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BOOKLET


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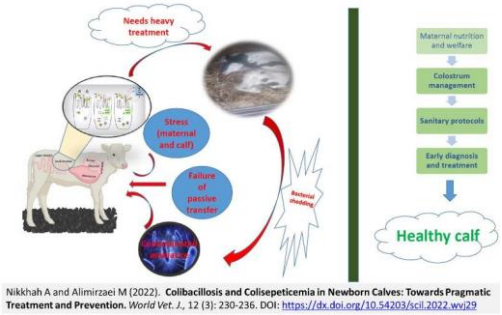
# Volume 12 (3); September 25, 2022 [EndNote XML for Agris]

## Review

### Colibacillosis and Colisepeticemia in Newborn Calves: Towards Pragmatic Treatment and Prevention

*World Vet. J.* 12(3): 230-236, 2022; pii:S232245682200029-12

DOI: https://dx.doi.org/10.54203/scil.2022.wvj29




**ABSTRACT:** Diarrhea is the most perturbing disease in dairy and beef industries worldwide, leading to significant rates of morbidity and mortality as well as economic losses. The objective of this review article was to delineate the pathophysiology and practical biology of colisepticemia in neonatal calves. Preventive and therapeutic protocols were also presented and discussed from a new integrative perspective. Notably, the situation can be the most deleterious in case diarrhea turns into septicemia. Under such circumstances, the mortality rate may be remarkably high and hard to control. *Escherichia coli* (*E. coli*) is an invasive and opportunistic bacteria causing severe diarrhea (colibacillosis) and colisepticemia in newborn calves. Colisepticemia is commonly prevalent in 2-5 days old calves, and colostral immunity is considered the first defensive line against *E. coli* infection. In addition to colostrum feeding quality and management, other management factors, such as dry cow nutrition and welfare, newborn calf welfare and nutrition, housing system, sanitation protocols, as well as early identification and treatment of sick calves, are important in preventing colisepticemia. In conclusion, understanding the mechanism of action and transmission routes of pathogenic *E. coli* will provide scientific and practical insight to plan preventive and therapeutic protocols decisively and successfully.
**Keywords**: Diarrhea, Mortality, Newborn calf, Pragmatic Prevention, Septicemia

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## Research Paper

### Improved Dot-ELISA Assay Using Purified Sheep *Coenurus cerebralis* Antigenic Fractions for the Diagnosis of Zoonotic Coenurosis

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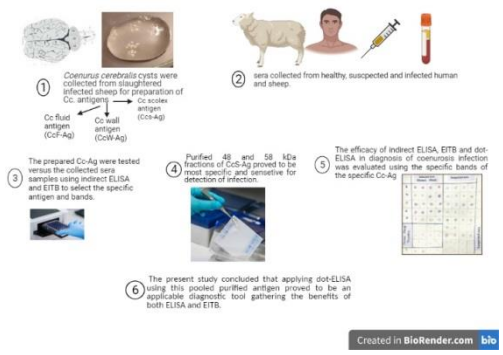
*World Vet. J.* 12(3): 237-249, 2022; pii:S232245682200030-12

DOI: https://dx.doi.org/10.54203/scil.2022.wvj30




**ABSTRACT:** Clinicians face significant problems in the diagnosis of zoonotic coenurosis. The current study aimed to develop an improved dot-Enzyme-linked-immunosorbent assay (dot-ELISA) for the diagnosis of zoonotic coenurosis using sheep *Coenurus cerebralis* scolices purified antigen (CcS-Ag) and to compare the obtained results with those of indirect ELISA and Enzyme-linked immunoelectrotransfer blot technique (EITB). Sera were collected from humans and sheep infected or suspected of infection with *Coenurus cerebralis*, control cases, and cases infected with other parasites. The CcS-Ag was proved to be the most specific antigen. This antigen was fractionated, and its specific polypeptides against anti-*C. cerebralis* antibodies (ACc-Ab) were identified using EITB. Fractions at the molecular weight (MW) of 48 and 58 kDa were proved as the only specific ones, eluted from the gel and concentrated, then dotted on the NC sheet as pooled antigen before its evaluation in the diagnosis of infection using dot-ELISA. Dot-ELISA demonstrated absolute 100% sensitivity and 100% specificity as recorded by EITB, compared to both fractions on a nitrocellulose (NC) sheet using surgically proved infected human or sheep sera as a gold standard. Diagnosis by ELISA using crude CcS-Ag revealed similar sensitivity but lower specificity (75%). The diagnostic accuracy of dot-ELISA was proved by comparing its results with postmortem data obtained post slaughtering of 20 suspected sheep and patients investigated by computed tomography (CT) and magnetic

resonance imaging (MRI). In conclusion, the selection of specific fractions after EITB to be used in dot-ELISA improved the diagnostic value of the test as a diagnostic tool gathering the benefits of ELISA and EITB.
**Keywords:** Antigen,*Coenurus cerebralis,* Dot-ELISA, Human, Sheep, Scolices

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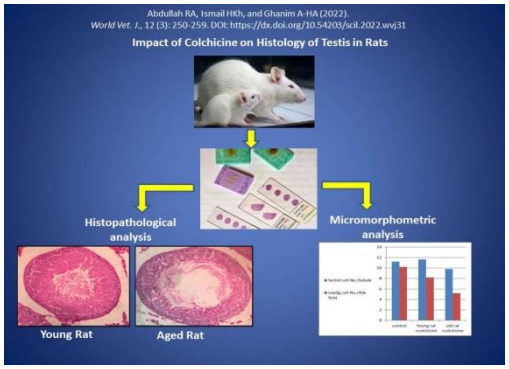
**Research Paper**

## Impact of Colchicine on Histology of Testis in Rats

*World Vet. J.* 12(3): 250-259, 2022; pii:S232245682200031-12

DOI: https://dx.doi.org/10.54203/scil.2022.wvj31




**ABSTRACT:** Colchicine is a drug widely used for the management of many disorders, such as acute gout and Behçet's disease. It is also prescribed for the treatment of pericarditis, atrial fibrillation coronary artery diseases, and secondary amyloidosis. In case this drug is used at the early stages of coronavirus infection, its anti-inflammatory properties may reduce the severe inflammatory reactions related to a cytokine storm by affecting the inflammasome. The purpose of the present study was to determine the toxicity of Colchicine on testis in rats from different age groups for 10 days. A total of 27 male Wistar rats were divided into three groups. The rats in group I (control group) were administered distilled water by oral gavage. Group II consisted of young rats (5-6 months old) who orally received Colchicine 3 mg/kg body weight. Group III entailed rats of 14-16 months who were orally administered colchicine 3 mg/kg body weight. The testis of the treated groups was dissected and examined for histological changes and morphometrical analysis. The obtained results indicated that high doses of Colchicine (3 mg/kg body weight) could induce tissue damage to the testis, including degeneration and necrosis of both Sertoli and Leydig cells with irregular divisions of germinal epithelium, even when it was used for short periods (10 days). In the elderly treated rats, there were severe tissue damages, including degeneration and necrosis of germinal epithelium with irregular divisions of germ cells, necrosis of Sertoli and Leydig cells with sloughing of germinal epithelium toward the lumen of the tubule. Therefore, there is a need to conduct more studies to investigate the side effect of Colchicine as it is excessively used in the management of coronavirus.
**Keywords**: Colchicine, Histology, Morphometric trait, Rat, Testis

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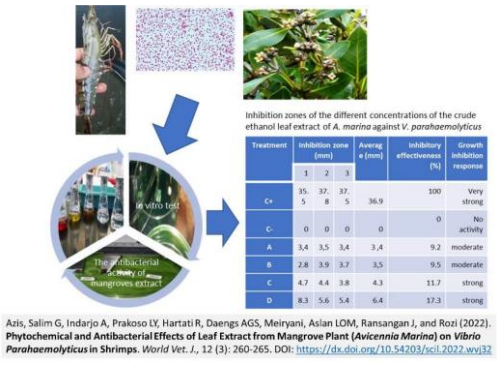
**Research Paper**

## Phytochemical and Antibacterial Effects of Leaf Extract from Mangrove Plant (*Avicennia Marina*) on Vibrio Parahaemolyticus in Shrimps

Azis, Salim G, Indarjo A, Prakoso LY, Hartati R, Daengs AGS, Meiryani, Aslan LOM, Ransangan J, and Rozi.

*World Vet. J.* 12(3): 260-265, 2022; pii:S232245682200032-12

DOI: https://dx.doi.org/10.54203/scil.2022.wvj32




**ABSTRACT:** Recently, there has been a tremendous increase in the studies addressing the application of bioactive compounds from the natural ecosystem, particularly for medical purposes. Hence, the present study investigated the antibacterial properties of the secondary metabolites possibly contained in the leaves of *Avicennia marina* (*A. marina*) for possible prevention of *Vibrio parahaemolyticus* (*V. parahaemolyticus*), a devastating bacterial pathogen in shrimp aquaculture. In the current study, secondary metabolites were extracted from the leaves of mangrove plant using ethanol extraction method. The ethanolic extracts were then subjected to phytochemical and antibacterial activity tests. The results from the phytochemical analysis demonstrated that the ethanolic extract from the mangrove plant contained varying amounts of flavonoids, tannins, saponins, polyphenols, alkaloids, steroids, and triterpenoids. However, the number of flavonoids and alkaloids seemed to be higher than the other metabolites. The antibacterial activity analysis through the agar diffusion method has shown that different concentrations (50 ppm, 100 ppm, 200 ppm, and 300 ppm) of the ethanolic extract of *A. marina* inhibited the *V. parahaemolyticus.* At 300 ppm, the

plant extract exhibited 17.3% antibacterial effectiveness, compared to the antibacterial activity of chloramphenicol. The findings indicated that the secondary metabolites of *A. marina* have the potential that can be developed as an alternative treatment for aquatic animal diseases in the future.
**Keywords:** Aquaculture, Bioactive compounds, Mangrove ecosystem, Treatment

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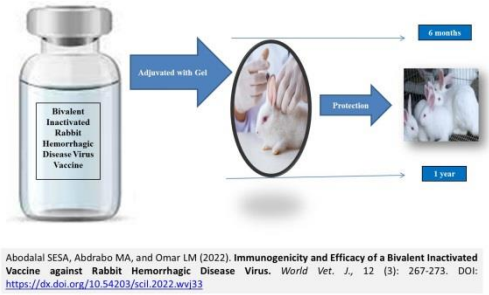
**Research Paper**

## Immunogenicity and Efficacy of a Bivalent Inactivated Vaccine against Rabbit Hemorrhagic Disease Virus

Abodalal SEA, Abdrabo MA, and Omar LM.

*World Vet. J.* 12(3): 266-273, 2022; pii:S232245682200033-12

DOI: https://dx.doi.org/10.54203/scil.2022.wvj33



Abodalal SESA, Abdrabo MA, and Omar LM (2022). **Immunogenicity and Efficacy of a Bivalent Inactivated Vaccine against Rabbit Hemorrhagic Disease Virus.** *World Vet. J.*, 12 (3): 267-273. DOI: https://dx.doi.org/10.54203/scil.2022.wvj33

**ABSTRACT:** Rabbit hemorrhagic disease is a fatal threat to rabbits that causes sustainability problems and substantial economic losses. The aim of the current study was to compare the immuno-enhancing effects of a bivalent inactivated rabbit hemorrhagic disease virus (RHDV) vaccine adjuvanted with Montanide with an inactivated RHDV vaccine with an aluminum hydroxide gel. Montanide incomplete seppic adjuvant 71 VG was prepared as an oil emulsion, and several batches adjuvanted with an aluminum hydroxide gel were prepared. Then, 250 New Zealand rabbits aged 6 weeks were randomly allocated to three groups. Group 1 was subjected to the bivalent inactivated RHDV adjuvanted with an aluminum hydroxide gel, Group 2 received the oil-emulsion vaccine adjuvanted with Montanide, and Group 3 was left unvaccinated as a negative control group. Efficacy was determined using a hemagglutination inhibition test, and resistance was determined using virulent RHDVa and RHDV2. The clinical signs included sudden death, nervous manifestations, aimless running, lateral recumbence, and crying before death. The mortality rates were recorded at 3 weeks, 3 months, 6 months, and 12 months after vaccination. In addition, blood samples were collected on the first day as well as 1, 2, 3, 4, 6 weeks post-vaccination (WPV), and 2, 3, 4 month post-vaccination (MPV) until 12 MPV. Serological analysis indicated that the bivalent inactivated RHDV oil-emulsion vaccine was more effective than the bivalent inactivated RHDV aluminum hydroxide gel vaccine, resulting in improved immune responses and longer-lasting protective immunological responses in vaccinated rabbits. The bivalent inactivated RHDV oil-emulsion vaccine was also sterile and safe and helped the protection of the rabbits against RHDVa and RHDV2, hence reducing the time and effort required during the vaccination process and reducing the levels of discomfort for the rabbits.
**Keywords**: Immunity, Inactivated vaccine, Oil emulsion, Rabbit hemorrhagic disease virus

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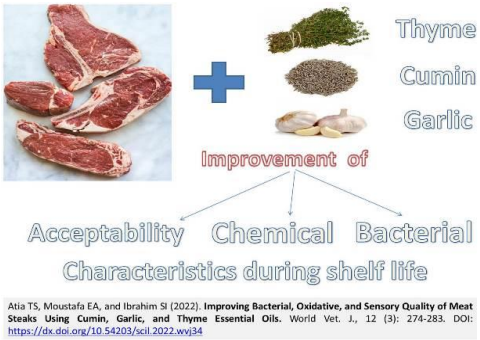
**Research Paper**

## Improving Bacterial, Oxidative, and Sensory Quality of Meat Steaks Using Cumin, Garlic, and Thyme Essential Oils

Atia TS, Moustafa EA, and Ibrahim SI.

*World Vet. J.* 12(3): 274-283, 2022; pii:S232245682200034-12

DOI: https://dx.doi.org/10.54203/scil.2022.wvj34



Atia TS, Moustafa EA, and Ibrahim SI (2022). **Improving Bacterial, Oxidative, and Sensory Quality of Meat Steaks Using Cumin, Garlic, and Thyme Essential Oils.** World Vet. J., 12 (3): 274-283. DOI: https://dx.doi.org/10.54203/scil.2022.wvj34

**ABSTRACT:** The meat industry increasingly considers meat shelf life as a critical problem. Some essential oils contain antibacterial and antioxidant characteristics that help to keep meat safe. Therefore, the purpose of this study was to evaluate the preservation benefits, including antibacterial and antioxidant properties, of cumin, garlic, and thyme essential oils at 1% on chilled beef meat steaks, as well as their effects on pH, total volatile basic nitrogen (TVBN), thiobarbituric acid (TBA), and related sensory aspects (color, odor, appearance, consistency, and overall acceptability). The results of the current study showed that pretreating beef meat steaks with EOs of cumin, garlic, and thyme at a concentration of 1% effectively reduced levels of APC, coliform count, *staph aureus* count, TVBN, and TBA while extending shelf life to 12, 15, and 18 days when stored at 4°C. In terms of antibacterial and antioxidant properties, shelf life, and sensory quality on beef meat steaks, the thyme essential oil group outperformed cumin and garlic essential oils. The current study introduced an effective natural preservative alternative that could replace undesirable synthetic substances in the future while also lowering antibiotic resistance.

**Keywords:** Coliforms, Cumin, Garlic, Preservation, Shelf life, Thyme

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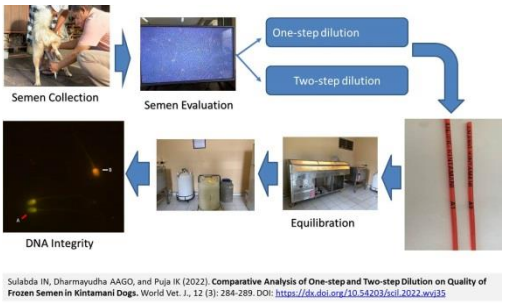
**Research Paper**

## Comparative Analysis of One-step and Two-step Dilution on Quality of Frozen Semen in Kintamani Dogs

Sulabda IN, Dharmayudha AAGO, and Puja IK.

*World Vet. J.* 12(3): 284-289, 2022; pii:S232245682200035-12

DOI: https://dx.doi.org/10.54203/scil.2022.wvj35




Sulabda IN, Dharmayudha AAGO, and Puja IK (2022). **Comparative Analysis of One-step and Two-step Dilution on Quality of Frozen Semen in Kintamani Dogs.** World Vet. J., 12 (3): 284-289. DOI: https://dx.doi.org/10.54203/scil.2022.wvj35

**ABSTRACT:** Preservation of sperm by freezing allows breeding dogs that are separated over long distances. To increase the fertility of frozen and then thawed spermatozoa, they must be able to survive the process. The current study aimed to evaluate the sperm motility and DNA integrity of Kintamani dogs extended in extenders with one-step and two-step dilution techniques. Ejaculates collected from four dogs were used in the current study. The semen was divided into two equal parts and diluted with extenders using two different dilution techniques, namely One-step dilution in Tris egg yolk containing 7% glycerol, and a two-step dilution technique diluted in an initial 2:1 with an extender, containing 20% egg yolk without glycerol. The same volume of the second extender was added, including 14% glycerol. The sample was loaded into 0.25 ml straws, cooled to 4°C for 4 hours, equilibrated, and then plunged into the liquid nitrogen. The sperm motility was evaluated using Computer-Assisted Sperm Analysis (CASA), and DNA integrity was assessed using Acridine Orange (AO) stained. Results showed that the sperm motility of Kintamani dogs in extenders using two-step dilution was significantly higher compared to the one-step dilution technique. In addition, the obtained results indicated that two types of dilution steps in Kintamani dog semen were not detrimental to the sperm DNA integrity during the freezing process. In conclusion, extenders with two types of dilution techniques could maintain sperm motility above 30%, and no difference between one and two steps dilution was detected.
**Keywords:** Dilution techniques, DNA integrity, Egg yolk, Kintamani dog, Motility

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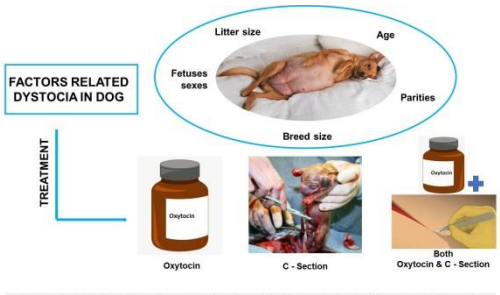
**Research Paper**

## Canine Dystocia: The Risk Factors and Treatment Methods in Dogs of Hanoi, Vietnam

Long STh, Hien NThTh, Hang PhTh, Hoai NTh, and Bach PhX.

*World Vet. J.* 12(3): 290-295, 2022; pii:S232245682200036-12

DOI: https://dx.doi.org/10.54203/scil.2022.wvj36




Long STh, Hien NThTh, Hang PhTh, Hoai NTh, and Bach PhX (2022). **Canine Dystocia: The Risk Factors and Treatment Methods in Dogs of Hanoi, Vietnam.** *World Vet. J.*, 12 (3): 290-295. DOI: https://dx.doi.org/10.54203/scil.2022.wvj36

**ABSTRACT:** Dystocia is a common disorder that can cause harmful health risks to bitch and puppies. The aim of the current study was to evaluate some risk factors related to canine dystocia and the application of treatment methods to 612 diagnosed cases in Gaia Pets Clinic and Resort, Hanoi, Vietnam, from December 2013 to May 2020. The investigated factors comprised age, parity and breed size, and litter size, as well as fetal sex in relation to the proportion of dystocia in female canines. Dystocia was frequently observed in female dogs aged 1-3 years, with rates of 76.1%. The highest proportion of dystocia was found in the first litter group (80.21%). The incidence of dystocia increased as the weight of the dog decreased, and it was prevalent in the small breed (61.93%). Dystocia risk decreased as the litter size increased. The interventions used in this study were medical treatment with the hormone oxytocin (1.8%), surgical management with cesarean section (86.11%), and a combination of oxytocin and cesarean section (12.09%), with the success rates of each treatment method as 100%, 98.86%, and 100%, respectively. Some risk factors, such as age, parity, breed size, and litter size identified in the present research, could be used as prognostic indicators in the veterinary practice to optimize the survival rate of female dogs and puppies.
**Keywords:** Age, Breed, Dystocia, Fetus sex, Litter size, Parities

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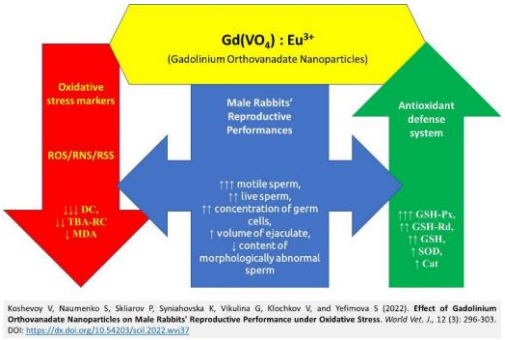
**Research Paper**

## Effect of Gadolinium Orthovanadate Nanoparticles on Male Rabbits' Reproductive Performance under Oxidative Stress

Koshevoy V, Naumenko S, Skliarov P, Syniahovska K, Vikulina G, Klochkov V, and Yefimova S.

*World Vet. J.* 12(3): 296-303, 2022; pii:S232245682200037-12

DOI: https://dx.doi.org/10.54203/scil.2022.wvj37




**ABSTRACT:** Oxidative stress as a leading factor of male infertility requires correction with modern pharmacological agents, particularly redox-active nanoparticles, to improve sperm quality and hormonal balance. The current experimental study aimed to investigate the effect of orthovanadate nanoparticles of rare earth elements, particularly Gadolinium, with pronounced redox properties on the reproductive function of male rabbits under oxidative stress. A total of 36 mature male Hyla rabbits were divided into three groups of intact control (n = 12) and two experimental groups, including rabbits ubder oxidative stress (n = 12), induced by the introduction of tert-Butyl hydroperoxide, and those under oxidative stress plus hydrosol of gadolinium orthovanadate nanoparticles (NPs, n = 12) intake for 14 days. There were four rabbits per three replicates in each group. Animals of all groups were kept on the same diet and had free access to water. The use of NPs led to an improvement in sperm quality indicators. There was an improvement in motility and ejaculate volume indicators (by 14.6% and 39.2%, respectively), a reduction of the content of morphologically abnormal sperm by 26.7%; normalization of sex hormones, an increase in the level of total testosterone (by 113%) with a decrease in 17-β-estradiol (by 16.5%). This sex hormones improvement led to an increase in the androgen saturation of the rabbit's body (free androgen index at the end of the experiment was 36.5%). The obtained changes were accompanied by a decrease in the oxidative load, as evidenced by a reduced content of diene conjugates and thio-barbituric acid-reactive compounds in the blood serum of rabbits by 30.4% and 26.8%, compared to the control. At the same time, there was an increase in the antioxidant potential, especially its glutathione link – the activity of glutathione peroxidase and glutathione reductase (by 42.5% and 34.2%, respectively), and the content of reduced glutathione increased by 62.3%, compared to the indicators before the introduction of NPs. The results of the study confirmed the effectiveness of using gadolinium orthovanadate NPs to correct the reproductive function of males under oxidative stress.
**Keywords**: Gadolinium orthovanadate, Male rabbits, Nanoparticles, Oxidative stress, Reproductive performances

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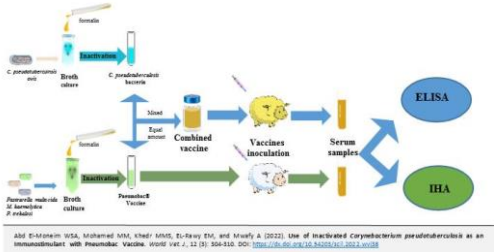
**Research Paper**

## Use of Inactivated Corynebacterium pseudotuberculosis as an Immunostimulant with Pneumobac Vaccine

Abd El-Moneim WSA, Mohamed MM, Khedr MMS, EL-Rawy EM, and Mwafy A.

*World Vet. J.* 12(3): 304-310, 2022; pii:S232245682200038-12

DOI: https://dx.doi.org/10.54203/scil.2022.wvj38




**ABSTRACT:** Sheep breeders in Egypt suffer from pneumonic pasteurellosis caused by *Pasteurella trehalosi, Pasteurella multocida*, and *Mannheimia haemolytica*. The disease is responsible for significant economic losses in the sheep industry according to the high mortality rate and reduced carcass values. Pneumobac® is the primary vaccine in Egypt used to control pasteurellosis in sheep. Therefore, the aim of the present study was to estimate the nonspecific immune stimulating impact of *Corynebacterium pseudotuberculosis ovis* against *Pasteurella* in sheep vaccinated with Pneumobac®. Nine sheep were classified into three groups, each with three animals. The sheep in the first and second groups were inoculated with the inactivated culture of Pneumobac® and a combined inactivated culture of Pneumobac® with *Corynebacterium pseudotuberculosis ovis* bacterin, respectively. The third group was nonvaccinated and kept in control. Indirect haemagglutination test (IHA) and enzyme-linked immunosorbent assay (ELISA) were used to measure the humoral immune response to the produced vaccines. The results of the present study confirmed that the antibodies titer against *Pasteurella multocida* type A, D, and B6, *Pasteurella trehalosi* type T, and *Mannheimia haemolytica* type A significantly increased in sheep vaccinated with a combined vaccine (Pneumobac® and *Corynebacterium pseudotuberculosis ovis* bacterin), compared to those vaccinated with Pneumobac® alone. It was concluded that the

addition of *Corynebacterium pseudotuberculosis ovis* bacterin to inactivated Pneumobac® vaccine could increase the immune response against pneumonic pasteurellosis.
**Keywords:** *Corynebacterium pseudotuberculosis*, *Pasteurella multocida*, Pasteurellosis, Pneumobac®

[Full text-PDF]

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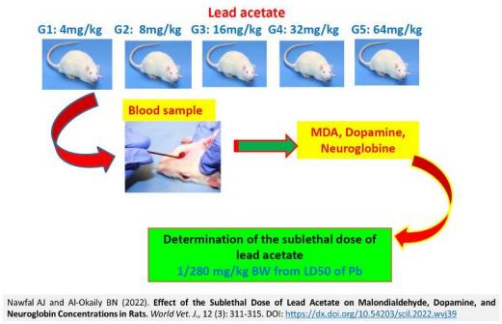
**Research Paper**

## Effect of the Sublethal Dose of Lead Acetate on Malondialdehyde, Dopamine, and Neuroglobin Concentrations in Rats

Nawfal AJ and Al-Okaily BN.

*World Vet. J.* 12(3): 311-315, 2022; pii:S232245682200039-12

DOI: https://dx.doi.org/10.54203/scil.2022.wvj39




**ABSTRACT:** Lead can have detrimental behavioral, biochemical, and physiological effects on the body. The current experiment was designed to estimate the sublethal dose of lead acetate that induce oxidative stress on the central nervous system (CNS) in adult using the probit analysis. Moreover, the current study examined the dose-response curve by successive doses of lead acetate on some parameters related to oxidative stress for 28 days. A total of 36 adult male rats were randomly selected and divided equally into six experimental groups and treated for 28 days. Rats in the control group received distilled sterile water, and those in G1, G2, G3, G4, and G5 were gavaged with 4, 8, 16, 32, and 64 mg/kg of lead acetate, respectively. The result indicated a positive correlation between the successive doses of lead acetate. Malondialdehyde concentration decreased dopamine and neuroglobin by increasing the dose of lead acetate in experimental groups (G3, G4, and G5), compared to the control group. In conclusion, exposure to the sublethal dose of 16 mg/kg of lead acetate significantly alters the levels of the neurotransmitters and increases the production of oxidative stress in the CNS tissue.
**Keywords**: Central nervous system, Dopamine and Neuroglobin, Lead acetate, Malondialdehyde, Rat

[Full text-PDF]

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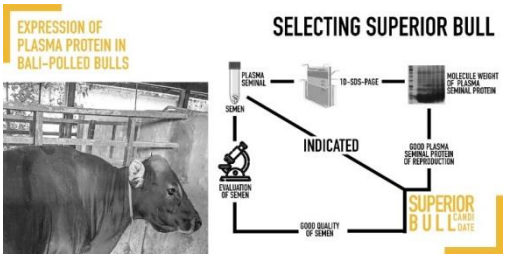
**Research Paper**

## The Expression of Plasma Protein in Bali-polled Bulls Using 1D-SDS-PAGE

Diansyah AM, Yusuf M, Toleng AL, Dagong MIA, and Maulana T.

*World Vet. J.* 12(3): 316-322, 2022; pii:S232245682200040-12

DOI: https://dx.doi.org/10.54203/scil.2022.wvj40




**ABSTRACT:** The fertility rate of bulls in a breeding program is not only described by the quantity and quality of semen. Factors, such as the interstice factor of the sperm and the plasma component of semen, affect the fertility rate of bulls. The fertility rate can also be determined by identifying the protein content of semen plasma. Therefore, the current study aimed to identify the relationship between seminal plasma protein molecular weight and semen quality of Bali-polled bulls. The study was conducted at the Laboratory of Semen Processing, Faculty of Animal Science, Hasanuddin University, Makassar, Indonesia, the Research Center for Applied Zoology, National Research and Innovation Agency, Cibinong, Indonesia and the Laboratory of Animal Biotechnology Center, IPB University, Bogor, Indonesia from November 2021 to January 2022. The samples came from 5 Bali-polled and 5 Bali-horned bulls. Semen collection was conducted twice a week using an artificial vagina. The concentration of seminal plasma protein was determined by the Bradford method of 1D-SDS-PAGE. The study results showed that fresh semen of Bali-polled and Bali-horned bulls was considered a normal category. Seminal plasma proteins of Bali-polled and Bali-horned bulls were classified using 8 bands to categorize molecular weight; 150 kD (IGF-1), 110 kD (A-kinase anchoring protein 3), 93 kD (A-kinase anchoring protein 4), 54-87 kD (Arylsulfatase-a), 44-62 kD (N-Acetyl-ß-Guicosaminidase), 44kD (Phosphoglycerate kinase), 15-30 kD (BSP A1/A2, BSP-A3 and BSP-30 [BSP1, BSP3, and BSP5]) and 12-14 kD (Acidic seminal fluid proteins). The findings indicated that both Bali-polled and Bali-horned bulls could have a high reproductive rate. In conclusion, protein analysis based on molecular weight using 1D-SDS-PAGE can be used as a biomarker for semen quality in Bali-polled bulls. Therefore, evaluating the semen quality with a molecular basis as an additional indicator of superior bull in the selection process is an alternative method.

**Keywords**: Bali-polled bull, Seminal protein plasma, Sperm, 1D-SDS-PAGE

[Full text-PDF]

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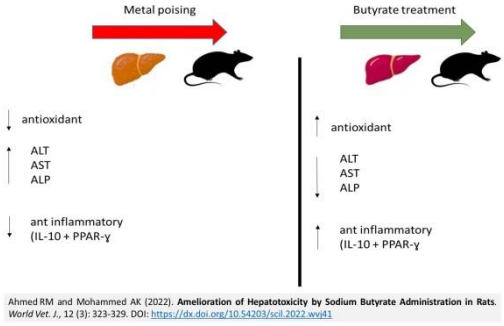
**Research Paper**

## Amelioration of Hepatotoxicity by Sodium Butyrate Administration in Rats

Ahmed RM and Mohammed AK.

*World Vet. J.* 12(3): 323-329, 2022; pii:S232245682200041-12

DOI:https://dx.doi.org/10.54203/scil.2022.wvj41




**ABSTRACT:** Lead poisoning is a serious environmental issue with life-threatening consequences. Lead poisoning increases the risk of cancers, gastrointestinal disorders, hepatotoxicity, central nervous system diseases, nephropathy, and cardiovascular diseases in animals and humans. The current study aimed to investigate the effect of sodium butyrate, as an antioxidant, on protecting female adult rats from the harmful effects of lead acetate. A total of 40 adult female albino rats were divided randomly into four equal groups. The first group dealt as the control. The second group received lead acetate at a dose of 200 mg/kg daily orally. The third group received lead acetate at a dose of 50 mg/kg daily orally, and the fourth group received both sodium butyrate and lead acetate orally/day for 35 days. The result indicated that sodium butyrate reduced the concentration of liver enzymes (ALT, AST, and ALP) which were elevated by lead acetate poising. Moreover, sodium butyrate ameliorates the redux status by decreasing malondialdehyde and increasing total antioxidant capacity. Additionally, sodium butyrate-treated rats showed significant alterations in the expression of peroxisome proliferator-activated receptor gamma and interleukin -10 genes. In conclusion, this study reveals an unrecognized role for peroxisome proliferator-activated receptor gamma and Interleukin-10 signaling after sodium butyrate treatment in regulating the immunopathology that occurs during lead acetate poising.
**Keywords**: Interleukin-10, Lead acetate toxicity, Sodium butyrate, PPAR-gamma, Rat

[Full text-PDF]

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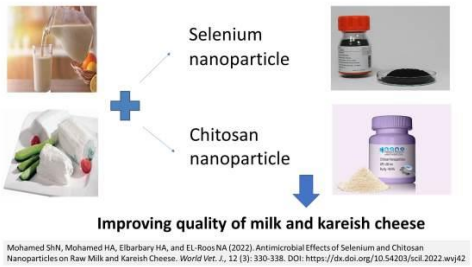
**Research Paper**

## Antimicrobial Effects of Selenium and Chitosan Nanoparticles on Raw Milk and Kareish Cheese

Mohamed ShN, Mohamed HA, Elbarbary HA, and EL-Roos NA.

*World Vet. J.* 12(3): 330-338, 2022; pii:S232245682200042-12

DOI: https://dx.doi.org/10.54203/scil.2022.wvj42




**ABSTRACT:** The contamination of milk and its dairy products with different microorganisms could cause public health hazards. Antibacterial nanoparticles (NPs) are a novel way to ensure that milk and milk products are safe. The present study investigated the effect of chitosan NPs (CS-NPs) and selenium NPs (Se-NPs) on some microorganisms, which consequently affect raw milk and Kareish cheese. Small-sized nanomaterials of Se-NPs and CS-NPs at the size of approximately 20 nm were used in this study. The samples were 700 ml raw milk and 700g Kareish cheese manufactured from 3000 mg milk. The concentrations of used nanoparticles were 0.5%, 1%, and 1.5% for Se-NPs and 2.5%, 5%, and 10% for CS-NPs. They were used to improve the microbial properties of milk and Kareish cheese samples during storage at the refrigerated temperature of 4°C. The aerobic plate count, Enterobacteriaceae count, Staphylococcus count, and mold count were significantly reduced in milk and Kareish cheese samples treated with CS-NPs and Se-NPs. The study has confirmed that CS-NPs and Se-NPs indicated high antimicrobial activity against the studied microorganisms at all concentrations although CS-NPs were more effective than Se-NPs. It can be concluded that these NPs can be used as preservatives in milk and milk products, such as Kareish cheese. In addition, increasing the concentrations of these NPs by 10% for CS-NPS and 1.5% for Se-NPS boosted their effects.
**Keywords:** Chitosan, *Enterobacteriaceae,* Kareish cheese, Nanoparticle, Selenium, *Staphylococcus aureus*

[Full text-PDF]

**Research Paper**

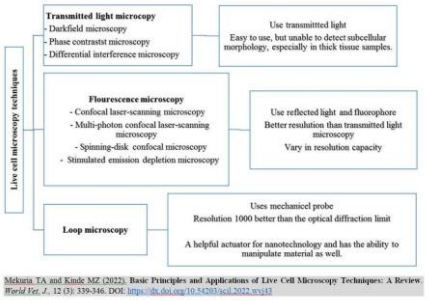
# Basic Principles and Applications of Live Cell Microscopy Techniques: A Review

Mekuria TA and Kinde MZ.

*World Vet. J.* 12(3): 339-346, 2022; pii:S232245682200043-12

DOI: https://dx.doi.org/10.54203/scil.2022.wvj43

**ABSTRACT:** Live cell imaging has provided great benefits in studying multiple processes and molecular interactions within and/or between cells. This review aimed to describe the common live cell microscopy techniques and briefly explain their principles and applications. A wide range of microscopic techniques, from conventional transmitted light to an array of fluorescence microscopy techniques, including advanced super-resolution techniques, can be applied for live-cell imaging. Transmitted light microscopy uses focused transmitted light that goes through a condenser to achieve a very high illumination on the specimen. On the other hand, fluorescence microscopy uses reflected light to capture images of cells or molecules that have been fluorescently dyed. Techniques for transmitted light microscopy are simple to use but have poor resolution. Although the resolution of fluorescent microscopy techniques is only approximately 200-300 nm, this is nevertheless an improvement over conventional transmitted methods. Conventional light microscopy's resolution was improved by the introduction of the super-resolution microscopy technology family. These methods "break" the diffraction limit, enabling fluorescence imaging with resolutions up to ten times higher than those possible with traditional methods. Each live cell imaging method has advantages and drawbacks. The primary deciding criteria for choosing the type of microscope are the study's objectives, previous experience, the researcher's interests, and financial viability. Hence, a thorough understanding of the technique and application of the various live-cell microscopy methods is paramount in life science studies.




**Keywords**: Application, Fluorescence, Imaging, Laser-Scanning, Live cell, Microscopy

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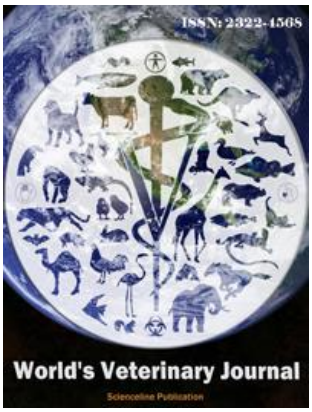


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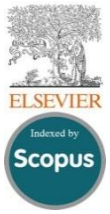


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# Designing a Recombinant Multi-epitope DNA Vaccine as Candidate for Protection against Pathogenic *Leptospira* Infection in Animals

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ORIGINAL ARTICLE
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**ABSTRACT**

*Leptospirosis* can cause severe disease and probable death in humans. Antigenic epitopes from pathogenic strains of the bacteria have shown potential for serving as vaccine candidates and play a key role in the sensitivity and specificity of immunodiagnostic tests. This *in-vitro* analysis was undertaken to develop a prototype recombinant DNA vaccine using *in-silico* epitope prediction method. Epitope prediction software programs predicted the most antigenic linear B-cell epitopes of *OmpL1*, *LipL32*, *LipL41*, *Loa22*, and *LigA*. Thirteen epitopes were predicted, connected by the Gly-Ser linker, and synthesized. The purity of the concentrated recombinant multi-epitope protein was assessed by restriction enzyme digestion and gel electrophoresis. *In-vitro* expression on mammalian Chinese Hamster Ovary cell line indicated strong cytoplasmic fluorescence produced based on an indirect immunofluorescence antibody test. The green color of the cytoplasm indicates successful transcribed and translated DNA as against the blue-stained nucleus observed in the un-transfected control group based on the indirect immunofluorescence antibody test. The findings of the current study showed high antibody binding potentials of the vaccine constructs, which could be used for diagnostic applications or as polyvalent vaccine candidates.

**Keywords:** B-cell epitopes, Indirect immunofluorescence antibody test, Leptospirosis, Multi-epitope vaccine, Recombinant vaccine

## INTRODUCTION

Leptospirosis is a zoonosis affecting a wide range of mammals, including humans, with significant public health implications (Garba et al., 2018a; Wang et al., 2022). The disease has a worldwide distribution, with varying severity depending on the infecting serovar, economic status, and prevailing environmental conditions (Azhari et al., 2018; Neela et al., 2019; Chacko et al., 2021). Leptospirosis is recognized as an important public health problem due to the increasing incidence of the disease and its occurrence in epidemic proportions in both developing and developed countries (Hasoun et al., 2017; Garba and Moussa, 2021). Like many tropical diseases, most cases of the illness are characterized by mild self-limited disease episodes (Al-Rasheed et al., 2018; Abdulhaleem et al., 2019; Jesse et al., 2020; Zakaria et al., 2020). However, in severe cases, degenerative changes can be seen in the liver and kidney dysfunction (Garba and Moussa, 2021).

Currently available vaccines for both medical and veterinary applications, which are predominantly whole-cell killed bacterins, can not protect against serovars not included in the vaccine preparations (Bashiru and Bahaman, 2018). Also, experimental evaluations have shown that Leptospiral vaccines can effectively prevent disease and reduce shedding after challenges with the serovar included in the vaccine (Lin et al., 2016; Garba et al., 2018b). However, there is a lack of cross-reactivity among antigenically distinct serovars, which further limits the ability of available vaccines to provide heterologous protection (Branger et al., 2005; Garba et al., 2018b). DNA vaccines are a novel approach for inducing an immune response. They are the simplest embodiment of vaccines that, rather than consisting of the antigen itself, provides genes encoding the antigen (Donnelly et al., 2005). In this approach, purified plasmid DNA containing the coding sequences of an immunogenic gene and the essential regulatory elements to transcribe and translate them is introduced into the tissue parenterally. This is followed by tissue expression and induction of potent, long-lasting heterologous immune response. The efficiency of Leptospiral DNA vaccines has been demonstrated in animal models (Branger et al., 2005; Maneewatch et al., 2007; Garba et al., 2018b). This is seen as a positive development in the efforts



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to prevent leptospirosis for which conventional vaccines have failed. In addition, DNA vaccines also allow for the inclusion of multiple genes to improve the coverage and the ability to protect against a wide range of serovar infections.

The *Leptospira* outer membrane proteins *OmpL1*, *LipL32*, *LipL41*, *Loa22*, as well as the immunoglobulin-like proteins; *LigA*, LigB, and LigC are among the major markers of pathogenicity among *Leptospira* specie (Koizumi and Watanabe, 2005). These proteins are conserved among the pathogenic *Leptospira* specie and can confer immunogenic protection, as reported in some animal models (Wang et al., 2007; Alia et al., 2019).

