets live, and always keep the environment clean.

Keywords: Dog, Heartworm, Hematology, Serum biochemistry

INTRODUCTION

Heartworm disease is caused by *Dirofilaria immitis*, a parasite carried by mosquitoes, such as *Culex theileri* and *Anopheles maculipennis* (Ferreira et al., 2015). Infection can be found in many animals, including dogs, cats, ferrets, and humans (McCall et al., 2008; Hoch and Strickland, 2008). Presently, heartworm infection in dogs has been reported worldwide, including in Italy (Magi et al., 2012), Brazil (Alves et al., 1999), and Korea (Lee et al., 1996). Heartworm is a zoonosis through which humans are accidentally infected by mosquito bites from dogs that carry heartworms. Human clinical signs are mostly respiratory symptoms (McCall et al., 2008; Polak et al., 2014; Little et al., 2018).

The infected dogs are usually asymptomatic; however, they might show symptoms when there is a large amount of heartworm and a disturbance of the blood vessels (Hoch and Strickland, 2008). Clinical signs in infected dogs include emaciation, weight loss, inability to exercise for long periods, coughing, dyspnoea, panting, and sudden loss of consciousness (Hoch and Strickland, 2008; Lu et al., 2017). As the symptoms of infected dogs are not specific, detecting heartworm infection requires a combination of several examination methods, such as Enzyme-Linked Immunosorbent Assay (ELISA), radiography, echocardiography, and molecular detection (Kamyingkird et al., 2017; Lu et al., 2017; Kim et al., 2020).

Dogs infected with heartworm have been reported to suffer hematologic changes, such as anemia, thrombocytopenia, leucocytosis, and increased liver and renal enzymes (Niwetpathomwat et al., 2007; Kim et al., 2020). Therefore, hematological tests could be helpful in monitoring or evaluating subclinical heartworm infection or assessing the severity of the occurrence (Kim et al., 2020). There is still a lack of evidence about hematologic changes in heartworm-infected dogs, factors affecting infection, and infection incidence. Therefore, the current study aimed to survey the incidence of heartworm infection and hematological changes in dogs referred to a pet clinic in Thonburi district, Bangkok, Thailand, during 2020-2022.

MATERIALS AND METHODS

Ethical approval

The current study followed the Institutional Animal Care and Use Committee (IACUC) of Suan Sunandha Rajabhat University (SSRU), Bangkok, Thailand. The researchers were trained in using animals for research under the training code U1-08960-2563. Information about animals has been disclosed with consent from the owners.

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Database collection

The dogs (n = 980) came to an animal clinic in Thonburi district, Bangkok Province, Thailand (establishment license 01-957/2562, latitude 13.707529, longitude 100.478054) from 2020 to 2022. Twenty-one dogs were found with heartworm infection. Diagnosis of heartworm infection in the dogs using blood smear and rapid enzyme immunoassay testing (IDEXX SNAP® 4Dx®, United States (was made following the manufacturer's instructions. Clinical signs of infected dogs included anorexia, depression, coughing, panting, hind legs weakness, and vomiting. Physical examination of the dogs revealed fever (39.4 ± 0.27° Celsius, normal dogs temperature is 38 ± 0.88° Celsius, Cichocki et al., 2017), increased heart and lung sounds, emaciation, and dehydration. A vet specialist in small animal internal medicine collected the data. Data included gender, age, breed, rectal temperature, close-open husbandry pattern, ectoparasites prevention program, such as topical (*Frontline*®, *France*), chewing (NexGard®, Brazil), or injection (Baymec®, Korea), and heartworm prevention program. The dog's history data comes from the history taken from the dog's owner.

Clinical hematology and serum biochemistry collection

Blood was collected from the cephalic vein (1.5 ml) in an EDTA (Ethylenediaminetetraacetic acid, China) tube for hematology, blood smear, and rapid enzyme immunoassay testing. Moreover, 1.5 ml of blood was collected in a heparin tube for serum biochemistry testing. Hematology used automatic hematology cell counter (MS 4, Melet Schloesing laboratories, Cergy-Pontoise Cedex, France) evaluation included White Blood Cells (WBC), Hematocrits (Hct), and Platelets (PLT). Serum biochemistry used an automatic analyzer machine (BT 2000, Biotechnica Instruments, Rome, Italy) evaluation consisted of Alanine Aminotransferase (ALT), Blood Urea Nitrogen (BUN), and creatinine. Hematology, serum biochemistry, and blood smears were examined at a standard laboratory (Laboratory of Vet Clinical Center, Bangkok, Thailand). The blood samples were collected aseptically following the study conducted by Sirois (2014).

Statistical analysis

Descriptive analysis was used for the study of database collection, clinical hematology, and serum biochemistry recording using IBM SPSS statistics, version 29 (USA).

RESULTS

From 980 dogs referred to a pet clinic, dogs infected with heartworm involved 21 mixed-breed dogs (2.14%), comprising 12 males (57.14%) and 9 females (42.86%). The average age and rectal temperature of the dogs were 5.62 ± 2.48 years and 39.4 ± 0.27° Celsius, respectively. The husbandry system indicated that all dogs infected with heartworm were classified as an open system (100%). Of the investigated dogs, 10 (47.62%) with heartworm infections were regularly administered with a product to prevent ectoparasites. Moreover, 16 dogs (76.19%) were found to receive the heartworm prevention medication (Ivermectin 6 µg/kg, Heartgard Plus™, Venco et al., 2004) for more than 4 months, while 5 dogs (23.81%) had never received such medication (such as the macrocyclic lactone group, Table 1).

The thin blood smear and the rapid enzyme immunoassay test indicated positive dogs (Figure 1). The average total WBC count in the infected dogs was 17.16 ± 5.65 ($\times 10^3$ cells/µL). The Hct and PLT values were found to be 39.45 ± 7.27% and 301.14 ± 84.90 ($\times 10^3$ cells/µL) in the infected dogs, respectively. The serum biochemistry of infected dogs, ALT, BUN, and creatinine were recorded as 182.33 ± 198.78 IU/L, 71.19 ± 23.83 mg/dL, and 2.09 ± 0.82 mg/dL, respectively (Table 2).

Table 1. Associated factors with heartworm infection in infected dogs of Thonburi district, Bangkok, Thailand, during 2020-2022

Criteria	Dogs infected with *Dirofilaria immitis*	No.	Percentage
Breed	Mixed-breed dogs	21	2.14
	Gender		
	Male	12	57.14
	Female	9	42.86
Age (years)	2	1	4.76
	3	5	23.81
	4	1	4.76
	5	4	19.05
	6	4	19.05
	7	1	4.76
	8	2	9.52
	10	3	14.29
Husbandry system	Close	0	0
	Open	21	100
	Prevention ectoparasites		
	Consistent	10	47.62
	Never/Sometimes	11	53.38
	Prevention heartworm		
	Consistent	0	0
	Never	5	23.81
	Sometimes	16	76.19

Data collection (n=980), No: Number of dogs, Never: Dogs never used preventive products, Sometimes: Dogs use preventive products sometimes but not regularly, Prevention ectoparasites: Dogs use effective drugs against fleas or ticks, Prevention heartworm: Dogs use effective drugs to eliminate heartworm larvae.

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Table 2. Clinical hematology and serum biochemistry of dogs infected with *Dirofilaria immitis* in Thonburi district, Bangkok Province, Thailand during 2020-2022

Parameters	Infected dogs (n = 21)		Normal Range	
	Mean	Observation*	Mean	Range
WBC ($\times 10^3$ cells/µL)	17.16	8.79-32.40	12.05	5.00-14.10
Hct (%)	39.45	21.40-46.60	63.50	35.00-57.00
PLT ($\times 10^3$ cells/µL)	301.14	61.00-497.00	416	211.00-621.00
ALT (IU/L)	182.33	30.00-984.00	64.50	10.00-109.00
BUN (mg/dL)	71.19	35.00-120.00	18	8.00-28.00
Creatinine (mg/dL)	2.09	1.16-4.96	1.1	0.50-1.70

*Observation= range of data observed from raw data. Normal range References (Cyntia, 2011).

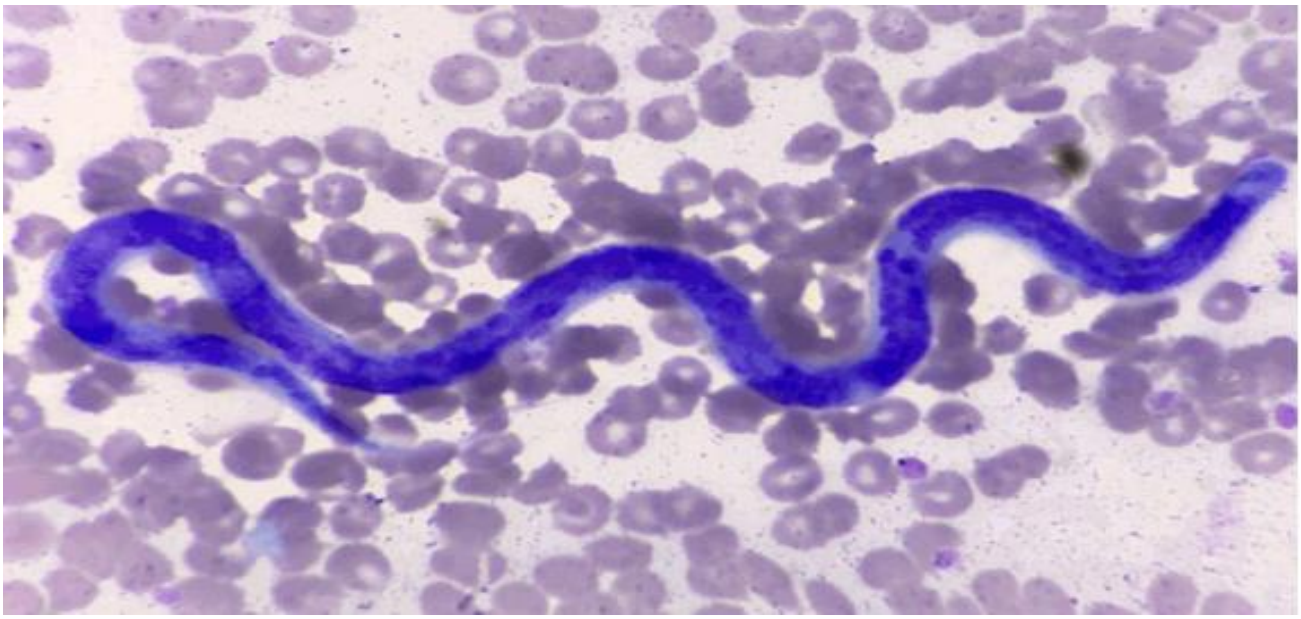

Figure 1. Microfilaria of *Dirofilaria immitis* in an infected dog detected by thin blood smear method under Giemsa staining, 1000× magnification in Thonburi district, Bangkok Province, Thailand

DISCUSSION

The detection of heartworm in dogs has been reported in many countries, such as Italy (Little et al., 2018), the United States (Little et al., 2018), and Thailand (Niwetpathomwat et al., 2007; Kamyingkird et al., 2017). In the current study, all dogs infected with heartworm were found to be mixed breeds. According to a previous report, heartworms can be found in all breeds of dogs (Vieira et al., 2014). The infected dogs were dominantly male dogs (n = 12). However, Boonyapakorn et al. (2008) report indicated no gender difference in heartworm infection in dogs. The average age of the heartworm-infected dogs was 5.62 ± 2.48 years, ranging from 2-10 years. In previous studies, the infected dogs aged were between 2- 6years and over 10 years of age (Boonyapakorn et al., 2008).

Regarding the husbandry system, it was found that all infected dogs (n = 21) were in an open system, which is consistent with previous studies, indicating that infected dogs were often located outside the home and were at greater risk of being bitten by mosquitoes (Borthakur et al., 2015). No differences were between the groups of dogs who consistently used prevention ectoparasites products and those who never, or only occasionally, used prevention ectoparasites products. The reason can be the chosen product was ineffective or inadequate in preventing heartworm, for instance, using only fipronil to control ectoparasites. Therefore, prevention should include other drugs, such as the macrocyclic lactone group (Noack et al., 2021). Previous studies have reported that using moxidectin in combination with doxycycline effectively eliminates dogs' larvae and adults of heartworms (Genchi et al., 2019).

Regarding the history, it was found that five infected dogs that had never been administered the heartworm prevention product were strays brought in by compassionate people. The remaining 16infected dogs had owners to take care of them. The results are consistent with previous reports that dogs without heartworm prevention products were at greater risk of infection (Boonyapakorn et al., 2008).

The hematology and serum biochemistry changes results indicated that the average WBC count was higher in the infected dogs than in the reference range (17.16 $\times 10^3$ cells/µL). The current study result showed all dogs' mean Hct were in the normal range (39.45%), and these data disagree with Kim et al. (2020), reporting anemia and found that

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anisocytosis in dogs with severe status resulted from hemolysis and red blood cell destruction from passing through the worm (Kim et al., 2020). In this study, infected dogs have no sign of severe anemia may be due to a low number of heartworms in infected dogs. The PLT data revealed that infected dogs averaged within the normal range, compared to the reference values. The mean ALT in the dogs infected in the current studywas higher than the reference value (182.33 IU/L). This is related to previous studies by Niwetpathomwat et al. (2007) that have also found elevated ALT in heartworm-infected dogs, which may be associated with intracellular damage, leading to enzyme release (Niwetpathomwat et al., 2007). The BUN and creatinine mean values were higher than the reference values (71.19 and 2.09 mg/dL). The increase in these two values is commonly associated with renal dysfunction, dietary intake, and dehydration (Niwetpathomwat et al., 2007). Previous studies have found that heartworm-infected dogs have elevated BUN and creatinine values, which may be related to immune-mediated glomerulonephropathy (Rawlings and Calvert, 1989; Niwetpathomwat et al., 2007).

Heartworm infection in dogs by carrier mosquitoes can indicate the quality of life in dogs. Infection means that dogs have been subjected to improper handling of vector defenses and inappropriate animal welfare standards (Merck, 2012). Mosquito control, a clean environment, and a lack of stagnant water can improve the environment where dogs are raised; moreover, the living area of pets should have anti-mosquito nets, using chemical eliminates, such as organophosphate (Benelli, 2015). Future studies are needed to explore strategies to educate dog owners about the severity and importance of heartworm prevention to attain better animal welfare management.

CONCLUSION

The study found that heartworm-infected dogs in the Thonburi district, Bangkok province, Thailand, were male and female mixed-breed dogs aged 2 to 10 years. All infected dogs were classified as open husbandry systems and did not consistently use heartworm prevention products. However, despite the use of ectoparasites products, heartworm-infected dogs can still be detected. Heartworm detected in dogs with regular administration of ectoparasites products may result from the inefficacy of the preventative products that do not eliminate heartworm larvae in the bloodstream. The hematological changes in infected dogs included leucocytosis as well as increased levels of ALT, BUN, and creatinine. Owners should keep the dog's area clean, install mosquito nets, and employ regular heartworm prevention strategies.

DECLARATIONS

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Competing interests
The authors declare no competing interests.

Ethical consideration
The authors extensively considered the ethical concerns, including plagiarism, fabrication, falsification, double publication or submission, and consent to publication.

Authors' contribution
Narong Kulnides suggested research guidelines and data analysis, presented information and wrote a manuscript. Athip Lorsirigool conducts research data collection, analyzes data, presents data, and wrote a manuscript. Natapol Pumipuntu recommends and revised content in a manuscript. Chaikamon Chantrarasmee conducted research data collection. Nopparuj Janthong performed data collection. All authors read and approved the final manuscripts.

Availability of data and materials
The authors confirm that the data supporting the findings of this study are available.

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Knowledge, Attitudes, and Practices of Red Meat Handlers in Moroccan Slaughterhouses

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ABSTRACT

Meat handlers are vectors of pathogens in slaughterhouses and can play a major role in the microbiological contamination of meat. The level of knowledge of meat handlers in slaughterhouses is a critical factor in food safety. Good hygienic practices in the slaughterhouse are required to reduce the risk of microbiological contamination while handling meat. This study evaluated workers' knowledge, attitudes, and practices in four municipal slaughterhouses in Morocco. A total of 267 employees were evaluated using a structured survey. The results showed that workers had acceptable knowledge and practices, and their attitudes were very satisfactory, averaging 52.87%, 50.9%, and 63.07%, respectively. A positive correlation between the workers' level of knowledge and education was found in all studied slaughterhouses. Similarly, the results indicated a positive correlation between knowledge and attitudes at Meknes and Kenitra slaughterhouses. The impact of the studied sociodemographic characteristics may vary from one slaughterhouse to another. In conclusion, the study suggested that although the knowledge, attitudes, and level of practice of food handlers were very satisfactory, some aspects related to the control of the health status of the handlers and personal protective equipment had to be underlined. Ongoing food safety training should become mandatory to enhance food safety in the slaughterhouses of study locations.

Keywords: Attitudes, Food Safety, Hygienic practices, Knowledge, Slaughterhouse

INTRODUCTION

The increasing number of epidemic outbreaks of food poisoning and food crisis worldwide forces authorities to apply the best hygiene and quality practices (Baş et al., 2006). According to the world health Organization, Global estimates on foodborne diseases indicate the annual infection of more than 600 million people by consuming contaminated food with bacteria, viruses, or parasites, and 420000 deaths (WHO, 2017). Poor hygiene and sanitation conditions in meat processing establishments, such as slaughterhouses, contribute significantly to the high incidence of community-acquired foodborne diseases, which are frequent and sometimes severe (Egan et al., 2007; Lues and Van Tonder, 2007; WHO, 2006).

In Morocco, more than 90% of collective food poisoning is due to bacteria, of which meat products constitute 11% (Belomaria et al., 2007). In 2016, the Moroccan Poison Control Center (CAPM) recorded 2723 cases of food poisoning, 53% of which were cases of collective food poisoning (Ghailani et al., 2020). Butchers working in slaughterhouses are the main vectors for meat contamination (Todd et al., 2010; Jianu and Chiș, 2012; Matchawe et al., 2019). Investigations into foodborne disease outbreaks have shown that poor personal hygiene plays a major role in the passive transmission of pathogens, such as Noroviruses, *Salmonella* species, *Staphylococcus aureus*, and *Shigella* species. These pathogens are often present in handlers' wounds, mouth, skin, and hair. Furthermore, the unwashed hands of workers can also transmit pathogens, particularly fecal pathogens (Sharif et al., 2013; Lambrechts et al., 2014).

Morocco has 180 municipal slaughterhouses, 3 approved private slaughterhouses, and 223 (out of 702) uncontrolled rural slaughterhouses (Annual report of the court of auditors of Morocco, 2018). Most of these slaughterhouses do not fulfill the hygienic requirements recommended by the National Office for Sanitary Safety of Food Products (ONSSA), such as a lack of hygiene training for working staff (Annual report of the court of auditors of Morocco, 2018). A knowledge, attitude, and practice (KAP) survey is a structured, standardized questionnaire used for a particular population to collect information about what is known, believed, and done on a specific topic (WHO, 2008). This type of investigation serves as an educational diagnosis of populations (Salih et al., 2019). Surveys on KAP have become a widely used means of research worldwide for public health studies to propose reflections on the training of food handlers, given their responsibility for food safety and consumer health (Ahmed et al., 2020). Several studies have been conducted to evaluate these processes in food safety in international slaughterhouses (Annor and Baiden, 2011; Abdul-Mutalib et al., 2012; Soares et al., 2012).

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The current study is a complement to previous research published by the Laboratory of Microbiology and Molecular Biology, Mohammed V University, Rabat, Morocco, providing information on the level of knowledge, attitudes, and practices of workers, as well as on the microbiological situation of the slaughter areas of the municipal slaughterhouses of Marrakech city located in southern Morocco (Bahir et al., 2022). This study aimed to assess the knowledge, attitudes, and practices of food handlers in four Moroccan municipal slaughterhouses in Rabat, Salé, Kenitra, and Meknes, situated in Morocco.

MATERIALS AND METHODS

Ethical approval

All the information in this study was based on the recommendations of the Faculty of Sciences, Mohammed V University, Rabat, Morocco. Authorizations were requested from the administration of each slaughterhouse, which granted permission to carry out the surveys. The manipulators involved in the current study were reported anonymously.

Study area

Present work concerned four municipal slaughterhouses located in the northwest of Morocco (Rabat, Sale, Kenitra, and Meknes). In Morocco, the sanitary inspection of municipal slaughterhouses is carried out by the veterinarians of ONSSA, while the administrative management is under the supervision of the Ministry of Interior. The choice of the studied slaughterhouses was based on the accessibility to their geolocation. The authorization of the responsible authorities was obtained for the realization of this survey. For this purpose, the interviewed participants included 267 randomly selected male employees working in cattle or sheep slaughterhouses, cutting rooms, or the carcass transportation sector. The number of interviewed participants in each slaughterhouse varied depending on the size of the abattoirs and their willingness to participate in the surveys. Meat handlers were voluntarily and randomly selected. A structured questionnaire with different sections was administered to all participants. The questions were explained and read aloud to the participants, who were given sufficient time to answer each question. No one was forced to participate in the survey.

Survey

The survey was conducted between January and April 2021. Based on previous research, a structured four-part study was developed to assess the sociodemographic characteristics, knowledge, attitudes, and slaughter practices of food handlers (Angelillo et al., 2001). Information on the sociodemographic characteristics of the handlers was mainly related to their work area in the slaughterhouse, age, level of education, work experience, training in meat hygiene, and health status. The information on workers' knowledge of food safety included 10 questions related to microbiological risks of carcass contamination, the importance of refrigeration and personal hygiene, and risks related to foodborne illness, to which participants were given the option of answering "Agree" or "Disagree". Following the same, respondents' attitudes toward food safety were also assessed by 10 questions addressing their personal hygiene and the cleaning of surfaces used during the slaughter process, as well as the standard cleaning of the used equipment. The participants could be scored 0-10 for the knowledge and attitude sections. The obtained results have been converted to 100%. Scores between 40% and 70% were considered acceptable, while scores more than 70% were considered excellent. The slaughter practices section included 18 questions on topics related to hygiene and the wearing of personal protective equipment during work. Participants were asked to answer "yes" or "no". A score was assigned to each correct answer and converted to a percentage. An average was calculated for each section to get a global idea of the handlers' knowledge, attitudes, and practices.

Statistical analysis

The statistical analysis was performed using version 2021 of XLSTAT Life Sciences, UK. The relationship between the sociodemographic profile of the carcass handlers and their KAP levels was determined using the chi-squared test. Differences in the mean of participants within each group studied were analyzed using a one-way ANOVA integrated post hoc test to show that particular differences between pairs of means are significant. Statistical significance for all tests was set at $p \leq 0.05$ using Ducan Test.

RESULTS

Sociodemographic characteristics of respondents

Tables 1 to 4 summarize the results concerning sociodemographic characteristics. Information on the sociodemographic characteristics of the carcass handlers included their work area in the slaughterhouse, age, education level, work experience, and hygiene training. The findings indicated that 56% (n = 45), 65% (n = 26), and 66% (n = 56) of participants were practicing cattle slaughter in Rabat, Salé, and Meknes, respectively. However, almost all

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respondents (93.55%, n = 58) were sheep slaughters in Kenitra. A minority in Rabat worked in trip cleaning (6.25%) or transporting the carcasses (17.25%) to cold storage (Table 1).

The majority of handlers aged between 18 and 40 years, according to their recruitment, physical strength, and performance during the different slaughter operations. A few workers were under 18 years of age in Rabat (4%) and Kenitra (4.84%, Table 2). College and primary levels of education were 26.25% and 38.5% in Rabat, 60% and 20% in Sale, 30.59% and 32.94% in Meknes, and 35.48% and 27.42% in Kenitra, respectively (Table 3). The majority of handlers had good professional experience and worked for more than 20 years, compromising 41.25% in Rabat, 50% in Sale, 31.76% in Meknes, and 47.77% in Kenitra slaughterhouse (Table 4).

Table 1. Distribution of the work section of handlers in the municipal slaughterhouses of Rabat, Salé, Meknes, and Kenitra in Morocco

Working area	Rabat percentage (n)	Sale percentage (n)	Meknes percentage (n)	Kenitra percentage (n)	Total (n)
Sheep	20 (16)	30 (12)	22.35 (19)	93.54 (58)	105
Cattle	56.5(45)	65 (26)	65.88 (56)	3.23 (2)	129
Cleaning Tripe	6.25 (5)	5 (2)	2.35 (2)	3.23 (2)	11
Transport of carcasses	17.5 (14)	0 (0)	9.42 (8)	0 (0)	22
Total	100 (80)	100 (40)	100 (85)	100 (62)	267

N: Number of workers

Table 2. Age distribution of handlers in the municipal slaughterhouses of Rabat, Salé, Meknes, and Kenitra in Morocco

Age categories	Rabat percentage (n)	Sale percentage (n)	Meknes percentage (n)	Kenitra percentage (n)	Total
< 18 YO	5 (4)	0 (0)	0 (0)	4.84 (3)	7
18 -30 YO	28.75 (23)	25 (10)	25.88 (22)	25.81 (16)	71
31-40 YO	23.75 (19)	30 (12)	37.65 (32)	19.35 (12)	75
41-60 YO	22.50% (18)	35 (14)	23.53 (20)	37.10 (23)	75
> 60 YO	20 (16)	10 (4)	12.94 (11)	12.90 (8)	39
Total	100 (80)	100 (40)	100 (85)	100 (62)	267

YO: Years old, n: Number of workers

Table 3. Distribution of handler's education level in the municipal slaughterhouses of Rabat, Salé, Meknes, and Kenitra in Morocco

Education level	Rabat percentage (n)	Sale percentage (n)	Meknes percentage (n)	Kenitra percentage (n)	Total (n)
Informal	13.75 (11)	20 (8)	17.65 (15)	17.74 (11)	45
Primary	38.75 (31)	20 (8)	32.94 (28)	27.42 (17)	84
College	26.25 (21)	60 (24)	30.59 (26)	35.49 (22)	93
High school	16.25 (13)	0 (0)	12.94 (11)	17.74 (11)	35
Academic	5 (4)	0 (0)	5.88 (5)	1.61 (1)	10
Total	100 (80)	100 (40)	100 (85)	100 (62)	267

Informal: Manipulators who never had any basic education, n: Number of workers

Table 4. Distribution of work period of handlers in the municipal slaughterhouses of Rabat, Salé, Meknes and Kenitra in Morocco

Working period	Rabat percentage (n)	Sale percentage (n)	Meknes percentage (n)	Kenitra percentage (n)	Total (n)
0-4 years	8.75 (7)	0 (0)	11.77 (10)	14.52 (9)	26
5-10 years	17.50 (14)	20 (8)	21.18 (18)	11.29 (7)	47
11-15 years	10 (8)	15 (6)	12.94 (11)	11,29 (7)	32
15-20 years	22.5 (18)	15 (6)	22.35 (19)	16,13 (10)	53
>20 years	41.25 (33)	50 (20)	31.76 (27)	46.77 (29)	109
Total	100 (80)	100 (40)	100 (85)	100 (62)	267

n: Number of workers

Handler training in meat hygiene and handling

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In the four slaughterhouses, the majority of workers had no training in hygiene and meat handling. Only a minority of workers received this type of training, including 12.5% in Rabat, 20% in Sale and 7.06% in Meknes, and 0% in Kenitra (Table 5). With regard to the number of training sessions, the majority were rarely trained (1 to 4 times per year). Thus, of the 10 workers who received training, 70% (n = 7) rarely received training when offered by private companies in the Rabat slaughterhouse, and 30% (n = 3) received training 1 to 4 times per year. In Salé slaughterhouse, workers (n = 8) rarely received training. For Meknes, only 5% (n = 4) of the workers had training once a year 2.5% (n = 2) barely received any hygiene training (Table 6). On the other hand, 100% (n = 24) of the workers in all slaughterhouses who received training considered training as effective (Table 7), and intended to conduct such training in the near future (Table 8).

Table 5. Distribution of handlers training in hygiene and handling meat in the municipal slaughterhouses of Rabat, Salé, Meknes, and Kenitra in Morocco

Hygiene-training	Rabat percentage (n)	Sale percentage (n)	Meknes percentage (n)	Kenitra percentage (n)	Total (n)
Yes	12.50 (10)	20 (8)	7.06 (6)	0 (0)	24
No	87.50 (70)	80 (32)	92.94 (79)	100 (62)	243
Total	100 (80)	100 (40)	100 (85)	100 (62)	267

n: Number of workers

Table 6. Variation of training courses taken by workers in the municipal slaughterhouses of Rabat, Salé, Meknes, and Kenitra in Morocco

Number of hygiene-training	Rabat percentage (n)	Sale percentage (n)	Meknes percentage (n)	Kenitra percentage (n)	Total (n)
One per month	0 (0)	0 (0)	0 (0)	0 (0)	0
4 per year	20 (2)	0 (0)	0 (0)	0 (0)	2
1 per year	10 (1)	0 (0)	66.66 (4)	0 (0)	5
Rarely	70 (7)	100 (8)	33.34 (2)	0 (0)	17
Total	100 (10)	100 (8)	100 (6)	0 (0)	24

n: Number of workers

Table 7. Appreciation of the quality of hygiene training received by workers in the municipal slaughterhouses of Rabat, Salé, Meknes, and Kenitra in Morocco

Quality of hygiene-training	Rabat percentage (n)	Sale percentage (n)	Meknes percentage (n)	Kenitra percentage (n)	Total (n)
Effective	100 (10)	100 (8)	100 (6)	0 (0)	24
Not effective	0 (0)	0 (0)	0 (0)	0 (0)	0
Total	100 (10)	100 (8)	100(6)	0 (0)	24

n: Number of workers

Table 8. Worker's appreciation of the willingness to follow hygiene training in the municipal slaughterhouses of Rabat, Salé, Meknes, and Kenitra in Morocco

Willingness to follow hygiene-training	Rabat percentage (n)	Sale percentage (n)	Meknes percentage (n)	Kenitra percentage (n)	Total (n)
Yes	82.50 (66)	75 (30)	94.12 (80)	85.48 (53)	229
No	17.50 (14)	25 (10)	5.88 (5)	14.52 (9)	38
Total	100 (80)	100 (40)	100 (85)	100 (62)	267

n: Number of workers

The medical situation of the workers

The majority of handlers did not have a health certificate. According to the current regulation in Morocco, there should be medical files for all meat handlers, and the files must be updated annually. These files must be made available to the veterinary inspector. This document indicates the medical aptitude to perform slaughter activities. However, it has been observed that staff are rarely provided with medical certificates, which are not updated in Rabat, Sale, Meknes, and Kenitra at rates of 93.75% (n = 75), 82.5% (n = 33), 78.82% (n = 67), and 88.71% (n = 55), respectively (Table 9). On the other hand, 41.18% of the workers had a medical check-up before being hired in the slaughterhouse of Meknes (Table 10), and a very small number of workers did this operation before in Rabat, Sale, and in Kenitra slaughterhouses. Only 20% of the workers in the Kenitra slaughterhouse continued these check-ups (Table 11). The interval of the sanitary control of workers varied from one slaughterhouse to another. In Rabat, the majority of workers (41.25%, n =

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33) checked their health status every 3 months, and 20% (n = 10) and 17.5% (n = 14) of them reported having consulted a doctor between 6 months to a year, respectively. In Meknes and Kenitra, the majority of handlers declared having checked their health status between 6 months and a year. In the Salé slaughterhouse, 50% (n = 20) of the workers never checked their health status (Table 13). Except for the Kenitra slaughterhouse, many workers never consulted a doctor in their life, 21.25% (n = 17), 25% (n = 10), and 16.47% (n = 14) of workers in Rabat, Salé, and Mèknes, respectively (Table 13).

Table 9. Possession of medical certificates by workers in the municipal slaughterhouses of Rabat, Salé, Meknes, and Kenitra in Morocco

Possession of a medical certificate	Rabat percentage (n)	Sale percentage (n)	Meknes percentage (n)	Kenitra percentage (n)	Total (n)
Yes	6.25 (5)	17.5 (7)	21.18 (18)	11.29 (7)	37
No	93.75 (75)	82.50 (33)	78.82 (67)	88.71 (55)	230
Total	100 (80)	100 (40)	100 (85)	100 (62)	267

n: Number of workers

Table 10. Administrative requirement of a pre-employment medical check-up for workers at the municipal slaughterhouses of Rabat, Salé, Meknes, and Kenitra in Morocco

Pre-employment medical check-Up	Rabat percentage (n)	Sale percentage (n)	Meknes percentage (n)	Kenitra percentage (n)	Total (n)
Yes	17.5 (14)	15 (6)	41.18 (35)	24.19 (15)	70
No	82.5 (66)	85 (34)	58.82 (50)	75.81 (47)	197
Total	100 (80)	100 (40)	100 (85)	100 (62)	267

n: Number of workers

Table 11. Continuity of medical checks in the slaughterhouse by workers in the municipal slaughterhouses of Rabat, Salé, Meknes, and Kenitra in Morocco

Respondents who followed medical checks in the slaughterhouse	Rabat percentage (n)	Sale percentage (n)	Meknes percentage (n)	Kenitra percentage (n)	Total (n)
Yes	0 (0)	0 (0)	0 (0)	20 (3)	3
No	100 (14)	100 (6)	100 (35)	80 (12)	67
Total	100 (14)	100 (6)	100 (35)	100 (15)	70

n: Number of workers

Table 12. Last health check carried out by workers in the municipal slaughterhouses of Rabat, Salé, Meknes, and Kenitra in Morocco

The last health check carried out	Rabat percentage (n)	Sale percentage (n)	Meknes percentage (n)	Kenitra percentage (n)	Total (n)
1 month	17.5 (14)	2.5 (1)	10.59 (9)	4.84 (3)	27
3 months	8.75 (7)	2.5 (1)	11.76 (10)	27.42 (17)	35
6 months	13.75 (11)	15 (6)	20 (17)	11.29 (7)	41
12 months	12.5 (10)	17.5 (7)	16.47 (14)	12.9 (8)	39
>12 months	26.25 (21)	37.5 (15)	24.71 (21)	43.55 (27)	84
Never	21.25 (17)	25 (10)	16.47 (14)	0(0)	41
Total	100 (80)	100 (40)	100 (85)	100 (62)	267

n: Number of workers

Table 13. Health check interval by workers in the municipal slaughterhouses of Rabat, Salé, Meknes, and Kenitra in Morocco

Health Check Interval	Rabat percentage (n)	Sale percentage (n)	Meknes percentage (n)	Kenitra percentage (n)	Total (n)
Every 3 months	41.25 (33)	0 (0)	17.65 (15)	11.29 (7)	55
Every 6 months	17.5 (14)	2.5 (1)	21.18 (18)	17.74 (11)	44
Every 12 months	20 (16)	10(4)	27.05 (23)	24.19 (15)	58
If necessary	15 (12)	37.5(15)	23.53 (20)	46.77 (29)	76
Never checked before	6.25 (5)	50 (20)	10.59 (9)	0 (0)	34
Total	100 (80)	100 (40)	100 (85)	100 (62)	267

n: Number of workers

Handlers' hygiene knowledge

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Generally, a good knowledge of hygiene was approached by half of the workers (54.16%, 55.8%, 48.7%, and 52.9% in the slaughterhouses of Rabat, Sale, Meknes, and Kenitra, respectively). Most workers were aware of the microbiological risks, the importance of good hygiene practices in the workplace, and the impact of shelf life on meat contamination. In the Kenitra slaughterhouse, only 37% (n = 23) of the workers knew that unhygienic handling could be a source of meat contamination. In the slaughterhouses of Rabat and Kenitra, 59% (n = 47) and 55% (n = 34) of workers knew that refrigeration delays contamination, respectively, while this was not the case for workers in Salé (25%) and the slaughterhouse in Meknes (18.18%). In the Rabat and Meknes slaughterhouses, 51.3% (n = 41) and 56.5% (n = 48) of the workers were aware that microbial contamination of meat could cause serious illnesses leading to hospitalization and sometimes death, respectively. The level of knowledge of handlers about the risk of microbial contamination by a person suffering from diarrhea was generally very low at 46% (n = 37), 32.5% (n = 13), 35.3% (n = 30), and 44% (n = 27), in Rabat, Sale, Meknes, and Kenitra, respectively (Table 14).

Table 14. Hygiene knowledge of handlers in the municipal slaughterhouses of Rabat, Salé, Meknes, and Kenitra in Morocco

Percentage of correct answers (n) / The statements	Rabat (n = 80)	Sale (n = 40)	Meknes (n = 85)	Kenitra (n = 62)
Can meat spoilage be caused by microorganisms?	69 (55)	75 (30)	61.18 (52)	71 (44)
Is the contamination of meat very risky due to the shelf life?	75 (60)	85 (34)	68.2 (58)	55 (34)
Could unsanitary practices be a source of carcass contamination?	68 (54)	65 (26)	70.6 (60)	37 (23)
Can contamination be caused by direct contact between bare hands and animals or materials?	70 (56)	70 (28)	68.2 (58)	65 (40)
Does the chilling of meat at temperatures below 20°C contribute to delaying microbial deterioration?	59 (47)	25 (10)	18.18 (15)	55 (34)
Can microbial contamination cause serious illness leading to hospitalization and sometimes death?	51.3 (41)	47.5 (19)	56.5 (48)	35 (22)
Can healthy carriers carry microbes?	31.3 (25)	75 (30)	63.5 (54)	68 (42)
Can a handler with diarrhoeal syndromes be a source of risk?	46 (37)	32.5 (13)	35.3 (30)	44 (27)
Can water be a source of microbial contamination?	38 (30)	48 (19)	23.5 (20)	55 (34)
Can water from hoses used for cleaning be a source of contamination of carcasses?	34 (27)	35 (14)	21.18 (18)	44 (27)
Average knowledge estimate*	67%	55.8%	58.3%	52.9%

*Average knowledge estimate: Represents the average value of obtained data percentage for each bellow investigated response**,** n: Number of workers

Table 15. Attitudes of handlers towards hygiene and meat handling in the municipal slaughterhouses of Rabat, Salé, Meknes and Kenitra in Morocco

Percentage of correct answers (n) / The statements	Rabat (n = 80)	Sale (n = 40)	Meknes (n = 85)	Kenitra (n = 62)
Hand washing after the toilet with a disinfectant is mandatory	77.5 (62)	80 (32)	79 (67)	80.64 (50)
The handler must check his state of health	87.5 (70)	50 (20)	89 (76)	53.22 (33)
Handling meat with lesions on the hand is a risk of contamination	20 (16)	10 (4)	26 (22)	11.29 (7)
The state of health must be checked before employment	82.5 (66)	15 (6)	59 (50)	24.19 (15)
Training is very interesting for me	82.5 (66)	75 (30)	94 (80)	85.48 (53)
Disinfecting abattoir premises is a way to avoid contamination	87.5 (70)	85 (34)	76 (65)	96.77 (60)
The wearing of protective equipment (Apron) is necessary	11.25 (9)	32.5 (13)	52 (44)	53.22 (33)
Cleaning the slaughter area before slaughter operations	87.5 (70)	85 (34)	76 (65)	96.77 (60)
Cleaning of equipment before slaughter is desirable	71.25 (57)	50 (20)	79 (67)	80.64 (50)
The deposit of organ meats on the ground is prohibited	62.5 (50)	67.5 (27)	55 (47)	32.25 (20)
Average attitudes estimate*	67%	55%	58.3%	61.44%

*Average attitude estimate: represents the average value of obtained data percentage for each bellow investigated response**,** n: Number of workers

Hygiene attitudes

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Generally, the various handler statements regarding attitudes, hygiene, and meat handling were satisfactory. The mean values of correct answers were significantly positive in each slaughterhouse at 67.3% in Rabat, 55.1% in Salé, 68.5% in Meknes, and 61.44% in Kenitra. The majority of handlers in Rabat slaughterhouse (82.5%) agreed with the obligation of continuous monitoring using medical tests before hiring. On the other hand, handlers at Salé (85%) and Kenitra slaughterhouse (75.81%) considered it unnecessary. The majority of workers agreed with the continuous monitoring of medical tests. Attitudes in relation to hand washing and the importance of disinfecting premises were very satisfactory. The attitudes of the majority of workers concerning wearing personal protective equipment were weak. In Rabat and Salé, 88.75% (n = 71) and 67.5% (n = 27) of slaughterers considered using an apron during slaughtering operations as unnecessary, respectively. In the same direction, 80% (n = 64), 90% (n = 40), 74.11% (n = 22), and 88.71% of the handlers in Rabat, Salé, Meknes, and Kenitra slaughterhouses considered the handling of carcasses with lesions on the hands did not constitute a risk of contamination, respectively. In the Salé slaughterhouse, 67.74% (n = 20) of handlers considered that the deposit of meats on the floor did not contain any risk of contamination (Table 15).

Hygiene practices

Regarding slaughter practices of handlers, the average of correct responses obtained was between 47.9% and 54.12%. Most handlers reported that they washed their hands before the slaughter. Their responses were at the rates of 77.5% (n = 62) in Rabat, 80% (n = 32) in Salé, 78.8% (n = 67) in Meknes, and 100% (n = 62) in Kenitra, but they did not generally use a disinfectant except for one employee in Kenitra. Similarly, most handlers washed their hands after using the toilet but did not use sanitizer. In the 4 slaughterhouses, the majority of workers declared that they handled carcasses with lesions on their hands at response rates of 80% (n = 64) in Rabat, 90% (n = 36) in Salé, 74.1% (n = 63) in Meknes and 88.7% (n = 55) in Kenitra. More than half of the handlers in Rabat and Meknes slaughterhouses announced that they handled carcasses with diarrheal syndrome (57.5% and 56.5%). On the other hand, the majority of workers declared this fact in Salé and Kenitra, 85% (n = 34) and 61.3% (n = 38), respectively. As for the cleanliness of the handlers, the majority cut their nails and used boots, but most of them did not use gloves and aprons during slaughter operations. The majority of the handlers in Salé slaughterhouse (75%) announced that meat did not affect the working environment, unlike in Rabat, Meknes, and Kenitra slaughterhouses, 62.5% (n = 50), 89.4% (n = 76) and 69.4% (n = 43), respectively. Most slaughterers followed good practices to clean the slaughter area and equipment before and after the slaughter process (Table 16).

Table 16. Food handlers' practices toward food hygiene and sanitation in the municipal slaughterhouses of Rabat, Salé, Meknes and Kenitra in Morocco

Percentages of answers (n) / Slaughterhouse's station	Rabat percentage (n=80)		Sale percentage (n=40)		Meknes percentage (n=85)		Kenitra percentage (n=62)	
Questions	**Correct**	**Wrong**	**Correct**	**Wrong**	**Correct**	**Wrong**	**Correct**	**Wrong**
Hand washing before handling	77.5 (62)	22.5 (18)	80 (32)	20 (8)	78.8 (67)	21,2(18)	100 (62)	0 (0)
If so, do you wash them with one of the disinfectants?	37.10 (23)	62.90 (39)	25 (8)	75(24)	24.7 (21)	54.11 (46)	0 (0)	100 (100)
Do you wash your hands every time you use the restroom?	83.75 (67)	16.25 (13)	85 (34)	15 (6)	81.2 (69)	18.8 (16)	100 (62)	0
With or without soap?	28.36 (19)	60 (48)	30 (12)	70 (28)	16.5 (14)	83.5 (71)	3.22 (2)	96.8 (60)
Do you handle carcasses when you have injuries on your hands?	20 (16)	80 (64)	10 (4)	90 (36)	25.9 (22)	74.1 (63)	11.29 (7)	88.7 (55)
Do you handle carcasses when you are sick or suffering from diarrhoeal syndromes?	42.5 (34)	57.5 (46)	15 (6)	85 (34)	43.5 (37)	56.5(48)	38.7 (24)	61.3 (38)
Do you keep your fingernails long?	81.25 (65)	18.75 (15)	85 (34)	15 (6)	42.4 (36)	57.6 (49)	87.1 (54)	12.9 (8)
Do you wear gloves during slaughter?	8.75 (7)	91.25 (73)	5 (2)	95 (38)	17.6 (15)	82.4 (70)	0 (0)	62 (100)
During slaughter, is there any contact with the skin, walls, floor, or equipment?	37.5 (30)	62.5 (50)	75 (30)	25 (10)	10.6 (9)	89.4 (76)	30.6 (19)	69.4 (43)
Do you use an apron during the process?	15 (12)	85 (68)	10 (4)	90 (36)	20 (17)	80 (68)	13 (21)	49 (79)
Do you use boots during slaughter?	85 (68)	15 (12)	100 (40)	0 (0)	94.1 (80)	5.9 (5)	74.2 (46)	25.8 (16)
Do you place the cutters and winches on the floor?	60 (48)	40 (32)	30 (12)	70 (28)	22.4 (19)	77.6 (66)	50 (31)	50 (31)
Are carcasses and offal placed in direct contact with floors, walls, or other equipment during hide removal and transport operations?	62.5 (50)	37.5 (30)	67.5 (27)	32.5 (13)	55.3 (47)	44.7 (38)	32.3 (20)	67.7 (42)
Do you clean slaughter equipment daily?	26.25 (21)	62.5 (50)	55 (22)	45 (18)	18.8 (16)	81.2 (69)	0 (0)	100 (62)
Cleaning of the area before	87.5 (70)	12.5 (10)	85 (34)	15 (6)	76.5 (65)	24.7% (21)	96.8 (60)	3.2 (2)
The cleaning of the area after	88.75 (71)	11.25 (9)	72.5 (29)	27.5 (11)	96.5 (82)	3.5 (3)	96.8 (60)	3.2 (2)
Cleaning the front knives	71.25 (57)	28.75 (23)	50 (20)	50 (20)	78.8 (67)	22.2 (19)	80.6 (50)	19.4 (12)
Cleaning the knives after	61.25 (49)	38.75 (31)	50 (20)	50 (20)	51.8 (44)	48.2 (41)	62 (100)	0(0)
Average practices estimate*	54.12%	45.25%	51.7%	47.5%	47.9%	51.1%	51.3%	48.7%

*Average practices estimate: represents the average value of obtained data percentage for each bellow investigated response**,** n: Number of workers

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Correlation between the level of knowledge and sociodemographic factors of handlers in the municipal slaughterhouses of Rabat, Salé, Meknes, and Kenitra in Morocco

Rabat slaughterhouse

The obtained results in Rabat slaughterhouse showed a positive correlation between the handlers' level of knowledge and their education level (X^2=3.09, $p > 0.05$), as well as with the training handlers (X^2=1.87). No correlation was reported between the age and professional experience of the handlers (Table 17).

Sale slaughterhouse

A significant correlation was observed between the level of knowledge and the level of education ($X^2 = 1.61$). The other statistical indices show no correlation with the level of knowledge (Table 18).

Meknes slaughterhouse

In Meknes slaughterhouse, there was a highly significant correlation between the handlers' level of knowledge and their level of education (X^2=11.85; $p < 0.01$; Odds ratio =8.21). A positive correlation was also observed between their knowledge level and professional experience (X^2=1.67; Odds ration=3.74). The other statistical indices almost show no correlation between age, slaughter area, training, and level of hygiene knowledge (Table 19).

Kenitra slaughterhouse

The statistical indices obtained in Kenitra slaughterhouse showed a positive relationship between the knowledge of the handlers and their slaughter area, and their level of education with X^2=1.4 and X^2=1.07, respectively. No other statistical indices were significant (Table 20).

Table 17. Relationship between the level of knowledge of the handlers and their sociodemographic characteristics in Rabat slaughterhouse achieved on January 2021 in Morocco

Variables		Level of knowledge		No.	X^2	P-value	Prevalence Ratio	Odds Ratio	CI
		Acceptable	Excellent						
Slaughter area	Cattle + Sheep	34	30	64	0.05	0.82	0.94	0.88	0.43-0.59
	Cleaning Trips + Transport of carcasses	9	7	16					
Age	Young people (18 – 40)	28	18	46	0.12	0.73	0.94	0.85	0.42-0.58
	Age 40 - 60	22	12	34					
Level of education	Low: Informal or primary	28	10	38	3.09	0.08	1.35	2.31	0.42-0.57
	High: middle school, high school, or university	23	19	42					
Work experience	Low period (0-4 years)	6	2	8	0.16	0.69	1.10	1.41	0.40-0.55
	High period (4-more than 4 years)	49	23	72					
Training	Yes	5	5	10	1.87	0.17	0.70	0.40	0.40-0.55
	No	50	20	70					

CI: Confidence interval

Table 18. Relationship between the level of knowledge of the manipulators and their sociodemographic characteristics in Sale slaughterhouse achieved on January 2021 in Morocco

Variables		Level of knowledge		No.	X^2	P-value	Prevalence Ratio	Odds Ratio	CI
		Acceptable	Excellent						
Slaughter area	Cattle + Sheep	30	8	38	0.53	0.47	0.79	0.00	0.33-0.52
	Cleaning Trips + Transport of carcasses	2	0	2					
Age	Young people (18 – 40)	16	6	22	0.13	0.71	0.94	0.76	0.36-0.56
	Age 40 - 60	14	4	18					
Level of education	Low: Informal or primary	13	3	16	1.61	0.20	1.30	2.60	0.38-0.60
	High: middle school, high school, or university	15	9	24					
Work experience	Low period (0-4 years)	0	0	0	-	-	-	-	0.32-0.49
	High period (4-more than 4 years)	33	7	40					
Training	Yes	13	3	16	0.56	0.46	1.15	1.78	0.36 -0.56
	No	17	7	24					

CI: Confidence interval

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Table 19. Relationship between the level of knowledge of handlers and their sociodemographic characteristics in Meknes slaughterhouse achieved on March 2021 in Morocco

Variables		Level of knowledge		No.	X^2	P-value	Prevalence Ratio	Odds Ratio	CI
		Acceptable	Excellent						
Slaughter area	Cattle + Sheep	56	19	75	0.96	0.33	1.24	1.96	0.39-0.53
	Cleaning Trips + Transport of carcasses	6	4	10					
Age	Young people (18 – 40)	43	11	54	0.01	0.91	0.99	0.94	0.35-0.47
	Age 40 - 60	25	6	31					
Level of education	Low: Informal or primary	40	3	43	11.85	0.01	1.50	8.21	0.36-0.49
	High: middle school, high school, or university	26	16	42					
Work experience	Low period (0-4 years)	9	1	10	1.67	0.20	1.27	3.74	0.39-0.53
	High period (4-more than 4 years)	53	22	75					
Training	Yes	4	2	6	0.19	0.67	0.89	0.68	0.38-0.52
	No	59	20	79					

CI: Confidence interval

Table 20. Relationship between the level of knowledge of the handlers and their sociodemographic characteristics in the municipal slaughterhouse of Kenitra achieved on March 2021 in Morocco

Variables		Level of knowledge		No.	X^2	P-value	Prevalence Ratio	Odds Ratio	CI
		Acceptable	Excellent						
Slaughter area	Cattle + Sheep	35	25	60	1.40	0.24	0,58	0.00	0.42-0.60
	Cleaning Trips + Transport of carcasses	2	0	2					
Age	Young people (18 – 40)	20	11	31	0.30	0.59	0.91	0.74	0.40-0.57
	Age 40 - 60	22	9	31					
Level of education	Low: Informal or primary	19	9	28	1.07	0.30	0.85	0.55	0.37-0.54
	High: middle school, high school, or university	27	7	34					
Work experience	Low period (0-4 years)	7	2	9	0.81	0.37	1.25	2.12	0.41-0.59
	High period (4-more than 4 years)	33	20	53					
Training	Yes	0	0	0	-	-	-	-	0.42-0.60
	No	36	26	62					

CI: Confidence interval

Correlation between respondents' attitude level, knowledge, and sociodemographic characteristics in the municipal slaughterhouses of Rabat, Salé, Meknes, and Kenitra in Morocco

Rabat slaughterhouse

The relationship between attitude level and sociodemographic characteristics in Rabat slaughterhouse showed a strong significant correlation ($X^2 = 5.96$; $p < 0.05$) between slaughter area and attitude level. An excellent attitude is related to the cattle slaughter zone. The odds ratio presented a value of 0.25, indicating a negative attitude effect for the cleaning trips and transporting carcasses. The chi-square, prevalence, and odds-ratio indices indicated no relationship between age and attitude level. There was also a significant correlation between education and attitude level (low education is related to an excellent attitude). This can be explained by the correlation between attitude level and work experience (Odds ratio = 4.20, $p > 0,05$, Table 21).

Sale slaughterhouse

In Sale slaughterhouse, the results showed a correlation between age and attitude level (the youngest manipulators had a positive attitude, compared to the oldest manipulators) with $X^2 = 3.30$. There was no correlation between knowledge and attitude level ($X^2 = 0$, $p > 0.05$).

Meknes slaughterhouse

There was a correlation between knowledge and attitude in Meknes with $X^2 = 2.63$. The statistical indices showed almost no correlation between age and attitude level (Table 23).

Kenitra slaughterhouse

In Kenitra slaughterhouse, knowledge correlated with attitude ($X^2= 3.21$). The statistical indices indicated almost no correlation between attitude level and the other variables (Table 24).

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Correlation between hygiene and meat handling practices and sociodemographic characteristics of handlers in Rabat, Sale, Meknes, and Kenitra slaughterhouses

Rabat slaughterhouse

The results regarding the correlation between sociodemographic factors and slaughter practices showed a highly significant correlation between practice level and slaughter area (X^2 =11.55; p < 0,05). Prevalence and Odds-ratio confirmed this correlation, showing a negative effect between the level of practices and the washing of the entrails and the transport area with 0.16 and 0.31, respectively. The results also revealed a positive correlation between the level of practice and the education level of handlers (X^2 = 3.17). Unacceptable slaughter practices were associated with low education levels (Table 25).

Sale slaughterhouse

The results in the Sale slaughterhouse showed a positive correlation between slaughter practices and handlers' level of education (X^2 = 1.78, prevalence = 0.55, and Odds-Ratio = 0.39). The results of the slaughter area, age, and work experience showed no significant relationship between these factors and the level of handlers' practices.

Meknes slaughterhouse

In the slaughterhouse of Meknes, the results indicated that an acceptable level of slaughter practices was related to a young age range of handlers with an X^2=2.37. The other statistical indices showed almost no significant relationship between slaughter practices and other sociodemographic factors.

Kenitra slaughterhouse

In the slaughterhouse of Kenitra, the obtained results showed almost no positive correlation between slaughter practices and sociodemographic factors.