This research aimed to develop a synthetic B-cell epitope gene from conserved pathogenic *Leptospira* genes (*OmpL1*, *LipL32*, *LipL41*, *Loa22*, and *LigA*) using an *in-silico* bioinformatics approach to identify highly antigenic B-cell epitopes for construction of the multi-epitope chimeric gene. The study will also examine the *in-vitro* expression and reactivity using the indirect immunofluorescence antibody test.

## MATERIALS AND METHODS

### Ethical approval

All the experimental procedure was approved by the Institutional Animal Care and Use Committee (IACUC) of the Universiti Putra Malaysia (UPM/IACUC/AUP-R0012/ 2016).

### Selection of the epitopes

The criteria for selection of B-cell epitopes for inclusion in the multivalent vaccine include the ability for spontaneous clearance of *Leptospira* from animals with infection and prevention of renal colonization, conservation of the sequence among the most isolated *Leptospira* serovars and potential for the epitopes to induce an immune response (Vijayachari et al., 2015; Dellagostin et al., 2017). The complete amino acid sequences of genes *LipL32* (*L. interrogans* serovar Icterohaemorrhagiae), *LipL41* and *OmpL1* (*L. interrogans* serovar Lai), *Loa22* (*L. interrogans* serovar Grippotyphosa) and *LigA* (*Leptospira kirschneri* serovar Grippotyphosa) were retrieved from the UniProt knowledgebase (UniprotKB) NCBI database using the following search parameters and filters.

- Database: UniProtKB/Swiss-Prot- non-redundant protein sequences (nr) DBSOURCE: UniProtKB: locus Q72SM7_LEPIC, accession Q72SM; Max E-value: 1e-1. The search was conducted on all five genes under the entry UniProtKB/TrEMBLNCBI, and protein accession numbers for each were retrieved (Q72SM7, AAP04735, AAT48511, AAT48493, AGH20068). All the sequences were analyzed on BLAST using UniProtKB BLASTP, Matrix: Blossum 62, and threshold 10 (Boutet et al., 2007).

### Construction of multi-epitope containing plasmid

The selected epitopes were engineered together and linked using the glycine-rich linker (Trinh et al., 2004). These chimeric genes encoding multiple *Leptospiral* epitopes were codon optimized according to preferred codon usage for hamsters to enhance their expression (Chung and Lee, 2012). To enhance their immune-stimulatory capabilities and improve expression, a CpG motif (Class C) was added at both the N and C terminus, and a Kozak sequence was incorporated at the initiator terminal, respectively. The epitopes were combined and assembled in the following manner: epitopes from *LipL32* and *LipL41* together to give LipDNA01; epitopes from *OmpL1* and *Loa22* combined and designated OmpDNA02; epitopes from *LigA* were named LigDNA03, and the final construct is CompDNA04 which is the overall combination of all the epitopes. All the multi-epitope DNA constructs were designed with suitable restriction enzyme sites at the 5' and 3' ends, respectively (*LipDNA01- NotI* and *XhoI*; *OmpDNA02- SalI* and *BamHI*; *LigDNA03- KpnI* and *XhoI*; *CompDNA04- HindIII* and *Xbal*). The DNA molecule was synthesized by GeneScript (860 Centennial Ave. Piscataway, NJ 08854, USA) and was supplied cloned in pUC57 standard vector transformed in *E. coli* Top10 cells.

### Cloning of multi-epitope gene(s) in pBudCE4.1 mammalian expression vector

The mammalian expression vector pBudCE4.1 is a 4.6 kb vector designed with the ability for simultaneous expression of two genes independently. It contains a human cytomegalovirus (CMV) immediate-early promoter and a human elongation factor 1α-subunit (EF-1α) promoter for high-level, constitutive, independent expression of two recombinant proteins. In addition to the CMV and human elongation factor 1α sub-unit, it contains a C-terminal peptide encoding the *myc* (*c-myc*) epitope, the V5 epitope, and a polyhistidine (6xHis) metal-binding tag that allows the detection and purification of expressed proteins. The vector also contains a Zeocin™ resistance gene for selection in *E. coli* and the creation of stable mammalian cell lines (Ly et al., 2005). Although the pBudCE4.1 plasmid is a bicistronic vector, only one synthesized gene fragment was cloned to each plasmid (monocistronic), resulting in the construction of four different plasmids designated LipDNA-pBudCE01 (520bp), OmpDNA-pBudCE02 (609bp), LigDNA-pBudCE03 (236) and CompDNA-pBudCE04 (1299bp). Furthermore, LipDNA-pBudCE01 and LigDNA-pBudCE03 were cloned upstream of the V5 epitope tag at the 5' end, while OmpDNA-pBudCE02 and CompDNA-pBudCE04 were fused upstream of the cmyc epitope tag at the 5' end as well.



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**Transformation and sub-cloning of multi-epitope genes in pBudCE4.1 expression vector**

The standard $CaCl_2$ chemical transformation protocol (Sambrook et al., 1989) was used with essential modifications. Then, 1.25µl of pBudCE4.1 plasmid from the frozen stock was mixed with 25µl competent *E. coli* cells in a 1.5ml centrifuge tube. The tubes were swirled and flicked gently to suspend the cells and then incubated on ice for 30 minutes, followed by exposure to heat shock using a water bath at 42°C for 40-45 seconds. The tubes were placed on ice for 2-5 minutes, and 900µl of SOC medium was added. The suspended bacteria were then incubated at 37°C with gentle shaking (200-250*g*) for 1 hour. Cells were centrifuged (Thermo Scientific™, USA) at 4000 *g* for 2 minutes, the supernatant was discarded, and then the cells were re-suspended in 1 mL of phosphate-buffered saline (PBS), and 200µl was plated on LB agar plate containing 25 µg $mL^{-1}$ Zeocin. The plates were incubated at 37°C for 12-16 hours, and the resulting colonies were selected using the blue-white screening (Green and Sambrook, 2019).

Similarly, 1µg of purified pBudCE4.1 plasmid was digested using a combination of *NotI/XhoI*, *BamHI/SalI*, *KpnI/XhoI*, and *HindIII/XbaI* for genes 1, 2, 3, and 4, respectively. The compatibility of the restriction enzymes and their presence within the multiple cloning site of the plasmid vector was ascertained with the help of the New England Biolab NEB double digest finder. The optimized protocol for 40µl restriction enzyme digestion reaction comprises 5µl DNA (1µg), 2µl 10X NEB buffer, 0.5µl Bovine Serum Albumin (BSA), 1 µl each of restriction enzyme RE I and II, and nuclease-free water 30.5 µl.

The reaction mixtures were mixed thoroughly by finger tapping the tubes and then shortly spun down before incubating at 37°C for 1hr. After incubation, the reaction was deactivated by heating at 65°C for 15 minutes on a heating block. The digested products were separated in 0.8% agarose, stained with Gel red stain (Biotium, USA), and visualized using an Alpha imager™ gel documentation system (Alpha Innotech, Germany). Gel fragments were excised and purified using the QIAquick Gel extraction kit according to the manufacturer's instructions (QIAquick®, Germany).

The *NotI-XhoI*, *BamHI-SalI*, *kpnI-XhoI*, and *HindIII-XbaI* fragments for genes LipDNA-01, OmpDNA-02, LigDNA-03, and CompDNA-04 earlier prepared from pUC57 cloning vector were ligated into the corresponding sites of the digested pBudCE4.1 expression vector, generating LipDNA01-pBudCE.4.1, OmpDNA02-pBudCE4.1, LigDNA03-pBudCE4.1 and CompDNA04-pBudCE4.1 respectively. The ligation of the insert DNA fragments to the expression vector was achieved using the NEB T4 DNA ligase quick ligation kit (New England Biolabs®, England) according to the manufacturer's instructions. Briefly, a 20µl reaction containing 10µl of insert DNA, 5µl of the expression vector, 2µl of 10X buffer, 2µl of T4 DNA ligase, and 1µl of nuclease-free water was incubated at room temperature for 5 minutes. The ligation reaction was chilled on ice before using to transform chemically competent Top10 *E. coli* cells.

**Confirmation of insert DNA in pBudCE4.1 eukaryotic expression vector**

***Colony PCR***

Colony PCR to determine the presence of insert DNA in pBudCE.4.1 plasmid constructs were conducted. Individually transformed bacterial colonies were added to the PCR reaction and lysed during the initial heating step. This initial heating step was extended to 5 minutes to have complete lysis and release of the plasmid DNA from the cell, thereby serving as a template for the amplification reaction (Walch et al., 2016). The reaction condition used according to the NEB OneTaq® Quick-Load® 2X Master Mix Kit involved initial denaturation at 95°C for 5 minutes, denaturation at 94°C for 30 seconds, annealing at 56°C for 30 seconds, extension at 72°C for 2 minutes and final extension at 72°C for 7 minutes.

The NEB OneTaq® (Quick-Load®, England) 2X master mix was used as the source of polymerase, dNTP, $MgCl_2$, buffer components, stabilizers, and tracking dyes. The manufacturer's instructions and reaction protocol are shown in Table 1.

**Table 1.** Gene-specific primers designed using Primer3 software (v. 0.4.0) for colony PCR confirmation

| Gene | Forward primer | Reverse primer | Size |
|---|---|---|---|
| LipDNA01-pBudCE | GCG GCC GCA CCA TGG GCT CCA GCT T | CTC GAG GCA GCG CGT CTA AAG | 520bp |
| OmpDNA02pBudCE | GTC GAC ACC ATG GGC AGC TCC | GGA TCC GGC GCG CCG GAA GGA GCT A | 609bp |
| LigDNA03-pBudCE | GGT ACC ACC ATG GGC AGC | CTC GAG GCG GCG CGC CGA AAG | 236bp |
| CompDNA04pBudCE | AAG CTT ACC ATG GGC AGC TCC TTC | TCT AGA GGC GCG CCG AAA AGA GGA GC | 1299bp |

Source of gene sequence: NCBI genebank (Q72SM).

**Restriction enzyme digestion**

Restriction enzyme digest was used to determine the presence of the insert DNA in the pBudCE4.1 plasmid vector (Ly et al., 2005). The restriction enzymes used were based on the REs inserted during the design and synthesis of the genes. The reaction mixes and incubation were based on NEB protocol, and the product was analyzed using gel electrophoresis in 0.8% agarose to determine both the insert and vector fragments.



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**Sequence analysis of cloned genes**

Sanger sequencing technique involves using a DNA polymerase, a primer, unlabelled deoxynucleotide triphosphates (dNTPs), and fluorescently labeled dideoxynucleotide triphosphates (ddNTPs) was used to determine the presence and orientation of the insert DNAs. The sequencing was done by Next gene Scientific, SDN. BHD, Malaysia.

**Transfection of the mammalian cell line with plasmid DNA-carrying gene insert**

The lipid-based Lipofectamin® LTX transfection method was used to transfect CHO cells grown on cover slips in a 6 well cell culture plate. Six well plates were seeded with $10^6$ cells per well re-suspended in F12-K1 medium containing 10% FBS and incubated for 24 hours until they became 70-90% confluent. pBudCE4.1 plasmid containing a multi-epitope gene and a control plasmid were used to transfect the confluent cells using the Lipofectamin® LTX transfection reagent. According to the protocol, 200 µl of the Lipofectamin® LTX reagent (Thermo Fisher, USA) was diluted with 200 µl of serum-free opti-MEM medium (Thermo Fisher, USA) in a 1.5ml Eppendorf tube and 3 µg of plasmid DNA was mixed with 2.5 µl PLUS™ reagent containing 200 µl opti-MEM medium in another 1.5ml Eppendorf tube. The diluted DNA was then added to the diluted Lipofectamin® LTX reagent in a ratio of 1:1 and the mixture was incubated for 5 minutes at room temperature. The DNA-lipid complex was then finally dispensed into the respective wells of LipDNA01-pBudCE.4.1, OmpDNA02-pBudCE4.1, LigDNA03-pBudCE4.1 and CompDNA04-pBudCE4.1 as well as an additional well containing un-transfected cell as control. The plates were incubated in a humidified 5% $CO_2$ incubator for 5 minutes while agitating it occasionally to prevent drying of the cells. After 4 hours of incubation, the transfection medium was replaced with F12-K1 medium containing 5% FBS. Finally, 24, 48, and 72 hours after transfection, the plates were withdrawn, and the functionality and expression of DNA plasmids were evaluated.

**Analysis of expression of plasmid by indirect immunofluorescence test IIFT**

The c-myc Tag and V5 Tag monoclonal antibodies (ThermoScientific, USA) were used as the primary antibodies to detect the LipDNA01-pBudCE.4.1, and LigDNA03-pBudCE4.1 fused to the V5 epitope tag region of the pBudCE 4.1 plasmid vector and the CompDNA04-pBudCE4.1 and OmpDNA02-pBudCE4.1 equally cloned at the c-Myc epitope tag. However, the secondary antibody used was the Goat anti-mouse IgG conjugated to fluorescein dye Alexa Fluor 488 (ThermoFisher Scientific, USA)**.**

The instructions of the manufacturer in each case were followed. Working concentrations for all the listed antibodies were reconstituted as recommended by the manufacturers. Briefly, spent media from transfected cells withdrawn after 24- and 48-hours incubation were discarded, and the cells were washed twice with an interval of 5 minutes between washes with sterile PBS; the cells were then fixed with 4% formaldehyde and incubated for 15 minutes at room temperature (Sambrook et al., 1989). The cells were rinsed with PBS 3-4 times after fixation and then permeabilized with 0.2% buffered Triton X100 for 5 minutes. The cells were further rinsed with PBS and then blocked using 1% Bovine Serum Albumin (BSA) diluted in PBST (sterile PBS in Tween 20), followed by 1-hour incubation with primary antibody at 37$^o$C. Unbound primary antibodies were removed by rinsing 3-4 times with PBS before incubation with secondary antibody for 1 hour at 37$^o$C. The primary and secondary antibody incubation were performed under humidified and dark conditions. After the incubation period with the secondary antibody, the cells were rinsed once again with PBS, but this time it was done with PBS containing Tween 20 (0.05%) and then counter-stained with DAPI (4',6-Diamidino-2-Phenylindole, Dihydrochloride) and incubated at room temperature for 5 minutes. The coverslips, upon which the cells were grown, were carefully lifted and placed on a clean microscope slide containing a drop of fluorescein mounting media with the cells facing downward. The mounted slides were kept drying in the dark before viewing with a confocal microscope.

## RESULTS

**Transformation of Top10 *E. coli* cells**

The synthesized genes used in this study were cloned in pUC57 cloning vector as glycerol stock of Top10 *E. coli* cells. The plasmid was purified and sequenced using the M13 forward and reverse primers. The result indicated 100% similarity with the sequence submitted for synthesis. Restriction digestion of the pUC57 plasmid yielded two fragments with plasmid size 2.7kb while the sizes of the genes were LipDNA01-520bp, OmpDNA02-609bp, LigDNA03-236bp, and CompDNA04-1299bp, respectively.

The genes were then cloned in pBudCE4.1 expression vector to serve as the carrier of the vaccine gene. The genes were inserted upstream of the *myc* (OmpDNA02 & CompDNA04) and V5 (LipDNA01 & LigDNA03) epitope tags under the regulation of CMV and EF1-α promoter (Figure 1).

Each of the digested genes was ligated to its corresponding pBudCE4.1 vector using T4 DNA ligase (New England Biolabs). The ligated product was used to transform chemically competent *E. coli* cells. After overnight incubation at 37oC, successfully transformed cells were selected and further analyzed by colony PCR, restriction enzyme digestion, and sequencing (Swords, 2003).



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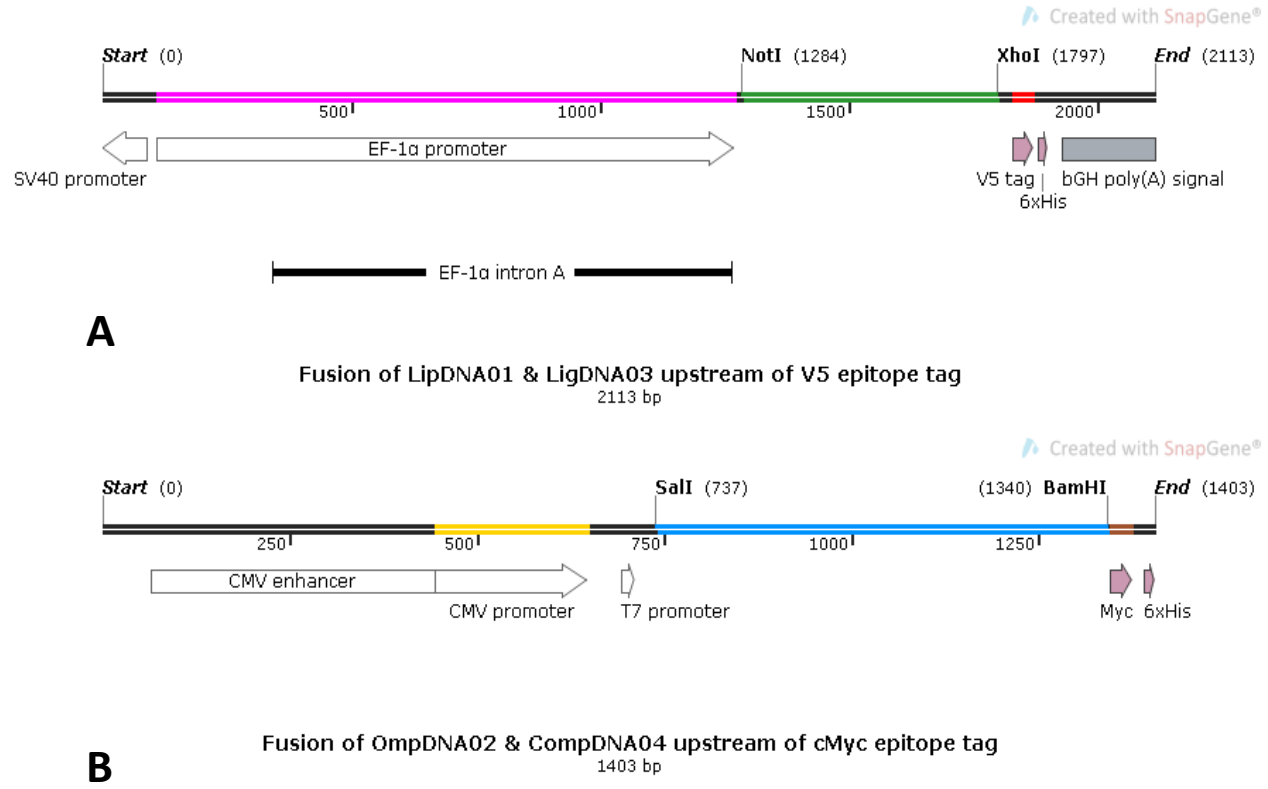


**Figure 1.** Schematic representation linear map of the segment of the plasmid vector showing V5 and cMyc epitope tags region. [A] fusion of LipDNA01 and LigDNA03 and [B] fusion of OmpDNA03 and CompDNA04 genes under the control of EF1α and CMV promoters, respectively (SnapGene® software).

**Confirmation of positive clones by colony PCR**

Using colony PCR, five bacterial colonies from each of the four plates with transformed Top10 E. coli cells were selected and screened for the presence of plasmid-carrying insert. The result (Figure 2) showed all colonies to be positive, indicating the incorporation of the inserted gene within the plasmid.

**Confirmation of insert DNA by restriction enzyme digestion**

Double digestion of purified plasmid DNA from bacterial colonies revealed that three colonies were positive for LipDNA01-520bp while OmpDNA02-609bp and LigDNA03-236bp had all clones carrying insert and for CompDNA04-1299bp, only four colonies were positive (Figure 3).


**Figure 2.** Showing bands for the screening of successfully cloned genes by colony PCR with positive bands against the 1kb DNA marker. Lane 1 DNA ladder; lane 2-4 LipDNA01 (520bp); lane 6 and 7 OmpDNA02 (609bp); lane 9 CompDNA04 (1299bp), lane 15-19 LigDNA03 (236bp), Lane 00 (empty)



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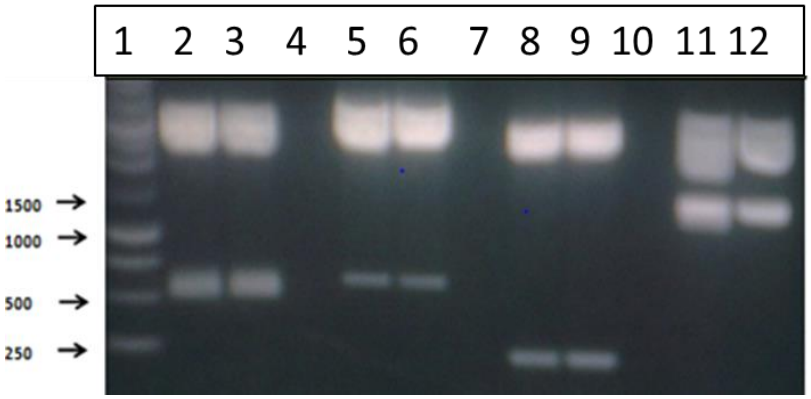


**Figure 3.** Gel electrophoresis of double-digested genes. Confirmation of bands from digested genes after subcloning in BudCE4.1 vector. Lane 1 is 1kb ladder, lane 2 and 3 is LiPDNA01 size 520bp, lane 5 and 6 is OmpDNA02 size 630bp, lane 8 and 9 is LigDNA03 size 236bp and lane 11 and 12 is CompDNA04 size 1299bp

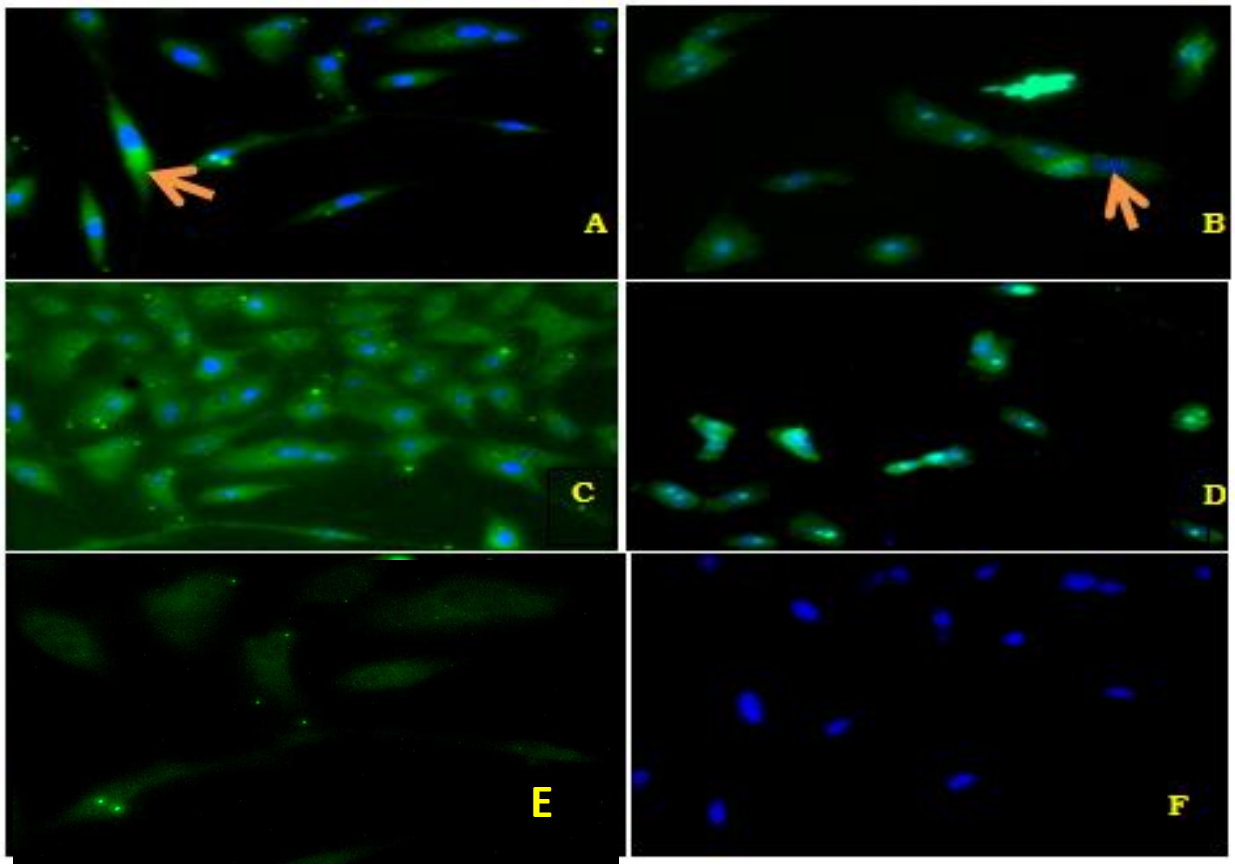


**Figure 4.** Demonstration of expression of plasmid DNA constructs in CHO-K1 cells at 48 hours' incubation after transfection with vaccine constructs as well as un-transfected control plasmid after counter-staining with DAPI. (A) LipDNA01-pBudCE.4.1 with an arrow showing expressed proteins in the cytoplasm indicated by green fluorescence emission of FITC (arrowhead-slide A). (B) OmpDNA02-pBudCE4.1; arrow showing the blue stained nucleus of the cells after picking the DAPI counter stain (arrowhead-slide B). (C) LigDNA03-pBudCE4.1 also well expressed due to the small size of the gene. (D) CompDNA04-pBudCE4.1 showing the green-coloured cytoplasm and the DAPI-stained nucleus. (E) Demonstration of expression of positive control GFP plasmid. (F) Negative control un-transfected CHO-K1 cells counter-stained with DAPI nucleic acid stain.

**Confirmation of insert DNA by sequence analysis**

Sequence analysis for pBudCE4.1 plasmid carrying each gene indicated a 100% homology with the reference sequence synthesized by Genescript (USA). The only exception was CompDNA04 with size 1299bp. Although, sequencing the gene in fragments would have sufficed. However, it should be noted that Sanger sequencing can only sequence DNA up to 1kb at a time. The result also revealed that the genes were inserted in the correct orientation as indicated by restriction enzyme sites inserted upstream and downstream of the insert DNA and the presence of nucleotide sequences of the plasmid flanking the N and C terminal portion of the insert.



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***In-vitro* expression of vaccine constructs**

The plasmid DNA constructs incubated for 48 hours gave the best result, with expression indicated by the strong cytoplasmic fluorescence produced (Figure 4). The green color of the cytoplasm indicates successful transcribed and translated DNA as against the blue-stained nucleus observed in the un-transfected control group. The fluorescence emission of FITC (Fluorescein isothiocyanate) conjugates produced green fluorescence, while DAPI, a nucleic acid stain that binds to A-T rich regions of DNA stains the nucleus blue. The positive control represented by GFP protein also produces solid green color, while the negative control represented by un-transfected cells only shows the blue stained nucleus material.

## DISCUSSION

This study was undertaken to determine the potential of recombinant DNA vaccine construct based on *LipL32*, *LipL41*, *OmpL1*, *LigA*, and *Loa22* gene to serve as a vaccine candidate that can render protection against pathogenic *Leptospira* infection. The genes used are antigenically conserved among pathogenic *Leptospira* species, and their potential as vaccine candidates is enhanced by their expression during infection of mammalian host (Haake et al., 1999; Wang et al., 2007) This technology is mooted to provide better protection compared the routinely used inactivated vaccines due to the wide antigenic variation common among pathogenic *Leptospira* as reported by Dellagostin et al. (2011).

The synthesized gene was cloned into a plasmid in this study to understand their immune responses against leptospirosis. The fusion of the genes to the *myc* epitope and the V5 epitope permits co-expression of the insert genes as fused proteins to the epitopes and gives room for the detection and monitoring of the expression level of the genes. This approach is in congruence with earlier studies where *myc* and V5 epitope tags were used for protein expression due to their versatility and the ability for detection by immune cells (Traenkle et al., 2020). Similarly, the genes were expressed with the help of an expression vector using cell culture. The *in-vitro* expression of foreign genes is influenced by the promoter strength, the ribosomal binding site, which regulates the frequency of translation of the messenger RNA, the stability of the cloned protein, and the codons utilized in the foreign gene. In this study, the synthetic gene was codon optimized according to preferred codon usage for hamsters which is the chosen animal model and the origin of the cell line (Chung and Lee, 2012). It has been reported that optimal codons help achieve higher accuracy translation rates. However, the scientific basis of mammalian codon optimization indicates that codon usage is rate-limiting for protein expression (Lanza et al., 2014; Mauro and Chappell, 2014). Furthermore, the addition of flexible spacer sequences, as done in this study, ensures the stability of the insert DNA in the plasmid, allowing independent interaction of different segments without having any adverse effect on the outcome (Trinh et al., 2004).

Following the digestion of both genes and their corresponding plasmid vector, both were ligated using the T4 DNA ligase. T4 DNA Ligase catalyzes the joining of two DNA strands between the 5´-phosphate end of the insert DNA and the 3´-hydroxyl end of the digested plasmid nucleotides in a cohesive-ended configuration (Dickson et al., 2000).

The chemical competence conferred on Top10 *E. coli* cells was to ease the passage of the plasmid-carrying gene construct through the hydrophobic cell membrane, as earlier reported during vaccine development using the outer-membrane protein *LipL32* (Khodaverdi Darian et al., 2013; Humphryes et al., 2014). Although the Top10 *E. coli* cells' transformation efficiency was not determined, LigDNA03-pBudCE4.1 with 236bp had the highest success rate, while CompDNA04-pBudCE4.1 with 1299bp had the least. This observation may be due to the large size of the CompDNA04-pBudCE4.1. This is like studies that report that transformation efficiency decreases with an increase in DNA size (Ohse et al., 1995; Kung et al., 2013).

PCR and restriction enzyme digestion confirmed successful insertion of the genes into the plasmid. This was further supported by the sequencing results, which indicate the insertion of genes in the correct orientation by virtue of the restriction enzyme sequence and nucleotide sequences from the plasmid upstream and downstream of our insert gene. The sequencing result also shows 100% similarity with the synthesized sequence, indicating that no mutation occurred, particularly after exposure to UV light during the excision of bands from the gel. This is important as UV light is known to have mutagenic tendencies on DNA, which could have a detrimental effect on the integrity of the gene (Pfeifer et al., 2005; Rastogi et al., 2010). Although the quality of sequence result for CompDNA04-pBudCE4.1 was poor, especially at the beginning and towards the end of the sequence, this is one of the challenges of Sanger sequencing, and its due to poor quality in the first 15-40 bases of the sequence because of primer binding and deteriorating quality of sequencing traces after 700-900 bases (Ledergerber and Dessimoz, 2011). Sanger method is a popular sequencing technology for sequencing short pieces of DNA. However, the quality of a Sanger sequence is often not very good in the first 15 to 40 bases because that is where the primer binds (Tomotoch-Serra et al., 2017).

During pathogenic *Leptospira* infection, protection is dependent on the stimulation of an appropriate antibody response; highly potent neutralizing antibodies can intercept pathogenic *Leptospira's* before it attaches to its target cell. This ability is based on the antibodies' specific recognition of antigen epitopes (Gershoni et al., 2007). Thus, it is essential to evaluate the immunogenicity of B-cell combined epitopes for the development of novel vaccines. In this


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study, B-cell combined epitopes in the outer membrane proteins *LipL32*, *LipL41*, *OmpL1*, *LigA*, and *Loa22* from pathogenic *Leptospira*, which can induce the immune response against *Leptospiral* infection, were identified and characterized, and these will be used to develop vaccines or therapeutic strategies.

The production of recombinant proteins is a very important and powerful technique with multiple applications in life sciences (Pollet et al., 2021). Recombinant proteins have been used in industrial processes, diagnosis of infectious diseases, and production of subunit vaccines. Depending on the requirement of the cells, proteins are expressed and regulated to meet these purposes. The machinery for protein synthesis is stored in DNA and decoded by highly regulated transcriptional processes to create messenger RNA (mRNA). The mRNA is then translated into a protein (Pollet et al., 2021). Expressing the target protein is essential in producing the antigen required to initiate an immune stimulatory reaction. The expressed proteins can stimulate a specific response against invading microorganisms, eventually resulting in the clearance and development of immunological memory (Fraga et al., 2011). Hence, *in-vitro* gene expression has been used to explore the potential of DNA-induced immune response (Moreno et al., 2004).

In the current study, the expression of multi-epitope plasmid DNA for the purpose of producing a multivalent leptospirosis vaccine was successfully demonstrated. The use of a mammalian expression system in this study is to permit post-translational modifications, which ensures the regulation of the activity of the polypeptides, their location, and the completeness of their structure which enhances their biological activity (Duan and Walther, 2015). The efficient expression of a gene generally depends on how well it is transcribed. Transcription usually occurs when the RNA polymerase complex interacts with the promoter moving in the 5' to 3' direction leading to the production of mRNA transcript that dissociates from the gene at the transcription signal for subsequent translation. The gene expression in eukaryotic cells requires a suitable cell line and appropriate vectors that will act as a vehicle to transport the gene into the required cell lines. The pBudCE4.1 plasmid used in this study is designed for the independent expression of two genes in a single plasmid. It also possesses the CMV promoter for high-level transcription of genes with c-myc and V5 epitope tags for rapid detection of expressed proteins as well as 6xHis sequence for easy purification. In addition, it also has the human EF-1α promoter for high-level expression of genes. The lack of cross-reactivity among pathogenic serovars of *Leptispira* causing human and animal leptospirosis has greatly hampered the successful clinical application of many experimental vaccines developed (Barazzone et al., 2022). The results of the present study show promise that this lack of cross-protective immunity can be overcome using recombinant polyvalent epitope-based vaccines, which have been previously reported by other authors (Lauretti-Ferreira et al., 2020; Teixeira et al., 2020).

CHO-K1 cells are among the most common cell line utilized for protein expression. Similarly, CHO-K1 cells have been used to express the *Leptospiral OmpL37* gene (Oliveira et al., 2015). From the immunofluorescence images, it is obvious that LigDNA03-pBudCE4.1, which is the smallest with 236bp was expressed better compared to CompDNA04-pBudCE4.1 which is the longest with 1.3kb size. However, the expression level was not quantified. Although the actual expression levels were not determined, the judgment made in this research was based on subjective visualization and the number of cells per microscope field. Proximity to neighboring genes and size have all been reported to play significant roles in gene expression, with smaller genes being highly expressed, compared to longer ones (Chiaromonte et al., 2003).

## CONCLUSION

In conclusion, this study was able to demonstrate the *in-vitro* expression of the synthetic genes and their reaction with antibodies against the *myc* and V5 epitope tags contained in the expression plasmid. This indicates the potential for these synthetic genes to serve as vaccine candidates for protection against multiple *Leptospira* infections in animals. It is, however, very important that further in-vivo evaluation be conducted using a suitable animal model to determine the efficacy and safety of the vaccine.

## DECLARATION

### Authors' contribution

Bashiru Garba conceptualized the idea and conducted the laboratory work. Both authors (Bashiru Garba and Najib Isse Dirie) contributed to the drafting, editing, and production of the final draft. All authors confirmed and consented to the final submission.

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### Competing interests

The authors declare that they have no competing interests.



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**Ethical considerations**

The authors ensure that all ethical issues concerning plagiarism, approval to publish, errors in fabrication, double publication, and submission are adhered to.

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# Effects of Tannin-containing Supplement on Enteric Methane Emissions, Total Digestible Nutrient, and Average Daily Gain of Local Indonesian Beef Cattle

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**ABSTRACT**

Reducing methane (CH4) emissions is one of the most critical goals in ruminant nutrition. This study aimed to evaluate the effect of concentrate and tannin supplementation on the mitigation of methane gas in Indonesian local beef cattle. The current study was conducted *in vivo* using 12 Bali cattle using a completely randomized design with four treatments and three replicates. Cattle were fed a basal ration with field grass (control), the addition of concentrate 25% dry matter (DM) ration no tannin as well as tannin supplemented in concentrate at levels of 0.12% and 0.18% of DM concentrate. The concentrate contains 7.5% crude protein and 71.25% total digestible nutrients and tannin supplementation using gambir (*Uncaria gambir* Indonesia) tannin extract. The parameters measured were apparent digestibility, total digestible nutrients, methane production, and average daily gain. The results showed that concentrate addition significantly increased DM consumption, crude protein digestibility, and total digestible nutrients. Supplementation of 0.18% tannin in concentrate can mitigate 49.7% methane gas production resulting in energy efficiency, which was reflected in the weight gain rate of 0.75 kg/day. In conclusion, present results suggest that the supplementation of 0.18% gambier tannin extract in concentrate could be considered a suitable feed additive to mitigate methane gas production and increase the average daily gain of Indonesian local beef cattle.

**Keywords:** Digestibility, Feed supplement, Gambir, Methane, Tannin

## INTRODUCTION

The population of beef cattle in Indonesia in 2021 was 18 million heads, increasing about 2.3% since 2016 (BPS, 2021). The increase in greenhouse gases in the atmosphere from components of carbon dioxide (CO2), methane (CH4), and nitrous oxide (N2O) is a challenge behind the increase in livestock population. Methane production in ruminants contributes 56% of total agricultural greenhouse gas emissions and 93% of total livestock emissions globally (Watts et al., 2021). In addition to impacting the environment as a greenhouse gas, methane emissions are a waste of energy that livestock should use for production. About 3-12% of the gross energy of feed consumed by ruminants is lost as methane (Ku-Vera et al., 2020). Strategies to reduce methane emissions are grouped into animal breeding, dietary manipulation, and rumen manipulation (Arndt et al., 2021). Meta-analysis showed that the addition of concentrate and tannin supplementation decreased CH4 Yield (g/kg DMI) by 13.7-17.4% and CH4/Gain by 20.1% (Congio et al., 2021). Thus, in the current study, the concentrate was added to tannin supplementation from gambir extract (*Uncaria gambir* Indonesia) to mitigate CH4 emission.

This study hypothesized that adding concentrate supplemented with tannins could mitigate CH4 emissions and increase the total digestible nutrients, thus reflecting an increase in average daily gain. Therefore, this study aimed to determine the effect of tannin concentrate and supplementation on enteric CH4 production, total digestible nutrient, and average daily gain in local Indonesian cattle.

## MATERIALS AND METHODS

### Ethical approval

This study was carried out according to standard protocols without causing discomfort or injury to the cattle. Furthermore, the experimental procedure was approved by the Center for Research and Community Service at Agricultural Polytechnic of Payakumbuh, referring to Government Regulation of the Republic of Indonesia No. 95 of 2012 concerning veterinary public health and animal welfare.



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**Animal and treatment diets**

The experiment was conducted in June-September 2021 at the Agricultural Polytechnic of Payakumbuh, Sumatera Barat, Indonesia. The climatic data referred to in AccuWeather (2021) recorded throughout the study is the end of summer and the beginning of the entry of the rainy season with a daily temperature range of 24-34 ºC. The study used 12 male Bali cattle (*Bos sondaicus*) of similar aged 1.5-1.8 years, with initial body weights of 237.31±4.85 kg. The cattle were placed in a metabolic cage measuring 1.8×1.5 m. Newly entered cattle were given the parasite drug Wormectin 1ml/ 50 kg of body weight (Medion, 2022). The animals and treatments were allocated randomly according to a completely randomized design with four treatments and three replications. The treatments were 100% basal ration (control), the addition concentrate of 25% dry matter (DM) ration without tannins (T0), and the addition of 0.12 (T2) and 0.18 (T3) % of DM concentrate gambir tannin extract. Tannin extract was obtained from the extraction of tannins from the gambir plant with water as a solvent in the gambier industry according to the method by Fauza (2014). Basal ration using mixed field grass from the genus Paspalum and the concentrate addition was composed of 30% bran, 20% coconut cake, 30% sago pith, and 20% cassava with a protein content of 7.5% and a total digestible nutrient (TDN) of 71.25%. Supplements containing tannins are made with a mixture of 15% brown sugar, 28% bran, 9% coconut cake, 15% tapioca, 20% soybean meal, 5% urea, 3% salt, 2.5% ultra-mineral, and 2.5% gambir tannin extract with a crude protein content of 23.69%, and TDN (78.55%). The chemical composition of the experimental feed is shown in Table 1.

**Table 1.** Nutritional content of treatment diets

| Chemical composition | Basal diet* (control) | Concentrate** Level tannin*** | | |
|---|---|---|---|---|
| | | T0 = 0.00 | T1 = 0.12 | T2 = 0.18 |
| Organic matter (%) | 90.33 | 91.27 | 90.52 | 90.14 |
| Crude protein (%) | 5.14 | 6.04 | 7.65 | 8.46 |
| Neutral detergent fiber (%) | 58.66 | 52.03 | 51.09 | 50.62 |
| Acid detergent fiber (%) | 37.85 | 33.13 | 32.27 | 31.84 |

*Basal diet: mixed field grass of the Paspalum genus; **add concentrate 25% of dry matter ration; ***Level tannin (percentage of dry matter concentrate)

**Sample collection and analysis**

The experimental period was 40 days, 26 days for the digestibility trial, and 14 days for the daily gain measurement. The digestibility experiment was started with a 10 days preliminary period; the cows were given 100% basal feed to eliminate the effect of the last feed. The next 10 days were the adaptation period to the treatment diet and 6 days to collect digestibility data. The number of rations offered and rejected was weighed to determine the total daily dry matter intake. The excreted feces are collected in a bag tied to the cattle during the collecting period. Then the samples of feed and feces (100 g as fed) were stored in the freezer (-20°C) to be analyzed for their nutritional content. The body weight of cattle was measured at the end of the digestibility data collection as the initial weight and 14 days later as the final weight. The weighing was carried out in the late morning, 16 hours after the previous day's ration was given. After completing the research, the feed and feces samples were dried in an oven at 60°C, then mashed using a 0.5 mm sieve. The concentration of dry matter, organic matter, and crude protein were determined by the method of AOAC (2005), while the fiber fraction was analyzed by the method of Van Soest et al. (1991). Methane gas production was predicted by performing the following Formula 1 according to Jentsch et al. (2007) and Ningrat et al. (2018).