Table 21. Relationship between the level of attitude of the manipulators, their sociodemographic characteristics, and their level of knowledge in Rabat slaughterhouse achieved on January 2021 in Morocco

Variables		Level of knowledge		No.	X^2	P-value	Prevalence Ratio	Odds Ratio	CI
		Acceptable	Excellent						
Slaughter area	Cattle + Sheep	35	25	60	5.96	0.01	0.48	0.25	0.42-0.57
	Cleaning Trips + Transport of carcasses	2	0	2					
Age	Young people (18 – 40)	20	11	31	0.001	0.97	0.99	0.98	0.38-0.52
	Age 40 - 60	22	9	31					
Level of education	Low: Informal or primary	19	9	28	3.86	0.05	0.55	0.39	0.42-0.58
	High: middle school, high school, or university	27	7	34					
Work experience	Low period (0-4 years)	7	2	9	3.53	0.06	3.00	4.20	0.31-0.43
	High period (4-more than 4 years)	33	20	53					
Training	Yes	0	0	0	1.13	0.29	1.52	2.04	0.42-0.57
	No	36	26	62					
Knowledge	Acceptable	10	20	30	0.49	0.48	1.28	1.42	0.39-0.54
	Excellent	13	37	50					

CI: Confidence interval

Tableau 22. The relationship between the level of attitude of the manipulators, their sociodemographic characteristics, and their level of knowledge in Sale slaughterhouses achieved on January 2021 in Morocco

Variables		Level of knowledge		No.	X^2	P-value	Prevalence Ratio	Odds Ratio	CI
		Acceptable	Excellent						
Slaughter area	Cattle + Sheep	15	23	38	1.26	0.26	-	-	0.40-0.63
	Cleaning Trips + Transport of carcasses	0	2	2					
Age	Young people (18 – 40)	6	16	22	3.30	0.07	0.49	0.30	0.41-0.64
	Age 40 - 60	10	8	18					
Level of education	Low: Informal or primary	6	10	16	0.07	0.79	0.90	0.84	0.41-0.64
	High: middle school, high school, or university	10	14	24					
Work experience	Low period (0-4 years)	0	0	0	-	-	-	-	0.41-0.65
	High period (4-more than 4 years)	18	22	40					
Training	Yes	8	8	16	1.11	0.29	0.75	0.50	0.41-0.64
	No	16	8	24					
Knowledge	Acceptable	8	8	16	0.00	1.00	1.00	1.00	0.41-0.65
	Excellent	12	12	24					

CI: Confidence interval

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Table 23. Relationship between the level of attitude of the manipulators, their sociodemographic characteristics, and their level of knowledge in Meknes slaughterhouse achieved on March 2021 in Morocco

Variables		Level of knowledge		No.	X^2	P-value	Prevalence Ratio	Odds Ratio	CI
		Acceptable	Excellent						
Slaughter area	Cattle + Sheep	34	41	75	0.84	0.36	1.51	1.93	0.43-0.59
	Cleaning Trips + Transport of carcasses	3	7	10					
Age	Young people (18 – 40)	22	32	54	0.23	0.63	1.15	1.25	0.43-0.58
	Age 40 - 60	11	20	31					
Level of education	Low: Informal or primary	16	27	43	0.94	0.33	0.78	0.65	0.43-0.59
	High: middle school, high school, or university	20	22	42					
Work experience	Low period (0-4 years)	7	3	10	2.15	0.14	1.54	2.81	0.44-0.59
	High period (4-more than 4 years)	34	41	75					
Training	Yes	3	3	6	0.11	0.74	1.16	1.32	0.43-0.59
	No	34	45	79					
Knowledge	Acceptable	10	25	35	2.63	0.10	0.62	0.47	0.43-0.58
	Excellent	23	27	50					

CI: Confidence interval

Table 24. Relationship between the level of attitude of the handlers, their sociodemographic characteristics, and their level of knowledge in Kenitra slaughterhouse achevied on March 2021 in Morocco

Variables		Level of knowledge		No.	X^2	P-value	Prevalence Ratio	Odds Ratio	CI
		Acceptable	Excellent						
Slaughter area	Cattle + Sheep	22	38	60	0.15	0.70	0.73	0.58	0.41-0.59
	Cleaning Trips + Transport of carcasses	1	1	2					
Age	Young people (18 – 40)	15	16	31	1.06	0.30	1.36	1.70	0.42-0.60
	Age 40 - 60	11	20	31					
Level of education	Low: Informal or primary	13	15	28	0.17	0.68	1.13	1.24	0.42-0.61
	High: middle school, high school, or university	14	20	34					
Work experience	Low period (0-4 years)	5	4	9	0.24	0.62	0.87	0.70	0.41-0.59
	High period (4-more than 4 years)	34	19	53					
Training	Yes	0	0	0	-	-	-	-	0.41-0.59
	No	22	40	62					
Knowledge	Acceptable	10	21	31	3.21	0.07	0.59	0.39	0.42-0.61
	Excellent	17	14	31					

CI: Confidence interval

Table 25. Relationship between the level of hygiene practices of handlers and their sociodemographic characteristics in Rabat slaughterhouse achieved on January 2021 in Morocco

Variables		Level of knowledge		No.	X^2	P-value	Prevalence Ratio	Odds Ratio	CI
		Acceptable	Excellent						
Slaughter area	Cattle + Sheep	11	50	61	11.55	0.01	0.31	0.16	0.39-0.53
	Cleaning Trips + Transport of carcasses	11	8	19					
Age	Young people (18-40)	12	34	46	1.34	0.25	0.68	0.57	0.40-0.55
	Age 40-60	13	21	34					
Level of education	Low: Informal or primary	14	24	38	3.17	0.07	1.93	2.48	0.39-0.53
	High: middle school, high school, or university	8	34	42					
Work experience	Low period (0-4 years)	1	6	7	0.67	0.41	0.50	0.41	0.39-0.53
	High period (4-more than 4 years)	21	52	73					

CI: Confidence interval

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Table 26. Relationship between the level of hygienic practices of handlers and their sociodemographic characteristics in Sale slaughterhouse achevied on January in Morcco

Variables		Level of knowledge		No.	X^2	P-value	Prevalence Ratio	Odds Ratio	CI
		Acceptable	Excellent						
Slaughter area	Cattle + Sheep	14	24	38	0.14	0.71	0.74	0.58	0.40-0.63
	Cleaning Trips + Transport of carcasses	1	1	2					
Age	Young people (18 – 40)	8	14	22	0.03	0.87	0.94	0.90	0.40-0.63
	Age 40 - 60	7	11	18					
Level of education	Low: Informal or primary	4	12	16	1.78	0.18	0.55	0.39	0.40-0.63
	High: middle school, high school, or university	11	13	24					
Work experience	Low period (0-4 years)	0	0	0	-	-	-	-	0.40-0.63
	High period (4-more than 4 years)	15	25	40					

CI: Confidence interval

Table 27. Relationship between the level of hygiene practices of handlers and their sociodemographic characteristics in Meknes slaughterhouse achieved on March 2021 in Morocco

Variables		Level of knowledge		No.	X^2	P-value	Prevalence Ratio	Odds Ratio	CI
		Acceptable	Excellent						
Slaughter area	Cattle + Sheep	39	36	75	0.23	0.63	0.87	0.72	0.44-0.59
	Cleaning Trips + Transport of carcasses	6	4	10					
Age	Young people (18-40)	32	22	54	2.37	0.12	1.41	2.01	0.44-0.59
	Age 40-60	13	18	31					
Level of education	Low: Informal or primary	24	19	43	0.29	0.59	1.12	1.26	0.44-0.59
	High: middle school, high school, or university	21	21	42					
Work experience	Low period (0-4 years)	4	6	10	0.76	0.38	0.73	0.55	0.44-0.59
	High period (4-more than 4 years)	41	34	75					

CI: Confidence interval

Table 28. Relationship between the level of hygiene practices of handlers and sociodemographic characteristics in Kenitra slaughterhouse achieved on March 2021 in Morocco

Variables		Level of knowledge		No.	X^2	P-value	Prevalence Ratio	Odds Ratio	CI
		Acceptable	Excellent						
Slaughter area	Cattle + Sheep	16	44	60	0.53	0.47	0.53	0.36	0.38-0.55
	Cleaning Trips + Transport of carcasses	1	1	2					
Age	Young people (18 – 40)	11	20	31	1.25	0.26	1.57	1.89	0.39-0.56
	Age 40 - 60	7	24	31					
Level of education	Low: Informal or primary	9	19	28	0.54	0.46	0.78	0.68	0.41-0.59
	High: middle school, high school, or university	20	33	53					
Work experience	Low period (0-4 years)	2	7	9	0.81	0.37	0.59	0.47	0.41-0.59
	High period (4-more than 4 years)	41	34	75					

CI: Confidence interval

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DISCUSSION

This study provided valuable information on the knowledge, attitudes, and practices of handlers regarding hygiene and meat handling in four municipal slaughterhouses in Morocco. The information collected on workers' knowledge of food safety highlighted the microbiological risks of contamination of carcasses, the importance of refrigeration and personal hygiene, and the risks related to foodborne diseases. Regarding handler attitudes, the study identified the handler's personal hygiene, cleaning of surfaces used during the slaughter process, and standard cleaning of equipment used. Considering slaughter practices, topics related to hygiene and wearing personal protective equipment during work should be focused. Several studies have reported that handlers working in meat processing factory directly impact foodborne disease outbreaks (Howes et al., 1996; Clayton et al., 2002). The results of this study showed adequate knowledge and practices, while attitudes were very satisfactory. In the four slaughterhouses, almost all respondents were aware of the microbiological contamination of carcasses and the risks associated with consuming contaminated meat. At the same time, they were not aware of the modes of disease transmission as most of them believed that working with diarrhoeal syndrome or using water from cleaning pipes during slaughter did not have any contamination risk. This is in agreement with the study by Matchawe et al. (2019), who reported that 43.1% of respondents in the slaughterhouse of Yaoundé, Cameroon, stated that disinfecting facilities were not important in reducing the risk of carcass contamination.

For meat refrigeration, 75% and 82% of respondents in the slaughterhouses of sale and Meknes did not have enough information, respectively. In Rabat and Kenitra, most respondents were also conscious of the importance of refrigeration in storing and reducing meat contamination. A study conducted in Turkey by Baş et al. (2006) showed that 63.1% of the respondents knew the importance of the refrigerator temperature to minimize food safety risks since temperature handling is often the critical control point in a production process.

Misunderstanding temperature can impede the implementation of any Hazard Analysis Critical Control Point (HACCP) policy (Walker et al., 2003). The results of a study conducted in Turkey by Baş et al. (2006) suggested that 63.1% of the surveyed individuals were aware of the importance of temperature control in minimizing food safety risks. Walker et al. (2003) highlighted that a lack of knowledge regarding temperature handling in a production process could be a major obstacle in implementing any Hazard Analysis Critical Control Point (HACCP) policy.

The results dealing with the attitudes of meat handlers were generally standard. According to Abdullah Sani and Siow (2014) and Al-Shabib et al. (2016), attitude is a critical factor that can impact the food safety behavior and practices of food handlers by reducing the incidence of foodborne illness. Respondents in the four slaughterhouses surveyed were aware of general hygiene measures, such as mandatory hand washing with disinfectant after using the toilet, disinfection of slaughter areas, as well as the prohibition of the deposit of offal on the floor. In contrast, the majority of respondents failed to answer the question correctly. Handlers believed that working with lesions on their hands does not pose a risk of carcass contamination. In contrast to the present study, Zanin et al. (2015) identified that nearly 85% of their staff were conscious of the risk of touching food with cuts on their hands or fingers. The Food and Drug Administration (FDA) (2017) reported that food handlers with illnesses or cuts and injuries on their hands had to stop work immediately and leave the workplace to prevent the transmission of foodborne illness. In Rabat and Sale, food handlers found it unnecessary to wear personal protective equipment. Similarly, respondents in Sale and Kenitra believed that health checks were not required before hiring. This is in disagreement with Ansari-Lari et al. (2010), where 93.8% of the handlers at the meat processing factory in Fars, Iran, found that wearing personal protective equipment was an indispensable act to avoid carcass contamination.

According to Zanin et al. (2017), attitude plays a critical role in slaughter practices, as there is a key link between knowledge and practice; workers with an acceptable level of knowledge are more likely to translate it into practice if they have a positive attitude. Hand washing before each manipulation and after each use of the toilet by the majority of handlers remained among the most encouraging results. Similar results have been reported in previous studies by Al-Kandari et al. (2019), showing that 99.5% of respondents washed their hands before and during food handling. Moreover, lack of hand hygiene is a very important risk factor in food poisoning, according to the Codex Alimentarius Commission (2003). In the present study, 62.5%, 25%, 90.6%, and 69.4% of respondents in Rabat, Salé, Meknes, and Kenitra, respectively, reported direct contact with bare hands with the wall, floor, and equipment during slaughter operations. A previous study by Al-Kandari et al. (2019) stated that 51.7% of the handlers used bare-hand contact with ready-to-eat foods. The lack of cleaning devices in the workplace, the absence of training and awareness programs, and the low level of education of the participants in this study may explain this.

A significant positive correlation existed between the handlers' knowledge and their education level in all studied slaughterhouses. This is consistent with the work of Ansari-Lari et al. (2010), Martins et al. (2012), and Abdul-Mutalib et al. (2012). The correlation of attitudes with sociodemographic factors varied in each slaughterhouse. A positive correlation between attitudes and knowledge was observed in Meknes and Kenitra. Similarly, there was a positive correlation between attitudes, education level, and slaughter area in Rabat.

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The results of the correlation between practices and sociodemographic factors are presented in tables 15-19. A positive correlation was reported in the slaughterhouse of Rabat between the practices and the slaughter area, as well as with the education level in Meknes. A positive correlation was detected with the age of the meat handlers in Meknes. In Salé and Kenitra, the obtained results were similar to these of Matchawe et al. (2019), who reported no correlation between practices and sociodemographic factors. Effective education of workers about food safety can minimize the risk of bacteriological contamination throughout the food chain. Foodborne illness cannot be reduced without food handling procedures and knowledge of food safety practices (Salih et al., 2019). Bahir et al. (2022) showed that meat could easily be contaminated during the slaughtering process by a low level of knowledge and poor practices of handlers, as well as the high bacterial loads and some pathogenic bacteria isolated from most of the studied surfaces in slaughterhouses of Marrakech, Morocco.

CONCLUSION

This study complemented previous research compiled by the current study research team. The work constituted an inventory of the red meat slaughterhouses in Morocco to get a maximum of information on the slaughtering sector in the country. The present study indicated that training and awareness programs for handlers on health risks and hygienic handling of meat must be strengthened to improve food safety in the country. The obtained results showed that while the level of KAP is satisfactory in the four studied slaughterhouses, other hygienic aspects need to be highlighted. Also, sociodemographic factors can directly impact the handlers' knowledge, attitudes, and practices. Microbiological studies should complement the studies to link the level of awareness of handlers on good hygiene practices with the risks and the dissemination of pathogens on slaughter surfaces. The authors of the current study recommended this type of research to contribute to the understanding of the current situation of handlers in slaughterhouses in Morocco and to implement authorities to take actions to reduce the occurrence of toxic infections among consumers.

DECLARATIONS

Data availability and materials

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

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Consent to publish

Not applicable in this section.

Authors' contribution

Mohammed amine Bahir and Ikram Errachidi collected samples used in this Survey. Mohammed amine Bahir and Mohamed Alami contributed data analysis. Mohammed amine Bahir, Asmaa Tantane, and Bouchaib Sarhnae wrote the original draft. Bouchra Belkadi and Abdelkarim Filali-Maltouf revised and edited the draft and generated the final version of the manuscript. All authors contributed to the article and approved the submitted version.

Competing interests

All authors confirm that there are no conflicts of interest associated with this publication and that this work received no financial support.

Ethical consideration

Ethical considerations (including plagiarism, consent to publish, misconduct, fabrication and/or falsification of data, dual publication and/or submission, and redundancy) were checked by all authors.

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The Effect of Sausage Tree Fruit (*Kigelia africana*) on Gonadal Development and Growth Performance of *Oreochromis andersonii*

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ABSTRACT

In Zambia fish farms, *Oreochromis andersonii* is an important common indigenous fish species. Naturally, safe phytochemicals can effectively improve fish reproduction performance and their production potential. Therefore, this study was conducted to determine the effect of *Kigelia africana* on the gonadal development and the performance of *Oreochromis andersonii*. A total of 96 male fingerlings were randomly assigned to four dietary treatments (D1-D4), and each treatment group had three replicates. The D1, D2, D3, and D4 groups were formulated to receive 0, 50, 100, and 150 g of powdered *Kigelia africana*/kg, respectively. The fish were fed the diets for 9 weeks, followed by the study parameter measurements at the end of the experiment. The highest mean body weight and gonadal weight were (29.8 ± 0.63 and 0.09 ± 0.010 g, respectively) for fish in the D2 group. There was no significant difference between the mean body weight of fish in different groups, but their mean gonadal weights differed significantly. The gonadal somatic index of fish differed significantly among treatment groups, with those in D2 having the highest mean value (0.36 ± 0.060). The highest mean standard length (103.3 ± 0.63 mm) and total length (126.0 ± 0.11 mm) of fish were observed for D1 and D2 groups, respectively. Additionally, the mean values for those parameters decreased with increasing *Kigelia africana* in the diet. The physicochemical parameters of water, including temperature and dissolved oxygen, ranged 16.8-23.1℃ and 0.6-2.2 mg/L, respectively; these were generally at low levels considering the optimum requirements for this fish species. In conclusion, *Kigelia africana* improved gonadal growth and development but did not promote overall fish growth. The best gonadal growth/development results of *Kigelia africana* powder were observed at a level of 50 g/kg, which can be used as a performance booster in the aquaculture production of *Oreochromis andersonii*.

Keywords: Aquaculture, Gonadal development, Growth, *Kigelia africana*, *Oreochromis andersonii*, Sausage tree

INTRODUCTION

Zambia is endowed with 12 million hectares of water bodies and 8 million hectares of wetlands (FAO, 2021) that can support the production of enough fish for consumption and export. Fish is a crucial source of income, food, and nutrition in Zambia; however, the estimated annual deficit of 108,000 tons of fish and fish products necessitates strategies to increase production (Genschick et al., 2017; FAO, 2021). Even with over 70% contribution of captured fish to the national catch, the existing supply shortfall presents an opportunity for aquaculture to flourish (Genschick et al., 2017; Avadí et al., 2022). Although Zambia presently ranks fifth in aquaculture production in Africa, with a 1.11% contribution to the regional share (Adeleke et al., 2021), there is a dire need to increase production to reduce the demand-supply gap. Additionally, this gap is projected to increase further by 2030, with a possibility of import as the primary contributor to fish supply for local consumption (Tran et al., 2019).

It is noteworthy that increased aquaculture production to reduce the fish demand-supply gap requires innovations in science and technology to improve the existing aquaculture production techniques (Maulu et al., 2019; Kaminski et al., 2022). Many factors constrain aquaculture production, inter alia, the cost of fingerlings and feed, the quality and quantity of fingerlings, diseases, and inadequate extension (Adeleke et al., 2021). The insufficient quantities of fish have been attributed to the low survival rate, low fecundity, and high mortality rate of hatchlings, especially among small-scale farmers (Adeleke et al., 2021; Avadí et al., 2022). The inadequacy in the quantity and quality of fingerlings is a concern that necessitates urgent interventions at every stage of fingerling production. Some crucial stages of fingerling production include induced egg spawning and milt production, egg fertilization, incubation, hatching, and nursery management (Emeka et al., 2014). Additionally, controlled production through the application of natural or synthetic substances, such as hormones and growth promoters, has been one of the interventions used to increase the

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quality/quantity of fingerlings (Emeka et al., 2014). These substances manipulate the sex, productive viability, and growth of fish.

Many synthetic drugs, including hormones (like 17α-methyl testosterone), and growth promoters, such as oxytetracycline, have improved fish production. However, the growing fears over drug/chemical residues in animal/fish products, which affect human health and cause antimicrobial resistance, have attracted attention to natural phytochemicals (Manosroi et al., 2004; Reda et al., 2013; Emeka et al., 2014; Abaho et al., 2021). Consequently, many beneficial phytochemicals have been evaluated and developed into drugs with little or no side effects (Adedeji et al., 2006; Emeka et al., 2014). In an effort to enhance production, medicinal plants, such as *Gercinia kola*, *Kigelia africana (K. africana)*, and *Macuna pruriens* have been experimented on fish, and the results revealed improved performance (Musthafa et al., 2018; Abaho et al., 2021). In particular, *K. africana*, an aphrodisiac, is reported to increase testosterone and enhance fertility and has been used to manage infertility, poor libido, and sexual asthenia in men (Nabatanzi et al., 2020). However, despite the reports of *K. africana* use in men, there is still a paucity of knowledge about its potential effects on fish biology. Hence, it is necessary to explore its potential role in influencing the reproductive and productive characteristics of farmed fish in Zambia.

Furthermore, Adeleke et al. (2021) found that many countries are currently focusing on indigenous catfish and tilapia species for aquaculture production. Similarly, Avadí et al. (2022) and Genschick et al. (2017) reported that Zambia is among the largest producers of tilapia fish, of which *Oreochromis andersonii (O. andersonii)* is regarded as the tastiest and preferred species by consumers in Zambia (Malumbe and Musuka, 2013). There have been deliberate calls for promoting *O. andersonii* production and establishing gene banks for its conservation (Kefi and Mwango, 2018). Despite the urgent need for improved productivity and/or production of tilapia species, particularly *O. andersonii* (Genschick et al., 2017; Kefi and Mwango, 2018), few studies have ventured into the manipulation of their reproduction to improve productivity through the application of safe phytochemicals. Hence, this study could contribute to the knowledge addressing the potential of *K. africana* for productivity improvement of *O. andersonii*. The objectives were to determine the gonadal somatic index of *O. andersonii* fed on different levels of dried *K. africana* fruit and assess the influence of *K. africana*-containing diet on the growth performance of *O. andersonii.*

MATERIALS AND METHODS

Ethical approval

This study was conducted with strict and routine supervision by the institutional committee on animal research, The University of Zambia. Additionally, fish handling and experimentation were performed in compliance with the guide for the care and use of agricultural animals in research and teaching (CCAC, 2005).

Study area

The research was carried out at the Chilanga fish farm, Lusaka, Zambia, from July to September 2021. The study area is located at latitude 15° 33’ 51’ 'S, longitude 28° 16’ 13’ ‘E, and altitude of 1205 meters above sea level. Chilanga fish farm is a government-owned farm under the Department of Fisheries, Ministry of Fisheries and Livestock, Lusaka, Zambia. Furthermore, the average annual precipitation in Zambia ranges 800-1400 mm, while the temperature during winter is within the range of 10-20℃, and during the hot, dry season, it ranges 20-30℃ (RCCC, 2021).

Fingerling collection and feed formulation

Oreochromis andersonii fingerlings were sourced from Kalimba farms in Lusaka, Zambia. The procured fish comprised an all-male population. Up on delivery, a few sample fish were weighed. Their average body weight, standard length, and total length were 28.7 ± 1.4 g, 9.01 mm, and 12.24 mm, respectively. All the fingerlings were then conditioned for 10 days in plastic fish tanks when fed on the formulated diet without *K. africana*. Fresh *K. africana* fruits were sourced from Milambo farms in Barlastone park, Lusaka, Zambia. Before the diet formulation, these fruits were washed using clean water, cut into smaller pieces, sun-dried, and ground into powder using a locally fabricated pounding machine (Zambia), and this was performed following Vipinkumar et al. (2019).

Experimental diets were formulated to contain 32% crude protein using maize meal (11.39% crude protein) and low-fat soya bean meal (44.76% crude protein) for each treatment. Diet formulation was done manually using the simultaneous equation method and algebraic expression (Afolayan and Afolayan, 2008). Then, all the ingredients were accurately measured using a digital scale (Sartorius, Lab Instruments GMbH and Co. KG, Gottingen, Germany) with a precision of 0.1 g, for processing. The processing procedure was performed following Vipinkumar et al. (2019). After measurements, the ingredients were accordingly mixed in a clean bucket. The resultant mash was pressed through an artisanal mincer for pelleting. Additionally, cassava flour (One Banja Co. Ltd, Lusaka, Zambia) was added as a binder. All the pelleted diets were air-dried at room temperature and kept frozen until the start of the experiment. The feed

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ingredients used, their corresponding proportions, and the composition of different dietary groups are summarized in Table 1.

Table 1. Ingredients in the experimental diets of tilapia (*O. andersonii)* fingerlings

Variables	**Experimental diets**			
Feed ingredients (kg)	**D1**	**D2**	**D3**	**D4**
Maize meal	1.88	1.88	1.88	1.88
Soya bean meal	2.94	2.94	2.94	2.94
Premix*	0.03	0.03	0.03	0.03
Methionine	0.04	0.04	0.04	0.04
Cassava (binder)	0.01	0.01	0.01	0.01
DCP	0.019	0.019	0.019	0.019
Vegetable oil	0.04	0.04	0.04	0.04
Salt	0.01	0.01	0.01	0.01
K. africana (g/kg)	0	50	100	150
****Chemical composition (DM %)**				
Crude protein	34.40	32.40	33.03	34.01
Ash	6.28	5.59	5.08	5.66
Moisture	7.14	7.94	6.65	6.14
Crude fat	4.44	4.45	4.94	4.35
Crude fiber	5.33	8.15	8.20	8.38
Metabolizable energy	3.28	3.27	3.34	3.51

DCP: DiCalcium Phosphate, *K. Africana: Kigelia africana*, Vit: Vitamin, *the composition per kg of premix: Vit. A: 4,000,000 I.U, vit. B1: 1,000 mg, vit. B2: 2,400 mg, vit. B6: 2,000 mg, vit. B12: 2 mg, folic acid: 400 mg, Niacin: 15,000 mg, vit. C: 10,000 mg, vit. D3: 400,000 mg, vit. E: 40,000 mg, vit. K3: 5,000 mg, Biotin: 25 mg, vit. B5: 4,000 mg, lysine: 2,800 mg, zinc: 6,000 mg, iron: 6,000 mg, selenium: 30 mg, copper: 1,000 mg, manganese: 10,000 mg, iodine: 250 mg, D1: 0 g/kg, D2: 50 g/kg, D3: 100 g/kg, and D4: 150 g/kg, **Determined based on standard methods (AOAC, 1995).

Study design and sampling

This study employed a completely randomized design and a positivism paradigm to generate data on the effect of *K. africana* on gonadal growth and development in fish. A total of 12 plastic fish tanks measuring 6 mm thick, with dimensions of 55 cm in length, 40 cm in width, and 33 cm in height, were used, and each tank contained plastic plates for holding the feed. The tanks were placed on 6-inch concrete bricks in a pond and filled with borehole water to a volume of 50 liters each. A total of 96 male fingerlings with an average weight of 28.7 ± 1.4 g were used for this study. These were randomly assigned to 12 tanks, with each tank containing eight fingerlings. In this case, four treatment groups, each with three replications (three tanks), were used. The treatment groups included control without supplementation of *K. africana* (Group D1), Group D2 (50 g/Kg *K. africana*), Group D3 (100 g/kg *K. africana*), and Group D4 (150 g/kg *K. africana*). The formulated diets were then assigned randomly to the tanks, and each group of fish was fed at 5% body weight/day in two equal portions in the morning (10:00-10:30 hours) and afternoon (15:00-15:30 hours) for 60 days. Every after feeding, a netting material with appropriate holes to allow aeration was used to prevent predation from birds and other predators. Furthermore, the tanks were thoroughly cleaned once a fortnight with water and clean burlap-like materials from gunny sacks.

Physico-chemical quality of water

During fish feeding experimentation with the treatment diets, the temperatures and dissolved oxygen were monitored daily, and their average values were obtained each week. These parameters were measured with a water quality checker (ProDSS, Miami, USA) according to the manufacturer's specifications. Data were recorded in ℃ and ml/L for temperatures and dissolved oxygen, respectively.

Body length and weight measurements

The standard and total lengths of *O. andersonii* were measured at the end of the experiment using a one-meter measuring board with a precision of 0.1 mm, as previously described by Önsoy et al. (2011). Every fortnight, samples were randomly weighed using a digital scale (AND HR200 Lab analytical balance, USA) with a precision of 0.0001 g to monitor the changes. At the end of the experiment, each fish was sacrificed by the pitching method using a needle and then wiped using a napkin before taking weight measurements. The body and gonadal weights were then measured following the previously described procedure (Yadav et al., 2016). The gonadal weights were obtained following body weight measurements. This involved dissecting fish to obtain the gonads and weighing using a digital weighing scale (AND HR200 Lab analytical balance, USA) with a precision of 0.0001 g.

Gonadal somatic index

The mean weights of the fish and gonads were used to compute the Gonadal somatic index (GSI) based on a formula (Sturm, 1978);

$$\text{Gonadal somatic index} = \frac{\text{weight of gonads}}{\text{weight of fish}} \text{ x } 100$$

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Gonadal development

At the end of the experiment, the gonadal maturity stages of the fish were determined based on macroscopic characteristics, including the gonadal color and condition and gonadal morphometric characteristics, as described by Kefi et al. (2012). The gonads were then classified into stages according to the previously described procedure (Nikolsky, 1963) that classify gonadal maturity into six stages, namely immature (Stage I), quiescent (Stage II), maturing (Stage III), mature (Stage IV), running (Stage V), and spent (Stage VI).

Data analysis

In the Statistical Analysis System Software package (SAS institute, 2004), data were analyzed using descriptive statistics, including means and standard errors of means (SE). The various fish traits, namely, body length, body weight, gonadal weight, and GSI, were analyzed by ANOVA test using the General Linear Model, a univariate analysis procedure. The considered model was; $X_{ijk} = u + A_i + B_j + AB_{ij} + e_{ijk}$

Where, X is the dependent variable representing the value of the measured trait, u denotes the overall mean, A_i refers to the effect of treatment (diet) groups with four levels (i = D1, D2, D3, and D4), B_j signifies the effect of the tank, AB_{ij} is the interaction between the main effects, e_{ijk} defines the random error term. The least-square difference test was used to determine the pairs whose means differed. In all cases, significance was taken at a level of $p < 0.05$. The data for maturity stages were descriptively analyzed using the measure of central tendency (frequencies).

RESULTS

Mean standard length and total length

The mean standard length (SL) and the total length (TL) of *O. andersonii*, fed on the experimental diet containing different amounts of *K. africana* fruit, are presented in Table 2. The highest mean SL (103.3 ± 0.09 mm) was observed for fish in the D1 group, while those in the D4 group had the lowest mean SL (91.71 ± 0.12 mm). Generally, fish in the D2 treatment had the highest mean TL (126.0 ± 0.11 mm), while D3 treatment had the lowest mean TL (122.8 ± 0.14 mm). The results revealed significant differences among the treatment groups in terms of the mean SL of fish ($p < 0.05$). The results revealed significant differences between the mean SL of fish in the D1, D2 with D3 and D4 groups ($p < 0.05$). Furthermore, there was no significant difference among the four groups with regard to the mean TL of fish ($p > 0.05$).

Mean weight and gonadal somatic index

The mean body weight, gonadal weight, and GSI of *O. andersonii*, fed on different experimental diets, are presented in Table 3. The *O. andersonii* in the D2 group had the highest mean body weight (29.8 ± 0.63 g), while those in the D3 group had the lowest mean body weight (27.4 ± 0.72 g). The mean fish gonadal weight from group D2 was numerically higher (0.09 ± 0.010 g) than other treatment groups. The *O. andersonii* in the D2 group had the highest GSI (0.36 ± 0.060 g), while those in the D1 group had the lowest mean GSI value (0.14 ± 0.032). The results indicated no significant difference among the treatment groups in terms of the mean body weight of *O. andersonii* ($p > 0.05$). The mean gonadal weight of *O. andersonii* differed significantly among dietary treatments ($p < 0.05$). Additionally, the results showed significant differences in the GSI of *O. andersonii* when dietary treatments were compared ($p < 0.05$). Regarding the mean gonadal weight of *O. andersonii*, the current study results revealed significant differences among D2, D3 with D1 and D4 ($p < 0.05$). The results showed no significant difference between the gonadal weight *O. andersonii* in the D2 and D3 groups ($p > 0.05$). The results also indicated that the GSI of *O. andersonii* in the D2 and D3 groups were significantly higher than the GSI of those in the D1 and D4 groups ($p < 0.05$). The results showed no significant difference between the GSI of *O. andersonii* in the D2 and D3 groups ($p > 0.05$).

Maturity status of the gonads from *Oreochromis andersonii*

The maturity status and occurrence frequency of gonads among *O. andersonii* fed with the different levels of *K. africana* are presented in Table 4. The gonads were classified as immature, active, or quiescent stages of development within each dietary treatment. The highest proportion of fish (53.33%) in the D1 group had quiescent gonads, while no fish in the same group had immature gonads. Most fish (56.25%) in the D2 group had active gonads, while a few (6.25%) in the same group had immature gonads. The majority of fish (60%) in the D3 group had active gonads, whereas 6.67% of the same group had immature gonads. Regarding the D4 group, the highest proportion (55.56%) had quiescent gonads, while 11.11% of *O. andersonii* had immature gonads.

Levels of dissolved oxygen in fish tanks

The average levels of dissolved oxygen (mg/L) in a week for all tanks are presented in Figure 1. For the morning measurements, the highest average level of dissolved oxygen (2.2 mg/L) in the tanks was observed in week 9, while the

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lowest level (0.6 mg/L) was in weeks 1, 4, and 6. Regarding the afternoon measurements, the highest average level of dissolved oxygen (2.8 mg/L) was recorded in week 2, whereas the lowest average value (0.7 mg/L) was noted in week 1.

Average temperature variations

The average weekly temperature readings for all tanks are presented in Figure 2. Regarding the morning readings, the highest average temperature (21.1℃) in the tanks was recorded in week 9, while the lowest average temperature value (16.8℃) was in week 2. With regard to the afternoon measurements, the highest average temperature value (23.1℃) was noted in week 8, whereas the lowest average temperature (17.9℃) was in week 4.

Table 2. The mean standard length and total length of *Oreochromis andersonii*, fed with different levels of *Kigelia africana* powder

Variables \ Dietary treatments	D1	D2	D3	D4
SL (mm)	103.3 ± 0.09[a]	102.9 ± 0.10[a]	98.0 ± 0.12[b]	91.71 ± 0.12[b]
TL (mm)	125.6 ± 0.10[a]	126.0 ± 0.11[a]	122.8 ± 0.14[a]	123.3 ± 0.15[a]

Values are Mean ± SE, [a, b] means with dissimilar superscripts within a row differ significantly ($p < 0.05$), SL: Standard length of fish, TL: Total length of fish, mm: Millimeters; D1: 0 g/kg, D2: 50 g/kg, D3: 100 g/kg, and D4: 150 g/kg

Table 3. The mean weights and gonadal somatic index of *Oreochromis andersonii* fed with different levels of *Kigelia africana* powder

Variables \ Dietary treatments	D1	D2	D3	D4
Body weight (g)	28.9 ± 0.65[a]	29.8 ± 0.63[a]	27.4 ± 0.72[a]	28.1 ± 0.87[a]
Gonadal weight (g)	0.04 ± 0.008[b]	0.09 ± 0.010[a]	0.08 ± 0.010[a]	0.04 ± 0.008[b]
GSI	0.14 ± 0.032[b]	0.36 ± 0.060[a]	0.33 ± 0.050[a]	0.18 ± 0.030[b]

Values are Mean ± SE, [a, b] means with dissimilar superscripts within a row differ significantly ($p < 0.05$), GSI: Gonadal somatic index, D1: 0 g/kg, D2: 50 g/kg, D3: 100 g/kg, and D4: 150 g/kg

Table 4. Gonadal maturity status among groups of *Oreochromis andersonii* fed with different levels of *Kigelia* powder

Gonadal maturity (stages) \ Dietary treatments	D1	D2	D3	D4
Immature	0 (0)	1 (6.25)	1(6.67)	2 (11.11)
Active	7 (46.67)	9 (56.25)	9 (60.00)	6 (33.33)
Quiescent	8 (53.33)	6 (37.50)	5 (33.33)	10 (55.56)
Total	15 (100)	16 (100)	15 (100)	18 (100)

The total number within dietary treatment was used to obtain percentages of gonads for each treatment, D1: 0 g/kg, D2: 50 g/kg, D3: 100 g/kg, and D4: 150 g/kg

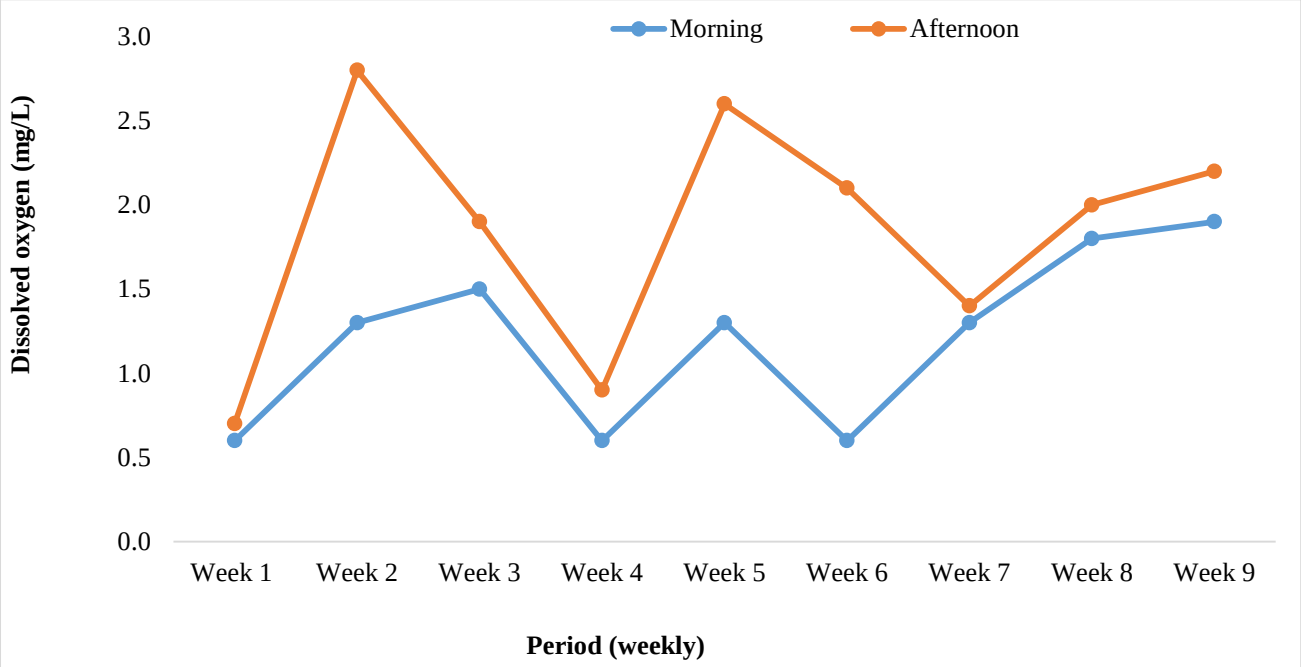

Figure 1. Average levels of dissolved oxygen in water tanks containing *Oreochromis andersonii* fish

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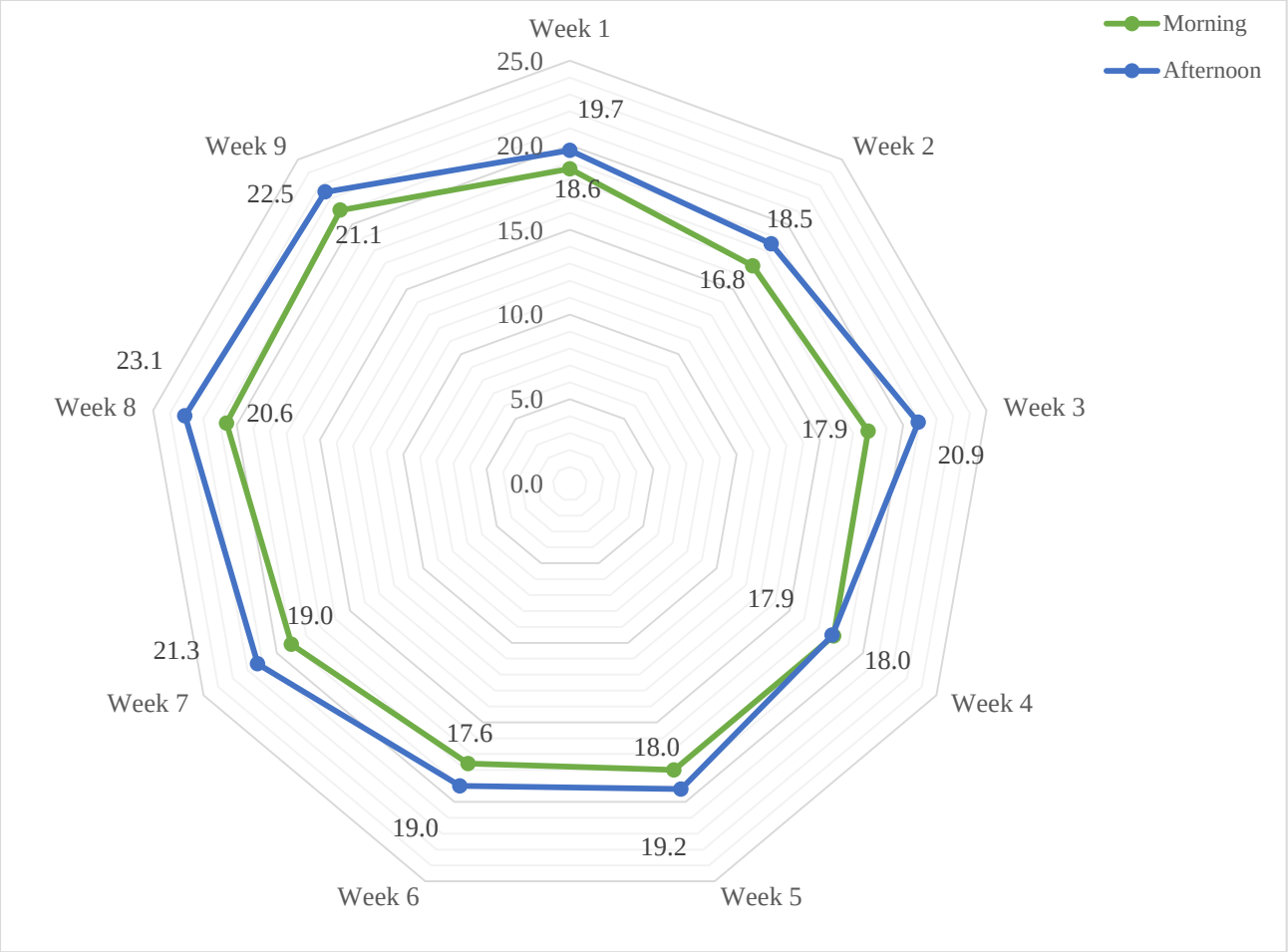

Figure 2. Temperature variations (℃) during the nine weeks of feeding *Oreochromis andersonii* fish with different levels of *Kigelia* powder

DISCUSSION

The results of the current study showed that *K. africana*-containing diet could affect some, but not all, biological parameters of *O. andersonii*. With regard to the SL and TL parameters, the observed higher mean values for *O. andersonii* in the D1 group compared to other treatment groups and the decrease in mean SL and TL of *O. andersonii* followed by an increase in *K. africana* levels indicated that increasing *K. africana* level did not significantly improve the SL and TL of male *O. andersonii*. Of note, the rate of fish growth can appropriately be identified through the rise in body weight and length (Awas et al., 2020). Thus, the inclusion of *K. africana*, at the levels used in the current study did not increase the growth of male *O. andersonii*. Chivandi et al. (2011) recommended *K. africana* as potential supplement for fish growth due to its substantial proportions of nutrients, such as lipid (49.2%) and crude protein (35.7%). Nevertheless, such nutrients can be directed towards gonadal development instead of overall growth since fish can prioritize the available resources for various body functions, such as basal metabolism, movement, immune system, growth, and reproduction (Salze et al., 2013). Furthermore, the contribution of other factors, such as the pharmacological dose, fish growth phase, feed availability and quality, and the general fish condition to the SL and TL cannot be underestimated (Önsoy et al., 2011).

The present results revealed no significant differences in the mean body weight of fish, which is consistent with a previous study report on average weights of *Oreochromis niloticus* (*O. niloticus*) in control and *K. africana*-containing diets (Ndour et al., 2021). Besides the resource prioritization strategy, the observed minimal or no growth of *O. andersonii* in the current study could be attributed to the low dissolved oxygen levels in the water tanks. A previous study by Li et al. (2020) indicated an increase in growth rate and weight gain for a closely related fish species, *O. niloticus*, which had a higher dissolved oxygen level (5 mg/L), compared to the current study. Again, another possible reason for the insignificant effect of *K. africana* in the current study was the ambient temperature (16.8-23.1℃), which was generally lower than the recommended optimal range (25-30℃) for the growth of *Oreochromis* species (El-Sherif and El-Feky, 2009). El-Sherif and El-Feky (2009) noted that temperatures lower than optimal reduced feed intake, feed conversion ratio, and weight gain. Accordingly, future studies may be needed to validate the effect of *K. africana*-containing diets on *O. andersonii*, particularly under the recommended temperature and dissolved oxygen levels.

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Although *K. africana*-containing diet did not affect the fish body weight, it could significantly affect their gonadal weights. The current findings disagree with an earlier study by Adeparusi et al. (2010) that reported no significant difference between the weight of testes among the dietary treatments. The observed disparity in the findings was most probably due to age and fish species differences. Nevertheless, the same authors reported a significant effect of *K. africana* on the sperm quality parameters of *Clarias gariepinus*, namely sperm count, motility, and fertilization ability, particularly in the 100 g/kg dietary group (Adeparusi et al., 2010). It is noteworthy that testicular weight is an anatomical indicator of fertility and a marker for reproductive capability, directly proportional to the level of testosterone produced (Banihani, 2018). In light of this, it is plausible that some phytochemicals present in *K. africana* influenced steroid/testosterone production in *O. andersonii*, which in turn promoted the gonadal development as observed in the D2 group. However, these phytochemicals perhaps possessed a dose-dependent characteristic in their pharmacological actions considering the observed lower gonadal weights of fish in the D3 and D4 groups.

Most earlier studies reported the presence of phytochemicals in *K. africana*, such as β-sitosterol, stigmasterol, Zinc, and vitamin E that are associated with enhanced reproductive performance (Chivandi et al., 2011; Oseni and Williams, 2018; Fagbohun et al., 2019; Nabatanzi et al., 2020). For example, stigmasterol stimulated gonadal development in *O. nilotucus*, similar to androgen hormones (Yusuf et al., 2019). On the other hand, some phytosterols may also be precursors for the de novo biosynthesis of steroid hormones (Tarkowská, 2019). For instance, sitosterol is a precursor for plant steroid hormones like brassinosteroids, as well as the animal steroids hormones like progesterone and testosterone and its derivatives (Tarkowská, 2019). Zinc influences the male reproductive system through the gonadotropic hormones, and is also required to convert testosterone into its active form, called dihydrotestosterone (Oseni and Williams, 2018; Baltaci et al., 2019). Furthermore, vitamin E was confirmed to improve gonadal development (Pamungkas et al., 2014). It is required for the synthesis and excretion of gonadotrophic hormones and also improves sperm quality through its antioxidant activity (Rajesh and Mendon, 2001; Chivandi et al., 2011; El-Sayed and Izquierdo, 2021). Considering the presence of these phytochemicals and the aforementioned roles, the observed gonadal weight increment in the current study could be ascribed to the use of *K. africana* in the trial diets.

One of the important parameters in reproductive biology is GSI, a means used to measure the sexual maturity of animals, including fish, in correlation to the ovary or testes development (Kiran, 2015). According to Yadav et al. (2016), this parameter is considered during the breeding mechanism and seed production; thus, assessment of the GSI, including *O. andersonii*, contributes to understanding the fish breeding mechanism for aquaculture production and fish conservation. The current findings agree with an earlier study that confirmed the GSI increment with gonadal development and maturation of male fish (Yadav et al., 2016). Based on the current GSI findings, it can be concluded that *K. africana* could be used to improve the development of *O. andersonii* gonads. It is noteworthy that the beneficial effects are significant at lower pharmacological doses considering the observed mean gonadal weight, GSI, and the proportion of active gonads of *O. andersonii* in the D2 group. The *K. africana* at lower doses (50 g/kg), as used in D2, could probably lead to better results. So far, this study has confirmed that increasing *K. africana* levels results in higher proportions of immature gonads in *O. andersonii*.

CONCLUSION

Oreochromis andersonii remains one of the important native fish species suitable for aquaculture production, and has the potential to improve the livelihoods of small-scale farmers in Zambia. However, the full productivity, production potential, and benefits of *O. andersonii* will only be harnessed by manipulating its reproductive performance. The current study has found that *K. africana* fruit can enhance the gonadal development and maturity of *O. andersonii* fingerlings. Additionally, the development and maturity of gonads were significantly enhanced by the dietary treatment containing 50 g/kg of *K. africana*. However, the plant was not found to enhance overall fish growth at the inclusion levels studied. It is recommended that further studies be conducted using the *K. africana* fruit at levels lower than 50 g/kg to investigate further the observed beneficial effects of this plant. A larger sample size is recommended in future studies to replicate the current study.

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Availability of data and materials

The data from the present study are available on request from the corresponding author.

Competing interests

Authors declare no conflict of interest regarding this publication.

Authors' contribution

Pharaoh Collins Sianangama conceived, designed, supervised the study, and reviewed the manuscript, Emeldah Nundwe designed the study and collected the data. Sylvia Jana Harrison and Eva Nambeye supervised the study and reviewed the manuscript. Rubaijaniza Abigaba analyzed data and wrote the manuscript. All authors read and approved the final manuscript for publication.

Ethical consideration

The authors declare that this manuscript is original and is not being considered elsewhere for publication. All authors have consented to publish it in this journal.

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The Relationship between Warm Weather and Milk Yield in Holstein Cows

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ABSTRACT

The increasing variability of weather conditions associated with global climate change is becoming a major problem for dairy farming. The present article provided the results of studies on the relationship between the milk production of Holstein cows and environmental parameters during the warm season. The study investigated whether the relationship between weather conditions (air temperature, relative humidity, wind direction, wind strength, and insolation) and daily milk yield, as well as its components (milk fat yield and milk protein), depended on the conditions comfortable for the cows. The temperature-humidity index was calculated based on air temperature and relative humidity data, which were recorded by the nearest weather station to the farm, which is subordinate to the Ukrainian Hydrometeorological Center. It was found that the relationship between environmental parameters and milk yield was weak concerning the increase in proportion to the growth of heat load. However, the factorial analysis indicated that the total influence of weather factors on milk yield, milk fat, and protein yield was 42-46%. Moreover, weather conditions could significantly impact dairy productivity when cows are kept in naturally ventilated barns. This suggests further investigation of issues related to the microclimate improvement in cowsheds in hot seasons using sprinkler systems for cooling dairy cows.

Keywords: Components of milk, Correlation, Cows, Hot weather, Milk yield, Naturally ventilated

INTRODUCTION

The dairy industry is susceptible to global climate change (Smith et al., 2007; Escarcha et al., 2018). In recent years, this has become a challenge for countries with hot climates and European countries with temperate continental climates (Tomczyk et al., 2019). The increasing variability of weather conditions and high summer temperatures lead to a drop in cow milk yields and a deterioration of milk quality as the main raw material for the dairy processing industry (Zazharska et al., 2018; Maggiolino et al., 2020).

An increase in the frequency of thermal stresses in the summer produces noticeable effects on dairy cattle, including various physiological and metabolic disorders (Polsky and von Keyserlingk, 2017; Danchuk et al., 2021). The reduced feed consumption by animals decreases milk yield during persistent heat (Nardone et al., 2010). However, this is only 35% associated with decreased appetite in cows, while the remaining 65% of losses are due to the direct effect of heat stress related to hormonal imbalance, rumen dysfunction, and decreased absorption of nutrients (Rhoads et al., 2009).

Dairy cattle are directly affected by extreme environmental factors when kept year-round in naturally ventilated barns (NVB) without the use of pastures during warm periods of the year (Hempel et al., 2019; Mylostyvyi et al., 2019). Natural ventilation (through the pulled-up blinds and skylights) and mechanical ventilation (using accelerating axial flow fans) during the summer heat cannot provide comfortable conditions for cows. Therefore, their milk yield can be reduced to one liter per head per day (Izhboldina et al., 2020; Mylostyvyi et al., 2021).

Therefore, an investigation into the impact of environmental parameters on dairy cow performance can anticipate the scale of losses in the dairy industry during high summer temperatures (Binsiya et al., 2017). Predictions based on such studies will pursue strategies to mitigate the adverse effects of global warming on milk production promptly (Gunn et al., 2019; Avtaeva et al., 2021).

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Considering the direct impact of environmental factors on the physiological state of productive animals, the comfort conditions for dairy cattle are assessed using particular indices. For example, the temperature and humidity index (THI) based on measurements of air temperature (AT) and relative humidity (RH) has been widely used to determine the severity of heat stress. The THI is informative and easy to calculate (Sejian et al., 2021). According to Rodriguez-Venegas et al. (2022), temperature-humidity index (THI) values classify heat stress in dairy cows as 68-71 THI (light stress), 72-76 THI (moderate stress), 77-79 THI (intense stress), and ≥80 THI (extreme stress). Data on the relationship between the productive qualities of cattle and environmental factors need constant clarification and supplementation under conditions of rapid global warming. On their basis, all new thermal indices are created, which have recently become the focus of many researchers (Mbuthia et al., 2022).

Therefore, this study aimed to determine the relationship between environmental parameters and milk productivity of Holstein cows kept in NVB during the warm season.

MATERIALS AND METHODS

Ethical approval

The study was conducted according to the guidelines of the Declaration of Helsinki and approved by the Commission on Bioethics of the Institute of Biotechnology and Animal Health (protocol No. 5 dated May 29, 2018).

Study design

The study was conducted on dairy cows at one of the largest commercial dairy complexes for breeding Holstein cattle in central Ukraine (48°28′44″ N, 35°36′46″ E). The work involved the study of the relationship between the dairy productivity of cows and environmental parameters for two years (2017, 2018), taking into account only the warm season (May to September).

Keeping, feeding, and recording the milk productivity of cows

Dairy cows were kept in NVB without tethering. These were Holstein cows of medium lactation (90-150 days of lactation) with an average milk yield of 25-26 kg/day. The cows' milk yield and the number of animals (ranging from 700 to 800 cows) did not differ significantly yearly. The barns had a four-row arrangement of cubicles (length of 2.25 m and width of 1.1 m), and sand was used as bedding. The cows received a total mixed ration, including corn silage, alfalfa hay, grain hay, wheat straw, barley grain, oats, and corn. Rapeseed, sunflower, and soybean meal, dried cellulose, and mineral and vitamin supplements were also included in the rations. The rations were balanced for essential nutrients according to the recommendations of the National Research Council (NRC, 2001). Feeding alleys and group water troughs were readily available in the facility. The ration was not changed during the year. The animals were kept indoors during the study without grazing. Information on the dairy productivity of cows at a commercial dairy complex was obtained using the DairyComp 305 herd management system (VAS, USA). Average milk production values for the herd of cows (daily milk yield [DMY]; milk fat [MF]; milk protein [MP]; percentage of milk fat [PMF] and protein [PMP]) were calculated using a standard set of statistical functions, recording the indicators daily after milking.

Recording of weather conditions and systematization of the data obtained

Weather data from the nearest weather station was taken from the website of the Ukrainian Hydrometeorological Center, receiving data from all meteorological stations in the country. The distance from the dairy farm to the meteorological station in Pavlograd (Dnipropetrovsk region) did not exceed 25 km in a straight line. Weather data (insolation conditions [IC], wind direction [WD], wind strength [WS], air temperature [AT], and relative humidity [RH]) were recorded continuously at one-hour intervals. Then, the average value of these indicators for a day was calculated (the data was used to find the Pearson correlation). Various meteorological data were systematized using codes described in more detail in the previously published study (Mylostyvyi and Chernenko, 2019). For example, the coding for wind strength (m/s) proceeded from gradually increasing values of the Beaufort wind strength scale, starting from calm (point 0) to high wind (point 7). A similar principle was employed by assigning numerical values as codes to each environmental parameter. The calculations of the THI (Kibler, 1964) and THI index for NVB (THI_{CHT}), which was tested by Mylostyvyi et al. (2020), were made according to the given equations:

$$THI = 1.8 \times T - (1 - RH/100) \times (T - 14.3) + 32$$

$$THI_{CHT} = 46.00549 + 1.04460 \times T,$$

Where, THI is the temperature-humidity index, THI_{CHT} denotes the temperature-humidity index in a hangar-type barn, T is air temperature (°C), and RH signifies relative humidity (%).

It should be noted that a similar approach to the systematization of data covering the warm period of 2017 has been previously outlined in the form of a data descriptor (Mylostyvyi and Chernenko, 2019). The decision to use this dataset again was driven by the need to combine the 2018 and datasets of the previous year. This is a distinctive feature of this article. The originality of the approach in this study was to determine the extent to which weather factors influence the dairy performance of cows using factorial ANOVA, as well as a new approach to determine the relationship between

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these traits, taking into account the extent to which cows were under heat stress (according to THI value), or they were in comfortable conditions.

Statistical analysis

The mean values (Mean) and standard deviations (SD) were calculated for each of the above weather and cow productivity indicators. Correlation analysis was performed using Spearman's rank correlation coefficient. A factorial ANOVA was used to determine the effect of environmental parameters on the dairy productivity of cows. The percent of exposure (%) of the meteorological factors on the productivity of dairy cows was determined by the method of biometric analysis (Kovalenko et al., 2010) based on the results of ANOVA in the program Statistica 12 (StatSoft, Inc., Tulsa, OK, USA). The difference with values of $p < 0.05$ was considered statistically significant.

RESULTS

Weather conditions during the study period from May 1 to August 31, 2018, are shown in Table 1. Of these 123 days (or 2952 hours) of warm weather, 2662 hours were estimated, representing about 90.2% of the duration of the specified warm period. It was found that clear weather, cloudy, rainy hours, and overcast conditions accounted for 2253 (84.6%), 294 (11.0%), 103 (3.9%), and 12 (0.5%) hours, respectively. May was the month with the most clouds, July was the rainiest, and the largest number of clear days was seen in August.

In some months of the year, the predominant wind was East (E) in May, North-East (NE) in June, and North (N) in July and August. During the warm period, the prevailing winds were in N, NE, and E directions (31.1, 25.1, and 23.9%, respectively). The total amount of time when the wind strength corresponded to 0 was 120 hours (4.5%), 1-7 denoted 58 (5.9%), 932 (35.0%), 844 (31.7%), 352 (13.2%), 103 (3.9%), 6 (0.2%) and 147 hours (5.5%), respectively. May was the windiest month when the period of wind strength from 3 to 7 lasted for 492 hours (64.7%) of the total time. The duration of the calm air period was the greatest in July (8.2%).

It should be noted that a rather large variability characterized the weather conditions during the warm period of years since the differences in clear days were up to 13%, in the strength and direction of the wind – up to 21%. The mean values of air temperature and relative humidity in the warm season are shown in Table 2. These data indicated the inconstancy of weather conditions during the warm period. Mean temperatures from May to July exceeded the indicators of the previous year by 0.9-3.4°C, while August was slightly cool (by 0.4°C). The same dynamics were observed in the mean indicators of the temperature and humidity index, and the differences were 1.6-4.6 and 1.0 units, respectively.

For a clearer picture of the duration of dairy cows under heat stress of varying severity, the data for the two-year observation period are presented in Table 3. It should be noted that the number of hours during which animals experienced heat load (THI > 68) during the warm season increased from 1,160 hours in 2017 to 1,351 hours in 2018 (by 191 hours). At the same time, the period during which cows could be under heat stress when kept in NVB (based on THI_{CHT} values) increased by 106 hours in 2018, compared to the previous year.

Compared with May as the most comfortable month for the cows, there were significant changes in the composition of cow milk during the summer heat of 2018 (Table 3). In some summer months, milk fat yield decreased by 38 g, milk protein yield decreased by 33 g, and percent milk fat and milk protein decreased by 0.1% and 0.02%, respectively ($p < 0.05$). The drop in daily milk yield during the summer months ranged from 0.3 to 0.9 kg/day, echoing last year's similar dynamics of milk production of cows on the dairy complex under the influence of heat stress (Table 4).

It was found that the correlation between insolation conditions and the milk productivity of cows was weak (Table 5). The correlation was expressed only between insolation conditions and milk fat percentage ($r = +0.3$; $p < 0.05$). The relationship between wind strength and daily milk yield was weak ($r = +0.2$; $p < 0.05$). The relationship between air temperature and daily milk yield/milk fat was weakly negative ($r = -0.2$-0.3; $p < 0.05$); the relationship between relative humidity and milk fat yield/percentage ($r = -0.2$-0.4; $p < 0.05$) was similar. The relationship between temperature and humidity indices (THI and THI_{CHT}) and milk fat yield was strongest (from $r = -0.3$ to $r = -0.5$; $p < 0.05$) in different years.

It should be noted that the strength of general trends in the relationships between weather factors and cows' milk productivity both in 2018 and in the previous year, was generally low, although reliable.

We hypothesized that the relationship between meteorological parameters and the milk production of cows should have depended on whether the animals were under conditions of comfort or heat stress of varying severity. The correlation between the indicators was assessed using data for two years (246 tests) by distributing them according to the value of the temperature and humidity index. Out of 246 tests, 138 tests belonged to the comfort zone; 72 tests corresponded to light heat stress and 36 tests corresponded to moderate heat stress, in which cows were kept for 138, 72, and 36 days during the hot period, respectively (Table 6).

Indeed, the data obtained indicated that weather conditions had a different relationship with cow productivity, depending on the conditions, in which the animals were kept. For example, IC positively correlated with milk yield and milk components during the period of thermal comfort, while during periods of low and especially moderate stress, the negative relationship between the indicators increased.

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A similar situation was observed with respect to wind strength when under conditions of low heat stress, the relationship between WS and DMY/MP became negative. The WD negatively correlated with the cows' milk productivity in the temperature comfort zone; however, this relationship was weakened, losing its significance under heat stress. The negative correlation between AT and DMY, and milk components (MF/MP) increased with higher heat stress on the animals, while the positive correlation between RH and milk yield/components decreased. With the increase of heat load beyond temperature comfort, the negative relation between temperature and moisture indices and milk productivity of the animals gradually increased. In this respect, THI_{CHT} was the most informative indicator, which correlates well with milk yield and milk components during heat stress.

Since the influence of weather conditions in hot periods of the year on milk yield/composition of milk was significant (although in some cases with a rather low correlation), it decided to determine the impact of meteorological factors on the milk productivity indicators based on the comfort state of animals, using four-factor analysis for this purpose.