Methane (MJ/day = 1.62(DCP) – 0.38(DF) + 3.78(DCF) + 1.49(DN-fe) + 1142 (Formula 1)

Where, DCP is a digestible crude protein (g), DF denotes digestible fat (g), DCF signifies digestible crude fiber (g), and DN-fe refers to digestible N-free extract (g)

**Data analysis**

The resulting data were tabulated and statistically processed using the statistical package for the social sciences (SPSS, Chicago, USA) was used for analyzing the data, and the one-way ANOVA test was chosen in this software. Duncan's test was chosen to determine the mean significant differences between treatments. The $p < 0.05$ was considered a significant difference between the groups.

## RESULTS AND DISCUSSION

The effects of supplementation of 25% concentrate (DM diet) and different levels of tannin supplementation on consumption, digestibility, methane production, and weight gain in Bali cattle are shown in Table 2. As can be seen in Table 2, the addition of concentrate can increase ($p < 0.05$) dry matter consumption, while tannin supplementation does not affect consumption ($p > 0.05$). The addition of concentrate caused a 6.36% reduction in neutral detergent fiber (NDF) in the ration (Control vs. T0-T3, Table 1). The decrease in NDF allows the ration to be digested more quickly in the



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rumen and accelerates the rate of rumen emptying. The NDF content can reduce dry matter intake (DMI). Souza et al. (2017) reported a negative correlation between forage NDF and DMI. Condensed tannins from various plant sources at moderate levels (1-4%) did not have a significant effect on feed intake (Bunglavan and Duta, 2013).

Regression analysis showed that organic matter digestibility and NDF digestibility had a close relationship with DM consumption ($p < 0.05$). This relationship showed that dry matter consumption is influenced by the digestibility of organic matter and NDF. Organic matter and NDF are the main components of rations in local beef cattle with 100% forage on traditional farms in Indonesia. Organic matter is all organic components of dietary protein, carbohydrates, fats, and vitamins, and NDF is a component of cell walls consisting of cellulose, hemicellulose, and lignin (Lardy, 2018).

**Table 2.** Dry matter intake, apparent digestibility, methane production, and weight gain measured in Bali beef cattle aged 1.5 years after supplementation of diet with different levels of tannin

| Items | Basal diet | Concentrate Level tannin ** | | | SEM | p value |
|---|---|---|---|---|---|---|
| | | 0.0 | 0.12 | 0.18 | | |
| **Dry matter intake** | | | | | | |
| Percentage of BW | 2.17[b] | 2.66[a] | 2.77[a] | 2.70[a] | 0.04 | <0.01 |
| Metabolic body weight (g/kg) | 74.20[b] | 88.35[a] | 93.49[a] | 90.91[a] | 0.09 | <0.01 |
| **Apparent digestibility (%)** | | | | | | |
| DM | 73.74 | 71.47 | 71.59 | 73.02 | 0.51 | 0.06 |
| Organic matter | 77.13 | 75.12 | 75.01 | 76.35 | 0.45 | 0.05 |
| Crude protein | 35.97[b] | 56.30[a] | 55.53[a] | 57.31[a] | 3.00 | 0.01 |
| Crude fiber | 77.36 | 76.28 | 68.89 | 78.29 | 2.03 | 0.05 |
| NDF | 66.97 | 66.59 | 67.08 | 67.09 | 2.83 | 0.36 |
| ADF | 67.55 | 60.94 | 60.17 | 64.38 | 1.84 | 0.05 |
| Cellulose | 73.13 | 69.37 | 67.20 | 68.09 | 1.82 | 0.08 |
| **Total digestible nutrients** | | | | | | |
| BW (g/kg) | 15.45[b] | 19.18[a] | 19.19[a] | 19.06[a] | 0.56 | 0.01 |
| Percentage of dry matter intake | 67.89[b] | 72.77[a] | 72.02[a] | 75.35[a] | 0.01 | 0.04 |
| **Methane production (MJ/day)*** | 10.06[a] | 10.40[a] | 9.79[a] | 8.41[b] | 0.15 | <0.01 |
| **ADG (kg/d)** | 0.37[c] | 0.47[bc] | 0.59[b] | 0.75[a] | 0.04 | 0.013 |

[abc]mean data with different lowercase letters on the same row show significantly different ($p < 0.05$). SEM: standard error mean, BW: body weight, DM: Dry matter, BW: Body weight, NDF: Neutral detergent fiber, ADF: Acid detergent fiber, MJ: Megajoule, TDN: Total digestible nutrient ADG: Average daily gain. *Methane is predicted using the method of Jentsch et al. (2007); **Percentage of DM concentrate

The addition of concentrate and supplementation of tannins had no effect ($p > 0.05$) on apparent digestibility except for crude protein. The digestibility of crude protein increased with tannin supplementations ($p < 0.05$). In the current study, protein digestibility increased due to an increase in the protein level of the diet after the addition of concentrates and tannin supplementation. Basal diets using tropical forages had a crude protein content of 5.14%, a value considered limiting adequate rumen microbial activity, implying sub-optimal conditions in the rumen (Sampaio et al., 2010). Rira et al. (2022) reported that condensed tannins did not affect feed degradability but interfered with microbial colonization; tannin-rich feeds had a relatively lower abundance of fibrinolytic microbes, especially *Fibrobacter* spp. Furthermore, it is supported by the report of Souza et al. (2010) that the protein content below 7 g/kg DM in the basal ration limits the degradation of rumen microorganisms. The same finding was also reported by van Kuijk et al. (2022).

Tannin compounds can bind strongly to proteins (Le Bourvellec and Renard, 2018) which causes a decrease in crude protein digestibility in the rumen. According to Ramaiyulis (2021), diet containing 0.12% tannin can reduce protein degradation in the rumen from 1.17 to 0.99% per hour *in vitro*. However, in the present study, the opposite results were obtained due to the *in vivo* nature of the study. The tannin-protein complexes formed in the rumen (pH 3.5-7) make the protein resistant to rumen microbial degradation. However, these complexes should dissociate in the pH environment of the duodenum and proximal abomasum (Getachew et al., 2006). Some studies also indicated the effect of tannins in reducing crude protein degradation in the rumen. However, it did not affect total post-rumen digestibility (Jolazadeh et al., 2015; Canadianti et al., 2020).

Total digestible nutrients showed an increase ($p < 0.05$) due to the addition of concentrate and were not affected by tannin supplementation ($p > 0.05$). The addition of concentrate plays a role in supplying additional nutrients because the basal feed is classified as low-quality grass (basal feed with crude protein < 8% and NDF > 50%). Rufino et al. (2016) stated that low-quality basal feed requires crude protein supplementation to meet the needs of rumen microbes to develop. The concentrates contain essential nutrients for rumen microbial growth, such as soluble carbohydrates, crude protein sources from urea (non-protein nitrogen), and macro and micro minerals. The fulfillment of rumen microbial nutrition can increase microbial activity to digest feed in the rumen (Ramaiyulis et al., 2019).

Table 2 shows that methane production per head of livestock ranged from 8.41 to 10.40 (MJ/d). Methane production is suppressed with 0.18% tannin supplementation ($p < 0.05$). Previous studies also found a 44-49% decrease in the proportion of methane with the addition of supplements containing tannins in forage or fermented straw basal feed



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(Liu et al., 2019; Ramaiyulis et al., 2021). A dairy cattle diet containing condensed and hydrolyzed tannin compounds can be considered a sustainable approach to reducing the environmental impact of rumination because the feed containing tannins can reduce enteric CH4 emissions (Stewart et al., 2019).

The ratio of Methane/TDN decreased simultaneously with the addition of supplements 0.12 and 0.18 % tannins ($p < 0.05$). Mitigation of methane gas is energy efficient, considering that 5-15% of energy is lost in the form of methane gas during the rumen fermentation process (Wanapat and Kang, 2015). This efficiency was reflected in the increase of body weight gain in beef cattle from 0.37kg/d (control) to 0.75kg/d with the addition of concentrate and 0.18% tannin supplementation.

## CONCLUSION

The supplemented concentrate with gambir extract tannins can mitigate CH4 emissions and increase total digestible nutrients. The highest CH4 mitigation (49.7%) occurred in the basal diets of field grass with the addition of concentrate (75:25% DM) and 0.18% tannin supplementation. The highest CH4 mitigation can lead to the optimal weight gain of 0.75 kg/d in local Indonesian cattle.

## DECLARATIONS


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### Authors' contribution

Ramaiyulis supervised the experiment and wrote the original manuscript. Devi Kumala Sari, Dihan Kurnia, Debby Syukriani, and Irzal Irda conducted the experiment and data analysis. Yurma Metri prepared tables and finalized draft. All authors have read and agreed to the data analysis and the final version of the manuscript.

### Competing interests

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

### Ethical considerations

The authors carefully examined all ethical issues concerning plagiarism, approval to publish, errors in fabrication, double publication, and submission.

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# Multiple Drug Resistance *Salmonella* and Antibiotic Residues in Egyptian Animal Products

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**ABSTRACT**

Food of animal origin is considered a primary source of foodborne diseases. The misuse of antibiotics to treat and control many bacterial diseases in farm animals has led to multiple antibiotic-resistant pathogens in contaminated food that can seriously threaten public health. The present study aimed to highlight the impact of antimicrobial misuse in Egyptian beef meat, poultry, and dairy farms on the emergence of multiple antibiotic resistance *Salmonella* and the detection of antibiotic residues in milk. A total of 1050 samples were collected randomly from poultry (liver, intestinal content, and bone marrow), meat, and milk products from different Egyptian governorates. *Salmonellae* were isolated from the collected samples and subjected to antimicrobial sensitivity testing through disk diffusion test using the most commonly used seven antibiotics in veterinary fields (cefradine, ciprofloxacin, oxytetracycline, erythromycin, amoxicillin, ampicillin, and streptomycin). The detection of oxytetracycline residue in milk samples was performed by high-performance liquid chromatography (HPLC). Most isolated *Salmonella*e were multiple drug resistant with an incidence rate of 8.6%, 15.4%, and 4% from poultry, meat-associated products, and milk-associated products, respectively, from different governorates. Antibiogram test showed that the isolated *Salmonella* from poultry, meat, and milk samples were resistant to oxytetracycline at 100%, 31.4%, and 43%, to amoxicillin at 73.3%, 31%, and 50%, and to ampicillin 66.6%, 50%, and 57%, respectively. No resistance to ciprofloxacin was detected in *Salmonella* isolates from all samples. Using HPLC, oxytetracycline residues were detected in milk samples. In conclusion, more attention should be paid to the connection between the widespread emergence of antibiotic-resistant *Salmonella* in Egypt and the overuse of antimicrobials in poultry, dairy, and meat farms. This connection affects consumer health and increases the likelihood of resistance genes spreading between different bacterial species.

**Keywords:** Antibiogram, High-performance liquid chromatography, Multiple drug resistance, *Salmonella*

## INTRODUCTION

Food poisoning caused by bacterial infection is a serious public health hazard worldwide, and most countries invest significant resources to combat it. *Salmonella* is one of the most prevalent food poisoning causes in Europe (Callejón et al., 2015; Myintzaw et al., 2020), leading to 91,857 human illnesses in Europe in 2018 (ECDC, 2020).

Salmonellosis is usually associated with consuming food products contaminated with *Salmonella*, particularly poultry, meat, and egg products. Contamination can occur through various means, including poor hand washing or contact with infected pets (Munck et al., 2020). Food handlers of "meat processing" and "ready-to-eat" foods are critical in the spread of *Salmonella* (Ehuwa et al., 2021). The marketing of improper food items and products produced under poor quality measures are considered the primary sources of the disease spread (FAO/WHO, 2004; Bettridge et al., 2014).

Multidrug resistance refers to bacteria's ability to withstand several types of antibiotics (three or more classes) that are structurally distinct and target different microorganisms (Nikaido, 2009). Antibiotic resistance (AR) is usually caused by antibiotic overuse, through which more bacterial species may acquire AR due to increased antibiotic use (Gelband et al., 2015). Misuse of antibiotics can result in bacterial resistance, increasing the burden of infectious diseases and healthcare costs (Nhung et al., 2017). Direct interaction with animals, exposure to animal waste, ingestion of raw meat, and contact with meat surfaces are common ways of transmitting resistant bacteria to humans (Marshall and Levy, 2011).

Farmers have resorted to the indiscriminate use of antibiotics as prophylactic and growth enhancement to fulfill the rising demand for poultry meat and eggs, which are the most important source of animal protein (Adesokan et al., 2015). This has resulted in antimicrobial-resistant strains of various pathogens, including *Salmonella* (Musawa et al., 2021). Different food animal species received antibacterial drugs, mostly in poultry. Animal production methods in Africa employ many antibiotics, including tetracycline, aminoglycosides, and penicillin. Therefore, the existing high levels of MDR and AR on the continent are worsened (Kimera et al., 2020).



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Antimicrobial tolerance is a growing concern in animal and human Salmonellosis (Su et al., 2004). *Salmonella* strains resistant to antibiotics are common in most parts of the world and have increased sharply in the last decade (WHO, 2018). Transmission from animal to human is rapidly becoming more frequent. The issue of antimicrobial drug resistance is more problematic in developing countries (Shrestha et al., 2017). Multidrug resistance is most commonly seen in *Salmonella* strains obtained from food (Gargano et al., 2021), especially poultry and poultry products (Ehuwa et al., 2021; Raji et al., 2021). The ability of these bacteria to transfer their resistance genes to a human bacterial pathogen is one of the significant concerns (Musawa et al., 2021).

Alarming multidrug resistance of the *Salmonella* serovars isolated from chicken embryos in Henan province, China, calls for an immediate reduction in the usage of antimicrobial medicines in chicken hatcheries. Additionally, various patterns among the Salmonella serovars by pulsed-field gel electrophoresis indicate the presence of several contamination sources (Xu et al., 2021).

Allergies, sensitization, and development of MDR bacteria are the most common human risks occurred by eating foods of animal origin containing antibiotic residues (Donoghue, 2003). Antibiotic residues can cause various severe health problems, including antimicrobial tolerance, immunopathological effects, autoimmunity, carcinogenicity, mutagenicity, nephropathy, hepatotoxicity, reproductive abnormalities, bone marrow toxicity, or allergy (Nisha, 2008). It is critical in food safety programs to monitor veterinary drug residues in raw animal products, such as milk, eggs, and meat, to ensure public health preventative measures (Botsoglou and Fletouris, 2001; FAO, 2015).

High-performance liquid chromatography (HPLC) is one of the most effective analytical chemical instruments, capable of distinguishing, recognizing, and quantifying antibiotics found in food, its use in the field of residual analysis is growing by the day due to the diversity of mobile stages, the availability of a wide range of column packings, and the variety of operating modes (Kebede et al., 2014). Several studies using HPLC technology for the characterization and identification of veterinary pharmaceuticals in premixes and medicated feeds are documented (Krasucka et al., 2010; Han et al., 2020). The present study aimed to detect MDR *Salmonella* from foods of animal origin as well as the detection of antibiotic residues in milk samples.

## MATERIALS AND METHODS

### Ethical approval

The current study was conducted on animal specimens, and no invasive procedures were performed on animals. This study did not involve any *in vivo* experiments. Poultry, milk and milk products, meat and meat by product samples were collected from the market for microbiological studies

### Samples collection

A total number of 1050 poultry, milk and milk products, meat, and meat by-product samples were collected randomly from seven Egyptian governorate markets, including Giza, Cairo, Bani Suef, Fayoum, Alexandria, Menofia, and Qalyubia. These samples were divided as shown in Table 1.

**Table 1.** Source, type, and number of poultry, meat, and milk samples obtained from Egyptian markets

| Source of samples | Type of samples | Number of examined samples |
|---|---|---|
| Poultry | Liver, intestinal content, and bone marrow | 350 |
| Meat and meat by-products | Beef meat, minced meat and liver, luncheon, sausage, hotdog, and kofta | 350 |
| Milk and milk by-products | Raw cow's milk, yogurt, and Kareesh cheese | 350 |
| **Total** | -- | **1050** |

### Isolation and identification of *Salmonella*

All samples were collected aseptically and placed in separate sterile plastic bags, and transferred to the laboratory as soon as possible under hygienic conditions in an ice box. The samples were screened for isolation of *Salmonella* spp. The incubated samples were streaked over the surface of the SS agar medium (Oxoid) and incubated for 24 hours at 37°C before being analyzed for colony characteristics and cellular morphology. All bacterial isolates were identified by traditional methods, including morphological, biochemical, and colonial characters, according to Cruickshank et al. (1973) and Quinn et al. (2011), and confirmed by a test kit (BioMérieux, France).



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**Antibiogram assay**

The antimicrobial sensitivity tests were performed on confirmed *salmonella* strains using the disk diffusion technique according to Fine gold and Martin (1982) and the clinical laboratory standards institute (CLSI, 2020). Antibiotics used in the study included cefradine, ciprofloxacin, oxytetracycline, erythromycin, amoxicillin, ampicillin, and streptomycin. All antibiotic disks were procured from HI media laboratories (Mumbai, India).

**High-performance liquid chromatography**

Seven raw cow milk samples (15 ml) were randomly collected for testing oxytetracycline residues using the HPLC technique (Abbasi et al., 2011).

**Analysis of analytical standards of oxytetracycline in milk samples**

***Solvents, reagents, and certified standards***

Analytical standards of oxytetracycline, HPLC grade acetonitrile, methanol, and oxalic acid were obtained (Sigma Aldrich Company, USA), and the solid phase extraction (SPE) column (Bond Elut C18, 500 mg, 6 ml, Varian) were used for detection of oxytetracycline in milk. The water used for HPLC analysis was purified through Milli-Q water generated by a Milli-Q Plus Water Purification System (Millipore, USA).

***Extraction of oxytetracycline from milk samples***

Milk samples were prepared according to the methods by Abbasi et al. (2011). In brief, in a 50 ml plastic centrifuge bottle, a 15 ml milk sample was homogenized and combined with 25 ml Mcllvaine Buffer (Mixed citrate/phosphate, pH 4.1 with EDTA). The solution was vortexed for 1 minute before being centrifuged (Germany) at 10000 × g for 12 minutes at 4°C. The floating lipid layer and precipitate were removed, and the residual supernatant was extracted using SPE cartridges. The SPE cartridge was conditioned with 3 ml of methanol at a flow rate of 3 ml/minute before being washed with 2 ml of deionized water. At a flow rate of 5 ml/minute, the prepared mixture (centrifuged sample solution) was put into the SPE cartridge. The cartridge was rinsed with 1.5 milliliters of 5% methanol in deionized water. Elution was carried out at a rate of 4 ml/minute with 2 ml of HPLC-grade methanol. Drying samples by lyophilizing and reconstituting with 1 ml mobile phase, and 50 µl of the sample was injected into the HPLC column.

***Chromatographic condition***

High-performance liquid chromatography Agilent Series 1200 quaternary gradient pump, Series 1200 autosampler, and Series 1200 fluorescence detector with excitation and emission wavelengths were 255 and 365 nm, respectively. The HPLC software used was HPLC 2D Chemstation software (Hewlett-Packard, Les Ulis, France). The analytical column (stationary phase) was a reversed-phase C18 (25×04.6 mm, 5 µm) Teknorama (Spain). The mobile phase was a mixture of methanol, acetonitrile, and 50 mM oxalic acid (10: 20: 70% V/V). The mobile phase was mixed and filtered through a 0.45 µ filter (Nalgene, USA) and sonicated for 5 minutes to degas. The flow rate was 0.8 mL/minute. The retention time was 7.8 minutes.

**Statistical analysis**

The data were analyzed using IBM SPSS 25 program. The independent variables were tested for significance using the chi-square test, and the variables were found to be significant at $p < 0.05$.

## RESULTS

**Isolation and identification of *Salmonella* isolates**

The suspected isolates were motile, Gram-negative, non-sporulated, and bacilli. It was a non-lactose fermenter on MacConkey agar medium while appeared as a colorless colony with a black center on SS agar media, and red colonies with black center colonies on Xylose lysine deoxycholate (XLD) agar medium. The suspected isolates were unable to ferment lactose, oxidase, indole production, Voges Proskauer, and urea hydrolysis were negative. Meanwhile, catalase and methyl red tests were positive and confirmed using a test kit (BioMérieux, France). Regarding the incidence rate of *Salmonella* spp. from different animal sources, the recovery rate of *Salmonella* spp. from poultry, meat and meat products, and milk and milk products were 8.6%, 15.4%, and 4%, respectively, from different governorates (Table 2).

**Antibiotic sensitivity test of *Salmonella* isolated from different sources**

The results of the antibiotic sensitivity test for *Salmonella* isolated from different sources are shown in Table 3 and Graph 1. *Salmonella* isolates from poultry samples revealed a high incidence of multiple AR. The highest resistance was found against oxytetracycline at 100% followed by amoxicillin at 73.3% and ampicillin at 66.6%. *Salmonella* isolates from meat and meat by-products showed a high incidence of multiple AR. The highest resistance was against cefradine



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at 53.7%, followed by ampicillin at 50%, then oxytetracycline and amoxicillin at 31.4%. Nearly the same results of the high incidence of MDR *Salmonella* were obtained from *Salmonella* isolates recovered from milk and milk by-products. The highest resistance incidence was found against erythromycin at 100%, followed by ampicillin at 57%, then cefradine and amoxicillin at 50%. All observed Chi-square values were higher than the expected value of the Chi-square test at 12 degrees of freedom. Thus, there was a strong relationship between the antibiotic and sensitivity.

**Quantification of oxytetracycline concentration in milk samples**

As shown in Table 5, the calibration curves of peak area relative to oxytetracycline concentration were plotted using data from 7 concentrations (0.05-5 g/ml milk). Using this technique, the standard curve was shown to be linear (R2 > 0.9995). The level of detection and limit of quantification for oxytetracycline were 0.0167 and 0.05 g/ml, respectively. Figures 1, 2, 3, and Table 4 provide the HPLC chromatogram for validation and standardization of oxytetracycline residues, the standard, blank milk sample, and positive sample with the standard, respectively.

**Table 2.** Incidence of *Salmonella* from different sources

| Governorate | Poultry | | | Beef meat and meat by-products | | | Cow's milk and milk by-products | | |
|---|---|---|---|---|---|---|---|---|---|
| | Percentage | +ve | Total | Percentage | +ve | Total | Percentage | +ve | Total |
| Cairo | 50 | 5 | 10 | 50 | 8 | 16 | 50 | 1 | 2 |
| Giza | 50 | 4 | 8 | 50 | 6 | 12 | 50 | 2 | 4 |
| Fayoum | 50 | 6 | 12 | 50 | 9 | 18 | 50 | 2 | 4 |
| Bany suif | 50 | 3 | 6 | 50 | 7 | 14 | 50 | 2 | 4 |
| Menofia | 50 | 6 | 12 | 50 | 8 | 16 | 50 | 3 | 6 |
| Alexandria | 50 | 2 | 4 | 50 | 5 | 10 | 50 | 1 | 2 |
| Qalyubia | 50 | 4 | 8 | 50 | 11 | 22 | 50 | 3 | 6 |
| Total | 350 | 30 | 8.6 | 350 | 54 | 15.4 | 350 | 14 | 4 |
| p value | 0.753 | | | 0.731 | | | 0.911 | | |
| Test statistics | 3.427 | | | 3.591 | | | 2.083 | | |

Milk and meat were obtained from Cow, +ve: Positive for *Salmonella* isolation

**Table 3.** Antibiotic sensitivity test of *Salmonella* isolated from different sources of veterinary products in Egypt

| Antibiotics | Poultry (30 isolates) | | | | | | Meat and meat by-products (54 isolates) | | | | | | Milk and milk by-product (14 isolates) | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Resistance | | Sensitive | | Intermediate | | Resistance | | Sensitive | | Intermediate | | Resistance | | Sensitive | | Intermediate | |
| | No. | % | No. | % | No. | % | No. | % | No. | % | No. | % | No. | % | No. | % | No. | % |
| Cefradine | 18 | 60 | 10 | 33.3 | 2 | 6 | 29 | 53.7 | 25 | 46.3 | 0 | 0 | 7 | 50 | 5 | 35.7 | 2 | 14 |
| Ciprofloxacin | 0 | 0 | 18 | 60 | 12 | 40 | 0 | 0 | 54 | 100 | 0 | 0 | 0 | 0 | 13 | 93 | 1 | 7 |
| Oxytetracycline | 30 | 100 | 0 | 0 | 0 | 0 | 17 | 31.4 | 33 | 61.1 | 4 | 7.4 | 6 | 43 | 7 | 50 | 1 | 7 |
| Erythromycin | 19 | 63.3 | 2 | 6 | 9 | 30 | 13 | 24 | 4 | 7.4 | 37 | 68.5 | 14 | 100 | 0 | 0 | 0 | 0 |
| Amoxicillin | 22 | 73.3 | 3 | 10 | 5 | 16.6 | 17 | 31.4 | 30 | 55 | 7 | 12.9 | 7 | 50 | 7 | 50 | 0 | 0 |
| Ampicillin | 20 | 66.6 | 8 | 26.6 | 2 | 6.6 | 27 | 50 | 25 | 46.3 | 2 | 3.7 | 8 | 57 | 4 | 28.5 | 2 | 14 |
| Streptomycin | 3 | 10 | 17 | 56.6 | 10 | 33.3 | 7 | 12.9 | 33 | 61 | 14 | 25.9 | 6 | 42.8 | 7 | 50 | 1 | 7 |
| p value | 49.04 | | | | | | 200.4 | | | | | | 101.26 | | | | | |

No: Number; %: percent

**Table 4.** The concentrations of Oxytetracycline concentration spiking in blank milk (µg/ml) and their corresponding peak response automatically using by high-performance liquid chromatography with recovery

| Level | Amount of oxytetracycline in standard (µg/ml) | Area under peak | Concentration (µg/ml) | Recovery (%) |
|---|---|---|---|---|
| 1 | 0.05 | 7.2 | 0.055981 | 111.96 |
| 2 | 0.1 | 15.75 | 0.117272 | 117.27 |
| 3 | 0.25 | 35.48 | 0.258705 | 103.48 |
| 4 | 0.5 | 73.179 | 0.528949 | 105.78 |
| 5 | 1 | 135.62 | 0.976555 | 97.65 |
| 6 | 2.5 | 337.45 | 2.423365 | 96.93459498 |
| 7 | 5 | 702.4 | 5.039494 | 100.7898781 |



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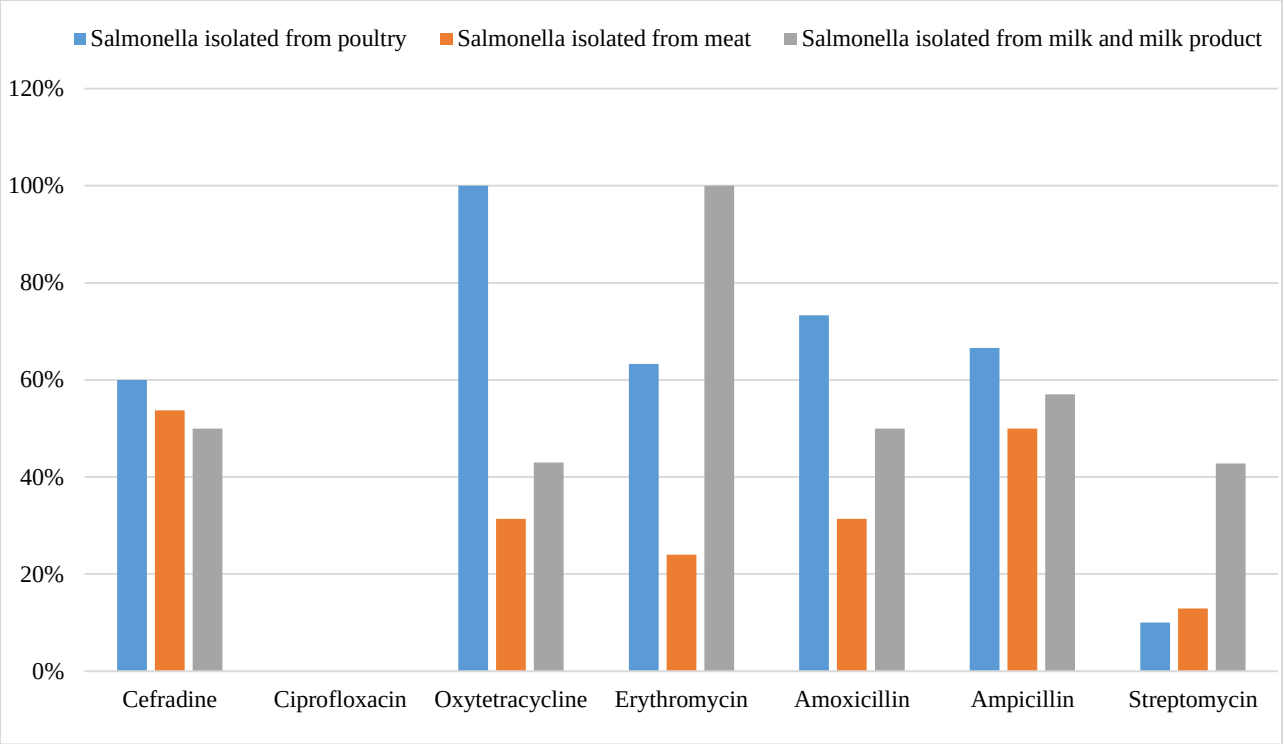


**Graph 1.** Antibiotic resistance of *Salmonella* isolated from different types of veterinary products in Egypt

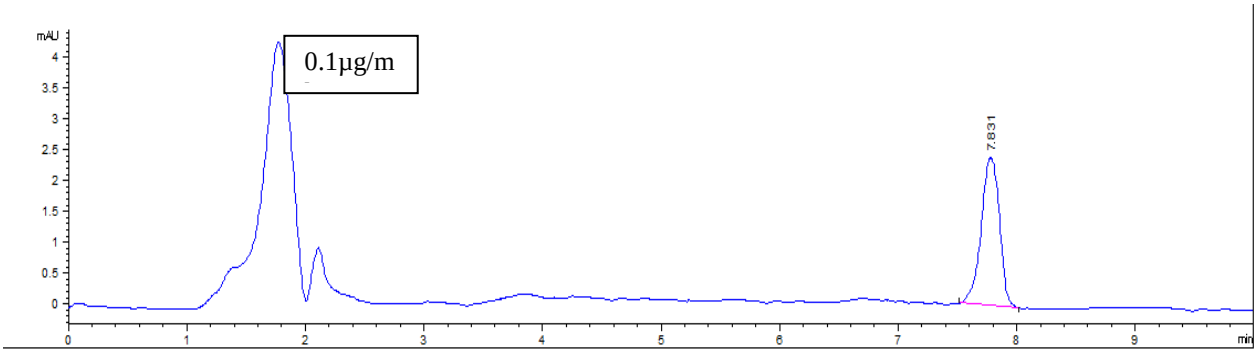


**Figure 1.** Cow milk from local Egyptian markets with oxytetracycline at a concentration of 0.1 µg/ml

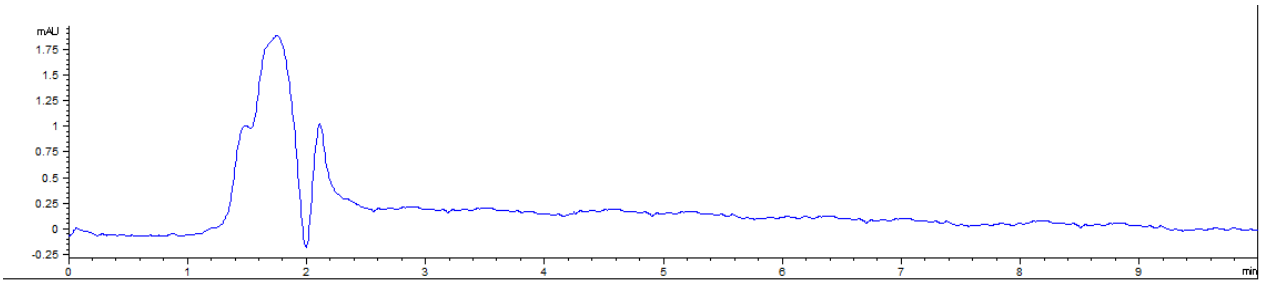


**Figure 2.** Blank cow milk sample from local Egyptian markets

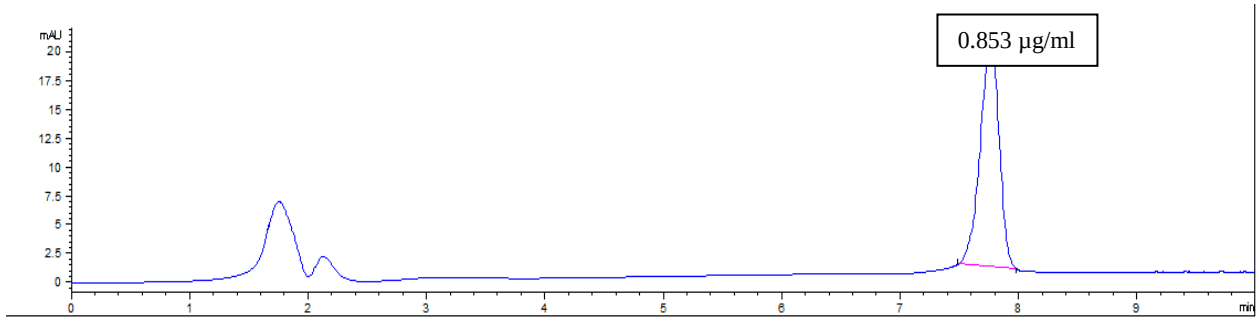


**Figure 3.** Cow milk sample from local Egyptian markets at a concentration of 0.853 µg/ml of oxytetracycline



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## DISCUSSION

The use of antimicrobials in animals, particularly food-producing animals, has serious implications for both human and animal health since it can lead to bacterial resistance. The antibiotic-resistant bacteria (with resistance genes) developed in animals can be transmitted to humans by food intake, direct contact with food-producing animals, or environmental dissemination. Therefore, this section discusses the effect of antimicrobial use in Egyptian poultry, dairy and meat farms on the emergence of multiple AR *Salmonella*, and the detection of antibiotic residue in milk.

The results of the present study showed that the recovery rate of *Salmonella* spp. from poultry was 8.6% in different Egyptian governorates (Table 2). Raji et al. (2021) recorded nearly the same recovery rate as an overall *Salmonella* prevalence rate. However, higher results were reported by Endris et al. (2013), as the cultural prevalence of Salmonella among seropositive chicken was 35.7%. Fajilade et al. (2021) found that the incidence rate of *Salmonell*a in chickens was up to 100% in Ado-Ekiti, South Western Nigeria. In addition, the prevalence of Salmonellosis was 16.1% in poultry farms of Hawassa, Ethiopia (Endris et al., 2013), and it was 14.6%, as reported in Ethiopia by Ali et al. (2020). Ibrahim et al. (2014) indicated that the incidence of *Salmonellae* among local chicks was 21.67%, compared to 11.67% among imported chicks. Snow et al. (2007) found a rate of 10.7% for *Salmonella* recovered from poultry in the United Kingdom, and Ibrahim et al. (2013) isolated *Salmonell*a from broiler chickens at a rate of 16.66% in Beni-Suef governorate, Egypt.

The present results showed that the *Salmonella* incidence rate in meat and meat products was 15.4%. This result nearly agreed with Barrel (1982), who found a *salmonella* incidence rate of 17.6% for sausage, and Mrema et al. (2006), who reported a *Salmonella* prevalence rate of 20% for meat. In contrast to Abu Elnaga et al. (2021), who found that zero recovery rate of *Salmonellae* from raw meat of cows, goats, and sheep gathered from various retail marketplaces in Egypt. While a lower incidence rate was recorded by Abd El-Tawab et al. (2015), the obtained results indicated that the incidence of *Salmonell*a *enteritidis* in the examined samples of minced meat, sausage, and beef burger were 1/70 (1.4%), 1/40 (2.5%), and 0/40 (0%), respectively. Also, a high incidence rate of *Salmonell*a was reported by Fajilade et al. (2021) in Ado-Ekiti, South Western Nigeria, indicating recovered *Salmonella* from pork meat (100%), meat pie (71%), and Gala sausage (14%). Malkawi (2003) found a significant *Salmonella* prevalence of 81% for the studied minced meat.

The present results revealed that the recovery rate of *Salmonella* from milk and milk by-products was 4%. In the same line, Kunadu et al. (2018) found that MDR *Salmonella enterica* serovars Muenster and Legon were recovered as 11.8% and 5.9%, respectively, from unfermented cheese samples. While a higher prevalence was observed by Yasmin et al. (2015), their inspection divulged the presence of microorganisms with the harmful multidrug-resistant *Salmonella* spp. in 9 out of 35 samples from milk and the milk-based product collected from Dhaka metropolis, Bangladesh.

Elafify et al. (2019) found nine *Salmonella* isolates were recovered from raw milk (4/9, 44.4%) and Kariesh cheese (5/9, 55.5%), respectively. The antibiotic sensitivity testing showed that all isolates were resistant to Erythromycin and Streptomycin. Elafify et al. (2022) found that the highest *Salmonella* incidence rate was recovered from Kariesh cheese (16.67%), followed by market raw milk (6.66%), and soft white cheese (3.33%). In addition, Fajilade et al. (2021) observed a high prevalence of Salmonella in yogurt from Ado-Ekiti, South Western Nigeria (92%). Mhone et al. (2012) reported negative isolation of *Salmonella* spp. from processed and raw milk samples.

The cause of positive *Salmonella* isolation in food includes contamination due to infected people, environmental contamination (soil, vegetation, water) as well as animal food products, such as meat meal, bone meal eggs, or fish (Kariuki et al., 2006; Corry et al., 2002). Milk contamination may occur due to the fecal material of subclinically infected cows (Radke et al., 2002). *Salmonella* in milk poses a lower risk to public health through proper hygienic measures and pasteurization (Bankole et al., 2011).

This discrepancy in results might be attributed to differences in sample procedures, geographic location, and technique used. In Egypt, the most common serotype varies depending on where you live. This might be caused to contamination during the manufacturing, handling, packing, and storage of the product (Rabie et al., 2012).

Results of antibiotic sensitivity test of *Salmonella* isolated from different sources, *Salmonella* from poultry sources revealed a high incidence of multiple AR, the highest incidence of *Salmonella* resistance was against oxytetracycline 100% followed by amoxicillin 73.3% then ampicillin 66.6%, MDR isolates from poultry were found by Shrestha et al. (2017). Agada et al. (2014) announced that *Salmonella* recovered from poultry in Jos, Nigeria, was resistant to oxytetracycline (63%), ceftazidime (84%), and ampicillin (96%). The current study showed resistance to amoxicillin (73.3%), while that of Raj et al. (2021) was (100%).

While no ciprofloxacin resistance was detected, Raj et al. (2021) found ciprofloxacin resistance to *salmonella* isolated was 100% from the intestinal contents of slaughtered chickens and ready-to-eat chicken gizzards in Ilorin, Kwara State, Nigeria. Agada et al. (2014) reported that *Salmonella* isolated from poultry (poultry droppings, feeds, feces, and hand swabs from poultry farm workers and swabs from surfaces of intact eggshells) showed sensitivity to ciprofloxacin (81.6%) in Jos, Nigeria. However, Fashae et al. (2010) recorded a 3% resistance against ciprofloxacin in Ibadan, Nigeria. The isolated strains of the present study showed resistance to streptomycin (10%) in poultry samples,



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while Adesiji et al. (2011) revealed that the examined *Salmonella* isolates from pork meat samples were sensitive to ciprofloxacin and tetracycline by 100% but all were resistant to amoxicillin.

Mion et al. (2016) reported the sensitivity of *Salmonella* isolates isolated from poultry processing plants against ciprofloxacin by 94% and ampicillin by 77%, respectively. The current study showed resistance against ampicillin and erythromycin in 66.6% and 63.6%, respectively, in contrast to 100% resistivity of ampicillin and erythromycin in Dorgham et al. (2019). Ogu et al. (2021) found that *Salmonella* isolates from raw chicken meat were sensitive against gentamycin (40.39%) and resistant against ampicillin (96.15%). The multidrug-resistant *Salmonella* strains found in the study by Xu et al. (2020) in Henan, China, could pose a major threat to human and animal health. Thus, in order to prevent the spread of resistance to current antimicrobial agents, it is essential to monitor, regulate, and optimize the use of antimicrobial agents in chicken farms.

The results of antibiotic sensitivity of bacterial isolates from meat and meat by-products revealed a high incidence of multiple AR found between *Salmonella* isolates. The highest incidence of *Salmonella* resistance was against cefradine at 53.7%, followed by ampicillin at 50%, then oxytetracycline and amoxicillin at 31.4%. The antibiogram sensitivity test of *Salmonella enteritidis* isolates by Abd El-Tawab et al. (2015) indicated resistance to oxytetracycline and sensitivity to chloramphenicol, amoxicillin, levofloxacin, ciprofloxacin, enrofloxacin, and gentamycin, while Mezali and Hamdi (2012) recorded the resistance of 56 (90.32%) *Salmonella* isolates against at least one antibiotic, of which 20 isolates (32.26%) were MDR. The overall number of collected samples was 314 (128 raw chicken meat and poultry products, 144 raw red meat and meat products, and 42 processed meat products) from different market outlets. The *Salmonella* recovery rate was 61 (19.43%). The most significant recovery rate was recorded for red meat (23.61%, n = 34) and poultry (raw poultry meat and poultry products, 17.97%, n = 23).