It was found that the total impact of environmental factors on cow milk yield was 46% (Figure 1). The impact of individual factors did not exceed 1–10%, namely WD (10%) and WS (7%) had a sound effect on the daily milk yield. The effect of IC and THI on DMI (9%) was not proven.

The total impact of weather factors on milk components (MF/MP) was 42–45% (Figure 2 and Figure 3). The effect of WD, WS, and THI on MF was 5-8% ($p < 0.05$). The impact of the combination of IC and THI and WD and THI was slight (4-5%), and it was insignificant ($p > 0.05$). The individual influence of WD, WS, and THI on MP was within 4-5% ($p < 0.05$), while the combination of IC and THI (9%) and WD and THI (5%) had no significant effect ($p > 0.05$).

It should be noted that only THI values had a significant ($p < 0.05$) influence on PMP and PMF (7% and 4%, respectively), while the percentage of individual factors fluctuated within 2-3%. Generally, the effect of weather factors on the percentage of fat and protein in cow's milk was estimated at 30-36%.

Regarding the design features of the premises (which are already included in THI_{CHT}), they had a significantly greater effect on cows' milk productivity. The microclimate of the barn also affects the productivity of cows, along with environmental factors. Indeed, the obtained results indicated an increase in the effect of indoor temperature and humidity on DMI by 7.2%, MF by 4.2%, MP by 4.3%, and PMF by 5.7% (Figure 4).

Table 1. Environmental conditions at a Holstein cattle dairy farm in central Ukraine from May to August 2018

Parameters	May		June		July		August	
	hours	(%)	hours	(%)	hours	(%)	hours	(%)
Weather characteristic								
Clear	598	83.8	381	80.4	550	74.7	724	98.1
Mostly cloudy	81	11.3	63	13.3	138	18.8	12	1.6
Mostly cloudy, rain	26	3.6	27	5.7	48	6.5	2	0.3
Overcast	9	1.3	3	0.6	-	0	-	0
Wind direction								
North	195	27.3	136	28.7	263	35.7	234	31.7
North-East	170	23.8	152	32.1	131	17.8	215	29.1
East	240	33.6	76	16.0	95	12.9	225	30.5
South-East	-	0	-	0	-	0	-	0
South	37	5.2	32	6.8	29	3.9	14	1.9
South-West	32	4.5	30	6.3	35	4.8	2	0.3
West	28	3.9	18	3.8	120	16.3	21	2.8
North-West	12	1.7	30	6.3	63	8.6	27	3.7
Wind strength, forces[1]								
0	26	3.7	7	1.5	60	8.2	27	3.7
1	31	4.3	22	4.6	62	8.4	43	5.8
2	165	23.1	160	33.8	323	43.9	284	38.5
3	185	25.9	151	31.9	196	26.6	312	42.3
4	100	14.0	104	21.9	77	10.5	71	9.6
5	54	7.6	30	6.3	18	2.4	1	0.1
6	6	0.8	-	0	-	0	-	0
7	147	20.6	-	0	-	0	-	0

[1]Numerical values from 0 to 7 characterize the wind force, similar to the Beaufort wind strength scale described earlier (Mylostyvyi and Chernenko, 2019).

Table 2. Temperature and humidity conditions at a Holstein cattle dairy farm in central Ukraine in 2018

Month	Air temperature	Relative humidity	THI	THI_{CHT}
	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD
May	19.0±3.05	56.2±14.23	63.4±3.93	65.9±3.19
June	22.3±3.49	52.4±11.08	67.9±4.59	69.3±3.65
July	22.5±1.66	69.5±9.58	69.4±2.27	69.5±1.73
August	23.8±1.87	47.3±9.36	68.9±2.62	70.6±1.94

THI: Temperature–humidity index (Kibler, 1964), THI_{CHT}: Temperature–humidity index in the hangar-type barn (Mylostyvyi et al., 2020). SD: Standard deviation

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Table 3. Distribution of temperature-humidity index values depending on the severity of heat stress in the center of Ukraine in the warm months of 2017[1]/2018

Month	THI (hours)				THI_{CHT} (hours)			
	<68	68.0-71.9	72.0-79.9	80.0-89.9	<68	68.0-71.9	72.0-79.9	80.0-89.9
May	649 / 502	73 / 132	15 / 80	-/-	606 / 476	87 / 95	44 / 143	-/-
June	429 / 224	152 / 97	133 / 146	1 / 7	418 / 217	123 / 89	168 / 154	6 / 14
July	386 / 276	146 / 220	188 / 238	5 / 2	391 / 324	131 / 175	184 / 237	19 / -
August	288 / 309	148 / 186	245 / 243	54 / -	253 / 295	156 / 130	206 / 290	120 / 23

[1]The data of 2017 in the table were taken from the data descriptor (Mylostyvyi and Chernenko, 2019). THI: Temperature–humidity index (Kibler, 1964), THI_{CHT}: Temperature–humidity index in the hangar-type barn (Mylostyvyi et al., 2020). The gradation of temperature and humidity index values was as follows, values below 68 corresponded to comfortable conditions for cows, 68-71 to light stress, 72-79 to moderate stress, and 80-89 to strong stress. No data (-) indicates that no hours have been recorded with this temperature and humidity index value

Table 4. Average daily milk yield and milk components of Ukrainian Holstein cows

Month	Daily milk yield (kg)	Milk fat yield (kg)	Milk protein yield (kg)	Milk fat content (%)	Milk protein content (%)
	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD
May	25.1 ± 0.12	0.888 ± 0.02	0.803 ± 0.02	3.53 ± 0.09	3.20 ± 0.07
June	25.2 ± 0.14	0.874 ± 0.02	0.804 ± 0.02	3.46 ± 0.08	3.19 ± 0.08
July	24.8 ± 0.76	0.850 ± 0.03	0.788 ± 0.03	3.43 ± 0.04	3.18 ± 0.09
August	24.2 ± 0.23	0.852 ± 0.02	0.770 ± 0.02	3.53 ± 0.06	3.18 ± 0.08

SD: Standard deviation

Table 5. Correlation between environmental conditions and milk productivity of Ukrainian Holstein cows for 2017[1]/2018

	DMY	MF	MP	PMF	PMP
IC	-0.20*/-0.12	-0.17/+0.11	-0.11/+0.003	-0.09/0.27*	-0.05/+0.11
WD	+0.09/+0.19	+0.02/-0.01	-0.01/+0.16	-0.02/-0.21	-0.04/+0.04
WS	-0.41*/+0.18*	-0.21*/+0.09	-0.19*/+0.08	-0.05/-0.06	-0.06/-0.06
AT	-0.19*/-0.23*	-0.46*/-0.26*	-0.37*/-0.17	-0.44*/-0.11	-0.33*/-0.02
RH	+0.40*/+0.08	+0.23*/-0.20*	+0.13/-0.05	+0.07/-0.35*	-0.01/-0.14
THI	-0.113/-0.21*	-0.45*/-0.33*	-0.35*/-0.19*	-0.46*/-0.22*	-0.35*/-0.06
THI_{CHT}	-0.19*/-0.22*	-0.47*/-0.26*	-0.37*/-0.16	-0.44*/-0.11	-0.33*/-0.02

The data of 2017 in this table were taken from the data descriptor (Mylostyvyi and Chernenko, 2019). DMY: Daily milk yield, MF: Yield of milk fat, MP: Yield of milk protein, PMF: Percentage of milk fat, PMP: Percentage of milk protein, IC: Insolation conditions, WD: Wind direction, WS: Wind strength, AT: Air temperature, RH: Relative humidity, THI: Temperature–humidity index (Kibler, 1964), THI_{CHT}: Temperature–humidity index in the hangar-type barn (Mylostyvyi et al., 2020).

Table 6. Correlation between weather conditions and cows' milk productivity depending on the THI values of Ukrainian Holstein cows for 2017[1]/2018

	DMY	MF	MP	PMF	PMP
Comfort zone. THI<68.0[1]					
IC	+0.17*	+0.12	+0.14	-0.01	+0.04
WD	-0.20*	-0.25*	-0.23*	-0.13	-0.13
WS	+0.20*	+0.07	+0.12	-0.10	-0.02
AT	+0.29*	-0.04	+0.09	-0.35*	-0.15
RH	-0.15	-0.12*	-0.24*	-0.10	-0.19*
THI	+0.28*	-0.07	+0.05	-0.39*	-0.19*
THI_{CHT}	+0.29*	-0.04	+0.09	-0.35*	-0.15
Light heat stress (THI = 68.1-72.0[2])					
IC	-0.14	+0.05	-0.09	+0.28*	0.00
WD	-0.05	-0.15	-0.05	-0.17	-0.02
WS	-0.23*	-0.19	-0.32*	+0.03	-0.26*
AT	-0.44*	-0.28*	-0.36*	+0.18	-0.11
RH	+0.40*	+0.16	+0.33*	-0.32*	+0.09
THI	-0.22	-0.22	-0.19	-0.04	-0.06
THI_{CHT}	-0.44*	-0.28*	-0.36*	+0.18	-0.11
Moderate to severe heat stress (THI = 72.1 and>[3])					
IC	-0.45*	-0.36*	-0.16	+0.05	+0.17
WD	0.00	-0.01	-0.03	-0.02	-0.04
WS	+0.12	-0.03	+0.09	-0.26	+0.02
AT	-0.32	-0.37	-0.22	-0.16	-0.04
RH	+0.17	+0.19	+0.09	+0.08	-0.03
THI	+0.27	-0.34*	-0.23	-0.18	-0.10
THI_{CHT}	-0.32	-0.37*	-0.23	-0.16	-0.04

[1]n=138, [2]n=72, [3]n=36. *p<0.05. DMY: Daily milk yield, MF: Yield of milk fat, MP: Yield of milk protein, PMF: Percentage of milk fat, PMP: Percentage of milk protein. IC: Insolation conditions, WD: Wind direction, WS: Wind strength, AT: Air temperature, RH: Relative humidity. THI: Temperature–humidity index (Kibler, 1964), THICHT: Temperature–humidity index in the hangar-type barn (Mylostyvyi et al., 2020).

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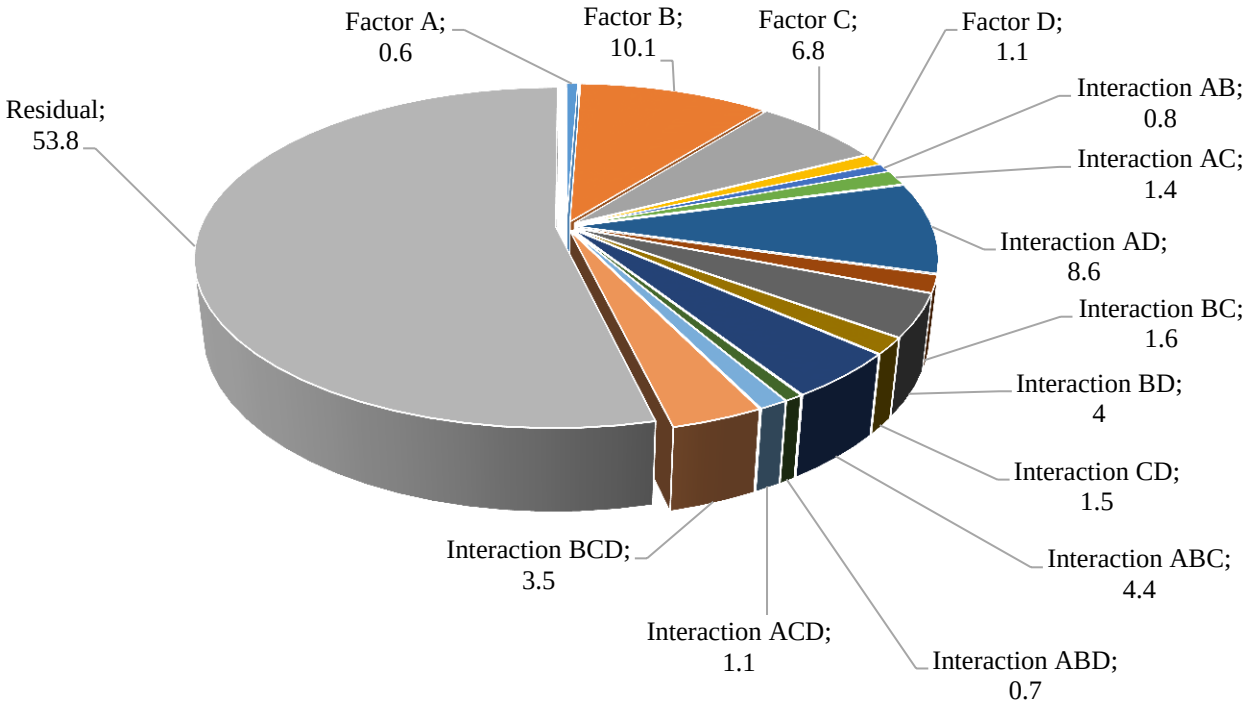

Figure 1. The influence (percentage) of weather factors on daily milk yield of Ukrainian Holstein cows. Factor **A**: Insolation conditions, Factor **B**: Wind direction, Factor **C**: Wind force, Factor **D**: Temperature and humidity index

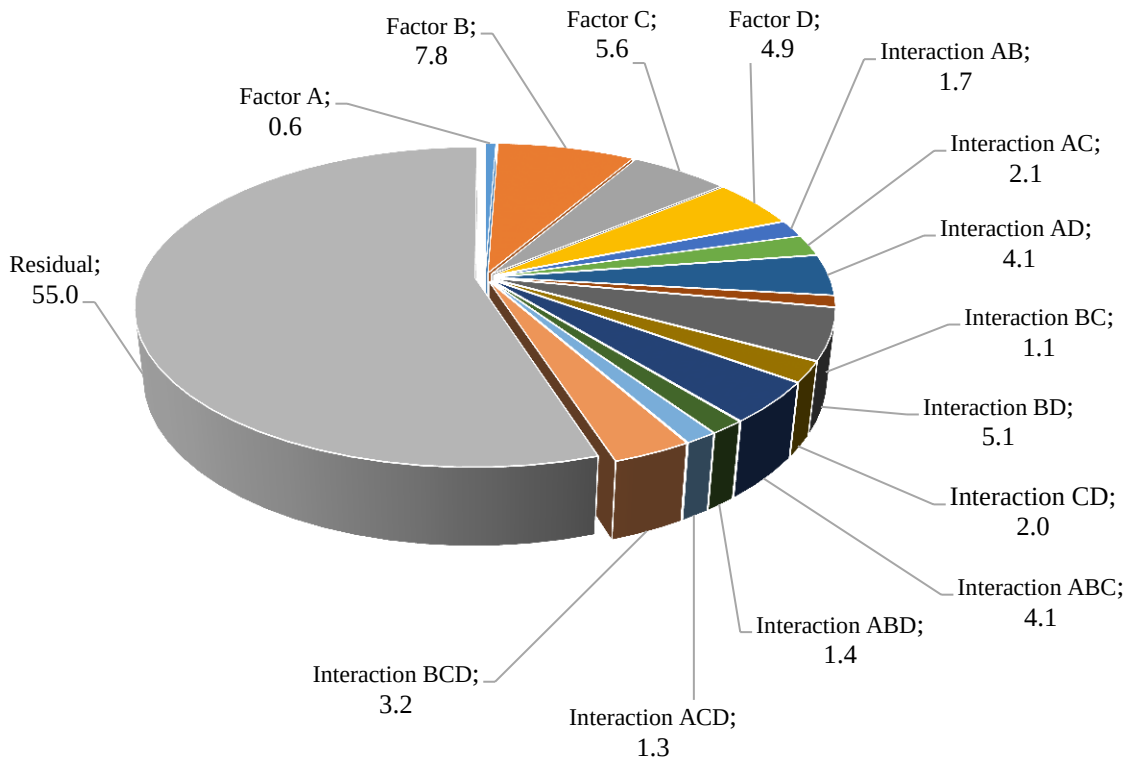

Figure 2. The influence (percentage) of weather factors on the milk fat yield of Ukrainian Holstein cows. Factor **A**: Insolation conditions, Factor **B**: Wind direction, Factor **C**: Wind force, Factor **D**: Temperature and humidity index

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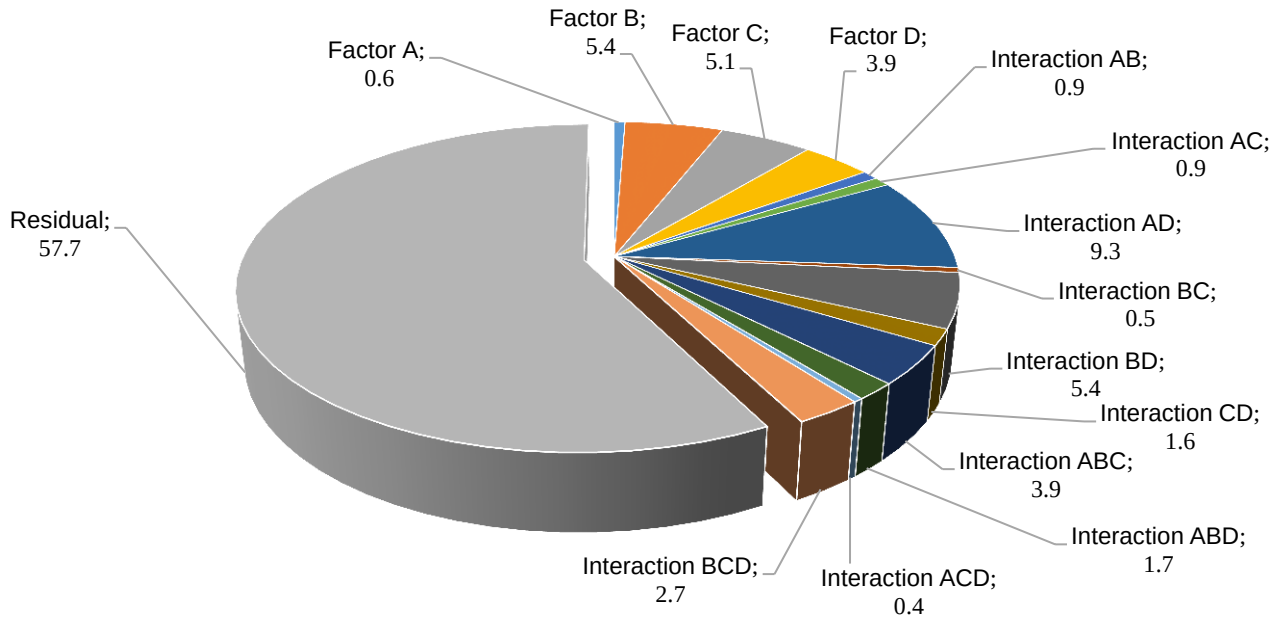

Figure 3. The influence (percentage) of weather factors on the milk protein yield of Ukrainian Holstein cows. Factor **A**: Insolation conditions, Factor **B**: Wind direction, Factor **C**: Wind force, Factor **D**: Temperature and humidity index

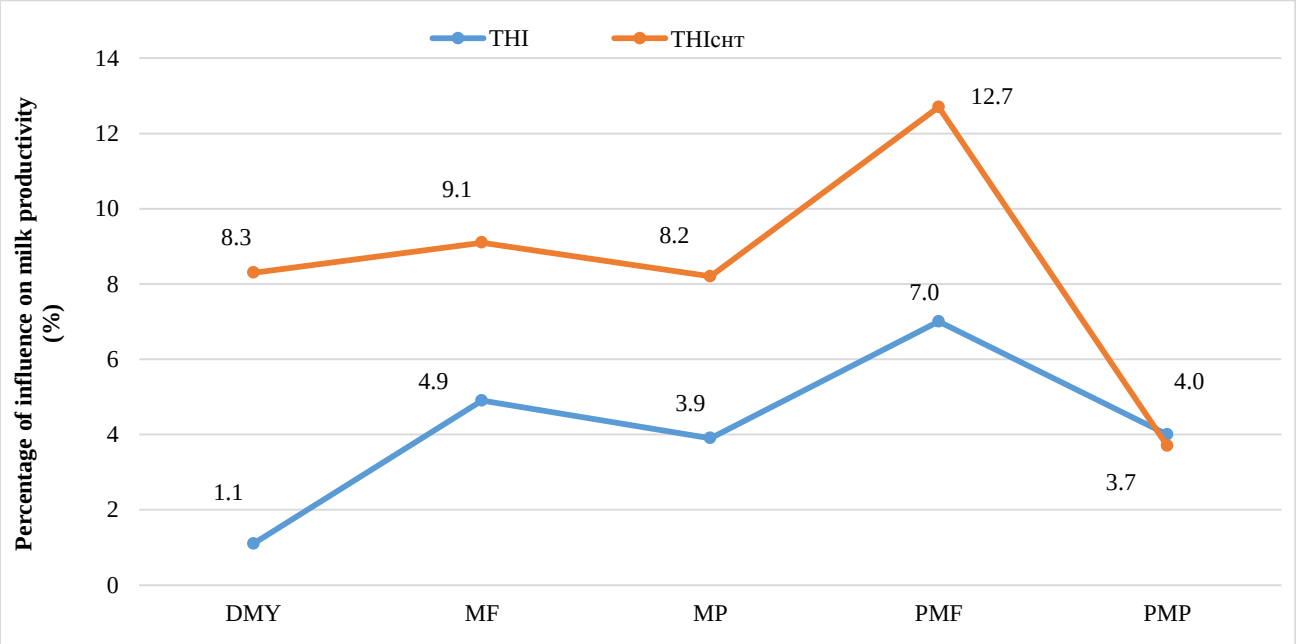

Figure 4. The percentage of temperature and humidity indices influence the milk productivity of Ukrainian Holstein cows. DMY: Daily milk yield, MF: Yield of milk fat, MP: Yield of milk protein, PMF: Percentage of milk fat, PMP: Percentage of milk protein. THI: Temperature–humidity index, THI_{CHT}: Temperature–humidity index in the hangar-type barn

DISCUSSION

The growth of average global temperatures and changing rainfall patterns contribute to extreme weather events that significantly challenge agriculture. More extreme conditions and greater regional climate variability are predicted, accompanied by an increase in the number of heat waves and their duration in the eastern and southeastern regions of Europe (Tomczyk et al., 2019). In Ukraine, critical temperatures in cold and hot seasons negatively impact the degree of realization of the genetic potential of cattle (Baschenko et al., 2020). Therefore, optimization of the indoor microclimate with year-round intensive loose housing of dairy cows in conditions of seasonal hypo- and hyperthermia is a reserve for the development of animal breeding.

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The body of an animal can homeostasis and regulate physiological processes. In a certain range of conditions, it manages to compensate for changes and disturbances in physiological equilibrium and, therefore, maintain physiological constancy. However, a significant increase in air temperature was followed by heat stress, leading to a decrease in milk yield and changes in milk components (Polsky and von Keyserlingk, 2017).

In addition to managerial decisions (Kismul et al., 2018), feeding strategies (Conte and al., 2018) and technical means (Gunn et al., 2019) aimed at reducing exposure to high temperatures by creating comfortable conditions for animals during short-term heat waves, great attention is also paid to the selection of livestock for resistance to high temperatures in the long term (Izhboldina et al., 2020).

In this case, the practical significance of the correlation analysis of the indices lies in the fact that, when selecting animals, it leverages positive qualities and reduces the undesirable ones and selects a smaller number of traits, which significantly accelerates the rate of genetic improvement of the herd.

The literature provides rather contradictory reports on the correlation between weather conditions and cows' productivity. For example, a weak negative correlation (r = -0.28) was found between air temperature and milk yield (Baschenko et al., 2020), while Herbut et al. (2018) reported a strong relationship between cow productivity and ambient temperature (r = -0.89). It was also reported by Baschenko et al. **(**2020) that the relationship between the fat content of cows' milk and high air temperature (r = -0.63), relative humidity (r = -0.38), and atmospheric pressure (r = -0.22), while the correlation between milk fat and air movement was relatively low (r = -0.06). It was reported that there was a strong negative correlation between THI and milk yield (r = -0.88), protein (r = -0.79), and fat (r = -0.86) in the hot season (Cheruiyot et al., 2020).

The discrepancies in the data reported by the researchers may be due to the fact that weather conditions are characterized by great variability and inconstancy, which complicates the assessment of the influence of individual parameters on the animals' productivity.

It should also be noted that the impact of weather on animals depends on the animal management system, especially if the cows are in naturally ventilated premises, the climate directly depends on environmental conditions (Hempel et al., 2019). Despite the high correlation between the temperature and humidity conditions in non-insulated premises and the ambient conditions (r = 0.95; R^2 = 0.90) reported by Mylostyvyi et al. (2019), the influence of premises factor on cows' milk productivity turned out to be quite significant (Figure 4). However, it should be taken into account when assessing the relationship between the indices.

On a final note, science recognizes climate change as one of the key causes of changes in the productivity of dairy cattle (Sejian et al., 2021). However, the complexity of the relationships between weather conditions and individual indicators of animal productivity makes it difficult to assess their impact on future losses since the impacts associated with climate change can be very diverse (Lees et al., 2022). Recent studies indicated an increase in the strength of the correlation between the productivity of dairy cows and the environmental conditions in connection with global warming, which confirms the demand for further research in this direction (Cheruiyot et al., 2020). To solve the problem of global climate change associated with the anthropogenic factor, it is necessary to rethink the relationship of the world community to the planet and the emergence of a new Culture of humanity.

CONCLUSION

It has been established that the productivity of dairy cows can be conditioned by almost 50% by environmental conditions when kept in naturally ventilated barns in the warm period of the year. The combination of meteorological parameters and microclimate conditions associated with barn design can increase the influence of these factors on milk yield and milk components. This shows the importance of using indices considering the combined effects of different environmental factors on dairy cattle to find effective strategies for mitigating global climate change.

DECLARATIONS

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Authors' contribution

All authors approved the final version of the article before publication in the journal. Conceptualization, writing-original draft preparation, Roman Mylostyvyi, and Olena Izhboldina; developed an experiment, analyzed data, Svitlana Midyk, and Bogdan Gutyj; writing-review and editing and supervision, Oleh Marenkov, and Volodymyr Kozyr.

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Ethical consideration

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.

Availability of data and materials

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

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Competing interests

The authors declare no conflict of interest.

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The Effect of Shrimp Shell (*Litopenaeus vannamei*) Extract on Testicular Parameters of Streptozotocin-induced Diabetic Rats

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ABSTRACT

Diabetes mellitus (DM) is a chronic metabolic disorder that has become a major health problem worldwide. Reproductive dysfunction is one of the main complications of DM, particularly in men. However, as is known, shrimp shell extract contains nutrients, such as astaxanthin, that affect reproductive traits. The present study aimed to evaluate the effect of shrimp shell extract on the volume, weight, and histological features of the testes of a DM rat model. Fifteen adult male rats were randomly divided into three groups. Group A (n = 5) was a healthy control group, group B (n = 5) was a DM control group, and group C (n = 5) was a DM group treated with shrimp shell extract. Rats in groups B and C were treated with streptozotocin to induce DM. Rats in group C were given shrimp shell extract at 25 mg/kg body weight for 30 consecutive days after DM induction. Testicles were collected and submitted to dimension, weight, and histological examinations. The testicle volume and weight of rats in group C were significantly higher and heavier, respectively, than rats in group B and did not differ from rats in group A. The seminiferous tubule diameter of rats in group C was significantly larger than rats in group B and did not differ from rats in group A. Rats in group B had a lower testicle volume and lighter testicle weight as well as a shorter seminiferous tubule diameter than rats in groups A and C. In conclusion, shrimp shell extract could improve male fertility parameters in a DM rat model. However, the mechanism of action needs to be studied further.

Keywords: Astaxanthin, Diabetes mellitus, Fertility, Seminiferous tubule, Testis

INTRODUCTION

Diabetes mellitus (DM) is a chronic metabolic disorder that has become a major health problem worldwide. A previous study reported that in 2015, there were 415 million people with DM worldwide; this number is predicted to rise to over 642 million by 2040 (Ogurtsova et al., 2017). In 2012, there were 1.5 million deaths due to DM (Ogurtsova et al., 2017). This disease could affect the quality of a patient's life due to its many complications. One of the complications is reproductive dysfunction (Shi et al., 2017). It has been recognized that abnormal blood glucose levels can impair reproductive function in men with DM (Maresch et al., 2018). DM can affect reproductive function, including ejaculation, penile erection, fertility, sperm maturation, and spermatogenesis (Ding et al., 2015; Nna et al., 2017). Insulin-based therapy was introduced in the 1920s and is still the primary treatment for patients with type 1 DM and some patients with advanced stages of type 2 DM (Tavares et al., 2018). In addition, there are some antidiabetic drugs for type 2 DM, such as sulfonylureas, meglitinide, biguanides, and thiazolidinediones (Skliros et al., 2016). Studies regarding the effectiveness of existing antidiabetic drugs on the male reproductive system have been carried out in animal models of DM (Adaramoye and Lawal, 2014; Alves et al., 2014; Ayuob et al., 2015; Zaidi et al., 2017; He et al., 2021). Although the use of antidiabetic drugs is relatively safe and they have been widely prescribed in patients with DM, some side effects, such as hypoglycemia, hyperlactatemia, or metabolic acidosis, may still result from long-term use of currently available antidiabetic drugs (Anagnostis et al., 2018; Wang and Hoyte, 2019).

Many studies have concerned the role of natural products on DM and male reproductive functions (Tran et al., 2020; Swelum et al., 2021; Thikekar et al., 2021; Fu et al., 2022). Some researchers have used whole plants or part of plant extracts, such as *Chlorophytum borivilianum* (root), *Amaranthus spinosus* (stem), *Danae racemosa* (leaves), and *Nigella sativa* (seeds), while others have used just specific compounds such as phenols, flavonoids, and flavanones isolated from plants (Nna et al., 2017). However, most of those natural products are not included in the food ingredients consumed daily by people. In this study, *Litopenaeus vannamei* shell extract was used in an animal model of DM to evaluate its effect on male reproductive organs. *Litopenaeus vannamei* is one of the most widely cultivated shrimp species besides *Penaeus monodon* and *Penaeus chinensis* (FAO, 2014). Shrimp is commercialized as seafood and is usually sold whole or sometimes only the meat of shrimp. Thus, shrimp shells are abundant in solid waste and underutilized in the food industry. Shrimp shells still contain some nutrients, such as minerals, proteins, chitin, and

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chitosan (Cavalcanti et al., 2016). Chitin has a variety of biological and biomedical uses, including tissue healing. Chitosan is also known for its potential therapeutic effects, including anti-inflammatory, antioxidant, antidiarrheal, and anti-Alzheimer's disease effects (Satitsri and Muanprasat, 2020). Chitosan can enhance the size of the antral follicle, the number of endometrial arterioles, and the endometrial thickness of female rats exposed to lead acetate (Purwitasari et al., 2019). Although mammals do not have endogenous chitin (Ohno et al., 2013), a previous study demonstrated that chitosan, a derivative of a natural carbohydrate biopolymer derived from chitin deacetylation, could improve sperm count and the motility of progressive sperm of lead-acetate-induced rats (Marianti et al., 2020). This suggests that chitin and/or chitosan may be involved in the male reproductive system. In addition, shrimp shell extract contains astaxanthin, which is known to have good effects on the male reproductive system in some species, such as rainbow trout and discus (Ahmadi et al., 2006; Haque et al., 2023). Astaxanthin could have a protective effect on sperm mitochondrial function and also ameliorate testicular heat stress and reproductive poison damage (Liu et al., 2016). However, research concerning the effect of astaxanthin supplementation via shrimp shell extract on the male reproductive organs in the DM condition is still limited. The present study aimed to evaluate the effect of shrimp shell extract on the volume, weight, and histological features of the testicular organ of a DM rat model.

MATERIALS AND METHODS

Ethical approval

The experimental protocols carried out in this study had been approved by Universitas Kristen Duta Wacana (UKDW) Medical Research Ethics Committee with Ethical Clearance Certificate Number: 1265/C.16/FK/2021.

Shrimp shell extraction

Fresh shrimp (*L. vannamei*) was obtained from a fish market located on the south beach of Java, Indonesia, during the rainy season in July 2021. Shrimp shell extraction was carried out at the Biotechnology for Health Laboratory (Indonesia). Shrimp shells were separated from the flesh manually and washed with running water. Shrimp shells were dried using an oven at 40°C for approximately a day. Then, shrimp shells were ground into powder. Dried shrimp shell powder was subjected to a 3-day extraction using a maceration method as described by Najoan et al. (2021). Ethanol (70%) was used as the solvent at a 1:10 (v/v) ratio with water. The maceration was repeated two times for 3 days, respectively. Subsequently, evaporation was carried out using a rotary evaporator at 5 rpm and 40°C, and then continued in an oven at 40°C until the consistency was like a paste (Najoan et al., 2021).

Experimental animals

A total of 15 male Wistar rats (*Rattus norvegicus*) aged 8-12 weeks with an average body weight of 190 g from the Faculty of Biology, Universitas Gadjah Mada, Indonesia, were used in this study. Before the experiment began, acclimatization was carried out for 7 days. The rats were given access to water and food *ad libitum* during this period. The rats were maintained in five plastic boxes, each with three rats at room temperature in a tropical environment with a 12-h photoperiod. After acclimatization, the rats were divided randomly into three groups. Group A (n = 5) was a healthy control group, group B (n = 5) was a DM control group, and group C (n = 5) was a DM group treated with shrimp shell extract. Group A did not receive any treatment during the experiment. In groups B and C, DM was induced by intraperitoneally injecting 50 mg/kg body weight (BW) of streptozotocin (STZ) (Cayman Chemical, USA) diluted in citrate buffer. The STZ dosage for DM induction was previously described by Suman et al. (2016). Three days after DM induction, a blood sample was collected via the caudal vena cava to measure blood glucose levels using glucose meters (OneTouch, USA). The diabetic condition was proven by high blood glucose levels (≥ 150 mg/dL) (Furman, 2021). After inducing DM, rats in group C were given shrimp shell extract at the dosage of 25 mg/kg BW, and rats in group B were given 1 ml of sterile water as a placebo. The shrimp shell extract dosage was chosen according to a previous study by Wisaksono et al. (2021). Sterile water for the rats in group B and shrimp shell extract for the rats in group C was administered orally via gavage for 30 consecutive days.

Sample collection and histology slide preparation

After 30 days of oral treatment using shrimp shell extract, all rats were euthanized for sample collection. Before euthanasia, rats were anesthetized using tiletamine and zolazepam (Zoletil, Virbac, India) at 20 mg/kg BW (Limprasutr et al., 2021). After the rat was fully anesthetized, indicated by the absence of a pedal reflex (Sivula and Suckow, 2018), it was euthanized by cutting the respiratory tract and carotid vessel in the cervix. The testicles were removed from their scrotum and fixed using a 10% formalin solution. The testicles were measured for dimension and weight before being processed for histological staining. Testicle tissue was trimmed, processed with paraffin, and cut at 5 µm thickness. The tissue slides were placed on the slide warmer for 30 minutes. Subsequently, tissue slides were deparaffinized using xylene and rehydrated using a graded series of alcohol. Haematoxylin and eosin (HE) staining was performed before the

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dehydration and clearing processes. Then, the slide was mounted with a cover slip. Histological examination was carried out using a light microscope (Olympus, Tokyo, Japan) with 40× magnification.

Data collection

The dimensions of the testicle, including length (l), width (w), and thickness (t) were measured using a vernier calliper. These dimensions were used to calculate the volume of the testicle by using the formula of volume (v) for ellipsoid ($v = [\pi/6] \times l \times w \times t$) (Van der Plas et al., 2013). The testicle weight was measured with a digital scale (Camry Scale, USA). The seminiferous tubule diameter was determined from histology slides. Three photomicrographs were taken for each histology slide using a digital camera connected to a light microscope. The objective lens was 4× magnification, and the total magnification was 40×. The diameter of 10 seminiferous tubules in each photomicrograph was measured using the Image Raster 3.0 software (Optilab, AZ 85012, USA). Finally, the mean seminiferous tubule for each sample was calculated.

Data analysis

Statistical analyses were conducted using SAS version 9.4 (SAS Inst. Cary, NC, USA.). The data are presented as mean ± standard error. Descriptive statistics were analyzed by using the MEANS Procedure. The testicle volume, testicle weight, and seminiferous tubule diameter of each group were analyzed by multiple analyses of variance using the generalized linear model procedure. Least-squares means were obtained from each group of the variables and were compared by using Tukey-Kramer adjustment for multiple comparisons. Correlation analysis between seminiferous tubule diameter and testicle volume, and testicle weight was carried out using Pearson correlation analysis of SAS. For all the statistical analyses, $p < 0.05$ was considered statistically significant.

RESULTS

There were 14 rats included at the end of this study. One rat from group C was excluded due to inferiority within the group. The average testicle volume and testicle weight of the rats differed significantly between the three groups ($p < 0.05$, Table 1). The testicle volume of rats in group B was significantly lower compared with the testicle volume of rats in groups A and C ($p < 0.05$). The rats in group C had the highest testicle volume, but it was not significantly different compared with the testicle volume of the rats in group A ($p > 0.05$). The testicle weight of rats in group B was significantly lower compared with the testicle weight of the rats in group A and group C ($p < 0.05$; Table 1). The rats in group C had the highest testicle weight, but it was not significantly different compared with the testicle weight of the rats in group A ($p > 0.05$).

The average seminiferous tubule diameter of the rats differed significantly between the three groups ($p < 0.05$) (Table 1). The seminiferous tubule diameter of the rats in group B was significantly shorter than that of the rats in groups A and C ($p < 0.05$). The rats in group C had the largest seminiferous tubule diameter, but it was not significantly different compared with the seminiferous tubule diameter of the rats in group A ($p > 0.05$). Pearson correlation analysis showed a strong and significant relationship between the seminiferous tubule diameter and testicle volume, and testicle weight ($p < 0.05$, Table 2).Histological observation showed that the rats in groups A and C had rounder seminiferous tubules compared with the rats in group B. Some grooved surfaces of seminiferous tubules could be observed in the rats in group B (Figure 1, black arrows).

Table 1. Testicular parameters in a diabetic rat model treated with shrimp shell (*Litopenaeus vannamei*) extract in Indonesia

Testicular parameters	Group A	Group B	Group C
Number of samples	5	5	4
Testicle volume (cm^3)	1.07 ± 0.07^a	0.54 ± 0.07^b	1.21 ± 0.08^a
Testicle weight (g)	1.17 ± 0.08^a	0.62 ± 0.08^b	1.26 ± 0.09^a
Seminiferous tubule diameter (µm)	329 ± 9^a	240 ± 9^b	339 ± 10^a

The values are presented as the mean ± standard error. Group A: healthy control group; group B: Diabetes mellitus control group; group C: Diabetes mellitus group treated with shrimp shell extract. Different superscripts indicate significant differences in rows ($p < 0.05$).

Table 2. Pearson correlation analysis of seminiferous tubule diameter, testicle volume, and testicle weight in a diabetic rat model treated with shrimp shell (*Litopenaeus vannamei*) extract in Indonesia

Variables \ Seminiferous tubule diameter	Correlation coefficient (r)	p value
Testicle volume	0.940	<0.001
Testicle weight	0.937	<0.001

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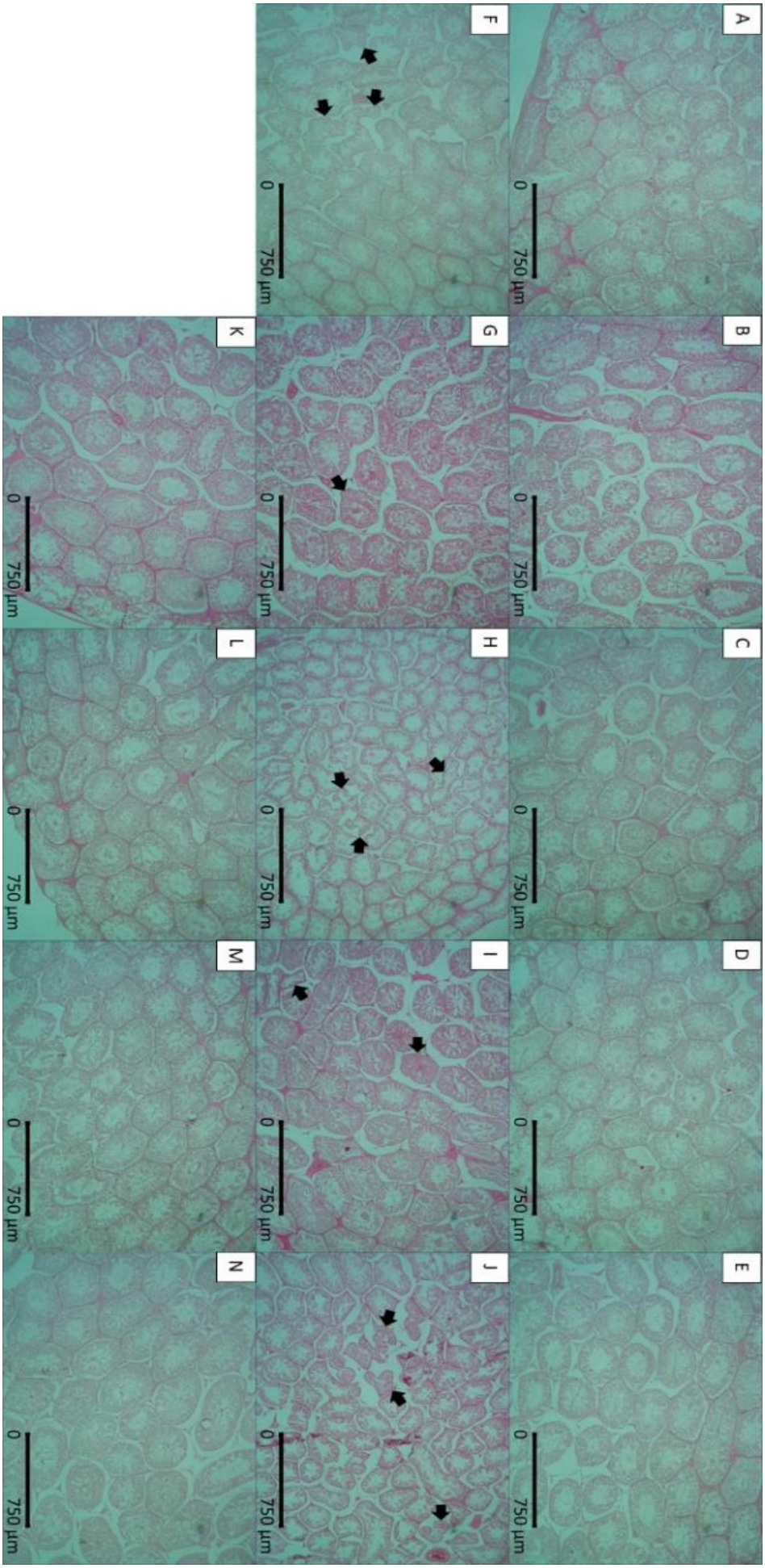

Figure 1. Histological changes in seminiferous tubules of diabetic rats. **A-E:** The healthy control rats (Group A); **F-J**: The diabetes mellitus (DM) rats (Group B); **K-N:** The diabetic rats treated with shrimp shell extract (Group C).

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DISCUSSION

The protocols for STZ-induced insulin deficiency and hyperglycemia in mice and rats have been well established (Furman, 2021). Suman et al. (2016) used the same STZ dosage to induce type 2 DM in combination with a high-fat diet. STZ injection increases glucose, insulin, free fatty acid, and triglyceride concentrations. A single intraperitoneal injection of a low STZ dose (30 mg/kg BW) in adult male Wistar rats affects pancreatic β-cells as well as the reproductive system via its diabetogenic effect (Omolaoye et al., 2018). Researchers have also reported adverse effects of STZ-induced DM on the male reproductive system in experimental animals (Omolaoye et al., 2018; Kotian et al., 2019; Sampannang et al., 2020). Maresch et al. (2019) demonstrated two major pathways of hyperglycemia-induced organ damage in the testis and epididymis, namely the diacylglycerol-protein kinase C pathway and the polyol pathway. The present study demonstrated that compared with the rats in group A, the rats in group B showed a significant decrease ($p < 0.05$) in the testicle volume, testicle weight, and seminiferous tubule diameter after 30 days of STZ injection from 1.07 cm^3, 1.17 g, and 329 µm, respectively, to 0.54 cm^3, 0.62 g, and 240 µm, respectively.

Shrimp shells are a waste product in the food industry. However, some nutrients contained in shrimp shells are still useful, such as chitosan (de Queiroz et al., 2017). *N,O*-Carboxymethyl chitosan, a derivative of chitosan, can be used to increase fiber contents; the resilience of food storage; and the stability of nutrients, including lowering the levels of dry substances, lowering the ash content, increasing the protein content, maintaining the fat content, and increasing the level of nitrogen-free extract (Kusuma et al., 2015). Nadapdap et al. (2014) demonstrated that supplementation with chitosan derived from shrimp shells could improve sperm count, normal sperm morphology, sperm motility, and sperm viability of Wistar rats treated with lead. The possible mechanism for this is that chitosan binds to the lead and forms bonds that make it hydrophilic and thus excretable via urine, thus reducing the male reproductive side effects of lead. However, the direct mechanism of action of chitosan on the male reproductive system is still unclear. Abd El-Hakim et al. (2020) demonstrated that a combination of chitosan-stabilized selenium nanoparticles and metformin could increase sperm motility, sperm viability, and sperm concentration and reduce sperm abnormality in an STZ-induced DM rat model. This suggests that chitosan may act as a delivery agent for the other substance. In addition, astaxanthin can be found in the shrimp shell when extracted using a maceration method with 70% ethanol as the solvent (Wisaksono et al., 2021). Astaxanthin is a xanthophyll carotenoid found in various microorganisms, marine animals, and crustaceans, including shrimp shells (Higuera-Ciapara et al., 2006; Ambati et al., 2014; Wisaksono et al., 2021). Astaxanthin has many biological activities and health benefits, such as antioxidant, anti-lipid peroxidation, anti-inflammatory, and anticancer activities; cardiovascular disease prevention; and immunomodulation (Visioli and Artaria, 2017; Faraone et al., 2020; Fouad et al., 2021). Martínez-Álvarez et al. (2020) stated that the use of astaxanthin and astaxanthin-containing lipid extracts as a food ingredient might have a double function: a technological function because they can provide foods with attractive reddish color and a bioactive function (for example, antioxidant activity) when consumed. Moreover, astaxanthin is safe to consume daily at a dosage ranging from 2 to 24 mg (Brendler and Williamson, 2019).

The use of astaxanthin in DM has been studied by many researchers (Feng et al., 2020; Landon et al., 2020; Ahriyasna et al., 2021; Wisaksono et al., 2021). However, the reproductive aspect in such studies has not been evaluated. The present study revealed that shrimp shell extract could protect STZ-induced rats from testicular damage. This was denoted by the improvement in testicle volume, testicle weight, and seminiferous tubule diameter in STZ-induced rats that were supplemented with shrimp shell extract for 30 days. However, the effect of shrimp shell extract on sperm parameters and reproductive hormones still needs to be clarified. Bašković et al. (2021) reported that intraperitoneal injection of astaxanthin has a favorable effect on histological morphometric testicular parameters (mean seminiferous tubule diameter, mean seminiferous lumen diameter, epithelial height, tubular area, luminal area, and Johnsen score) in testicular torsion/detorsion-induced rats. This effect is mediated by the antioxidant activity of astaxanthin (Demir et al., 2022). Astaxanthin supplementation of 50-100 mg/kg feed for 6 weeks to improve reproductive performance has been reported in many studies in various species such as *Nodipecten nodosus* (Linnaeus, 1758), *Procambarus clarkia*, and layer breeder roosters (Suhnel et al., 2014; Zhenhua et al., 2020; Gao et al., 2021). Wisaksono et al. (2021) reported that supplementation with shrimp shell extract could reduce blood glucose levels in STZ-induced rats. This strengthens the notion that the mechanism by which shrimp shell extract protects STZ-induced rats from reproductive organ damage not only comes from its astaxanthin content, which has bioactive activity but might also be caused by lowering hyperglycemia.

It has been reported that long-term hyperglycemia can increase levels of reactive oxygen species and advanced glycation end products, inhibits endothelial nitric oxide synthase metabolism, and decrease endothelial synthesis and the release of nitric oxide, which leads to erectile dysfunction in patients with DM (He et al., 2021). In addition, hyperglycemia interferes with gonadotropin-releasing hormone secretion, thus reducing gonadotropin and prolactin secretion, which in turn leads to a significant decrease in testosterone secretion from Leydig cells and ultimately to spermatogenesis disorders (He et al., 2021).

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CONCLUSION

In conclusion, supplementation of shrimp shell extract in STZ-induced rats could improve testicle volume, testicle weight, and seminiferous tubule diameter, which are fertility parameters in males. Shrimp shells are a waste product of the food industry that might be useful in preventing reproductive problems in patients with DM in the future. However, the mechanism of action in reproductive health, especially in pathological conditions, needs to be studied further.

DECLARATIONS

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Authors' contribution

Aniek Prasetyaningsih and Vinsa Cantya Prakasita designed the research project and obtained the funding. Aniek Prasetyaningsih, Abner Amadeuz Wicaksono, and Yosua Kristian Adi conducted the experiments and collected the samples. Vinsa Cantya Prakasita analyzed the data and prepared the manuscript. All authors read and contributed to evaluating the manuscript.

Competing interests

The authors have not declared any conflict of interest.

Ethical consideration

Plagiarism, consent to publish, misconduct, data fabrication and/or falsification, double publication and/or submission, and redundancy have been checked by the authors.

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Investigation of Ovarian and Non-ovarian Associated Factors Related to Follicular Population and Oocyte Maturation of Chadian Cattle Breeds

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ABSTRACT

A cow can give birth to an average of 6-7 calves in her entire reproductive period. The remaining oocytes could be used for the *in vitro* production of embryos. The present study was conducted to evaluate the effects of ovarian and non-ovarian factors on the follicular population and oocyte maturation of three Chadian cattle breeds (Arab, Kouri, and Toupouri). For this purpose, the ovaries of 166 cycled cows were collected at the Farcha slaughterhouse of Chad and placed individually in labeled conical tubes containing 0.9% NaCl and 0.5 mg/ml penicillin-streptomycin. After clearing the ovaries of tissue debris, they were weighed, and the follicles were counted. The diameter of each follicle was measured and classified into three categories. A total of 2734 oocytes were collected in 28 days with a minimum of 97 per day by the slicing method using a 10X stereoscope. They were then classified into four groups according to the structure of their cumulus oophorus. Immature oocytes (class 1 and 2 [1455]) were placed in different culture media consisting of Minimum Essential Medium (MEM) alone, MEM with 10% follicular fluid, and MEM with 50% follicular fluid for oocyte maturation. The results indicated that the mean follicular population and mean oocyte yield were 24.71 ± 0.88 and 11.65 ± 0.94, respectively. The mean oocyte index and the number of cultivable oocytes for *in vitro* embryo production (class 1 and 2) were 1.03 ± 0.23 and 1.65 ± 0.94, respectively. The number of follicles observed in the age group of 6-9 years was higher than in other age groups. Oocyte yield was significantly higher in cows with a body condition score of 4-5 compared to average and lean cows. Among the different culture media used for oocyte maturation, the medium consisting of MEM plus 10% follicular fluid recorded a higher maturation rate than the other culture media. Cows aged 6-9 years had a higher maturation rate than other age groups. In conclusion, the good follicle (follicle that produced oocyte) and appropriate oocyte performance were observed in cows with body condition score 3-5 and an age range of 6-9 years.

Keywords: Age, Breed, Cattle, Maturation, Oocyte

INTRODUCTION

Livestock significantly influences the economies of sub-Saharan African countries (Tacher and Letenneur, 1999). It can reduce poverty and increase food disponibility. In Chad, the livestock sector, with more than 20 million ruminants, including cattle, supports 40% of the population's needs for meat (MDPPA, 2011). With more than 10 million heads, the cattle sector plays a vital role in national animal production (PNDE, 2017). It provides almost 87,000 tons of meat and 89% of the milk supply annually (FAO and CEEAC 2021). Despite this high representativeness of cattle and their contribution to the bioavailability of meat in Chad, their numerical productivity remains very low, and the oocyte reserve of a heifer at birth is estimated at around 100,000 oocytes (Hanzen et al., 2000). A cow can give birth to an average of 6-7 calves in during her entire reproductive period. The remaining oocytes could be used for the *in vitro* production of embryos.

In this regard, there are new modern reproduction techniques, such as embryo *in vitro* production and transfer to the recipient mother (Manik et al., 2003; Huang and Rosenwarks, 2012). These new techniques contribute to the intensification of the genetic improvement of the herd (Chukwuka et al., 2010) and the usefulness of slaughtered pregnant cows' oocytes.

The production of embryos *in vitro* and transfer to recipient females act as alternatives to artificial insemination (Huang and Rosenwarks, 2012). These techniques allow the preservation of the genetic potential of sub-fertile or dead animals (Deuleuze et al., 2009) through the creation of a gene bank (Ducos et al., 2021) and the production of embryos from the oocytes of slaughtered animals to promote or multiply of this species (Guignot, 2005). Ovaries collected from animals after slaughter are the greatest source of inexpensive primary oocytes that could be incubated until maturation, followed by *in vitro* fertilization (Agrawal et al., 1995). This technique has not yet been sufficiently explored in Chad

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although many diverse cattle species exist. This study was conducted to highlight the use of the ovarian oocytes of slaughtered cows at the slaughterhouse.

MATERIALS AND METHODS

Ethical approval

The work was conducted based on the ethical rules of the National Institute of Science and Technology of Abeche, Chad.

Study site

The study was carried out from October 2020 to the end of September 2021 in the Farcha slaughterhouse, Chad, and the laboratory of the Institute of Livestock Research for Development in the Chari baguirmi region, in the peri-urban area of Ndjamena, Chad (13°49'59''N and 20°50'05''E). Arid climate and rainfall are nil for five months, from November to March, while July and August are well watered, with 144 mm and 175 mm, respectively.

Animal

A total of 166 cycled cows were divided into three breeds, namely Arab (n = 59), Kouri (n = 57), and Toupouri (n = 50). In this study, 40 pregnant cows were grouped into Arab (n = 11), Kouri (n = 15), and Toupouri (n = 14). The study was conducted in two seasons, a dry season (October to June) and a rainy season (July to September).

Age determination

The age of each animal (pregnant or not) was determined by simultaneous analysis of the dentition and the horn (Garba et al., 2013, Table 1). When a female was pregnant, the approximate age of the fetus was determined using the following formula: $Y = X(X+2)$

where, X represents the number of months of gestation, and Y denotes the nape-rump length in centimeters (Santos et al. 2013).

For age by horns, the following formula was used: Age (in years) = N + 2,

where, N represents the number of furrows and 2 is constant (Garba et al., 2013).

Table 1. Method of determining the age of the cows (Arab, Kouri, and Toupouri) in Chad

Teething	Age of the cow
Full development of the first intermediate pair of permanent incisors and corners	3-5 years
Permanent pinchers show noticeable wear	6 -9 years
Corners show dental stars	10 and more

Body condition score

The body condition score (BCS) was determined before the animal was slaughtered according to a study by Vall et al. (2004). As proposed by Natumanya et al. (2008), the cows were classified into three categories. Scores 1-2 represent lean, score 3 refers to average, and scores 4-5 are for fat.

Ovary collection

After the slaughter of the animal, the two ovaries were removed separately by incising the broad ligament with a chisel. These were identified (left and right ovary) and individually introduced into tubes containing 0.9% NaCl isotonic medium and penicillin-streptomycin (0.5 mg/ml). The samples (ovaries) were placed in an isothermal container at a temperature of 30-32°C and transported immediately to the laboratory in less than 20 minutes, as the slaughterhouse and the laboratory were situated in the same company. Ovaries with pathologies (cysts) were excluded (Wang et al., 2007).

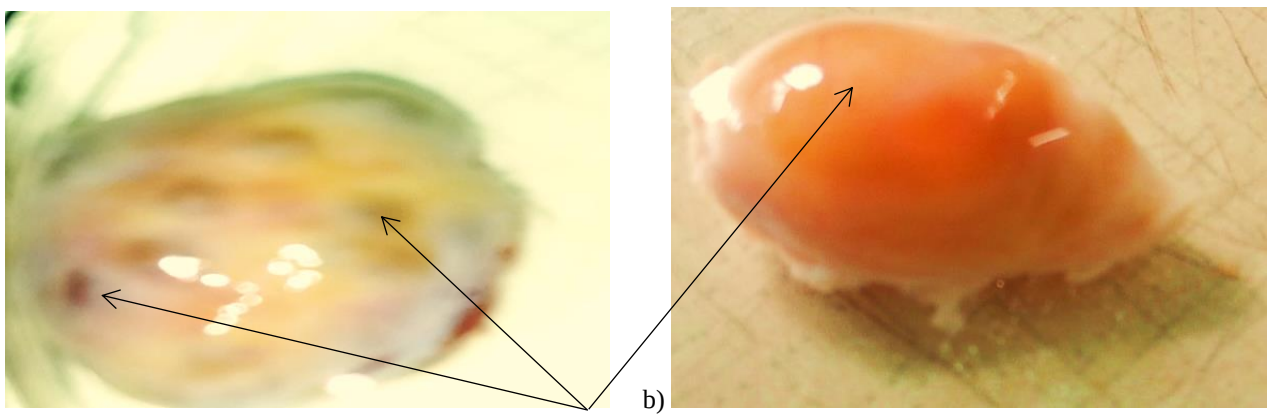

Figure 1. Ovaries bearing follicles in a cow. **a**: small and medium; **b**: large (Source: Azafack, 2019)

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Determination of follicular population

In the laboratory, ovaries were cleaned 4 times using 0.9 % of sodium chloride and cleared of their tissue debris using a chisel. Each ovary was weighed. The surface area of each ovary was observed, the present follicles were counted and their diameters (Φ) were measured using electronic stainless hardened calipers, then classified into three categories of small ($\Phi < 3$ mm), medium ($3 \leq \Phi \leq 8$ mm), and large ($\Phi > 8$ mm) following a study by Duygu et al. (2013). The color and shape of the corpus luteum were observed to determine the stage of each cow's sexual cycle, as described by Houmadi (2007).

Oocyte collection and classification

Ovaries were incised using the slicing technique (Wang et al., 2007). Into a petri dish containing 5ml of 0.9% NaCl solution to collect oocytes.