The antibiotic sensitivity testing of *salmonella* isolates from milk and milk products in the current study revealed the highest incidence of resistance against erythromycin (100%), followed by ampicillin (57%), and then cefradine and amoxicillin (50%). Kunadu et al. (2018) found resistance to ciprofloxacin (100%), unlike the present results that were not resistant. Yasmin et al. (2015) found that 100% of *Salmonella* isolates showed resistance against ampicillin and ciprofloxacin.

Elafify et al. (2019) found that all *Salmonella* isolates from dairy products were resistant to erythromycin and streptomycin. In addition, Elafify et al. (2022) reported that all *Salmonella* spp. isolated from retailed dairies in Egypt, were resistant to oxacillin and nalidixic acid.

The results of AR of *Salmonella* isolates from all samples in the current study revealed a high resistance incidence of most *Salmonella* isolates from foods of animal origin. In the present study, erythromycin resistance from milk samples (100%) and poultry samples (63%) was recorded. High incidence of AR was recorded in milk samples (43% against oxytetracycline, 57% against ampicillin, and 50% against cefradin), poultry samples (100% against oxytetracycline, 66.6% against ampicillin, and 60% against cefradin), and meat samples (31.4% against oxytetracycline, 50% against ampicillin, and 53.7% against cefradin). It has been suggested that the increasing incidence of AR of Salmonellosis may result from misusing these antibiotics in veterinary fields (Economou and Gousia, 2015).

Rakitin et al. (2022) investigated the incidence of antimicrobial resistance among *salmonella enterica* strains obtained from food; their findings indicated that MDR against routinely used antibiotics as most of the strains (68.75%) exhibited multiple AR against the most commonly used antibiotics. The rise of MDR *Salmonella* strains is a worldwide public health concern. In Bangkok, Thailand, antimicrobial-resistant and virulent bacteria were obtained from retail food samples using the disc diffusion technique. It was found that the isolates were susceptible to amikacin and carbapenems. More than 30% of the isolates were resistant to ciprofloxacin, ampicillin, and tetracycline. Twenty isolates were resistant to at least three antimicrobial classes. Minimum inhibitory concentration revealed that about 12.07% of the isolates β-Lactam were resistant (Kong-Ngoen et al., 2022).

In the present study, using HPLC determination was one of the most used antibiotics in the field of oxytetracycline residues, six milk samples out of 7 (85.7%) contained residues exceeding the permissible limit (100 ug/l). The concentrations of oxytetracycline in seven samples were found to be 0.055 ug/ml, 0.011 µg/ml, 0.258 µg/ml, 0.528 µg/ml, 0.976 µg/ml, 2.423 µg/ml and 5.639 µg/ml. Abbasi et al. (2011) The mean of total tetracycline residues in all samples (114 samples) was 97.6 ± 16.9 ng/g, and that of pasteurized, sterilized, and raw milk samples were 87.1 ± 17.7, 112.0 ± 57.3, and 154.0 ± 66.3 ng/g, respectively. It was reported that the incidence of samples containing antibiotic residues was 25.4% of the total samples and 24.4%, 30%, and 28.6% of the sterilized, pasteurized, and raw milk samples, respectively. Al-Mazeedi et al. (2010) mentioned that more than 18% of milk samples had tetracyclines residues above the permissible limit in Kuwait. Navratilova et al. (2009) analyzed raw cow milk in Czech Republic and discovered tetracycline residues in 100% and oxytetracycline residues in 50.6% of tested samples. Ghimpeteanu et al. (2022) declared that all available food groups evaluated in different studies, including meat and meat products, milk and dairy products, eggs, honey, and non-animal-origin commodities, exhibit evidence of antibiotic residues.



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## CONCLUSION

Misuse of antibiotics for treatment and control of bacterial infection in veterinary farms may give rise to multiple drug resistance *Salmonella* as well as increasing antibiotic residues in foods of animal origin which can be a major threat to public health. Health and veterinary authorities should prevent using any antibiotics on veterinary farms without the direct supervision of veterinarians and apply a strict application of suitable withdrawal period for different antibiotics before consuming food of animal origin. Therefore, periodic surveillance is strongly to find microorganisms with various forms of antibiotic resistance, advised.

## DECLARATIONS


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### Author's contribution

Ayman Ameen Samy designed the study and critically revised the manuscript. Amany Ahmed Arafa collected samples and performed bacterial isolation and biochemical typing. Riham Hassan Hedia participated in isolation, antibiotic sensitivity, and writing. Eman Shafeek Ibrahim took part in isolation, sensitivity, and revising the manuscript. All authors checked and approved the final version of the manuscript for publishing in the present journal.

### Competing interests

There are no stated conflicts of interest by the authors.

### Ethical considerations

All of the authors have reviewed the manuscripts for ethical concerns, such as plagiarism, consent to publish, misconduct, data fabrication and/or falsification, double publishing and/or submission, and redundancy.

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# The Effects of Antimicrobial Residues on Microbiological Content and the Antibiotic Resistance in Frozen Fish

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**ABSTRACT**

As fish are perishable foods, their storage conditions require appropriate sanitary and temperature regimes. The producers commonly use various antibiotics to stop fish's microbiological and biochemical processes. The current research aimed to examine antibacterial residues in frozen fish (Argentina, flounder, lackerda, mackerel, capelin, salka, saithe, herring, dorado, and pink salmon) to find their influence on the quantitative content of microorganisms and to determine the sensitivity of isolated psychrotrophic bacteria to antibiotics. A total of 75 samples were collected from the fillets of frozen fish species. These fish were imported from Norway (16 samples), Vietnam (24 samples), Russian Federation (8 samples), China (14 samples), New Zealand (2 samples), Italy (2 samples), United States (4 samples), and United Kingdom (5 samples). The obtained results revealed that aminoglycosides (Gentamicin, Kanamycin, Spectinomycin, Dihydrostreptomycin, Paromomycin, and Apramycin) were in 45.6 ± 1.4% of frozen fish. The findings indicated the presence of some antibacterial residues (Nalidixic acid, antibiotics: Apramycin, Kanamycin, Tiamulin, and Nafcillin) in frozen fish, the definition of which has not been specified in the EU Regulation. This gives grounds to prohibit the use or develop standards for the maximum permissible concentration of these antibacterial substances in fish. The most common psychrotrophic bacteria isolated from frozen fish without antibacterial residues were highly sensitive to antibiotics, including Penicillin, Tetracycline groups, and Aminoglycosides. Therefore, it can be concluded that the residual levels of various biocides found in fish are a source for the expression of multi-resistance genes, which can be transmitted to consumers in the food chain.

**Keywords:** Antibacterial residues, Antibiotic resistances, Frozen fish, Multi-resistance genes, Psychrotrophic microorganisms

## INTRODUCTION

Fish and seafood are a source of easily digestible protein and contain fats, a valuable source of energy, as well as macro- and micronutrients (Al-Jasser and Al-Jasass, 2014; Nirmal et al., 2022). Fish is a nutrient medium for the development of microorganisms of all kinds (EC, 2002; Nirmal et al., 2022). They are classified as perishable foods, so the conditions and terms of their storage require appropriate sanitary, hygienic, and temperature regimes (Feng et al., 2017; Farag et al., 2021).

Fish and imported fish products enter the Ukrainian market in a frozen state. They are controlled according to criteria set for microbiological safety (presence of *Salmonella* spp., *Listeria monocytogenes*) and hygiene of the technological process (bacteria of the coliform group, mesophilic aerobic and facultative anaerobic microorganisms, *Staphylococcus aureus* (DSTU, 2007). Food stored in the refrigerator is dominated by psychrotrophic microflora (Grynevych et al., 2018; Zhang et al., 2019). Its development is related to product organoleptic and chemical defects (Ercolini et al., 2009; Moschonas et al., 2011; Malimon et al., 2018). Chilled and frozen fish are commonly contaminated with psychrotrophic microorganisms, such as *Pseudomonas* spp., *Acinetobacter* spp., *Flavobacterium* spp., *Moraxella* spp., *Shewanella* spp., and *Aeromonas* spp., which can perish the fish (Franzetti and Scarpellini, 2007; Popelka et al., 2016) and cause food poisoning. Accordingly, producers widely use various antibacterials to stop microbiological and biochemical processes in the fish chain from catch to consumer (Akinbowale et al., 2007; Bayer et al., 2017). Antibacterial drugs are used to feed fish in aquaculture and to prevent and treat diseases (Samanidou and



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Evaggelopoulou, 2007; Rico et al., 2012; Grynevych et al., 2018; Zhang et al., 2019; Nasr-Eldahan et al., 2021). Researchers have investigated the antibacterial residues of different types of fish in Ukraine (Grynevych et al., 2018), China (Chen, 2014; Maan et al., 2021), Croatia (Kolda et al., 2020), and Australia (Al-Jasser and Al-Jasass, 2014). It is believed that excessive amounts of antibiotics lead to antibiotic-resistant bacteria forming in the aquatic environment, which can cause fish diseases (Grigorakis and Rigos, 2011; Su et al., 2011; Miller and Harbottle, 2018). Consequently, there is a possibility of resistance transmission to microorganisms that cause human food infections.

Thus, it is of utmost significance to study microbiological indicators, particularly psychrotrophic bacteria's content and residual amounts of antibacterial substances in frozen fish imported to Ukraine. Such studies can improve preventive measures to monitor and control residual levels of antibacterial drugs in aquatic products entering the fish market. With this in mind, the current research was an attempt to study antibacterial residues in frozen fish, their influence on the quantitative content of microorganisms, and to determine the sensitivity of isolated psychrotrophic bacteria to antibiotics.

## MATERIALS AND METHODS

### Sampling

A total of 75 samples were collected from the fillets of frozen fish species (Argentina, flounder, lackerda, mackerel, capelin, salka, saithe, herring, dorado, and pink salmon). The frozen fish were purchased from the trade network in Lviv and Ternopil, Ukraine. These fish were imported from Norway (16 samples), Vietnam (24 samples), the Russian Federation (8 samples), China (14 samples), New Zealand (2 samples), Italy (2 samples), the United States (4 samples), and the United Kingdom (5 samples). All samples of frozen fish had an acceptable shelf life of 6-9 months and were stored at a temperature of -18°C.

### Microbiological study of fish

In this phase of the study, 48 cultures (18 *Pseudomonas* spp., 15 *Acinetobacter* spp., and 15 *Alcaligenes* spp.) from fish samples in the absence of antibacterial residues and 80 cultures (30 *Pseudomonas* spp., 25 *Acinetobacter* spp., and 25 *Alcaligenes* spp.) in the presence of antibacterial residues were studied.

The presence of antibacterial residues was determined by the chromatographic method (Waters mass spectrometer, TQD ACQITY system, USA). The antibiotics included Sulfaguanidine, Sulfacetamide, Sulfapyridine, Sulfadiazine, Sulfamethoxazole, Sulfathiazole, Sulfamerazine, Sulfamethizol, Sulfabenzamide, Sulfamethazine, Sulfinoxolin, Sulfadoxin, Sulfadimethoxine, Penicillin G, Cephalexin, Ampicillin, Penicillin V, Amoxicillin, Trimethoprim, Nafcillin, Oxacillin, Josamycin, Spiramycin, Nalidixic Acid, Flumequine, Oxalic Acid, Norfloxacin, Ciprofloxacin, Spectinomycin, Danofloxacin, Enrofloxacin, Marbofloxacin, Sarafloxacin, Difloxacin, Lincomycin, Gentamicin, Doxycycline, Chlortetracycline, Tetracycline, Ostetracycline, Kanamycin, Apramycin, Streptomycin, Dihydrostreptomycin, Paromomycin, Sulfamoxol, Sulfaphenazole, Sulfamethoxypyridazine, Sulfamonomethoxine, and Tiamulin (Di Corcia and Nazzari, 2002).

The number of mesophilic microorganisms was determined at a temperature of 30ºC incubation for 72 hours of Nutrient Agar (Pharmactive, Ukraine). The number of psychrotrophic microorganisms was calculated at a temperature of 6.5ºC incubation for 10 days on Nutrient Agar (Pharmactive, Ukraine). The NEFERM test 24 was used to identify isolated cultures of psychrotrophic microorganisms (Lachema, Czech Republic). The bacteria species were isolated according to the Bergey's Manual of Systematic Bacteriology (Vos et al., 2011). The psychrotrophic bacteria *Acinetobacter* spp., *Pseudomonas* spp., *Enterobacter* spp., *Alcaligenes* spp., and *Aeromonas* spp were identified. The sensitivity of the isolated bacteria to 14 antibiotics was tested by the classical disk-diffusion method Kirby-Bauer (NCCLS, 2003). The classical antibacterial disks used in this experiment consisted of Nalidixic acid, Tiamulin, Apromycin, Kanamycin, Sulfaphenazole, Gentamicin, Penicillin V, Difloxacin, Dihydrostreptomycin, Amoxicillin, Naphcillin, Spectinomycin, Tetracycline, Streptomycin, Paromomycin (Pharmactive, Ukraine). All procedures of this experiment were repeated three times.

### Statistical analysis

The results were expressed as mean value and standard deviation (SD) of three measurements were calculated. Statistical processing was performed by analysis of variance (ANOVA) using SAS (Version 9.2). P value less than 0.05 was considered statistically significant.

## RESULTS

In previous research, 10% of frozen fish samples imported into Ukraine contain residues of antibacterial substances. Figure 1 shows the chemical composition of the available residual antibacterial amounts in fish fillets. As can be seen in Figure 1, the antibacterial residues of Nalidixic acid was most often detected in frozen fish imported to Ukraine in 17.8 ±



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0.3% of samples. Apramycin and Kanamycin from Aminoglycosides antibiotics were detected in 16.3 ± 0.3% and 15.1 ± 0.3% of cases, respectively. The sulfonamide drug Sulfaphenazole was detected in 10% of the samples. In the investigated fish samples, Gentamicin and Tiamulin antibiotics were detected in almost the same amount of 6.9 ± 0.2 and 6.5 ± 0.2%, respectively. Such aminoglycoside antibiotics as Dihydrostreptomycin and Streptomycin were in a smaller number of fish samples (4.1 ± 0.2 and 1.6 ± 0.2%, respectively).

Among antibiotics of the Penicillin series, Penicillin V was most often detected in fish in 5.0 ± 0.2% of samples, and Amoxicillin and Nafcillin contained an average of 3.3 ± 0.1% of fish samples. Tetracycline and Paromomycin were detected in 1.7 ± 0.2% and 1.3 ± 0.1% of frozen fish samples, respectively.

Table 1 shows the permissible level of antibacterial residuals based on EU Regulation No. 37/2010. According to Table 1, Nalidixic acid, Apramycin, Kanamycin, Tiamulin, and Nafcilin antibacterials found in frozen fish were not based on regulations set by European legislation (EU Regulation No. 37/2010). In addition, the level of Gentamicin, Difloxacin, and Paromomycin found in the fish almost reached the maximum permissible amount allowed for these antibiotics. Amoxicillin and Penicillin V in the fish were twice lower than the maximum permissible amount according to EU Regulation 37/2010. At the same time, it was found that Tetracycline and Spectinomycin were above the maximum permissible amount of antibiotics in fish by 10% by 12.4%, respectively.

In the next phase of the study, microbiological indicators of frozen fish were determined based on antibacterial residuals. Various chemical preservatives influence the quantitative content of microorganisms in raw materials and food products. Figure 2 shows the number of microorganisms (mesophilic and psychrotrophic microflora) in frozen fish based on the detected antibacterial residues.

The findings revealed that the number of microorganisms in fish containing antibacterial residues of various pharmacological groups was 1.3-1.6 times lower than that of microflora in fish without antibiotics (Figure 2). Notably, in fish meat containing Tetracycline and Fluoroquinolone, the number of mesophilic microorganisms was 51.7 and 43.5 times ($p < 0.05$) less than in fish without antibiotics, respectively. This is probably due to the significant antibacterial effect of these antibiotics on mesophilic microflora. Regarding number of mesophilic microorganisms, all frozen fish samples met the requirements of the microbiological standard up to $5\times10^4$ CFU/g to SSU 4868: 2007 Frozen fish (DSTU, 2007).

In addition, it was found that in frozen fish without antibiotic residuals, the number of psychrotrophic microflora was 2.1 times greater than the number of mesophilic bacteria ($p < 0.05$), and in fish with antibiotic residuals, it was 1.3-1.7 times greater than the mesophilic bacteria ($p < 0.05$). For this reason, the microbiological control of frozen fish by psychrotrophic microflora content can determine the hygiene of the technological process involved in fish production and storage.

Regardless of the antibiotics in frozen fish, the dominant microflora is psychrotrophic microorganisms. Therefore, psychrotrophic microorganisms isolated from frozen fish were identified. It was found that the psychrotrophic microflora of frozen fish consists of *Acinetobacter* spp for 35-40%, *Pseudomonas* spp. for up to 30%, *Enterobacter* genus for 7-10%, and up to 20% for *Alcaligenes*, *Aeromonas*, cocci bacteria, and fungal microflora.

The current research was also conducted to determine the resistance of psychrotrophic microorganisms to antibiotics. The results are shown in Figures 3-7. Figure 3 illustrates the resistance of bacteria to antibiotics isolated from frozen fish without antibacterial residues. It can be inferred that in fish with no antibacterial residues, the isolated psychrotrophic microflora was mostly sensitive to antibiotics. The level of antibiotic-resistant strains of *Pseudomonas* spp., *Acinetobacter* spp., and *Alcaligenes* spp. did not exceed 20%. An exception was the Penicillin antibiotic Amoxicillin, which did not affect *Pseudomonas* spp. cultures, due to the natural resistance of these bacteria to Penicillins. Therefore, it was found that the most common psychrotrophic microorganisms isolated from frozen fish free from antibacterial preparations are mainly sensitive to antibiotics.

Figure 4 shows the results of antibiotic resistance of psychrotrophic bacteria isolated from frozen fish containing Tetracycline. As can be seen, isolated psychrotrophic microorganisms from fish containing Tetracycline were more resistant to antibiotics, compared to microorganisms isolated from fish without antibiotics. In particular, the level of bacterial cultures resistant to Tetracycline was 86.6-93.4%. At the same time, Ciprofloxacin, Ceftriaxone, and Gentamicin showed a stable antimicrobial action against isolated bacteria of the genus *Pseudomonas, Acinetobacter*, and *Alcaligenes*; the level of resistant strains did not exceed 26.7%.

The cephalosporin antibiotic Ceftazidime and the nitrofuran drug Furamag showed high antimicrobial activity. Since resistant bacteria of the genera *Acinetobacter* were not detected to these drugs, and the resistance of strains of *Pseudomonas* spp. was from 6.7 to 13.3%. Therefore, it was established that in case of the presence of antibiotics of the Tetracycline group in frozen fish, microflora resistant to drugs of this pharmacological group is released from the fish.



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The results of antibiotic resistance of psychrotrophic microorganisms isolated from fish containing Penicillin group preparations are shown in Figure 5.It was set that bacteria of the genera *Acinetobacter* and *Alcaligenes* developed resistance to antibiotics of the Penicillin group, as the number of resistant strains was 86.7-93.3% (Figure 5). The content of antibiotics can explain this in the Penicillin group in frozen fish. At the same time, antibiotics of other pharmacological groups showed high activity against isolated bacteria. The results of the research on antibiotic resistance of psychrotrophic microorganisms isolated from fish containing Gentamicin are shown in Figure 6. It was found that resistance to some specific antibiotics in frozen fish of samples containing Gentamicin residual is formed in the isolated microflora. In particular, the antibiotic Gentamicin was detected in frozen fish, as a result of which the resistance level of isolated bacteria of the genera *Pseudomonas*, *Acinetobacter*, and *Alcaligenes* were 86.7% and 93.3%, respectively (Figure 6).

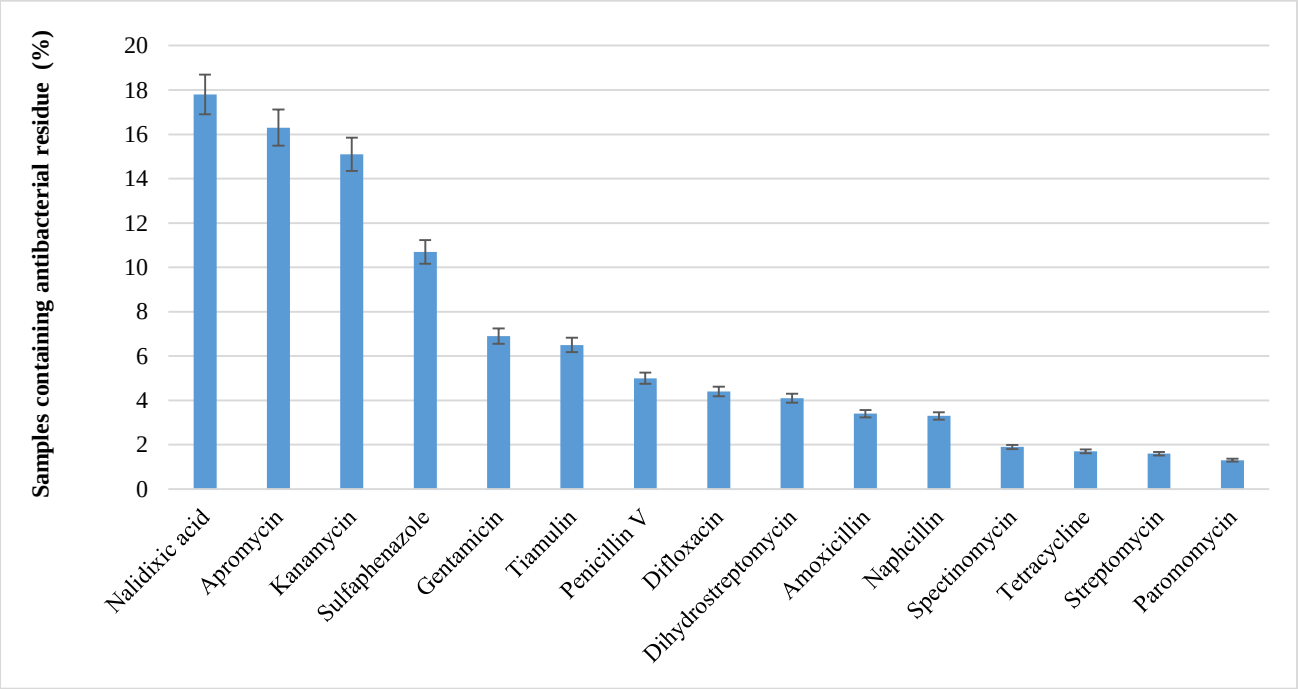


**Figure 1.** The percentage of fish samples containing antibacterial residue (n = 75, mean ± standard deviation)

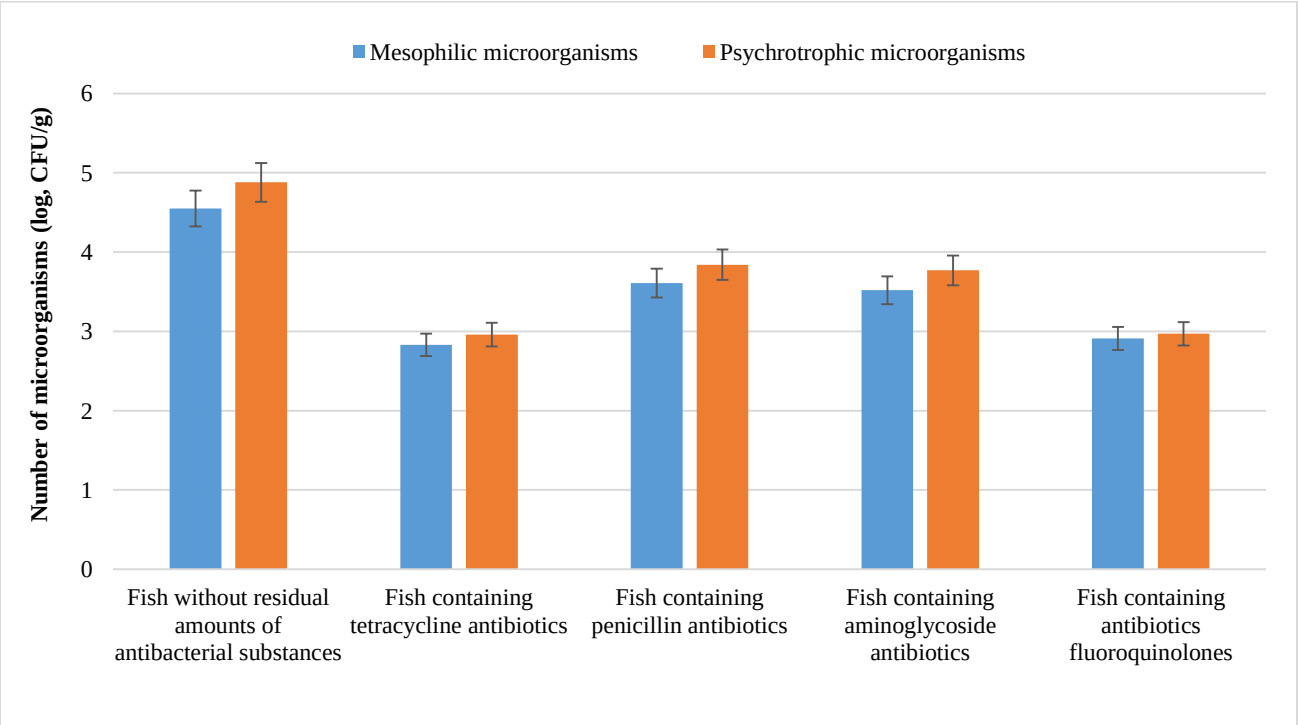


**Figure 2.** The number of mesophilic and psychrotrophic microflora in frozen fish fillets (n = 75) containing antibacterial residuals (mean ± standard deviation).



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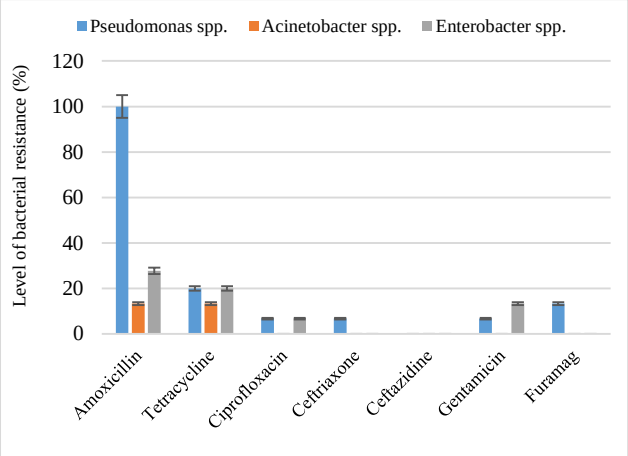


**Figure 3**. Antibiotic resistance of psychrotrophic microorganisms (mean ± standard deviation) isolated from fillets of frozen fish (n = 75) in the absence of antibacterial residues

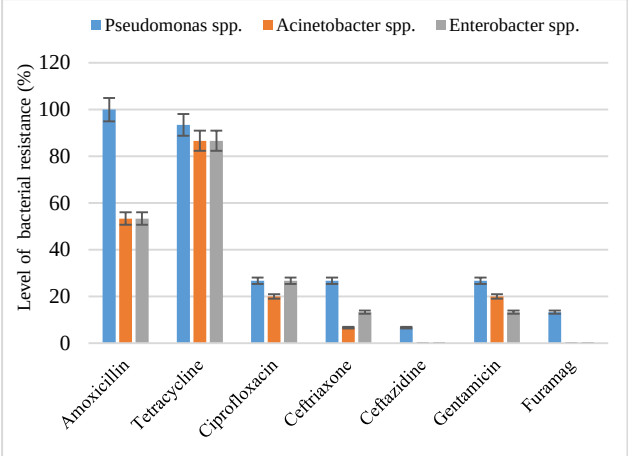


**Figure 4.** Antibiotic resistance of psychrotrophic microorganisms (mean ± standard deviation) isolated from frozen fish fillets (n = 75) with antibacterial residual of Tetracycline

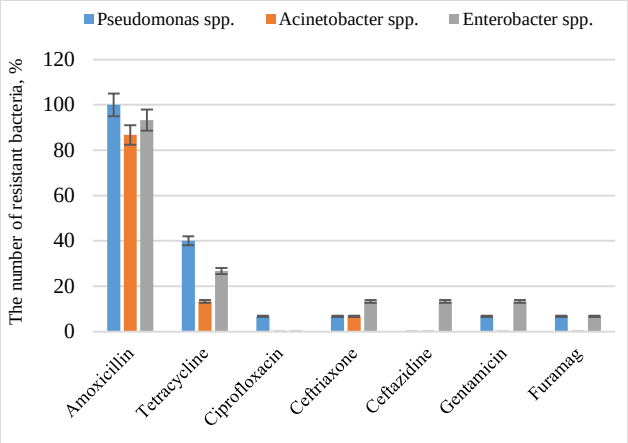


**Figure 5.** Antibiotic resistance (mean ± standard deviation) of psychrotrophic microorganisms (n = 75) isolated from frozen fish fillets containing antibacterial residual of the Penicillin group

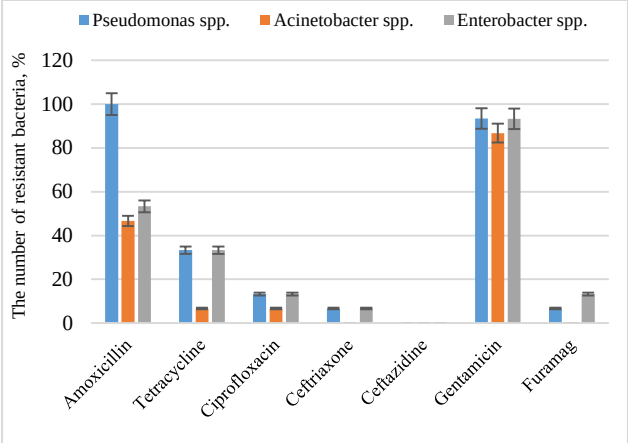


**Figure 6**. Antibiotic resistance (mean ± standard deviation) of psychrotrophic microorganisms (n = 75) isolated from frozen fish fillets containing antibacterial residual of Gentamicin

**Table 1.** Detection of antibacterial residuals in the fillets of frozen fish (n = 75)

| **Antimicrobial drugs** | **Concentration in fillet (mg.kg$^{-1}$)** | **The maximum permissible level (mg.kg$^{-1}$) according to EU Regulation No. 37/2010** |
|---|---|---|
| Nalidixic acid | 90.5 ± 4.0 | Not regulated in fish |
| Apramycin | 881.3 ± 72.1 | Not regulated in fish |
| Kanamycin | 117.4 ± 50.3 | Not regulated in fish |
| Sulfafenazole | 75.2 ± 4.8 | 100 |
| Gentamicin | 94.9 ± 6.5 | 100 |
| Tiamulin | 78.9 ± 4.3 | Not regulated in fish |
| Penicillin V | 23.9 ± 2.0 | 50 |
| Difloxacin | 97.8 ± 4.3 | 100 |
| Dihydrostreptomycin | 361.5 ± 23.1 | 500 |
| Amoxicillin | 27.2 ± 2.2 | 50 |
| Nafcillin | 163.5 ± 10.6 | Not regulated in fish, only for ruminants – 300 |
| Spectinomycin | 337.2 ± 18.2 | 300 |
| Tetracycline | 110.4 ± 6.5 | 100 |
| Paromomycin | 459.7 ± 28.1 | 500 |

## DISCUSSION

The basic principles of food safety set out in the EU Regulation (EC, 2002) provide that food products are required to ensure traceability throughout the production and circulation chain. The obtained results of imported fish for residual



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amounts of antibacterial residues showed that the most commonly found antibiotics (45.6 ± 1.4 percentage of cases from the collected samples) are aminoglycosides (Apramycin, Kanamycin, Gentamicin, Spectinomycin, Paromomycin, Dihydrostreptomycin). The detection of almost 50% of residual amounts of antibiotics in this group is probably due to a wide range of antimicrobial action on Gram-negative and Gram-positive microorganisms, which are the causative agents of fish spoilage and human food poisoning. In addition, in almost 18% of cases, nalidixic acid was detected, which is probably due to its good activity against bacteria of the family *Enterobacteriaceae* (Casagrande Proietti et al., 2022). Other studies indicated the significant use of aminoglycosides and macrolides in animal husbandry, fish farming, and poultry farming, which can be detected through the residual analysis of these biocides in raw materials and products (Cabello, 2004; Su et al., 2011; Horiuk et al., 2019). The results are consistent with those of other researchers reported for foods, antibiotics, sulfonamides, and nitrofuran preparations (Akinbowale et al., 2007; Bayer et al., 2017).

In addition, it should be noted that the EU Regulation does not regulate residues of nalidixic acid, antibiotics such as apramycin, kanamycin, tiamulin and nafcillin in meat (EC, 2010). Exceeding the permissible content values was found for antibiotics such as Tetracycline by 10 mg.kg-1 and spectinomycin by 37.2 $mg.kg^{-1}$ (EC, 2010). It is reported that the maximum allowable concentration of antibiotics in Tetracycline products has been exceeded (Miranda et al., 2003; Akinbowale et al., 2007; Su et al., 2011).

Thus, the results revealed that during the production of frozen fish, antibacterial substances are used, which is not provided by the EU Regulation (EC, 2010). This gives grounds to prohibit the use or development of standards for the maximum permissible concentration of these antibacterial substances in fish. In addition, the maximum allowable amount for antibiotics such as Tetracycline and Spectinomycin was exceeded. The present study also necessiates the need for careful control of frozen fish imported to Ukraine for the presence of residual amounts of antibacterial substances.

In the presence of residues of antibacterial substances in frozen fish, microbiological indicators did not exceed the standards (DSTU, 2007). The lowest microbial contamination of fish was detected in the presence of antimicrobial residues of Fluoroquinolone, Tetracycline groups, and Aminoglycosides. The obtained data are consistent with studies by Popelka et al. (2016), and Sheng and Wang (2021), which report higher significant contamination of frozen fish with psychrotrophic microflora, compared to mesophilic. Therefore, it can be considered that the microbiological characteristics of frozen fish in terms of the content of psychrotrophic microorganisms are more indicative and reliable for assessing compliance with the hygiene of production and refrigerated storage.

Various biocides are widely used in aquaculture to prevent and treat infectious diseases (Grynevych et al., 2018). The use of antibacterial substances in modern animal husbandry and aquaculture causes a global problem of antibiotic resistance of microorganisms (Kemper, 2008; Mulcahy, 2011; WHO, 2011; Horiuk et al., 2019). Studies on determining the sensitivity of the identified microflora to antibiotics have shown that in fish in which there are no antibacterial drugs, there are psychrotrophic bacteria, which are mainly sensitive to antibiotics. The resistance level of *Pseudomonas* spp., *Acinetobacter* spp., and *Alcaligenes* spp. did not exceed 20%. At the same time, in the presence of residual amounts of antibiotics of the Tetracycline group in frozen fish, microflora resistant to these antibiotics was isolated. A similar pattern was found in the presence of antibiotics in other groups. Thus, Gentamicin residues were detected in the meat of frozen fish and, as a result, isolated from psychrotrophic microorganisms showed resistance to Gentamicin in 86.7-93.3%t. Some studies also reported antibiotic resistance in microorganisms isolated from aquaculture (Miranda et al., 2003; Akinbowale et al., 2007; Su et al., 2011). In particular, resistant bacteria to antibiotics of the Penicillin group (ampicillin), Tetracycline group (Tetracycline and oxytetracycline), and florfenicol were isolated. The researchers believe that the presence of residues of antibacterial substances in raw materials or products causes antibiotic resistance in bacteria (Kemper, 2008; Mulcahy, 2011; Horiuk et al., 2019).

Therefore, the results of the current studies could support the findings of other studies indicating that the residual amounts of various biocides found in fish are a source of microorganisms with multi-resistance genes to antibiotics.

## CONCLUSION

Aminoglycoside antibiotics (Gentamicin, Kanamycin, Spectinomycin, Dihydrostreptomycin, Paromomycin, and Apramycin) were most often detected in frozen fish in a total amount of 45.6 ± 1.4% of the tested samples. Antibacterial preparations (Nalidixic acid, Apramycin, Kanamycin, Tiamulin, and Nafcillin) were detected in frozen fish, which is not specified in the EU Regulation (No. 37/2010). Moreover, the amount of antibiotics, such as Tetracycline and Spectinomycin in fish exceeds the maximum permissible amount. In the absence of antibacterial preparations in frozen fish, the existing microflora is highly sensitive to most antibiotics. At the same time, microflora resistant to the identified antibiotics forms in frozen fish with the remains of antibacterial preparations. Therefore, to prevent the receipt of fish with antibacterial substances to consumers and the spread of antibiotic-resistant strains of bacteria through fish, it is necessary to introduce careful control over the safety of frozen fish. Future experiments can be conducted to determine antibiotic residues in frozen seafood (mussels, shrimps, rapans) exported to Ukraine.



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## DECLARATIONS


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### Authors' contribution

Mykola Kukhtyn, Zoya Malimon, Volodymyr Salata, Igor Rogalskyy, Bogdan Gutyj developed an experiment, analyzed data, and wrote the manuscript. Larysa Kladnytska, Khrystyna Kravcheniuk, and Yulia Horiuk helped with the manuscript writing setting and data analysis. All authors checked and approved the final version of the manuscript for publication in the present journal.

### Competing interests

The authors declare that they have no conflict of interest.

### Ethical considerations

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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# Immunopathological Assessment of *Hydatid* Cyst and *Cysticercus Tenuicollis* Sonicated Protoscoilces Antigens in Mice

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**ABSTRACT**

The present study was designed to investigate the cross-protection (protective immunity) between Hydatid cyst and Bladder worm and evaluate the immunologic response of both humerol and cellular immunity in mice. To achieve these goals, 120 mice were used and equally divided into four groups immunized subcutaneously with 2 doses of antigen at the first and 14 days of the experiment. Mice in the first group (n=30) were immunized with 0.3 ml of hydatid cyst sonicated protoscolex antigen. Those in the second group (n=30) were immunized s/c with 0.3 ml of *Cysticercus tenuicollis* sonicated protscolex antigen. The third group (n=30) was immunized with 0.3 ml of both antigens (0.15 + 0.15), and the fourth group was a control group in which the mice were intraperitoneally injected with 0.2 ml of phosphate buffer solution. At the end of the experiment (30 days), blood samples were taken from the hearts of mice in all groups after being anesthetized by intramuscular injection of Ketamine 60 mg/kg, and Xylazine 12 mg/kg for the assessment of mouse Interleukin-12, IgG, and tumor necrosis alpha levels. The skin test results 24 hours (day 28) post-immunization showed an increase in the skin thickness against both antigens in the treatments, compared to the control. However, there was a decrease at 48 hours (day 29) post-examination in all groups. The results of TNFα titer showed higher titer in the third group, compared to the first, second, and fourth groups. Interleukin 12 concentration showed a higher titer in the third group than in the first, second, and fourth groups. The IgG concentration showed higher titer in the third group compared to the first, second, and fourth groups. In conclusion, immunopathological studies have shown that Ags used in the study, induce humoral and cellular immunity, compared to each Ag alone, and the mixed antigens were much more immunogenic. This cross-reactivity and synergistic interactions between the two parasites may be the cause of their antigenic activities.

**Keywords:** Immunopathological cross reaction, Interlukin-12, Parasitic antigens, Sonicated protoscolex antigens, TNFα

## INTRODUCTION

Both *Taenia hydatigena* cysticercosis and echinococcosis caused by *Echinococcus granulosus*, also known as hydatidosis, are common parasitic diseases that affect many wild and domestic animals as well as humans (Gessese 2020). They are regarded as one of the major causes of economic losses and livestock productivity in both the developing and industrialized worlds (Oryan et al., 2012). Proglottids or eggs carried in dog feces that pollute the pasture or feeding areas cause the intermediate host to become infected (Murell, 2005). Some canids, including wolves, jackals, and foxes, are natural hosts for cysts and can spread them (Parija, 2004). The loss is related to the condemnation of organs induced by hydatid and *Cysticercus tenuicollis* cysts in small ruminants. *Cysticercus tenuicollis*, a cystic development made up of many liquid-filled cysts that are specifically found on the fascia of the abdominal organs of ruminants, harbors the invaginated scolex of the future tapeworm. Generally, the causative organism is subclinically and mildly infected (Torgerson et al., 2008).

A hydatid cyst infection can produce T helper 1 and T helper 2 (cytokines). In echinococcosis, Th 1 cytokines are linked to a protective response, but Th 2 cytokines induce susceptibility to the disease. The immune response will promote parasite proliferation and development if cytokine response skews Th 1/Th 2 ratios in favor of a preferred immunopathology-associated Th 2 polarization. Additionally, cysts' illness, formation, and development are linked to (or indicated by) the serum antibody response (Zhang and McManus, 2008). It has been demonstrated that lambs



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generate both IgG1 and IgG2 as the primary immunoglobulins after exposure *to Cysticercus tenuicollis* infection (Craig and Rickard, 1982). IgG2 has a lower magnitude than IgG1, but it has a much more pronounced effect in terms of preventing infection. The main immunoglobulin subclass present in ruminant colostrums and milk is IgG. Many IgG subclasses, like IgG1, serve as the primary immunoglobulin in colostrums (Hurley and Theil, 2011).

The role of antibodies in the protection of sheep infection against several Taeniid metacestodes was examined using the passive transfer of immunoglobulin. In *Cysticercus tenuicollis*, a 70-80% reduction in cyst numbers was achieved by transferring 100-120 ml of serum from immunized sheep with *Cysticercus tenuicollis* oncospheres to recipients (Jacobs *et al*., 1994**).** Cross-reactivity with different cestodes is seen in parasites due to the high number of shared antigens (El-Moghazy and Abdel- Rahman 2012). Numerous physical and antigenic similarities exist among different cestodes. *T. hydatigena* was employed as a model organism for *Taenia saginata, Echinococcus granulosus*, and other cestodes (Mcmanus, 2014; Miquel et al., 2015).

Crude protein from the fluid of *Taenia hydatigena* cysts was employed as an ELISA antigen to detect antibodies against *Taenia saginata* cysticercosis, *Echinococcus granulosus* hydatidosis, and *Taenia solium* cysticercosis (Kamanga-Sollo et al., 1987; Rhoads et al., 1991 Bogh et al., 1995; Kara et al., 2003). Additionally, antigens can lead to immune-mediated cross-protection in the intermediate host for infections with *Cysticercus ovis*, *Cysticercus bovis*, *Cysticercus cellulose*, and Hydatid cyst. The development of metacestodes in these infections can be reduced by pre-exposure to *C. tenuicollis* (Conlan et al., 2012). Thus, this study aimed to investigate the cross-protection (protective immunity) between hydatid cyst and bladder worm and evaluate the immunologic response of both humerol and cellular immunity in mice.

## MATERIALS AND METHODS

### Ethical approval

All experiences were approved by the ethical committee at the College of Veterinary Medicine, Kerbala, Iraq (ethical approval number: COVM-6341).

### Study design

*Hydatid cyst* were collected randomly during inspection of the sheep carcasses in the abattoirs (Kerbala, Iraq) and transferred into the laboratory (Veterinary Medicine College in Kerbalaa, Iraq) for examination, by a cooling box. Hydatid protoscolices antigen was prepared following the method of Nasrieh and Abdel-Hafez (2004) with certain modifications. Collected hydatid fluid was clarified by centrifugation at 10000 rpm at $4^0$C for 60 minutes. The deposit containing protoscolices of hydatid cysts was washed thoroughly in phosphate buffer saline (PBS) three times to remove cyst wall debris and dead protoscolices. About 1.8 g of the washed pellet was suspended in 3 ml of PBS in which 40 µl of 0.2 molar phenyl methyl sulphonyl Fluoride (Sigma, USA) in isopropanol was added. The mixture was homogenized using a glass homogenizer (B-Braun Biotech International, Germany) for 20-30 strokes. The homogenate was sonicated at 50 cycles /s at the maximum tune of 1.8 µm peak to peak for 30 seconds four times in an ice bath using an ultra Sonicator (B-Braun Biotech International, Germany). The sonicate was centrifuged at 15,000 rpm at $4^o$C for 20 minutes. The supernatant was used as protoscolices antigen of hydatid protoscolex sonicated antigen (HPSA) and stored at -20°C. Similarly, *Cysticercus tenuicollis* scolex antigen (CTSA) was also prepared.

### Experimental design

A total of 120 mice of both sexes (80 males and 40 females separated), aged from 4-6 weeks with 22 g mean weight were obtained from the Iraqi Center for Cancer and Medical Research), they were adopted at the animal house of Veterinary Medicine College in Baghdad, Iraq for 2 weeks before starting the experiment. The mice were divided into four groups with three replicates (10 mice in each replicate). Each group received 2 doses of the antigen for 14 days. In the first group, mice were immunized s/c with 0.3 ml of *Hydatid cyst* sonicated protoscolex Ag. In second group mice were immunized s/c with 0.3 ml of *Cysticercus tenuicollis* sonicated protscolex Ag. In the Third group, mice were immunized s/c with 0.3 ml of both Ag, and in the fourth group mice were injected intraperitoneal with 0.2 ml PBS as the control negative group. Delayed-type hypersensitivity test (Skin test) was performed according to Jacysyn et al. (2003) and Silva et al. (2021) on mice groups after immunization. For all groups, the left hind footpad was injected with 0.1 ml sterile PBS. The thickness of the skin was measured by a vernier caliper on day 28, and day 29 of the experiment

Blood samples were taken one-time from the hearts of the mouse groups after anesthetized rats by intramuscular injection of Ketamine 60 mg/kg and Xylazine 12 mg/kg, at the end of the experiment (30 days). The blood samples were centrifuged at 1500 rpm for 15 minutes, and the serum was transferred into an Eppendorf tube. It was kept frozen at -20°C until use.

Assessment of mouse Interleukin-12, IgG, and tumor necrosis alpha levels (TNFα) were conducted using ELISA KIT ´(KOMA BIOTECH INC, South Korea) based on manufacturing protocol.



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**Statistical analysis**

The statistical analysis was done using SPSS software (version 22). A two-way analysis of variance (ANOVA) was used to analyze the data statistically. Duncan's test was chosen to determine the mean significant differences between treatments, and $p \leq 0.05$ considered significant (Snedecor and Cochran, 1980).

## RESULTS

**Delayed-type hypersensitivity**

The skin test findings of mice post-immunization showed the means of skin thickness against both Ag were significantly high in the first (3.35 ± 0.18), second (2.40 ± 0.14), and third (3.62 ± 0.11) groups, compared to the fourth group (1.87 ± 0.08, $p \leq 0.05$). However, these values decreased 48 hours (day 29) post-examination in all groups (Table 1).

**Determination of immunized parameters**

Table 2 shows the result of TNFα titer 30 days post-immunization. The findings indicated that TNFα titer significantly increased ($p < 0.05$) in the third (603.88 ± 21.6) and the first groups (598.05 ± 13.56), compared to the second (280.97 ± 23.83) and fourth groups (270.27 ± 140.65). The results of IL 12 titer also showed a significant ($p < 0.05$) difference in the third group (1382.27 ± 27.37), compared to other groups, and the control group was the lowest (252.12 ± 59.96). There was a significant difference in IgG concentration between groups first (6.80 ± 0.71) and third (6.79 ± 0.20A) with other groups, and the control group was the lowest (2.54 ± 0.13).

**Table 1.** Skin thickness in immunized mice at days 28 and 29 of the experiment

| Groups | day 28 | day 29 |
|---|---|---|
| Immunize with hydatid cyst sonicated protoscolex Ag | $3.35 \pm 0.18^{Aa}$ | $1.92 \pm 0.08^{Bb}$ |
| Immunize with *cysticercus tenuicollis* sonicated protoscolex Ag | $2.40 \pm 0.14^{Ba}$ | $1.88 \pm 0.07^{Bb}$ |
| Immunize with both Ag | $3.62 \pm 0.11^{Aa}$ | $2.57 \pm 0.13^{Ab}$ |
| Control | $1.87 \pm 0.08^{Ca}$ | $1.41 \pm 0.13^{Cb}$ |

Values are expressed as mean ± standard error. Different superscript letters ($^{A,B,C}$) means significant differences in a column ($p < 0.05$). Different superscript letters ($^{a,b,c}$) means significant differences in a row ($p < 0.05$). Ag: Antigene.

**Table 2.** Determination of immunized parameters (TNF, IL-12, and IgG) in mice within 30 days of the experiment

| Groups | TNFα (pg/ml) | IL-12 (pg/ml) | IgG (ng/ml) |
|---|---|---|---|
| Immunize with hydatid cyst sonicated protoscolex Ag | $598.05 \pm 13.56^{A}$ | $772.4 \pm 210.28^{B}$ | $6.80 \pm 0.71^{A}$ |
| Immunize with *cysticercus tenuicollis* sonicated protoscolex Ag | $280.97 \pm 23.83^{B}$ | $604.23 \pm 32.83^{B}$ | $4.01 \pm 0.45^{B}$ |
| Immunize with both Ag | $603.88 \pm 21.6^{A}$ | $1382.27 \pm 27.37^{A}$ | $6.79 \pm 0.20^{A}$ |
| Control | $270.27 \pm 140.65^{B}$ | $252.12 \pm 59.96^{C}$ | $2.54 \pm 0.13^{C}$ |

Values are expressed as mean ± standard error. Different superscript letters ($^{A,B,C}$) means significant differences in a column ($p < 0.05$). Different superscript letters ($^{a,b,c}$) means significant differences in a row ($p < 0.05$). Ag: Antigene.

## DISCUSSION

All immunized groups showed a significant increase in the thickness of mice footpads compared to the control group. The highest mean of thickness was on day 28 of the experiment. This result may be due to the higher sensitivity of antigens, epitopes, or peptides responsible for delayed-type hypersensitivity and effective immune responses in skin testing. Specific antigens could be easily engulfed, processed by antigen-presenting cells, then recognized by T-cells, and induce a strong hypersensitivity reaction. Therefore, the main cause of skin thickness is the aggregation of a large number of lymphocytes which may reach to hundred times more than in normal conditions, especially sensitized T lymphocyte that releases chemokine and attracts phagocytic cells. The early thickness of the skin may be due to the release of chemical mediator, which induces edema, congestion of blood vessels, and swelling at the inoculation site (Helou et al., 2021). According to Mahmoudzadeh-Niknam et al. (2007), the early delayed-type hypersensitivity (DTH) reaction to Ag in mice is caused by eosinophils, basophilic mast cells. As a result, the current findings might be the result of Ags-activated CD4+ and CD8+ T-cells, which play a role in triggering DTH. Following exposure to dendritic cells and Langerhans cells, the site moves from the epidermal layers to the lymph nodes, where they present antigens to T lymphocytes that secrete interferon via major histocompatibility entities (Hemmi et al., 2001).



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An obvious marked cellular immune response with a significant increase in thickness was revealed in the mice group immunized with mixed Ag (*Fasciola hepatica* and *hydatid cyst* fluid antigen) before and after the challenge dose (Al-malki, 2012). A number of studies have also revealed new information on the effective response of the immune system showing that Antigen B (AgB, the major antigens of *Echinococcus granulosus*), and cytokine response in a study on rabbits immunized with *Echinococcus granulosus* AgB (Chemale et al., 2005; Nisreen and Wafaa 2017), which can skew type 1-type 2 cytokines toward a preferred Th2 polarization (Rigano et al., 2001; Al-malki, 2012).

A regulatory role for IL-12 in innate resistance in intermediate host infection has been suggested, and this was accompanied by a Th1 response (Al-malki, 2012). The role of AgB in the human inflammatory response is determined by its effect on polymorphonuclear cells (PMN) and its action in acquired immunity (Riganò et al.,2001). This result may be due to factors IL-2 and IL-12 dependent on the activation of T-cells (Spiering 2015; Condotta and Richer, 2017).

The AgB stimulates intricate immunological responses of IL 12. Among these are polarized Th2 reactions paired with Th1 reactions. It has been proposed that IL-12 regulates innate resistance in the infection of intermediate hosts, which is accompanied by a Th1 response (Al-malki, 2012). The primary immune system regulators are CD4+ cells, which come in two subtypes (Th1 and Th2) with distinct roles (Toes et al., 1997). Th1 activation can promote the cellular immune response by inducing CD8+ T-cell lymphocytes to release IL-2, IL-12, and IFN-γ (Gómez et al., 2021).

Al-Qaoud et al. (2008) revealed that immunization of mice with AgB led to elevated IgG1 and IgG2a. AgB induced more IL-4 when given intraperitoneally (IP). Moreover, polarization towards the Th2 response showed that subcutaneous AgB vaccination of Balb/c mice caused the formation of a significantly important amount of total IgG (especially IgG1) linked with other routes of immunization, such as intramuscular and intraperitoneal, which coincided with the Th2 response. The earliest quantifiable IgG response to AgB occurs 2 to 11 weeks after immunization in mice and sheep, and 4 weeks in vervet monkeys (Zhang et al., 2012). Early infections can cause a high cellular inflammatory response and pathologic changes. Elevated eosinophils, lymphocytes, and macrophages cause leukocytosis. Oncospheres produce necrosis, neutrophil, and macrophage invasion for 3-5 days (Finkelman et al., 1991). According to some studies, exposure to or immunization with Taenia hydatigena protects against *Taenia ovis* (Heath et al., 1979; El-Moghazy and Abdel-Rahman, 2012).

*Taenia hydatigena* infection has been shown to protect mice from *Taenia taeniaeformis*, *Taenia saginata* in calves, and *Fasciola hepatica* in sheep (Muku et al., 2020; Jansen et al., 2021). It should be highlighted that *Leishmania donovani* antigens are combined with *Cysticercus tenuicollis* fluid antigen as an adjuvant, which may help to explain why antibodies are produced. Adjuvants are chemicals added to vaccinations to boost their potency by changing the immune system's reaction to certain immune cells (NCI, 2010). The outcome of this study is the production of antibodies, which may be a result of the adjuvants used to boost a vaccine's effectiveness by modifying the immunological response to certain immune system cell types.

## CONCLUSION

The two types and mixes of Ag have been demonstrated to generate humoral and cellular immunity in immunopathological investigations. The combined antigens (*Hydatid cyst* and *Cysticercus tenuicolis*) may be significantly more immunogenic than each Ag alone. The cross-reactivity and synergistic interactions might bring about the antigenic activity for the two investigated parasites. It is therefore suggested to apply different substances to vaccines to increase their effectiveness by altering the immunological response to certain immune system cells**.**

## DECLARATIONS


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### Authors' contribution

The final manuscript draft was reviewed by all authors, who also gave their approval.

### Competing interests

There is no conflict of interest.

### Ethical considerations

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



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# Seroprevalence and Associated Risk Factors of Porcine Cysticercosis in Boucle Du Mouhoun Region of Burkina Faso: A Cross-sectional Survey

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**ABSTRACT**

*Taenia solium* cysticercosis is a neglected tropical zoonosis with economic and public health importance. Cysticercosis is widely present in low-income countries with extensive pig breeding systems and poor human hygiene practices. In Burkina Faso, a study on porcine cysticercosis has been done only in Boulkiemde province. There is a lack of serological data on this disease in other areas, such as Balés province in the Boucle du Mouhoun region. This study aimed to assess the prevalence and risk factors of porcine cysticercosis in Boucle du Mouhoun, Burkina Faso. A total of 373 serum samples were collected from local breed pigs that were randomly selected from four villages in the mentioned region. Data were also collected using a structured questionnaire to determine explanatory factors for the infection. Serum samples were tested using an antigen ELISA test to detect circulating antigens of *Tænia solium*. The prevalence of the disease was 54.9% (95% CI = 49.8-59.9). Following univariate and multivariate logistic regression analysis, pigs originating from Kombia were found to be a protective factor (OR=0.54, 95% CI = 0.33-0.89). Male pigs were more likely to be infected than females (OR= 1.7, 95% CI = 1.09-2.64). The prevalence and factors associated with *Taenia solium* cysticercosis were identified and according to these data, porcine cysticercosis had a high prevalence in this area. Therefore, it is important to implement control actions focusing on disease control and public health for people infected with *Taenia solium*.

**Keywords:** Burkina Faso, Prevalence, Pigs, *Taenia solium*, Zoonosis

## INTRODUCTION

Cysticercosis caused by *Taenia solium* (*T. solium*) has important public health and socioeconomic impact, mainly in developing countries (Murrell et al., 2005). Human is the definitive natural host and harbors the adult tapeworm and becomes infected by ingesting undercooked or raw pig meat with the parasite cysts. Pigs act as intermediate hosts. The transmission to pigs occurs when they ingest human feces or water/feed contaminated by *T. solium* eggs. Dogs can also act as intermediate hosts, and transmission to dogs occurs in the same conditions as pigs (Wandra et al., 2015; Ito et al., 2016). Humans, as accidental intermediate hosts, are infected when they accidentally ingest the parasite eggs with water or food or during auto-infestation (García et al., 2003; Murrell et al., 2005). As accidental intermediate host, the cysts might locate in the eyes and central nervous system, and this lead respectively to ocular cysticercosis and neurocysticercosis (Murrell et al., 2005). In animals, cysticercosis is commonly asymptomatic, but human infection can cause headaches and varying focal neurological manifestations, hydrocephalus, chronic meningitis, lacunar infarct syndromes, neuropsychiatric manifestations, and blindness (Murrell et al., 2005). Different prevention and control methods are described in the literature, including pig meat inspection, improved pigs husbandry practices, pigs vaccination, basic sanitary facilities, health education, etc. (Murrell et al., 2005). Regarding risk factors, extensive or free-range pig husbandry, open human defecation, consumption of human feces by pigs, deliberate use of human feces as pig feed, the connection of pig pens to human latrines, involvement of human carriers of the parasite in pig rearing and care has been described (Murrell et al., 2005).

In Burkina Faso, many studies have established the prevalence in humans between 0 and 37.5% (Carabin et al., 2009, 2015; Millogo et al., 2012; Nitiéma et al., 2012) in the Midwest region and Nayala province of Boucle du Mouhoun region. In pigs, the prevalence was estimated to range between 0 and 39.6% in the Midwest region, and



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infection was associated with the free-range keeping of pigs (Ganaba et al., 2011) as noticed in other studies in Africa (Sikasunge et al., 2007; Pondja et al., 2010; Ngwing et al., 2012; Assana et al., 2013). The national pig population was estimated to 2,345,803 and the Boucle du Mouhoun region pig population counted for 11.5% of the national pig population (INSD, 2016). Farming practices are mainly traditional with free roaming of pigs during the dry season but tethered or kept in small houses during the rainy season (FAO, 2012). According to these pig farming practices, pigs may be infested by ingesting human feces contaminated by *T. solium* eggs. Also, according to information collected from veterinary services in some villages of this region, pig meat is sometimes seized because of cysticercosis. However, no epidemiological study has been carried out to know the prevalence of porcine cysticercosis in this area and the factors associated with infestation. So, this study aimed to describe the prevalence and factors associated with *T. solium* cysticercosis in pigs from four villages in Boucle du Mouhoun region in Burkina Faso.

## MATERIAL AND METHODS

### Ethical approval

The study has been approved by the research board of Inter States School of Veterinary Sciences and Medicine (University of Dedougou, Burkina Faso) before its implementation. Informed consent was asked of each farmer included in the present study. Also, non-pregnant animals were dewormed using albendazole. All applicable international, national, and/or institutional guidelines for the use of animals were followed.

### Study area

This cross-sectional study was implemented from December to April 2017 during the dry season. It took place in the province of the Balés, located in the Boucle du Mouhoun region in Burkina Faso. Four villages in Fara county were chosen for this study (Figure 1). The villages are set on regional road 11 between Poura Carrefour and the border with Ghana. Mainly animists are noted in Toné, Sadon Bobo, and Koumbia villages, where an important pig population was noted, except for Kabourou where there are many Muslims, with a small pig population. The area has a tropical climate with two seasons. The rainy season goes from May to September, with an average rainfall of 871 mm. Pigs are tethered or kept in little pens during the rainy season, while free roaming of pigs is noted in villages during the dry season. Human open defecation was noted in villages despite the presence of latrines in some households.

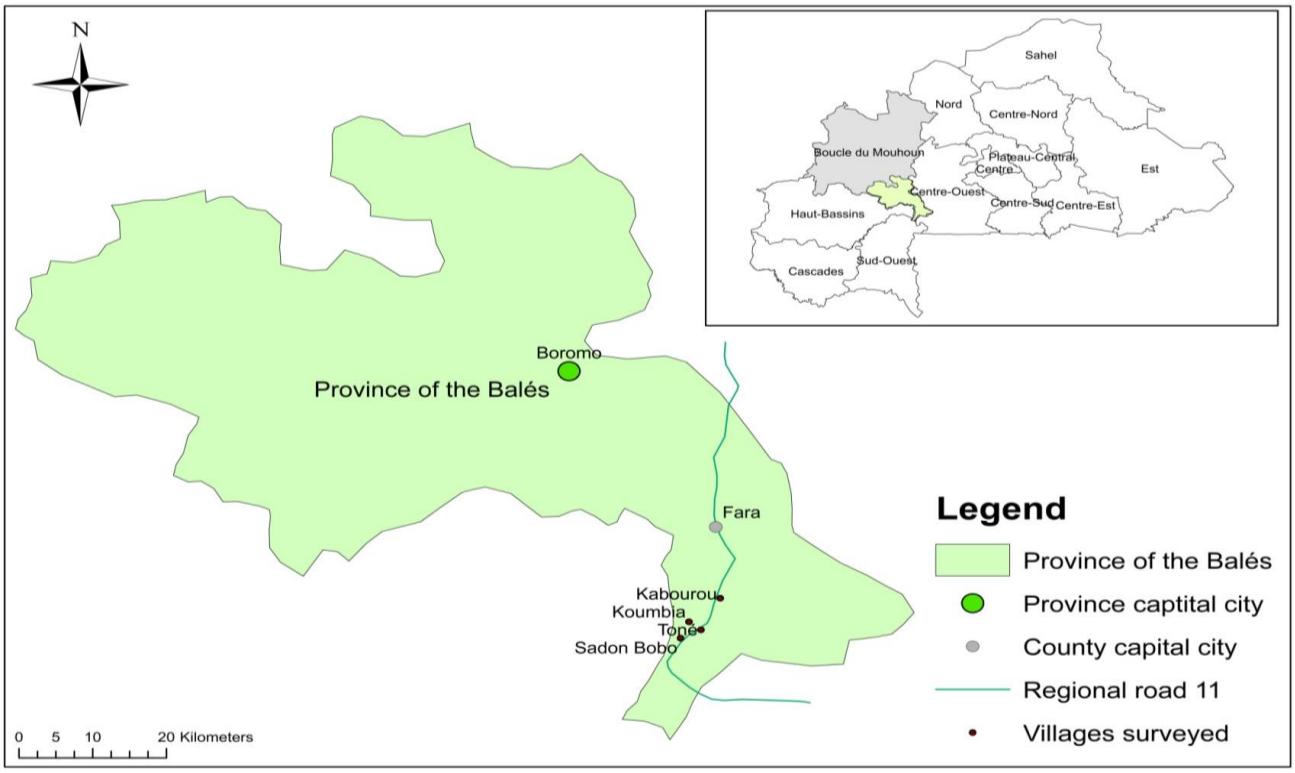


**Figure 1**. Location of the study area in the country

### Sample size determination and samples collection

The sampling size was calculated using Win Episcope 2.0 software (Australia) with a precision of 5 %, a confidence level of 95 %, and expected prevalence of 32.5 % (Ganaba et al., 2011) and pig population of 270,504 (INSD, 2016). In the region, local and exotic pig breed was noted, but only local and mixed pig breed was considered for



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the sampling area. The minimum sample for this study was 338, but 376 blood samples were collected. Blood was collected using a 2ml blood collection tube (heparin) from the jugular vein. In each village, households were randomly selected by moving on roads in villages and selecting each pig-keeping household at intervals of 100 m. In each selected household, one pig was randomly chosen. From each animal sampled, blood was taken, and serum was harvested using centrifugation at 3000 RPM for 15 minutes. Because of the ELISA kit availability, only 373 serums were analyzed.

**Questionnaire survey and direct observations**

For each animal sampled, a survey was made in the household to identify factors that could be associated with pigs' infection. The presence of latrines, farmer gender, and the use of pig houses as latrines, pigs' sex, age, and breed were investigated. Also, information on pig access to human feces, anthelmintic use, and history of cysticercosis in the farm was noticed. All data were mentioned on a record sheet with the same ID as on the blood sampling tube.

**Laboratory analysis**

Laboratory analyses were made in the laboratory of Parasitology and Mycology of Inter State School of Veterinary Sciences and Medicine in Dakar (Senegal). The serums samples were tested for circulating antigens of the metacestode of *T. solium* detection with an Ag-ELISA (commercial Kit from ApDia, Belgium) test with the monoclonal antibody B158/B60 as described previously (Dorny et al., 2004). This test is used to detect viable cysticerci of *T. solium* based on manufacturer instructions. The test is known to have high specificity and sensitivity in pigs at 86.7% and 94.7%, respectively (Dorny et al., 2004).

**Statistical analysis**

After performing tests, investigations, observations, and serological tests, data were saved in a Microsoft (2007) Excel sheet. The overall apparent prevalence was calculated. Statistical software used was STATA 11 (StataCorp, USA). Respondents and pig characteristics were determined using descriptive statistics. First, a univariable logistic regression analysis was done to determine the association between each factor and *T. solium* infection. Factors with p-values $\leq 0.1$ were included in a multivariable logistic stepwise regression analysis. For excluding factors one at a time, using $p > 0.05$ as the criterion, a backward elimination procedure was used. For all analyses, the significance level was set at 0.05. Only Farmer gender, the village of origin, and pig sex were included in the final model for multivariable logistic regression analysis. Prevalence and odds ratio are given with their confidence interval (CI).

## RESULTS

**Pig husbandry characterization**

Among pigs' husbandries visited, 95.7 % of owners were women, and most (65.7%) sampled animals were sows (Table 1). The average age of sampled animals was 18.8 months. The local breed was most important (77.2%), and all pigs were roaming free during the dry season and enclosed in small houses (74%) or tethered at a pole or a tree (26%) during the rainy season. During the dry season, 25.5% of pigs' owners said that village people defecate in their piggery. Most of the household (87.7%) had latrines but only 15.5% had doors and 3% was clean. All pigs get access to human feces, and only 1.3 % were dewormed. More than two-thirds of pigs' owners (69.2%) noted that they had seen cysts on their pig slaughtered.

**Serological data**

Out of the 373 pigs' samples tested, 205 were positive, so the overall prevalence based on the detection of circulating antigens was 54.9% (95 % CI = 49.9 - 59.9%). The prevalence was higher at Koumbia (60.7% [95% CI = 50.7 - 70.7%]) than in other villages (Table 1). Prevalence was significantly higher at Toné, Koumbia, and Sadon Bobo than the one found at Kabourou ($p < 0.05$). Otherwise, the prevalence was significantly higher ($p < 0.05$) in boars (63.3%) than in sows (50.6%). Regarding farmer gender, the prevalence was significantly higher in pigs owned by men than those owned by women ($p < 0.05$) (Table 1). The prevalence was 52.9 %, 54.7% 55.1%, respectively, for pigs aged less than four months, between four and nine months, and for pigs aged more than nine months, but the variation was not significant ($p > 0.05$). Regarding the pig breed, the prevalence was 54.9 % and 55.3%, respectively, for a local and mixed breed, but no significant variation was noted ($p > 0.05$).

**Risk factors associated to porcine cysticercosis**

According to the multiple regression analysis, pigs which were males were 1.7 times more likely to have *T. solium* cysticercosis than females (Table 2 and Table 3). Likewise, only pigs from Koumbia were less likely to be infected with *T. solium* when compared with pigs from Kabourou (Table 2 and Table 3). Other variables included in the multivariable logistic regression were insignificantly different ($p > 0.05$).



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**Table 1.** Descriptive characteristics of farmers sampled local breed pigs with associated pig seroprevalences in four villages of Boucle Du Mouhoun region in Burkina Faso

| Variables | Category | Number | Seroprevalence (95 % CI) | p value |
|---|---|---|---|---|
| Village | Kabourou | 84 | 39.3 [29.3-49.3] | 0.01 |
| | Koumbia | 79 | 60.7 [49.7-71.7] | |
| | Sadon Bobo | 80 | 60 [49-70] | |
| | Toné | 130 | 58.4 [49.9-66.9] | |
| Farmer experience | Less than 5 years | 24 | 58.3 [38.5-78.0] | 0.76 |
| | Between 5 and 10 years | 170 | 52.9 [45.4-60.4] | |
| | Above 10 years | 179 | 56.4 [49.1-63.7] | |
| Defecation in pen | Yes | 95 | 50.5 [40.4-60.5] | 0.29 |
| | No | 278 | 56.5 [50.7-62.3] | |
| Farmer gender | Men | 16 | 81.2 [62.1- 100] | 0.04 |
| | Women | 357 | 53.8 [48.7-58.9] | |
| Pig sex | Boar | 128 | 63.3 [55-71.6] | 0.02 |
| | Sow | 245 | 50.6 [44.4-56.8] | |
| Pig breed | Local | 288 | 54.9 [49.2-60.6] | 0.94 |
| | Mixed | 85 | 55.3 [44.7-65.9] | |
| Age class | [0 - 4 months] | 9 | 52.9 [29.9-75.9] | 0.98 |
| | [ 4 - 9 months] | 60 | 54.7 [41.7-67.7] | |
| | > 9 months | 304 | 55.1 [49.5-60.7] | |
| Presence of latrines in the households | Yes | 327 | 56.3 [50.8-61.8] | 0.29 |
| | No | 46 | 45.7 [31.3-60.1] | |
| Reared near latrine | Yes | 327 | 56.3 [50.9-61.7] | 0.17 |
| | No | 46 | 45.7 [31.3-60.1] | |
| Pigs' deworming | Yes | 5 | 60 [17-100] | 0.71 |
| | No | 368 | 54.9 [49.9-59.9] | |
| Former infestation of *T. solium* in the husbandry | Yes | 258 | 56.6 [50.6 - 62.6] | 0.34 |
| | No | 115 | 51.3 [2.2-60.4] | |
| Total animal sampled | - | 373 | 54.9 [49.8-59.9] | |

The significance level was set at 0.05

**Table 2.** Univariable logistic regression analysis of potential explanatory of *Taenia solium* cysticercosis in local breed pigs in Boucle du Mouhoun region in Burkina Faso

| Variables | B-coefficient | p value at 95%CI |
|---|---|---|
| Farmer gender | 1.32 | 0.04 |
| Farmer experience | 0.05 | 0.76 |
| Village | 0.78 | 0.01 |
| Pig sex | 0.52 | 0.02 |
| Pig breed | 0.02 | 0.94 |
| Type of rearing | 0.10 | 0.69 |
| Reared near latrine | 0.43 | 0.18 |
| Defecate in pen | 0.25 | 0.29 |
| Deworm pigs | 0.35 | 0.71 |

The significance level was set at 0.05

**Table 3.** Multivariable logistic regression analysis of selected potential predictors of *Taenia solium* cysticercosis in local breed pigs in Boucle du Mouhoun region in Burkina Faso

| Variable | Category | B-coefficient | p value | Odds Ratio | 95% Confident Interval for Odds Ratio |
|---|---|---|---|---|---|
| Village of origin | Toné | 0.137 | 0.48 | 1.15 | 0.79-1.67 |
| | Sadon Bobo | 0.156 | 0.53 | 1.17 | 0.72-1.91 |
| | Koumbia | -0.611 | 0.01 | 0.54 | 0.33-0.87 |
| | Kabourou | Reference | - | 1.00 | - |
| Farmer gender | Male | 1.136 | 0.08 | 3.11 | 0.87-11.17 |
| | Female | Reference | - | 1.00 | - |
| Pig sex | Male | -0.53 | 0.02 | 1.70 | 1.09-2.64 |
| | Female | Reference | - | 1.00 | - |

The significance level was set at 0.05



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## DISCUSSION

This study described the prevalence and the risk factors of *T. solium* cysticercosis in pigs in Boucle du Mouhoun region in Burkina Faso. The overall prevalence based on the detection of circulating antigens was 54.9%. This high prevalence could be linked to pig farming systems in villages. Also, most people practice open defecation in the area (Dahourou et al., 2018) and this situation allows pigs to get access to human feces infected by *T. solium* eggs (Sreedevi et al., 2012). This high prevalence could also be linked to the assay used as the Ag ELISA test does not allow differentiation between infection of different Taenia species (*T. solium, T. asiatica*, and *T. hydatigena*, Dorny et al., 2004). Even if *T. asiatica* is most located in Asia (Eom et al., 2009), *T. hydatigena* has been found to be prevalent in Burkina Faso (Dermauw et al., 2016). As dogs were found in the study area, some positive samples could be cross-reactions with *T. hydatigena*, so these data must be interpreted carefully. However, according to some observations that authors made in slaughter and tongue palpation during the field survey, most infections with *T. solium* were suspected according to cyst presence on the tongue and also cysts' location, and morphology on pigs' carcasses. The overall prevalence was higher compared to the prevalence found (0 to 39.6% according to the villages) by a previous study in the province of Boulkiemdé using the same Ag-ELISA assay (Ganaba et al., 2011). This difference could be associated with a higher number of carriers of *T. solium* and poor hygiene behaviors in the study area compared to their area. In this study, the prevalence was higher than the prevalence found by Kungu et al. (2017) in Uganda using the same technique and ELISA HP10 (12.2%), and also by Thomas et al. (2016) in Kenya, using the Ag-ELISA HP10 method, which was 37.6%. It was nevertheless lower than the prevalence found by Pondja et al. (2015) in the northwest of Mozambique (66.7%), in Benin (72.22%) by Goussanou et al. (2014), and South Africa (57%) by Krecek et al. (2012) with the same methods of the present study. The prevalence was significantly higher in boars compared to sows, as mentioned by Sikasunge et al. (2008) in Zambia.
For this study, some factors like farming system (free roaming or not) and access to human feces were not included in statistical analysis because all pigs were free roaming and had access to human feces while it has been described as risk factors in the country (Ganaba et al., 2011).

The prevalence was significantly lower in Kabourou than in the villages of Koumbia, Sadon Bobo, and Toné. The prevalence found was also significantly higher in pigs held by men. In the study area, pigs bred by men are not well feed; in this case, pigs spent much time looking for food and are therefore more exposed to infected human feces. There was no significant variation in infestation according to the pigs' age or their breed. Regardless of the breed or age of the animals, they are found wandering with the same risks of being in contact with infected human feces. According to Pondja et al. (2010) and Ngwing et al. (2012), the prevalence increases significantly with the age of the pigs in Mozambique and Cameroon, respectively.

Belonging to the village of Koumbia seems to be a protective factor. At this level, it is difficult to explain this situation, and further studies could clarify some. Also, being male was identified as a risk factor. Males might be more active in looking for a feed, so they are more exposed to ingesting human feces containing eggs of *T. solium*.

Findings noted that factors like rearing pigs near latrines, the existence of latrines in the household, human defecation in pen, and pig deworming were not significantly associated with infection. The use of latrines is an important factor to be assessed as, in some area, latrines could be present in a household, but people practice open defecation. This situation was previously described in Cameroon rural areas (Pouedet et al., 2002; Assana et al., 2010) and Mozambique (Pondja et al., 2010). In families with access to latrines, some people prefer open defecation, and in Zambia this is associated with taboos related to the use of latrines (Thys et al., 2015). Some farmers said that people used their pens as latrines, mainly during the rainy season. This situation seems to be very common in the study area, and maybe some wrong answers have been collected during field activities about this question. Pig deworming is not very frequent in the area; anthelmintic used are often out-of-date or not from official veterinary services. So, most of the time, farmers buy fraudulent drugs with no or low efficacy.

## CONCLUSION

This study found a high prevalence of *T. solium* infection in pigs. Different factors were found to be associated with the infection. This is a serious public health problem for the people who consume pork and people living in the study area. It is important to design and implement control measures for this important zoonotic parasite in this area. Also, health education programs need to be implemented in this study area to reduce the risk of transmission of the disease to pigs and humans.

## DECLARATIONS

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**Authors' contribution**

Laibané Dieudonné Dahourou, Oubri Bassa Gbati and Athanase Millogo designed the study. Laibané Dieudonné Dahourou, Kacou Martial NDA, Arnaud R. Stéphane TAPSOBA made field survey and laboratory analysis. Laibané Dieudonné Dahourou did statistical analysis validated by Amadou Traore. Laibané Dieudonné Dahourou proposed the draft of the manuscript. All authors made contributions to the revision of the manuscript. All authors read and approved the final manuscript.

**Availability of data and material**

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

**Competing interests**

For this study, authors declare that there is no competing interest.

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**Ethical considerations**

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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# Deterioration of Frozen Semen of Bali Cattle after Cooling at 5°C

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## ABSTRACT

Frozen semen is produced through several stages, which deteriorate spermatozoa. This research aimed to evaluate the deterioration degree of frozen semen after 5 °C cooling and freezing of Bali cattle. The samples included 10 male Bali cattle with a body weight of 542-668 kg, from which semen was collected once a week for five weeks. The deterioration of each individual's sperm was determined by observing two distinct straws. The parameters observed included viability, abnormalities, intact plasma membrane, and intact acrosome cap. Initial observations of the parameters were conducted following the addition of semen to diluent A1 (AD) as much as the volume of fresh semen. The semen in the AD group was not cooled and frozen. The A1 semen was then divided into two, namely, those with cooling at 5 °C for 4 hours (PT1) and at 5°C for 22 hours (PT2). The results showed that individual variations in Bali cattle caused significant differences in viability and intact plasma membrane of AD and PT1 groups, while PT2 did not differ in viability and intact plasma membrane spermatozoa. Abnormalities were significantly different between AD and PT2 groups, however PT1 did not differ in abnormalities spermatozoa. Intact acrosomal cap was significantly different in the AD, PT1, and PT2 groups. In conclusion, individual variations, including viability, abnormalities, intact plasma membrane, and acrosome cap of spermatozoa, were better at 4 hours compared to cooling at 5°C for 22 hours. A Cooling time of 4 hours at 5°C can be recommended for frozen semen processing.

**Keywords:** Abnormalities, Bali cattle, Intact acrosome cap, Intact plasma membrane, Viability

## INTRODUCTION

Bali cattle are one of the original beef cattle in Indonesia. They should be developed, utilized, and preserved as native livestock resources with certain characteristics, and the ability to thrive in diverse environments. Bali cattle also have quite varied production performance and high reproductive capacity (Saili, 2020), which makes them suitable for producing frozen semen. Artificial insemination (AI) is one of the reproductive techniques used to exploit superior males. Therefore, the genetic dissemination of superior males is carried out as an effort to improve their performance. Implementing AI in cattle depends on the quality and quantity of semen ejaculated by a male, as well as the ability to maintain the quality and increase the volume of semen for a longer time. Hence, more female acceptors will be inseminated (Nyuwita et al., 2015). The use of frozen semen is one of the success factors for the AI program, which optimizes the functioning of the males and saves the cost of raising male cattle. The reason is that, frozen semen is more durable and can be used after several years, which necessitates the quality evaluation of semen (Zuidema et al., 2021).

Frozen semen is made using certain stages. The steps include a dilution process at 37 °C, cooling at 5 °C, adding cryoprotectants and equilibration, freezing in liquid nitrogen at -196 °C, and thawing (Zampini et al., 2020). Semen is stored for a certain time during the cooling process until it reaches a temperature of 5 °C. Long cold storage time increases lactic acid (Kowalczyk et al., 2020) and forms reactive oxygen species (Silvestre et al., 2021). Reactive oxygen species cause damage to polyunsaturated fatty acids in spermatozoa membranes (Aitken, 2017). Spermatozoa pass through various extreme changes in temperature and osmolarity, hence triggering the production of reactive oxygen species (Aitken and Drevet, 2020). High concentrations of reactive oxygen species in cells produce the oxidation of lipids and proteins (Lundgren et al., 2018). This process that sperm undergo can lead to cold shock, osmotic stress, and the formation of ice crystals, thereby reducing their quality in motility, permeability changes, and lipid components of the membrane (Peris-Frau et al., 2020). Furthermore, the ability of sperm to fertilize an egg is affected by the sperm cell membrane (Anifandis et al., 2014). Changes in the lipid components of the spermatozoa membrane can disrupt the stability and cause acrosomal damage (Nofa et al., 2018).

Individual variation is a genetic factor in the ability of males. Each cattle has a different genetic potential, which affects its semen quality. According to Fazrien et al. (2020), the quality of frozen semen produced by each cow is different. Bali cattle have different characteristics of viability values, intact plasma membranes, and abnormalities



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(Indriastuti et al., 2020). Furthermore, the value of viability decreases, when the abnormality increases after freezing (Indriastuti et al., 2020). Spermatozoa produced by each cattle have a different membrane composition and resistance, affecting their ability to withstand freezing and heat shock during thawing (Zamuna et al., 2016). The plasma membrane is needed as a protective organelle in the cell and a filter for exchanging intracellular and extracellular substances. The differences in the components of spermatozoa and the character of each individual affect the quality of produced frozen semen (Fazrien et al., 2020).

Frozen semen deterioration is often evaluated based on motility, but it is important to consider other factors, including viability, abnormality, intact plasma membrane, and intact acrosome cap (Santoso et al., 2021). The determinant of fertilization success depends not only on sperm motility, but also on the condition of an intact plasma membrane and sperm acrosome cap. Therefore, the current research aimed to evaluate the deterioration degree of frozen semen as an effect of the 5°C cooling and freezing process from different individual Bali cattle.

## MATERIALS AND METHODS

### Ethical approval

The study was conducted at the Singosari AI Center, Indonesia, according to the standard procedure SNI ISO 9001:2015 NO.G.01-ID0139-VIII-2019 and supervised by a veterinarian. The ethics committee of the Singosari AI Center provides ethical guidance and approval for responsible behavior when using bulls for semen collection. This research was approved by the Brawijaya University Ethical Committee number 121-KEP-UB-2022.

### Experimental animal

The cattle used were male Bali cattle from the Singosari AI center, Malang, East Java, Indonesia. Semen was collected once a week for 5 weeks using an artificial vagina (Minitube, Germany). Semen was collected using a teaser to increase libido. Furthermore, the semen was obtained from 10 male cattle. The cattle samples were within the age range of 7-12 years with a body weight of 542-668 kg. The deterioration of each individual's sperm was determined by observing two distinct straws. Males were housed under the care of a veterinarian and provided with grass (22 kg), silage (3 kg), concentrates (4 kg), hay (1 kg), and minerals, including calcium and phosphor (0.06 kg).