Morphological evaluation of oocyte quality

The collected oocytes were examined and counted under stereoscope at (10X) objective and then classified into four qualities taking into account the homogeneity of the cytoplasm or layers of cumulus oophorus cells according to Alves et al. (2014). Quality 1 entails compacted cumulus having more than three layers with homogeneous cytoplasm. Quality 2 entails compacted cumulus with one or two layers with homogeneous cytoplasm. Quality 3 encompasses cumulus not very compacted with irregular cytoplasm with dark zones, and quality 4 includes no cumulus with irregular cytoplasm for those of quality 1 and 2, these oocytes were recovered with a pipette for *in vitro* maturation. The average oocyte yield per cow was determined as the ratio of the total number of oocytes to the total number of cows. The oocyte index (In) was calculated using the following formula:

In = ([quality I x 1+ quality II x 2 + quality III x 3 + quality IV x 4] / total number of oocytes) to assess the overall oocyte quality (Duygu et al., 2013). A value tending towards 1 reflects good overall oocyte quality.

Maturation of oocytes

Media used for maturation

The different culture media used for oocyte maturation were composed. Medium 1 included minimum essential medium (MEM), Medium 2 contained MEM + 10% follicular fluid, and Medium 3 consisted of MEM + 50% follicular fluid.

Follicular fluid

Follicular fluid was collected by puncturing the antral follicles, as described by Fahiminiya et al. (2010).

Oocyte collection and culture

After examination, counting under a stereoscope, and classification of the oocytes, the cultivable oocytes (class 1 and 2) were removed with a pipette and placed in a multi-well dish with 5-6 oocytes per well. Each well contained 1.5 ml of culture medium. The dish was wrapped in a plastic bag and incubated in an oven at 38°C under 5% CO2 for 24 hours.

Oocyte reading

After 24 hours of incubation, the oocytes were observed in their wells using an inverted microscope at 400 X magnification to observe the maturation rate. Oocytes considered mature were those with expanded cumuli (Figure 1). On the other hand, those whose cumuli persisted were considered immature (Figure 2).

Statistical analysis

All data were entered into Excel® and subjected to multifactor analysis of variance (multifactor ANOVA) using SPSS (Statistical data analysis software Package for Social Scientists, USA), version 20. Duncan's t-test was used to separate the means where there was a difference ($p < 0.05$).

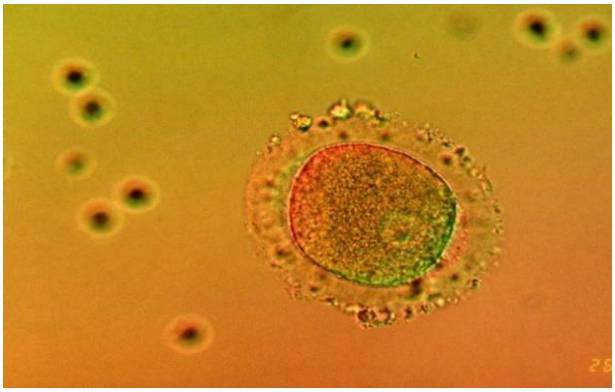

Figure 2. Mature oocyte in a cow

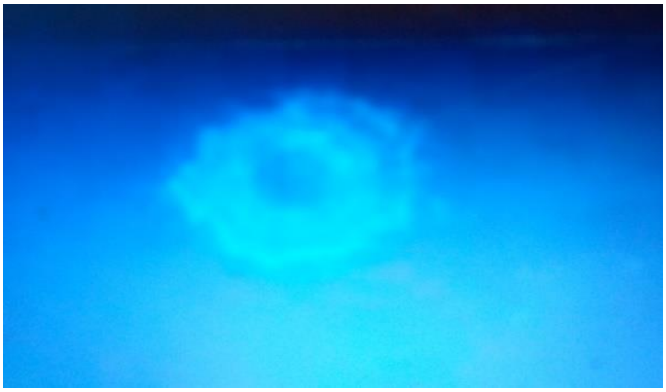

Figure 3. Immature oocytes in a cow

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RESULTS

Characterization of cows according to breed, age, body condition score, and physiological status

According to Table 2, the rate of pregnant cows was 13%. Regarding BCS, 31.66 of the slaughtered cows had BCS 3. The average age of the cows in the current study was 8 ± 0.88.

Determination of the follicular population

Figure 4 shows the follicle population in different classes. It can be seen that the number of follicles was inversely higher according to their size (small follicles: 14.32 ± 0.72, medium follicles: 10.25 ± 0.43, and large follicles: 0.61 ± 0.04).

Determination of oocyte class yield

Table 3 present the number of oocytes per class. As it can be seen, the number of oocytes was not spread out in different classes although a slight increase was observed in class I.

Table 2. Distributions of cows by breed, age, body condition score, and physiological status in Chad

Factors	Breed	Arab	Kouri	Toupouri	Average
BCS	[1-2]	17	19	15	17
	[3]	34	38	23	31.66
	[4-5]	19	15	26	20
	Total	70	72	64	-
Age (years)	[3-5]	42	43	39	41
	[6-9]	20	21	18	19.66
	[10 and more]	8	8	7	7
	Total	70	72	64	-
Physiological status	Not pregnant	59	57	51	55.66
	Pregnant	11	15	13	13
	Total	70	72	64	-

BCS: Body Condition Score

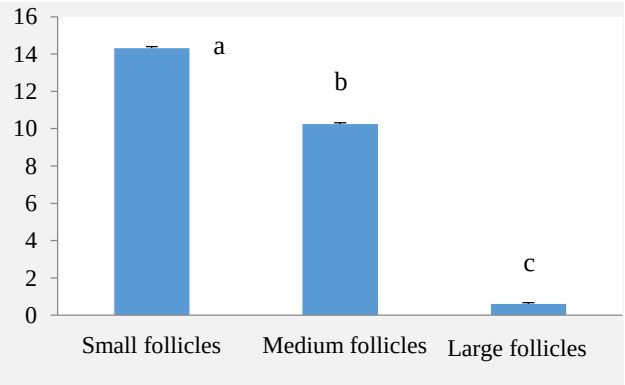

Figure 4. Determination of a follicular population of cows (Arab, Kouri, and Toupouri) in Chad as a function of size.

Table 3. Determination of oocyte class I, II, III, and IV yields of cows (Arab, Kouri, and Toupouri) in Chad

Oocyte class	Number
Class I	766
Class II	689
Class III	626
Class IV	653

Effects of breed and stage of the sexual cycle on follicle number

The breed of cattle did not significantly ($p > 0.05$) affect the average number of follicles. Furthermore, the follicle numbers recorded in metestrus and diestrus were comparable but significantly ($p < 0.05$) higher than the values recorded in proestrus and estrus independently of the breed of animals (Table 4).

Determination of oocyte yield by breed and stage of the sexual cycle

Table 5 shows the oocyte number according to breed and stage of the sexual cycle of the cows. The breed and stage of the sexual cycle did not significantly affect the oocyte average number ($p > 0.05$). Regardless of the sexual cycle stage, the comparison of the breeds indicated that Arab and Toupouri breeds recorded lower numbers of the oocyte in

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diestrus ($p < 0.05$) in reference to that recorded in Kouri. On the other hand, the number of oocytes recorded a significantly lower value in the Toupouri breed during the estrus stage when sexual cycle stages were considered regardless of breed ($p < 0.05$).

Determination of oocyte classes by breed and stage of the sexual cycle

Table 6 summarizes the effects of race and sexual cycle stage on oocyte classes. It is shown that race and stage of the sexual cycle did not significantly affect oocyte classes ($p > 0.05$). The number of oocytes generally increased with the evolution of the sexual cycle of the cows, but the difference was not significant ($p > 0.05$).

Effects of breed and body condition score on egg yield in cows

Table 7 shows the effects of breed and BCS on egg yield. The result indicated that breed and BCS did not significantly affect egg yield ($p > 0.05$). Cows with medium and fat BCS had significantly higher oocyte yields than lean cows ($p < 0.05$).

Effects of breed and body condition score on oocyte class

The effects of breed and body condition score on the oocyte class of cows are presented in Table 8. The breed and body condition scores did not significantly affect the oocyte class ($p > 0.05$). Cows with medium and high body reserves had significantly higher numbers of class I and IV oocytes, compared to lean cows ($p < 0.05$).

Effects of breed and body condition score on egg quality

The effects of breed and body condition score on egg quality is in Table 9. According to the table, the breed and body condition scores did not significantly affect oocyte quality ($p > 0.05$). Good quality oocytes (class I and II) were compared to poor ones (class III and IV, $p > 0.05$).

Effects of breed and age on egg yield

Table 10 shows the effects of breed and age on the egg yield of cows. The results revealed that breed did not significantly affect average egg yield ($p > 0.05$). However, cows aged 6-9 and 10-15 years had a significantly higher oocyte count than the 3-5 age group ($p < 0.05$).

Effects of breed and age on egg yield by class

The effects of breed and age on egg yield with regard to cow class, breed, and age are presented in Table 11. As it can be seen, breed and age did not significantly affect oocyte yield ($p > 0.05$). However, class II oocytes were significantly higher in Arab cows aged 10-15 years compared to other age groups ($p < 0.05$).

Number of oocytes collected for oocyte maturation

Table 12 shows the number of follicles and oocytes collected for oocyte maturation. Of 14, 9, and 13 ovaries selected from the Arab, Kouri, and Toupouri breeds, 210, 126, and 205 follicles were counted, respectively. From these follicles, a total of 113, 84, and 130 oocytes were recovered for oocyte maturation in the Arab, Kouri, and Toupouri breeds, respectively.

Distribution of oocytes by breed and class

The distribution of oocytes collected from a cow ovary sample by breed and class is listed in Table 13. In all breeds studied, good-quality oocytes (class I and II) were generally more than poor-quality oocytes (class III and IV). However, this rate was slightly higher with Arabian cows (56.97%), compared to Kouri and Toupouri (59.05% and 51.53%, respectively).

Oocyte maturation rate by breeds

Table 14 shows the oocyte maturation rate by breed and according to the different culture media. The culture medium of MEM + 10% follicular fluid showed a higher maturation rate for all breeds. This value was higher in the Arabian breed than in the Kouri and Toupouri breeds.

Effects of race and age on oocyte maturation rate

The effects of breed and age on oocyte maturation rate are shown in Table 15. It can be seen that the oocyte maturation rate was not significantly affected by breed ($p > 0.05$). On the other hand, cows belonging to the age group of 6-9 years showed a significantly higher oocyte maturation rate, compared to other age groups ($p < 0.05$).

Effects of breed and Body condition score on oocyte maturation rate

The effects of breed and body condition score on oocyte maturation rate are presented in Table 16. It is seen that the maturation rate was not significantly affected by breed and BCS ($p > 0.05$).

Relationships between different cow parameters

Positive and significant correlations were found between BSC and average ovarian weight ($r = 0.67$, $p < 0.01$) and between the total number of follicles and the total number of small follicles ($r = 0.93$, $p < 0.01$).

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Table 4. Effects of breed and sexual cycle phases on the number of follicles of cows (Arab, Kouri, and Toupouri) in Chad

	Races	Stage of the sexual cycle					p-value
		Prestrus (n = 35)	Estrus (n = 50)	Metestrus (n = 43)	Diestrus (n = 38)	Mean ± Standard deviation	
Total follicles	Arab (59)	23.93 ± 3.31^{aA}	25.67 ± 4.95^{abAB}	25.00 ± 17.79^{abA}	30.25 ± 4.16^{bA}	$25.98 \pm 1.89^{\alpha}$	0.02
	Kouri (57)	24.14 ± 2.39^{aA}	30.17 ± 3.50^{aB}	28.18 ± 3.45^{aAB}	30.63 ± 3.16^{aA}	$27.42 \pm 1.71^{\alpha}$	0.30
	Toupouri (50)	19.25 ± 2.64^{abA}	15.65 ± 1.62^{aA}	33.88 ± 3.02^{cB}	24.56 ± 1.90^{bA}	$24.47 \pm 13.86^{\alpha}$	0.04
	Mean ± Standard deviation	$21.81 \pm 1.68^{\alpha}$	$23.88 \pm 2.18^{\alpha}$	$29.91 \pm 1.81^{\beta}$	$28.63 \pm 1.54^{\beta}$	$25.37 \pm 11.36^{\alpha}$	-
	P-value	0.44	0.02	0.01	0.40	-	-

$^{a\,b\,c}$ Values with the same letter in a row do not differ significantly ($p > 0.05$). $^{A\,B\,C}$ Values with the same letter in a column do not differ significantly ($p > 0.05$). $^{\alpha\,\beta}$ Values with the same letter in a column or a row do not differ significantly ($p > 0.05$).

Table 5. Total number of oocytes according to breed and stage of the sexual cycle of cows (Arab, Kouri, and Toupouri) in Chad

	Races	Stage of the sexual cycle					p-value
		Prestrus (n =38 35)	Estrus (n = 50)	Metestrus (n = 43)	Diestrus (n = 38)	Mean ± Standard deviation	
Total oocyte	Arab	13.71 ± 1.77^{abA}	12.93 ± 2.45^{aAB}	12.33 ± 1.02^{aA}	14.17 ± 1.56^{bA}	$13.19 \pm 0.89^{\alpha}$	0.02
	Kouri	12.36 ± 1.00^{aA}	17.44 ± 2.24^{aB}	14.18 ± 1.58^{aAB}	16.13 ± 2.26^{aA}	$15.04 \pm 0.95^{\alpha}$	0.3
	Toupouri	11.25 ± 1.00^{aA}	9.12 ± 0.97^{aA}	17.25 ± 1.68^{cB}	13.33 ± 1.80^{bA}	$12.22 \pm 0.76^{\alpha}$	0.03
	Mean ± Standard deviation	$12.64 \pm 0.98^{\alpha}$	$13.26 \pm 1.22^{\alpha}$	$13.98 \pm 0.84^{\alpha}$	$14.63 \pm 1.67^{\alpha}$	$13.37 \pm 11.36^{\alpha}$	-
	P-value	0.44	0.01	0.20	0.4	-	-

$^{a\,b\,c}$ Values with the same letter in a row do not differ significantly ($p > 0.05$). $^{A\,B\,C}$ Values with the same letter in a column do not differ significantly ($p > 0.05$). $^{\alpha\,\beta}$ Values with the same letter in a column or a row do not differ significantly ($p > 0.05$).

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Table 6. Variation in oocyte class according to breed and stage of the sexual cycle of cows (Arab, Kouri, and Toupouri) in Chad

Classes	Race	Stage of the sexual cycle				Mean ± Standard deviation	p-value
		Proestrus (n = 35)	Eostrus (n = 50)	Metoestrus (n = 43)	Diestrus (n= 38)		
Class I	Arab	8.29 ± 1.16^{aB}	9.07 ± 1.97^{bAB}	8.28 ± 0.85^{aA}	9.43 ± 0.96^{bA}	$8.71 \pm 0.63^{\alpha}$	0.03
	Kouri	8.00 ± 0.85^{aAB}	11.22 ± 1.60^{aB}	9.29 ± 1.31^{aA}	9.80 ± 0.57^{aA}	$9.67 \pm 0.69^{\alpha}$	0.3
	Toupouri	6.88 ± 0.93^{aA}	6.00 ± 0.72^{aA}	10.88 ± 1.44^{aA}	8.89 ± 0.82^{aA}	$7.92 \pm 0.51^{\alpha}$	0.23
	Mean ± Standard deviation	$7.86 \pm 0.59^{\alpha}$	$8.80 \pm 0.86^{\alpha}$	$9.16 \pm 0.88^{\alpha}$	$9.26 \pm 0.51^{\alpha}$	$8.88 \pm 2.41^{\alpha}$	0.42
	P-value	0.03	0.01	0.23	0.3	-	-
Class II	Arab	3.36 ± 0.70^{aB}	3.27 ± 0.70^{aA}	3.33 ± 0.44^{aA}	2.53 ± 0.46^{aA}	$3.15 \pm 0.23^{\alpha}$	0.03
	Kouri	3.21 ± 0.28^{aAB}	4.28 ± 0.80^{aA}	3.35 ± 0.46^{aA}	4.63 ± 1.37^{aB}	$3.77 \pm 0.35^{\alpha}$	0.3
	Toupouri	2.88 ± 0.93^{aA}	3.18 ± 0.68^{aA}	4.38 ± 0.46^{aA}	3.23 ± 0.61^{aAB}	$3.33 \pm 0.35^{\alpha}$	0.23
	Mean ± Standard deviation	$3.19 \pm 0.34^{\alpha}$	$3.60 \pm 0.42^{\alpha}$	$3.53 \pm 0.22^{\alpha}$	$3.29 \pm 0.41^{\alpha}$	$3.88 \pm 2.41^{\alpha}$	0.42
	P-value	0.03	0.01	0.23	0.03	-	-
Class III	Arab	2.59 ± 0.58^{aA}	2.67 ± 0.53^{abAB}	2.61 ± 0.26^{aA}	3.27 ± 0.63^{bA}	$2.73 \pm 0.24^{\alpha}$	0.03
	Kouri	3.36 ± 0.55^{aA}	4.44 ± 0.66^{aB}	3.71 ± 0.57^{aAB}	2.23 ± 0.33^{aA}	$3.67 \pm 0.22^{\alpha}$	0.3
	Toupouri	3.25 ± 0.70^{bcA}	2.41 ± 0.43^{aA}	4.38 ± 0.42^{cB}	3.26 ± 0.63^{bcA}	$3.16 \pm 0.29^{\alpha}$	0.02
	Mean ± Standard deviation	$3.00 \pm 0.30^{\alpha}$	$3.22 \pm 0.32^{\alpha}$	$3.37 \pm 0.27^{\alpha}$	$3.08 \pm 0.37^{\alpha}$	$3.27 \pm 3.26^{\alpha}$	0.2
	P-value	0.40	0.02	0.03	0.69	-	-
Class IV	Arab	3.50 ± 0.65^{aB}	2.60 ± 0.57^{aAB}	3.17 ± 0.39^{aA}	3.75 ± 0.58^{aA}	$3.22 \pm 0.27^{\alpha}$	0.34
	Kouri	2.50 ± 0.56^{aAB}	4.00 ± 0.56^{bB}	3.82 ± 0.72^{baA}	5.35 ± 0.86^{cA}	$3.77 \pm 0.34^{\alpha}$	0.3
	Toupouri	2.00 ± 0.50^{aA}	1.710 ± 0.28^{aA}	4.00 ± 0.50^{bA}	3.61 ± 0.46^{bA}	$2.72 \pm 0.25^{\alpha}$	0.23
	Mean ± Standard deviation	$2.78 \pm 0.35^{\alpha}$	$2.80 \pm 0.30^{\alpha}$	$3.58 \pm 0.34^{\alpha}$	$4.02 \pm 0.34^{\alpha}$	$3.38 \pm 0.21^{\alpha}$	0.03
	P value	0.02	0.17	0.22	0.41	-	-

$^{a\,b\,c}$ Values with the same letter in a row do not differ significantly ($p > 0.05$). $^{A\,B\,C}$ Values with the same letter in a column do not differ significantly ($P > 0.05$). $^{\alpha\,\beta}$ Values with the same letter in a column or a raw do not differ significantly ($p > 0.05$).

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Table 7. Effects of breed and body condition score on egg yield of cows (Arab, Kouri, and Toupouri) in Chad

Egg yield	Race	Body condition score			Mean ± Standard deviation	p-value
		[1-2] n = 51	[3] n = 95	[4-5] n = 60		
Total egg	Arab	9.97 ± 1.23^{aA}	14.80 ± 1.29^{bA}	14.83 ± 1.78^{bA}	$13.13 \pm 0.89^{\alpha}$	0.02
	Kouri	8.50 ± 1.51^{aA}	13.39 ± 1.55^{bA}	15.45 ± 1.10^{bA}	$12.43 \pm 1.10^{\alpha}$	0.04
	Toupouri	7.44 ± 1.13^{aA}	11.41 ± 0.91^{bA}	16.19 ± 1.16^{cA}	$12.22 \pm 0.78^{\alpha}$	0.03
	Mean ± SD	$10.39 \pm 0.87^{\alpha}$	$13.90 \pm 0.73^{\beta}$	$13.62 \pm 0.89^{\beta}$	$11.71 \pm 0.19^{\alpha}$	-
	p-value	0.2	0.36	0.21	-	-

$^{a\,b\,c}$ Values with the same letter in a row do not differ significantly (P > 0.05). $^{A\,B\,C}$ Values with the same letter in a column do not differ significantly (P > 0.05). $^{\alpha\,\beta}$ Values with the same letter in a column or a raw do not differ significantly (P > 0.05), SD: Standard deviation

Table 8. Effects of breed and body condition score on oocyte class of cows (Arab, Kouri, and Toupouri) in Chad

Size of the follicles	Race	Body condition score			Mean ± Standard deviation	p-value
		[1-2] n = 51	[3] n = 95	[4-5] n = 60		
Class I	Arab	5.56 ± 0.70^{aA}	10.17 ± 0.99^{bA}	10.10 ± 1.09^{bA}	$8.793 \pm 0.68^{\alpha}$	0.03
	Kouri	8.88 ± 0.70^{abB}	9.91 ± 1.1^{aAB}	10.37 ± 1.4^{aA}	$9.67 \pm 0.69^{\alpha}$	0.64
	Toupouri	4.78 ± 0.66^{aA}	7.85 ± 0.64^{bB}	9.96 ± 0.69^{bA}	$7.95 \pm 0.52^{\alpha}$	0.04
	Mean ± SD	$6.51 \pm 0.50^{\alpha}$	$9.24 \pm 0.55^{\beta}$	$10.04 \pm 0.64^{\beta}$	$8.91 \pm 11.89^{\alpha}$	-
	p-value	0.03	0.02	0.36	-	-
Class II	Arab	2.78 ± 0.43^{aA}	3.37 ± 0.44^{aAB}	3.39 ± 0.45^{aA}	$3.12 \pm 0.42^{\alpha}$	0.78
	Kouri	3.79 ± 0.82^{aA}	3.57 ± 0.43^{aA}	4.20 ± 0.44^{aA}	$3.79 \pm 0.51^{\alpha}$	0.47
	Toupouri	2.00 ± 0.54^{aA}	3.15 ± 0.58^{abB}	4.38 ± 0.54^{bA}	$3.33 \pm 0.31^{\alpha}$	0.03
	Mean ± SD	$2.91 \pm 0.33^{\alpha}$	$3.35 \pm 0.26^{\alpha}$	$\pm 0.33^{\alpha}$	$3.52 \pm 0.21^{\alpha}$	-
	p-value	0.98	0.02	0.31	-	-
Classe III	Arab	1.77 ± 0.23^{aA}	3.27 ± 0.49^{bA}	3.00 ± 0.48^{bA}	$2.73 \pm 0.24^{\alpha}$	0.02
	Kouri	3.34 ± 0.39^{aB}	3.70 ± 0.35^{aA}	3.80 ± 0.29^{aA}	$3.64 \pm 0.79^{\alpha}$	0.76
	Toupouri	2.00 ± 0.45^{aA}	2.85 ± 0.36^{aA}	4.31 ± 0.46^{bA}	$3.17 \pm 0.28^{\alpha}$	0.02
	Mean ± SD	$2.37 \pm 0.22^{\alpha}$	$3.28 \pm 0.23^{\alpha}$	$3.72 \pm 0.31^{\alpha}$	$3.41 \pm 0.19^{\alpha}$	-
	p-value	0.02	0.36	0.21	-	-
Class IV	Arab	2.67 ± 0.48^{aAB}	3.29 ± 0.40^{bA}	3.78 ± 0.43^{bA}	$3.28 \pm 0.29^{\alpha}$	0.02
	Kouri	3.50 ± 0.68^{abB}	4.04 ± 0.54^{bB}	3.47 ± 0.55^{aA}	$3.77 \pm 0.30^{\alpha}$	0.03
	Toupouri	1.63 ± 0.35^{aA}	2.87 ± 0.35^{abA}	3.34 ± 0.51^{bA}	$2.78 \pm 0.28^{\alpha}$	0.52
	Mean ± SD	$2.73 \pm 0.29^{\alpha}$	$3.42 \pm 0.23^{\beta}$	$3.51 \pm 0.22^{\beta}$	$3.37 \pm 0.92^{\alpha}$	-
	P-value	0.01	0.03	0.06	-	-

$^{a\,b\,c}$ Values with the same letter in a row do not differ significantly (P > 0.05). $^{A\,B\,C}$ Values with the same letter in a column do not differ significantly (P > 0.05). $^{\alpha\,\beta}$ Values with the same letter in a column do not differ significantly (P > 0.05), SD: Standard deviation

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Table 9. Effects of breed and body condition score on egg quality of cows (Arab, Kouri, and Toupouri) in Chad

Egg quality	Race	Body condition score			Mean ± Standard deviation	p-value
		[1-2] n = 51	[3] n = 95	[4-5] n = 60		
Class I and II	Arab	16.54 ± 2.90^{aA}	15.47 ± 1.89^{aA}	16.15 ± 1.89^{aA}	$15.93 \pm 1.38^{\alpha}$	0.46
	Kouri	17.93 ± 2.60^{bA}	16.71 ± 2.3^{abA}	13.27 ± 2.49^{aA}	$16.12 \pm 2.39^{\alpha}$	0.04
	Toupouri	8.22 ± 1.55^{aA}	10.40 ± 1.04^{bA}	13.96 ± 1.22^{cA}	$11.65 \pm 0.92^{\beta}$	0.04
	Mean ± SD	$14.60 \pm 2.18^{\alpha}$	$14.26 \pm 1.41^{\alpha}$	$14.73 \pm 1.46^{\alpha}$	$14.51 \pm 1.59^{\alpha}$	-
	P-value	0.43	0.25	0.36	-	-
Class III and IV	Arab	6.78 ± 0.83^{bA}	10.27 ± 1.94^{aA}	10.79 ± 1.75^{aA}	$9.32 \pm 0.62^{\alpha}$	0.78
	Kouri	9.79 ± 1.12^{aA}	13.00 ± 1.19^{bA}	10.87 ± 1.51^{aA}	$11.65 \pm 1.21^{\alpha}$	0.47
	Toupouri	6.69 ± 1.04^{aA}	10.69 ± 1.06^{bA}	13.00 ± 0.60^{cA}	$10.67 \pm 0.64^{\alpha}$	0.03
	Mean ± SD	$8.31 \pm 1.63^{\alpha}$	$11.46 \pm 1.56^{\alpha\beta}$	$11.57 \pm 1.53^{\beta}$	$10.32 \pm 0.21^{\alpha}$	-
	P-value	0.98	0.34	0.31	-	-

$^{a\ b\ c}$ Values with the same letter in a row do not differ significantly (P > 0.05). $^{A\ B\ C}$ Values with the same letter in a column do not differ significantly (P > 0.05). $^{\alpha\ \beta}$ Values with the same letter in a column or a row do not differ significantly (P > 0.05), SD: Standard deviation

Table 10. Effects of breed and age on egg yield of cows **(**Arab, Kouri, and Toupouri) in Chad

Oocyte yield	Race	Age (years)			Mean ± Standard deviation	p-value
		[3-5] n = 123	[6-9] n = 60	[10-15] n = 23		
Total oocyte	Arab	12.00 ± 0.99^{aA}	13.76 ± 1.24^{abA}	18.25 ± 1.69^{bB}	$13.72 \pm 0.72^{\alpha}$	0.03
	Kouri	13.21 ± 0.99^{abB}	17.45 ± 1.79^{bA}	12.00 ± 0.70^{aA}	$14.34 \pm 0.84^{\alpha}$	0.02
	Toupouri	11.33 ± 0.74^{aA}	14.44 ± 1.51^{bA}	13.00 ± 2.70^{abA}	$13.43 \pm 0.64^{\alpha}$	0.03
	Mean ± SD	$12.23 \pm 0.53^{\alpha}$	$15.20 \pm 0.78^{\beta}$	$14.34 \pm 1.00^{\beta}$	-	-
	P-value	0.98	0.34	0.02	-	-

$^{a\ b\ c}$ Values with the same letter in a row do not differ significantly (p > 0.05). $^{A\ B\ C}$ Values with the same letter in a column do not differ significantly (P > 0.05). $^{\alpha\ \beta}$ Values with the same letter in a row do not differ significantly (P > 0.05). $^{\alpha.\ \beta}$ Values with the same letter in a column do not differ significantly (p > 0.05). SD: Standard deviation

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Table 11. Effects of breed and age on egg yield by class of cows **(**Arab, Kouri, and Toupouri) in Chad

Oocyte class	Race	Age (years)			Mean ± Standard deviation	p-value
		[3-5] n = 123	[6-9] n = 60	[10-15] n = 23		
Class I	Arab	3.33 ± 0.38^{aA}	6.75 ± 1.19^{bB}	4.69 ± 0.59^{abA}	$4.27 \pm 0.48^{\alpha}$	0.03
	Kouri	3.64 ± 0.50^{abA}	4.32 ± 0.47^{bA}	3.17 ± 0.69^{aA}	$3.78 \pm 0.39^{\alpha}$	0.64
	Toupouri	3.00 ± 0.29^{aA}	3.00 ± 0.40^{aA}	3.75 ± 0.92^{aA}	$3.05 \pm 0.22^{\alpha}$	0.42
	Mean ± Standard deviation	$3.28 \pm 0.28^{\alpha}$	$4.30 \pm 0.341^{\alpha}$	$4.51 \pm 0.74^{\alpha}$	$3.94 \pm 0.29^{\alpha}$	-
	P-value	0.43	0.25	0.36	-	
Class II	Arab	3.00 ± 0.39^{abA}	4.50 ± 0.56^{bAB}	4.50 ± 0.56^{bB}	$3.10 \pm 0.62^{\alpha}$	0.02
	Kouri	3.31 ± 0.38^{aA}	4.95 ± 0.69^{bB}	$2.91 \pm .48^{aA}$	$3.75 \pm 0.21^{\alpha}$	0.47
	Toupouri	3.02 ± 0.34^{aA}	4.44 ± 0.60^{bA}	4.00 ± 0.70^{bAB}	$3.30 \pm 0.24^{\alpha}$	0.03
	Mean ± Standard deviation	$3.11 \pm 0.225^{\alpha}$	$3.87 \pm 0.37^{\alpha}$	$3.65 \pm 0.33^{\alpha}$	$3.82 \pm 0.31^{\alpha}$	-
	P-value	0.98	0.04	0.03	-	-
Class III	Arab	2.61 ± 0.23^{aA}	3.00 ± 0.39^{aA}	3.13 ± 0.62^{aB}	$2.83 \pm 0.37^{\alpha}$	0.52
	Kouri	3.14 ± 0.30^{aA}	3.77 ± 0.14^{aA}	2.82 ± 0.45^{aA}	$3.31 \pm 0.26^{\alpha}$	0.76
	Toupouri	2.75 ± 0.20^{abA}	4.44 ± 0.39^{bA}	1.79 ± 0.86^{aA}	$2.97 \pm 0.26^{\alpha}$	0.02
	Mean ± Standard deviation	2.86 ± 0.16	$3.52 \pm 0.29^{\alpha}$	$2.76 \pm 0.33^{\alpha}$	$2.871 \pm 0.23^{\alpha}$	-
	P-value	0.12	0.36	0.21	-	-
Class IV	Arab	3.06 ± 0.35^{aA}	2.97 ± 0.380^{aAB}	4.13 ± 0.38^{bA}	$3.14 \pm 0.24^{\alpha}$	0.69
	Kouri	3.07 ± 0.32^{aA}	4.47 ± 0.62^{bB}	3.09 ± 0.63^{aA}	$3.50 \pm 0.20^{\alpha}$	0.03
	Toupouri	2.82 ± 0.25^{aA}	2.54 ± 2.90^{aA}	3.53 ± 0.51^{aA}	$2.82 \pm 0.38^{\alpha}$	0.52
	Mean ± Standard deviation	$2.96 \pm 0.18\alpha$	$3.43 \pm 0.32\alpha$	$3.53 \pm 0.32\alpha$	$3.57 \pm 0.52\alpha$	-
	P-value	0.6	0.02	0.6	0.5	-

$^{a\,b\,c}$ Values with the same letter in a row do not differ significantly ($p > 0.05$). $^{A\,B\,C}$ Values with the same letter in a column do not differ significantly ($p > 0.05$). $^{\alpha\,\beta}$ Values with the same letter in a column do not differ significantly ($p > 0.05$).

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Table 12. Number of follicles and oocytes recovered per breed of cows **(**Arab, Kouri, and Toupouri) in Chad

Breed of cows	Number of ovaries	Number of follicles	Number of oocytes
Arab	04	59	21
	06	88	52
	04	63	40
Total	**14**	**210**	**113**
Kouri	02	36	25
	04	51	39
	03	39	20
Total	**09**	**126**	**84**
Toupouri	03	55	31
	07	102	79
	03	48	20
Total	**13**	**205**	**130**

Table 13. Distribution of collected oocytes by race and class of cows **(**Arab, Kouri, and Toupouri) in Chad

Race	Oocyte numbers	Oocytes class 1	Oocytes class 2	Oocytes class 3	Oocytes class 4	Oocytes For cultivation (Class 1 and 2)	Proportion (%)
Arab	21	07	06	05	03	13	61.90
	52	15	17	13	07	32	61.52
	40	08	11	09	12	19	47.5
Total	**113**	**30**	**34**	**27**	**22**	**64**	**56.97**
Kouri	25	8	6	8	3	14	56
	39	7	11	9	12	18	46.15
	20	6	9	1	4	15	75
Total	**84**	**21**	**26**	**18**	**19**	**47**	**59.05**
Toupouri	31	12	4	7	8	16	51.61
	79	19	21	22	17	40	50.63
	21	6	5	5	5	11	52.38
Total	**131**	**37**	**30**	**34**	**30**	**67**	**51.53**

Table 14. Oocyte maturation rates per breed and according to the different culture media of cows **(**Arab, Kouri, and Toupouri) in Chad

Race	Composition of medium	Number of oocytes in culture	Number of expanded oocytes	Number of immature oocytes	Rate of maturation (%)
Arab	MEM only	13	4	9	30.76
	MEM + 10% follicular liquid	32	13	19	40.62
	MEM + 50% follicular liquid	19	3	16	15.78
Kouri	MEM only	14	3	11	35.71
	MEM + 10% follicular liquid	18	7	11	38.88
	MEM + 50% follicular liquid	15	4	11	26.66
Toupouri	MEM only	16	4	12	25
	MEM + 10% follicular liquid	40	12	28	30
	MEM + 50% follicular liquid	11	3	8	27.27

MEM: Minimum essential medium

Table 15. Effects of breeds and age on oocyte maturation rate of cows **(**Arab, Kouri, and Toupouri) in Chad

Maturation	Breeds	Age (years)			Mean ± Standard deviation	p-value
		[3-5] n=12	[6-9] n=12	[10-15] n=12		
Rate of maturation (%)	Arab	12.50 ± 7.50^{aA}	43.75 ± 6.20^{bB}	31.25 ± 11.66^{abA}	$28.17 \pm 6.02^{\alpha}$	0.04
	Kouri	25.00 ± 10.43^{aAB}	37.50 ± 7.20^{aAB}	25.00 ± 9.35^{aA}	$29.37 \pm 5.19^{\alpha}$	0.64
	Toupouri	31.25 ± 11.25^{aB}	25.00 ± 7.21^{aA}	31.25 ± 6.95^{aA}	$29.17 \pm 5.78^{\alpha}$	0.3
	Mean ± SD	$22.92 \pm 7.78^{\alpha}$	$35.47 \pm 6.541^{\beta}$	$29.17 \pm 7.44^{\alpha\beta}$	$29.27 \pm 5.79^{\alpha}$	-
	P-value	0.04	0.02	0.36	-	-

$^{a, b c}$ Values with the same letter in a row do not differ significantly (P > 0.05). $^{A, B C}$ Values with the same letter in a column do not differ significantly (P > 0.05). $^{\alpha\beta}$ Values with the same letter in a column or row do not differ significantly (p > 0.05).

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Table 16. Effects of race and body condition score on oocyte maturation rate of cows (Arab, Kouri, and Toupouri) in Chad

Maturation	Breeds	Body condition score			Mean ± Standard deviation	p-value
		[1-2] n=12	[3] n=12	[4-5] n=12		
Rate of maturation (%)	Arab	25.00 ± 11.80^{aA}	25.08 ± 6.20^{aA}	31.25 ± 16.66^{aA}	$27.07 \pm 5.02^{\alpha}$	0.46
	Kouri	32.25 ± 6.50^{aA}	37.50 ± 6.20^{aA}	31.25 ± 7.35^{aA}	$33.33 \pm 3.19^{\alpha}$	0.06
	Toupouri	33.00 ± 9.25^{bB}	30.00 ± 9.21^{abA}	18.75 ± 11.95^{aA}	$27.08 \pm 7.78^{\alpha}$	0.02
	Mean ± SD	$29.17 \pm 6.78^{\alpha}$	$29.17 \pm 9.541^{\alpha}$	$29.17 \pm 22.44^{\alpha}$	$29.17 \pm 5.79^{\alpha}$	-
	P-value	0.03	0.25	0.36	-	-

$^{a\,b\,c}$ Values with the same letter in a row do not differ significantly ($p > 0.05$). $^{A\,B\,C}$ Values with the same letter in a column do not differ significantly ($P>0.05$). $^{\alpha\,\beta}$ Values with the same letter in a column do not differ significantly ($p > 0.05$).

DISCUSSION

The present study on the effects of ovarian and non-ovarian factors on the follicle population and oocyte maturation of three cattle breeds in Chad (Arab, Kouri, and Toupouri) revealed that the cows in the current study had a BCS of 3.3 ± 0.34. This value is higher than those reported by Azafack et al. (2019), which was 2.93 ± 0.64 in Cameroon. The reason can be the long duration of the dry season and the scarcity of pasture in Chad. Indeed, BCS offers a good estimate of the quantity of lipids stored, and its variations are a good indicator of the energy balance (Lefebvre et al., 2022). It also allows an indirect assessment of the animal's nutritional status (Leperre et al., 1992). In Chad, the animals do not receive food supplements. The livestock system in this area has retained a traditional character characterized by extensive herd management on natural pasture (Zampaligré et al., 2019), which could negatively affect animal performance. The animal is finally sold in poor condition to butchers or slaughterers who take it to the slaughterhouse. The present study showed that most of the cows slaughtered were young (3 to 5 years old). The decision to send young cows to the slaughterhouse could be explained by the fact that older cows (10-15 years old) were lighter due to their poor nutritional status. The proportion of pregnant cows slaughtered at the slaughterhouse during our study was 13%, lower than the 21.34% reported by Alaku and Orjiude (1991). The slaughter of pregnant cows is against state veterinary legislation and reflects the negligence of the antemortem examination, which must be applied to animals before their referrals to the slaughterhouse.

The average number of follicles per cow in this study was 24.71 ± 0.88. This value is higher than the 5.20 reported by Kumar et al. (1997) in India and 23 examined by Taneja et al. (2000) in the same country but lower than the 37.5 ± 25.2 follicles estimated by Kouamo et al. (2014) in Cameroun and 32 follicles measured by Takaji et al. (1992) for the ovaries of a cow reared in Japan. This variation in the number of follicles would be linked to parity (number of births) and the breed of the cow (Rhodes et al., 1995). The average oocyte yield per cow was 13.27 ± 0.14, lower than the results reported by Humblot et al. (2005). This difference can be related to the collection technique. Indeed, Cognié and Baril (2002) noted that 4 to 5 additional oocytes could be obtained after cutting the ovary with a razor blade (slicing technique), compared to other techniques, such as aspiration and the ovum pick-up techniques. In the present study, the overall maturation rate (32.25%) was lower than that of Margaux (2022, 67%) using the same technique. This low maturation rate could be explained by the temperature at which the ovaries were stored during transport. Indeed, keeping the ovaries at a low temperature increases the maturation rate. Bohlooli et al. (2015) reported a higher maturation rate when transporting ovaries stored at 4°C, compared to 25°C and 38°C. According to Wang et al. (2007), low temperature would reduce cellular metabolism, but at 15°C, it would reduce the apoptosis index. The preservation of the ovaries during transport is a key element in maintaining the maturation competence of oocytes (Margaux, 2022). The low rate of maturation obtained in the current work could be due to the enrichment of the culture medium by the follicular fluid of the large follicles (mature follicles). According to Choi et al. (1998), follicular fluid from mature follicles has a reduced inhibitory effect on oocyte maturation, compared to follicular fluid collected from small and medium follicles. For Takahashi (1982), certain substances, such as hormones in the follicular fluid, prevent meiosis's resumption during maturation. Choi et al. (1998) showed that bovine follicular fluid would inhibit nuclear maturation and instead increase cytoplasmic maturation as indicated by pronuclear formation. According to Sadeesh et al. (2014), slowing nuclear maturation would give the oocyte more time to synthesize, modify and store new proteins and ribonucleoproteins and thus improve its competence. A recent study found that follicular fluid could promote cytoplasmic maturation of oocytes during in vitro maturation (Armstrong, 2001). Cows between 6 and 9 years old had a higher oocyte maturation rate than cows over 10 years old. This result agrees with that of Natumanya et al. (2008) and Kouamo et al. (2014), indicating a significantly higher oocyte maturation rate in cows belonging to the 6-9-year age group.

The current study revealed that cows with average and high body condition scores had significantly higher oocyte yields than lean cows ($p < 0.05$). For example, an oocyte yield of 13.90 ± 0.73 was observed in cows with an average BCS (BSC = 3) and an oocyte yield of 13.62 ± 0.89 in fat cows (BSC = 4-5), compared to an oocyte yield of 10.39 ± 0.87 observed in thin cows (BSC = 1-2). The positive correlation observed between BCS and oocyte yield in this study could be explained by the fact that BSC significantly affects the number of follicles, yield, and oocyte quality. Rhind et

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al. (1989), Dominguez (1995), and Kumar et al. (1997) support the effect of nutrition on reproductive processes at the ovarian level. It should be noted that diet influences all reproductive parameters, including cyclicity, fecundity, fertility, prolificacy, and embryonic development (Celine, 2022).

CONCLUSION

Regarding the effects of ovarian and non-ovarian factors on the follicular population and oocyte maturation of three bovine breeds (Arab, Kouri, and Toupouri) in Chad, the ovaries collected from slaughtered cows are an important source of oocytes for *in vitro* production. Age, body condition score, ovarian weight, and stage of the sexual cycle of cows influence the follicular population and oocyte maturation. The culture medium with reduced follicular fluid (10%) increases the oocyte maturation rate.

DECLARATIONS

Authors' contributions

Souleyman Hachim and Ferdinand Ngoula conceived, designed the research, and reviewed the manuscript. Hervé Tchoffo and Dorice Kana Azafack collected the data, carried out data analysis, and wrote the manuscript. All authors read and approved the final manuscript.

Ethical consideration

The work was conducted based on the ethical rules of the National Institute of Science and Technology of Abeche, Chad. The authors thoroughly examined all ethical concerns surrounding plagiarism, consent to publish, misconduct, data fabrication, falsification, duplicate publishing or submission, and manuscript redundancy.

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Competing interests

There was no competing interest in the submission and processing of this article.

Competing interests

The authors declare no conflict of interest.

Availability of data and materials

The datasets generated for this study are available on request to the corresponding author.

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Antibiotic Resistance of *Escherichia coli* and *Salmonella* Species Isolated from Table Eggs in Morocco

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ABSTRACT

The development of antimicrobial resistance has become a severe global public health emergency. Foods of animal origin are considered possible drivers of resistant bacteria, including *Escherichia coli* (*E. coli)* and *Salmonella* spp. It is associated with the indiscriminate use of antibiotics, resulting in the inability to treat patients infected with antibiotic-resistant pathogens and a high risk of transmission of these resistant pathogens. The current study aimed to determine the prevalence and antibiotic resistance of *E. coli* and *Salmonella* spp. in raw table eggs in Morocco. A total of 870 table eggs resulting from 290 samples (3 eggs = 1 sample), were purchased from ambulatory sellers, street vendors, kiosks, and neighborhood markets from different cities in Morocco and transferred to the laboratory in the Hassan II Agronomy and Veterinary Medicine Institute of Rabat, Morocco. The egg shells and contents were tested separately then the isolation and identification of bacterial pathogens were performed according to the Moroccan Standard Norms. The bacterial isolates were tested for susceptibility to six commonly used antibiotics, namely nalidixic acid (30 µg), kanamycin (30 µg), gentamycin (15 µg), ciprofloxacin (15 µg), tetracycline (30 µg), and amoxicillin (10 µg). The findings revealed that 38 samples (13%) tested positive for *E. coli* of which 9% were on egg shells, and 4% were in egg content, while for *Salmonella enteritidis* (*S. enteritidis*), 5 samples (2%) tested positive and only in the egg contents. *Escherichia coli* showed the highest resistance to amoxicillin, followed by tetracycline and nalidixic acid with 92.10%, 84.21%, and 50%, respectively, and was sensitive to ciprofloxacin (84.21%), kanamycin (65.79%), and gentamicin (60.54%). *Salmonella enteritidis* had the highest resistance against tetracycline (80%), followed by ciprofloxacin and nalidixic acid with 40% each. The highest sensitivity rates of *S. enteritidis* were for gentamicin, amoxicillin, and kanamycin at 80%, 80%, and 40%, respectively. Given that these resistant bacteria could potentially be transferred to humans through eggs or egg products, it is necessary to employ strict hygiene measures and provide a wise and legal use of antibiotics in animal breeding.

Keywords: Antibiotic resistance, *Escherichia coli*, *Salmonella enteritidis*, Table egg

INTRODUCTION

Antimicrobial resistance (AMR) is one of the world's most challenging problems today (WHO, 2021). The inappropriate use of antibiotics, including their use in animal production systems as growth promoters and their overuse in clinical treatments, has created selective pressure on bacteria in recent years leading to defense systems against these antibiotics and a therapeutic impasse (Zhao et al., 2012; Roca et al., 2015; Zwe et al., 2018). It is estimated that AMR leads to the annual death of approximately 700,000 people worldwide (Clifford et al., 2018). These resistant bacteria may contaminate humans directly through cross-contamination or/and handling of contaminated food or indirectly when contaminated food or food products are consumed (Collignon et al., 2016; Lambrecht et al., 2018).

Among the foods responsible for AMR transmission, poultry, and poultry products act as the primary vector for transferring antimicrobial-resistant bacteria and antimicrobial-resistance genes to humans (de Mesquita Souza Saraiva et al., 2022) since the inappropriate use of antibiotics as a treatment and growth promoters at sub-therapeutic doses can lead to their development. However, many bacteria are isolated from poultry meats and products, WHO considers *Salmonella* spp. and *Escherichia coli* (*E. coli*) as the most responsible bacteria for AMR transmission (WHO, 2021). These two bacteria have been shown to cause major infectious diseases in both poultry and humans. The *E. coli* is the main causative agent of cellulitis, septicemia, and aerosacculitis in poultry, and *Salmonella* spp. is the causative agent of

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pullorum disease, avian typhoid, and avian paratyphoid (Gomis et al., 1997). In addition to mild to severe gastrointestinal diseases, *E. coli* can cause urinary tract infections, pneumonia, meningitis, and peritonitis in humans (Schoeni and Doyle, 1994). *Salmonella* spp. can also cause human foodborne gastroenteritis, a disease characterized by intestinal inflammation and self-limited diarrhea (Winter et al., 2010).

Moreover, *E. coli* and *Salmonella enterica* subsp. *enterica* serovars are the most common avian pathogens that can be vertically transmitted through eggs (Singh et al., 2010). Many studies have isolated *Salmonella* spp. and *E. coli* from eggs (Adesiyun et al., 2005; Stepien, 2010; El ftouhy et al., 2022), and several egg-borne outbreaks of salmonellosis have been reported (Guerra-Centeno et al., 2020), considering eggs a possible vehicle for resistant bacteria and genes.

In Morocco, despite the increasing demand for the production (5.5 billion units in 2020, according to FISA) and consumption of table eggs, there is a lack of information on their microbiological quality, foodborne pathogens, and AMR, particularly *E. coli* and *Salmonella enteritidis* (*S. enteritidis*), isolated from eggs or egg products. Also, previous studies have demonstrated that treatment failures were linked to reports of increased antimicrobial resistance (Filali et al., 1988; Amara et al., 1995). Therefore, the current study aimed to investigate the occurrence and the antimicrobial resistance of *Salmonella* spp. and *E. coli* in fresh table eggs in Morocco.

MATERIALS AND METHODS

Ethical approval

All procedures in the present study were carried out following the Hassan II Agronomic and Veterinary Institute of Rabat, Morocco, and Moroccan Ministry of Agriculture recommendations, which are in accordance with international ethical standards (European Union Directive 2010/63/EU) legislation and ARRIVE (Animal Research Reporting of *in vivo* Experiments) guidelines.

Sample collection

A total of 870 table eggs, resulting from 290 samples of 3 eggs each, were purchased from ambulatory sellers, street vendors, kiosks, and neighborhood markets from January to September 2021. Eggs were kept under ambient temperature on the markets. Samples were collected from different locations in Morocco, namely Kenitra, Sale, Rabat, Temara, Mohammedia, Casablanca, and Benslimane. Once collected, eggs were transferred aseptically to the microbiology laboratory of the Avian Pathology Unit at the Hassan II Agronomy and Veterinary Medicine Institute in Rabat, Morocco, to run different analyses for egg shells and contents.

Egg shells

A swab technique was applied. The surface of the whole eggs was aseptically swabbed with a sterile cotton swab moistened in a sterile distilled water solution (Adesiyun et al., 2005).

Egg content

Eggs were soaked in 70% ethanol for 5-10 seconds to disinfect and then air-dried near the Bunsen burner. Then, the egg contents (3 eggs) were decanted and pooled into a sterile stomacher bag before finally being mixed manually for 30 seconds to obtain a homogeneous mixture. Both swabs and a mixture of egg contents were used separately to inoculate 9 ml of water peptone buffer and incubated at 37°C for 18-24 hours (Adabara et al., 2020).

Isolation of *Salmonella*

For *Salmonella* spp. isolation, 0.1 ml of the pre-inoculated buffered peptone water (CM 0509 Oxoid, Oxoid LTD, Basingstoke, Hampshire, England) was transferred to 10 ml of Rappaport-Vassiliadis Soja (RVS; BK148HA Biokar diagnostics, Zac de Ther, France) and incubated at 42°C for 24 hours. A loopful of RVS was transferred to Xylose lysine deoxycholate (BK058HA Biokar diagnostics, Zac de Ther, France) agar and incubated at 37°C for 24-48 hours according to the Moroccan standard NM ISO 6579, 2007 (NM 08.0.103), which is similar to the international Norm (ISO, 2002).

Isolation of *Escherichia coli*

For *E. coli*, a loopful of the broth water peptone buffer was subcultured on Eosin Methylene Blue agar (EMB agar; CM 0069 Oxoid, Oxoid LTD, Basingstoke, Hampshire, England) and then incubated at 37°C for 24 hours (Siriporn et al., 2015). All isolated bacteria were identified according to their colony, color, shape, morphology, and color change of the culture media. They were also dye-stained with Gram stains and examined under a × 100 light microscope (OPTIKA B-151, ITALY) using oil immersion. In addition, the performed biochemical tests included coagulase (6BR0020, Biokar diagnostics, Zac de Ther, France), catalase (1840, SOLVAPUR**,** SOLVACHIM, Morocco), oxidase (MICROBAT Oxoid LTD, United Kingdom), and motility tests using Mannitol Motility Nitrate Medium (M1320, HI Media laboratories,

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Mumbai, India). The semisolid nature of this medium helps to detect motility due to 0.35% agar. This detection was also confirmed by API 20E (20 100, biomerieux, Marcy-l'Etoile, France) for further biochemical identification (Abdullah, 2010).

Antibiotic sensitivity tests

Individual colonies of confirmed isolates of *S. enteritidis* and *E. coli* spp. were suspended in normal saline to McFarland standards of 0.5 and then were inoculated onto the Mueller-Hinton agar surface (Bk048HA Biokar diagnostics zac de ther, France). Antibiotic discs were aseptically placed on the inoculation medium with sterile forceps and incubated at 37°C for 24 hours. After incubation, the inhibition zone diameter around the antibiotic discs was measured, and sensitivity was determined. Results were interpreted later according to the criteria of the Clinical and Laboratory Standards Institute Performance Standards for Antimicrobial Susceptibility Testing (CLSI, WEINSTEIN, 2018). Antibiotic susceptibility testing was performed against six antibiotics of nalidixic acid (30 µg), kanamycin (30 µg), gentamycin (15 µg), ciprofloxacin (15 µg), tetracycline (30 µg), and amoxicillin (10 µg) purchased from Oxoid LTD, England. These antibiotics were selected according to their availability on the market and everyday use in the poultry industry worldwide.

Statistical analysis

The data obtained in this study were analyzed using the Statistical Package for the Social Sciences (SPSS, version 22). One-way analysis of variance (ANOVA) and Duncan's test were used to determine significant differences between eggshell and egg content contamination for each bacterium studied. The $p < 0.05$ was considered statistically significant. Results were also calculated and presented as a percentage using Excel spreadsheets.

RESULTS AND DISCUSSION

The table egg is a major food in the human diet due to its high nutritional value, availability, and low cost. However, it can be contaminated with harmful bacteria leading to serious foodborne diseases. The obtained results of the current study revealed that 15% of the samples tested positive for microbial contamination. Among them, 38 samples (13%) tested positive for *E. coli*, and 5 samples (2%) for *S. enteritidis* (Table 1).

The presence of *E. coli* in eggs taken from different sites in the present study may result from poor sanitary practices and conditions since *E. coli* is an indicator of hygienic quality (Carter and Cole, 1990). The results indicated that the prevalence rates of *E. coli* isolated from egg shells and egg contents were 9% and 4%, respectively. There was a significant difference between the contamination of the shells and the contents ($p < 0.05$) since this bacterium commonly contaminates the surface of eggs. As previously reported, the bacteria most frequently isolated from egg shells are Gram-negative bacteria, such as *E. coli* (Papadopoulou et al., 1997; Musgrove et al., 2004). The presence of this bacterium in the eggshell more than in the egg content can be explained by the fact that the eggs are supposed to be sterile due to their defense system (USDA, 2011).

The detection of *E. coli* in the shell may be due to the presence of feces, soil, dust, poor hygienic conditions during manipulation, contaminated egg crates, transportation, and commercialization, while the contamination recorded in the egg contents of the examined samples may be attributed to the fact that the laying hens are carriers of the pathogen before the shell formation (Gantois et al., 2009). Dirt in the nests can also contaminate the egg shells; therefore, the pathogen can move from the outside to the inside of the egg (USDA, 2011).

Several studies on different parts of the world, including Bangladesh (Haque et al., 2021), India (Arathy et al., 2009), Egypt (Mansour et al., 2015), Nigeria (Adabara et al., 2020), and South Africa (Jambalang et al., 2017) have revealed the presence of *E. coli* in table eggs. The same prevalence of *E. coli* in egg shells found in this study was previously reported in farm eggs in Nigeria (9.1%, Adabara et al., 2020). Regarding the egg shells, the prevalence of *E. coli* was detected in Zambia (Kapena et al., 2020), and Thailand (Siriporn et al., 2015). Besides, *E. coli* was isolated from egg contents in several previous studies conducted in Trinidad, Egypt, and Nigeria (Adesiyun et al., 2005; Mansour et al., 2015; Adabara et al., 2020).

The isolation rate of *S. enteritidis* in the table eggs tested was 2%. This prevalence was only observed in the egg contents since the shells were *S. enteritidis* free; therefore, the difference was not statistically significant ($p > 0.05$). The findings of the present study suggest that during the egg-laying process, the egg passes through the common part of the reproductive and digestive tracts where contamination may occur. In addition, the existence of *S. enteritidis* in the hen's ovary or oviduct (prior to shell formation) may also result in its presence in the egg contents (Gantois et al., 2009).

The absence of *S. enteritidis* on the shell can be attributed to the fact that poultry farmers practice strict hygiene during handling, transport, and marketing. These findings are supported by previous studies that showed the presence of *Salmonella* spp. and *Salmonella enteritidis* in table eggs (Islam et al., 2018; Hai et al., 2020; Haque et al., 2021).

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In Nigeria, analyses of egg contents from farms revealed the presence of 1.5% *Salmonella* spp. (Okorie-Kanu et al., 2016). In contrast to the present findings, some studies demonstrated the presence of *Salmonella* spp. in the egg shells purchased from street vendors and directly from farms (Arathy et al., 2009; Siriporn et al., 2015; Kapena et al., 2020).

The higher contamination of *E. coli* compared to *S. enteritidis* observed in this study could be explained by poor sanitary practices of farmers during egg handling, by the presence of excreta on eggs or by dust in the environment.

The presence of these microorganisms (*E. coli* and *S. enteritidis*) outside and inside of the egg (content and eggshell) is significant for public health since people consume table eggs at all ages (Réhault-Godbert et al., 2019).

Egg contamination can happen due to different factors, including poultry droppings, moist and warm bedding, dirt in the nest, dust, a highly contaminated environment, poor handling of eggs by farmers and their dirty clothes, poor transportation method, poor storage in stores where these eggs are sold (humidity), unhygienic handling conditions by sellers (De Reu et al., 2005).

In addition, the hen may carry the pathogen and transfer it to the egg contents inside the reproductive system. Contaminated egg crates may also contaminate the shell, and consequently, the bacteria may translocate to the egg contents through the pores of the shell over time (Musgrove et al., 2009).

Table 1. Distribution of *Escherichia coli* and *Salmonella enteritidis* contaminations in raw table eggs of Morocco from January to September 2021

Number of samples	Isolation of pathogens (%)			
	Escherichia coli		*Salmonella enteritidis*	
	Eggshell (%)	Egg content (%)	Eggshell (%)	Egg content (%)
290	9%	4%	-	2%

Antibiogram study

The *in-vitro* antibiotic sensitivity test results indicated that the isolated *E. coli* had the highest resistance to amoxicillin, followed by tetracycline and nalidixic acid with 92.10%, 84.21%, and 50%, respectively. On the other hand, this bacterium showed the maximum sensitivity to ciprofloxacin (84.21%), followed by kanamycin (65.79%), and gentamicin (60.54%, Table 2).

In the present study, *S. enteritidis* had the highest resistance patterns against tetracycline (80%), followed by ciprofloxacin and nalidixic acid with 40% each. While gentamicin, amoxicillin, and kanamycin had the highest sensitivity rates of 80%, 80%, and 40%, respectively (Table 3).