### Diluent preparation

The diluent used was tris-egg yolk consisting of 17.25 g tris aminomethane (Merck, Germany), 9.65 g citric acid (Serva, USA), 15.55 g lactose (Serva, USA), 27.95 g raffinose (Serva, USA), egg yolk, streptomycin (Meiji, Indonesia) 1 g/liter, penicillin (Meiji, Indonesia) 1,000,000 IU/liter, distilled water, and 13% glycerol (Merck, Germany) of the total required diluent. In the preparation procedure, tris aminomethane, citric acid, lactose, and raffinose were put into an erlenmeyer containing distilled water, then homogenized and heated to a temperature of 40°C for 10 minutes. Egg yolks 20% were added after the solution was cooled, then streptomycin and penicillin were added. Diluent was stored in the refrigerator for three days, after which the supernatant and pellet were separated. Approximately 13% glycerol was added according to the total required diluent (Tethool et al., 2021).

### Research procedure

The fresh semen produced was observed macroscopically (color, consistency, pH, volume) and microscopically (concentration, abnormality, and motility) using a microscope (Olympus CX-23, Japan) to determine the feasibility. Diluent was added three times to each sample, namely A1, A2, and B. The following formulas 1-4 indicate the measurement of the diluent amount at the Singosari AI center following a study by Arif et al. (2020):

$$\text{Total volume} = \frac{\text{semen volume x concentration}}{25 \text{ million}} \quad \text{Formula 1}$$

$$\text{A1 volume} = \text{semen volume} \quad \text{Formula 2}$$

$$\text{A2 volume} = \frac{\text{Total volume} - (\text{A1 volume} + \text{semen volume})}{2} \quad \text{Formula 3}$$

$$\text{B volume} = \frac{\text{Total volume}}{2} \quad \text{Formula 4}$$

The deterioration was initially observed after semen was added to A1 diluent (AD). It was then divided into semen with cooling at 5°C for 4 hours (PT1) and 22 hours (PT2). Diluent A2 was added before storage for 4 and 22 hours. Meanwhile, diluent B was added after cooling for 4 and 22 hours, and equilibration was performed for 2 hours. Using 0.25 ml straws, sperm was filled and sealed into each straw before packaging and freezing in liquid nitrogen at -196°C. Finally, the frozen semen deterioration was observed after thawing at 37°C for 30 seconds (Santoso et al., 2021).



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**Observation of semen deterioration**

Spermatozoa viability was assessed by placing one drop (5 µl) of semen on the edge of the object glass and one drop of eosin-nigrosin (20 µl) on the semen in a ratio of 1:4, and then homogenized the mixture (Santoso et al., 2021). Furthermore, the mixture was prepared for analysis. The viability percentage was determined by dividing the number of live spermatozoa by the total sperm cells and multiplying by 100. Live and dead spermatozoa were indicated by transparent (colorless) and red heads, respectively (Mohamed et al., 2015).

Spermatozoa abnormalities were assessed using eosin-nigrosin staining. The semen was dripped onto the object glass and stained with eosin-nigrosin, and then thin smear preparations were made (Suhardi et al., 2020). Using a microscope (Olympus CX-23, Japan) with 400x magnification, 200 spermatozoa were counted in each of the five fields of view to determine the sperm count. The percentage of abnormality was obtained by counting the abnormal spermatozoa divided by the total number of sperm cells multiplied by 100 (Suhardi et al., 2020).

The acrosome cap was observed by making a thin slide of semen on the object glass, after which the preparations were dried and put into a 5% formalin solution at 37°C for 30 minutes. The slide was removed, washed using distilled water, and dried. Giemsa staining solution was prepared by adding 3 ml of Giemsa solution (Merck, Germany) drop by drop into 2 ml of standard pH 7 phosphate buffer solution, then adding 35 ml of aquabidest (Jayamas Medica, Indonesia). Subsequently, this solution was mixed until evenly distributed. The slide fixed in 5% formaldehyde (Merck, Germany) was put into the ready Giemsa staining solution for 4 hours in a water bath at 37°C. The stained slide was washed with distilled water and observed using a microscope (Olympus CX23, Japan - Optilab advanced V2, Indonesia) by counting 200 spermatozoa. Acrosomes of intact sperm were indicated by the purple top of the head, while those with light color imply deterioration (Chowdhury et al., 2014; Prihantoko et al., 2020).

The integrity of the spermatozoa membranes was tested using a hypoosmotic swelling test solution, consisting of 0.31 g of sodium citrate (Merck, Germany) and 0.565 g of fructose (Merck, Germany) dissolved in 50 mL of aquadest (Purwoistri et al., 2013). The test was conducted using 1 ml of the hypoosmotic solution from 150 ml osmol added to 0.1 ml of spermatozoa and incubated at 37°C for 30 minutes. In the next step, observation was performed with a microscope (Olympus CX23, Japan) with 400x magnification (Yendraliza et al., 2019). A circular tail characterized sperm cells with intact membranes at the end. Meanwhile, those with incomplete membranes were characterized by straight-tail conditions (Rajashri et al., 2017).

**Statistical analysis**

Statistical analysis was performed using SPSS software (version 25 IBM). Individual differences among Balinese cattle were obtained by one-way ANOVA and Duncan's multiple-range test. P value less than 0.05 was considered significant.

## RESULTS AND DISCUSSION

**Semen quality**

Table 1 shows the characteristics of fresh semen produced by each Bali cattle. The fresh semen in the current research had the lowest and highest motility values of 73.24 ± 2.28% (Individual H) and 84.88 ± 1.17% (Individual C), respectively. The lowest and highest concentrations were 1226.8 ± 279.5 million/ml (Individual J) and 1982.2 ± 227.4 million/ml (Individual D), respectively. These differed from previous research, reporting the lowest and highest motility rates as 79.7 ± 0.09% and 85.6 ± 0.08%, respectively (Tethool et al., 2021). Moreover, in a study conducted by Tethool et al. (2021), the lowest and highest concentrations were 876.2 ± 225.3 million/ml and 1459.6 ± 294.1 million/ml, respectively. According to National Standard number 4869-1:2021, the suitable semen to be processed as frozen should have a motility value of ≥70% (Santoso et al., 2021). Therefore, the means values produced by each individual meet the criteria, indicating a suitable condition for processing frozen semen.

**Table 1.** Fresh semen quality of Bali cattle aged 7-12 years

| Individuals | Color | Consistency | pH | Volume (ml) | Concentration (million/ml) | Abnormality (%) | Motility (%) |
|---|---|---|---|---|---|---|---|
| A | Milky white | Thick | 6.40 ± 0.001 | 3.96 ± 0.78 | 1421.8 ± 240.7 | 4.80 ± 2.52 | 75.40 ± 7.46 |
| B | Milky white | Thick | 6.56 ± 0.09 | 2.10 ± 1.81 | 1453.0 ± 208.6 | 5.28 ± 3.02 | 77.40 ± 9.22 |
| C | Milky white | Thick | 6.56 ± 0.17 | 7.12 ± 1.78 | 1606.0 ± 95.7 | 4.68 ± 1.87 | 84.88 ± 1.17 |
| D | Milky white | Thick | 6.44 ± 0.17 | 3.88 ± 0.77 | 1982.2 ± 227.4 | 3.92 ± 1.47 | 80.96 ± 4.87 |
| E | Milky white | Medium | 6.36 ± 0.09 | 5.20 ± 2.12 | 1409.8 ± 255.9 | 3.68 ± 1.51 | 79.72 ± 4.03 |
| F | Milky white | Medium | 6.52 ± 0.11 | 5.20 ± 1.11 | 1323.4 ± 336.9 | 2.74 ± 1.33 | 81.48 ± 4.55 |
| G | Milky white | Thick | 6.40 ± 0.14 | 4.88 ± 1.03 | 1782.8 ± 153.9 | 3.66 ± 1.14 | 80.52 ± 6.42 |
| H | Milky white | Thick | 6.52 ± 0.11 | 2.72 ± 1.11 | 1758.4 ± 309.4 | 6.24 ± 1.70 | 73.24 ± 2.28 |
| I | Milky white | Medium | 6.56 ± 0.17 | 4.72 ± 2.67 | 1411.2 ± 218.4 | 6.48 ± 3.74 | 74.38 ± 6.95 |
| J | Milky white | Medium | 6.60 ± 0.14 | 7.00 ± 4.79 | 1226.8 ± 279.5 | 3.30 ± 0.60 | 79.34 ± 11.06 |



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**Viability and abnormalities**

As can be seen in Table 2, individual variations caused significant differences in the viability of spermatozoa in AD and PT1 groups ($p < 0.05$), while in the PT2 group there was no significant in the viability of spermatozoa. The highest values of individual variations in AD, PT1, and PT2 sperm viability were 92.43 ± 3.12% (Individual C), 72.90 ± 6.69% (Individual F), and 67.52 ± 5.14% (Individual B), respectively. Meanwhile, the lowest values were recorded for Individual F (84.84 ± 5.58%), followed by Individual G (65.74 ± 4.89%), and H (62.32 ± 6.36%). The viability of spermatozoa before freezing was higher than 71.82 ± 7.38% - 72.08 ± 6.63%, as reported by Hapsari et al. (2018). However, after freezing, it was lower than the amount of 75.79 ± 0.84% - 79.9 ± 20.84% in a study by Indriastuti et al. (2020).

**Table 2.** Viability and abnormalities value of spermatozoa in different Bali cattle individuals aged 7-12 years

| Individuals | Viability (%) | | | Abnormality (%) | | |
|---|---|---|---|---|---|---|
| | AD | PT1 | PT2 | AD | PT1 | PT2 |
| A | 86.69 ± 5.26$^{ab}$ | 70.63 ± 5.49$^{abc}$ | 64. 85 ± 7.26 | 5.57 ± 1.11$^{abc}$ | 8.97 ± 1.29 | 13.58 ± 4.66$^{b}$ |
| B | 91.24 ± 3.93$^{c}$ | 72.65 ± 5.90$^{c}$ | 67.52 ± 5.14 | 6.46 ± 2.33$^{c}$ | 9.32 ± 1.37 | 10.93 ± 1.39$^{a}$ |
| C | 92.43 ± 3.12$^{c}$ | 67.23 ± 4.13$^{ab}$ | 63.69 ± 3.88 | 4.64 ± 2.61$^{ab}$ | 9.66 ± 1.04 | 10.46 ± 0.78$^{a}$ |
| D | 90.54 ± 3.42$^{bc}$ | 70.09 ± 6.65$^{abc}$ | 64.74 ± 4.01 | 5.45 ± 0.97$^{abc}$ | 9.49 ± 0.97 | 10.27 ± 1.25$^{a}$ |
| E | 88.48 ± 3.97$^{abc}$ | 70.81 ± 4.57$^{abc}$ | 65.77 ± 4.18 | 6.33 ± 1.76$^{bc}$ | 9.56 ± 1.57 | 10.59 ± 1.34$^{a}$ |
| F | 84.84 ± 5.58$^{a}$ | 72.90 ± 6.69$^{c}$ | 63.26 ± 7.14 | 4.91 ± 1.56$^{abc}$ | 9.48 ± 0.47 | 10.92 ± 1.30$^{a}$ |
| G | 90.66 ± 5.44$^{bc}$ | 65.74 ± 4.89$^{a}$ | 63.16 ± 5.01 | 4.46 ± 1.27$^{a}$ | 9.60 ± 1.37 | 10.45 ± 0.70$^{a}$ |
| H | 90.29 ± 2.34$^{bc}$ | 68.49 ± 6.89$^{abc}$ | 62.32 ± 6.32 | 4.83 ± 1.13$^{abc}$ | 9.65 ± 1.50 | 10.38 ± 1.45$^{a}$ |
| I | 88.62 ± 4.09$^{abc}$ | 71.66 ± 5.65$^{bc}$ | 64.43 ± 4.29 | 6.50 ± 2.99$^{c}$ | 9.25 ± 1.00 | 10.32 ± 1.36$^{a}$ |
| J | 91.49 ± 3.24$^{c}$ | 71.28 ± 4.73$^{bc}$ | 62.65 ± 4.95 | 4.97 ± 1.47$^{abc}$ | 9.25 ± 0.80 | 10.12 ± 1.24$^{a}$ |

$^{a,b,c}$Different superscripts in the same column were significantly different ($p < 0.05$). AD: After adding A1 diluent; PT1: Post-thawing cooling time of 4 hours; PT2: Post-thawing cooling time of 22 hours.

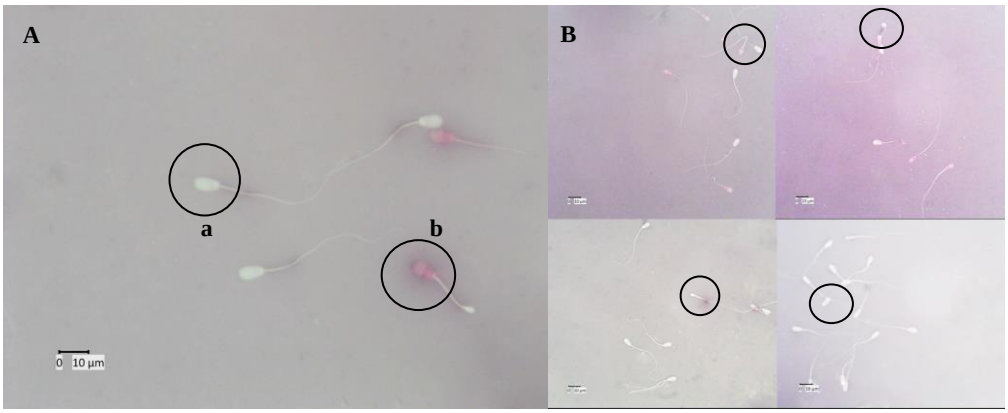


**Figure 1.** Identification of viability and abnormalities of spermatozoa Bali cattle aged 7-12 years. **A:** (**a:** viable [the head does not absorb color], **b:** nonviable), **B:** Abnormalities of sperm

Spermatozoa viability is one of the primary requirements and factors used for quality testing that shows fertilizing ability (Tanga et al., 2021) based on the number of sperm cells that can survive. It is assessed by exposing spermatozoa to eosin-nigrosine staining. Live spermatozoa cells are characterized by colorless spermatozoa heads (Figure 1A) since the function of protecting cell organelles and regulating the entry, and discarding of the required substances cannot occur properly. Damage to the plasma membrane of spermatozoa results in the disruption of metabolic processes, and consequently its death. The dead sperm cells cannot filter the staining liquid, leading to a colored head for the dead cells (Tanga et al., 2021). According to the results of the present study, individual variation in Bali cattle caused differences in the viability of the spermatozoa AD groups and PT1 groups because individuals in these groups had different cholesterol characteristics in their membranes. As reported in previous studies, individual variations affect spermatozoa viability due to the cholesterol characteristics that form condensation and plasma membrane integrity in each individual (Saez and Drevet, 2019; Indriastuti et al., 2020). Therefore, individuals with the highest viability values before freezing (AD) differed from those after freezing (PT1 and PT2). The low cholesterol content in the membrane results in the low resistance of spermatozoa to the freezing process, so the spermatozoa membrane will lose its function (Cornelius et al., 2015). Ice crystals are formed due to extreme temperature changes, and the freezing process in spermatozoa in this context causes deterioration of structure and plasma membrane (Sharma et al., 2015). Variations in male cattle affect spermatozoa viability (Mohammed et al. 2015). The ability of sperm cells to survive freezing varies between species and individuals of the same species (Yánez-Ortiz et al., 2021). This difference results from variations in each individual's biochemical properties and cell metabolism (Ali et al., 2022).

The value of spermatozoa viability is closely related to the integrity of the plasma membrane (Palacin et al, 2020). It was reported that the loss of spermatozoa viability was due to membrane damage induced by lipid peroxidation in the



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plasma membrane (Alahmar, 2019). The mechanism of membrane deterioration is initiated through the transition phase at the beginning of cooling. Furthermore, the cooling process changes the molecular structure of lipids, proteins, and nucleic acids, leading to a decrease in membrane fluidity (Sieme et al., 2015). During the freezing process, extracellular ice crystals are formed from diluent medium spermatozoa and an increase in the concentration of electrolytes in the spermatozoa cells (Öztürk et al., 2020). The formation of extracellular ice crystals increases the concentration of solutes contained. This osmotic gradient causes the water in the spermatozoa to diffuse out of the head through the plasma membrane, dehydrating the sperm cells. This condition causes the plasma membrane to be susceptible to lipid peroxidation by the activity of reactive oxygen species (Sobeh et al., 2020). According to Ramírez-Reveco et al. (2016), the percentage of post-thawing viability of sperm cells declared as the best value for artificial insemination is 64-80.

Individual variations of Bali cattle caused significant differences ($p < 0.05$) in the abnormalities values of AD and PT2 groups, while the PT1 group did not differ significantly ($p > 0.05$, Table 2). The highest values of the average abnormality obtained in AD, PT1, and PT2 were 6.50 ± 2.99% (Individual I), 9.66 ± 1.04% (Individual C), and 13.58 ± 4.66% (Individual A), respectively, while the lowest were 4.46 ± 1.27% (Individual G), 8.97 ± 1.29% (Individual A), and 10.12 ± 1.24% (Individual J), respectively. The results obtained were higher than that of Indriastuti et al. (2020), which were 3.45 ± 0.79 - 5.00 ± 0.37% and 4.15 ± 0.93 - 7.80 ± 1.29% before and after freezing. However, it was lower than the study by Surahman et al. (2021), which was 23.2% in semen after freezing.

The abnormality value indicates the percentage of spermatozoa with abnormal morphology throughout spermatogenesis or in the reproductive tract till ejaculation. Individual variations in the AD and PT2 groups led to significant differences in the abnormalities (Figure 1B), while the PT1 group did not differ in the abnormalities (Table 2). The differences in each of these individuals can be caused by the ability of each to maintain the stability of their cell membranes differently, hence, giving a different response to the abnormality of the spermatozoa produced. According to Parameswari and Sridharan (2019), the deterioration of the morphology can be avoided when the stability of the spermatozoa cell membrane is maintained. Furthermore, long cold storage time causes pressure changes and the production of free radicals (Bustani and Baiee, 2021). Extreme changes in osmotic pressure and ice crystals during cooling and freezing experienced by spermatozoa result in deterioration and abnormalities (Upadhyay et al., 2021). As a result of the freezing and thawing process, abnormalities in the midpiece of spermatozoa are acceptable (Ghirardosi et al., 2018). According to the 2021 Indonesian National Standard, a maximum of 20% spermatozoa abnormality is a requirement for frozen semen (Nugraha et al., 2021). Perry (2021) stated that the quality of semen could be doubted when it has a spermatozoa abnormality value of more than 20%. A high value of sperm abnormality could affect fertilization ability (Perry, 2021). According to a study of Firhamsah et al. (2022), Bali cattle with low spermatozoa abnormality values had a higher chance of successful insemination due to the number of cattle that do not return to heat for 60-90 days after mating or insemination.

**Plasma membrane and intact acrosome**

Individual variations caused significant differences in the intact plasma membrane of spermatozoa in AD and PT1 ($p < 0.05$), such a difference was not observed in the PT2 group ($p > 0.05$). Individual D had the highest intact plasma membrane values in the AD and PT1 groups, with 84.88 ± 6.07% and 67.8 ± 33.29%, while Individual B had the lowest intact plasma membrane values in the PT2 group, with 61.4 ± 51.95%, as shown in Table 3. The results obtained were in the same range as those by Indriastuti et al. (2020), which were 77.88 ± 1.64% - 86.22 ± 0.82% (before freezing) and 68.58 ± 0.86% - 77.09 ± 0.58%. However, it was higher than other research, which reported that Bali cattle's intact plasma membrane values after freezing were 43% (Diansyah et al., 2021) and 44.60 ± 0.6% - 48.21 ± 0.9% (Yendraliza et al., 2019).

The plasma membrane protects the spermatozoa against external conditions, such as entering certain substances (Diansyah et al., 2021). Sperm cells exposed to a solution with a higher osmotic pressure caused the tail to swell and coil, as shown in Figure 2A. Deterioration of the plasma membrane results in disruption of the metabolic process of spermatozoa cells, decreasing viability and increasing abnormalities (Ugur et al., 2019). Intact plasma membranes are also needed to perform normal functions and metabolism and induce capacitation and acrosome reactions (Cunha et al., 2017), enabling sperm cells to interact with oocytes (Tulake et al., 2015). The best value of intact plasma membrane was obtained in individual C. According to Indriastuti et al. (2020), variations of Bali cattle affect the proportion of intact plasma membranes. The proportion of the plasma membrane is influenced by the components of the membrane consisting of phospholipids, proteins, and carbohydrates (Öztürk et al., 2020).

Cell membranes containing cholesterol become sensitive to changes in temperature (De Toni et al., 2021). The condition of the spermatozoa cells membrane is influenced by the fatty acid composition and lipid ratio (Mandal et al., 2014). During freezing, extreme temperatures and osmotic pressure alter the structure and lipid composition of the plasma membrane in each part of the sperm cells (Cheng et al., 2022), which causes functional deterioration, resulting in decreased motility, circular movement, and premature death (Reis et al., 2016; Shan et al., 2021). The condition of the plasma membrane is related to the intact acrosome cap. This is because the deterioration of the plasma membrane is usually accompanied by that of the organelles of the intact acrosome cap cells, causing the release of enzymes needed during the fertilization process (Arvioges et al., 2021).



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The highest mean values of intact acrosomes of Bali cattle semen in AD, PT1, and PT2 groups were 88.58 ± 3.57% (Individual J), 74.04 ± 4.76% (Individual E), and 71.28 ± 9.99% (Individual B), respectively. Individual variations of Bali cattle caused significant differences in the intact acrosome cap values of AD, PT1, and PT2 groups ($p < 0.05$, Table 3). Before freezing, Bali cattle's average intact acrosome cap was 91.06% (Damayanti et al., 2021), while frozen semen was 64.12 ± 1.21-76.82 ± 1.55% (Prihantoko et al., 2020).

**Table 3.** Plasma membrane and intact acrosome value in different Bali cattle individuals aged 7-12 years

| Individuals | Intact plasma membrane (%) | | | Intact acrosome (%) | | |
|---|---|---|---|---|---|---|
| | AD | PT1 | PT2 | AD | PT1 | PT2 |
| A | 79.05 ± 2.92$^{ab}$ | 57.17 ± 5.36$^{a}$ | 60.86 ± 2.13 | 81.14 ± 6.79$^{a}$ | 68.85 ± 6.49$^{a}$ | 65.61 ± 7.51$^{abc}$ |
| B | 84.10 ± 4.75$^{cd}$ | 66.33 ± 3.28$^{cd}$ | 61.45 ± 1.95 | 83.43 ± 2.63$^{abc}$ | 70.85 ± 4.96$^{ab}$ | 71.28 ± 9.99$^{c}$ |
| C | 84.88 ± 6.07$^{d}$ | 67.83 ± 3.29$^{d}$ | 60.23 ± 3.34 | 83.62 ± 6.45$^{abc}$ | 67.88 ± 4.08$^{a}$ | 60.91 ± 5.26$^{a}$ |
| D | 80.23 ± 6.19$^{abc}$ | 65.09 ± 4.09$^{bcd}$ | 60.97 ± 3.02 | 84.96 ± 5.41$^{abc}$ | 68.62 ± 5.68$^{a}$ | 62.74 ± 8.52$^{ab}$ |
| E | 82.88 ± 5.64$^{bcd}$ | 65.19 ± 4.51$^{bcd}$ | 60.37 ± 2.78 | 87.09 ± 6.12$^{bc}$ | 74.04 ± 4.76$^{b}$ | 65.56 ± 7.51$^{abc}$ |
| F | 81.80 ± 4.12$^{abcd}$ | 65.09 ± 4.53$^{b}$ | 60.30 ± 2.78 | 86.97 ± 6.55$^{bc}$ | 72.27 ± 6.27$^{ab}$ | 66.67 ± 8.34$_{abc}$ |
| G | 79.73 ± 5.07$^{abc}$ | 67.53 ± 3.24$^{d}$ | 60.23 ± 2.63 | 87.09 ± 5.29$^{bc}$ | 69.25 ± 4.98$^{ab}$ | 60.55 ± 2.67$^{a}$ |
| H | 82.18 ± 4.05$^{bcd}$ | 66.94 ± 3.25$^{d}$ | 60.05 ± 3.01 | 82.49 ± 7.39$^{ab}$ | 67.25 ± 5.67$^{a}$ | 63.34 ± 6.03$^{ab}$ |
| I | 77.61 ± 4.79$^{a}$ | 62.96 ± 2.97$^{bc}$ | 58.72 ± 3.54 | 84.27 ± 6.86$^{abc}$ | 68.88 ± 3.07$^{a}$ | 69.77 ± 9.74$^{bc}$ |
| J | 83.14 ± 6.62$^{bcd}$ | 66.93 ± 4.23$^{d}$ | 61.01 ± 2.50 | 88.58 ± 3.57$^{c}$ | 73.89 ± 5.10$^{b}$ | 59.55 ± 7.81$^{a}$ |

$^{a,b,c}$Different superscripts in the same column were significantly different ($p < 0.05$). AD: After adding A1 diluent; PT1: Post-thawing cooling time of 4 hours; PT2: Post-thawing cooling time of 22 hours.

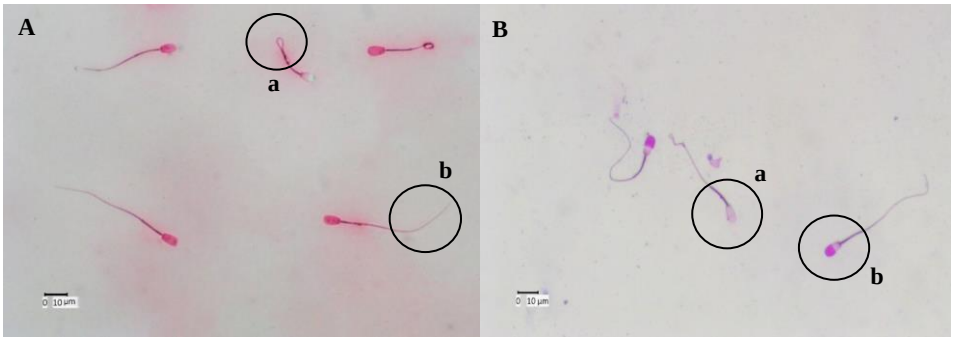


**Figure 2.** Identification of plasma membrane integrity using HOS test solution and acrosome cap using Giemsa staining in Bali cattle sperm aged 7-12 years. **A:** Intact plasma membrane (**a**), Deterioration plasma membrane (**b**), **B**: Intact acrosome cap (**a**), Deterioration acrosome cap (**b**)

The best percentage of intact acrosome cap in the PT1 group was for Individual E, with a value of 74.04%, but it was not different from individual J with a value of 73.89%. Before freezing, individual J had the highest intact acrosome cap value of 88.58%; hence, it maintained the condition of the acrosome cap after freezing. The differences obtained can result from variations in the protein profile of each individual. The distribution of tyrosine-phosphorylated acrosome protein from each male is different, resulting in individual differences in maintaining acrosome stability after the freezing process (Arai et al., 2017). Due to its small molecular weight, Giemsa staining can bind to proteins on the membrane and can pass through cell membranes that protect the acrosomes (Nofa et al., 2018; Prihantoko et al., 2020). Furthermore, the integrity acrosome is needed to ensure the success of spermatozoa in fertilizing the egg because the cap protects the enzymes contained (Sun et al., 2020).

The male sperm cells with a fertility rate of more than 53% have a high percentage of acrosome integrity (Yániz et al., 2021). Acrosome integrity of Bali cattle spermatozoa was observed using Giemsa staining. The dark purple color of the head indicated an intact acrosome cap, while the light purple or even colorless showed spermatozoa with incomplete acrosome caps (Figure 2B). The acrosome is a structure located in the apical part of the head, which plays a vital role in fertilization. Therefore, its deterioration results in the release of enzymes from the interior and directly causes the spermatozoa to lose their fertilizing ability (Hirose et al., 2020). The acrosome contains glycohydrolases and acrosins responsible for binding and penetrating the zona pellucida (Nagdas et al., 2016; Adrian et al., 2019). The binding of spermatozoa to the zona pellucida results in acrosome reactions and the release and activation of its enzymes, which allows penetration (Sawada and Saito, 2022). The percentage of intact acrosome caps in each individual is influenced by the ability of the membrane function to protect the sperm acrosome (Sitepu and Marisa, 2019).

## CONCLUSION

The individual variations in semen before freezing can affect the parameters of spermatozoa deterioration, including viability, abnormalities, intact plasma membrane, and intact acrosome. Individual variations at 5°C in the cooling time of



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4 hours had better viability, abnormalities, intact plasma membrane, and acrosome caps of spermatozoa, compared to cooling at 5°C for 22 hours.

## DECLARATIONS


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### Authors' contribution

Angelina Novita Tethool, Gatot Ciptadi, Sri Wahjuningsih, and Trinil Susilawati contributed to the study design, data analysis, and manuscript writing. Angelina Novita Tethool collected samples from the field and performed laboratory analysis. All authors reviewed the data from this study, performed statistical analyses, and approved the final draft of the manuscript. The authors reviewed and approved the final manuscript prior to submission to the journal.

### Competing interests

The authors declare that there are no competing financial, and personal interests that might influence the research presented here.

### Ethical considerations

The authors declare that this manuscript is original, has been checked by all the authors, and is not currently being considered for publication elsewhere.

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# Effects of Heat Stress on Growth Performance, Carcass Traits, Physiological Components, and Biochemical Parameters in Local Algerian Growing Rabbits

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**ABSTRACT**

Heat stress is a detrimental factor affecting the welfare of all livestock, especially rabbits, as they are sensitive to high temperatures. The current study investigated the effect of high ambient temperature on growth performance, slaughter traits, physiological indicators, and some hematological and biochemical parameters in Algerian local growing rabbits. A total of 48 local rabbits of both sexes (35 days old) were allotted into two groups (24 per group). The control group rabbits were exposed to an ambient temperature and humidity, averaging 21.8 ± 1.3°C and 51.7 ± 3.6%, respectively. Rabbits in the heat stress group were subjected to a warm ambient temperature and humidity of 30.5 ± 1.82°C and 65.5 ± 7.2%, respectively. The growth performance was measured and calculated from 35 to 91 days of age. Physiological indicators (rectal, skin, and ear temperatures, respiratory, and heart rates) were examined at 88 days of age. The carcass traits, blood metabolites, and hematological parameters of rabbits were measured and calculated at slaughter (92 days of age). The obtained results indicated a decrease in body weight, daily gain, and daily feed intake of rabbits in heat stress rabbits, compared to the control group. However, feed conversion ratio was significantly higher in the heat stress group, compared to the control. Heat stress group rabbits showed significantly higher blood metabolite levels, except the glycemia, which was similar in both groups. No significant effect of heat stress was found on the carcass yield, anterior, posterior, and intermediate parts of the carcass. However, the yield of the other components of the carcass (liver, kidney, peritoneal and inter-scapular fat) was significantly lower in the heat stress group. In the heat stress group, rectal, skin, and ear temperatures as well as heart and respiratory rates, were significantly higher than those of the control group. In the present experimental conditions, exposure of local rabbits to chronic heat stress could induce some changes to biological, physiological, and biochemical parameters leading to altered growth performance.

**Keywords:** Carcass yield, Growth performance, Heat stress, Local rabbit, Metabolic profile, Thermoregulation

## INTRODUCTION

Rational rabbit farming is currently of great importance due to its potential contribution to satisfying the growing human population's need for animal proteins (Dalle Zotte, 2014; Cherfaoui, 2015). Rabbit production is advantageous due to the high prolificacy and short biological cycle of rabbits as well as nutritional and organoleptic qualities of rabbit meat (Lebas, 2007; Ibitoye et al., 2010; Dalle Zotte, 2014).

Despite the importance of rabbit breeding in Algeria, its breading system remains very traditional (Saidj et al., 2013). Production is almost entirely restricted to the local rabbit population and commercial hybrid rabbit descendants (Cherfaoui, 2015; Zerrouki et al., 2014; Belabbas et al., 2019). A few modern farms rear the selected rabbit strains in a small proportion (Moula and Yakhlef, 2007).

Algeria has a long hot climate season from May to October, with an ambient temperature ranging from 28 to 35°C corresponding to chronic heat stress with frequent acute peaks of about 40-45°C (Temim, 2000; Zerrouki et al., 2005). During the last three decades, the most important topic of animal production research has been the impact of heat stress on productivity, and studies have been concerned with ways to improve production under these conditions (Gonzalez-Rivas et al., 2020; Thornton et al., 2021), for different species such as cow (Srikandakumar and Johonson., 2004), chicken (Dahmani., 2009), pig (Mayorga et al., 2018) and rabbit (Ajao and Ola, 2021)

Rabbits are very sensitive to extreme environmental conditions, particularly high temperatures, due to their heavy fur coats and non-functional sweat glands, which complicate the excess body heat elimination processes (Verga et al., 2007; Adelodun, 2015; Khaled, 2017). With high tolerance to low temperatures (Fayez et al., 1994a; Verga et al., 2007; Ashour et al., 2017), the ideal safest temperature for rabbits ranges 16-21°C (thermo-neutrality zone, Fayez et al., 1994a; Marai and Habeeb, 1994; Ashour et al., 2017). When the rabbits are exposed to high temperatures (above 25-30°C), they try to dissipate the excessive heat by various mechanisms, including thermoregulatory reactions. These thermoregulatory



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reactions affect rabbits by disrupting their physiological functions and behavioral performance (El Sabry et al., 2021; Mutwedu et al., 2021; Liang et al., 2022), such as impairment of appetite, alteration of the feed efficiency and growth performance, milk yield, and reproduction in rabbits, leading to considerable production and economic losses (Plá et al., 1994; Zeferino et al., 2011). It was reported that a reduction of body temperature to 1°C or less suppresses both physiological and growth performance in livestock (Manca et al., 2018).

Several researchers have investigated the characterization of the local rabbit population in Algeria since it is a popular population used by family farms with low sensitivity to heat (Ilès, 2017; Belabbas et al., 2019; Saidj et al., 2019). However, only a few studies have focused on the effect of heat stress on rabbits' production and productivity traits. These studies investigated the impact of heat stress on zootechnical performance while overlooking the adaptive capacity of thermoregulation as well as physiological, biochemical, and hematological responses. Therefore, the current study aimed to investigate the effects of environmental temperature (thermo-neutrality and chronic heat stress) on growth performance, carcass yield, thermoregulatory parameters, plasma hematological parameters, and biochemical parameters in local Algerian rabbits during the growth and fattening period.

## MATERIALS AND METHODS

### Ethical approval

This research was approved by the scientific council of the High National Veterinary School of Algiers, Algeria, with certificate reference 204/FDRS/2022.

### Study design

The present work was carried out at the rabbitry of the High National Veterinary School in Algiers, Algeria. The trial lasted 8 weeks during the hot months (July to August 2018). A total of 48 rabbits aged 35 days from the local Algerian population were checked by a veterinarian, then weighed and divided into two main groups of 24 rabbits with an average weight of 662 ± 9 g. These rabbits were born and bred within the rabbitry of the High National Veterinary school of Algiers and were subjected to a week of adaption (28-35 days). Each group was divided into six cages with a mean of four rabbits per cage (six replicates for each group). The animals were housed in standard fattening cages of 54 cm × 59 cm × 35 cm. The first group acted as the control (C) group and was kept in a partially air-conditioned house at thermo-neutrality with an average temperature of 21.8 ± 1.3°C and an average humidity of 51.7 ± 3.6%. The second group (HS) was kept in a different hutch and exposed to seasonal variations in the ambient temperature during the summe with an average temperature of 30.5 ± 1.82°C and an average humidity of 65.5 ± 7.19 %. Two hygro-thermometers were placed in the middle of the building for each hutch. To determine the average Temperature-humidity index (THI) values, ambient temperature and relative humidity (RH) were recorded five times daily at 8, 10,12,14, and 16 hours. The THI, an indicator of thermal comfort level for animals, was calculated using the following formula (Marai et al., 2001):

$$THI = db°C - [(0.31 - 0.31\times RH\%) \times (db°C - 14.4)]$$

Where, db°C is the dry bulb temperature in centigrade and RH denotes relative humidity (%).

The THI values were calculated to evaluate the intensity and severity of heat stress (HS) under the environmental conditions classified as the absence of heat stress (< 27.8 centigrade), moderate heat stress (27.8-28.8 centigrade), severe heat stress (28.9-29.9 centigrade), and very severe heat stress (> 30 centigrade). The rabbits in both groups had access to the same food and water *ad libitum* throughout the trial (which lasted from 28 to 91 days, including one week of adaptation from 28 to 35 days). A commercially available, well-balanced pelleted meal was fed to the rabbits. The composition and chemical analysis of the food were carried out using AFNOR-(1985) recommendations (Table1).

### Growth performance

During the experimental period, the growth performance of rabbits was recorded and calculated as initial and final live body weights, the daily body weight gain, and the daily feed intake. Finally, the feed conversion ratio (FCR) was calculated for each group.

### Physiological indicators

Thermoregulatory parameters of rabbits recorded include rectal temperature, skin temperature, ear temperature, respiratory rate, and heart rate. All efforts were made to ensure that measurements were taken under no additional environmental stress. Measurements were taken on day 88 (between 12 and 2 p.m. as the hottest time of the day) in 10 rabbits (with an average weight representative of the batch) from each group. The temperature measurements were taken using a digital medical thermometer (Thermoval Hartmann, Belgium). The rectal temperature was taken by introducing the thermometer 2-3 cm approximately in the rectum of the rabbit (Askar and Ismail, 2012). For the skin temperature, the thermometer was inserted inside a fold of skin, and the ear temperature was measured by placing the thermometer into the central internal surface area of the auricle (Marai et al., 2004). The respiratory rate was measured by counting the rabbit's flank abdominal movements for one minute using a stopwatch (Clock Mark1, Chine, Mousa-Balabel, 2004;



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Abdalla and Intsar, 2009). Finally, the heart rate was measured using a stethoscope (KaWe, Germany) for one minute (Mousa-Balabel, 2004).

**Carcass traits**

On the day after the experiment was over (day 92), 10 rabbits from each group were weighed and slaughtered (without fasting) to record slaughter yield and carcass quality measurements. The carcass dissection procedures and the carcass characteristics evaluation were carried out according to the World Rabbit Science Association (WRSA) recommendations, as described by Blasco and Ouhayoun (1993). Slaughter weight (SW) was recorded just after slaughter. After complete bleeding, slaughtered rabbits were skinned, and skin weight (S) was recorded, then the skin yield (S/SW) was calculated (skin weight as SW%). The slaughtered rabbits were eviscerated (the digestive tract and urogenital organs were removed), and their digestive tract was weighed. The intermediate and the fore and hind legs were kept to conform to the regulations of commercial carcass presentation in Algerian markets (Lounaouci, 2001). The remaining parts, which were considered hot carcasses, were chilled at 4ºC for 24 hours. After chilling, the carcasses were weighed. Chilled carcass (CC) and the carcass yield was calculated (CC weight as SW percentage). Subsequently, the liver, kidneys, perirenal fat, and inter-scapular fats were removed and weighed, and then the head was separated. The remaining parts of the carcasses were dissected into three anatomical parts (between the seventh and eighth thoracic vertebrae, and between the sixth and seventh lumbar vertebrae), also known as the fore part, intermediate part, and hind part, respectively. These parts were also weighed. Finally, the proportions of the different organs and parts (liver, kidneys, perirenal and inter-scapular fats, fore, intermediate and hind parts) to CC were calculated.

**Blood sample**

On day 92, 10 rabbits with similar average weights were slaughtered (in the fed state) and used to determine blood parameters. The blood samples were taken at the hottest time of the day (between 12-2 p.m.). Two blood samples, each 5 ml, were collected from each rabbit. The first blood samples from all the rabbits were put into tubes containing Ethylene diamine tetraacetic acid. They were analyzed shortly after collection for hematological parameters, namely hemoglobin concentration (Hb), hematocrit percentage (Hct), red blood cell count (RBC), white blood cell count (WBC), lymphocyte percentage, and monocytes percentage. These parameters were measured on fresh blood using automated hematology analyzers (automate Scil Vet abc Plus, France) as described by Post et al. (2003). The second blood samples were collected into heparinized tubes and centrifuged at 3000 rpm for 10 minutes (Sigma, Germany). The blood plasma was collected and put away at -20°C awaiting investigation. To determine the biochemical parameters, a Spectrophotometer (LKB Novastec, Austria) and a commercial kit (SPINREACT, SA, Spain) were used to analyze the blood plasma's glucose, cholesterol, triglycerides, total proteins, urea, and creatinine.

**Statistical analysis**

Statview software (Abacus Concepts, 1996, Incorporation, Berkeley, CA94704-1014, USA) was used to analyze all the measured parameters to find out how heat stress affects the parameters subjected to one-factor analysis (ANOVA). Fisher test was performed, and the data are shown as means ± standard error and the level of significance at $p < 0.05$.

**Table 1.** Composition and chemical analysis of the diet of local Algerian rabbits given to both groups

| **Ingredients** | **Percentage** |
|---|---|
| Corn grain | 4 |
| Barley grain | 17.7 |
| Wheat bran | 30 |
| Soybean meal | 8 |
| Alfalfa | 38 |
| Limestone | 0.5 |
| Dicalcium phosphate | 0.3 |
| Sodium chloride | 0.5 |
| Premix* | 1 |
| **Chemical composition (%DM)** | **Percentage** |
| Dry matter | 90.4 |
| Crude protein | 17.3 |
| Crude fiber | 13.9 |
| Fat | 2.2 |
| Minerals | 7 |
| Crude energy (Kcal/kg) | 3460 |
| Digestible energy (Kcal/kg) | 2460 |

DM: Dry matter, Premix: Mineral and vitamin complement. *1kg premix: Methionine (%) 10, Sodium (%) 9.9, Calcium (%) 20.3, Chlorine (%) 15.3, Vitamin A (IU/kg) 1000000, Vitamin D3 (IU/kg) 150000, Vitamin E (mg/kg)1000, Vitamin K3 (mg/kg)100, Vitamin B1 (mg/kg) 100, Vitamin B2 (mg/kg) 300, Vitamin B3(mg/kg) 2000, Vitamin B5 (mg/kg) 600, B6 (mg/kg)150, B9 (mg/kg) 20, Vit B12 (mcg) 1000, Choline Chloride (mg/kg) 25000, Iron (mg/kg) 5000, Manganese (mg/kg) 7000, Copper (mg/kg) 1000, Zinc (mg/kg) 5000; Iodine (mg/kg) 100, Selenium (mg/kg) 25, Antioxidant (mg/kg) 41.6.



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## RESULTS

### The temperature-humidity index

The average, maximum, minimum, ambient temperature, Relative humidity, and THI values are shown in Table 2. The daily average THI is shown in Figure 1 for the whole experimental period (35-91 days). The estimated average THI values were 31.3 and 22 for the HS and C groups, respectively. Minimum and maximum THI averages in both hutches were 27.4 and 34.7 for the HS group and 19 and 24.2 for the C group, respectively.

### Physiological indicators

Heart rate, respiratory rate, rectal temperature, skin temperature, and ear temperature are shown in Table 3. Heat stress negatively affected all thermoregulatory parameters. Rectal temperature, skin temperature, ear temperature, and heart and respiratory rates were +1.34°C, +1.09°C, + 3.44°C, 34.8 beats/minute and 15.7 breaths/minute higher in the HS group than the control, respectively ($p < 0.05$).

### Growth performance

The effects of chronic heat stress on body weight, body weight gain, feed intake, and FCR in local Algerian rabbits are presented in Table 4. At the beginning of the experiment (age of 35 days), the rabbits of both groups had almost similar initial live weights (control versus heat stress = 655.61 ± 23.19 g versus 668.88 ± 30.8 g, $p > 0.05$). At the end of the fattening period (day 91), decreases of 10%, 14%, and 13% were respectively recorded in the body weight, average daily gain, and average daily feed intake of the HS group, compared to the C group ($p < 0.05$). However, FCR was significantly higher in the HS group compared to the C group (+11%, $p < 0.05$).

### Carcass traits

Table 5 compares carcass yield, weights and proportions of the perirenal and inter-scapular fats, kidney, liver, and different parts of the carcass. Statistical analysis revealed that rabbits subjected to heat stress had a lower average live weight at slaughter than rabbits reared at thermo-neutrality (-9%, $p < 0.05$). It has also been shown that the skin and the full digestive tract were both lighter in weight (-13% and -18%, $p < 0.05$, respectively). However, both groups recorded similar results for the chilled carcass weight, carcass yield, and skin yield. Average weights of the liver, kidneys, inter-scapular, and perirenal fats were lower (26%, 19%, 26%, and 40%, $p < 0.05$) in rabbits of the HS group than in the C group. The proportions of the liver, kidney, inter-scapular and perirenal fats to chilled carcass were significantly higher in the C group, compared to the HS group (19%, 12%, 19, 33%, $p < 0.05$, respectively). No differences were recorded in weights and proportions of different parts of the carcass except in the weight of the hind part, which was reduced by 9% in the HS group, compared to the C group (453.18g versus 498.51, $p < 0.05$).

### Biochemical and hematological parameters

The effect of heat stress on blood biochemistry and hematological parameters is presented in Table 6. A significant increase in the concentrations of plasma triglyceride (36%, $p < 0.05$), cholesterol (21%, $p < 0.05$), total proteins (11%, $p < 0.05$), urea (11%, $p < 0.05$) and creatinine (15%, $p < 0.05$) were recorded in the HS group, compared to C group, whereas a significant difference was not observed in plasma glucose concentration between the two groups ($p < 0.05$). The hematological parameters were significantly influenced by the heat stress of local Algerian rabbits, compared to those under thermos-neutrality ($p < 0.05$). The present results are shown in Table 6. A significant increase in RBC count, Hb concentration, Hct percentage, and monocyte rate was recorded in the HS group, compared to the C group (36%, 9%, 5%, and 16%, $p < 0.05$, respectively). However, a decrease in WBC count (25%, $p < 0.05$) and an insignificant decrease in the lymphocyte level (10%, $P > 0.05$) were registered in HS group.

**Table 2.** The average temperature, humidity, and temperature-humidity index during the experimental period in rabbitries

| Group | Temperature (°C) | | | Relative humidity (%) | | | THI | | |
|---|---|---|---|---|---|---|---|---|---|
| | Min | Max | Av | Min | Max | Av | Min | Max | Av |
| Control | 19.00 | 23.8 | 21.8 ± 1.28 | 44.4 | 60.6 | 51.7 ± 3.50 | 19 | 24.2 | 22 ± 1.37 |
| Heat stress | 26.9 | 33.6 | 30.5 ± 1.82 | 48.5 | 78.4 | 65.5 ± 7.19 | 27.4 | 34.7 | 31.3 ± 1.91 |

Min: Minimum, Max: Maximum, Av: Average, THI: Temperature-humidity index



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**Table 3.** Effect of heat stress on the thermoregulatory parameters of local Algerian rabbits

| Items | Control | Heat stress | SEM | p-value |
|---|---|---|---|---|
| Heart rate (beats/minute) | 85.2 ± 1.36 | 120 ± 2.50 | 1.93 | p < 0.05 |
| Respiratory rate (breaths/minute) | 68 ± 2.18 | 83.7 ± 0.98 | 1.58 | p < 0.05 |
| Rectal temperature (°C) | 38.74 ± 0.09 | 40.08 ± 0.12 | 0.10 | p < 0.05 |
| Skin surface temperature (°C) | 38.15 ± 0.17 | 39.24 ± 0.17 | 0.17 | p < 0.05 |
| Ear surface temperature (°C) | 33.46 ± 0.18 | 36.9 ± 0.15 | 0.16 | p < 0.05 |

SEM: Standard error of the mean

**Table 4.** Growth performance of local Algerian rabbits subjected to heat stress

| Traits | Control | Heat stress | SEM | p-value |
|---|---|---|---|---|
| Body weight at 35 days (g) | 655.61 ± 23.19 | 668.88 ± 30.8 | 27 | p > 0.05 |
| Body weight at 91 days (g) | 2269.67 ± 53.26 | 2052.28 ± 38.13 | 45.49 | p < 0.05 |
| Body weight gain (g) | 28.88 ± 0.40 | 24.74 ± 0.57 | 0.48 | p < 0.05 |
| Feed intake (g) | 87.79 ± 3.59 | 76.27 ± 2.77 | 6.36 | p < 0.05 |
| Feed conversion ratio | 3.01 ± 0.06 | 3.39 ± 0.05 | 0.05 | p < 0.05 |

SEM: Standard error of the mean

**Table 5.** Effect of heat stress on carcass traits of local Algerian rabbits

| Traits | Control | Heat stress | SEM | p-value |
|---|---|---|---|---|
| **Weight (g)** | | | | |
| Average live weight at slaughter | 2259.49 ± 68.20 | 2045.82 ± 45.75 | 56.97 | p < 0.05 |
| Skin weight | 211.89 ± 8.44 | 183.18 ± 4.83 | 6.63 | p < 0.05 |
| Full digestive tract weight | 428.28 ± 15.07 | 349.58 ± 15.68 | 15.37 | p < 0.05 |
| Chilled carcass | 1423.22 ± 48.69 | 1314.26 ± 31.09 | 39.89 | p > 0.05 |
| Liver | 77.41 ± 2.58 | 56.94 ± 2.72 | 2.67 | p < 0.05 |
| Kidney | 12.42 ± 0.44 | 10.09 ± 0.29 | 0.36 | p < 0.05 |
| Interscapular fat | 7.01 ± 0.64 | 5.18 ± 0.43 | 0.53 | p > 0.05 |
| Perirenal fat | 22.71 ± 2.20 | 13.59 ± 0.98 | 1.59 | p < 0.05 |
| Fore part | 458.58 ± 13.59 | 425.65 ± 11.37 | 12.48 | p > 0.05 |
| Intermediate part | 228.61 ± 12.07 | 220.08 ± 8.84 | 10.45 | p > 0.05 |
| Hind part | 498.51 ± 17.59 | 453.18 ± 9.98 | 13.78 | p < 0.05 |
| **Yield (%)** | | | | |
| CC/SW | 62.89 ± 0.05 | 64.67±1.90 | 1.20 | p > 0.05 |
| S/SW | 9.35 ± 0.17 | 8.96±0.13 | 0.15 | p > 0.05 |
| **Proportion (%)** | | | | |
| L/CC | 5.50 ± 0.21 | 4.42 ± 0.29 | 0.25 | p < 0.05 |
| K/CC | 0.88 ± 0.02 | 0.77 ± 0.02 | 0.02 | p < 0.05 |
| PF/CC | 1.55 ± 0.11 | 1.03 ± 0.06 | 0.08 | p < 0.05 |
| ISF/CC | 0.48 ± 0.03 | 0.39 ± 0.03 | 0.03 | p < 0.05 |
| FP/CC | 32.33 ± 0.28 | 31.53 ± 0.81 | 0.54 | p > 0.05 |
| IP/CC | 15.90 ± 0.42 | 16.65 ± 0.41 | 0.41 | p > 0.05 |
| HP/CC | 35.03 ± 0.25 | 34.54 ± 0.3 | 0.27 | p > 0.05 |

CC: Chilled carcass, FP: Fore part, HP: Hind part, ISF: Interscapular fat, IP: Intermediate part, K: Kidney, L: Liver, PF: Perirenal fat, S: Skin, SW: Live weight at slaughter, SEM: Standard error of the mean

**Table 6.** Effect of heat stress on the biochemistry and hematological parameters of local Algerian rabbits

| Parameters | Control | Heat stress | SEM | p-value |
|---|---|---|---|---|
| **Biochemistry** | | | | |
| Glucose (mmol/L) | 8.15 ± 0.33 | 7.77 ± 0.22 | 0.27 | p > 0.05 |
| Triglycerides (mmol/L) | 1.36 ± 0.04 | 2.12 ± 0.06 | 0.05 | p < 0.05 |
| Cholesterol (mmol/L) | 2.13±0.09 | 2.69 ± 0.11 | 0.10 | p < 0.05 |
| Total Proteins (g/L) | 74.08 ± 2.08 | 83.43 ± 2.51 | 2.29 | p < 0.05 |
| Urea (mmol/L) | 6.60 ± 0.22 | 7.42 ± 0.27 | 0.24 | p < 0.05 |
| Creatinine (mg/dl) | 1.37 ± 0.04 | 1.61 ± 0.05 | 0.04 | p < 0.05 |
| **Hematology** | | | | |
| Hemoglobin (g/dl) | 10.46 ± 0.25 | 11.44 ± 0.19 | 0.22 | p < 0.05 |
| Haematocrit (%) | 35.11 ± 0.63 | 36.94 ± 0.59 | 0.61 | p < 0.05 |
| Red blood cells count (*$10^3$/µl) | 1.41 ± 0.09 | 2.21 ± 0.28 | 0.18 | p < 0.05 |
| White blood cells count (*$10^3$/µl) | 8.23 ± 0.64 | 6.19 ± 0.56 | 0.60 | p < 0.05 |
| Lymphocytes (%) | 65.91 ± 2.95 | 59.32 ± 2.21 | 2.58 | p > 0.05 |
| Monocytes (%) | 13.51 ± 0.78 | 16.07 ± 0.73 | 0.75 | p < 0.05 |

SEM: Standard error of the mean



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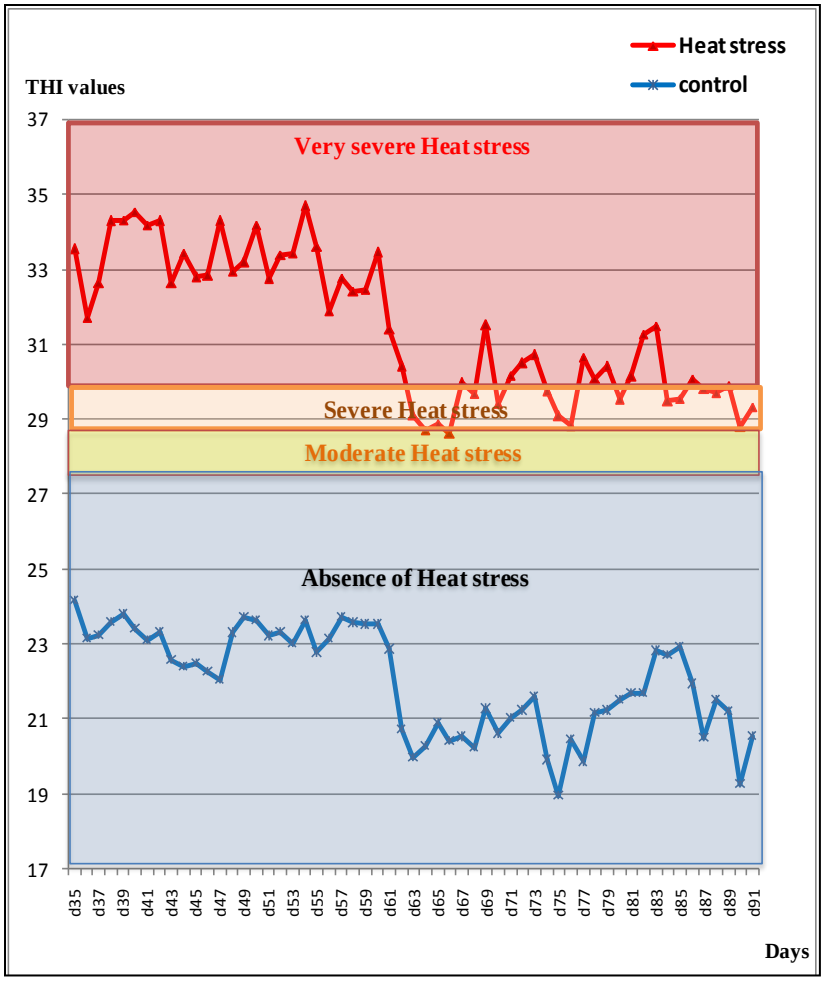


**Figure1.** Evolution of daily mean temperature-humidity index during the experimental period (35-91 days)

## DISCUSSION

### Temperature-humidity index

The recorded values of THI during this experiment strongly show that the control group was indeed raised in thermo-neutrality (22 ± 1.37) whereas the rabbits of the heat stress group were exposed to severe heat stress (31.3 ± 1.91). Similarly, a study by Marai et al. (2001) revealed that THI values <27.8 corresponded to the absence of heat stress, whereas THI values more than >30 indicated the presence of heat stress. The recorded temperatures for both groups showed that the control group was subjected to temperatures within the range of 18-21°C corresponding to thermoneutrality (comfort zone for rabbits) whereas the rabbits of the second group were indeed subjected to temperatures exceeding 30°C which in turn correspond to chronic heat stress (Fayez et al., 1994a; Marai and Habeeb, 1994).

### Physiological indicators

All thermoregulatory parameters are affected by heat stress (Heart rate, respiratory rate, and body, skin, and ear temperatures). Abdelnour et al. (2020) considered that rabbits could keep their body temperature constant by regulating heat loss using physical and morphological processes. The results obtained in this study are similar to some previous studies (Khalil et al., 2014; Adelodun., 2015; Sabah et al., 2017). During heat stress, both indicators (rectal temperature and respiratory rate) suggested a compensatory response of the animals to the imposed thermal stress (Yamani and Khalil 1994). Verma et al. (2000) considered rectal temperature as one of the most sensitive indicators of heat tolerance This has also been confirmed by many other studies, concerned with physiological reactions conditions on animal species, such as cows (Srikandakumar and Johonson., 2004), chickens (Dahmani., 2009), pigs (Waltz et al ., 2014) and rabbits (Ajao and Ola, 2021)

Rabbits are very sensitive to extreme environmental conditions, particularly ear temperatures due to rabbit exposure to severe heat stress has been confirmed by previous studies on fattening rabbits during chronic heat stress (Adelodun, (2015), on adult rabbits in chronic heat stress (Asemota et al., 2017; Sabah et al., 2017; Ajao and Ola, 2021), and young rabbits during acute heat stress (Amici et al., 2000; Khalil et al., 2014).



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The rectal temperature values obtained in the current study are not in agreement with the values recorded by Zeferino et al. (2011), who showed no effect of heat stress on rectal temperature, and they assumed that rabbits have an efficient thermoregulatory system. Furthermore, rabbits raised in warm conditions increased their respiratory rate (16 breaths/minutes, $p < 0.05$), compared to rabbits raised in optimal conditions. Present results are in agreement with several studies on growing rabbit (Zeferino et al., 2011; Adelodun, 2015) and on adult rabbits (Popoola et al., 2014; Jimoh and Ewuola, 2016; Asemota et al., 2017). However, Marai et al. (1999) did not find a significant difference in respiration rate between rabbits raised in hot conditions and those raised under thermo-neutrality conditions.

It was reported that hyperthermia is associated with tachycardia (Juneet et al., 2013). Hyperthermia produces a hyper-metabolic state with increased catecholamine stimulation, tachycardia, and possibly an increased risk of ventricular fibrillation and ventricular tachycardia. The present study reported an accelerated heart rate in the rabbits subjected to chronic heat stress (35 beats/minutes). These results agree with the reports of Adelodun (2015), Jimoh and Ewuola (2016), Asemota et al. (2017), and Sabah et al. (2017). However, the study by Abdalla and Intsar (2009) reported a slower heart rate for rabbits raised in summer than in winter.

Rabbits are very sensitive to high temperatures (above 25-30°C) since they have few functional sweat glands limiting their ability to eliminate excess body heat (Adelodun, 2015; Abd El-Monem et al., 2016, Mousa-Balabel et al., 2017), in addition to their perspiration being hindered by their fur (Marai et al., 2001). When animals are exposed to high temperatures above 25-30°C, their body temperature rises. Rabbits try to balance the excessive heat load using different means to dissipate it as much as possible (Abdel-Hamid and Dawod, 2015; El Sabry et al., 2021; Mutwedu et al., 2021).

In order to dissipate heat, rabbits respond in a number of mechanisms, such as increasing vasodilatation with increased blood flow to the skin surface. Sweating and speeding their respiratory rate are other means that help rabbits release heat by vaporizing high moisture through respiratory air accounting for 30% of total heat dissipation (Mousa-Balabel, 2004). Rabbits stretch their ears to dissipate heat through radiation and convection, similar in function to a radiator (Marai et al., 2007). These responses make the heart work hard and result in the body's loss of salt and water through perspiration and urination, affecting the rabbit's efficiency, and causing haemo-concentration (Farghly et al., 2021; Oladimeji et al., 2022). Exposing rabbits to severe heat stress activates physiological mechanisms to balance the excessive heat load, leading to an increase in the thermoregulatory parameters (Adelodun et al., 2015; Jimoh and Ewuola, 2016; Abd El-Monem et al., 2016).

**Growth performance**

All growth performance parameters were lower in heat stress than in thermo-neutrality. A significant decrease was observed in final body weight, daily weight gain, and feed intake ($p < 0.05$).

The animals raised under heat stress had noticeably lost weight, especially during the last two weeks of the experiment, which is in agreement with the findings of the studies of Lakabi (2010), who reported a decrease of 13% in the live weight of local Algerian rabbits aged 11-14 weeks raised in summer, compared to those raised in optimal conditions. Similar results have already been reported in other studies conducted on rabbits of selected breeds during the growing period (Dalle Zotte and Paci, 2014; Terhes et al., 2018; Matics et al., 2021), and on the adult rabbits by Okab et al. (2008) and Khaled (2017) confirming that all rabbits show the same response regardless of breed, age or gender. The FCR was significantly higher in HS rabbits ($p < 0.05$) than in thermoneutrality rabbits during 35-91 days, which can be explained by better feed efficiency in thermoneutrality rabbits. The results are echoed in studies of Marai et al. (1999) and Ali and Abdel-Wareth (2014), who have reported a lower feed efficiency in HS rabbits leading to a higher FCR. On the other hand, Fayez et al. (1994b), Ayyat and Marai (1997), and Zeferino et al. (2011) did not find a significant effect of heat stress on FCR. On the contrary, other studies have found a lower FCR in rabbits raised in heat stress conditions leading to higher feed efficiency. Ondruska et al., 2011; Terhes et al., 2018; Matics et al., 2021)

The failure in the zootechnical performance of rabbits subjected to thermal stress is probably due to the reduction in feed intake (Ali and Abdel-Wareth, 2014; Liang et al., 2022). A decrease in feed consumption is a common reaction to heat stress conditions (Ali and Abdel-Wareth, 2014; Okab et al., 2008). This reduction is the result of the peripheral thermal receptors stimulation, which transmit suppressive nerve impulses to the appetite center in the hypothalamus, causing a decrease in feed intake (Dalle Zotte and Paci, 2014; Terhes et al., 2018; Liang et al., 2022), thereby a decreased feed efficiency and live weight (Farghly et al., 2021; Oladimeji et al., 2022). Ali and Abdel-Wareth (2014) suggested that the lower body weight may be due to the increase in energy consumption by increasing the respiratory rate during heat stress. Hence, low metabolizable energy is left for growth requirements, which explains the low weight of animals exposed to heat stress.

**Carcass traits**

The HS rabbits recorded a significantly lighter live weight at slaughter and lower skin and digestive tract weights than thermoneutrality rabbits. These findings probably resulted from a decrease in feed intake and poor feed efficiency, leading to the harmful effects of chronic heat stress ($p < 0.05$). Lakabi et al. (2004) reported similar results to those of



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the present study, indicating that local Algerian rabbits raised under heat stress recorded lighter weights of skin and digestive tract as well as a lighter live weight at slaughter. Other studies conducted on different rabbit breeds have revealed that heat-stressed rabbits had a lighter live weight and skin and digestive tract weights at slaughter (Dalle Zotte and Paci., 2014; Terhes et al., 2018).

However, Lakabi (2010) worked on the local Algerian population and did not find the same results and did not record a significant effect of the season on local rabbits' slaughter weight. At the same time, the present study recorded a similar carcass yield (Cf/PV) and chilled carcass weight in rabbits raised in optimal conditions to those reared in a warm climate ($p > 0.05$). According to the results of the current study, the similarity in the weight of the carcass yield while the slaughter weight is different was due to the low weights of the skin and the digestive tract in the HS group in comparison to the control group, this is also attributed to the loss of water during the carcass bleeding as well as the blood volume after bleeding and draining of the carcass which was more significant in the control group.

In the heat-stressed rabbits, the low weights of the perirenal and inter-scapular fat and their proportions recorded during the experiment could be explained by their low feed intake, which reduced the amount of energy available for the animal to meet its maintenance requirements and to regulate its internal temperature better. It decreased adipogenesis and increased adipolysis by hydrolysis, reducing fatty deposits and favouring the loss of water, consequently resulting in less adiposity (Marai et al., 1999; Ayyat and Marai, 1997; Chiericato et al., 1996). These results are in agreement with previous studies, indicating that rabbits raised under heat stress had less perirenal and interscapular fat weights than those raised under thermoneutrality (Marai et al., 1999; Terhes et al., 2018). On the other hand, Lakabi (2010), Matics et al. (2021), and Zeferino et al. (2013) have reported no significant effect of heat stress on adiposity in fattening rabbits.

In the present study, heat stress significantly reduced liver and kidney weights and their proportions ($p < 0.05$). Some studies reported negative effects of heat stress on rabbits' organs yields in fattening rabbits of selected breeds (Chiericato et al., 1993; Bhatt et al., 2002; Zeferino et al., 2013) and in the local Algerian population fattening rabbits (Lakabi et al., 2004). In accordance with the present results, Bhatt et al. (2002) found that the weights of livers and kidneys were directly proportional to their respective chilled carcass weights on day 84, but no such trend existed for liver weight on day 98. In contrast to the present findings, other studies showed no significant effect of heat stress on liver weight and yield, but they found lower kidney weight and proportion in heat group rabbits compared to those in the control group (Ayyat and Marai, 1997; Marai et al., 1999). According to Chiericato et al. (1993) and Bhatt et al. (2002), the reduced weights and proportions of the liver and kidneys in rabbits subjected to chronic heat stress are probably due to them being proportional to the live weight of the animal at slaughter. At high ambient temperatures (30.5°C), the current study revealed that rabbits reduced their feed intake, and consequently, fewer quantities of nutrients were available for the internal organs, which compromised their development.

Concerning the different parts of the carcass (fore part, Intermediate part; andhind part ), the proportions and the recorded weights were similar for the two batches except for the hind part weight, which was slightly reduced in the heat stress batch ($p < 0.05$) These findings are in agreement with those found by Marai et al. (1999) and Zeferino et al. (2013), as well as Terheset al. (2018), who did not find differences in the proportions and weights of different parts of the carcass as a result of season. In contrast to the current findings, Ayyat and Marai (1996) noted a significant effect on the proportions of the fore and the hind parts, which were higher in rabbits reared in thermoneutrality, with no effect recorded on the intermediate part.

**Biochemical and hematological parameters**

Results of the present study clearly showed that heat stress significantly ($p < 0.05$) affected almost all of the biochemical and hematological parameters (blood plasma's cholesterol, triglycerides, total proteins, urea, and creatinine, Hb, Hct, RBC, WBC, and monocytes percentage). Heat stress significantly increased blood metabolites, compared to those recorded under control temperature, except glycemia which was similar for both groups ($p < 0.05$). The increase in total proteins in hyperthermic animals recorded during the current study is in accordance with the results reported by Okab and El-Banna (2008), which indicated that this increase helps rabbits resist heat stress by helping the body retain water in the intravascular fluids, and so sustain the blood viscosity which compensates for the water that is lost through evaporation. This non-evaporative heat dissipation mechanism efficiently shifts the heat from inside the skin (holding the water inside the body to make up for evaporated lost water). On the other hand, the results of the present study do not corroborate with the results of some studies, which affirm the reduced level of the total plasma proteins of animals in conditions of heat stress. As these studies suggested, this decrease is linked to the decline of globulin levels and the concentration of T4 during heat stress which could significantly affect the reduction of protein biosynthesis (Fayez et al., 1994b; Marai et al., 1999; Okab et al., 2008). Another explanation is that higher water consumption leads to plasma dilution and thus lowers the concentration of proteins (Ondruska et al., 2011; Abdel-Hamid and Farahat, 2015).

Heat stress significantly increased plasma triglycerides and cholesterol concentration ($p < 0.05$). These results are similar to those shown by Ondruska et al. (2011) and Okab et al. (2008), who reported that plasma cholesterol and total lipid concentrations were significantly higher during the summer than in winter. Ondruska et al. (2011) explained that



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the increase might be related to the increased activity of hydroxy-methyl-glutaryl coenzyme (A HMG-CoA) reductase and the stimulation of cholesterol synthesis.

The plasma creatinine and urea levels are considered indicators of renal function. Changes in their levels reveal a dysfunction of the glomerular filtration of the kidneys (Mostafa et al., 2007). Marai et al. (2004) and Mostafa et al. (2007) recorded results similar to the results of the current study. Marai et al. (2004) have also found an increase in the plasma urea and creatinine levels of rabbits raised in warm conditions (THI = 33.9), compared to animals raised in thermo-neutrality (THI = 18.5) (47 ± 2.1 mg/dl versus 38.2 ± 0.9 mg/dl and 1.6 ± 0.1 mg/dl versus 1.4 mg/dl, respectively). The increase in the concentration of urea and creatinine may be a result of two factors. The first is the increase in protein catabolism, which leads to an increase in glucocorticoid hormones, and the second factor is the decrease in protein anabolism which results from the decrease in T3 hormone Marai et al. (2004). In the present study, a very high proteinemia was recorded; hence the former explanation does not justify the increase of urea and creatinine. Therefore, more probable that the hemoconcentration is due to hyperventilation. Okab et al. (2008) did not find a significant effect of heat stress on creatinemia, although they recorded a decrease in uremia in summer, compared to winter in adult male rabbits. Nevertheless, Marai et al. (1999) recorded a reduction in creatinine and blood urea levels.

This study did not find a significant effect of heat stress on glycemia ($p > 0.05$), while Ondruska et al. (2011) recorded a significant effect on growing New Zealand rabbits, and indicated a difference of +7.5% in rabbits raised in heat, compared to rabbits raised in thermoneutrality. According to Ondruska et al. (2011), the increase in glycemia is due to the decrease in the use of glucose as a source of energy in order to reduce heat production. However, Mostafa et al. (2007) and Okab et al. (2008) noted a decrease in glycemia in rabbits subjected to heat due to increased respiration rate. This increase in respiration rate caused a rapid utilization of blood glucose by the respiratory muscles. Thus, it decreased blood glucose under heat stress (Okab et al. (2008). In the present study, it is speculated that rabbits subjected to chronic heat stress have used blood glucose during acceleration of respiration, while at the same time, they have decreased the use of glucose by reducing their movements inside their cages, and therefore they produced less heat, which helped to balance glycemia. This could also be due to hemoconcentration.

Hematological parameters were significantly influenced by heat stress in the growing rabbits ($p < 0.05$). The results obtained by Waltz et al. (2014) on growing pigs exposed to thermal stress are in agreement with results of the present study on local rabbits raised in warmth. They observed an increase in the levels of RBC, Hb, and Hct. The same authors explained this increase by the fact that heat stress increases blood circulation in the skin to promote heat loss, which can cause a reduction in blood flow to other tissues and lead to tissue hypoxia. Consequently, an increase in the synthesis of reticulocytes and their liberation takes place to increase the level of Hb and protect the tissues from hypoxia, which results in a high level of Hct. They have also found that the elevations of these parameters were in a positive correlation with physiological parameters. Other authors have explained this increase as a result of an increase in blood viscosity due to the excessive water loss induced by hyperventilation (acceleration of respiration) and urinary loss, which caused dehydration and hemoconcentration in rabbits (Nakyinsige et al., 2013). However, Askar and Ismail (2012) noted a significant decrease in the level of hemoglobin, red blood cells, and white blood cells of New Zealand rabbits raised in chronic heat stress conditions ( 7%, -4%, and -9%, respectively). Similarly, Mostafa et al. (2007) and Okab et al. (2008) recorded a decrease in the level of Hb, RBC and Hct, but they noted an increase in WBC in summer compared to winter. On the other hand, Ondruska et al. (2011) did not record a heat stress effect on the RBC and WBC counts and the rate of monocytes in the growing rabbits, but they recorded a significant decrease in the rate of lymphocytes in hot-growing males, and WBC count in growing females. The studies of Khalil et al. (2014) and Dyavolova et al. (2014) during acute heat stress did not reveal a significant effect of heat on WBC, RBC, Hct, and monocytes except for the lymphocyte and Hb levels which decreased significantly. The decline in WBC recorded during present experimentation in growing local rabbits subjected to a hot climate can be considered an indicator of stress, as described by Dhabhar et al. (1995). In fact, it has been described that chronic heat stress can negatively affect the immune response in several production animal species (Ferrian et al., 2012). According to Khalil et al. (2014), the reduction in WBC can be interpreted either by the destruction of these cells (cell apoptosis), or probably by the redistribution of leukocytes to other organs to enhance the animal's immunity of target organs. A decrease in WBC can be due to the redistribution of leukocytes between the blood and other immune compartments (Dhabhar et al., 1995). Such redistribution may significantly affect the ability of the immune system to respond to potential or ongoing immune challenges.

Overall, this study recorded a concentration of most biological components of the blood (metabolites and blood cells), which can be attributed to one of two factors or both. First, it can be due to the fact that the blood samples were taken during the hottest hours of the day. The results can be explained by the acceleration of the respiratory rate, loss of water by evaporation, and decrease in blood volume leading to an increase in the concentration of the various metabolites and hematological parameters. Second, it can be attributed to the increase in the heart's workload due to the rise of blood flow to the skin, leading to a loss of salt and water from the body. These two factors impair working efficiency, overload the heart, and cause haemo-concentration. According to Fortun-Lamothe et al. (2015), the respiratory rate accelerates when the ambient temperature rises, allowing the rabbit to evaporate more water when the



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ambient temperature passes from 18°C to 30°C. This acceleration of the respiratory rate makes it possible to increase the quantity of water evaporated in 24 hours from 95 to 150 ml, which participates in the thermoregulation of the rabbit. This elevated water loss can therefore cause a high concentration of all blood elements (Okab et al., 2008).

## CONCLUSION

The obtained results of the present study affirm that the exposure of local Algerian rabbits to chronic heat stress deteriorates the growth performance by reducing feed intake and the average daily gain and hence results in poor feed efficiency with a low final slaughter weight. This study substantiates the fact that thermoregulation is considered a priority physiological function. It can lead rabbits to mobilize all thermoregulatory parameters to resist high ambient temperatures to regulate their internal temperature by modifying physiological parameters (acceleration of respiratory rate, heart rate, and losing as much heat as possible by radiation and convection). Thus, biochemical and hematological parameters are altered by exhausting its adiposity without recourse to the exhaustion of its muscle mass and without affecting the carcass quality. Nevertheless, technical solutions, feed, or therapeutic solutions by the use of additives seem necessary to minimise animal stress, improve production, and maintain animal welfare.

## DECLARATIONS


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### Authors' contribution

Dahmani Yamina, Benali Nadia, Ain Baziz Hacina, and Temim Soraya designed the study, and the experiment was carried out by Dahmani Yamina, Benali Nadia, Saidj Dyhia, Chirane Manel, Ain Baziz Hacina, and Temim Soraya curated the data. Laboratory analyses were done by Dahmani Yamina, Benali Nadia, Saidj Dyhia, and Chirane Manel. Data analyses by Dahmani Yamina and Benali Nadia. Dahmani Yamina wrote the draft of the manuscript. Dahmani Yamina, Saidj Dyhia, and Temim Soraya revised the manuscript. All authors checked and approved the final version of the manuscript for publishing in the present journal.

### Competing interests

The authors have not declared any conflict of interest.

### Ethical consideration

All authors have checked ethical issues (including plagiarism, double publication and/or submission, and redundancy, data fabrication and/or falsification, consent to publish, misconduct)

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# Effects of Ascorbic Acid on Maturation Rate, Morphology, and Gene Expression of Vitrified *In Vitro* Matured Dromedary Camel Oocytes

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## ABSTRACT

*In vitro* embryo generation, cryopreservation, and embryo transfer are examples of assisted reproductive technologies that can be used to improve camel genetic performance and fertility. The aim of this study was to investigate the impact of ascorbic acid supplementation to *in vitro* maturation media on the maturation rate, morphology, and gene expression of fresh and vitrified *in vitro* matured dromedary camel oocytes. In the current study, 810 oocytes of excellent and good quality were *in vitro* matured in maturation medium (TCM-199 + 10 ug/ml follicle stimulated hormone + 10% fetal calf serum + 100 IU/ml Pregnant mare serum + 50 µg/ml gentamycin) without any additives to act as a control group (C) and with 50 µg/ml ascorbic acid group (AA) and incubation in a CO2 incubator (38.5℃, 5% CO2, 20% O2 and 95% humidity) for 40 hours. *In vitro* matured dromedary camel oocytes with the first polar body (n = 210) in C group and AA group (n = 250) were placed in basic medium (BM) and then placed in vitrification solution1 (VS1) for one minute, followed by the transfer of oocytes to VS2 (double concentration of VS1, containing 20% Ethyl Glycol (EG) and +20% Dimethyl sulfoxide) for 30 seconds. Oocytes were then loaded into sterile 0.25 ml straws and stored in liquid nitrogen for 7-10 days. The normal fresh and vitrified /thawed *in vitro* matured dromedary camel oocytes were kept in RNA later at a -80°C freezer for gene expression analysis. The maturation rate of dromedary camel oocytes in the *in vitro* matured AA group was significantly higher than that of the C group. The percentage of normally recovered vitrified/thawed oocytes was higher in the *in vitro* matured with ascorbic acid (VAA) than in the control (VC) group. The expression pattern of the SOD1 gene and GDF9 gene was upregulated in fresh AA and VAA groups than in the fresh C and VC groups. The profile of the SOD1 gene was more abundant in the vitrified/thawed oocytes VAA group than in the VC group. All vitrified/thawed groups, whether control or ascorbic acid supplemented, had lower levels of SOD1, GDF9, and BMP15 expression, compared to the fresh groups. In conclusion, the supplementation of the maturation medium with ascorbic acid has an increased maturation rate, and normal morphology of vitrified/ thawed oocytes which was linked with upregulation of SOD1, GDF9 genes expression.

**Keywords:** Dromedary camel**,** Gene expression, *In vitro* maturation, Morphology, Vitrification

## INTRODUCTION

Dromedary camels are among the most economically important animals. They are used for various purposes, including entertainment, transportation, racing competitions, and beauty pageants (Faraz, 2019). The limited reproductive patterns of female dromedary camels in natural conditions (induced ovulatory, seasonal breeding, infertility delays, prolonged calving period, poor expressions of estrus signs) have been highlighted as a problem (Bello and Bodinga, 2020). Therefore, progress has been made in using assisted reproductive technologies, including *in vitro* production, cryopreservation, and embryo transfer, to optimize the fertility rate and enhance the performance of genetics in camels (Tukur et al., 2020).

A crucial stage in producing mature oocytes capable of effective embryonic development is *in vitro* maturation (Hashimoto, 2009). Camel oocytes transferred from the germinal vesicle (GV) stage to the metaphase II (M II) stage. The main obstacle to oocyte maturation is reactive oxygen species (ROS) production because of an imbalance between free radical production and internal antioxidants in oocytes. This is especially true in camel oocytes because of the long maturation period of about 40 hours, which can severely damage cell membrane integrity and other critical cellular organelles, as well as genetic material (Abdelkhalek et al., 2017).



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Various approaches, such as the supplementation of external antioxidants to the maturation medium, are considered the essential defense factor against oxidative stress (Khattab et al., 2020). Several studies have indicated that supplementing the maturation medium with ascorbic acid (AA) as an antioxidant is effective in reducing or scavenging the negative effect of ROS production and increasing glutathione, which allows better nuclear maturation and subsequent embryo development in camels (Ashour et al., 2021; Kandil et al., 2022).

Vitrification is a promising cryopreservation method that enables the preservation of genetic material from germ cells (oocytes and embryos) to create gene banks and expand relevant databases for breeding and animal research programs (Arav and Natan, 2019). To solidify the cell into a glass-like state without of developing ice crystals, it is necessary to subject the oocyte to a high concentration of cryoprotectants (Penzias et al., 2021). Cryoprotectants and vitrification have been extensively used for humans (Fabbri, 2006) as well as other species, such as porcine (Zhou and Li, 2009), bovine (Hwang and Hochi, 2014) and goat (Purohit et al., 2012). Some recent studies on the verification of immature camel oocytes discovered that vitrification caused mechanical damage and reduces the potential for oocyte development (Moawad et al., 2019; Yaqout et al., 2022). Moreover, vitrification has adverse effects on mitochondrial functions, gene transcript, and camel oocyte development (Saadeldin et al., 2020; Moulavi et al., 2021).

The addition of antioxidants to the maturation medium, vitrification medium, or culture media has a good effect on the viability and development of the oocytes after thawing as well as the gene transcript (Castillo-Martín et al., 2014). Several enzymes eliminate the stress in the oocytes; therefore, superoxide oxygen anion (O2) is changed into hydrogen peroxide (H2O2) by the antioxidant enzyme Cu-Zn-Superoxide Dismutase (SOD1, Ighodaro, and Akinloye, 2018). The Transforming Growth Factor (TGF-family) of genes includes the genes for bone morphogenetic protein 15 (*BMP15*) and growth differentiation factor 9 (GDF9), which are crucial regulators for follicle development, oocyte maturation, cell proliferation, and differentiation, as well as lowering the quality of oocytes following vitrification (Paulini and Melo, 2011).

Given the importance of this issue, there are few studies on the impact of ascorbic acid (AA) supplementation on the viability and gene expression of vitrified/thawed mature dromedary camel oocytes, as well as its impact on the maturation medium. Therefore, the current study aimed to study the effect of supplementation of ascorbic acid (AA) to *in vitro* maturation medium on the viability and gene expression of vitrified/thawed *in vitro* matured dromedary camel oocytes.

## MATERIALS AND METHODS

### Ethical approval

This study was carried out according to standard protocols without causing discomfort or injury to the camel. Furthermore, the experimental procedure was approved by the Centre for Research and Community Service at National Research Centre, Dokki, Cairo, Egypt.

Unless otherwise noted, all the chemicals and media used in this experiment were bought from Sigma-Aldrich (St. Louis, MO, USA). The National Research Center in Cairo, Egypt's Embryo and Genetic Resources Conversation Bank, was the site of conducting the current investigation.

### Collection of dromedary camel ovaries

Dromedary camel's Ovaries (n = 400) from 200 animals were taken from a slaughterhouse in El-Warraq abattoirs, Giza, Egypt, during the breeding season between December 2019 and 2020 to May 2020 and 2021. The animals were within the age range of 7-10 years and weighed 500-700 kg. The ovaries were kept in sterile normal saline solution (0.9% NaCl) with antibiotics (100 IU penicillin and 100 µg/ml streptomycin/ml) at 37°C for 1 hour before being transported to the lab. After removing extra tissues, the ovaries were washed in the laboratory three times in warm saline (0.9% NaCl). All ovaries were then promptly washed with ethanol (70%) and then with fresh normal saline and kept in a water bath (37°C). Camel oocytes were aspirated from follicles in diameter 2-8 mm using a 22-gauge needle connected to a 5-mL syringe. The aspiration medium was modified phosphate buffer saline (m-PBS) supplemented with 4 mg/ml of bovine serum albumin (BSA) and 50 µg /ml of gentamicin. The aspirated follicular fluid containing cumulus oocyte complex (COCs) was put into 15 mL conical tubes in a water bath at 37°C to allow COCs to settle for 20-25 minutes in the bottom of the conical tube. The aspirated COCs was ejected into a sterile dish (100 mm) for evaluation of the oocytes using Zeiss stereomicroscope (90 x).