Drug resistance continues to threaten public health, challenging the treatment of infectious diseases (WHO, 2021). In the current study, *E. coli* showed the highest resistance rates to amoxicillin, tetracycline, and nalidixic acid (92.10%, 84.21%, and 50%, respectively). The resistance rate to amoxicillin in this study agrees with previous studies that found that *E. coli* isolated from eggs were highly resistant to this antibiotic, with 90.1% and 88.89% of resistance (Rahmatallah et al., 2017; Ashish and Rajesh, 2017). The same is true for tetracycline resistance. Different studies recorded high rates of *E. coli* isolated from table eggs resistant to tetracycline with almost identical rates (Kapena et al., 2020, Haque et al., 2021). Regarding nalidixic acid resistance, a study conducted in Zambia showed that *E. coli* isolated from table eggs were resistant to nalidixic acid with a rate of 32.4% (Kapena et al., 2020). In contrast to the present study, a study in Bangladesh indicated that *E. coli* isolated from eggs were susceptible to this antibiotic with a rate of 44.44% (Islam et al., 2018). Of the 6 antibiotics, isolated *E. coli* showed the greatest sensitivity to ciprofloxacin, followed by gentamycin and kanamycin. Some studies have reported sensitivity to these antibiotics at a rate of 100% for each (Ashish and Rajesh, 2017; Islam et al., 2018; Adabara et al., 2020).

The resistance to tetracycline and amoxicillin could be partly attributed to the historical use of these antimicrobial classes in aviculture, resulting in antibiotic resistance development of the bacteria over time (Okorie-Kanu et al., 2016). The susceptibility to ciprofloxacin, gentamicin, and kanamycin could be attributed to the increased cost of these drugs, leading to their reduced use in poultry production (Okorie-Kanu et al., 2016).

The aforementioned results showed notable resistance rates of *S. enteritidis* to tetracycline, ciprofloxacin, and nalidixic acid: 80%, 40%, and 40%, respectively. A Moroccan study was conducted in 2016 to evaluate the antimicrobial resistance of *Salmonella* spp. isolates in Moroccan laying hens. The findings indicated that *S. enteritidis* was only resistant to nalidixic acid at a rate of 37.5%, meaning that the resistance in Morocco is increasing (Ziyate et al., 2016). Apart from tetracycline resistance, the present results disagree with reports from similar studies where *Salmonella* spp. isolated from table eggs were susceptible to ciprofloxacin and nalidixic acid (Okorie-Kanu et al., 2016; Islam et al., 2018; Kapena et al., 2020).

The noted susceptibility of *S. enteritidis* to gentamicin (80%) recorded during the present investigation was in line with the findings reported in an Egyptian study that reported a 90.9% of susceptible *Salmonella* spp. isolated from layer farms in Egypt (Diab et al., 2019). Concerning amoxicillin sensibility, a study conducted in Rajshahi found a rate of 76.47% of *S. enteritidis* isolated from eggs (Islam et al., 2018). Finally, previous studies conducted in Ethiopia and Egypt revealed the susceptibility of *Salmonella* isolated from eggs against kanamycin at 91% and 95.5% (Tessema et al.,

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2017; Diab et al., 2019). The uncontrolled, random, and repeated use of antibiotics in chicken farming worldwide may lead to the development and growth of resistant bacteria (Diab et al., 2019). For example, tetracycline was used for years to one-day-old chickens against *Salmonella* and *E. coli*, leading to its resistance to both antibiotics (Ekperigin et al., 1983; Lutful Kabir, 2010). Ciprofloxacin also belongs to the group of fluoroquinolones that have a rapid bactericidal action against *Salmonella* spp. Fluoroquinolones are widely used to treat salmonellosis in humans and animals (Folster et al., 2015) and be useful for treating infections caused by multidrug-resistant strains (Barnass et al., 1990). Therefore, its overuse may explain the high resistance observed in this study (Diab et al., 2019). Cross-resistance can also explain resistance to ciprofloxacin and nalidixic acid, as they are concrete examples of such resistance. When bacteria develop resistance to ciprofloxacin, they may also develop resistance to nalidixic acid because both drugs inhibit topoisomerase, a key enzyme in DNA replication (Périchon and Courvalin, 2009). On the other hand, some antimicrobials are rarely recommended because their high cost may explain the high susceptibility rate noted above (Okorie-Kanu et al., 2016).

Most of the *E. coli* strains (60.5%) were found to be multidrug-resistant (MDR), as they showed resistance to three antibiotics tested. In the same context, several studies have reported that *E. coli* is an MDR bacterium that has shown resistance to three, five, or more antibiotics, including tetracycline, amoxicillin, trimethoprim sulfate, streptomycin, and doxycycline (Adesiyun et al., 2007; Eid et al., 2015).

Regarding *S. enteritidis*, the present study revealed that 60% of the isolates were resistant to at least one antibiotic. In comparison, no MDR was detected, which agrees with a Moroccan study that revealed that 65.6% of *Salmonella* spp. strains were resistant to at least one antibiotic tested (Ziyate et al., 2016). This rate of multidrug resistance to antibiotics is most likely due to inappropriate use of treatment, either overuse, short treatment, or even misuse of these antibiotics (WHO, 2021).

The results were not surprising considering the range of antibiotics available in Morocco. The variation in susceptibility and resistance patterns may be due to the blinded use of antibiotics in poultry feed and poultry itself as prophylactic, therapeutic, and growth promoter agents since the farmers have unlimited and free access to antibiotics in Morocco (Rahmatallah et al., 2018).

In recent years, antibiotic-resistant bacteria have received considerable attention because they constitute an immediate risk to public health by increasing the incidence of overall hospitalizations and the risk of invasive infections and mortality (Verraes et al., 2013). They can lead to many consequences, such as the failure of medical treatments, including modern medicine. This is because surgical procedures and cancer chemotherapy would be compromised, the choice of antibiotics for treatment would be limited, and resistant gastrointestinal bacteria will gain an advantage when patients are treated with antibiotics for other medical reasons (Verraes et al., 2013). Furthermore, it has been experimentally proven that the genes conferring antibiotic resistance are easily transferable between normal flora, pathogenic *E. coli* and *Salmonella* spp. (Blake et al., 2003). Hence, antibiotic resistance in microorganisms from table eggs should be considered a significant public health hazard, as eggs can serve as a vector for transferring antimicrobial-resistant bacteria and genes to humans.

Table 2. Antibacterial sensitivity and resistant pattern of *Escherichia coli* isolated from eggs in Morocco from January to September 2021

Number of samples tested	Antibiotics	Sensitivity pattern		
		Sensitive (%)	Intermediate (%)	Resistant (%)
290	Tetracycline	10.53	5.26	84.21
	Nalidixic acid	44.74	5.26	50
	Amoxicillin	5.26	2.64	92.10
	Kanamycin	65.79	13.16	21.05
	Gentamycin	60.54	13.15	26.31
	Ciprofloxacin	84.21	5.26	10.53

Table 3. Antibacterial sensitivity and resistant pattern of *Salmonella enteritidis* isolated from eggs in Morocco from January to September 2021

Number of samples tested	Antibiotics	Sensitivity pattern		
		Sensitive (%)	Intermediate (%)	Resistant (%)
290	Tetracycline	20	0	80
	Nalidixic acid	40	20	40
	Amoxicillin	80	20	0
	Kanamycin	40	40	20
	Gentamycin	80	20	0
	Ciprofloxacin	20	40	40

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CONCLUSION

This study concludes that table eggs marketed for human consumption in Morocco could be infected with antibiotic-resistant *E. coli* and *S. enteritidis*, especially tetracycline. These resistant bacteria could potentially be transmitted to consumers through eggs or egg products, which could have significant public health consequences which require a one-health approach to combat the threat. Therefore, further studies can be conducted to assess the potential spread of antibiotic resistance from foodborne pathogens to humans.

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Authors' contributions

Fatima Zahra El Ftouhy, Sabrine Nacer, Sophia Derqaoui, and Nadia Charrat collected the samples used in this study. Fatima Zahra El Ftouhy performed the bacterial isolation and antibiotic susceptibility testing. Fatima Zahra El Ftouhy and Asma Fagrach contributed to the data analysis. Fatima Zahra El Ftouhy wrote the original manuscript. Saâdia Nassik, Abdelaziz Hmyene, and Ahlam Kadiri revised and generated the final version of the manuscript. All authors contributed to the article and approved the submitted version.

Competing interests

The authors declare that there is no conflict of interest regarding the publication of this paper.

Ethical consideration

All authors have checked and vetted the manuscript for ethical considerations, namely plagiarism, consent to publication, misconduct, fabrication and/or falsification of data, dual publication and/or submission, and redundancy.

Availability of data and materials

The authors declare that they have all the necessary data and are available where appropriate or requested by the editor.

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A Retrospective Study on Dairy Cattle Mortality Patterns in Two Farms of South-eastern Botswana

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ABSTRACT

Generally, high mortalities of dairy cattle due to infectious and non-infectious diseases cause huge economic losses, unprofitability, and low productivity in the dairy industry. The present study aimed at determining the mortality rates, their causes, and risk factors among 1779 cattle at two dairy farms belonging to the Department of Agricultural Research, Botswana. An 8-year retrospective study was conducted using farm records during 2005-2012. Monthly and annual records of the farms were examined regarding the total dairy cattle population, sex, breed, age, cattle deaths, and causes of death. Mortality was calculated from the total cattle population and expressed as a percentage, and it was analyzed with respect to farm, breed, age, sex, year, season, and mortality causes. The overall mortality rate was 8.5%. The semi-intensively managed Farm II, as well as young stock (<12 months old), and males recorded significantly higher mortalities than their counterparts. Dairy crosses of pure exotic and indigenous Tswana cattle had higher mortalities than the Friesians and Jerseys, and the wet season accounted for over 70% of the total deaths. Only two years (2010 and 2012) out of the 8-year study period had a mortality rate < 5%. Notably, 28.1 % of mortalities with a known cause were due to heartwater disease (n = 57), but most deaths (62.3%) were due to unknown causes. In conclusion, to improve farm herd health and husbandry practices, more efforts should be devoted to preventing heartwater and mortalities in young stock and male animals, particularly during the hot-wet season.

Keywords: Dairy cattle, Heartwater, Mortality, Risk factor

INTRODUCTION

In Botswana, cattle are the main source of milk production for human consumption. Indigenous Tswana cattle and other local beef breeds dominate the traditional dairy subsector in rural communities despite their low potential for milk production (APRRD, 2001). Commercial milk production developed recently in Botswana and is dominated by exotic Friesians, Jersey, Brown Swiss, and dairy crosses (Mosielele, 2005). Dairy goat milk is an alternative livestock enterprise suitable for small-scale livestock operations (Norris et al., 2011), although it is not a significant source of milk in the country. National milk demand is about 65 million liters annually, while local production accounts for only 11% of the demand, indicating that 89% of milk is imported (DAP, 2009). The reliance on milk importation has a negative impact on local production and has caused the average Motswana (citizen) to be unable to afford the high cost of milk.

The dairy sector experiences challenges regarding fodder and feed production, breeding, and dairy cattle management, leading to low productivity. One contributor to low milk production in the dairy sector of Botswana is the mortality of high-quality dairy animals. The losses of dairy animals occur during the different stages of their growth and is attributable to many factors (Jousan et al., 2005; Yitagesu et al., 2022). Dairy animal mortality results in huge financial losses through decreased production, high treatment and heifer replacement costs, and general loss of livestock (Raboisson et al., 2011). High mortality rates indicate sub-optimal health and welfare.

A recent study in Botswana showed high mortalities of dairy cattle (LEA, 2011). The reported main causes were pasteurellosis, heartwater, mastitis, brucellosis, anthrax, dystocia, milk fever, botulism, bloating, and unknown diseases (LEA, 2011). Other causes included inappropriate feeding, starvation due to droughts and road-traffic accidents (LEA, 2011). High mortalities of dairy calves aged 0-6 months (26.3%) and those aged between 6-12 months (32.6%) have been reported in Botswana (Mahabile and de Waal, 2011). Internal and external parasites were also noted as a problem in lactating cows (Jousan et al., 2005; Aldomy et al., 2009). Other potential causes of mortality are the physiological changes associated with high milk production, improper management, and feeding, especially in dairy calves (Lopez-Gatius et al., 2002; Silke et al., 2002). Unfavorable environmental and poor housing conditions may also contribute to

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high dairy cattle mortality, especially in Botswana, where ambient temperatures are high, with summer temperatures of 30-35^{o}C. These temperatures are high for exotic dairy cattle breeds developed and bred to produce in cooler environments and may cause lethal heat stress (Burhans et al., 2022).

High mortalities of dairy cattle cause huge economic losses leading to unprofitability and low productivity of the sub-sector in Botswana. Despite previous reports of high dairy cattle mortality in the Department of Agricultural Research (DAR) farms in Botswana (Mpapho, 2011), the mortality causes have not been fully studied and documented. For the DAR to find a solid remedy for the current high dairy cattle mortalities, there is a need to carry out a study to determine the mortality rates, their causes, and risk factors. This will also assist dairy farmers currently encountering losses due to livestock mortality in the country and similar environments elsewhere. Reduced mortalities could enable further opportunities to sustainably increase the national dairy herd, thus increasing milk yields and subsequently improving household food nutrition and security. Therefore, the aim of the study was to determine the level of mortality among the dairy cattle population on two dairy farms and to establish the causes of such mortality.

MATERIALS AND METHODS

Ethical approval

The study followed the Management Guide for Dairy Production in Botswana (DAR, 2002).

Study sites

The study was carried out at two farms belonging to the DAR. Farm I is located 10km north of Gaborone in the south-eastern part of Botswana; latitude 24^{o} 33' S, longitude 25^{o}57' E, and altitude 994 m. The mean rainfall for the area is 550 mm with a monthly average minimum and maximum temperature of 4.1^{o}C and 34.4^{o}C, respectively. The farm is almost flat with gently undulating plains, kopjes or small hills, and associated pediments. It is characterized by granite soils. The pasture availability is affected by season, being plentiful in the months of December-May and inadequate in June-November. The vegetation is a mixture of *Acacia savanna* with *Combretum apiculatum* and *Burkea africana*. The herbaceous layer consists of *Eragrostis rigidior* (*E. rigidior*) and other grasses including *Panicum maximum* (*P. maximum*), *Digitaria milanjiana* (*D. milanjiana*), *Urochloa mosambicensis* and *Urochloa trichopus*.

Farm II is located approximately 45 km south of Gaborone, latitude 25^{o} 06' S and longitude 25^{o} 44' E. The area receives erratic rainfall between October and May that averages 517 mm and is characterized by soils that are eutric regosols skeletic (pH of 6.2, Organic Carbon (OC) 0.2%, available Phosphorus (P) 3ppm, and Calcium (Ca) 2.7, Magnesium (Mg) 0.5, Potassium (K) 0.7 and Cation Exchange Capacity CEC 5.1 meq/100g) (APRRD, 2001). The vegetation can be described as Tree Savanna – semi-sweet mixed bushveld. The dominating tree species are *Peltoforum africanum*, *Acacia tortilis*, *Terminalia sericea*, and *Combretum imberbe*. The shrub layer is characterized by *Ziziphus mucronata*, *Acacia karoo*, *Acacia mellifera*, and *Grewia bicolor*. The species can provide desirable fodder to browsers during the dry season. Grass species, such as *E. rigidior*, Aristida congesta, and Schmidtia pappophoroides dominate the lower layer. The grasses have intermediate to good nutritional value as livestock feed except for *A. congesta*, which has poor nutritional value.

Farm I had 765 exotic *taurus* Friesian and Jersey breeds while Farm II hosted 1014 dairy cattle crossbreds; dairy *taurus* breed bulls sired with indigenous *indicus* Tswana cows and comprised of Friesian (FxT), Jersey (JxT) and Brown Swiss (BSxT) crosses. Animals at Farm I were intensively managed at zero-grazing and were fed locally made concentrates (3 types that include calf starter, grower, and production diets, Table 1) with grass-hay, maize stover, or maize silage as roughage. The Farm II animals were semi-intensively managed and were allowed to graze natural pastures comprising *E. rigidior*, *P. maximum*, *D. milanjiana*, and *Urochloa* grass species and had minimal supplementation with concentrate diets. Supplementation was done mainly during the dry season. Calves were allowed to suckle for 3 weeks before being offered calf starter and grower meals and allowed to graze. The nipple bottle and bucket feeding methods were used, where calves were fed 4 liters of whole milk per day (2 liters each in the morning and afternoon) for 3 months. The feeding was gradually reduced to 2 liters in the morning only in the last month. The weaning age was 3 months. At both farms, portable water was given ad libitum. Cows were bred through artificial insemination, and calves were housed in conventional calf pens for up to 3 months. The animal health management guide for DAR cattle was followed and used in the two farms. A veterinarian attended to any animal showing signs of disease appropriately, and treatment sheets were recorded by the farm manager.

Manual animal records at both farms included data on birth dates and weights, monthly weights, milk yields, feed types and intakes, animal breeds, reproduction parameters, pasture assessments, deaths, and causes. With respect to cases of animal diseases and deaths, the farms engaged the Department of Veterinary Services for advice on diagnoses, treatments, control and prevention. Samples from sick and dead animals were submitted to the Botswana National Veterinary Laboratory for diagnosis to confirm the cause of sickness or death.

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Table 1. Ingredients and composition of starter, grower, and production meals for dairy cattle during the study in Botswana.

Ingredient \ Diet	Calf starter (Percentage of Dry Matter)	Calf grower (Percentage of Dry Matter)	Production meal (Percentage of Dry Matter)
Ground maize	47.5	39.5	73.0
Maize stover	-	20.0	-
Sunflower-seed cake	34.0	-	15.0
Soyabean meal	-	22.0	-
Feed grade urea	-	-	1.5
Limestone	-	-	2.0
Dicalcium Phosphate (DCP)	0.5	0.5	0.5
Bran (wheat)	10.0	10.0	-
Molasses liquid	7.0	7.0	7.0
Salt	0.5	0.5	0.5
Vitamin and mineral premix*	0.5	0.5	0.5
Total	100	100	100

*Vitamin A 45000 IU. Vitamin D3 20000 IU, Vitamin E 125 mg, Vitamin B1 25 mg, Chromium 2 mg, Cobalt 18 mg, Copper 200 mg, Iodine 12 mg, Manganese 325 mg, Magnesium 25 mg, Selenium 4 mg, Sulphur 250 mg, Zinc 700 mg and Antioxidant 160 mg

Data collection and analysis

A retrospective study covering 8 years (2005-2012) was conducted using farm records of the two farms to determine dairy cattle mortality, causes, and risk factors. The study population included all dairy cattle at the two farms during the 8-year study period. Monthly and annual records of the two farms were examined regarding total dairy cattle population, sex, breed, age, cattle deaths, and causes of death. Data were captured into Microsoft Excel 2016 spreadsheet. Crude mortality rate (Number of deaths/Total number of animals x 100) was calculated from the total dairy cattle population and expressed as a percentage. The crude mortality rate was examined in relation to location, breed, sex, age, year, season, and causes of death. Categories were generated as follows: two for location (Farms I and II), five for the breed (Friesian, Jersey, FxT, JxT, and BSxT crosses), two for sex (male and female), three for age (< 12 months, 12-24 months and > 24 months), two for the season (wet and dry) and 8 for years (2005-2012). Comparisons were made for the different categories and a statistical package, EpiCalc 2000 (version 2) was used to measure the percentage differences between categories, and p values (analyzed by the Chi-square test for proportions) less than 0.05 were considered as significant. Associations between crude mortality rate and different categories of studied variables were assessed by calculating the odds ratio (OR) using the EpiCalc 2000 (version 2) statistical package.

RESULTS

The crude mortality according to different categories is presented in Table 2. A total of 1779 dairy cattle were studied, and the overall mortality rate was 8.5%, with Farm II recording a significantly higher mortality rate than Farm I ($p < 0.05$). Farm II was found to have a significantly higher odds ratio (OR = 2.2) of crude mortality than the other farm.

A significantly higher crude mortality rate was recorded for male (11.5%) compared to female (7.3%) dairy cattle ($p < 0.05$). The odds of mortality rate were approximately twice in males (OR = 1.7), compared to females. Regarding age, the crude mortality rate differed significantly among the groups ($p < 0.05$), with young dairy cattle (< 12 months) recording the highest (29.9%) and adults the lowest (2.4%). Young cattle (less than 12 months) were significantly associated with higher odds of crude mortality compared to the 12-24 months old (OR = 2.4) and older than 24 months old (OR = 17.2) age groups.

There was an insignificant difference in crude mortality rate between Friesians and Jerseys ($p > 0.05$), and similarly, the mortality rates of dairy crosses were not significantly different ($p > 0.05$). However, dairy crosses recorded higher mortality rates compared to pure breeds, with the JxT crosses (12.3%) recording a significantly ($p < 0.05$) higher mortality rate than the Friesians (5.2%) and Jerseys (5.9%).

The wet season accounted for over 70% (73.5%, n = 111) of the total deaths. The crude mortality rate varied annually, with the year 2011 (16%) recording the highest and 2012 (0.4%) the lowest. Out of the 8-year study period, only two years (2010 and 2012) recorded less than 5% crude mortality rates. The causes of death are shown in Table 3. Most of the deaths (62.3%, n = 94) had unknown causes, and for death with known causes, most (28.1%) were due to heartwater, followed by predation (12.3%) and coccidiosis (10.5%).

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Table 2. Summary of crude mortality in Farms I and II of Friesian, Jersey, and crossbred dairy cattle in Botswana from 2005 to 2012

Variable	Level/Category	Number of animals	Number of deaths	Crude mortality (95% CI)	Odds ratio (95% CI)	* p-value
	All animals	1779	151	8.5 (7.3-9.9)	-	-
Location	Farm I	765	41	5.4[a] (3.9-7.3)	-	-
	Farm II	1014	110	10.9[b] (9.0-13.0)	2.2 (1.5-3.1)	0.0001
Sex	Female	1276	93	7.3[a] (6.0-8.9)	-	
	Male	503	58	11.5[b] (8.8-14.7)	1.7 (1.2-2.3)	0.003
Breed	Friesian	544	28	5.2[a] (3.5-7.4)	-	-
	Jersey	221	13	5.9[a] (3.3-10.1)	1.2 (0.6-2.3)	0.82
	#FxT cross	312	31	9.9[ab] (7.0-13.9)	2.0 (1.2-3.5)	0.012
	#BSxT cross	353	36	10.2[ab] (7.3-14.0)	2.1 (1.4-3.5)	0.006
	#JxT cross	349	43	12.3[b] (9.2-16.3)	2.6 (1.6-4.3)	0.0002
Age	> 24 months	1034	25	2.4[a] (1.6-3.6)	-	-
	12-24 months	397	22	5.5[b] (3.6-8.4)	2.4 (1.3-4.3)	0.005
	< 12 months	348	104	29.9[c] (25.2-35.0)	17.2 (10.9-27.2)	0.00001
Years	2005	245	32	13.1[a] (9.2-18.1)	-	-
	2006	251	32	12.8[a] (9.0-17,7)	35.3 (4.8-260.6)	0.00001
	2007	277	16	5.8[b] (3.5-9.4)	34.3 (4.7-353.4)	0.00001
	2008	184	10	5.4[b] (2.8-10.1)	14.4 (1.9-109.5)	0.002
	2009	238	26	10.9[a] (7.4-15.8)	13.5 (1.7-106.5)	0.004
	2010	173	6	3.5[b] (1.4-7.7)	28.8 (3.9-214.3)	0.00001
	2011	175	28	16.0[a] (11.1-22.5)	8.4 (1.0-70.8)	0.05
	2012	236	1	0.4[c] (0.02-2-7)	44.8 (6.0-332.5)	0.00001
Season	Dry	882	40	4.5[a] (3.3-6.2)	-	-
	Wet	897	111	12.4[b] (10.3-14.8)	3.0 (2.0-4.3)	0.00001

[abc] Different superscript letters in a column mean significant differences ($p < 0.05$), CI: Confidence interval #FxT: Friesian x Tswana cross, #BSxT: Brown Swiss x Tswana cross and #JxT: Jersey x Tswana cross, *p-values are for the odds ratios and calculated for categories

Table 3. Summary of the causes of deaths in Farms I and II of Friesian, Jersey, and crossbred dairy cattle in Botswana from 2005 to 2012

Causes	Numbers	Percentage
Unknown	94	62.25
Heartwater	16	10.60
Predation	7	4.64
Coccidiosis	6	3.97
Dystocia	4	2.65
Pasteurellosis	4	2.65
Accidents	3	1.99
Bloat	3	1.99
Endoparasites	3	1.99
Rabies	3	1.99
Abscessation (liver)	2	1.32
Botulism	2	1.32
Uterine prolapse	2	1.32
Starvation	2	1.32
Total	151	100

DISCUSSION

The findings of this study should be viewed in light of its limitations. Due to missing data on the exact date of death for most animals, the animal time at risk was not calculated. Animal time at risk is an important parameter for calculating a more accurate mortality rate. Nevertheless, the available data showed some important mortality trends worth noting. Management practices could possibly explain the observed significant difference between the studied farms. Farm I, with intensive management with zero-grazing, had a lower mortality rate than Farm II, with semi-intensive management and pasture grazing. Individual animal attention is likely reduced on semi-intensively animals grazing on pasture, leading to a higher mortality risk. In addition, grazing is associated with an increase in mortality rate (Thomsen et al., 2006).

Breed has been previously reported to have a strong relation to mortality in dairy cattle (Alvasen et al., 2014; Pannwitz, 2015). For example, the high-producing Friesian breed is associated with increased mortality rates compared

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to other exotic dairy breeds (Thomsen et al., 2006; Raboisson et al., 2011; Alvasen et al., 2012, 2014; Pannwitz, 2015). However, the current study found no differences in mortality between Jersey and the Friesian breeds. Unexpectedly, the crossbreds recorded higher mortality rates than the exotic Friesian and Jersey breeds. This might be due to different management practices; exotic breeds were under an intensive system with zero-grazin,g while the crossbreds were under a semi-intensive system with pasture grazing and limited supplementation. In Farm II, animals can freely graze the natural pastures, leading to stress and exposure to harsh climatic conditions like excessive summer heat and cold winter temperatures. In addition to inadequate grazing, ingestion of poisonous plants like *Dichapetalum cymosum* and *Pavetta harborii* and possible malnutrition could also likely have contributed to the observed higher mortality at Farm II. While out freely grazing, some unattended crossbred animals at Farm II were killed by predators, such as jackals and hyenas. In Botswana, livestock depredation has been reported as a challenge by livestock farmers, especially during the spring calving season or drought when the predators' natural prey numbers are limited (Schiess-Meier et al., 2007; Mosalagae and Mogotsi, 2013).

The high mortality rate observed in male more than female animals agrees with previous findings (Swai et al., 2010; Pannwitz, 2015; Reimus et al., 2017). This probably reflects biological features (Raboisson et al., 2013; Pannwitz, 2015), such as heavier weights at birth leading to dystocia and higher mortality (Linden et al., 2009; Johanson et al., 2011). The relatively higher economic value of female animals as future replacements or animals for sale (Swai et al., 2010; Pannwitz, 2015) may also contribute to higher mortality of male animals since more management attention is shifted towards female animals, which are the mainstay of the dairy enterprise. The high economic cost of feeding male calves with milk in the dairy farming industry could also not be ruled out (Gitau et al., 1994; French et al., 2001), thus contributing to suboptimal management practices, with repercussions for mortality. Hossain et al. (2014) noted a higher mortality rate in females, possibly due to their higher proportion at the studied farm. According to Moran (2011), 20-25% of cows are generally anticipated to be replaced yearly in dairy farms. Therefore, since the mortality of female animals was 7.3 % in the current study, there is a possibility of raising enough replacement animals, and therefore the herd can be expanded.

The young age group (< 12 months old) had a higher mortality rate than the older animals. This is in agreement with earlier findings in Botswana (Mahabile and de Waal, 2011), other African countries (Gitau et al., 1994; French et al., 2001; Swai et al., 2010; Fentie et al., 2020) and elsewhere (Prasad et al.,2004; Hossain et al., 2014; Reimus et al., 2017). The observed pattern is likely attributable to poor management practices, including bucket feeding of calves and poor hygiene conditions that could lead to outbreaks like diarrhea or coccidiosis (Duguma et al., 2012). Calves also have an increased susceptibility to diseases and environmental stress than adults. It could be because some calves had compromised immune systems due to inadequate colostrum intake shortly after birth and subsequently experienced poor nutrition. However, this reason is also not expected to be too widespread since calves in the study area were allowed access to their mother's colostrum during the first few hours of birth for up to 3 weeks. Well-managed dairy farms in the USA have shown that mortality in young stock does not exceed 5% (Speicer and Hepp, 1973). The high young animal and annual mortality rates (only two years had a mortality rate < 5%) might be due to poor management practices on the studied farms, such as inadequate colostrum feeding, inadequate tick control programs, inadequate use of heat stress prevention methods (for example using shades on the farms) among others. The higher rate of unknown causes of death reported in this study (>60%) supports the need to improve farm husbandry practices, such as complete investigations of animal carcasses, recording accurate and complete individual animal records, and adopting electronic capturing of farm data.

The season was a factor that influenced the mortality, with the hot-wet season accounting for over 70% of the cases. Similar observations have been reported elsewhere (Reimus et al., 2017; Armengol and Fraile, 2018). The hot-wet season in Botswana is characterized by extreme weather conditions that include high ambient temperatures and erratic but heavy rainfalls exacerbated by climate change. High temperatures predispose livestock to heat stress, the effects of which may be influenced by lactation stage, breed, and age (Crescio et al., 2010), as well as a management approach. This may increase heat-related dairy cattle mortality (Crescio et al., 2010; Alvasen et al., 2012; Bishop-Williams et al., 2015; Cox et al., 2016; Reimus et al., 2017; Armengol and Fraile, 2018). Wet weather also increases the risk of infection with various pathogenic microorganisms. The study shows the importance of putting more emphasis on devising and implementing preventive measures during the hot-wet season. Strategies to mitigate heat stress at the studied farms need to be considered, and the farms should adapt their production systems to changing climate conditions.

The observed higher percentage of unknown causes of death reported in this study is similar to earlier findings in the region. Phiri et al. (2010) showed that many mortalities reported on smallholder dairy farms in Eastern and Southern Africa had undiagnosed causes. Except for drowning (0.01%) and snakebite (1%), the cause of death for most dairy cows (n = 1774) on dairy farms in the Eastern Cape Province of South Africa was not given (Diniso and Jaja, 2021). In contrast, lower proportions of unknown causes of death in dairy farms (4-20%) are reported from developed countries (Thomsen et al., 2004; Pinedo et al., 2010; Fusi et al., 2017; Armengol and Fraile, 2018). During the present study, some cases of sudden deaths occurred outside of normal staff working hours, and cases were decomposed, and no cause of

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death was reported. Farmers' knowledge of mortality causes would enable them to prioritize their resources on preventing the predisposing factors associated with morbidity and mortality (Reimus et al., 2017). Therefore, the two farms in the current study should prioritize herd health improvement plans.

Heartwater, a tick-borne disease, was the most important of the known causes of death in dairy cows. Tick-borne diseases are reported to be the major causes of mortality on smallholder dairy farms in eastern and southern Africa (Phiri et al., 2010). The culling of dairy cows in the Eastern Cape province of South Africa was due to Redwater and heartwater (Diniso and Jaja, 2021). In Botswana, heartwater and gall sickness are the two main tick-borne diseases of economic importance, and young calves may be especially vulnerable during their first grazing season after weaning as they encounter heavy parasitic loads for the first time (Batisani et al., 2012; Ramabu et al., 2018; Raboloko et al., 2020). Our observation is an indication of the need to revise the tick control strategies at the studied farms.

CONCLUSION

The study results showed that farm management practices, sex, and age of the animal as well as the season, influenced mortality. Males, young stock (<12 months old), and the hot-wet season were found to be associated with higher mortalities. Only two years (2010 and 2012) of the 8-year study period had a mortality rate of less than 5%. The percentage of unknown causes of death was very high (> 60%). Of the known causes of death, heartwater was the most important disease. Adequate colostrum feeding is recommended to reduce calfhood diseases due to malnutrition and immune deficiency. Following the recommended dipping regime for tick control is paramount to reducing heartwater cases. The provision of shades (none of the farms had shades) and automated cooling systems is recommended to mitigate heat stress. Electronic, accurate, and complete data recording is also an important activity that the studied farms need to consider.

DECLARATIONS

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Authors' contributions

Diphetogo Mosalagae contributed to the study conceptualization, data analysis, and manuscript writing. Kabo Mogotsi, Innocent Ithuteng, Onkemetse Basinyi, and Davies Pfukenyi contributed to data cleaning, data analysis, and manuscript writing. All authors read and approved the final manuscript and agreed to submit the manuscript to the current journal.

Competing interests

All the authors declare that there are no competing interests regarding this work.

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Ethical considerations

Before submission of the manuscript, all authors checked for ethical issues, including plagiarism, data fabrication, duplicate publishing, or submission.

Availability of data and materials

The authors declare that they will prepare all the necessary data upon reasonable request.

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Zootechnical Performance and Growth Curve Modelling of the Niamey Local Chickens in Niger

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ABSTRACT

The Niamey region in Niger depends on imports to meet its chicken meat needs. Although consumers appreciate local poultry products, they cannot fulfill their needs. The reluctance of modern producers to use local chickens on their farms is linked to a lack of knowledge of the production characteristics of local strains, which have been little studied. Thus, this study aimed to determine the growth profile of traditional chickens from villages in the Niamey region (Niger). In doing so, 100 local chicks whose parents were collected in the surrounding villages of the Niamey region were followed from hatching until the age of 140 days. The chickens were raised in cages with 10 per compartment of 3 m length and 1.5 m width. Food consumption was recorded daily, and weights were measured weekly. The parameters of the growth curves were obtained using the Gompertz equation. Female and male chickens had a significant weight difference at the third week of age. The mean weight of chicks at hatching was 24.90 ± 0.36 g. At the end of the follow-up, males, with a mean weight of 1523.05 ± 26.22 g were significantly heavier than females (1052.73 ± 14.04 g). Over the entire period of the experiment, the average daily gain and consumption indices were 9.5 g/d and 5.12, respectively. Asymptotic weights were 2096.78 g and 1313.26 g for males and females, respectively. The maturation factor of the Gompertz equation was higher in females (0.0196 g/d) than in males (0.0181 g/d), and the inflection age averaged 75 days for both sexes. In conclusion, Niamey local chickens are slow growing and have a high feed conversion ratio compared to the modern broiler or layer strains.

Keywords: Average daily gain, Feed conversion ratio, Growth curve, Local chicken, Weight gain

INTRODUCTION

In West Africa, chicken meat consumption is highly dependent on imports. Thus, in 2020, the import of chicken meat amounted to 488 tons (FAOSTAT, 2020). The low levels of production partly explain this large import of chicken meat in the traditional production systems (Ayssiwede et al., 2013). The viability of West African poultry farming, therefore, depends on the modernization of production systems through the improvement of their breeding conditions (Moula et al., 2009a) and of genetic potential because it is adapted to African environmental conditions (Keambou et al., 2014), it could replace imported exotic strains if their production performances were improved. Therefore, it is necessary to gain more knowledge about breeders' skills. Indeed, the optimization of food and sanitary behaviors leads to better performance of local chickens than those observed in extensive systems, even if these performances remain lower than those of highly specialized commercial strains that have undergone extreme genetic selection pressure (Youssao et al., 2012; Ndofor-Foleng et al., 2015; Dahloum and Hadjoudj, 2016).

The Niamey region depends on imports for its chicken meat needs. Indeed, local poultry products, although better appreciated, are far from covering consumer demand, especially during religious holiday periods (Assoumane and Ousseini, 2009). This deficit in local chicken meat is linked to the non-use of these local chickens in the modern farms of Niamey. Indeed, the local poultry products available in the markets of the region prepare from the surrounding villages (Mato et al., 2020). The reluctance of modern producers to use local chickens on their farms is linked to the lack of knowledge of the production characteristics of local strains. For these reasons, the productive performance of local chickens is poorly documented in Niger, although they contain 54.7% of Niger's poultry farms (RGAC, 2007).

The research and development program called Improvement of the Poultry Sector of the Niamey Region (PRD-AFARNi) was born in this context. Through a multidisciplinary approach, this program aimed to establish a differentiated local chicken breed with characteristics known and appreciated by local consumers. The present study dealt with the zootechnical aspects of the AFARNi program and aimed to describe the growth characteristics of local salmon-golden plumage chicken in the Niamey region, Niger.

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MATERIALS AND METHODS

Ethical approval

This study has received ethical approval from the Department of Animal Productions, the Faculty of Agronomy, University of Niamey, Niamey, Niger (Authorization N°: AFARNi/FA/DPA-003).

Study site

The study was performed at the experimental farm of the Faculty of Agronomy of the Abdou Moumouni University of Niamey (UAM/N), Niamey, Niger. The livestock building has a dimension of 30 m by 20 m. The sides are wire mesh to perfect the natural circulation of air. The interior of the boxes is lined with a wood-based litter 10 cm thick. Chickens were raised under natural lighting except for the starter period. Cages have been specially designed for the starter phase with dimensions of 2 m length, 1 m width, and 0.8 m height. They were made of plank and mesh and had a lighting and heating system (Figure 1). Lighting and heating are controlled manually through switches. The heating bulb was on during this phase from 6 a.m. to 8 p.m. The relative humidity varied between 40% and 60%, and the temperature inside the cages was maintained between 37 and 38°C.

Figure 1. Cages used for the starter period

Biological material

The chicks monitored in this study were descendants of 65 hens and 22 roosters collected in 8 villages around Niamey. This breeding group was formed based on morphological criteria established during a survey of sellers and consumers in poultry markets in the Niamey region, Niger (Mato et al., 2020). Figures 2 and 3 show the male and female chickens of this study. These chicks were obtained by artificial incubation of 126 eggs collected from the parents. The duration and storage period of the eggs before the beginning of the artificial incubation was 5 days to limit the quality deterioration of the eggs. Incubation lasted about 22 days. A first candling was done after 7 days to eliminate unfertilized eggs, which were 4. The second check was done on day 14 to identify eggs with embryos that had stopped developing. Thus, at the end of the incubation, 108 chicks were hatched, and only 100 were kept for experimentation. The startup phase lasted 4 weeks. For the first two weeks, the chicks were distributed in two cages at a rate of 50 chicks per cage. At the end of the second week, the chicks were distributed in 4 starter cages at a rate of 25 subjects per cage. At the end of the startup period, the non-sexed chicks were randomly distributed into 10 breeding units. The identification was made using plastic rings. In each breeding unit, all the subjects wore rings of different colors. This device made it possible to follow individually the evolution of the weight of each subject and to assign the sex at week 12 of age. Thus, the experimental group consisted of 46 males and 54 females. No mortality was recorded during the experiment.

Figure 2. Red roosters from the Niamey region, Niger

Figure 3. Golden Salmon hens from the Niamey region, Niger

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Feeding and health monitoring

The experiment lasted 20 weeks during which the chickens were weighed every week. The chicks were vaccinated against Newcastle disease (NC) and Gumboro disease (GB), which are endemic avian diseases in Niger. For Newcastle disease, the chicks were vaccinated orally, in the drinking water, at hatching, and a booster at day 8 with LaSota vaccine (Zoetis INC, US). A second vaccination against NC was performed at 8 weeks of age by subcutaneous injection with ITA-New vaccine (Laprovet, Hungary). Vaccination against GB (by drinking water) was done on day 5 after hatching and booster vaccination on day 14 with Gumboror-Vac (Elanco, Netherlands) was performed. The chickens were fed and watered *ad libitum*. Food consumption was measured by the difference between the quantity of distributed food and that food did not consume until the next day. The chickens were fed with 3 types of feed depending on the rearing phase which were the starter, grower, and pre-layer feeds (Table 1). The bromatological composition of these feeds was determined at the animal nutrition laboratory of the Faculty of Agronomy, University of Niamey, Niger. The amounts of non-nitrogen extracts (NNE) and gross (GE) and metabolizable (ME) energies were calculated according to formulas 1, 2, and 3 (Larbier and Leclercq, 1992).

NNE = DM% - (CP% + F%+ CF% + TMM%) (Formula 1)

GE = 57.2%CP + 95.0%F + 47.9%CF + 41.7%NNE (Formula 2)

EM = 0.64GE (Formula 3)

DM: Dry matter; F: Fat; CP: Crude protein; CF: Crude fiber and TMM: Total Mineral matter, GE: Gross energy

Table 1. Bromatological compositions as a percentage of dry matter of food in local chicken of Niamey, Niger

Content	Starter (0 to 28 days)	Grower (28 to 84 days)	Pre-layer (84 to 140 days)
Physical composition			
Corn (%)	50	50	50
Wheat bran (%)	30	30	30
Commercial concentrate[1] (%)	20	20	20
Chemical composition			
Dry matter (%)	92.90	93.59	93.40
Fat[2] (%)	6.09	6.51	4.54
Crude protein[2] (%)	21.35	16.17	16.53
Total mineral matter (ash)[2] (%)	6.81	11.93	10.67
Crude fiber[2] (%)	0.98	1.45	3.72
Non-nitrogenous extract (%)	64.77	63.93	64.54
Gross energy (Kcal/Kg)	4547.94	4279.22	4246.18
Metabolizable energy (Kcal/Kg)	2910.68	2738.70	2717.55

[1]Animal Care Services Konsult, Ogere, Nigeria; [2]Related to dry matter

Statistical analysis

The R software (version 4.0.5) was used for all statistical analyses. The parameters of the Gompertz model (Formula 4) were obtained with the easynls package. This model best describes the growth of *Gallus gallus domesticus* (Darmani Kuhi et al., 2010; Firas Rashad, 2015; Bashiru et al., 2019; Akinsola et al., 2021). The equation is written as follows:

$Y_t = Ae^{(-Be^{(-k_t)})}$ (Formula 4)

Where, the parameter B equals to L/k, Y_t denotes weight at time t in grams, A signifies asymptotic weight (in grams), L determines the specific initial growth rate (g/d), and K is the maturation factor.

The age at the inflection (T_I) is calculated as follows: $T_I = 1/k*\ln|B|$

The average daily gain (ADG) and consumption index (CI) were calculated using the following formulas:

ADG = Weight gain of the period (g) / Number of days in the period (Formula 5)

CI = Food consumption in the week (g) / Weight gain in the same week (g) (Formula 6)

Welch's t-test was used to compare means of ADG, CI, and weights between both sexes. The significance level was set at $p \leq 0.05$.

RESULTS AND DISCUSSION

Growth performance

At hatching, the mean weight of chicks of both sexes was 24.90 ± 0.36 g (mean ± standard error). This hatching weight of the chicks of the Niamey salmon-golden hen is slightly lower than those of the chicks of the ecotypes of local hens of Benin, which ranged from 26.2 to 26.8 g (Youssao et al., 2012). This gap is a little larger between the chicks of Niamey and those of the local hen of Congo, which was 28.38 ± 2.3 g. Despite this discrepancy, the Niamey chicks showed incredible growth during the startup period than the Congo chicks (Akouango et al., 2010). The latter had

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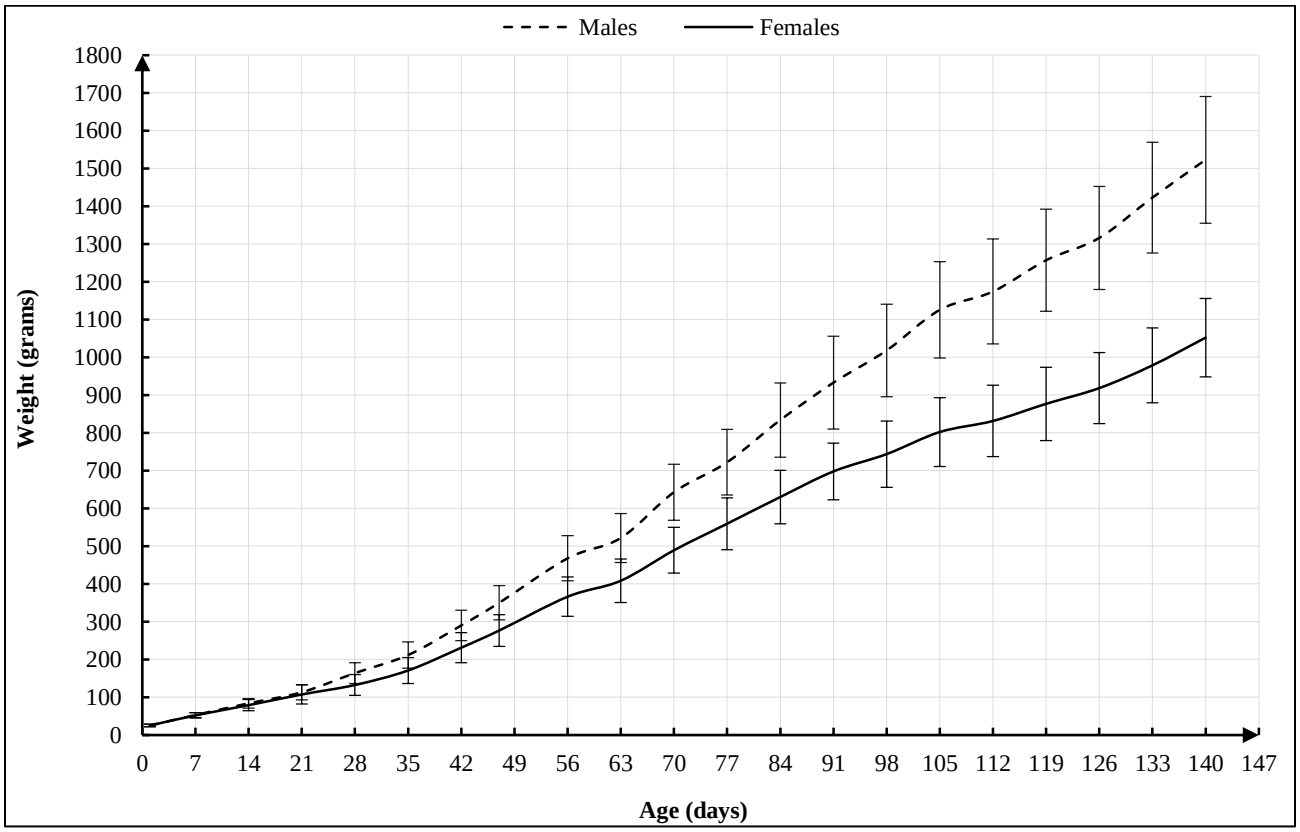
multiplied their weight by 4.2 after one month of rearing, while this ratio was 5.96 for the chicks in the present study. The good growth measured during the startup period in this study would be the result of the high nutritional quality of the feed but also of the breeding environment of the chicks during the startup phase. Indeed, the nutritional balance as well as the appropriate particle size of the food; the adequacy of lighting and heating, allow a better success in chick rearing (Bigot et al., 2001; Laborie et al., 2013; Boussaâda, 2016).

At 140 days of rearing, the males and females in this study weighed 1523.05 ± 26.22 g and 1052.73 ± 14.04 g, respectively. The weights at 140 days of the Niamey chickens were higher than those of the males (1239.36 ± 119 g) and females (897.3 ± 95.54 g) of the local Congolese chicken at the same age (Akouango et al., 2010). A similarity was observed between Niamey chickens and the Forest and Savannah ecotypes of Côte d'Ivoire whose males weighed 1536.8 ± 0.11 g and 1545.1 ± 0.09 g, respectively, and females weighed 1112.0.0 ± 0.12 g and 1089.3 ± 0.12 g at 5 months of age (Yapi-Gnaore et al., 2011). However, for the same age, the weights of the males (1865.13 g) and females (1361.71 g) of the local Kabyle chicken from Algeria were higher than those of the chickens in the present study (Moula et al., 2009b). However, these authors specified that crosses partly explain the high weight of Kabyle chickens with commercial broiler strains carried out by the breeders to increase the production of their chickens. The weights of males and females of the Kolonto ecotype of Niger in rural areas were 1641.2 ± 360 g and 1253.5 ± 275 g (Ousseini et al., 2019). These weights are slightly higher than those obtained in this study. However, it should be noted that the main feature of this Kolonto ecotype is its large size (Ousseini et al., 2020). In this study, the authors reported weight averages of 1484.24 ± 45.86 g for males and 1266.64 ± 45.05 g for females over the entire local chicken population of Niger. These measurements were conducted in rural areas of Gaya region (Dosso-Niger) on subjects whose ages can be well beyond the 140 days to which the present study had limited.

Weight sexual dimorphism

The growth of Niamey chicken follows different curves depending on sex, as shown in Graph 1. In addition, statistical tests revealed significant differences ($p < 0.05$) between males and females beyond the startup phase of the growth. This difference, described as dimorphism, has been identified in several local African strains (Moula et al., 2009b, Ait Kaki and Moula, 2013; N'Dri et al., 2018). This sexual dimorphism always favors males in the species Gallus gallus domesticus and affects weight growth in proportions ranging from 5 to 10% (Mignon-Grasteau and Beaumont, 2000). Such as weight growth, sexual dimorphism is also expressed in other qualitative characteristics called secondary sexual characteristics. For example, sexual dimorphism is expressed through the level of crest development or the appearance of specific colors (Coquerelle, 2000). Thus, it is this sexual dimorphism that explains the difference in plumage color between males and females in this study.



Graph 1. Growth curve of males and females of local chickens in Niamey, Niger, in 2022

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Food valorization

The average daily gain (ADG) and consumption index (CI) values, regardless of gender, were 9.5 g/d and 5.12, respectively. At the $p < 0.05$ threshold, the ADG of roosters (11.56 g/d) was higher than that of hens (7.85 g/d) for the CI, and females had a higher mean (5.87) than males (4.36). The mean ADG of Niamey chicken is higher than those reported by Guédou et al. (2016), which ranged from 5.89 to 6.78 g/d. In contrast, the average ADG in this study is similar to that of the exotic strain (RIR) studied in Ethiopia, which was 8.8 g/d for a rearing period from hatching to 22 weeks (Hassen et al. 2006). The mean CI obtained in this study was slightly lower than those (5.84 to 6.18) of the local chicken from Benin (Guédou et al., 2016). However, the mean CI obtained in this study was comparable to those of local Cameroonian chicken ecotypes that ranged from 4.13 to 5.34 (N'Dri et al., 2018). The local hen of Basse-Kabylie (Algeria) had a consumption index of 7.86 which was much higher than that of the chicken of Niamey (Moula et al., 2009b).

Table 2 shows the weekly evolution of ADG and the CI by sex. For these two parameters, the difference between the sexes was not significant during the first three weeks of rearing ($p > 0.05$); however, for the rest of the time, roosters had significantly higher averages of ADG and CI than hens ($p < 0.05$). The evolution of the ADG has been gradual and nonlinear. However, a substantial increase in CI was observed from day 91 for females and from day 98 for males. This increase in CI at these ages could be partly caused by food waste related to the change in food that occurred on day 84. Indeed, the pre-laying feed had a larger particle size than the grower feed, which could cause animals to look for the intermediate-sized particles they were used to while ejecting the larger particles into the litter. Another explanation would be the triggering of the sexual maturation process of these chickens, which occurs between 18 and 20 weeks of age for the chicken breed of the present study (Guisso Taffa et al., 2022a). Indeed, sexual maturity is a qualitative phenomenon requiring nutrients and energy (Larbier and Leclercq, 1992). This suggests that a part of the consumed food was used for unquantified physiological needs that are not considered in estimating the consumption index.

Thus, the local chicken of Niamey was characterized by a food valorization like that of other strains of local chickens in Africa. The observed performance is partly explained by the genetics of the strain but also by the improvement of the breeding conditions for food and habitat can improve the performance records. Better control when switching from a floury to a coarse food could reduce food waste and thus improve the consumption index during sexual maturation.

Table 2. Average daily gain and consumption index of local salmon-golden chicken in Niamey, Niger, from 7 to 140 days of age

Ages (days)	Average daily gain (g/d) (mean ± SE)		Consumption index (g/g) (mean ± SE)	
	Male	Female	Male	Female
7	3.99 ± 0.11 [a]	3.83 ± 0.13 [a]	1.59 ± 0.05 [a]	1.79 ± 0.14 [a]
14	4.44 ± 0.19 [a]	3.93 ± 0.17 [a]	2.61 ± 0.16 [a]	2.95 ± 0.16 [a]
21	4.18 ± 0.23 [a]	4.33 ± 0.24 [a]	4.89 ± 0.27 [a]	4.60 ± 0.21 [a]
28	8.28 ± 0.67 [a]	5.93 ± 0.50 [b]	3.13 ± 0.25 [a]	3.69 ± 0.23 [a]
35	6.83 ± 0.25 [a]	5.49 ± 0.23 [b]	3.55 ± 0.15 [a]	4.45 ± 0.17 [b]
42	11.20 ± 0.28 [a]	8.62 ± 0.23 [b]	2.52 ± 0.07 [a]	3.32 ± 0.10 [b]
49	8.57 ± 0.32 [a]	6.64 ± 0.23 [b]	3.46 ± 0.14 [a]	4.43 ± 0.14 [b]
56	16.81 ± 0.55 [a]	12.78 ± 0.38 [b]	2.28 ± 0.12 [a]	2.98 ± 0.12 [b]
63	7.98 ± 0.35 [a]	6.47 ± 0.35 [b]	4.91 ± 0.21 [a]	6.01 ± 0.21 [b]
70	17.30 ± 0.64 [a]	11.80 ± 0.34 [b]	2.83 ± 0.13 [a]	4.11 ± 0.15 [b]
77	12.93 ± 0.99 [a]	10.29 ± 0.41 [b]	3.81 ± 0.29 [a]	4.79 ± 0.19 [b]
84	16.45 ± 0.79 [a]	10.10 ± 0.41 [b]	3.17 ± 0.16 [a]	4.82 ± 0.15 [b]
91	15.37 ± 0.84 [a]	9.69 ± 0.47 [b]	3.51 ± 0.20 [a]	5.31 ± 0.22 [b]
98	12.93 ± 0.99 [a]	8.11 ± 0.54 [b]	4.96 ± 0.34 [a]	5.15 ± 0.26 [b]
105	15.77 ± 0.89 [a]	8.73 ± 0.55 [b]	5.28 ± 0.49 [a]	9.97 ± 0.75 [b]
112	9.46 ± 0.85 [a]	6.46 ± 0.54 [b]	8.17 ± 0.66 [a]	10.24 ± 0.63 [b]
119	12.19 ± 0.86 [a]	7.65 ± 0.42 [b]	6.30 ± 0.45 [a]	10.38 ± 0.60 [b]
126	8.94 ± 0.54 [a]	6.40 ± 0.53 [b]	9.59 ± 0.74 [a]	12.96 ± 0.89 [b]
133	15.4 ± 1.16 [a]	9.26 ± 0.60 [b]	6.05 ± 0.56 [a]	9.28 ± 0.75 [b]
140	14.32 ± 0.93 [a]	10.48 ± 0.61 [b]	4.67 ± 0.31 [a]	6.15 ± 0.28 [b]

[a,b] the averages bearing the same superscript letters on the same row are not significantly different at the 5% threshold

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Characteristics of the growth curve

The growth curve parameters estimated between 1 and 140 days are presented in Table 3. The data reported in this Table 3 indicate that in the Niamey chicken, the asymptotic weight (A), age of inflection (TI), and initial specific growth rate (L) of roosters were higher than those of hens. Contrary, the maturation factor (K) of hens was higher than that of roosters. Thus, the general characteristics of the growth curve in the local chicken of Niamey are well in line with the growth characteristics of poultry species marked by sexual dimorphism between males and females (Mignon-Grasteau and Beaumont, 2000).

In comparison with other chicken strains in Africa, the growth curve parameters obtained in this study follow the same trend as those reported by Yapi-Gnaore et al. (2011) on forest and savannah ecotypes in Côte d'Ivoire as well as those of Moula et al. (2009a) on the Kabyle hen in Algeria. The maturation rate (k) of Niamey chicken was lower than that of the chickens studied by N'Dri et al. (2018) and Moula et al. (2009a) but similar to the report of Yapi-Gnaore et al. (2011) and higher than the values reported by Moujahed et al. (2011). These results indicated that Niamey chicken, similar to most African chicken strains, belongs to light strains with slow juvenile growth and maturation compared to highly selected industrial strains (Mignon-Grasteau and Beaumont, 2000; Hassen et al., 2006; Moujahed and Haddad, 2013).

However, the asymptotic weight of the roosters indicates that it is possible to improve their growth by selection and increasing their specific initial growth rate. In addition, Guisso Taffa et al. (2022b) found that selection for growth improvement from 8 weeks of age would give good results for the same chicken strain. The low asymptotic weight of the hens combined with their precocity (high maturation factor) can lead to the production of small size eggs at the beginning of laying. Indeed, the weight of an egg is positively correlated with the age and weight of the hen (Bouvarel et al., 2010). In addition, the precocity of these hens can considerably reduce their growth during the laying phase. Indeed, during the reproductive phase (egg-laying for hens), the female organism preferentially redirects energy and food proteins toward the reproductive process (egg production) at the expense of growth (Whittemore, 2008).

Table 3. Parameters of the growth curve of the local golden salmon chicken in Niamey, Niger

Parameters	Female	Male	Average
A (g)	1313.26	2096.78	1638.06
B	3.91	4.29	4.08
k (g/d)	0.0196	0.0181	0.0188
L (g/d)	0.0765	0.0776	0.0767
T_I (d)	69.69	80.51	74.90

A: Asymptotic weight; B: Integration constant; L: Specific initial growth rate; K: Maturation factor; T_I: Age at the inflection

CONCLUSION

This study is one of the first reports on the growth performance of Niamey chicken in a semi-intensive rearing system. Thus, Niamey chicken exhibited the same growth characteristics as other local African strains. These chickens have a low hatching weight and a high feed conversion ratio due to poor feed conversion. Overall, the results of this study demonstrated that it is possible to improve the growth performance of the local Chicken of Niamey by improving the breeding conditions. Therefore, the use of Niamey's local chicken for profitable production would presuppose an improvement in rearing conditions, then in their consumption index, and later in their weight growth through genetic selection or crossbreeding. With attention to the conditions of hygiene level and farm management in Niger, crossbreeding would be the best way to improve the productivity of local chickens. The interest would be to obtain a hybrid strain that performs well and is acclimatized to the local conditions of Niger. In addition to this study, it would be necessary to conduct other studies to know the reproductive characteristics of this local chicken breed.

DECLARATIONS

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Authors' contributions

Guisso Taffa Adamou, Salissou Issa, Johann Detilleux, Chaibou Mahamadou, and Nassim Moula participated in the design and planning of the study. Guisso Taffa Adamou and Bachir Hamani collected the data. Guisso Taffa Adamou, Salissou Issa, and Nassim Moula participated in the analysis and interpretation of the data. Guisso Taffa Adamou wrote the first version of the manuscript. Salissou Issa, Chaibou Mahamadou, Johann Detilleux, Nassim Moula, and Bachir Hamani contributed to the critical revision of the manuscript. All authors checked and approved the final draft of the manuscript for submission to the present journal.

Conflict of interests

The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of the data; in the writing of the manuscript; or in the decision to publish the results.

Ethical considerations

The authors confirm that all ethical aspects of publishing an original article have been taken into account in the elaboration of this manuscript.

Availability of data and materials

The authors declare that they will be ready to provide all the necessary data upon reasonable request.

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Risk Factors Associated with Brucellosis Seropositivity in Goat Farms of Sing Buri Province, Thailand

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ABSTRACT

During 2012 - 2016, goat farms in Sing Buri province were growing rapidly with support from the Thai government. In the following three years (2017-2019), the analysis of brucellosis surveillance data indicated that the seropositivity of brucellosis in goats increased. Therefore, this study attempted to identify possible risk factors associated with brucellosis seropositivity in meat goats raised in Sing Buri province of Thailand. A case-control study was conducted in a random sampling of 72 goat farms in Sing Buri province, Thailand. Questionnaires were used to collect information regarding farm production types, husbandry, goat health management, grazing management, breeding, carcass management, and goat purchasing. Bivariate and logistic regression analyses were used to determine the risk factors of *Brucella* seropositivity. Results revealed that the most frequent health complaint by the farmers was a stillbirth. *Brucella* seropositivity at the farm level was 26.4%. The two most probable risk factors for seropositivity included raising goats in a communal pasture and keeping goats with a history of clinical signs associated with brucellosis. In conclusion, approximately 25% of goat farms in Sing Buri province were infected by the bacteria genus *Brucella*. The farmers were recommended to attentively seek and cull for a brucellosis-suspected goat in their farms using clinical signs or symptoms together with active serosurveillance. Furthermore, communal pasture avoidance would also help prevent the goat from *Brucella* infection.

Keywords: Brucellosis, Meat goat, Risk factor

INTRODUCTION

Brucellosis is a zoonotic infectious disease, known as undulant, Mediterranean, or Malta fever, caused by the bacteria genus *Brucella* (Xavier et al., 2009). The disease in animals is characterized by abortion or reproductive failure (Samadi et al., 2010). The most common *Brucella* species that cause infection in goats is *Brucella melitensis*, which can also infect sheep, cattle, buffalos, swine, dogs, camels, horses, and rodents; or can contaminate their products (Xavier et al., 2009). The economic losses due to brucellosis were 6.8 US$ per cattle, 18.2 US$ per buffalo, 0.7 US$ per sheep, 0.5 US$ per goat, and 0.6 US$ per pig (Singh et al., 2015). Brucellosis in humans most often occurs as a result of drinking raw milk from infected animals (Fuquay, 2011). Humans are accidental hosts, and all age groups can be affected by this disease. Some evidence indicated that brucellosis is an occupational hazard for livestock officers and goat farmers (Te-Chaniyom et al., 2016). Human infections of *Brucella melitensis* were confirmed in Southern Vietnam (Campbell et al., 2017). The disease in humans may persist as relapse, chronic localized infection, or delayed convalescence (Nimri, 2003). Brucellosis continues to be a major public health concern worldwide. The disease is widely distributed throughout the developing world, considered to be a serious public health problem. Livestock prevalence of brucellosis in 2010 was 8.2% in East Africa, 15.5% in West Africa, 14.2% in South Africa, 13.8% in North Africa, 16.0% in South Asia, and 2.9% in South-East Asia (McDermott et al., 2013). From 2000 to 2009, the seroprevalence of brucellosis in Malaysia was 0.91% among goats and 7.09% among goat farms (Bamaiyi et al., 2015).

Seropositivity risk factors have been reported in different studies (Akhter et al., 2014; Tsegay et al., 2015; Rajala et al., 2016). In Northern Thailand, herd size, reproductive problems, brucellosis test program, source of the new goat, and disinfection in the farm played significant roles in *Brucella* seropositivity (Kladkempetch et al., 2017). A study in South China demonstrated that introduction in the past 12 months, improperly disposal of sick or dead goats, and poor hygiene in the lambing pen were the potent risk factors associated with *Brucella* seropositivity on local goat farms (Li et al., 2021).

Sing Buri province is divided into six districts, where all districts raise goats. During 2012-2016, goat farms in Sing Buri province are growing rapidly with support from the Thai government. The Department of Livestock

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Development (DLD) launched a nationwide brucellosis surveillance campaign on goats. This campaign monitored goat health status both at the provincial and national levels. From laboratory surveillance data of small ruminant brucellosis in 2013, seroprevalence was 12.1% among farms, 1.4% at the animal level for goats, and 1.6% for sheep (Sagarasaeranee et al., 2016). The previous results showed that after 2013 the seroprevalence seemed to be increasing year by year, which would increase the risk of poor goat production. *Brucella* could also contaminate the goat products and the environment; therefore, the risk of human infection increase (Te-Chaniyom et al., 2016; Maksimović et al., 2022). However, no previous study on identifying risk factors of seropositivity of brucellosis in goats was performed in Sing Buri province.

Therefore, this study was designed to investigate possible risk factors associated with brucellosis seropositivity in goat farms raised in Sing Buri province. The expected results could be used to recommend prevention and control measures for brucellosis in goat farms.

MATERIALS AND METHODS

Ethical approval

Institutional Animal Care and Use Committee, Kasetsart University (ACKU65-VET-048) approved all procedures in this study. However, the study did not involve animals as an experimental setup. The seropositive (case) and seronegative (control) farms were from the routine measures for the brucellosis surveillance system of the DLD, Thailand. The permission in a verbal form to conduct the study and to use the data was agreed upon by the relevant authorities involved in this study.

Study area

Sing Buri province has six districts (Inburi, Bang Rachan, Mueang, Khai Bang Rachan, Phrom Buri, and Tha Chang). The study area covered six districts of Sing Buri province (Figure 1). The global positioning system (GPS) coordinate of Sing Buri province is 14°53'20.99" N 100°24'25.19" E. The hot season lasts 2 months, from March 6 to May 5, with an average daily high temperature above 36°C. The hottest month of the year in Sing Buri is April, with a temperature range of 27-37°C. The cool season lasts 4.6 months, from August 29 to January 15, with an average daily temperature below 33°C. The coldest month of the year in Sing Buri is December, ranging from 21°C to 32°C. The rainy period of the year lasts for 8.9 months, from March 1 to November 28, with rainfall of at least 13 mm. The month with the most rain in Sing Buri is September, with an average rainfall of 210 mm (Weather Spark, 2022).

Study design

A case-control study between December 2016 and February 2017 was conducted. The case and the control goat farms were from the DLD brucellosis surveillance program. The DLD operated a brucellosis surveillance campaign in meat and dairy goats throughout Thailand. In brief, goats older than 6 months in all farms were tested for *Brucella* infection using a modified Rose Bengal Test (mRBT; Ferreira et al., 2003) at the provincial laboratory. If serum samples were positive, those serum samples would then be sent to the central laboratory at the National Institute for Animal Health of the DLD for further confirmation using the complement fixation test (CF test) and enzyme-linked immunosorbent assay (ELISA). When the confirmatory test results were positive, the goats must be culled. Other goats in the same herd of the positive goat had to be re-tested at least three times for the two-month interval. When the confirmatory tests were negative for three consecutive samplings, the test would be performed again after 6 months. When the last tests were negative, the outbreak was declared over. In Thailand, *Brucella* vaccines were not used to prevent brucellosis in small ruminants.

The laboratory records of brucellosis were critically reviewed for brucellosis testing results in each goat farm. The case was defined as the farm in which at least one goat was diagnosed as positive to the confirmation tests, while the control was defined as the farm without any goats that tested positive to the mRBT three consecutive times for the two-month interval.

Sample size calculation

The study unit was the farm. The sample size was calculated by Stat Calc of Epi Info program (Centers for Disease Control and Prevention, USA). Out of 156 farms under the brucellosis surveillance program, the sample size required for this study was 72 farms (case = 19; control = 53) to achieve 95% confidence level, 80% power, and an odds ratio of 4.8. All goat farms in the province were listed, then each goat farm was selected by simple random sampling.

Data collection

Face-to-face interview with goat farmers using questionnaires was conducted. The questions concerned with herd management, husbandry, and health care were asked, and relevant information was given by the farmers in charge of

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caring for the farm animals. Variables of interest for the herd level were included in the questionnaires. The interview process was performed by a veterinarian.

Data analysis

Descriptive and inferential statistics were applied to identify potential risk factors associated with seropositivity of brucellosis using Stat Calc of Epi Info program (Epi Info™ 7, USA). Bivariate analysis was performed to determine the impact of each variable on seropositivity. If any variables got $p \leq 0.1$ in the bivariate analysis (Chi-square test), those variables were recruited into the multiple logistic regression analysis. The odds ratios were used to interpret the risk factors associated with seropositivity in this study.

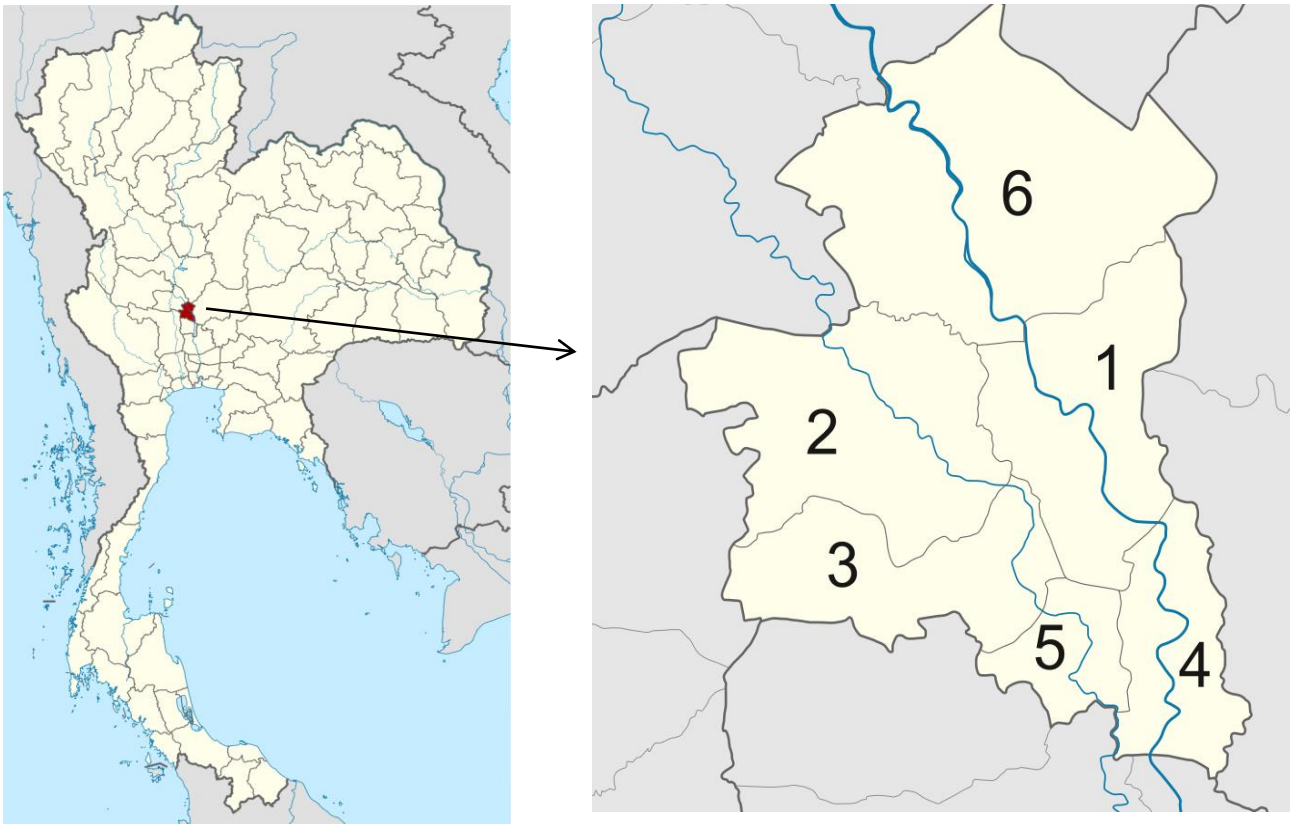

Figure 1. The map of the study area in Sing Buri province (Left), Thailand. The study site for identification of risk factors associated with seropositivity to goat brucellosis in six districts of Sing Buri province (Right); 1: Muang Sing Buri, 2: Bang Rachan, 3: Khai Bang Rachan, 4: Prom Buri, 5: Tha Chang, 6: Inburi. Source: Wikipedia

RESULTS

Table 1 demonstrates the overall brucellosis seropositivity in 72 goat farms according to the brucellosis surveillance program at the beginning of the study (December 2016). Out of 72 goat farms, Bang Ra Chan district had the highest prevalence (29.17%). Only one goat farm in Prom Buri and one in Tha Chang district were active in their business and were recruited into the study. The farm in Prom Buri was positive, and the farm in Tha Chang district was negative for brucellosis.

Table 2 presents the characteristics of goat farms and management in Sing Buri province. In total, 5,221 goats were raised in all 72 studied farms. All farms raised crossbred meat goats. There were two types of goat farms: fattening and breeding. The average herd size in infected farms was 73 goats/farm (range = 12-600), and in non-infected farms was 48 (range = 8-206). The average number of breeder males (buck) was 1.36 (range = 0-10), and the average number of breeder females (doe) was 35.9 (range = 2-300). The average number of goats categorized by age group in all farms was 18.75 goats/herd (range = 0-200), 16.65 goats/herd (range = 0-99), and 36.68 goats/herd (range = 3-302) for less than 6 months old, 6-12 months-old, and greater than 12 months old, respectively. The floor type of the barn was predominantly a combination of the slatted floor where the goats stayed during the night and the ground floor during the day. The main roughages included *leucaena*, straw, and para-grasses (*Brachiaria mutica*, Forsk). Most farms fed their goats with commercially available concentrates and offered the goats some mineral supplements. Most farms used the communal pasture, and only a few farms had their own pastures. Almost all farms consumed the water supply from tap water. For breeding purposes, 20% of the farms shared their bucks with others, while only one farm used artificial insemination.

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When the farmers found their dead goats, 83% of the farmers buried the carcass on their farms. About half of the farmers sold their goats to other farms, and the most frequent selling method was straight to the buyers on the farm site.

Table 1. Distribution of brucellosis seropositivity of goat farms according to the brucellosis surveillance program in Sing Buri province, Thailand, in December 2016

District	Number of farms	Number of seropositive farms	Percentage
Bang Rachan	24	7	29.17
Inburi	29	6	20.69
Khai Bang Rachan	5	2	40.00
Mueang	12	3	25.00
Phrom Buri	1	1	100.00
Tha Chang	1	0	0.00
Total	72	19	26.39

Table 2. Characteristics of goat farms and management in Sing Buri province, Thailand, between December 2016 and February 2017

Farm characteristics	Seropositive farms (n = 19)	Seronegative farms (n = 53)	Total (n = 72)
Farm production			
Fattening	19	46	65
Breeding	13	42	55
Average herd size (goat per farm)	73	48	60
Stall			
Cement floor	1	5	6
Ground floor	13	37	50
Slat floor	17	45	62
Feeding			
Leuceana	14	42	56
Straw	0	3	3
Grass	16	44	60
Concentrates	5	23	28
Mineral supplementation	15	47	62
Own pasture	1	7	8
Sharing pasture with other farms	1	3	4
Communal pasture	18	31	49
Water supply			
Tap water	18	49	67
Underground water	0	5	5
Surface water	2	5	7
River water	0	2	2
Breeding			
Artificial insemination	1	0	1
Sharing buck with other farms	1	14	15
Sharing doe with other farms	0	0	0
Dead goat management			
Bury	18	42	60
Consumed by farmers	0	1	1
Burn	0	1	1
Selling production to			
Local traders	1	2	3
Other farms	11	31	42
Co-operatives	0	0	0
Selling methods			
On-farm site	18	42	60
By mobile phone	0	10	10
At the live market	1	3	4

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The previous health problems concerning brucellosis are reported in Table 3. In this study, the most serious problems among goat farms included stillbirth, weak kids, abortion, mastitis, and lameness. In seropositive farms, the most complaint of health problems was stillbirth and abortion, while mastitis and stillbirth were the most frequent concerns in seronegative farms.

Twenty possible risk factors associated with brucellosis seropositivity were analyzed using bivariate analysis (Table 4). Farms that had flooding during 2012-2016 tended to have 3.1 times at risk of brucellosis seropositivity, compared with farms that did not have flooding. Farms that shared their bucks with others for mating tended to have 0.1 times at risk of seropositivity when compared with farms that did not share. Farms that had raising experience for more or equal to 60 months compared with farms that had less than 60 months tended to have 0.4 times at risk of seropositivity. Farms with previous health problems related to brucellosis were 5.1 times at risk of seropositivity, compared with farms without previous health problems. In addition, farms that used communal pasture were 12.8 times at risk of seropositivity when compared with farms that did not use it. Two factors were significant risk factors for brucellosis, were raising goats in communal pastures and goats with clinical signs of suspected brucellosis. In addition, goats that were not confined only in the barn tended to be 6.5 times at risk of seropositivity compared to goats confined in the barn.

For logistic regression analysis, the six important risk factors were recruited in the analysis, which was raising goat in the communal pasture, sharing bucks with other farms, quarantining newly introduced goats before entering the herd, receiving goats with previous health problems related to brucellosis, raising experience; and flooding occurrence. The results indicated that raising goats in communal pastures and the farm receiving goats with previous health problems related to brucellosis were most likely to be the significant risk factors for brucellosis seropositivity (Table 5).

Table 3. Previous health problem (clinical signs/symptoms) in relation to brucellosis among goat farms in Sing Buri province**,** Thailand between December 2016 and February 2017

Signs/symptoms	Seropositive (n = 19)		Seronegative (n = 53)		Total (n = 72)	
	No.	(%)	No.	(%)	No.	(%)
Stillbirth	6	31.6	6	11.3	12	16.7
Weak kid	2	10.5	9	17	11	15.3
Abortion	5	26.3	5	9.4	10	13.9
Mastitis	3	15.8	7	13.2	10	13.9
Lameness	2	10.5	4	7.6	6	8.3
Infertility	2	10.5	3	5.7	5	6.9
Arthritis	1	5.3	2	3.8	3	4.2
Metritis	1	5.3	2	3.8	3	4.2
Retained placenta	1	5.3	1	1.9	2	2.8
Orchitis	0	0	1	1.9	1	1.4

No: Number

Table 4. Bivariate analysis on possible risk factors for brucellosis seropositivity in goat farms, Sing Buri province, Thailand, between December 2016 and February 2017

Factors		Brucellosis test		Crude OR	95 %CI		p-value
		Seropositive	Seronegative		Lower	Upper	
Flooding occurrence	Yes	8	10	3.1	1.0	9.8	0.06
	No	11	43				
Raising experience	Low, <60 months	7	33	0.4	0.1	1.0	0.06
	High, ≥ 60	12	20				
Previous case of brucellosis on a farm during 2012-2015	Yes	4	0	NA*	2.0	NA	0.003
	No	15	53				
Raising goats and sheep on a farm	Yes	1	1	2 .9	0.2	48.6	0.46
	No	18	52				
Previous health problems related to brucellosis	Yes	9	8	5.1	1.6	16.4	0.009
	No	10	45				
Herd size	High, ≥ 73 goat	10	18	2.2	0.7	6.3	0.15
	Low, < 73 goat	9	35				
Purchase into the farm	Yes	11	38	0.5	0.2	1.6	0.27
	No	8	15				
Purchase out of the farm	Yes	19	41	NA	1.1	NA	0.02
	No	0	12				
Quarantine new goats before introducing	Yes	0	7	0	0	1.9	0.10
	No	19	46				
Testing brucellosis before introducing	Yes	9	20	1.5	0.5	4.3	0.46
	No	10	33				
Sharing bucks with other	Yes	1	15	0.1	0.02	1.1	0.05

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farms	No	18	38				
Using communal pasture	Yes	18	31	12.8	1.6	102.9	0.004
	No	1	22				
Confine only in the barn	No	18	39	6.5	0.8	53.0	0.10
	Yes	1	14				
Water canal	Yes	2	5	1.1	0.2	6.4	0.89
	No	17	48				
Contact with other goats outside the farm	Yes	4	8	1.5	0.3	6.6	0.55
	No	15	45				
Contact with other animals outside the farm	Yes	12	41	0.5	0.2	1.6	0.24
	No	7	12				
Cement floor	Yes	1	5	0.5	0.1	4.9	1.00
	No	18	48				
Ground floor	Yes	13	37	0.9	0.3	2.9	0.91
	No	6	16				
Disinfectant	Yes	15	39	1.3	0.4	4.7	0.76
	No	4	14				
Vehicle control	Yes	17	38	3.4	0.7	16.3	0.21
	No	2	15				

*NA: Not available for calculation; OR: Odds ratio

Table 5. Multiple logistic regression analysis on possible risk factors for brucellosis seropositivity in goat farms, Sing Buri province, Thailand, between December 2016 and February 2017

Factors	Crude OR	Adjusted OR	95%CI	
			Lower	Upper
Using communal pasture	12.8	14.8	1.5	140.6
Sharing bucks with other farms	0.1	0.1	0	1
Quarantine new goats before introducing them to the farm	0	0	0	$>1x10^{12}$
Previous health problems in relation to brucellosis	5.1	6.3	1 .3	30.6
Raising experience	0.4	2.9	0.7	12.2
Flooding occurrence	3.1	3	0.6	14.6

OR: Odds ratio, CI: Confidence interval

DISCUSSION

Sing Buri Province is located in the central part of Thailand, which is one of the most populated areas for goat farming. The farms are predominantly small-holder farms, where their raising practices might not be appropriate. However, the Provincial Livestock Office of the DLD assisted these farmers in disease treatment, prevention, and control. For brucellosis, the primary goal of the DLD was to eradicate this disease from goat farms. Therefore, a test-and-slaughter policy was implemented (Sagarasaeranee et al., 2016). The study was not designed to determine the seroprevalence; however, 26.39% of the studied farms were seropositive to *Brucella* infection. This seropositivity rate was higher than the seroprevalence of 16.67% reported by Kladkempetch et al. (2017) in Chiang Mai, the northern province of Thailand, and even higher than in Nakhon Si Thammarat, the southern province of Thailand (Te-Chaniyom et al., 2016). In a recent study in Ethiopia, the herd level seroprevalence of goat brucellosis was 46.61% (Teshome et al., 2022), which was rather high, compared to the present study. The underlying reason for the high prevalence was that no actual control plans for brucellosis were strictly implemented in some study areas. The *Brucella* infection could be expected to be relatively high if the farm had no effective control measures.

In this study stillbirth, weak kids, abortion, mastitis, and lameness were the most noticeable signs or symptoms associated with *Brucella* infection. Rerkyusuke et al. (2022) studied the clinical evidence and risk factors for reproductive disorders in meat goats in Northeastern Thailand and indicated that abortion with arthritis, orchitis, repeat breeder, sterile, and week kids have occurred in goat herds seropositive to either Q fever or chlamydiosis, or brucellosis. *Brucella* infection in goats is important in female reproductive disorders, especially abortion. In the study of Samadi et al. (2010) in Jordan, the prevalence rate of *Brucella* infection among aborted sheep and goats was 27.1%. It is also evident that *Brucella* was detected in a higher frequency in the samples such as blood, milk, supra mammary lymph nodes, udder tissue, aborted fetal organ, and placenta collected from the aborted animals than those samples collected from asymptomatic animals (Maksimović et al., 2022).

Sing Buri Province is one of the central-plains provinces in Thailand that has been affected by flooding during the monsoon season from July to October every year. During the flooding period, goat farmers had to rescue their goats to communal places where goats from several farms could most likely be contacted with each other. The flood situation might lead to the situation of poor management and overcrowding. It has been reported that a lack of separation of young, pregnant, or sick animals could increase the likelihood of *Brucella* seropositivity (Natesan et al., 2021). This might be one of the possible reasons why raising goats in a flooding area tended to increase the risk of *Brucella*

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seropositivity in this study. When a buck was used in an infected farm, it could transmit the infection to other farms. In this study, sharing bucks with other farms for mating showed an increased risk of seropositivity. In Thailand, it has been common for goat farmers to share their bucks for breeding purposes. The infected bucks could potentially be a source of disease transmission among goat farms (Te-Chaniyom et al., 2016; Rerkyusuke et al., 2022). This study demonstrated that farms with a longer period of goat farming, more than 5 years, were less likely to be seropositive to brucellosis. Farmers who have owned their farms for a longer time might have more concerns about brucellosis and might take action on prevention and control according to the guidance of the DLD officers. While relatively new farmers might have fewer concerns and might pay less attention to the brucellosis surveillance provided by the DLD. It could be noted that education and awareness of goat farmers play a key role in the effectiveness of brucellosis prevention and control (Natesan et al., 2021). Since the DLD has implemented the test and culling policy for goat brucellosis for several years, the seropositive farms were expectedly to decline yearly. The decline of *Brucella* infection depending largely on the testing and culling measures has been reported by Rerkyusuke et al. (2022). It could also be implied that farms that did not participate in the annual brucellosis testing and culling would most likely be seropositive (Rerkyusuke et al., 2022).

In this study, goat farms with previous health problems related to brucellosis, particularly reproductive problems, had more likely to be *Brucella* seropositive. This finding agreed with other studies (Samadi et al., 2010; Boukary et al., 2013; Kladkempetch et al., 2017). Boukary et el. (2013) found that the prevalence rate of *Brucella* seropositivity increased with the occurrence of abortion on the farm. In their study, farms with females that aborted among the animals had 4.2 times at risk for *Brucella* seropositive compared with the farms that did not have an abortion. Samadi et al. (2010) indicated that the number of *Brucella melitensis* cases is rather high among aborted animals. Likewise, Kladkempetch et al. (2017) found that reproductive problems significantly depended on *Brucella* seropositivity in goat farms. Teshome et al. (2022) also reported that a history of reproductive problems was a potential risk factor for the prevalence of brucellosis in goats in the Borana zone of Ethiopia. Farms with a previous history of reproductive problems closely related to brucellosis should be seriously monitored for the reservoir animals within the farm. Regular testing and culling measures could be helpful to completely eradicate the disease from the farm.

In addition, the present finding showed that farms that used communal pasture had an increased risk of seropositivity. The pasture-sharing practice could increase the potential for exposure to *Brucella* spp. in a contaminated environment or to the secretion of infected goats that share the same pasture (Reviriego et al., 2000). Samadi et al. (2010) provided evidence that grazing at a common pasture was a significant risk factor positively associated with brucellosis seropositivity. In Thailand, most goat farmers were small-scale holders. To reduce the feed costs, it was common for the farmers to share the communal pastures with others, for both small and large ruminants. Without any precautions, the pasture could be contaminated with *Brucella* spp., which could then be transmitted to other animals during grazing. In addition, the goats from different farms could have direct contact with each other, which was prone to receive the bacteria from the infected ones. From the logistic regression analysis results in this study, raising goats in communal pastures and the farm receiving goats with previous health problems related to brucellosis were most likely to be the significant risk factors for brucellosis seropositivity. Some other interesting risk factors for *Brucella* seropositivity were identified at the herd level. In India, brucellosis prevalence increased due to the frequent purchase of goats with an unknown background of brucellosis on the farm (Natesan et al., 2021). In Thailand, Te-chaniyom et al. (2016) found that goat farms that have dogs and/or rats on the farm were 5.12 times at risk of *Brucella* seropositive. Dogs and cats might spread the bacteria within the farm, and probably between farms if the dogs and cats were roaming freely.

CONCLUSION

Brucella spp. infected about 25% of goat farms in Sing Buri province; hence brucellosis was still a problem in goat production in Sing Buri province. The transmission of the disease could be reduced when the farmers carefully seek a suspected brucellosis goat using clinical signs/symptoms together with active serosurveillance. If any goats developed signs/symptoms of a case definition, most likely related to reproductive problems, or got seropositive by the test, they should be immediately culled by proper methods. Avoiding communal pastures or, if not possible, carefully managing the pasture with others should be considered to alleviate the risk of exposure to *Brucella* reservoir goats or contaminated pastures. Further studies on goat farmers' knowledge, attitude, and practice on communal pasture-sharing practices are also necessary to reduce goat brucellosis transmission in risky locations.

DECLARATIONS

Authors' contributions

Karoon Chanachai, Monaya Ekgatat, Tippawon Prarakamawonga, and Theera Rukkwamsuk conceived, designed, and supervised the project. Nattanan Thaumsuwan executed the experiment and analyze the data. Nattanan

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Thuamsuwan, Prakit Srisai, and Theera Rukkwamsuk interpreted the data and drafted the manuscript for intellectual content. Theera Rukkwamsuk and Nattanan Thuamsuwan critically revised the manuscript and all authors approved the final version. In addition, all authors had full access to all data in the study and took responsibility for the integrity of the data and accuracy of the data analysis. All authors checked and approved the results and the final version of the manuscript before publication.

Competing interests

The authors certified that there is no conflict of interest.

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Ethical consideration

The authors declared that this studied data had not been submitted previously for publication. The manuscript was original without plagiarism. The authors had scientifically conducted the research. Data were analyzed and taken care of for their fabrication and/or falsification. All authors agreed to publish the results.

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Availability of data and materials

The authors declare that they will prepare all the necessary data for the study upon reasonable request.

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Detection and Antimicrobial Susceptibility of *Salmonella* spp. Isolated From Commercial Eggs in Tiaret Province, Algeria

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ABSTRACT

Salmonellosis is a significant public health problem worldwide. The current study aimed to investigate the presence of *Salmonella* spp. in commercial eggs of Tiaret province, Algeria, and evaluate the susceptibility of isolated strains to different antimicrobial agents. A total of 180 commercial eggs collected from various retail outlets (groceries, butchers, wholesalers, street vendors) were analyzed by conventional methods, and 13 *Salmonella* spp. isolates were tested on a panel of 7 antimicrobial agents using the disc diffusion method. Of 180 chicken egg content samples examined, the findings indicated that 13 (7.22%) were positive for *Salmonella* spp. Regarding the collection site, 2 (1.11%), 4 (2.22%), and 7 (3.88%) of *Salmonella* spp. isolates were detected from butchers, wholesalers, and street vendors, respectively. Most antibiotic discs have demonstrated widespread resistance with an incidence rate of 100%, including amoxicillin + clavulanic acid, ampicillin, nalidixic acid, and erythromycin. However, colistin sulfate, gentamycin, and tetracycline were more effective against *Salmonella* isolates. It can be concluded that the highest detection rate of *Salmonella* spp. was observed for street vendors, and the highest resistance was recorded for commonly used antibiotics in poultry production.

Keywords: Antimicrobial, Chickens, Commercial eggs, *Salmonella*, Tiaret

INTRODUCTION

Salmonellosis is considered one of the most common foodborne diseases. The World Health Organization (WHO) reported 94 million cases of non-typhoid *Salmonella* gastroenteritis worldwide, with annual deaths reaching 500,000 in 2018 (WHO, 2018). Furthermore, the incidence and severity of cases of salmonellosis have increased significantly in both developed and developing countries (WHO, 2014). In Algeria, over 5000 human cases are reported yearly, but the real number of infections is likely higher (Fourar, 2019).

Poultry products are the most incriminated in most traceable foodborne diseases caused by *Salmonella* (Thorns, 2000). Chicken eggs, in particular, are considered the most potent food vectors of this bacterium for humans (Finstad et al., 2012). The contamination of eggshells and egg contents can occur during egg formation in the reproductive tract of laying hens or during processing and distribution due to poor hygienic practices (Moosavye et al., 2015).

Salmonella bacterium is a Gram-negative rod genus belonging to the *Enterobacteriaceae* family. Within two main species *Enterica* and *Bongoli*, over 2500 serotypes or serovars have been identified to date. *Enterica* species with serovars *Enteritidis* and *Typhimurium* are considered to be the most common foodborne species that cause human salmonellosis (Bahness et al., 2015).

Over the past decade, antimicrobial resistance has become a severe public health problem worldwide (Ali and Mohamed, 2020; Mirzaei et al., 2022). Several researchers have reported the emergence of multidrug-resistant bacteria isolated from poultry products (Almashhadany, 2019; Xie et al., 2019; Merati et al., 2020). Antimicrobial resistance is a natural consequence of adapting infectious agents to exposure to antimicrobials in medicine and food animals. Therefore, an increase in the resistance, particularly of *Salmonella*, to commonly used antimicrobials has been noted in both public health and veterinary sectors, and multidrug-resistant phenotypes have been increasingly described among *Salmonella* species worldwide (Castro-Vargas et al., 2020). Monitoring antibiotic resistance among *Salmonella* isolates and controlling its health risk is essential.

Due to the lack of updated and accurate data in Tiaret Province, Algeria, about the antimicrobial resistance pattern of *Salmonella* spp. associated with commercial eggs, the present study investigated the presence of *Salmonella* spp. in the commercial eggs in Tiaret Province, Algeria, and evaluated the susceptibility of isolated strains to different antimicrobial agents.

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MATERIALS AND METHODS

Ethical approval

This research was performed following the Veterinary Sciences Institute, University of Tiaret, Algeria guidelines.

Study area

The study was performed in Tiaret province, in Western Algeria. Geographically, the study site is located at a latitude of 35° 22' 15.71" N and a longitude of 1° 19' 1.16" E, and at an elevation average of 978 m above sea level.

Egg samples collection

A total of 180 unwashed chicken eggs were collected at random from different retail outlets (groceries [45], butchers [45], wholesalers [45], and street vendors [45]), located in Tiaret Province, under sterile hygienic conditions from January to May 2021. The samples were aseptically transported in an ice box to the hygiene and animal pathology laboratory, Tiaret, Algeria, for immediate bacteriological analysis.

Culture of *Salmonella*

Isolation procedures for *Salmonella* were carried out according to the recommendations of the International Organization for Standardization (ISO 6579, 2002). To collect the egg content, the surface of each chicken egg was disinfected with 75% alcohol, the shell was cracked with a sterile knife and carefully removed, each egg's content was mixed thoroughly and 25 g of the mixed egg content was inoculated into 225 ml of peptone broth and incubated at 37°C for 24 h. After the overnight incubation, 1 ml of the pre-enrichments broth was transferred aseptically into a tube containing 9 ml of Rappaport-Vassiliadis broth and incubated at 37°C overnight. Following incubation, a loopful of the broth was streaked onto one plate of xylose lysine desoxycholate agar (XLD; Biokar, France) and another plate of *Salmonella-Shigella* agar (SS; Biokar, France), and incubated at 37°C for 24 hr. Presumptive colonies for *Salmonella* isolates were then transferred onto nutrient agar (Oxoid, UK) and incubated aerobically at 37°C overnight (ISO, 2002).

Biochemical test

Salmonella suspected isolates were inoculated into triple sugar iron agar (TSI) slopes (Sigma-Aldrish, US) and incubated at 37°C for 24 hr. Typical *Salmonella* phenotypes were further confirmed with Analytic Profile Index (API) 20 E test strips (BioMerieux, France; Yang et al., 2015). All isolated strains were stored in peptone-glycerol solutions at -20°C for subsequent analysis.

Antibiotics susceptibility testing

The susceptibility of *Salmonella* isolates to antibiotics was examined by using the agar disc diffusion method on Mueller–Hinton Agar (MH; Biokar, France) according to the Standardization of Susceptibility to the National Scale Human and Veterinary (SSNSHV, 2011) guidelines. The following antibiotics manufactured for the analytical purpose were tested, amoxicillin + clavulanic acid (AMC: 30 µg; CYPRESS DIAGNOSTICS, Belgium), ampicillin (AM: 10 µg; CYPRESS DIAGNOSTICS, Belgium), tetracycline (TE: 30 µg; CYPRESS DIAGNOSTICS, Belgium), gentamycin (CN: 10 µg; Liofilchem, Italy), nalidixic acid (NA:30 µg; Liofilchem, Italy), erythromycin (E: 15 µg; Liofilchem, Italy), and colistin sulfate (CS:10 µg; Liofilchem, Italy).

Three to four colonies were suspended in 10 ml of 0.9% NaCl from a pure culture of each *Salmonella* isolate. The suspension was adjusted to match the turbidity standard of 0.5 McFarland units. Approximately 1 x 10^8 colony-forming units were streaked on MH agar plates using a sterile cotton swab, and the antibiotic discs were applied at the indicated doses. After aerobic incubation at 35°C for 20 h, the interpretation of the zones of inhibition was performed, and results were expressed as sensitive (S), intermediate (I), and resistant (R) according to the recommendation of SSNSHV (2011). An isolate was multidrug-resistant if it was resistant to at least one agent in three or more antimicrobial classes (Zhang et al., 2018).

Statistical analysis

Descriptive statistics were performed to compare proportions. The data were analyzed using Microsoft Excel 2016 (USA).

RESULTS

Bacteriological and biochemical examination

Out of 180 chicken egg samples examined, 13 (7.22%) were found positive for *Salmonella* spp. Regarding the collection site, 2 (1.11%), 4 (2.22%), and 7 (3.88%) of *Salmonella* spp. isolates were identified from butchers, wholesalers, and street vendors, respectively (Table 1). The isolates produced typical red-colored colonies with black

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centers on the XLD agar medium. Colorless colonies with black centers were also produced on SS agar medium. Biochemical characterization performed on API 20 E revealed that all the isolates were positive for lysine decarboxylase, ornithine decarboxylase, hydrogen sulfide, glucose, mannose, sorbitol, rhamnose, melibiose, and arabinose. In contrast, o-nitrophenyl-b-D-galactopyranoside, arginine dihydrolase, citrate, urease, Tryptophan deaminase, Indole, Voges-Proskauer test, gelatinase, inositol, sucrose, and amygdalin tests were negatives.

Antibiotic resistance of *Salmonella* spp. isolates

Salmonella isolates were tested against seven antibiotics. The results of susceptibility testing are illustrated in Table 2. All the isolates were resistant to amoxicillin + clavulanic acid, ampicillin, nalidixic acid, and erythromycin and immediately resistant to tetracycline. However, colistin sulfate and gentamycin were more effective against *Salmonella* isolates with 100% sensitivity. Regarding the multidrug-resistance results, all the isolates were resistant to three antimicrobial classes (Macrolide, Beta-lactam, and Quinolone); this means that 100% of the isolates were multidrug-resistant.

Table 1. Percentage of detected *Salmonella* spp. in different outlets isolated from commercial eggs in Tiaret, Algeria

Collection site	Number of examined samples	Number of Positive (%)
Groceries	45	00
Butchers	45	2 (1.11)
Wholesalers	45	4 (2.22)
Street vendors	45	7 (3.88)
Total	180	13 (7.22)

Table 2. Antimicrobial susceptibility results for 13 *Salmonella* spp. isolated from commercial eggs in Tiaret, Algeria

Antimicrobial agent	Disc concentration (µg)	*Salmonella* spp. isolates (n=13)			Percentage of resistance
		S	I	R	
Ampicillin	5	0	0	13	100
Amoxicillin + acide Clavulanique	10	0	0	13	100
Tetracycline	30	0	13	0	0
Gentamycin	10	13	0	0	0
Nalidixic acid	30	0	0	13	100
Erythromycin	15	0	0	13	100
Colistin sulfate	10	13	0	0	0

S: Sensitive, I: Intermediate, R: Resistant.

DISCUSSION

Salmonella has been the primary cause of the foodborne salmonellosis pandemic in humans over the last 20 years, during which egg products were the most often identified vehicle of the infection (Moosavy et al., 2015; Long et al., 2017). Over the past decades, the improper and uncontrolled use of antibiotics has allowed the appearance of strains of *Salmonella* with multiple drug resistance in many countries (Ben Salem et al., 2012; Nacer et al., 2022; Samy et al., 2022). For that reason, the antimicrobial resistance of *Salmonella* has become a matter of concern worldwide due to the significant threat that can present to public health (Wu and Hulme, 2021).

Considering the overall number of eggs collected, the results of the present study demonstrated that, of the 180 samples analyzed, 13 isolates of *Salmonella* spp. were detected at the rate of 7.22%. This partially agrees with the results shown by Hossain et al. (2019), who reported that the overall prevalence of *Salmonella* in the Naogaon district of Bangladesh was recorded as 7.78%, whereas 5.56% was on eggshell surfaces and 2.22% was on egg contents. In addition, Xie et al. (2019) found that 7.6% and 3.2% of farm and market egg samples were contaminated with *Salmonella* in Guangdong, China. In contrast, to the present findings, Zubair et al. (2017) reported that out of the 350 eggs analyzed, 17 (4.85%) samples of eggshells were found contaminated with *Salmonella* spp. and none of the egg content samples were contaminated with this bacterium. The same results were reported by Mansour et al. (2015) who recorded a total absence of *Salmonella* in the egg content samples. These observed variations can be explained by the different approaches used for the choice of samples (eggshell or egg content), and the methods used to isolate and identify the microorganism.

Regarding the detection rate of the bacterium with retail outlets, the street vendors presented the highest rate of *salmonella* contamination in relation to other outlets at 3.88%, followed by wholesalers and butchers at 2.22% and 1.11%, respectively. However, a complete absence of bacteria was noticed in grocery stores. It was suggested that street vendors' poor storage and marketing conditions may be the cause of the high level of egg contamination. Indeed, the egg's duration and storage temperature may have a disproportionate impact on subsequent contamination (Gantois et al., 2009).

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Antimicrobial susceptibility results of *Salmonella* spp. isolated from table eggs marketed in the Tiaret area revealed that all strains (100%) were resistant to amoxicillin + clavulanic acid, ampicillin, nalidixic acid, and erythromycin. Several studies have reported different resistance rates; Islam et al. (2018) recorded that 94.44% and 77.78% of the *Salmonella* strains isolated from table eggs were resistant to amoxicillin and ampicillin, respectively. Likewise, Hossain et al. (2019) demonstrated resistance rates of 92.86% and 71.42% for amoxicillin and ampicillin, respectively. Unlike the present results, Xie et al. (2019) reported in a study carried out in China lower resistance rates of 12.96%, 37.04%, and 59.26% against nalidixic acid, amoxicillin, and ampicillin, respectively. The high rate of resistance found in this study can be related to the improper and uncontrolled use of these drugs in treating and controlling respiratory and digestive infections in poultry.

However, the results of the current study showed that all isolates were susceptible to gentamycin, tetracycline, and colistin sulfate. Several studies have reported different resistance rates to these antibiotics; Islam et al. (2018) recorded a resistance rate of 22.22% for gentamycin. In addition, Xie et al. (2019) reported low resistance to gentamycin with a rate of 18.52%, and higher resistance to tetracycline, with a rate of 42.59%. In contrast, the Zubair et al. (2017) group isolated 17 *Salmonella* strains from 350 analyzed table eggs and showed that all isolated strains were susceptible to gentamycin and colistin sulfate. These differences could be explained by the fact that each country has applied various policies and interventions to control the use of antibiotics in poultry production.

CONCLUSION

In conclusion, the current study's findings showed, firstly, that *Salmonella* spp. is detected in table eggs marketed in the area of Tiaret, and the highest detection rate was observed for street vendors. Therefore, significant attention should be paid to guaranteeing good quality eggs to the consumer, as *Salmonella* is considered one of the most important causes of human foodborne illness. Secondly, all *Salmonella* spp. isolates were resistant to amoxicillin + clavulanic acid, ampicillin, and nalidixic acid, while they were all susceptible to gentamycin, tetracycline, and colistin sulfate. The current study's authors suggest that the highest rate of resistance was observed for commonly used antibiotics compared to those used in specific poultry production cases. The overuse and misuse of antibiotics should be avoided in layer farms, as they are considered the main factors in the development of antibiotic resistance.

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Authors' contributions

Rachid Merati contributed to the conception, design, data collection, analysis, and interpretation. Abdellatif Boudra contributed to the editing and writing the final draft of the manuscript. All authors approved the analyzed data and the last revised article.

Competing interests

The authors confirm that the data presented do not represent any conflict of interest.

Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Ethical consideration

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

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Effects of Phytogenic Feed Additives on Body Weight Gain and Gut Bacterial Load in Broiler Chickens

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ABSTRACT

Phytogenic feed additives (PFAs) have promising importance in chicken production as antibiotic alternatives to balance chicken gut microorganisms and improve productivity. The objectives of this study were to evaluate the body weight gain (BWG) and gut bacterial load of broiler chicks fed on selected herbs. For this experiment, 360 unsexed one-day-old broiler chicks of Cobb 500 with an average weight of 40.74 g were randomly allocated into six treatment groups with three replicates of 20 chicks in each pen. The treatment 1 (T1) group was fed by a basal diet alone. Chickens of T2, T3, T4, T5, and T6 were fed the basal diet containing 1% of basil, lemongrass, peppermint, rosemary, and thyme leaves powder, respectively for 49 days. Body weight (BW), BWG, and average daily weight gain (ADWG) data were recorded every week and at the end of every phase. On days 21 and 42, three chickens from each replicate were slaughtered for microbiological analysis (pathogenic and normal flora) of cecum contents aseptically. The obtained result showed that chickens kept on T3 had significantly higher BW, BWG, and ADWG during the starter and grower phases. Significantly highest final BW was recorded during the finisher phase on T3 and T6. Chickens that consumed T3 and T6 had significantly higher overall BWG and ADWG. The lowest *Escherichia coli* counts were seen in chickens fed on rosemary (T5) on both days 21 and 42 of the experimental time. Similarly, the highest *Lactobacilli* counts were recorded on chicken fed on T5 (day 21) and T3 (day 42). On the other hand, almost all treatment herbs showed a higher *Enterococcal* count, with the highest recorded for T3 (day 21) and T6 (day 42). The present findings suggest that supplementing lemongrass and thyme leaf powder improves BW performance and gut microbial composition. Likewise, rosemary leaf powder enhances the beneficial microbial composition and reduces pathogenic bacteria. However, the underlying detailed biological mechanisms and dose standardization of these herbs for inclusion in the diet of broiler chickens need to be studied further.

Keywords: Antimicrobial**,** Body weight gain, Broiler chicken, Feed additive, Gut bacteria, Phytogenic

INTRODUCTION

Poultry production has increased recently due to the increase in human population, incomes, and standard of living, which have pressured the poultry industry to expand and produce high-quality products for consumers (Farrell, 2013). Efficient nutrition improvements in the poultry sector have accelerated the utilization of antibiotic-based feed additives, which became widely used to increase eggs and meat production and improve the chickens' health by maintaining a healthy gut environment (Alloui et al., 2014). However, this extensive chemical antibiotic utilization increases the chance of their accumulation in animal products as residues and the environment and increases the resistance of pathogens to antibiotics (Haque et al., 2020; de Mesquita Souza Saraiva et al., 2022). These situations force the world to restrict the utilization of antibiotic growth promoters (AGPs) in animal feed (Alloui et al., 2014; Hady et al., 2016), and the international livestock feed industries have been directed toward non-antibiotic feed additives. Among them, the feed additives of plant origin, called Phytogenic Feed Additives (PFAs), are gained a lot of attention as a suitable alternative to AGP (Alloui et al., 2014; Abd El-Ghany, 2020).

Phytogenics and plant derivative products attract a lot of attention as safe alternatives for AGPs in poultry. Recently research has shifted away from chemical-based feed additives to the use of phytogenics that exists naturally in the environment (Alloui et al., 2014). These plant-origin PFAs are natural, less toxic, residue-free, and ideal feed additives in meat animal production when compared to inorganic antibiotics or organic chemicals (Hady et al., 2016). Evidence also suggests that herbs, spices, and various plant extracts have antimicrobial, appetizing, and digestion-stimulator properties (Diniz et al., 2020). Phytogenic compounds have antibacterial and immunostimulant properties and could be used as alternatives to AGP to enhance chicken production performance. Hence, phytogenics are currently

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considered as feed additives in poultry ration to improve body weight gain (BWG) and their antimicrobial activities (Hady et al., 2016; Abd El-Ghany, 2020).

Chicken producers currently face challenges in meeting the consumers' needs with zero antibiotic residues and improved production with optimum quality (Haque et al., 2020). To meet consumer needs, the producers are interested in producing chickens without antibiotic residues by utilizing feed scientifically through sustainable poultry farming principles (Madhupriya et al., 2018). Besides, feed costs share about 70% of the total variable costs of poultry production (Fathi et al., 2019). Hence, PFAs are assuming an apposition of prime importance in poultry nutrition for promoting growth and production with optimum cost (Alloui et al., 2014; Singh and Yadav, 2020)

Supplementation of basil, peppermint, lemongrass, thyme, and rosemary in broiler chicken feed significantly affected chicken health and production. For instance, the supplementation of basil leaf powder significantly improves BWG, nutrient absorption, and the immune system, and maintains normal intestinal microflora in broiler chicken due to its antibacterial activities (ELnaggar and El-Tahawy, 2018). Similarly, feeding peppermint improves growth performance (Gurbuz and Ismael, 2016) and increases the beneficial-to-harmful bacteria ratio (Petričević et al., 2021). Likewise, studies have also revealed that the inclusion of lemongrass in broiler feed improved BWG (Parade et al., 2019), increased the beneficial bacteria, and reduced pathogenic bacterial load in chickens (Weckesser et al., 2007; Alagawany et al., 2021). Utilization of thyme herb in a broiler diet improves BWG (Toghyani et al., 2010), increases nutrient utilization, and has antimicrobial activity against microbiota found in the gastrointestinal tract (El-Ghousein and Al-Beitawi, 2009; Wade et al., 2018). Rosemary powder supplementation in poultry diets resulted in higher BWG (Ghazalah and Ali, 2008), reduced pathogenic bacteria load, and had significant antimicrobial activities (Weckesser et al., 2007).

Several investigators elsewhere reported that phytogenics in broiler diets improved body weight (BW) and BWG (Alloui et al., 2014; Hady et al., 2016). However, there is limited published work conducted on phytogenics herbs from Ethiopia. Hence, evaluating the effects of locally available herbs on chicken performance and gut microbiota has paramount importance as PFAs enhance the productivity of chickens by improving their feeds for optimum utilization as well as by enhancing their well-being. Therefore, this study was conducted to determine the effect of supplementation of dried basil, lemongrass, peppermint, rosemary, and thyme leaves powder on the BWG and gut bacterial load of broiler chicken.

MATERIALS AND METHODS

Ethical approval

All procedures related to animal handling and their routine manipulations were carried out according to animal care guidelines and protocols approved by the institutional animal ethics committee of the College of Veterinary Medicine and Agriculture (VM/ERC/01/13/12/2020).

Study areas

The experiments were conducted in the College of Veterinary Medicine and Agriculture poultry house of Addis Ababa University, Bishoftu campus, Ethiopia. The area is situated 47 km East of Addis Ababa at an altitude of 1900 m above sea level, a latitude of 8.44`N, and a longitude of 38.57`E. The average annual rainfall is 686.9 mm with an average minimum and maximum temperature of 10.9^{o}C and 27^{o}C, respectively, and the average relative humidity is 60.0%.

Experimental herbal powders

The treatment herbs, namely basil (*Ocimum basilicum*), lemongrass (*Cymbopogon schoenanthus)*, peppermint (*Mentha piperita)*, rosemary (*Rosmarinus officinalis*), and thyme (*Thymus vulgaris)* green leaves, were purchased from Green mark herbs private limited company (PLC), horticulture farm found in Hawassa, Ethiopia, incorporate in the diets of broiler chickens as PFAs. The herbs were washed, and the leaves were detached from the stem and dried at room temperature. The dried leaves were prepared in powder form and were homogeneously mixed with the broiler diet manually (Nielsen, 2010**).**

Experimental design

In this study, 360 unsexed one-day-old broiler chicks of Cobb 500 with an average BW of 40.74 g were purchased from Bishoftu Alema poultry farms in Ethiopia. On arrival, all chicks were checked for any abnormalities and weighed. They were then randomly assigned to one of the 6 different feeding groups, each with three replicates with 18 pens containing 20 chicks each (Table 1) based on a completely randomized design. The chicks were reared in a wire-meshed wood partitioned (1.2 m × 1.8 m) deep litter floor housing system for 49 days of the experimental period. Before the arrival of the chicks, the watering equipment and feeding troughs were thoroughly cleaned, disinfected, and sprayed

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against pathogen and external parasites, and the room was fumigated by mixing formalin 10% solution with potassium permanganate ($KMnO_4$) powder.

The chicks were brooded with gradual height adjustment using 200-watt bulbs suspended as heat and light sources for each pen to ensure adequate and uniform distribution of heat and light. The lighting program was 23 hours of light and 1 hour of darkness for the first week, then gradually decreased and kept constant at 18 hours at the age of the second week. The room was well heated at a constant temperature of 32°C two days before and on the arrival of the chicks, then was gradually reduced after a week by 3°C per week from 32°C to a final temperature of 20°C on day 28 and then kept constant. Chicks of the control group were fed broiler commercial concentrate feed as a basal diet purchased from Alema Koudijs Feed PLC, Bishoftu, Ethiopia. In contrast, each treatment group was fed on a concentrate basal diet containing 1% of one of the five herbs prepared in powder form as treatment (Table 1). Chicks were fed their respective prepared diet at their respective ages (starter feed from day 1-10, grower feed from day 11-30, and finisher feed from day 31-49) throughout the study time, as per the recommendation by the feed supplier by Alema Koudijs Feed PLC. All diets were provided in mash form. The chicks freely accessed tap water using manual drinkers and weighed feed throughout the experimental time (for 49 days). The standard bio-security protocol was employed throughout the entire experimental period (SAPA, 2022; USDA 2014). All chicks were vaccinated against New Castle Disease (HB1 on day 1, NEW Lasota on days 12 and 24) and Infectious Bursal disease on their days 7 and 19 of age as per the recommended local vaccination schedule/program of the Alema farms hatchery.

Table 1. The starter, grower, and finisher broiler diets for treatment groups during the experiment

Treatments group	Experimental diet	Number of chicks per	
		Replication	Treatment
T1	Only concentrate feed (Basal diet)	20	60
T2	Basal diet + Basil leaf powder	20	60
T3	Basal diet + lemongrass leaf powder	20	60
T4	Basal diet + Peppermint leaf powder	20	60
T5	Basal diet + Rosemary leaf powder	20	60
T6	Basal diet + Thymus leaf powder	20	60

Nutrient composition of basal diets and treatment herbs

Nutrient compositions of basal diet and treatment herbs powder of the representative samples were analyzed at two different laboratories; 1) the Animal product, Veterinary Drug and Feed Quality Assessment Center, and 2) the Ethiopian Institute of Agriculture Laboratory (Table 2). Samples were analyzed for dry matter (DM), crude protein (CP), ether extract (EE), crude fiber (CF), phosphorus (P), and total ash (Ash). Nitrogen was determined by the Kjeldahl procedure, and CP was calculated by multiplying nitrogen content by 6.25 (AOAC, 2000). The metabolizable energy (ME) values of the sample were calculated indirectly from the EE, CF, and ash using Formula 1, adopting the equation proposed by Wiseman (1987).

$$\text{ME (Kcal/kg DM)} = 3951 + 54.4\,\text{EE} - 88.7\,\text{CF} - 40.8\,\text{Ash} \qquad \text{(Formula 1)}$$

Table 2. Nutrient composition of basal diets and different herbs in broiler chicken diets in during the experiment

Sample	DM	CP	EE	CF	P	Ash	ME (kcal/kg DM)
Treatment herb							
Basil	85.7	31.56	2.14	38.52	0.36	6.72	376.516
Lemongrass	88.17	14.9	4.6	19.52	0.45	14.21	1890.05
Peppermint	88.81	26.92	1.88	21.72	0.52	11.58	1654.24
Rosemary	90.22	12.45	7.62	42.95	0.17	6.76	280.055
Thyme	88.88	11.96	6.12	18.09	0.27	11.03	2229.32
Basal diet							
Starter diet	90.9	21.95	4.02	7.86	0.48	9.88	3069.4
Grower diet	90.79	20.51	4.85	7.62	0.39	8.95	3173.79
Finisher diet	91.08	19.0	5.63	7.25	0.32	9.42	3230.07

DM: Dry matter, CP: Crude protein, EE: Ether extract, CF: Crude Fiber, P: Phosphorus, Ash: Total ash, ME: Metabolizable energy

Data collection

Body weight and average body weight gain measurement

At the beginning of the experiment, chickens were weighed using a sensitive balance with a sensitivity of 0.01g in a group per pen, and the average weight of the pen was calculated and recorded as initial BW. To measure the chicken's BWG, the chicks' BW was weighed weekly and at the end of every phase (starter, grower, and finisher) to determine

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weight change. An average BWG for each phase was calculated for each treatment as the difference between the two successive weights divided by the number of chickens. The average daily body weight gain (ADWG) was determined by dividing the average body weight change by the number of experimental days (Formula 2, Kidane et al., 2017) for each phase.

$$\text{Average daily body weight gain (g)} = \frac{\text{Body weight gain (g)}}{\text{No. of experimental days}} \qquad \text{(Formula 2)}$$

Caecal bacterial load determination

On days 21 and 42 of the experiment, three representative chicks from each pen (nine chicks per treatment) were randomly selected (Petričević et al., 2021) for bacterial load determination. The selected chickens were humanely slaughtered by severing the jugular vein, exsanguination in a clean slaughter room, their viscera were exposed, and fresh caecal contents from representative chicks were collected immediately under aseptic conditions, pooled in a group on sterilized 25-ml screw cupped tubes respective to the treatment, ligated and carefully placed on sterile plastic bags and put on ice and transported to a laboratory for bacteriological assays. For enumeration of the *Enterococci*, *E. coli*, and *Lactobacilli* colonizing the intestinal tract of chickens among different treatment groups, Bile Esculin Azide agar, Violate Red Bile agar and de Man, Rogosa and Sharpe (MRS) agar (all from HiMedia, India) were used, respectively (Upadhaya and Kim, 2017). Culture plates of Bile Esculin Azide agar and Violate Red Bile agar were incubated at 37ºC for 24 hours, whereas MRS agar plates were incubated at 37 ºC for 72 hours in an anaerobic environment (Upadhaya and Kim, 2017). The bacterial colony counts were calculated as colony-forming units per milliliters (CFU/ml) of caecal digesta (Formula 3). The counted data were transformed into logarithmic form $\log_{10}$ CFU/ml (Oyeagu et al., 2019).

$$\text{Colony Forming}\left(\frac{\text{CFU}}{\text{ml}}\right) = \frac{\text{Average number of colonies}}{\text{Dilution factor x Volume plated}} \qquad \text{(Formula 3)}$$

Data analysis

The results of the treatment means were analyzed by one-way ANOVA using R tools (R project, 2020). When differences among the treatment effect were found significant, means were separately analyzed using Duncan's multiple comparison tests. Significance differences were considered at ($p < 0.05$). The following model was used to analyze the experiment where PFAs are the main effects (Formula 4, Gomez and Gomez, 1984).

$$Yij = \mu + \alpha i + \varepsilon ij \qquad \text{(Formula 4)}$$

Where, *Yij* represents an observation of chicken, μ is the overall mean of a response variable, αi denotes the effect due to treatments herb, and εij is the error term.

RESULTS

Body weight gain

The difference in BWG and ADWG between the treatment herbs is presented in Table 3. At the beginning of the experiment, BW of broiler chicks was similar in all treatments ($p > 0.05$, Table 3). BWG and ADWG were highly significant ($p < 0.05$) between treatments during the starter, grower, and finisher phases. Chickens kept on T3 and T6 had the highest BW, BWG, and ADWG, compared to others during the starter phase ($p < 0.05$). In contrast, chickens fed on T1, T4, and T5 had significantly ($p < 0.05$) lower mean BW, BWG, and ADWG, compared to T2 during the starter phase. During the grower phase, chickens kept on T3 had significantly highest BW, BWG, and ADWG than all other chickens. No significant difference ($p > 0.05$) between chickens consumed on T1, T4, and T5 and had the lowest BW, BWG, and ADWG than T2, T3, and T6.

Similarly, no significant difference was observed between T2 and T6 during the grower phase ($p > 0.05$). Significantly highest final body weight was recorded during the finisher phase on chickens that consumed T3 and T6. While chickens kept on T1 and T4 had the lowest final body weight compared to other treatments. Chickens kept on T6 showed significantly highest BWG and ADWG than all other treatments during the finisher phase, while chickens kept on T4 had the lowest BWG and ADWG among treatments. There was no significant difference in BWG and ADWG between T2 and T5 as well as T2 and T3, during the finisher phase ($p > 0.05$). At the end of the experiment, there was a significant difference between all treatments on overall BWG and ADWG. Chickens that consumed T3 and T6 had significantly higher overall BWG and ADWG than all other treatments, and chickens that consumed T4 and T1 had the lowest overall BWG and ADWG. A Similar trend was exhibited for BWG and ADWG gain records at the end of the experiment as previously obtained weights in T3 and T6.

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Table 3. Effect of inclusion of different herbs on body weight gain and average body weight gain of broiler chickens during starter, grower, and finisher phases

Parameter \ Treatment		T1	T2	T3	T4	T5	T6	SEM	P-value
Initial BW (g)		40.85	40.79	40.7	40.75	40.67	40.68	0.04	0.810
Starter phase	BW	211.96^{c}	220.38^{b}	235.26^{a}	209.71^{c}	209.65^{c}	231.23^{a}	18.60	0.000
	BWG	171.29^{c}	179.54^{b}	194.45^{a}	168.91^{c}	168.32^{c}	190.24^{a}	0.85	0.000
	ADWG	17.14^{c}	17.97^{b}	19.46^{a}	16.90^{c}	16.84^{c}	19.04^{a}	0.85	0.000
Grower phase	BW	864.76^{c}	901.15^{b}	936.87^{a}	875.18^{c}	867.74^{c}	908.58^{b}	2.10	0.000
	BWG	644.38^{c}	680.77^{b}	716.49^{a}	654.80^{c}	647.36^{c}	688.20^{b}	2.10	0.000
	ADWG	32.21^{c}	34.03^{b}	35.82^{a}	32.74^{c}	32.36^{c}	34.41^{b}	0.10	0.000
Finisher phase	BW	1963.51^{d}	2032.17^{b}	2074.78^{a}	1960.98^{d}	1988.61^{c}	2064.62^{a}	23.87	0.000
	BWG	1098.75^{d}	1131.02^{bc}	1137.91^{b}	1085.80^{e}	1120.87^{c}	1156.04^{a}	2.10	0.000
	ADWG	57.82^{d}	59.52^{bc}	59.89^{b}	57.14^{e}	58.99^{c}	60.84^{a}	0.11	0.000
Over-all	BWG	1922.84^{d}	1991.34^{b}	2033.97^{a}	1920.18^{d}	1947.65^{c}	2023.48^{a}	3.14	0.000
	ADWG	39.24^{d}	40.63^{b}	41.5^{a}	39.18^{d}	39.74^{c}	41.29^{a}	0.06	0.000

$^{a-e}$ Means within a row with different superscripts differ significantly ($p < 0.05$), SEM: Standard error of the mean, BW: Body weight, BWG: Body weight gain in, ADWG: Average daily weight gain, T1: Control (basal diet), T2: Basal diet + 1% basil leaf powder, T3: Basal diet +1% lemongrass leaf powder, T4: Basal diet + 1% peppermint leaf powder, T5: Basal diet + 1% rosemary leaf powder, T6: Basal diet + 1% thymus leaf powder

Caecum bacterial count

The results for caecal bacterial load for *Enterococci*, *Lactobacilli*, and *E. coli* are shown in Table 4. During this study, statistically significant differences were recorded in the mean logarithmic bacterial colony count between treatments during both days 21 and 42 of experimental time ($p < 0.05$). A statistically significant difference was noted in the *E. coli* colony count between treatments on day 21 ($p < 0.05$); the highest *E. coli* colony count was seen on chickens fed T1 and T6. However, the lowest *E. coli* counts were seen on chickens fed on T5 followed by T3. Chickens fed on T2 and T4 also reduced *E. coli* bacterial colony count. There was no significant difference in *E. coli* bacteria colony count between T1 and T6. There was a significant difference in *Enterococcal* count between treatment herbs and control during day 21 of bacterial colony count ($p < 0.05$). Almost all treatment herbs had shown higher caecal *Enterococcal* bacterial colony count, compared to the control. The highest *Enterococcal* count was recorded on chickens fed on T3 followed by T6. There was no significant difference in *Enterococcal* colony count between the treatment herbs ($p > 0.05$). Similarly, there was also a highly significant difference in *Lactobacilli* count between treatments during day 21 of experimental time ($p < 0.05$). The highest *Lactobacilli* colony counts were recorded from chickens fed T5 followed by T3; whereas the lowest *Lactobacilli* count was recorded from chickens fed T1. There was no significant difference in *Lactobacilli* bacterial colony count between chickens fed on T4 and T6 during 21 days of the experiment.

Table 4. Caecal bacterial count (CFU/ml) of *Enterococci*, *Lactobacilli*, and *Escherichia coli* on days 21 and 42 in broiler chickens

Experimental days \ Treatments	Bacterial count	T1	T2	T3	T4	T5	T6	SEM	P-value
Day 21	*Enterococci*	6.804^{b}	7.694^{a}	7.819^{a}	7.689^{a}	7.686^{a}	7.700^{a}	0.104	0.000
	Lactobacilli	7.238^{e}	8.234^{d}	9.977^{b}	9.504^{c}	10.375^{a}	9.554^{c}	0.325	0.000
	E. coli	8.168^{a}	7.987^{b}	6.127^{d}	7.203^{c}	5.329^{e}	8.145^{a}	0.327	0.000
Day 42	*Enterococci*	7.371^{b}	8.250^{b}	8.997^{ab}	9.123^{ab}	8.395^{b}	9.612^{a}	0.229	0.007
	Lactobacilli	9.796^{b}	10.025^{b}	12.695^{a}	10.246^{b}	12.462^{a}	11.842^{a}	0.360	0.000
	E. coli	8.08^{b}	7.661^{bc}	7.747^{bc}	8.421^{b}	7.034^{c}	9.225^{a}	0.201	0.004

$^{a-e}$ Means within a row with different superscripts differ significantly ($p < 0.05$), CFU/ml: Colony forming units/ml, SEM: Standard Error of the Mean, T1: Control (basal diet), T2: Basal diet + 1% basil leaf powder, T3: Basal diet +1% lemongrass leaf powder, T4: Basal diet + 1% peppermint leaf powder, T5: Basal diet + 1% rosemary leaf powder, T6: Basal diet + 1% thymus leaf powder

Similarly, on day 42 of the experiment, there was a significant difference in bacterial count between treatments ($p < 0.05$). The highest *E. coli* colony count was recorded in chickens fed on T6, compared to all other treatments. On the contrary, the lowest *E. coli* count was seen in chickens fed on T5. There was no significant difference in mean logarithmic bacterial colonies count between chickens fed on T1, T2, T3, and T4 ($p > 0.05$).

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There was also a significant mean difference in *Enterococci* count between treatments on day 42 of the experiment ($p < 0.05$). The highest *Enterococci* count was recorded on chickens fed T6, followed by T4 and T3, but there was no significant difference between chickens fed on T3, T4, and T6 ($p > 0.05$). On the other hand, the lowest *Enterococci* count was recorded on chickens fed on T1. The highest *Lactobacilli* counts during day 42 were recorded in chickens fed on T3 and T5, followed by T6. However, the lowest *Lactobacilli* counts were recorded in chickens fed on T1. No significant difference was recorded in chickens fed on T1, T2, and T4 in *Lactobacilli* count during day 42 of the experiment ($p > 0.05$).

DISCUSSION

Phytogenic feed additives considerably impact the gut environment, either directly or indirectly (Khan et al., 2022). In this study, the effect of the studied phytogenic herbs was found to be very effective in improving the performances of broiler chickens and exhibiting antimicrobial activities. Likewise, the findings of the present study and other reports suggest that phytogenics inclusion in the broiler diet improves the total number of beneficial bacteria, such as *Lactobacilli,* and reduces pathogenic bacteria growth, such as *E. coli* in the cecum (Riyazi et al., 2015a; Ahmed et al., 2016; Alagawany et al., 2021). These could lead to improved resistance to gut disease, enhance chickens' immunity, improve digestion, improve nutrient absorption, and in turn significantly improve the BWG of chickens.

Body weight gain

In this experiment, chickens fed on diets mixed with different phytogenic herbs showed higher BW, BWG, and ADWG. Chickens fed on lemongrass had higher BW, BWG, and ADWG values during the starter, grower, and overall period, compared to the control. This result was in line with Parade et al. (2019), who reported that the inclusion of 1.5% lemongrass leaf powder in the diet improved BWG and can reduce the time of harvesting broiler with proper slaughter weight. Similarly, another study also revealed that feeding a broiler with the inclusion of 2% lemongrass leaf powder improves their BWG compared to the standard diet (Shaheed, 2021). The difference in BW and BWG recorded in this study continues up to the end of the experiment. Higher BW improvement could be due to the active compounds found in lemongrass having antimicrobial and antioxidant activities. These compounds improve feed digestion and increase the secretion of digestive enzymes (Alagawany et al., 2021; Shaheed, 2021). These beneficial activities are reflected in the growth and BWG of the experimental chicks.

In this study, there was a statistically significant difference in BW and BWG in the rosemary-fed broiler chickens compared to the control during the finisher phase. The results obtained in this study were comparable with the investigation result obtained by Petričević et al. (2018), who reported that the inclusion of 0.6% rosemary in the broiler feed had no significant difference in ADWG during the starter and grower phase; however, significantly improve feed conversion ratio during the finishing phase. Contrary to the present findings, the inclusion of rosemary essential leaf powder in broiler feed had no significant difference in BWG compared to the control (El-Ghousein and Al-Beitawi, 2009). Rosemary herb has a strong flavor, and high crude fiber content, especially cellulose, tannin, and other potentially interfering substances, which may limit nutrient digestion and absorption in chicken (Loetscher et al., 2013).

The inclusion of basil leaf in the broiler diet in the current study significantly improves BW and ABWG more than the control group. This finding was in line with Onwurah et al. (2011), who reported that the utilization of basil leaf at 5g/kg in the diet of broilers had a beneficial effect on the BWG of broiler chicken. Feeding broiler with phytogenics herb can stimulate the digestive system of the animal by stimulating the secretion of endogenous digestive enzymes, improving the utilization of digestive products through enhanced liver function, and reducing bacterial load in the gastrointestinal tract, leading to the development of muscle in broiler chicken (Gurbuz and Ismael, 2016).

Supplementing peppermint in the broiler feed in the present study had no significant effects on BW and BWG compared to chickens fed on a control diet during the entire experimental period. This study is in line with the findings of Amasaib et al. (2013), reporting that supplementing chickens with 1% spearmint in a diet had no statistically significant effect on BWG. In contrast to this result, Witkowska et al. (2019) reported that peppermint oil mist could improve BW and BWG in broilers as the herb has a bactericidal effect and reduces infections.

The current study findings showed that the inclusion of thyme in the diet of broilers leads to the highest BW and BWG during the starter phase next to lemongrass. This result agrees with the previous findings of Toghyani et al. (2010), who reported higher BW in the broilers fed with a mixture of 0.5% thyme powder. Similarly, other studies also reported that higher BW was observed on broilers fed on a thyme essential oil-containing diet (Fotea et al., 2009). Contrary to the current study, thyme leaf powder supplementation had no significant difference in broiler performance compared to the control (Abdel-Ghaney et al., 2017). The BW and BWG performance seen in the broiler chicken supplemented with thyme in this study could be associated with the active principles of thymol and carvacrol found in the thyme herb. These active ingredients increase the secretion of digestive enzymes (Alagawany et al., 2021), have digestion stimulating (Al-

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Kassie, 2009) and antimicrobial activity against gut microbiota effects, and improve nutrient utilization through enhancing liver function (El-Ghousein and Al-Beitawi, 2009; Wade et al., 2018).

Caecum bacterial count

The caecum bacterial load analysis results of day 21 of the experiment showed that there was a higher mean logarithmic value of *Lactobacillus* in chickens that consume basil than in control. However, the *Lactobacillus* count was higher in all treatments compared to the control. This finding coincides with Riyazi et al. (2015a), who reported that the inclusion of 600 ppm basil essential oil reduces *E. coli* and increases *Lactobacillus* bacterial colonies count in broiler chicken. The presence of flavonoids in the *Ocimum* spp. improves the immune system, enhances BWG in the broiler, and maintains normal intestinal microflora due to its antibacterial activities (ELnaggar and El-Tahawy, 2018).

Lemongrass and rosemary inclusion significantly increased the *Lactobacilli* and *Enterococci* and reduced *E. coli* mean logarithmic bacterial count both during the days 21 and 42 of experimental time. This result aligns with (Weckesser et al., 2007), who reported that rosemary extract could reduce pathogenic bacteria load and had significant antimicrobial activities. The study also reported that the inclusion of lemongrass essential oil (450 mg/kg) in the quail's diet increased the *Lactobacillus* bacterial count and decreased the coliform, *E. coli*, and *Salmonella* bacterial count compared to the control group (Alagawany et al., 2021). Different literature exhibited that the reduction of such pathogenic bacteria in lemongrass-fed broilers could be due to the leaves and their essential oil rich in phytochemical compounds like tannins, flavonoids, and phenolic acid which have antimicrobial properties that can impair the integrity and function of the bacterial cell membranes and inhibit the growth of pathogenic bacteria (Parade et al., 2019; Alagawany et al., 2021; Shaheed, 2021).

In the current study, lower *E. coli* and higher values of *Enterococci* and *Lactobacilli* bacterial count were also recorded in chickens fed on thymes herb compared to the control. The findings of the current study are in line with the study (Saki, 2014) that reported that the inclusion of 0.20 ml/l thyme essential oil in drinking water significantly prevents the growth of *E. coli* in broiler chickens. Supplementation of thyme in the broiler diet affects the pathogenic bacteria by altering the cell wall permeability, and normal osmotic nature of the cell wall, which results in cytoplasmic membrane damage and protrusion of the content is a lethal condition to cell (Lee et al., 2004).

In addition, the results of the present study showed a significant reduction of *E. coli* and a higher number of *Enterococci* and *Lactobacillus* count in chickens fed peppermint leaf powder during day 21 of the experiment. This result coincides with the finding of Ahmed et al. (2016), who reported that a higher number of *Lactobacilli* and a lower value of some pathogenic bacteria count in the intestinal content of chickens fed on peppermint extract as a supplement. Likewise, the highest coliform bacteria count was reported in the intestine content of chickens fed on the control feed. Similarly, Petričević et al. (2021) found that the inclusion of 0.6% peppermint leaf powder in the broiler diet improves the beneficial-to-harmful bacteria ratio in the cecum. Contrary to our present findings, the study found no statistically significant difference in *Lactobacillus* and total numbers of aerobic bacteria count between control and peppermint powder-supplemented groups. Peppermint essential oil is rich in menthol content which is the main phenolic component and has antimicrobial activities (Schuhmacher et al., 2003), and it has the potential to be used as phytogenic feed additive in combating bacterial diseases in poultry (Hady et al., 2016; Abd El-Ghany, 2020). In general, phytogenic herbs have active compounds with selective antimicrobial activities. The active constituents of the phytogenic herbs diffuse through the cell wall and affect the microbial cell's normal physiology and reduce their growth. This inhibition of bacterial growth by the herb could be due to interference with the microbial enzymes, or by damaging the protein, affecting the DNA and RNA synthesis, and preventing nutrient uptake, transport systems, and energy production of the bacteria (Shan et al., 2007).

CONCLUSION

The finding of the present study indicated that lemongrass and thyme leaf powder supplementation in the broiler diet as PFAs improves growth performance and results in higher final body weight with beneficial cecal microbial composition. Likewise, supplementation of rosemary leaf powder enhances the broiler's gut-beneficial microbial composition and reduces pathogenic bacteria. Therefore, these herbs can have a promising potential to be effectively used as safe and natural growth promoter phytogenics in broiler chickens and might be replacing growth-promoting antibiotics. Furthermore, detailed studies should be conducted to determine other beneficiary effects and better understate the mechanism of action of these herbs on the growth performance and health of broilers.

DECLARATIONS

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Authors' contributions

Tesfaye Engida D. conceptualized the idea and methodology, performed the experiments, collected all samples, and analyzed, generated data, compiled information, and prepared the original and final manuscript. Mihretu Ayele contributed to the conceptualization and methodology of the study, performed the experiments and, collected the samples of bacteriological data, laboratory analysis, and drafted the manuscript. Hika Waktole contributed to the conceptualization and methodology of the study and reviewed the manuscript. Berhan Tamir and Fikru Regassa contributed to the conceptualization, methodology, validation, supervision, and review of the manuscript. Takele Beyene Tufa contributed to the conceptualization, methodology, validation, and supervision of the study, editing and reviewing the manuscript, project administration and funding acquisition. All authors approved the results of the study and the final version of the manuscript.

Competing interests

The authors have declared that no competing interest exists.

Ethical consideration

All Authors have checked the ethical issues, including plagiarism, consent to publish, misconduct, double submission, and redundancy.

Availability of data and materials

The authors declare that they prepared all necessary data for this study and are ready to send further documents related to this study upon reasonable request.

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NETosis and Calcium influx in Dromedary Camel Neutrophils after *In Vitro* Toll-like Receptor Stimulation

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ABSTRACT

Neutrophilic granulocytes are vital immune cells of the early response to pathogens. They contribute to the antimicrobial response through phagocytosis, production of reactive oxygen species, cytokine production, degranulation, and NET-formation. Neutrophil extracellular traps (NETs), also known as NETosis, are a critical antibacterial effector mechanism of cells of myeloid effector cells, including neutrophils and macrophages. Toll-like receptors (TLRs) are pattern recognition receptors (PRRs) that mediate pathogen sensing through the recognition of microbial structures known as pathogen-associated molecular patterns (PAMPs). The present study aimed to investigate the potential of several TLR ligands that mimic the sensing of bacterial and viral pathogens to stimulate NET-formation or Ca^{2+} influx in camel neutrophils. Neutrophils were purified from blood and were stimulated in vitro with ligands to TLR4, TLR2/1, TLR7/8, or TLR3. Net-formation was analyzed using the DNA-sensitive dye SYTOX™ Green and staining with antibodies to the neutrophil's granular enzyme myeloperoxidase. Real-time stimulation-induced Ca^{2+} influx was measured using the Ca^{2+}-sensitive dye Flou-4 and flow cytometry. Only the TLR4-ligand lipopolysaccharide (LPS) could induce NET-formation in camel neutrophils, while none of the investigated TLR agonists showed a Ca^{2+} influx-inducing effect in camel neutrophils. The current study represents the first report on the impact of direct activation of TLR on NET-formation and Ca^{2+} influx in camel neutrophils with a selective effect of LPS on NET-formation induction. Future studies may investigate the molecular mechanisms behind the different responsiveness of bovine and camel neutrophils to TLR stimulation.

Keywords: Camel, Ca^{2+} influx, Flow cytometry, Neutrophils, NETosis, Toll-like receptor

INTRODUCTION

Neutrophils are innate immune cells with a significant role in early defense against pathogens. They mainly contribute to antimicrobial response through the early detection of microbial structures and danger signals and the subsequent activation of other innate and adaptive immune cells essential for effectively eliminating pathogens (Soehnlein and Lindbom, 2010; Rosales et al., 2016). Neutrophils elicit their antimicrobial activity through several functions, including phagocytosis, production of reactive oxygen species, cytokine production, degranulation, and NET-formation (Akira and Takeda, 2004; Gordon, 2004; Tan et al., 2018).

Neutrophil extracellular traps (NETs), also known as NETosis, are a key antibacterial effector mechanism of cells of effector myeloid cells, including neutrophils and macrophages (Ciliberti et al., 2021). NETosis includes immobilizing intracellular DNA and nuclear chromatin to the extracellular space to build a network, where microbes are trapped and killed. The antimicrobial potential of NETs is mainly supported by many antimicrobial peptides released from their stores in neutrophil granules (Lippolis et al., 2006; Aulik et al., 2010; Remijsen et al., 2011). Although several models for NET-formation have been established for many veterinary species, including cattle, sheep, and goats (Worku et al., 2021), only a few studies investigated NET-formation in the dromedary camel (Hussen et al., 2022).

Toll-like receptors (TLR) are pattern recognition receptors (PRRs) expressed on and in different immune cells (Akira and Takeda, 2004; Beutler, 2004; Schmidt et al., 2004). They mediate the sense of pathogens through the recognition of microbial structures known as pathogen-associated molecular patterns (PAMPs, Ozinsky et al., 2000; Takeuchi and Akira, 2007; Radoshevich and Dussurget, 2016).

Together with a cluster of differentiation (CD)14, the LPS-binding protein (LBP), and MD-2, TLR4 is responsible for sensing gram-negative bacteria by the recognition of the PAMP lipopolysaccharide (LPS, Ohtsuka et al., 2001; Miyake, 2004; Johnzon et al., 2018). The interaction of the synthetic TLR-ligand Pam3CSK4 with TLR1/2 simulates innate sensing of Gram-positive bacteria (Mintz et al., 2013; Reid et al., 2021). Resiquimod (R848) and polyinosinic:

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polycytidylic acid (poly I:C) are synthetic agonists for the intracellular TLR8/TLR7 and TLR3, respectively, representing infection of viruses (Reid et al., 2021). The TLR-mediated release of NETs has been described after stimulation of neutrophils with different pathogens, including *Candida albicans* and *Staphylococcus aureus* (Pilsczek et al., 2010; Byrd et al., 2013; Block et al., 2022).

Changes in intracellular Ca^{2+} levels are a hallmark of several activation processes of neutrophils (Dixit and Simon, 2012). Under resting conditions, levels of neutrophils cytosolic Ca^{2+} are lower than in the extracellular compartment. After stimulation, neutrophils rapidly raise their intracellular Ca^{2+} levels through Ca^{2+} release from its cytosolic stores and/or Ca^{2+} influx from the extracellular milieu (Immler et al., 2018).

Several studies have investigated the impact of TLR activation on the phenotype and the function of neutrophils for humans and many other species (Byrd et al., 2013; Block et al., 2022). However, less is known about TLR activation in camel immune cells. The objective of the current study was to analyze the potential of several TLR ligands that mimic sensing of bacterial and viral pathogens to stimulate NET-formation or Ca^{2+} influx in camel neutrophils. The results of the present work would contribute to a better understanding of the interaction mechanisms of the camel immune response with different pathogen groups.

MATERIALS AND METHODS

Ethical approval

The study was approved by the Ethics Committee of King Faisal University (approval no KFU-REC-2021- DEC - EA000326).

Animals and blood sampling

Blood samples were collected from five clinically healthy (based on clinical examination) dromedary camels (Camelus dromedarius) that were randomly selected from 35 camels reared on a camel farm in the eastern proven of Saudi Arabia. All camels were males from the Almajaheem breed with ages between 10 and 12 years old and body weights between 325 and 365 Kg. Blood sample collection was performed without anesthesia using venipuncture of the jugular vein into EDTA tubes (BD Biosciences, San Jose, California, USA), and collected blood was kept cooled until used for cell separation in the immunology laboratory at King Faisal University (usually after 1 hour).

Purification of camel neutrophils

Camel neutrophils were separated as previously described by Hussen et al. (2023a). Briefly, 5 mL camel blood was diluted with 5 mL phosphate buffered saline (PBS), and diluted blood was then layered (carefully without mixing them) on 5 mL of the lymphocyte separation medium Lymphoprep™ (Stemcell Technologies, Vancouver, Canada) in Corning® 15 mL centrifuge tubes. The blood was then centrifuged for 30 min at 800 × g. After removing the peripheral blood mononuclear cells (PBMCs) from the inter-phase, neutrophils were separated after erythrolysis. For erythrolysis, aquadest (5 mL) was used for 20 sec to lyse the RBCs and 5 mL of a 2x solution of PBS was then used to restore cell osmosity. The RBC-lysis was repeated until having a pure white cell pellet. Neutrophils were suspended at 1 × 107 cells/mL in HBSS buffer (Hank's balanced salt solution; MOLEQULE-ON, Auckland, New Zealand).

Toll-like receptor stimulation in camel neutrophils *in vitro*

The TLR stimulation was performed as previously described (Hussen et al., 2023b). The TLR ligands lipopolysaccharide (LPS), Pam3CSK4, R848, and Poly IC were purchased from Invivogen (San Diego, USA). Phorbol myristate acetate (PMA) was purchased from Calbiochem (Merck Millipore, Darmstadt, Germany). For the *in vitro* stimulation, 1 × 106 neutrophils were incubated in Roswell Park Memorial Institute (RPMI) medium for 4 hours at 37 °C with LPS (1 µg/mL), Pam3CSK4 (1 µg/mL), R848 (0.2 µg/mL), Poly IC (10 µg/mL), or phorbol 12-myristate 13-acetate (PMA; 10 ng/mL), or were left in medium without stimulation (negative control).

Measurement of neutrophil extracellular traps formation by SytoxGreen

Stimulated and non-stimulated neutrophils (5 x 105 cells per well of a 96-well cell culture plate) were incubated with one drop of the DNA-sensitive dye SytoxGreen (Invitrogen, Germany). After 15 min incubation at room temperature, the labeled cells were analyzed by flow cytometry (Accuri C6; BD Biosciences) by the acquisition of at least 30.000 neutrophils (Masuda et al., 2017).

Measurement of membrane myeloperoxidase

Membrane myeloperoxidase (MPO) was detected by labeling the cells with a mouse monoclonal (Clone 5B8) antibody against MPO conjugated with phycoerythrin (PE, Raskovalova et al., 2019). The antibody was purchased from BD Biosciences (San Jose California, USA). For cell labeling, 100 µL cell suspension (5 x 105 cells) was incubated as a

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pellet with 20 µL anti-MPO antibody for 15 min at 4°C followed by washing the cells with cold PBS supplemented with bovine serum albumin (MOLEQULE-ON, Auckland, New Zealand). Finally, the labeled cells were analyzed by flow cytometry (Accuri C6; BD Biosciences, San Jose, California, USA).

Real-time analysis of calcium influx

Purified camel neutrophils (1 x 107 cells / µL) were incubated for 30 min at 37°C with 1 µmol/l Fluo-4 AM (Molecular Probes, Eugene OR) in Ca^{2+}/Mg^{2+} HBSS (MOLEQULE-ON, Auckland, New Zealand). Cells were washed three times with HBSS (8 minutes, 300 xg) and finally suspended in HBSS. Baseline Fluo-4 fluorescence was measured for 20 sec before TLR agonists were added to the cells. The cellular response towards HBSS and ionomycin (Sigma-Aldrich, Germany, 250 nmol/L final) were used as negative and positive control stimulation, respectively (Hussen et al., 2016).

Statistical analysis

GraphPad Prism (San Diego, USA) was used for statistical analysis. Data normality was tested using Shapiro–Wilk test. The 1-factorial analysis of variance (ANOVA) test was used in combination with Bonferroni's multiple comparison tests to analyze the effect of different stimuli on NET-formation and Ca^{2+} influx of neutrophils. P-values less than 0.05 indicate significant differences between the means.

RESULTS AND DISCUSSION

In the current work, Ca-influx and NETosis responses were investigated in purified camel neutrophils upon in-vitro stimulation with different synthetic TLR-ligands. Neutrophil purification was performed using density gradient centrifugation over Ficol-Histopaque (Figure 1, Hussen et al., 2016). This method resulted in a pure neutrophil population (always more than 93%) with a vitality rate above 95% (propidium iodide-negative cells).

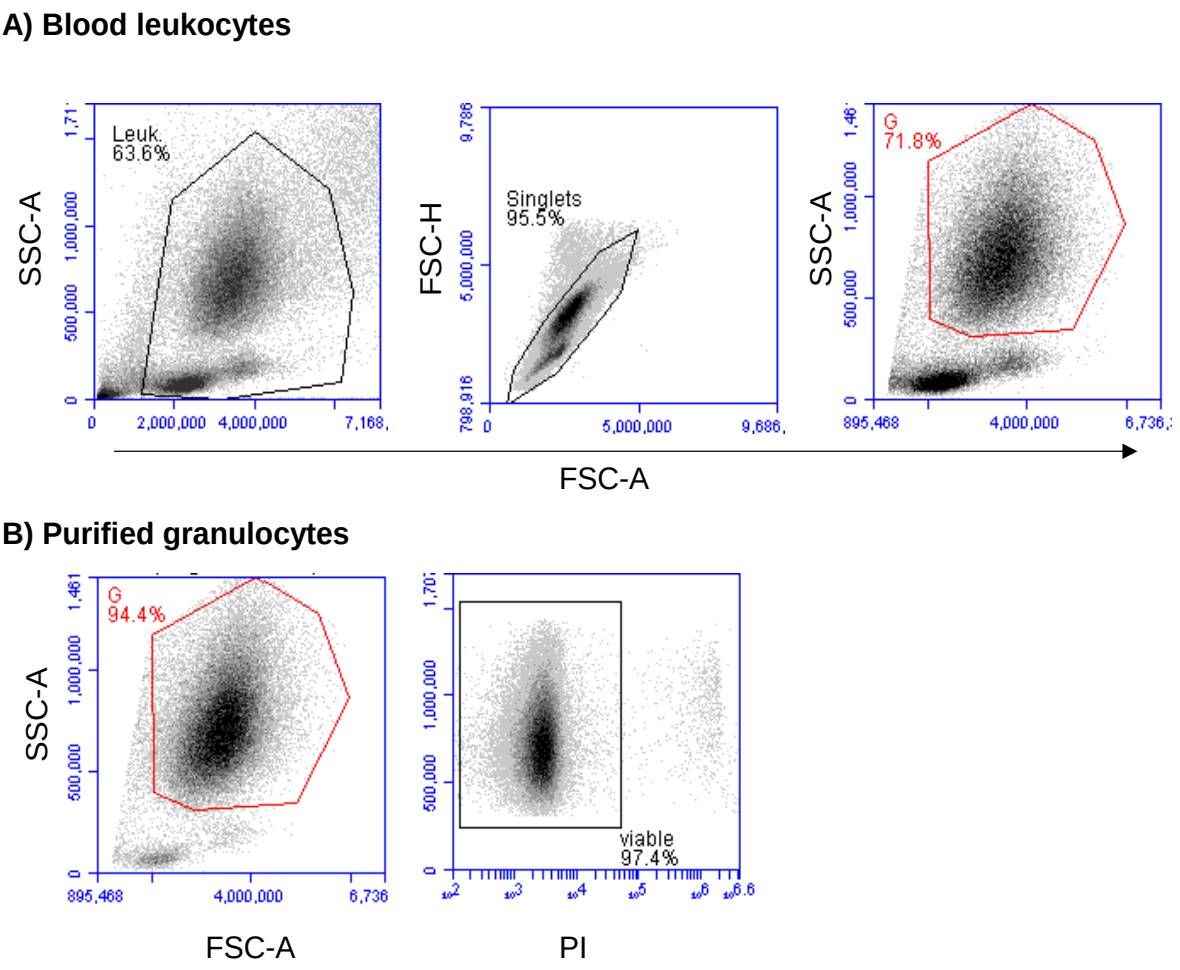

Figure 1. Purification of neutrophils from camel blood using density gradient centrifugation. **A**: After exclusion of cell debris (gate on leukocytes; Leuk) and cell duplicates (gate on singlets) in side scatter area against forward scatter area (SSC-A/FSC-A) and FSC-A/FSC-H dot plots, the fraction of neutrophilic granulocytes (G) was identified within leukocytes based on cell size (FSC-A) and granularity (SSC-A). **B**: Cell purity (percentage of neutrophils) and vitality (viability) of separated neutrophils were measured based on cell morphology (FSC-A/SSC-A) and staining with propidium iodide (PI), respectively.

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TLR-stimulation-induced NET-formation in camel neutrophils

Neutrophils NETosis (NET-formation) was measured based on the staining with the DNA binding dye SYTOX™ Green (Figure 2A-D). For control cells without stimulation, the percentage of neutrophils with enhanced SYTOX™ Green fluorescence was 2.0 % of total cells. Stimulation with PMA resulted in a 3-fold increase ($p < 0.05$) in the percentage of neutrophils with positive staining with SYTOX™ Green (7.01 %) as well as a 4-fold rise in the SYTOX™ Green mean fluorescence intensity (MFI: 1176 versus 4119 for non-stimulated cells) for the whole neutrophils population (Figure 2BC). For cells stimulated with TLR-ligands, only LPS stimulation resulted in a significant ($p < 0.05$) expansion in the SYTOX™ Green-positive cells (3.9 %) and enhanced MFI of total cells (MFI: 2555 versus 1176 for non-stimulated cells). The LPS-induced effect was, however, lower than that of PMA.

NET-formation was also confirmed by measuring the expression of the granular enzyme MPO on the surface of neutrophils (Figure 2E-H). For non-stimulated cells in medium control, the percentage of neutrophils with enhanced MPO staining was 3.6 % of total cells. Stimulation with PMA resulted in a 2-fold increase ($p < 0.05$) in the rate of neutrophils with positive staining with MPO (8.7 %) as well as a marked enhancement ($p < 0.05$) of the MPO mean fluorescence intensity (MFI: 950 versus 550 for non-stimulated cells) for the whole neutrophils population. With the exception of LPS, stimulation with TLR-ligands did not induce NET formation in neutrophils. In LPS-stimulated neutrophils, a marked ($p < 0.05$) expansion in the MPO-positive cells (6.07 %; Figure 2E) and an enhanced MPO MFI (MFI: 775) were observed (Figure 2F).

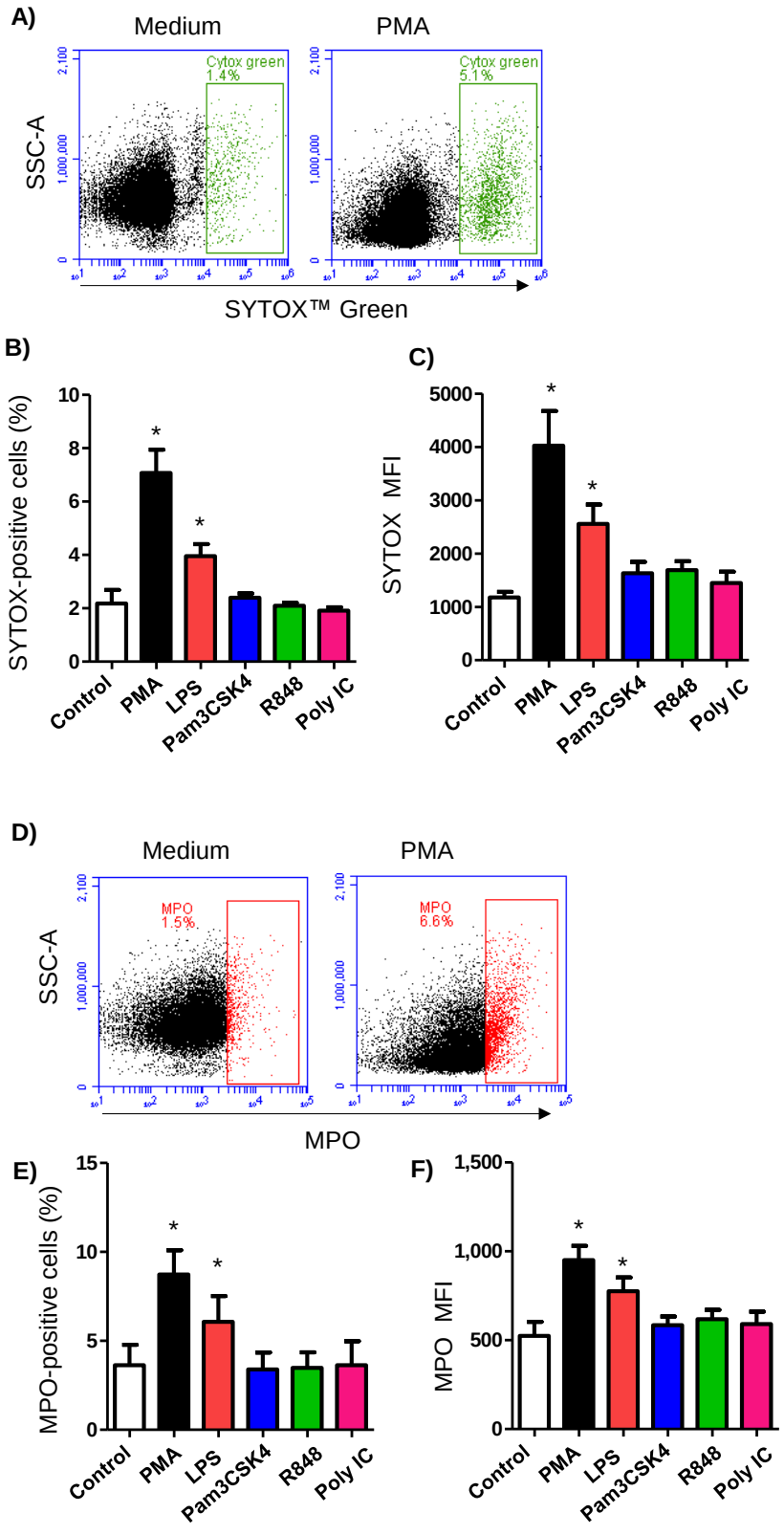

Figure 2. Neutrophils extracellular traps (NETs) in neutrophils. **A:** Purified neutrophils were stained with SYTOX™ Green and analyzed by flow cytometry. Cells with NET-formation were identified based on their positive staining with SYTOX™ Green. The percentage of SYTOX™ Green-positive cells (**B**) and the mean green fluorescence intensity (MFI) for all cells (**C**) were calculated and presented in graphs. **D:** Neutrophils were labeled with PE-conjugated monoclonal mouse antibodies to myeloperoxidase and labeled cells were analyzed using flow cytometry. Representative dot plots showing the percentage of MPO-positive neutrophils for non-stimulated and stimulated cells. The percentage of MPO-positive neutrophils (**E**), as well as MPO MFI for all neutrophils (**F**), were calculated and presented as mean and SEM ($n = 5$ camels; $* p < 0.05$ in comparison to control).

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Ca^{+2} influx in camel neutrophils after TLR stimulation

Stimulation-induced Ca^{+2} influx in purified camel neutrophils was analyzed using the Ca^{+2}-binding dey Fluo-4 (Figure 3A). Fluo-4 fluorescence was first explored for 20 seconds before adding a stimulant. For stimulation control, cells were stimulated with HBSS (negative control) and ionomycin (positive control). Stimulation with ionomycin resulted in a significant ($p < 0.05$) Ca^{+2} influx in neutrophils (71.3 ± 1.8 %) in comparison to non-stimulated (5.0 ± 1.9 %) cells (cells stimulated with HBSS, Figure 3B). In contrast to ionomycin, none of the TLR-ligands induced Ca^{+2} influx in purified camel neutrophils (Figure 3A, B).

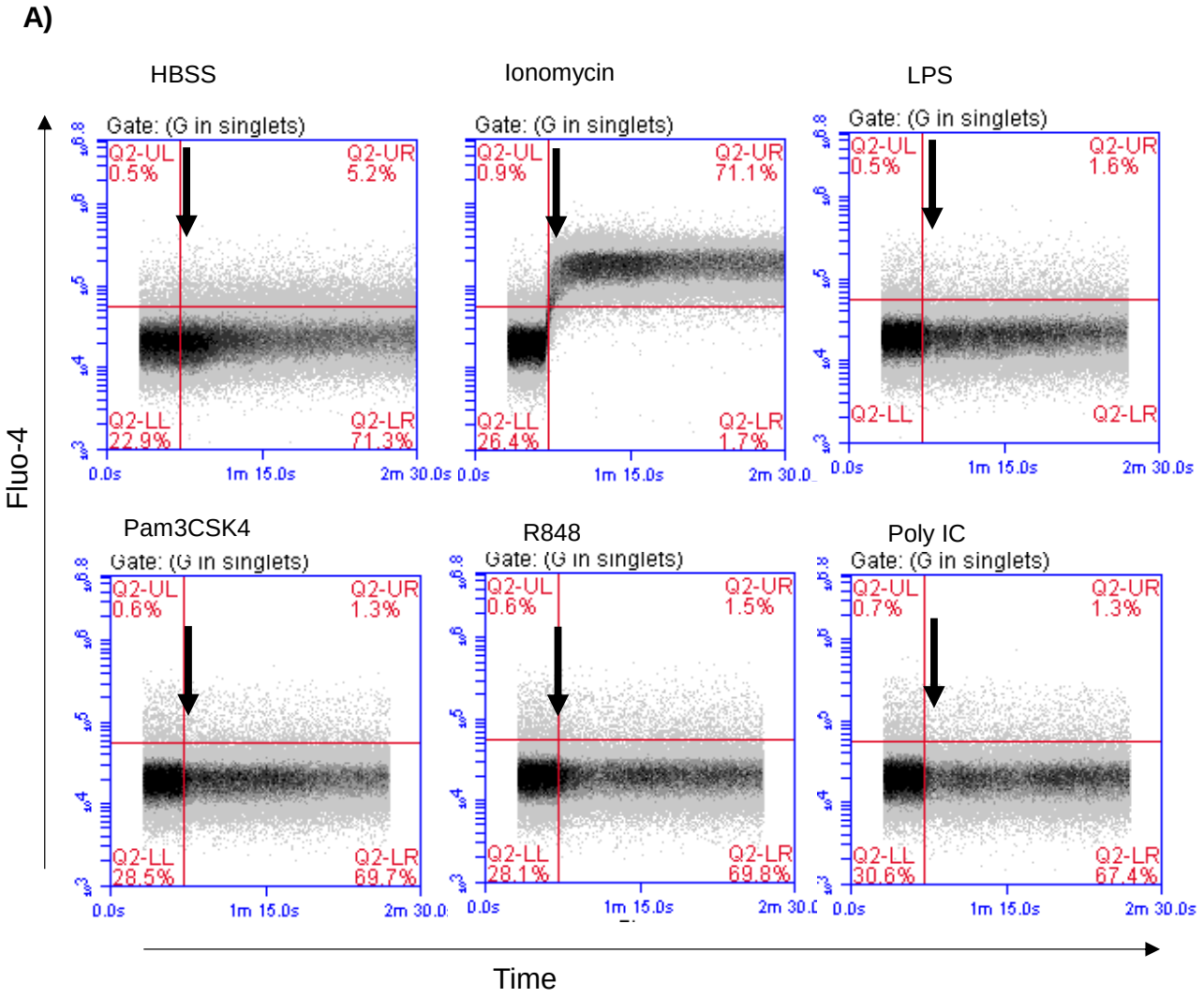

Figure 3. Purified camel neutrophils were stained with the calcium dey Fluo-4. **A:** For the measurement of real-time influx of calcium, Fluo-4 fluorescence height (FL1-H) was presented in relation to time. Neutrophils were defined based on their light scatter properties, and the influx of Ca^{2+} was measured as the fraction of cells that rose above the basic line after stimulation (arrows indicate the time point where stimuli were added). **B:** The fraction (%) of cells with the increased influx of Ca^{2+} in response to HBSS (negative control), ionomycin (Iono; positive control), or TLR ligands is presented graphically (n = 3 camels; * $p < 0.05$).

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Neutrophilic granulocytes are key immune cells in the early response to pathogens (Nathan, 2006; Mantovani et al., 2011; Kolaczkowska and Kubes, 2013; Malech et al., 2020; Burn et al., 2021). The interaction between neutrophils and pathogens is mediated through different receptors. TLRs are membrane and intracellular pattern recognition receptors interacting with PAMPs (Newton and Dixit, 2012; Zindel and Kubes, 2020). Although few recent studies analyzed the impact of some TLR-agonists on some functions of neutrophils in the dromedary camel (Hussen et al., 2023a; Hussen et al., 2023b), many questions still exist regarding the modulatory effect of TLR stimulation on neutrophils phenotype and function. Especially the potential of bacterial and viral TLR-agonists to induce NET-formation or Ca^{2+}influx in camel neutrophils has not been investigated so far. The present work investigated the effects of selected TLR agonists on NET-formation and Ca^{2+}influx in camel neutrophils.

Generation of neutrophils extracellular traps (NETs), also known as NETosis, is one of the effective mechanisms used by neutrophils for the extracellular killing of microbes (Brinkmann et al., 2004). During NETosis, neutrophils mobilize their DNA and nuclear proteins to build a network outside the cell. This network contains many antimicrobial peptides released from the neutrophil's granular stores. Microbes are trapped and killed inside this network (Brinkmann et al., 2004; Rada, 2019).

Receptor-mediated NET-formation has been recently described for human neutrophils. Receptors mediating NETosis in human neutrophils include TLRs, nod-like receptors, C-Type Lectin Receptors, FC receptors, and complement receptors (Chen et al., 2021). In the present study, only activation of TLR4 through the bacterial TLR4-ligand LPS showed the potential to induce NETosis in camel neutrophils. Studies in humans identified many TLRs that participate in NETosis by human neutrophils. This includes TLRs to viral, bacterial, fungal, and parasitic PAMPs (Chen et al., 2021). Although the results of the present work are in line with the reported TLR4-induced NETosis in human neutrophils, the lack of NET-formation after the stimulation of neutrophils with PAM3CSK4, R848, and Poly IC is in contrast to the human system, where TLR, TLR2, TLR7, and TLR8 were involved in the NETosis response to several pathogens in human neutrophils (Saitoh et al., 2012; Hiroki et al., 2019; Munoz-Caro et al., 2021).

The expression patterns of several members of the TLR group have been investigated for bovine and human neutrophils (Parker et al., 2005; Conejeros et al., 2015). Such studies are still lacking for camel neutrophils. In a recent study, activation of TLR-4, TLR-2/1, and TLR-7/8, but not of TLR-3, resulted in the activation of camel neutrophils with stimulation-induced shape change and modulation of activation markers expression (Hussen et al., 2023b).

A rise in cytosolic calcium levels represents an early step in the activation of neutrophils. It is associated with several functional activities, such as adhesion and migration to the site of infection, reactive oxygen species (ROS) production, and degranulation (Conejeros et al., 2015). To see whether TLR stimulation in camel neutrophils leads to Ca^{2+} influx, real-time flow cytometric measurement of changes in intracellular calcium concentrations in camel neutrophils was performed upon stimulation with TLR agonists. Present results showed that none of the used TLR ligands (LPS, Pam3CSK4, R838, or Poly IC) induced calcium influx in camel neutrophils. These results contradicted the reported increase of Ca^{2+} influx in bovine PMN exposed to Pam3CSK4 (Conejeros et al., 2015).

CONCLUSION

In conclusion, the present study evaluated the impact of selected TLR agonists representing PAMPs of bacterial and viral pathogens on NET-formation and Ca^{2+} influx in camel neutrophils. Only the TLR4-ligand LPS showed the potential to induce NET formation in camel neutrophils. None of the investigated TLR agonists showed a Ca^{2+} influx-inducing effect in camel neutrophils. The current study represents the first report on the impact of direct activation of TLR on NET-formation and Ca^{2+} influx in camel neutrophils. Further studies are required to investigate the molecular mechanisms behind the different responsiveness of bovine and camel neutrophils to TLR stimulation.

DECLARATIONS

Availability of data and materials

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

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None.

Authors' contributions

Khuzama Albahrani performed sample collection and preparation, manuscript revision. Jumanah Alessa did flow cytometry, manuscript revision. Baraa Falemban conducted data analysis, writing the original manuscript. Mayyadah

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Competing interests

No conflict of interest to disclose.

Ethical consideration

The authors declare that the manuscript has not been published before and is not currently being considered for publication elsewhere. The originality of the final draft of the manuscript has been checked by all the authors.

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Toxic Effects of Nanographene Oxide on Testes of Rats

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ABSTRACT

The current study aimed to examine the effects of nanographene oxide on the testes. A total of 48 male albino rats were randomly divided into 6 groups. The first, second, third, fourth, and sixth groups were treated with graphene oxide nanopowder at 20, 30, 40, 50, and 60 mg/kg concentrations, respectively. The sixth group was considered the control group. The results indicated a significant decrease in the average testis weight of rats treated with different nanographene oxide dosages, compared to the control group. There was also a significant decrease in the level of FSH and testosterone of treated rats with nanographene oxide, while there was no significant difference in the level of LH hormone when compared to the control group. The histological examination of the testes in the treated rats indicated hemorrhage, decreased sperm count, decreased thickness of the tubular epithelium, dissociation of connective tissue between the seminiferous tubules, in addition to hematological congestion, necrosis of the tubular epithelium, divergence of the seminal tubules, absence of sperm, shattering of the seminal tubule wall and degeneration sperm-forming cells and edema formation. Using the transmission electron microscope, the findings revealed a range of cellular changes, such as the presence of two-headed spermatids, the destruction of the nucleus membrane, spermatoblasts, the destruction of the cell membrane, and the denting of the nucleus membrane. It can be concluded that the nanographene oxide at 20-60 mg/kg concentrations can have harmful effects on spermatogenesis and normal function testis in rats.

Keywords: Laboratory rat, Nanographene oxide, Testes, Toxic effect

INTRODUCTION

Graphene is the thinnest electronic material which possesses distinctive chemical and physical properties. Graphene has remarkable properties, such as high surface area, thermal conductivity, electrical conductivity, and mechanical strength. These unique properties have led to an explosion of recent research in its composition, characterization, and development of its applications, especially in electronic devices, transparent electrodes for solar cells, plasma screens, and energy storage devices (Dhiman and Dhamija, 2014). The two-dimensional allotropic structure and bio-inherent properties of graphene make it applicable for biomedical and therapeutic purposes (Priyadarsini et al., 2018). The derivatives of graphene, such as graphene oxide, have received great attention due to their excellent solubility in physiological media, their good biocompatibility at the level of human exposure, and their ability to combine with other nanomaterials (Markovic et al., 2011). Graphene oxide has been widely applied in cellular imaging, drug and gene delivery, tissue engineering, and antibacterial therapies. It is a strong antibacterial alternative since it has severe toxic effects on bacteria, fungi, and other pathogens (Wu et al., 2015). The multiple applications of nanographene oxide, especially in the biomedical fields are increasing concerns about the potentially toxic effects of this substance on health and tissue cells. Therefore, the current study aimed to investigate the toxic effects of nanographene oxide on the testes of laboratory rats.

MATERIALS AND METHODS

A total of 48 male albino rats with an age range of 10-12 weeks and a mean weight of 170 g were examined for clinical health in the present study. The graphene oxide nanopowder was purchased from Sky Spring Nanomaterials, Inc., USA, in the form of black powder with 97% purity, 2-nm thickness, and an average diameter of 3-10 nm. Different concentrations of graphene oxide nanopowder were prepared by dissolving it in a normal saline solution. The animals were then divided into six groups and each group has a replicate (4 rats in each replicate). The first, second, third, fourth, and fifth groups were respectively treated with graphene oxide nanopowder at concentrations of 20, 30, 40, 50, and 60 mg/kg. The sixth group was considered as the control group. The solution was administered orally to the rats using a f*eeding tube* about 2-3 inches in length to prevent wounding the animal. The volume of administration was 0.1 ml per

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each animal, for 30 days. At the end of the experiment, the blood samples were collected from the lateral tail vein of each rat into an EDTA tube and a silicon-coating tube. The samples of silicon coating tubes remained at room temperature for up to 30 minutes to enable clotting. The clot is removed by centrifugation at 2000 x g for 10 minutes and the resulting supernatant immediately transfer to a polypropylene tube using a Pasteur pipette. The serum levels of LH, FSH, and testosterone hormones were measured immediately after receiving the serum samples by commercial kits (Beijing Northern Biotechnology Research Institute, Beijing, China) in an automated chemistry analyzer (BS 200, Mindray, China). The light and electron microscope slides were prepared based on the method proposed by Luna (1968). The microscopical studies were carried out using a light microscope (Olympus, Japan) and a transmission electron microscope (TESCAN company, China) to see the histological and cellular changes resulting from the effect of this substance. The weights of the testes in the different groups were measured using a sensitive scale (PCE-LSZ 200C, PCE- Deutschland Gmbh & Co, Germany), and the lengths of these samples were also measured using the ruler.

Statistical analysis

The statistical analysis was carried out using one-way ANOVA followed by L.S.D. The probability level of ≤ 0.05 was considered statistically significant.

RESULTS

Effects of graphene oxide on blood parameters

The results of the current study showed that there was a significant decrease in the average number of red blood cells in group 5 when compared with other groups ($p \leq 0.05$, Table 1). The results of the current study showed a significant decrease in the hemoglobin level of the group treated with a concentration of 60 mg/kg when compared to other groups ($p \leq 0.05$). Moreover, there was a non-significant increase in the average number of white blood cells when comparing the treated groups with concentrations of 20, 30, 40, and 50 mg/kg and the control group ($p > 0.05$). There was a significant increase in the rate of white blood cells when comparing treated rats with 60 mg/kg with other groups except for the fifth group ($p \leq 0.05$). The current study showed a significant increase in the mean number of platelets when comparing the groups treated with 40, 50, and 60 mg/kg of graphene oxide with other groups (Table 1).

Effects of graphene oxide nanoparticles on weight and length of testes

Comparing the control group with the experimental groups showed a significant decrease in the mean testis weight of treated rats ($p \leq 0.05$, Table 2). There was a significant decrease in the mean testicular length of all treated rats, compared to the control rats ($p \leq 0.05$, Table 2).

Effects of graphene oxide on the levels of sex hormones

There was a significant decrease in the FSH level when comparing all the treated groups with the control group ($p \leq 0.05$, Table 3). The results of the current study also showed an insignificant difference in the rate of LH hormone when comparing all the treated groups with the control group ($p > 0.05$). However, there was a significant decrease in the rate of testosterone hormone in all experimental groups, compared with the control group ($p \leq 0.05$).

Table 1. Effects of different levels of nanographene oxide on some blood parameters of rats

Groups	RBC	Hb	WBC	Platelets
Treatment 1 (20 mg/kg)	8.11 ± 0.47^{a}	14.12 ± 0.77^{a}	5.06 ± 0.52^{a}	437.20 ± 11.67^{a}
Treatment 2 (30 mg/kg)	8.11 ± 0.46^{a}	14.08 ± 0.29^{a}	5.06 ± 1.18^{a}	490.00 ± 18.95^{ab}
Treatment 3 (40 mg/kg)	7.71 ± 0.45^{ab}	13.46 ± 0.61^{a}	5.28 ± 0.83^{a}	597.60 ± 64.36^{e}
Treatment 4 (50 mg/kg)	7.69 ± 0.26^{ab}	12.64 ± 0.11^{ab}	5.56 ± 1.11^{ab}	632.80 ± 44.48^{e}
Treatment 5 (60 mg/kg)	6.82 ± 0.53^{c}	10.76 ± 0.61^{c}	6.46 ± 0.49^{b}	640.60 ± 96.94^{e}
Treatment 6 (Control)	8.71 ± 0.53^{a}	14.26 ± 1.25^{a}	4.40 ± 0.40^{a}	368.80 ± 60.76^{a}

The numbers in the table represent mean values ± standard deviation. RBC: Red blood cells, Hb: Hemoglobin, WBC: White blood cells. The different superscript letters in the same column indicate a significant difference at the probability level of $p \leq 0.05$.

Table 2. Effects of different levels of graphene oxide nanoparticles on the average weight and length of testes in rats

Groups	Weight of testis	Length of testis
Treatment 1 (20 mg/kg)	0.71 ± 0.04^{b}	1.71 ± 0.09^{b}
Treatment 2 (30 mg/kg)	0.69 ± 0.04^{c}	1.63 ± 0.05^{c}
Treatment 3 (40 mg/kg)	0.68 ± 0.03^{c}	1.50 ± 0.12^{d}
Treatment 4 (50 mg/kg)	0.67 ± 0.03^{cd}	1.48 ± 0.07^{e}
Treatment 5 (60 mg/kg)	0.63 ± 0.04^{d}	1.35 ± 0.05^{f}
Treatment 6 (Control)	0.80 ± 0.04^{a}	1.85 ± 0.05^{a}

The numbers in the table represent mean values ± standard deviation. The different superscript letters in the same column indicate a significant difference at the probability level of $p \leq 0.05$.

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Table 3. Effects of different levels of graphene oxide nanoparticles on sex hormones of rats

Groups	FSH	LH	Testosterone
Treatment 1 (20 mg/kg)	0.52 ± 0.29^{b}	1.15 ± 0.25	2.12 ± 0.04^{b}
Treatment 2 (30 mg/kg)	0.42 ± 0.29^{b}	1.14 ± 0.19	2.02 ± 0.29^{b}
Treatment 3 (40 mg/kg)	0.30 ± 0.11^{b}	1.06 ± 0.18	2.02 ± 0.16^{b}
Treatment 4 (50 mg/kg)	0.25 ± 0.05^{bc}	1.02 ± 0.13	1.86 ± 0.18^{b}
Treatment 5 (60 mg/kg)	0.24 ± 0.05^{bc}	1.01 ± 0.21	1.42 ± 0.20^{bc}
Treatment 6 (Control)	0.92 ± 0.17^{a}	1.22 ± 0.08	2.72 ± 0.57^{a}

The numbers in the table represent mean values ± standard deviation. The different superscript letters in the same column indicate a significant difference at the probability level of $p \leq 0.05$.

Histological examinations

Histological examinations on the control rats showed the normal structure of the testis. As observed in the control group, the seminiferous tubules contain germ cells that represent the different stages of sperm formation. In addition to the mature sperms present in the lumen of the seminiferous tubule, the seminiferous tubules are separated from each other by connective tissue as they are the testicle surrounded by the capsule (Figure 1). The histological examinations of the testes in the rats of the group treated with a concentration of 20 mg/kg showed bleeding, while those treated with a concentration of 30 mg/kg indicated a decrease in the number of sperms and thickness of the tubular epithelium as well as the dissociation of the connective tissue between the seminiferous tubules (Figures 2 and 3). Necrosis of the tubular epithelium and severe bloody congestion as shown in figures 3 and 4. The histological examinations of the testes in the rats treated with a concentration of 40 mg/kg showed bleeding, a decrease in the number of sperms, dissociation of the connective tissue between the seminiferous tubules, and spacing of the seminiferous tubules (Figures 5 and 6). The tubular epithelium, lack of sperm, and the destruction of the seminal tubule wall, as well as the dissociation of the connective tissue between the seminiferous tubules, and a decrease in the thickness of the tubular epithelium, were seen in treated rats with a concentration of 50 mg/kg (Figures 7 and 8). Histological examinations of the testes in the experimental rats administered a concentration of 60 mg/kg showed a lack of connective tissue between the seminiferous tubules and necrosis of the tubular epithelium, as well as hematopoietic congestion, lack of sperm, degeneration of sperm-forming cells and edema formation (Figures 9 and 10).

Examinations by electron microscopy

Transmission electron microscopy images of the testis in the rats of the control group revealed a normal structure of spermatids, where the cell membrane, cytoplasm, vesicle, and nucleus could be observed (Figure 11). Moreover, transmission electron microscope images of the testis in the rats of the treated group at a concentration of 20 mg/kg of nanographene oxide showed the presence of a spermatid with two heads (Figure 12); however, the images of the testis in the rats treated with nanographene oxide at a concentration of 30 mg/kg indicated the destructions in the nucleus membrane (Figure 13). The nucleus membrane and the cell membrane of the testis were destroyed in the rats treated with nanographene oxide at concentrations of 40 and 50 mg/kg, respectively (Figures 14 and 15). The transmission electron microscopy images of the testis in the rats treated with nanographene oxide at a concentration of 60 mg/kg showed that the nucleus wall and the nucleus were dented in the sertoli cell (Figure 16).

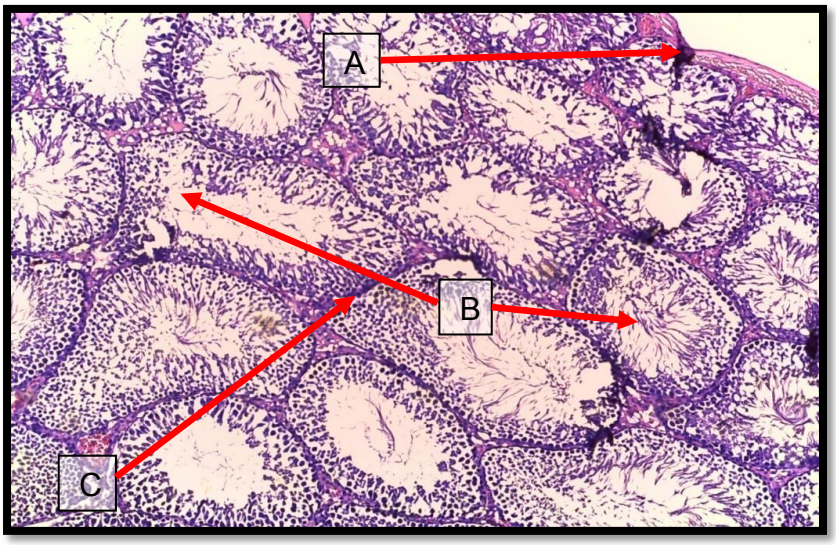

Figure 1. Normal testicular tissue of rats showing tunica albuginea (A), seminiferous tubules (B), the connective tissue between seminiferous tubules (C), H&E, 100X.

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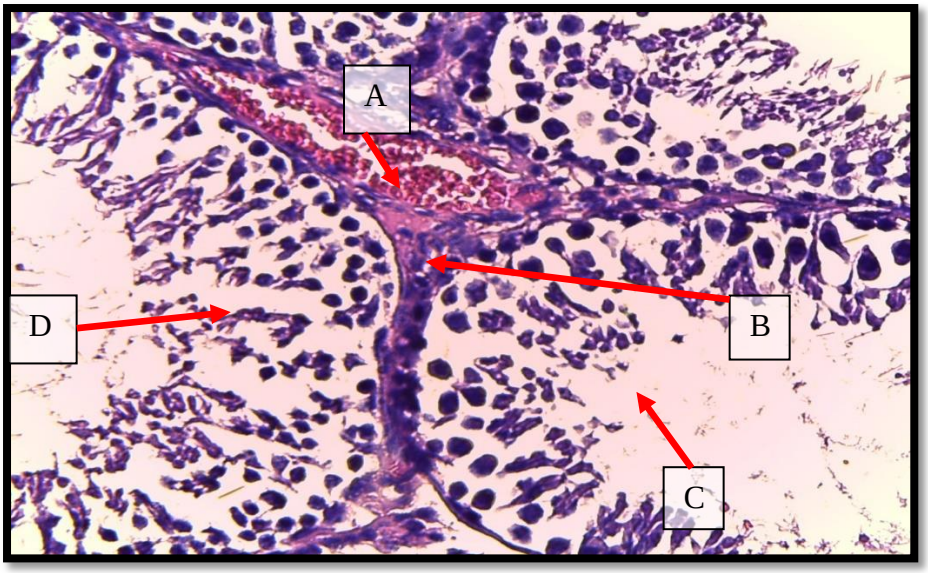

Figure 2. Testicular tissue of the rats treated with nanographene oxide at a concentration of 20 mg/kg indicates hemorrhagic bleed (A), progenitor sperm (B), the lumen of seminiferous tubule (C), spermatocytes in different stages of formation (D), H&E, 400X

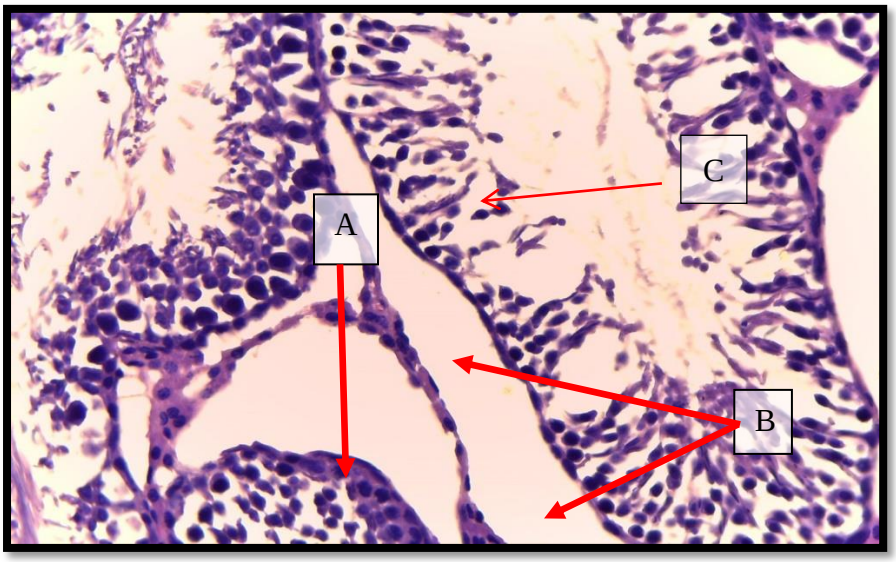

Figure 3. The testis tissue of the rats treated with nanographene oxide at a concentration of 30 mg/kg showis the dissociation of connective tissue between seminiferous tubules (A), necrosis of the tubular epithelium (B), decreased sperm count (C), H&E, 400X

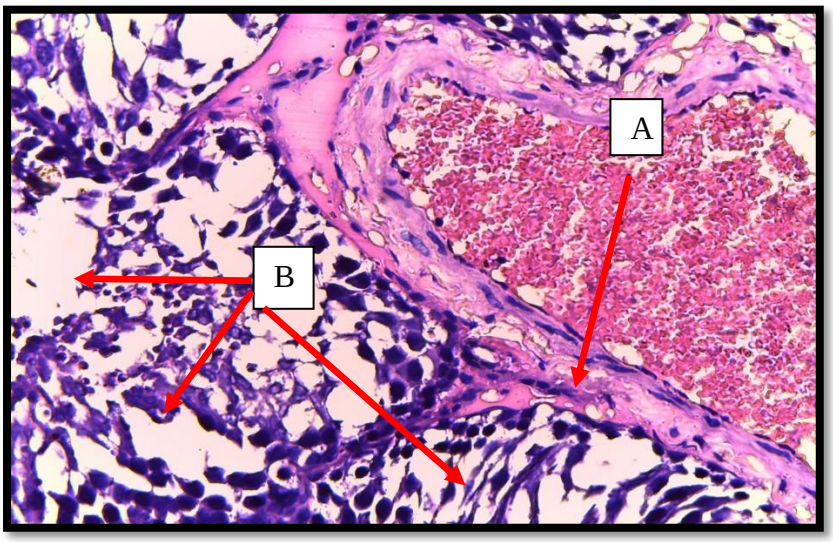

Figure 4. The testis tissue of the rats treated with nanographene oxide at a concentration of 30 mg/kg shows severe blood congestion (A) tubular epithelium necrosis (B) H&E, 400X

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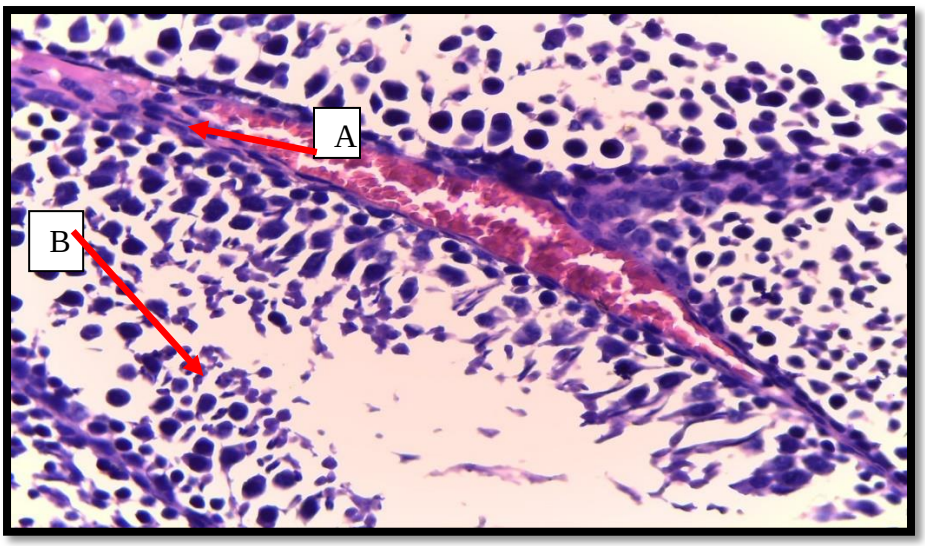

Figure 5. A cross-section of the testicular tissue of the rats treated with nanographene oxide at a concentration of 40 mg/kg indicates hemorrhage (A), decrease in sperm count (B) H&E, 400X

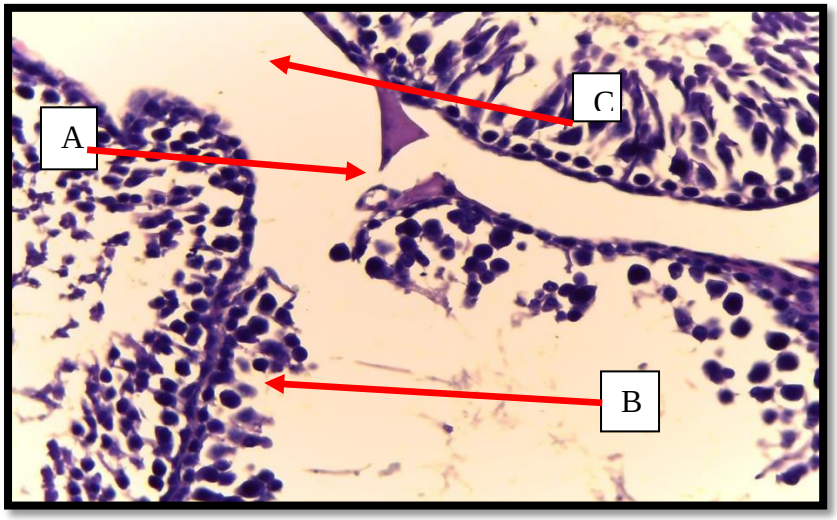

Figure 6. Testicular tissue of the rats treated with nanographene oxide at a concentration of 40 mg/kg reveals the dissociation of connective tissue between the seminiferous tubules (A), decrease in the number of sperm (B), spacing between the seminiferous tubules (C) H&E, 400X

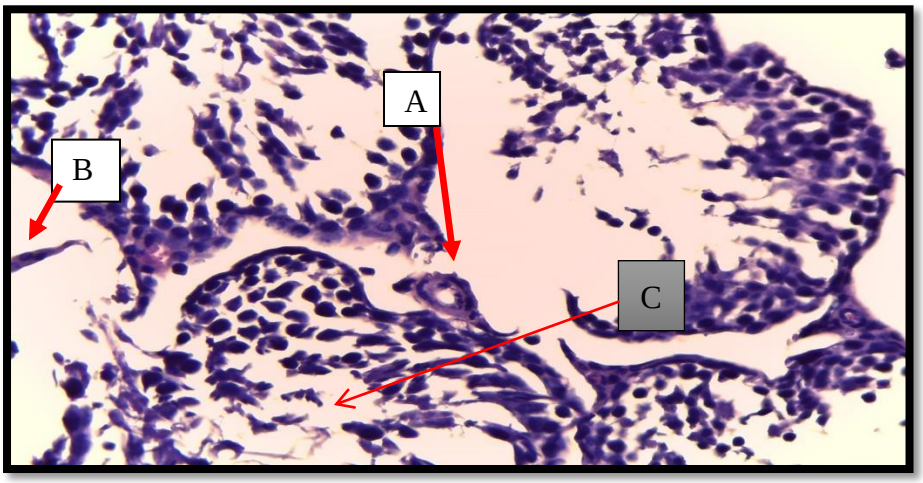

Figure 7. The testis tissue of the rats treated with nanographene oxide at a concentration of 50 mg/kg indicates the rupture of the wall of the seminiferous tubule (A), the dissociation of the connective tissue between the seminiferous tubules (B), Necrosis of the tubular epithelium (C), H&E, 400X

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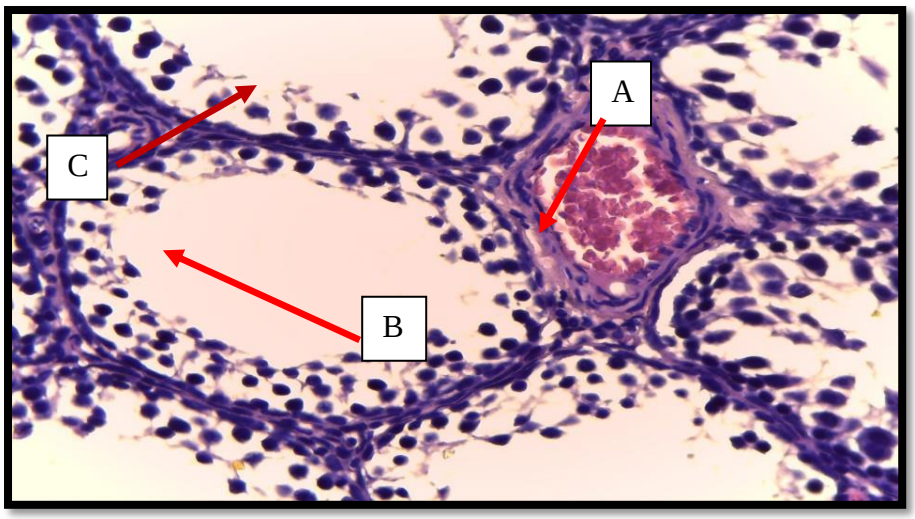

Figure 8. The testis tissue of the rats treated with nanographene oxide at a concentration of 50 mg/kg presents hemocongestion (A), lack of sperm (B), decrease in the tubular epithelium (C), H&E, 400X

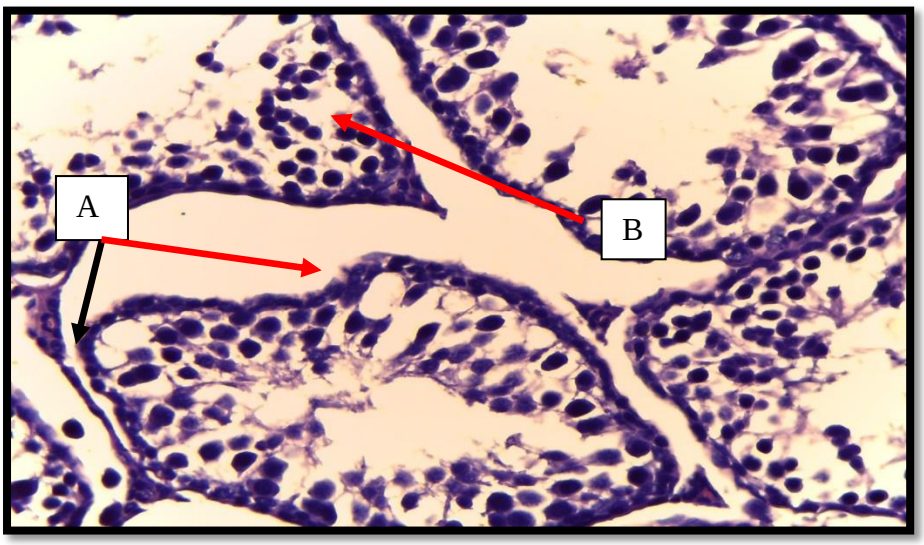

Figure 9. The testis tissue of the rats treated with nanographene oxide at a concentration of 60 mg/kg demonstrates a lack of connective tissue between the seminiferous tubules (A) necrosis of the tubular epithelium (B), H&E, 400X

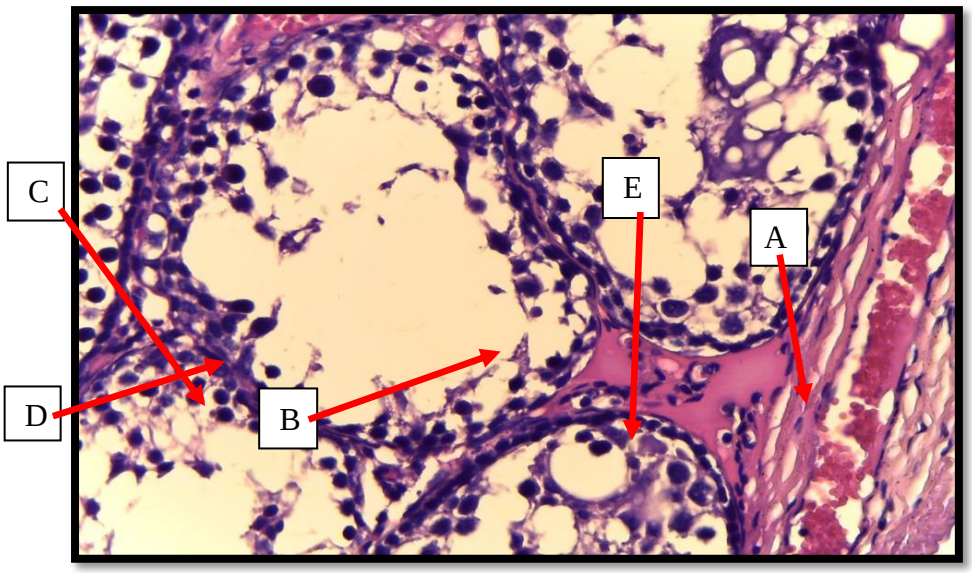

Figure 10. The testis tissue of the rats treated with nanographene oxide at a concentration of 60 mg/kg indicates blood congestion (A), necrosis of the tubular epithelium (B), lack of sperm (C), degeneration of sperm-forming cells (D), occurrence of edema (E), H&E, 400X

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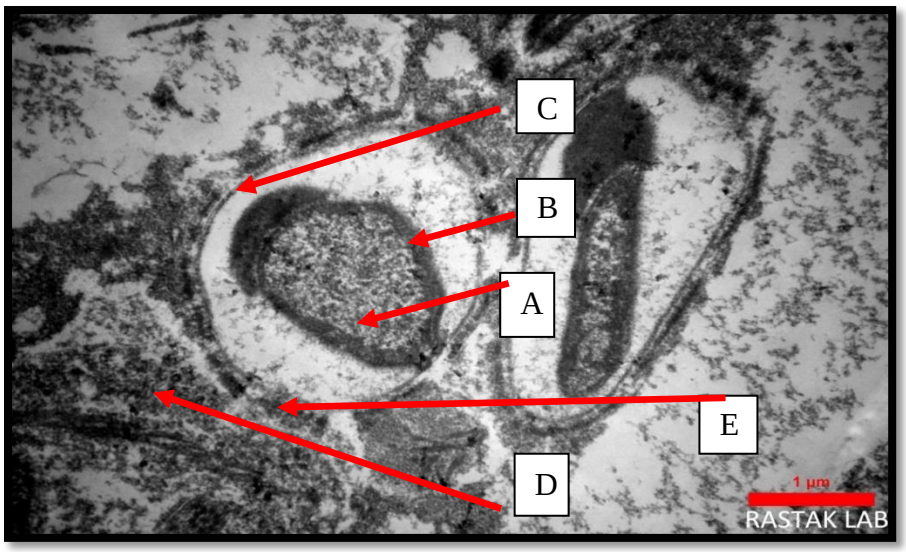

Figure 11. The testis tissue in control group rats showing the structure of the spermatid (A), cell membrane (B), cytoplasm (C), vesicle (D), centrioles (E), nucleus, transmission electron microscopy

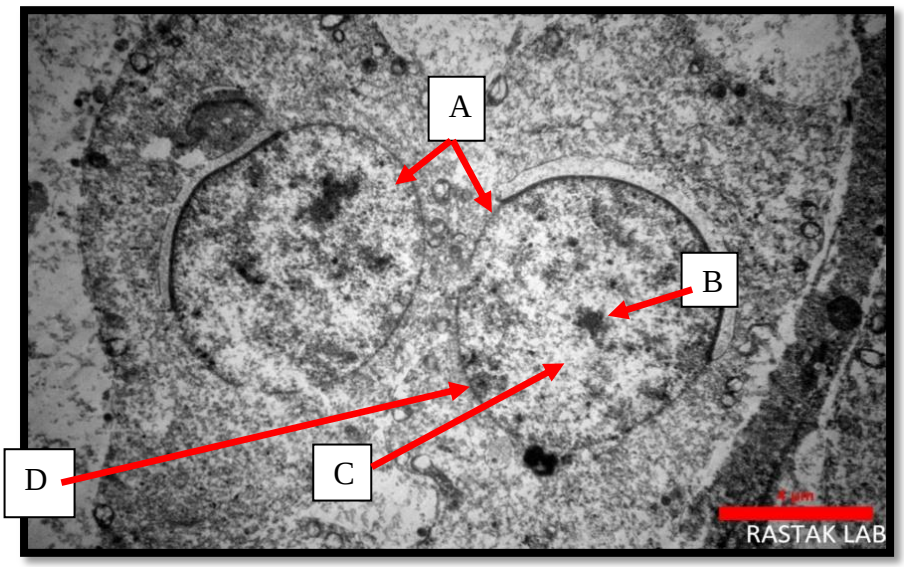

Figure 12. The testicular tissue of the rats treated with nanographene oxide at a concentration of 20 mg/kg indicates the structure of a double-headed spermatid (A), nucleus (B), cytoplasm (C), centriole (D), cell membrane, transmission electron microscopy

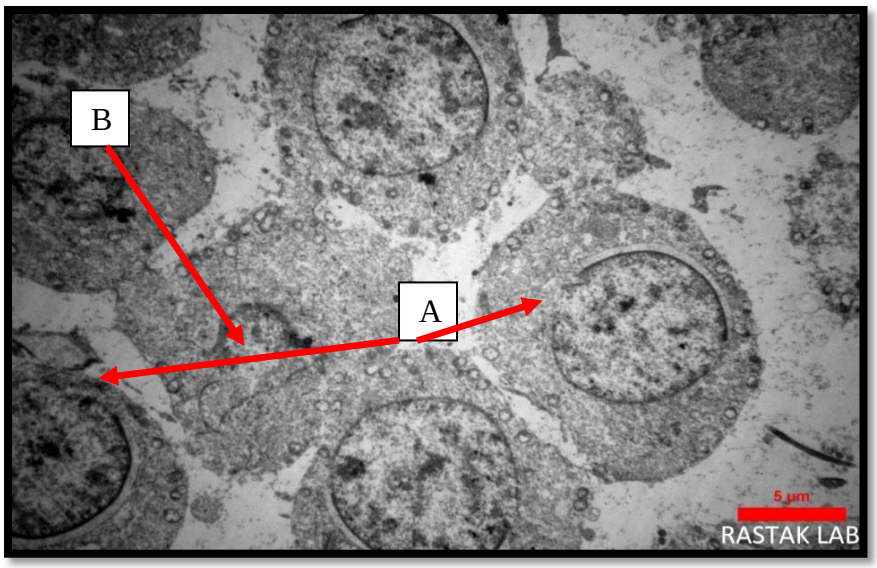

Figure 13. The testicular tissue of the treated rats with nanographene oxide at a concentration of 30 mg/kg. A nucleophilic breakdown in the cytoplasm (A, B), transmission electron microscopy

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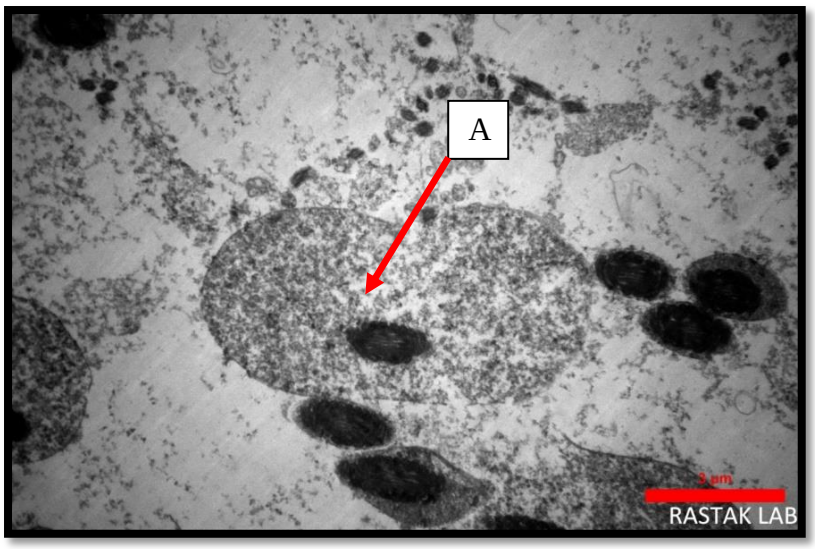

Figure 14. The testicular tissue of rats treated with nanographene oxide at a concentration of 40 mg/kg shows the breakdown of nucleus membrane (A), transmission electron microscopy

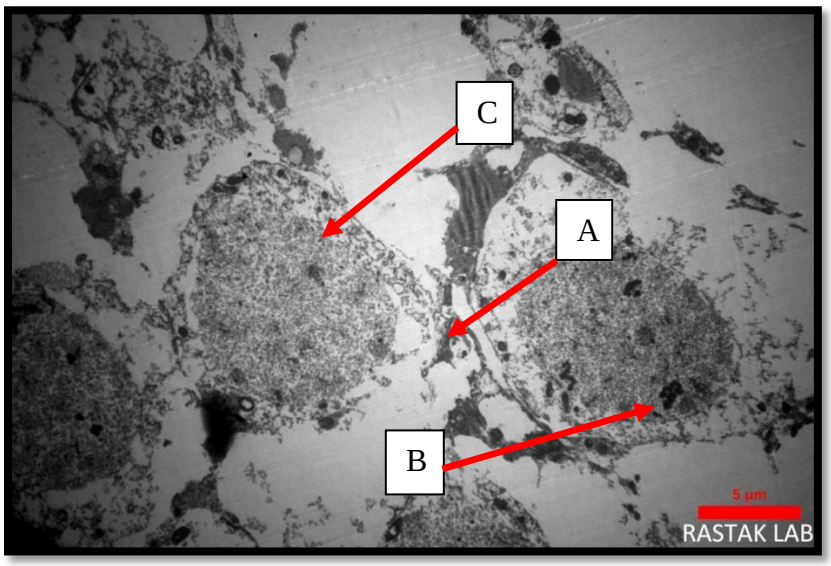

Figure 15. The testicular tissue of rats treated with nanographene oxide at a concentration of 50 mg/kg indicates cell membrane breakdown (A), nucleus (B), cytoplasm (C), transmission electron microscopy

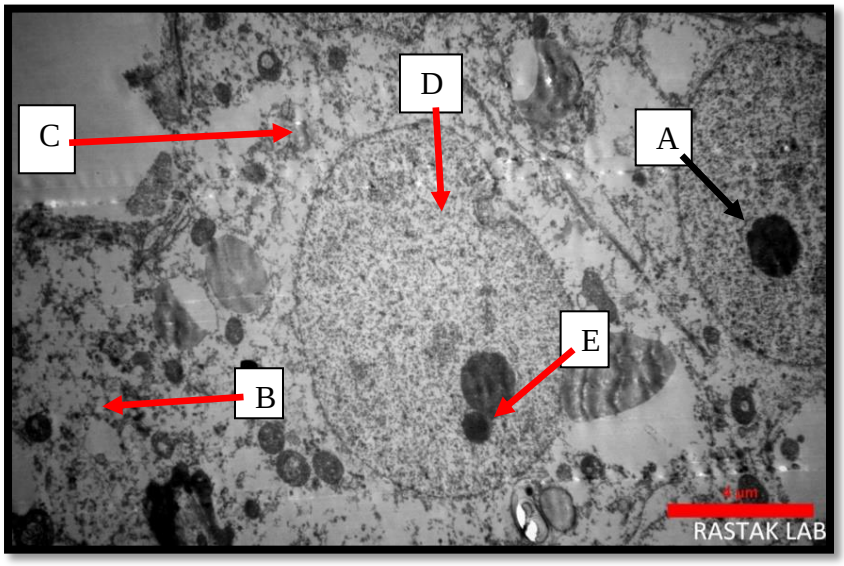

Figure 16. The testicular tissue of rats treated with nanographene oxide at a concentration of 60 mg/kg demonstrates buckling membrane of the nucleus (A), Golgi (B), Golgi apparatus (C), cytoplasm (D), nucleus (E), mitochondria in sertoli cell, transmission electron microscopy

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DISCUSSION

Effect of graphene oxide on the blood

The current study indicated a decrease in the number of red blood cells in all treated rats, especially in rats treated with 60 mg/kg of graphene oxide. The oxidative stress or pathological damage caused by the sharp edges of this material indicated that graphene oxide causes toxicity to the size and oxygen content of red blood cells leading to the dissolution of red blood cells (Liao et al. 2011). Moreover, this result agrees with a study of Puzyr et al. (2007), which showed the presence of toxic effects on blood parameters after intravenous injection, as graphene oxide interacts with the outer membrane of red blood cells through electrostatic interactions. This causes a disturbance in the polarity or permeability of the membrane and promotes hemolysis. These results are not in line with the obtained result of Qu et al. (2013), revealing that carbon nanoparticles do not cause any side effects to blood cells because they do not exert any toxic effects on blood cells. The results of the current study also revealed a decrease in the percentage of hemoglobin in the groups treated with nanographene oxide, compared to the control group. Hemolysis can be caused by the sharp edges of these nanomaterials that damage cell membranes, which was reported by Feng et al. (2015). The morphological changes, aggregation, and hemolytic effects on red blood cells when treated with graphene oxide are also indicated by Sasidharan et al. (2012). The reason behind the aggregation and hemolysis is the interaction between the hydrophobic surface of graphene and the lipid bilayer of the red blood cell membrane, or other interactions, such as hydroxyl and carboxylic groups in graphene oxide. These results also agree with those obtained by Stone et al. (2017), where a significant decrease was found in the hemoglobin percentage of groups treated with graphene oxide at concentrations of 80, 120, and 200 mg/kg, compared to the control group. In contrast, Escudero et al. (2019) reported that treating rats with graphene and graphene oxide could lead to smaller-sized red blood cells containing a high percentage of hemoglobin, justifying the reason for the high hemoglobin to compensate for the small size of red cells. The results of the current study also showed an increase in the numbers of white blood cells and platelets in the groups treated with graphene oxide, compared to the control group. There was an increase in the number of white blood cells although it decreased over time meaning that this effect was temporary. These results are consistent with a study by Vuppaladadium et al. (2020), where it was found that the number of white blood cells increased in mice treated with graphene oxide. This enhancement can be related to the foreign body, as it occurs after consuming the drug or graphene oxide. On the contrary, Rathnam et al. (2020) found that the hemoglobin content and the number of red blood cells were close to the control group. They also reported a slight increase in leukocytes, perhaps due to the response to nanomaterials during treatment. Pinto et al. (2013) and Zainab et al. (2021) also found that graphene-based nanomaterials were compatible with blood and did not cause hemolysis, platelet activation, changes in coagulation, or abnormalities in blood parameters. However, Lindstrom et al. (2015) reported that the number of white blood cells did not change in the animals treated with reduced graphene oxide.

Effect of graphene oxide on the testes

The results of the current study showed a decrease in the rate of testes' weight in the groups treated with graphene oxide. Compared with the control group, the histological examinations of the testes in rats treated with graphene oxide showed a group of changes represented by hemorrhage, a decrease in the number of sperms, a decrease in the thickness of the tubular epithelium, in addition to the dissociation of the connective tissue between the seminiferous tubules, necrosis of the tubular epithelium and blood congestion. The reason for the decrease in the weight of the testicles in the treated groups may be attributed to the lack of sperm and the suspension of the cell cycle, which resulted from a defect in the process of sperm formation in addition to the necrosis of the tubular epithelium leading to the loss of spermatozoa. These results were supported by Nirmal et al. (2017), indicating a decrease in the number of sperm in that rats exposed to graphene oxide nanoparticles for 15 and 30 days.

Histopathological changes in the testes, such as necrosis and a decrease in the thickness of the tubular epithelium, may be due to oxidative stress, as oxidative stress leads to the oxidation of cell membrane lipids, and consequently cell death. Adenosine triphosphate (ATP) is rapidly removed from the sperm, causing axonal damage and increasing sperm morphological abnormalities. These results were confirmed in a study by Mathur and Dcruz (2011), where there was a decrease in the number of sperms, their movement, and deformation due to a defect in the process of sperm formation as a result of the imbalance between oxidizing factors and antioxidant factors. Similarly, Cherian et al. (2014) found that oxidative stress and reactive oxygen species decreased cell proliferation, reduced steroid hormones, loss of germ cells, and cell death in the germinal epithelium. These results were also consistent with a study by Thakur et al. (2014), where nanomaterials cause the irregular appearance of the testis with atrophy of seminiferous tubules, loss of sperm-generating germ cells, necrosis of germ cells, and decrease or disappearance of sperms. In the same line, Hafsan et al. (2022), Huldani et al. (2022), and Li et al. (2016) found that Zno nanoparticles could cause reactive oxygen species (ROS) generation and DNA damage to germ cells and downregulation of the expression of proteins in sertoli cells, which may cause damage to the blood barrier in the testis. However, Liang et al. (2015) reported that the level of testosterone

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hormone in the serum did not change as it was shown that these nanomaterials did not stimulate the tissue damage of the testes and epididymis. These results do not agree with a study by Nirmal et al. (2017), indicating the concentrations of sex hormones in the serum of rats treated with graphene oxide did not change, compared to the control group after intravenous injection and the sexual behavior in male rats as reproductive structures were normal.

Results of transmission electron microscope

The findings of the transmission electron microscope showed changes at the cellular level of the testis, represented by the appearance of bi-headed and smashed-headed spermatids, in addition to the presence of sperms with broken membranes and other shrunken flagella walls in sertoli cells. This may be attributed to the ability of graphene oxide sheets to penetrate the cell membrane due to their nanoscale, causing damage to cell components through several mechanisms, including direct interaction with large biomolecules present in the cell or through oxidative stress that causes cell damage. The interaction of graphene oxide with large biological molecules, such as proteins, can result from functional groups with electrical charges on the surface of graphene oxide. Consequently, abnormalities occur in the intracellular signaling pathways that regulate cell growth, proliferation, differentiation, or survival. Similarly, Shi et al. (2012) reported that graphene oxide can weaken membrane proteins that act as carriers of nutrients or essential biomolecules, thus leading to a decrease in metabolic activity in cells treated with graphene derivatives. The mechanisms of action of graphene nanomaterials on living organisms include oxidative stress, inflammatory response, apoptosis, autophagy, and necrosis (Wu et al., 2021). Another study showed that treatment of some *in vitro* cultured cells, such as THP-1 and BEAS-2B cells, with graphene oxide, causes cytotoxicity by oxidizing lipids in the membrane and causing damage to the cell membrane (Li et al., 2018; Ansari et al., 2022). Another study showed that small parts of nanosheets of graphene derivatives could enter the nucleus, interact directly with DNA, and cause damage (Xu et al., 2018; Abolhasani Zadeh et al., 2022). In contrast to these results, some researchers stated that the graphene family of nanomaterials does not negatively affect the nucleus and DNA (Jarosz et al., 2016; Bokov et al., 2022).

CONCLUSION

It can be concluded that the nanographene oxide at 20-60 mg/kg concentrations is a hazardous material for testis heath in rats which can have dangerous effects on spermatogenesis, testosterone, and FSH levels and can destroy the testicular structure and impairment the testicular function in rats. The examined results of the transmission electron microscope confirmed the negative effects of this material in investigated dosages. The authors of the present study suggest that lower doses of nanographene oxide will consider for future histopathological studies in laboratory rats.

DECLARATIONS

Authors' contributions

The authors contributed equally to the study design, collecting the samples, statistical analysis, writing the first draft of the manuscript, and following the revisions of the last draft of the article for submission to the journal.

Conflicts of interests

The authors of the present study have no conflicts of interest to declare.

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 the authors.

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Availability of data and materials

The authors declare that they can prepare datasets for this study upon reasonable request.

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Administration of *Strobilanthes crispus* in an Angora Cat with Feline Lower Urinary Tract Disease

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ABSTRACT

The occurrence of feline lower urinary tract disease (FLUD) in Indonesia has not been widely reported. However, the incidence of the disease has increased due to dietary cat patterns. The diet habitually consists of commercial dry food only, without wet food, such as meat. FLUD often affects certain breeds of cats. Surgical therapy is the first option to remove kidney stones; however, rural areas in Indonesia often lack animal surgical facilities. This condition requires alternative therapies to cure the disease. A one-year-old male Angora cat was brought to Rumah Satwa veterinary clinic in Tanah Datar, Indonesia, for examination, with a history of urination difficulties or dysuria, pain in the abdomen when being handled, and lack of desire to mate. A macroscopic examination of urine showed a cloudy and dense appearance. The ultrasound examination revealed a stone (struvite) and a thickening of the urinary bladder wall. The angora cat was diagnosed with obstructive FLUD caused by urolithiasis. A capsule containing 125 mg Keji Beling (*Strobilanthes crispus*, Bl) extract was administered to the cat once daily to aid the struvite stone dissolution. Keji Beling is a herbal plant easily found in Indonesia and used to treat human kidney stones. After 32 days of therapy, the clinical condition of the cat improved. The ultrasound examination did not find any stones left in the bladder. In conclusion, based on local wisdom, Keji Beling leaves can potentially be an alternative therapy for FLUD in Angora cats with certain conditions.

Keywords: Angora cats, Keji Beling, Urinary bladder, Urolithiasis

INTRODUCTION

Indonesia is a tropical country with abundant biodiversities. One of its natural wealth is the diversity of plants that the community can use to treat various health conditions. Keji Beling (*Strobilanthes crispus* Bl) is a herb plant with various nutrients and active compounds; therefore, it can serve as an herbal remedy (Ismail et al., 2000). Keji Beling can be found easily and generally planted by the community as a home fence replacement. Keji Beling grows in clumps because it has many branches and leaves (Artanti and Fatimah, 2017).

Keji Beling is found in Madagascar, Africa, to Indonesia in Asia. Keji Beling is widely available in Asia, stretches from Japan and Korea in the north, from Afghanistan and Pakistan in the west, and even reaches only northern Australia in the south. Its diversity can be found in the Indian subcontinent, southern China, and mainland Southeast Asia, and it also occurs in Maritime Southeast Asia. A few species can be found in the Himalayan in altitudes above 3000 meters and in southwest China (Wood et al., 2003; Wood and Scotland, 2021).

Keji Beling is an herbal plant in Malaysia and is locally known as *Pecah Kaca*, *Pecah Beling*, *Karang Jin*, and *Bayam Karang*. It is also known as *Hei Mian Jiang Jun* to the local Chinese community (Ghasemzadeh et al., 2015). The part of the Keji Beling plant that is efficacious is the leaf which contains polyphenols, saponin, alkaloids, potassium, calcium, coumarin, flavonoid, and steroids (Ghasemzadeh et al., 2015; Ramadhani et al., 2021). However, the efficacy of Keji Beling is not well known by the public, whereas Keji Beling is a plant that is easy to grow, affordable, and does not need expensive treatment. Keji Beling, as herbal medicine, is generally available in capsule form and could be used for some diseases medications, such as kidney stones, obstipation, or diabetes mellitus (Fadzelly et al., 2006; Endrini et al., 2014; Artanti and Fatimah, 2017; Silalahi, 2020). In Indonesia, although Keji Beling is easy to grow, the utilization of this herbal plant is still rare. This also contributed to the infrequent publication of the efficacy of Keji Beling, especially as a remedy for feline lower urinary tract disease cases in pets.

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Feline lower urinary tract disease (FLUD) is a disease that causes disturbances in the bladder and/or urethra. Many factors are involved in the occurrence of FLUD (Abdel-Saeed et al., 2021). The disease can be classified into obstructive or non-obstructive types. The most common cause of non-obstructive FLUD is idiopathic cystitis at 65%. In comparison, the common cause of obstruction FLUD is a urethral plug at 59%, cystitis at 29%, urolithiasis at 10%, and urolithiasis with bacterial infection at 2% (Gunn-Moore, 2003).

Mainly, obstructions of the urinary tract are caused by kidney stones. Urolithiasis is the discovery of stones in the cat's urinary tract. Depending on the mineral content composition, urinary tract stones are divided into struvite and oxalate stones. Struvite stones commonly found in male cats comprise magnesium, ammonium, and phosphate. As a result of giving dry food containing high magnesium, there will be excessive magnesium absorption (Nikousefat et al., 2018; Abdel-Saeed et al., 2021). In addition, bacteria that infect the urinary tract can produce urease, ammonia, and carbonate ions that increase ammonium levels and pH. Increased concentrations of magnesium, ammonium, and phosphate in urine will cause supersaturation and struvite crystal formation (Dokuzeylül et al., 2015; Nikousefat et al., 2018). Struvites are colorless, shaped like a prism, and vary in size (Syme, 2012; Nururrozi et al., 2020). Appropriate treatments for FLUD are directed to restore the body's fluid balance, restore the urine flow and apply antibiotic therapy to eliminate the secondary infection (Prasetyo and Darmono, 2018). Surgery is a common method to remove kidney stones in felines. This case report described Keji Beling utilization, containing high sodium and potassium as alkaline properties to dissolve kidney stones and alkalinize the urine. Moreover, potassium in Keji Beling acted as a strong diuretic (Dharma et al., 2014). Keji Beling also contains cystolith, mainly composed of calcium carbonate, in which the infuse was alkaline. In addition, high saponin content in Keji Beling had impacts on oxalate crystal nucleation and aggregation in artificial urine solution, in an *in vitro* assay. The fraction of saponin also increased levels of glycosaminoglycan, a stone inhibitor macromolecule found in urine, and facilitated glomerular filtration (Patel et al., 2012; Ghasemzadeh et al., 2015).

MATERIALS AND METHODS

Case report

A one-year-old male Angora cat was brought to Rumah Satwa veterinarian clinic in Tanah Datar, Indonesia, on January 29, 2021, for examination (Figure 1). The cat has a history of Feline Rhinotracheitis, Calici-Panleukopenia, and *Chlamydia psittaci* vaccines (Felocell, Zoetis), according to the vaccination record book. Informed consent was approved and obtained from the owner before the examination. During the observation, the cat showed anorexia, anuria, and dysuria symptoms. The urinary bladder was distended and slightly enlarged. Other physical examinations indicated normal vital signs (body temperature was 37.6ºC, pulse was 104 beats/minute, respiratory rate was 28 times/minute, and normal turgor). The ultrasound examination of the urinary bladder indicated the presence of a prism-shaped stone and thickening of the vesica urinary wall (Figure 2). Therefore, the diagnosis was determined as FLUD caused by urolithiasis and cystitis. We preferred ultrasonography because it is more accurate to detect radioopaque struvite calculi compared to radiography (Hostutler et al., 2005)

Keji Beling capsules, made from Keji Beling's leaves, were given to the cat as a single dose/with a dose of 1 capsule/day for 3 consecutive days (dose was 80 mg/kg body weight, according to Package Leaflet, Keji Beling Capsule, Figure 4). Hematophan and biosolamine injections were also given as supportive therapy in the clinic. The patient was also prescribed glucosamine and vitamins for home therapy (Lew-Kojrys et al., 2017). The cat should consume a capsule of glucosamine three times daily. Glucosamine is also used to treat urinary tract inflammation. In addition, the daily feed was temporarily discontinued and replaced with diet feed supplemented with struvite stones dissolution formula, Royal Canin Urinary S/O (MARS Petcare, France). The food should have a low magnesium level and lead to more acidic urine; therefore, it can reduce the occurrence of struvite stones. The cat was previously given commercial dry cat food. This product contains a high level of phosphorus and magnesium that tend to form struvite stones in the urinary tract (Jukes et al., 2019; Tefft et al., 2021).

Eighteen days after the first visit, the cat returned–to the clinic. Anamnese, with the owner, revealed clinical symptoms of haematuria, anorexia, and pollakiuria. Ultrasound examination indicated decreased bladder stone size and reduced bladder wall thickness. The cat was administered the same therapies as the first clinic visit and given Lactate Ringer's solution infusion (contains electrolyte of Na^+ 130 mEq/L, Cl^+n3mEq/L, lactate 28 mEq/L) at 50 ml/kg/day to replace lost body fluids, maintain a balance of water levels in the body, stimulate nerves and support the metabolism process (Lavin et al., 2020).

After 32 days since the first visit, on February 29, 2021, the cat returned to the clinic for a follow-up visit. The clinical examination showed that the urine color was back to normal, and the patient had no urination difficulties. The cat's appetite recovered, and even the cat was mated with a female cat. The ultrasonography examination showed that the bladder stone had disappeared (Figure 5). The Keji Beling administration lasted 6 days, and the cat recovered from FLUD without performing any surgical procedures.

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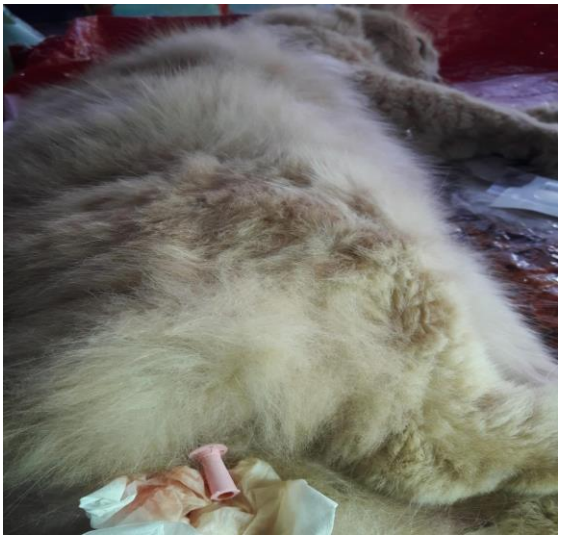

Figure 1. Physical examination of the male Angora cat with feline lower urinary tract disease caused by urolithiasis and cystitis

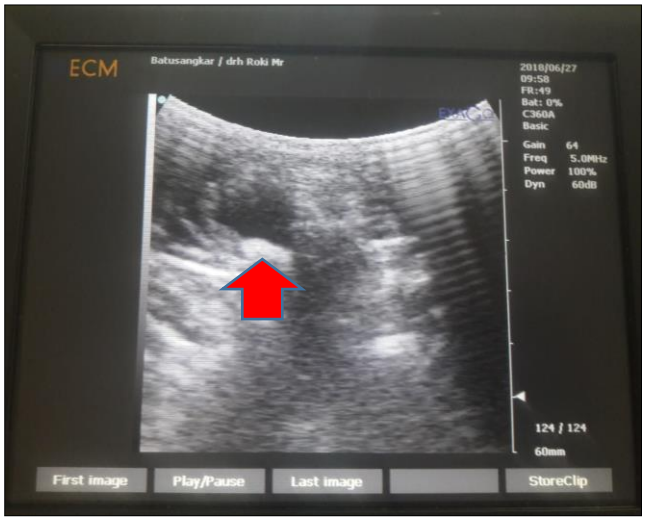

Figure 2. Ultrasonography examination (first day) of a male Angora cat. The presence of stones in the urinary bladder is indicated with the red arrow.

Figure 3. Keji Beling leaves (**A**) and flowers (**B**)

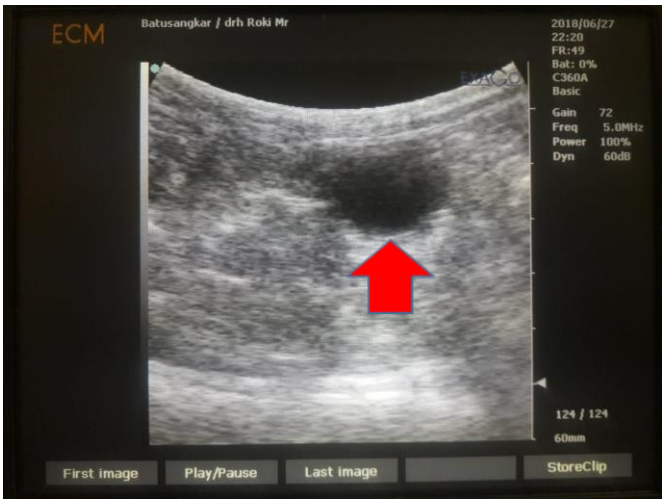

Figure 4. Ultrasonography examination of a male Angora cat on day 32 after treatment. No stone was seen in the urinary bladder (arrow).

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DISCUSSION

The diagnosis of obstructive FLUD was established by struvite finding in the cat's bladder and the presence of pollakiuria without polyuria (Lew-Kojrys et al., 2017; Abdel-Saeed et al., 2021). The administration of Keji Beling can facilitate kidney stone dissolution and improve the obstructions that occur (Setyawan et al., 2016). Kidney stones are formed by calcium ions reacting with oxalate and carbonate compounds and forming crystals. Kidney stones can be treated with high mineral content, such as sodium and potassium, in the Keji Beling leaves. The potassium content in Keji Beling leaves could reach 51% of the dry weight of leaves. Keji Beling leaves provide a diuretic effect that enables potassium or sodium to bind oxalate and carbonate compounds or calcium ions through the urine, therefore urine will be alkalinized. An infusion of dried Keji Beling leaves is mildly alkaline (Endrini et al., 2014).

After the bond formation between potassium, oxalate, and carbonate compounds, the strong diuretic effect on Keji Beling promotes the K_2CO_3 and Ca^{2+} binding formation to be excreted in the urine. This process will drive the urine to be alkaline, and this condition does not support the formation of kidney stones. As a result, in addition to treating kidney stones, Keji Beling can also inhibit the formation of new kidney stones (Fadzelly et al., 2006). Although the sodium content of Keji Beling leaves is not as high as potassium, only 24% of the total mineral, sodium also contributes therapeutic effects for treating kidney stones (Fadzelly et al., 2006).

Natrium performs a similar mechanism as potassium does. Natrium also binds oxalate and carbonate from calcium. With Keji Beling diuretic effect, the Natrium oxalate or natrium carbonate complexes will be excreted into urine and increase the urine pH. This strong diuretic effect acts on the epithelium of the kidney by inhibiting the reabsorption of electrolytes and increasing renal blood flow without an enhancement in the glomerular filtration rate. This results in a fluid decline, electrolyte reabsorption in the proximal tubular, and an increased diuretic effect (Iqbal et al., 2010; Dokuzeylül et al., 2015).

Keji Beling also increased K+, Ca++, and Mg++ excretion. The surge of electrolyte excretion will cause water excretion, leading to the elevation of urine excretion. In addition, the strong diuretic properties of Keji Beling inhibit the carbonic anhydrase enzyme. Carbonic anhydrase is an enzyme that catalyzes the reaction of $CO_2+H_20 \rightarrow H_2CO_3$. Balance of H_2CO_3 with H^+ and HCO_3^- ions is very important as a blood buffer in the body. These H^+ dan $HCO3^-$ ions are also important in the ions reabsorption process in the renal tubules, gastric acid secretion, and others. Due to carbonic anhydrase enzyme inhibition, the formation of H^+ and HCO_3^- in the tubular cells will be depleted. Therefore, it will decrease the H^+ ion secretion by the tubular cells and ultimately will inhibit the exchange of Na^+ and H^+ ions. As a result, bicarbonate, potassium, and sodium excretion in urine increase and drive the urine pH to alkaline. Elevation of electrolyte excretion also induces an increased water excretion (Iqbal et al., 2010; Dharma et al., 2014).

The diuretic property of Keji Beling leaves can inhibit the formation of kidney stones because it increases the excretion of electrolytes and water. Urinary tract stones can be determined based on the type of stone. The most common stones in the urinary tract of cats are struvite and calcium oxalate, and they can be differentiated by their morphology. Struvite is round or square-shaped, and has white, yellow, or brown color. Like chalk, struvite also has brittle consistency and a smooth or rough surface without protrusions. Calcium oxalates were square-shaped with X inside. It can also be described as looking like the back of an envelope and colorless (Syme, 2012).

The unpleasant odor of urine that contains struvite can be eliminated through modification in diet, but different treatments if the stone is oxalate, that 1 requires surgical procedures to remove them. The unpleasant odor originated from the urea breakdown process and the levels of erythrocytes in the cat urine.

CONCLUSION

In conclusion, Keji Beling can be suggested as an alternative therapy for feline urolithiasis caused by struvite stones. The authors suggest further studies on *Strobilanthes crispus*, Bl efficacy in different species of mammals, such as murine, canine, or even human, with more comprehensive clinical and laboratory evaluations.

DECLARATIONS

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Authors' contributions

I Gede Wempi Dody Surya Permadi and Risqa Novita contributed to the conception of this work, data interpretation, drafting, and revising the work; Roki Martarika contributed to the acquisition, analysis, and interpretation of data; Lisa Andriani Lienggonegoro contributed to draft revision. All authors approved the final version of the manuscript.

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Competing interests

The authors declare that there is no conflict of interest.

Ethical consideration

Ethical issues such as 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

The authors are ready to send the dataset of this study upon reasonable request.

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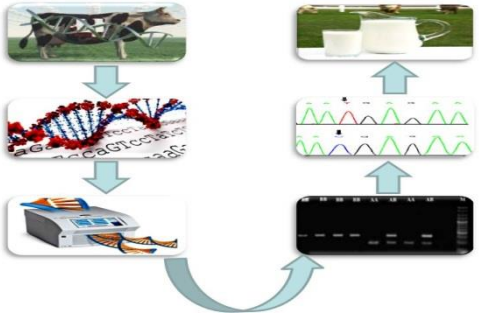

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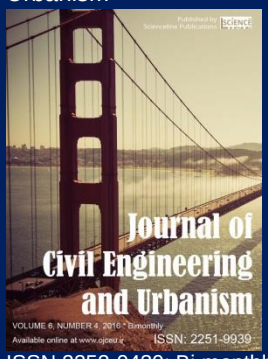

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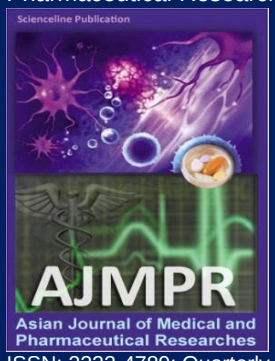

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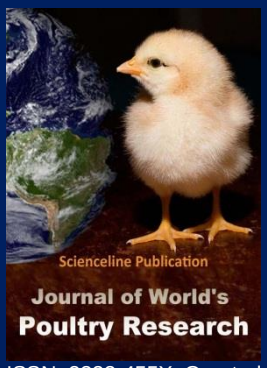

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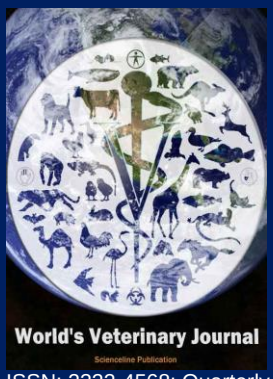

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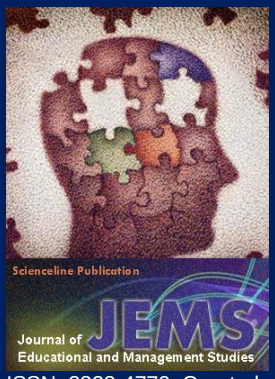

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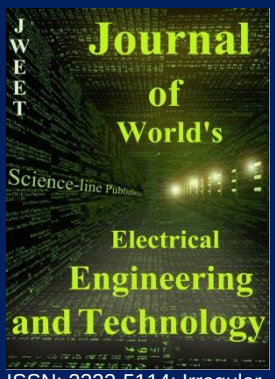

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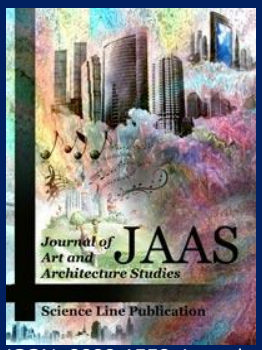

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