### Categorizing of oocytes

The oocytes were categorized into four groups of excellent, good, fair, and poor according to Kandil et al., (2014). Oocytes in the excellent group had five layers of compact cumulus cells surrounding their evenly granulated, homogeneous cytoplasm. Those in the good group had uniformly granulated, homogenous cytoplasm and 3–4 layers of compact cumulus cells. Oocytes with fragmented cytoplasm and partially surrounded by COCs were categorized as fair.



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Finally, denuded oocytes in the poor group had no granulation. Excellent and good-quality oocytes were used for further experiments.

**Oocytes *in vitro* maturation**

The maturation medium is composed of TCM-199 was used, supplemented with 10% heat-inactivated fetal calf serum (FCS), 50 µg/ml gentamycin, 10 µg/ml FSH (Foltrobin V, Bioniche, Canada), and 100 IU/ml pregnant mare serum (sterilized using syringe filter 0.22 um, thermos fisher). Selected excellent and good COCs (n = 810) oocytes with round and homogenous cytoplasm were washed twice in modified phosphate buffer saline (M-PBS) and three times in the maturation medium. Maturation medium without any additives acted as the control group (C) containing 420 oocytes. Another group (AA) matured in maturation media + 50 µg/ml ascorbic acid, containing 390 oocytes. Each group of oocytes was cultured in 500 µL of *in vitro* maturation medium in Nunc™ 4-well dishes (Thermo Fisher, Waltham, MA, USA) for 40 hours at 38.5°C in a 5% CO2 incubator with 95% humidity. Thirteen biological replicates were used to evaluate the *in vitro* maturation of camel oocytes. The first polar body (M II) in the perivitelline space indicated that the oocytes reached nuclear maturity. The first polar body detection was carried out using a 20X inverted microscope.

Maturation rate = (Number of matured oocytes (M II)/ Number of Excellent and Good quality oocytes) × 100.

**Vitrification/thawing procedures**

***Vitrification procedure***

*In vitro* matured dromedary camel oocytes with the first polar body in the control group (n = 210) and AA group (n = 250) were placed in BM (9.5 ml TCM 199 + 0.5 ml FCS + 50 µg/ml gentamicin). Oocytes were equilibrated for 1 minute, then moved to vitrification solution one (VS1, 10% Ethyl Glycol (EG) + 10% Dimethyl sulfoxide (DMSO), followed by vitrification solution two (VS2, 20% EG + 20% DMSO) for 30 seconds. Oocytes were loaded in holding media (BM + 0.5 Mol sucrose).

***Oocytes loading***

Oocytes were loaded into 0.25 ml French straw using a micro-classic pipette (Karl Hecht No. 558). The oocytes in the vitrification solution separated from the holding medium by two air bubbles from two sides, The loaded straw was sealed, and groups of around 25-30 oocytes were loaded into the straw. After 10 seconds of exposure to liquid nitrogen (LN2) vapor, the straw was submerged in LN2 and stored for 7 days (Ismail et al., 2022).

***Oocytes thawing***

Oocytes vitrified in straws were submerged in a water bath at 37°C for 10-15 seconds. Oocytes that had been vitrified and warmed were immediately transferred to a new medium comprising BM + 0.5 Mol (M) sucrose. Using a three-step process and a one-minute equilibration period in each solution, sucrose was successively diluted to concentrations of 0.5, 0.33, 0.17,0 M in BM. After that, three fresh BM washes were performed on the oocytes (Ismail et al., 2022).

**Morphological evaluation**

Oocytes that had been recovered were examined under an inverted microscope. The number of retrieved oocytes in the control group (n = 160) and AA group (n = 200) were evaluated. Oocytes with morphological modifications of membrane damage, swelling, degeneration, or leakage of cellular material, ruptured zona pellucida or vitelline membrane, and fragmented cytoplasm in control (n = 60) and AA groups (n = 50) were counted as abnormal. The numbers of normal morphology and surviving oocytes in the control group and the AA group were 100 and 150, respectively. The percentage of recovered vitrified oocytes= $\frac{\text{number of recovered oocytes after thawing}}{\text{total number of vitrified oocytes}} \times 100$

**Gene expression of fresh and vitrified-thawed *in vitro* matured camel oocytes**

***RNA extraction and cDNA synthesis***

Oocytes in the fresh group (90 oocytes for control and 65 for AA groups) and oocytes in the vitrified/ thawed group (110 for control and 150 for AA groups) were kept in RNA later in cryogenic vials (Corning Incorporated, Corning, NY, USA) and directly put into a -80°C freezer for later analysis. Following the kit's instructions, total RNA isolation was performed using a PicoPureTM RNA isolation kit (Arcturus, Thermo Fisher, Lithuania). All oocyte groups were mixed with extraction buffer and incubated for 30 minutes at 42°C in Thermo Block. The complete lysis was loaded into a pre-conditioned spin column and centrifuged for 2 minutes at 1000 rpm to allow the RNA to bind to the spin column, followed by 13250 rpm/30 seconds. DNA was removed using a column RNase-free DNase kit (Qiagen GmbH, Hilden, Germany). The column was washed twice using two different wash buffers (WB1 and WB2). Finally, RNA was eluted with 12 µl of RNase-free water. The extracted and quality of RNA were evaluated by NanoDrop 2000 (Thermo Fisher, USA) with 260/280 and 260/230 values ≥1.8. The QuantiTect Reverse Transcription (Qiagen, Germany) kit was used to



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perform cDNA, which was first adjusted to reach the same RNA concentration by adding RNase-free water. RNA sample of 14.0 µ1 was added to 4 µ1 5X RT buffer, 1 µ1 RT primer mix, and 1 µ1 Quantiscript reverse transcriptase. The mixture was incubated at 25°C for 10 minutes, 37°C for 120 min, 85°C for 5 minutes, and held at 4°C. The cDNA samples were stored at -20°C until real-time PCR quantification.

***Real-time polymerase chain reaction***

The real-time relative quantitative PCR was performed by QuantStudio RT-PCR (Applied Biosystems, USA) using Maxima SYBR Green QPCR Master Mix (Thermofisher, Lithuania). The reactions were 2 µl cDNA, 1µl each of forward (5 mM) and reverse primers (5 mM), 8.5 µl nuclease-free water, and 12.5 µl SYBR Green PCR Master Mix in a total reaction volume of 25 µl (96-well plates). RT-PCR was performed on the thermal cycler with the condition of 95 °C for 10 minutes, followed by 40 cycles of 95 °C for 15 seconds, 60 °C for 1 minute, and 72 °C for 40 seconds. The PCR reactions were run in triplicates, and *GAPDH* was used as a reference gene. The fold change and relative quantity of the target transcripts (SOD1, BMP15, GDF9) were calculated using the 2−ΔΔCt method (Schmittgen and Livak, 2008). Reactions without reverse transcriptase or cDNA template were used as negative controls, which resulted in no amplification. PCR primers were designed using the NCBI (Table 1).

**Table 1**. Primers sequences and GenBank accession numbers for dromedary camel

| Gene name | Forward primer (5′→3′) | Reverse primer (5′→3′) | Product size (bp) | Accession no. | References resources |
|---|---|---|---|---|---|
| GAPDH | AGGTCGGAGTGAACGGATTC | GGAAGATGGTGATGGCCTTT | 219 | XM_010990867.2 | MODEL Camelus dromedarius (Arabian camel) |
| CuZn-SOD (SOD1) | TGCAGGCCCTCACTTTAATC | CTGCCCAAGTCATCTGGTTT | 216 | JF758876.1 | MODEL Camelus dromedarius (Arabian camel) |
| BMP15 | GCCACTACTTTGCCCCTGAT | GGGGTGCAATGATCCAGTGA | 167 | XM_010998065.1 | MODEL Camelus dromedarius (Arabian camel) |
| GDF9 | CCATCAGTGGACCTGCTGTT | CACTGAGGGGTCGAGCTTTT | 131 | XM_010981399.1 | MODEL Camelus dromedarius (Arabian camel) |

bp: Base pair, no: Number

**Statistical analysis**

Data were expressed as mean ± standard error (SE). Statistical analyses were performed using SPSS version 16.0 and the ANOVA test (one-way analysis of variance). The significance of differences ($p < 0.01$) was tested using LSD.

## RESULTS

**Cytoplasmic and nuclear maturation of dromedary camel oocytes**

The present study showed no significant difference in the cumulus expansion rate of camel oocytes matured *in vitro* in a maturation medium containing 50 µg/ml AA and control group (55.38% and 60.71%, respectively). The extrusion of the first polar body significantly ($p < 0.01$) increased (80.77%,315/390) in camel oocytes matured *in vitro* in the maturation medium containing 50 µg/ml AA, compared to the control group (71.43%, 300/420) as shown in Table 2 and Figure 1.

**Viability and morphology of vitrified /thawed mature dromedary camel oocytes**

The results indicated that after thawing, the number of recovered *in vitro* matured dromedary camel oocytes in maturation medium containing 50 µg/ml AA was higher than that matured in a control medium (80% (200/250) vs. 76.19% (160/210), respectively, $p < 0.01$). This was reflected in the normal morphological rate, which significantly increased in oocytes matured *in vitro* in a maturation medium containing 50 µg/ml ascorbic acid ($p < 0.01$, 75 %), compared to that matured oocytes in the control group (62.5%, Table 3). The morphological abnormalities were higher in the vitrified warmed matured camel oocytes in the control group ($p < 0.01$, 37.5%), compared to mature camel oocytes supplemented *in vitro* maturation with 50 µg /ml ascorbic acid (25%). Cytoplasm shrinkage and fragmentation was observed as the most frequent abnormalities were observed in the vitrified/thawed control group less (50% and 33%,



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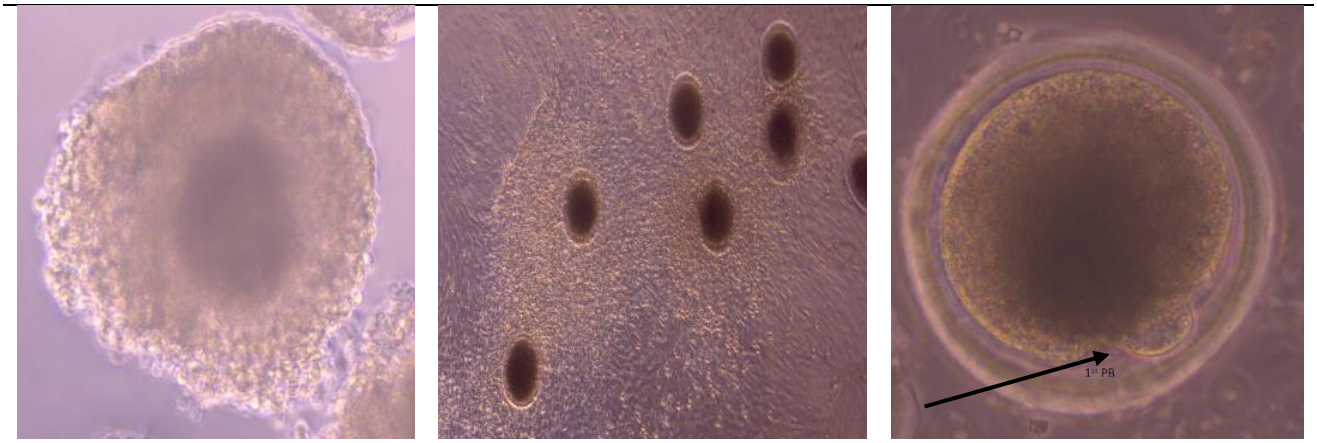
respectively) than vitrified/thawed supplemented oocytes in AA group (10% and 10%, respectively). As shown in Table 4 and Figure 2, zone breaking and cellular content leakage were lower in the vitrified/ thawed control group (8.33%, and 8.33%, respectively) than AA group (60% and 20%, respectively).

**Table 2.** The Effect of ascorbic acid supplementation to the maturation media on cumulus expansion, polar body extrusion of the dromedary camel oocytes

| Group | COC (n) | Maturation rate | | |
|---|---|---|---|---|
| | | Cumulus expansion rate (%) | (GII and GIII) | Polar body extrusion (%) |
| Control | 420 | 60.71%[a] | | 71.43%[a] |
| Ascorbic acid | 390 | 55.38%[a] | | 80.77%[b] |

n: Number of Oocytes, SE: Standard error, COC: Cumulus-oocyte complex; [a,b] Values with different superscripts within a column are significantly different at $p < 0.01$




Cumulus oocyte complex of camel oocytes (A) | Mature camel oocytes judged by the expansion of cumulus cells (B) | Extrusion of the first polar body of mature camel oocytes (C)

**Figure 1.** Steps of dromedary camel oocytes maturation using inverted microscope 20X (Zeiss). A: Cumulus oocyte complex, B: Mature camel oocytes judged by the expansion of cumulus cells, C: Extrusion of the first polar body

**Table 3**. Effects of the ascorbic acid on the viability and morphology of the vitrified/ thawed mature dromedary camel oocytes

| Group | No. of oocytes | No. of recovered oocytes | Morphologically normal oocytes % | Morphologically abnormal oocytes % |
|---|---|---|---|---|
| Vitrified/thawed control | 210 | 160 | 62.5%[b] | 37.5% [a] |
| Vitrified/thawed ascorbic acid | 250 | 200 | 75% [a] | 25% [b] |

n: Number of Oocytes; [a, b] Values with different superscripts within a column are significantly different at $p < 0.01$

**Table 4**. Effects of the ascorbic acid on types of the abnormal mature dromedary camel oocytes after vitrification/ thawing (percentage=%)

| Group | No. of abnormal oocytes | Types of oocytes abnormal after vitrification/ thawing % | | | |
|---|---|---|---|---|---|
| | | Zone breaking | Leakage of cellular content | Shrinking cytoplasm | Fragmented cytoplasm |
| Vitrified/ thawed control | 60 | 8.33%[b] | 8.33%[b] | 50%[a] | 33.33%[a] |
| Vitrified/ thawed ascorbic acid | 50 | 60% [a] | 20%[a] | 10 %[b] | 10%[b] |

[a,b] Values with different superscripts within a column are significantly different at $p < 0.01$



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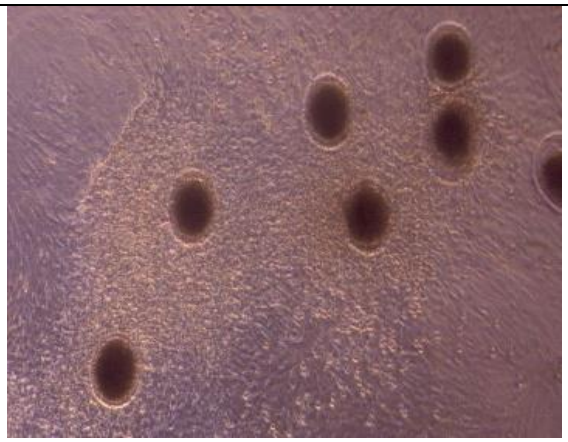
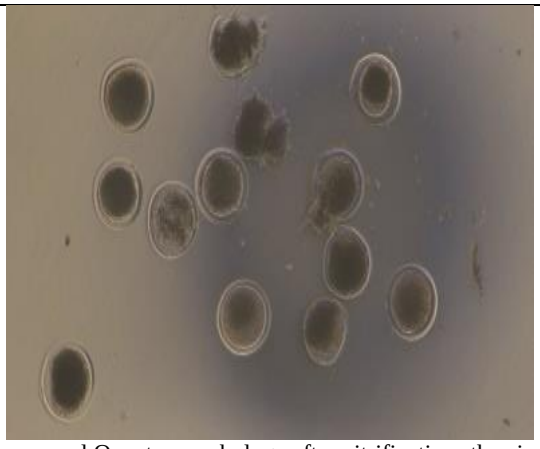
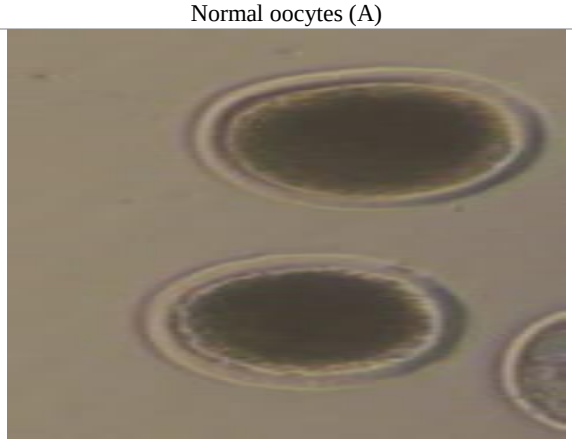


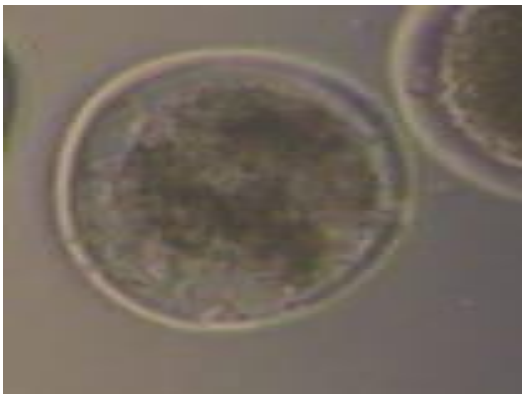
Normal oocytes (A)



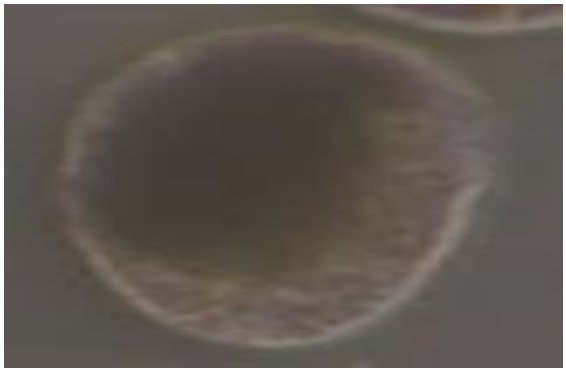
Mature camel Oocyte morphology after vitrification- thawing (B)



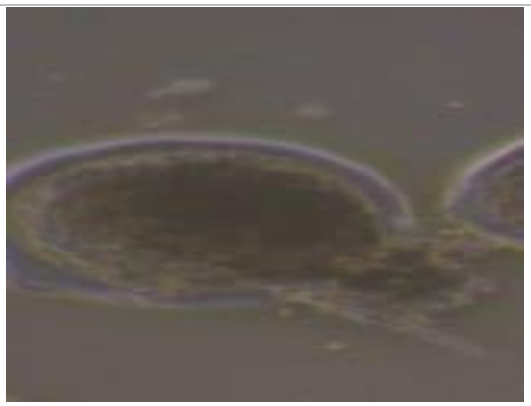
Normal vitrified-thawed mature camel oocyte with spherical shape (C)



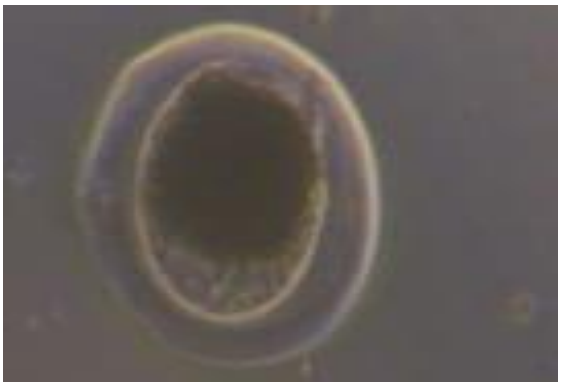
Abnormal vitrified-thawed mature camel oocyte with heterogeneous cytoplasm (D)



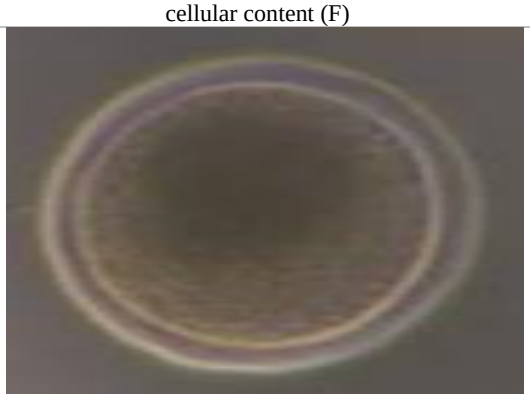
Abnormal vitrified-thawed mature camel oocyte with zone breaking (E)



Abnormal vitrified-thawed mature camel oocyte with leakage in cellular content (F)



Abnormal vitrified-thawed mature camel oocyte with shrined (G)



Abnormal vitrified-thawed mature camel oocyte with shrined fragmented cytoplasm (H)

**Figure 2**. Normal dromedary camel cumulus-oocyte complex COCs (A). Oocyte morphology after vitrification- thawing (B), Normal vitrified-thawed dromedary mature camel oocyte with spherical shape (C), Abnormal vitrified-thawed mature dromedary camel oocyte with heterogeneous cytoplasm (D), Abnormal vitrified-thawed mature dromedary camel oocyte with zone breaking (E), Abnormal vitrified-thawed mature dromedary camel oocyte with leakage in cellular content (F), Abnormal vitrified-thawed mature dromedary camel oocyte with shrined cytoplasm (G), Abnormal vitrified-thawed mature camel dromedary oocyte with fragmented cytoplasm (H). The pictures were taken with an inverted microscope (Zeiss) 20X.



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**SOD1 Gene expression of fresh and vitrified-thawed mature camel oocytes**

The effect of AA on the relative expression SOD1 gene on the fresh group (control vs. supplemented with 50 µg/ml AA) and the vitrified-thawed group (control vs. supplemented with 50 µg/ml AA) in the mature camel oocytes were investigated. The gene expression of *SOD1* in mature camel oocytes was significantly ($p < 0.01$) higher in fresh oocytes supplemented with AA (2.05 ± 0.01) when compared with the control group (1.0 ± 0.05). Moreover, SOD1 gene expression was significantly ($p < 0.01$) higher in vitrified /thawed oocytes supplemented with AA (0.54 ± 0.02) when compared with the vitrified/ thawed oocytes control group (0.22 ± 0.04). As shown in Table 5 and Figure 3, the gene expression of SOD1 in mature camel oocytes supplemented with AA in either fresh or vitrified/ thawed group was significantly double upregulated, compared to mature dromedary camel oocytes in the control group, either fresh or vitrified/ thawed ($p < 0.01$).

**GDF9 gene expression of the fresh group and vitrified-thawed group of the mature camel oocytes**

The relative expression GDF9 *gene* for fresh mature oocytes supplemented with AA was significantly higher (2.05 ± 0.21) than the fresh mature control (1.0 ± 0.01, $p < 0.01$). In addition, the expression of *GDF9* in vitrified mature oocytes supplemented with AA was significantly higher (0.75 ± 0.07), than the vitrified mature control group (0.65 ± 0.24, $p < 0.01$), as shown in Table 6 and Figure 4.

***BMP15* gene expression in the fresh group and a vitrified-thawed group of the mature camel oocytes**

There was no significant change in gene expression of *BMP15* in fresh mature camel oocytes in control (1.0±0.01) and fresh mature camel oocytes supplemented with AA (1.11 ± 0.03). Similarly, there was no significant change in the expression of *BMP15* in vitrified / thawed mature camel oocytes in control (0.25 ± 0.01) and vitrified / thawed mature camel oocytes supplemented with AA (0.24 ± 0.07) as shown in Table 7 and Figure 5. There was significant ($p < 0.01$) upregulation on relative BMP15 gene expression in fresh (C and AA group) when compared with vitrified/thawed *in vitro* matured camel oocytes (VC and VAA).

**Table 5**. The Effect of ascorbic acid on the SOD1 gene on the fresh group and vitrified-thawed group of the mature dromedary camel oocytes

| Groups | SOD1 gene (Fold change) |
|---|---|
| Fresh mature camel oocytes | $1.0\pm0.01^{b}$ |
| Fresh mature camel oocytes supplemented with ascorbic acid | $2.05\pm0.05^{a}$ |
| Vitrified mature camel oocytes | $0.22\pm0.02^{c}$ |
| Vitrified mature camel oocytes supplemented with ascorbic acid | $0.54\pm0.04^{d}$ |

[a,b,c,d] Values with different superscripts within a column are significantly different at $p < 0.01$

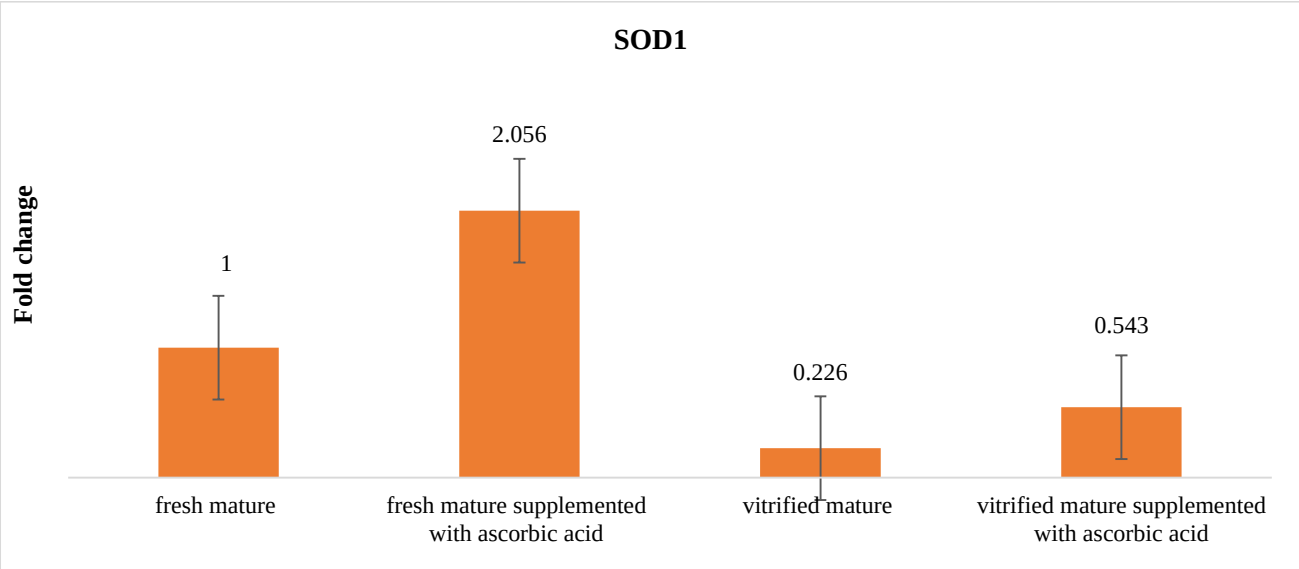


**Figure 3.** The effects of ascorbic acid on the SOD1 gene on the fresh group and a vitrified-thawed group of the mature camel oocytes



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**Table 6.** The effects of ascorbic acid on the GDF9 gene on the fresh group and vitrified-thawed group of mature camel oocytes

| Groups | GDF9 gene (Fold change) |
|---|---|
| Fresh mature camel oocytes | $1.0\pm0.01^{b}$ |
| Fresh mature camel oocytes supplemented with ascorbic acid | $2.05\pm0.07^{a}$ |
| Vitrified mature camel oocytes | $0.65\pm0.21^{c}$ |
| Vitrified mature camel oocytes supplemented with ascorbic acid | $0.75\pm0.24^{d}$ |

[a,b,c,d] Values with different superscripts within a column are significantly different at $p < 0.01$

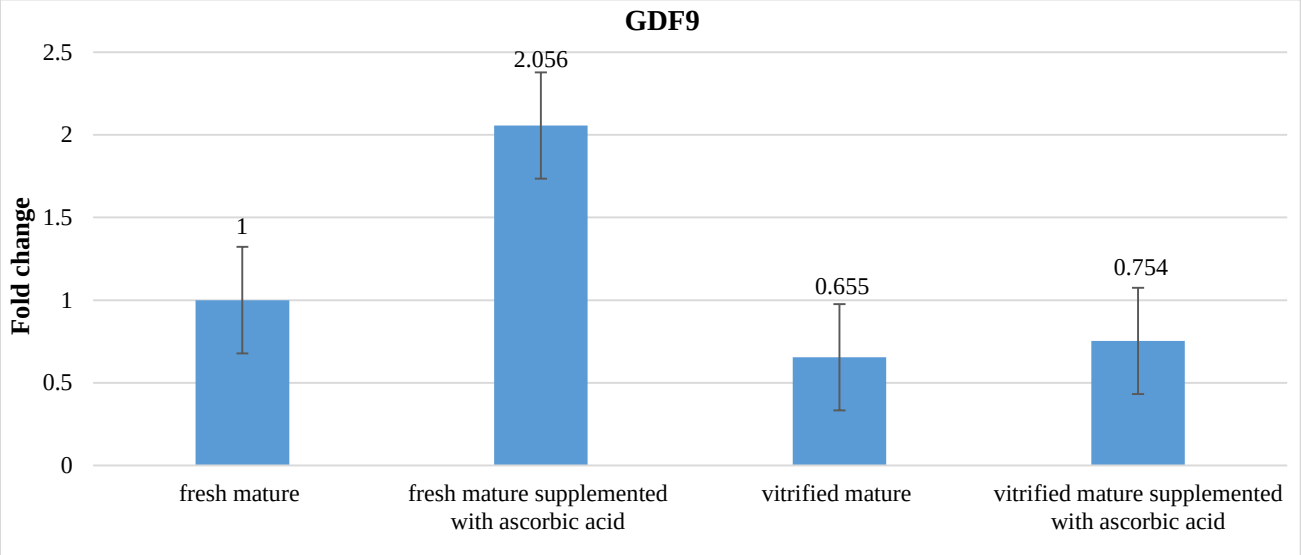


**Figure 4**. The effect of ascorbic acid on the GDF9 gene on the fresh group and a vitrified-thawed group of the mature camel oocytes

**Table 7**. The Effect of ascorbic acid on the BMP15 gene on the fresh group (control and supplemented with ascorbic acid) and vitrified-thawed group (control and supplemented with ascorbic acid) mature dromedary camel oocytes

| Groups | BMP15 gene (Fold change) |
|---|---|
| Fresh mature | $1.0 \pm 0.01^{a}$ |
| Fresh mature supplemented with ascorbic acid | $1.11 \pm 0.03^{a}$ |
| Vitrified mature | $0.24 \pm 0.07^{b}$ |
| Vitrified mature supplemented with ascorbic acid | $0.25 \pm 0.01^{b}$ |

[a,b] Values with different superscripts within a column are significantly different at $p < 0.01$

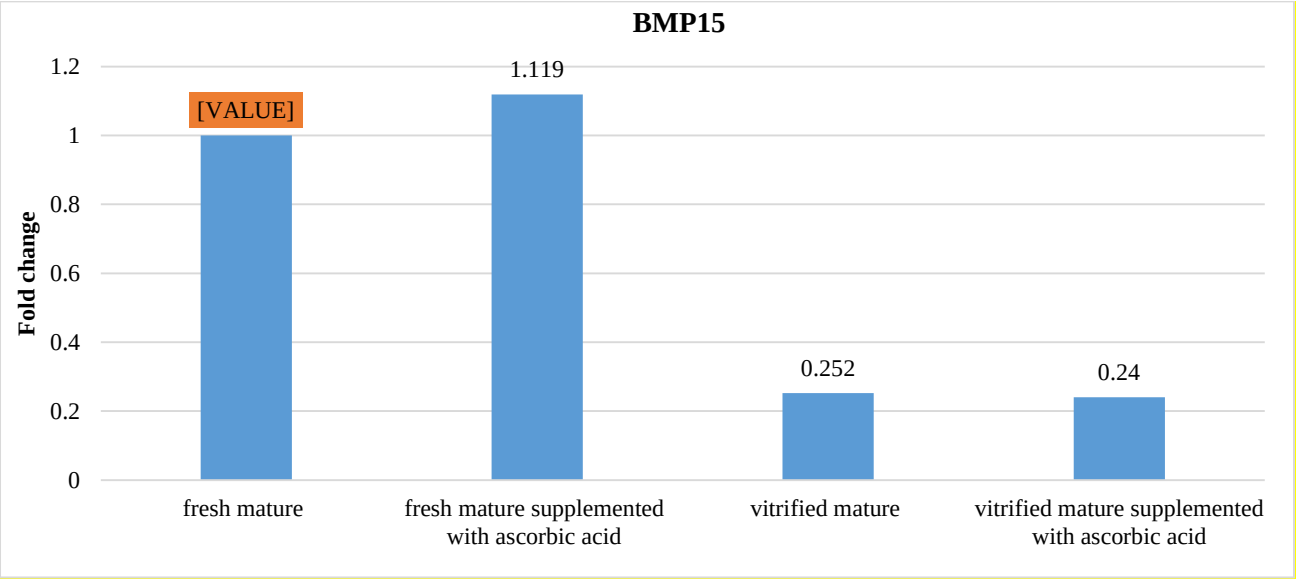


**Figure 5.** The effect of ascorbic acid on BMB15 gene expression of the fresh group and vitrified-thawed group of the mature camel oocytes



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## DISCUSSION

Due to its participation in the first line of antioxidant defense, AA plays a critical function in the prevention of oxidative damage to proteins and lipid membranes (Njus et al., 2020). Since vitamin C is a water-soluble molecule, it can work both within and outside of cells, neutralizing free radicals and reducing the production of ROS formation (Pehlivan, 2017). The results of the current study revealed a significant increase in the maturation rate of dromedary camel oocytes *in vitro* matured in a maturation medium containing 50 µg AA when compared with control group. These findings are in line with those of Kere et al. (2013) who found that adding 50 µg/ml of a ascorbic acid to porcine oocytes in maturation medium or culture medium and reduced apoptotic index and ROS content, improved the blastocyst and increase the number of cells overall. This supports the idea that AA is a potent antioxidant due to its ability to sustain redox status in different species and promote cell growth, proliferation of mammalian cells and decrease apoptosis (Moussa et al., 2020).

According to Sovernigo et al. (2017), bovine oocytes with considerably elevated intracellular glutathione had lower levels of oxidative stress, greater developmental competence, and a higher rate of blastocyst formation in bovines. Ascorbic acid dramatically improved the nuclear maturation of canine oocytes in metaphase I and M II at a concentration of 250 M (26.98% against 6.00%). However, AA (50 µg/ml) has no appreciable influence on maturation, fertilization, or embryo development parameters in porcine oocytes (Nohalez et al., 2018). All these variations in results could be attributed to genetic material, oocyte age, dosage effects on membrane integrity, and differences in species.

Cryopreservation is the process of preservation of living cells (germ cells, oocytes, sperm, and embryos) and tissues at extremely low temperatures. To preserve the genetic resource of the superior genetic animals in gene banks or for future use in *in vitro* fertilization and embryo transfer (Pegg, 2015). The development of the oocyte has been severely influenced by vitrification. The results of the current investigation showed that AA significantly improved the recovery rate of vitrified/thawed mature camel oocytes and reduced the appearance of abnormal oocyte morphology in comparison to the control. These findings are in agreement with Sonowal et al. (2017) who reported that the addition of a specific dose (100 M) of Vitamin C to *in vitro* maturation medium when used with immature bovine oocytes, increased the growth of vitrified-thawed bovine oocytes in terms of cumulus cell expansion and polar body formation after maturation. However, Chaves et al. (2017) found that immature COCs were more resistant to the effects of cryoprotectants than mature COCs and this result was confirmed by superior embryo growth (cleavage) after vitrification. According to Al-Soudy et al. (2016), mature camel oocytes were more resistant to cryo-injuries than immature oocytes and produced a high percentage of normal oocytes, which could be useful for future *in vitro* fertilization and camel improvement initiatives. Additionally, Castillo-Martín et al. (2015) revealed that L-ascorbic acid addition to culture and/or vitrification media increases porcine blastocyst survival rates. Nohalez et al. (2018) demonstrated that AA addition to vitrification and warming conditions increased the survival of *in vitro*-produced porcine blastocysts by reducing ROS production. Because vitrification has negative on morphology by increasing the frequency of defects including zona shattering, an increase in perivitelline space, leakage of cellular content, and cytoplasm fragmentation and non-symmetrical oocyte shape, it has a negative impact on oocyte morphology.

These abnormalities can be attributed to cooling and warming, which damaged the cytoskeleton and cause cellular degeneration (Allworth and Albertini, 1993). Osmotic stress is thought to be another factor that can induce damage in oocytes and alter their volume and negatively impact their viability (Mullen et al., 2007). This might be because camel oocyte cytoplasm is extremely susceptible to cryopreservation. In addition, there are many cryoprotectants (20% DMSO, 20% EG) in vitrification medium that could negatively affect oocyte viability. Furthermore, Yassen et al. (2020) reported that the survival rate, morphological characteristics, and ultrastructural quality were all improved when vitamin C or zinc chloride was added to the vitrification medium in bovine oocytes. These findings are consistent with the role of ascorbic acid in the reduction of abnormalities observed in the present results.

The results of this study indicate that SOD1 gene expression had dramatically increased in the groups supplemented with ascorbic acid (AA) either in fresh or vitrified/thawed oocytes. This is agreement with Fang et al. (2022), that reported upregulation of the antioxidant gene (SOD1) in porcine oocytes that in vitro matured in medium supplemented with ascorbic acid. According to the findings of the current study, vitrification causes lower expression in all groups whether control or AA-supplemented, than the fresh group. This suggests that vitrification can modify the mRNA content of the oocyte and change the expression of genes related to stress. This finding is consistent with Park and Kim (2014) who found an increase in the expression pattern of the SOD1 gene in fresh when compared with vitrified canine oocytes. Similarly, Habibi et al. (2010) found that expression of the SOD 1 gene was significantly up-regulated in vitrified IVM oocyte when compared with fresh IVM in immature human oocytes. On the contrary, Turathum et al. (2010) detected no variations in SOD1 gene expression in fresh or vitrified canine oocytes using real-time polymerase chain reaction (RT-PCR). Castillo-Martín et al. (2014), reported that addition of ascorbic acid during culture and vitrification/ warmed upregulated the expression of GPX1 and SOD1 genes in porcine blastocyst.



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GDF9 and BMP15 are important regulators of follicular development, oocyte maturation, and embryo quality. The results of this investigation showed that in the fresh and vitrified groups, AA supplementation significantly increase the relative expression of GDF9 when compared with to control groups and had no effect on the expression of BMP15 in control or other AA-supplemented oocytes. Roshan et al. (2021), reported that the addition of L-ascorbic acid to the *in vitro* maturation medium of porcine oocytes at both concentrations (50- and 100 M doses of L-ascorbic acid) did not significantly affect GDF9 and BMP15 genes expression. However, Yu et al. (2018) found that L-ascorbic acid (Vitamin C) can enhance meiotic maturation and developmental competence and increase BMP15 mRNA levels in porcine cells. In the current study the GDF9 and BMP15 genes relative expression pattern in fresh group was significantly increased than the vitrified /thawed group even in VC or VAA group. This results in agreement with Azari et al., (2017), who found that, bovine oocytes in the control group showed the highest expression level of GDF9 and BMP15 in compared to the vitrification groups. Unlike Di Pietro et al., (2010) revealed that the vitrification protocol keeps unaltered the human oocyte molecular profile including BMP15 and GDF9 does not cause messenger RNA degradation. These results deference might be due to the various breeds, vitrification protocol, variation in cryoprotectants and ascorbic acid concentrations employed during the *in vitro* maturation of oocytes.

## CONCLUSION

The addition of ascorbic acid in *in vitro* maturation media improves the maturation rate and sustained the normal morphology of vitrified/thawed dromedary camel oocytes. Moreover, ascorbic acid upregulates SOD1 and GDF9 gene expression either in fresh or vitrified/thawed *in vitro* matured dromedary camel oocytes that supported the viability after thawing.

## DECLARATIONS

### Competing interests

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


### Acknowledgments

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### Authors' contribution

Omaima Kandil designed the experiment, and supported all the equipment, chemicals, primers, and kits, Fatma Aboelwafa brought the samples and did lab work, Esraa Ismael did the gene expression and data analysis, Sahar Kandeel supplied the experiments with ascorbic acid and some chemicals for vitrification, Naser Ghanem support experiment with some primers and share in statistical analysis, Omaima Kandil, Fatma aboelwafa and Abd Elkader Gamal El-Dean write the manuscript and analysis of data. All authors confirmed the final analyzed data and the last revised article before publication in the present journal.

### Ethical considerations

Ethical considerations (e.g., plagiarism, consent to publish, misconduct, data fabrication and/or falsification, double publication and/or submission, and redundancy) have been made by the authors.


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# Prevalence and Antibiotic Resistance of *Salmonella* spp. and *Staphylococcus aureus* Isolated from Broiler Chicken Meat in Modern and Traditional Slaughterhouses of Morocco

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**ABSTRACT**

Handling and consuming contaminated meat can lead to food poisoning and the acquisition of antibiotic resistance genes. *Staphylococcus aureus* (*S. aureus*) and *Salmonella* spp. are the most isolated bacteria from broiler chicken meat, leading to serious foodborne diseases. The present study aimed to evaluate the presence and antibiogram profiles of *Salmonella* spp. and *S. aureus* strains in poultry meat purchased from modern and traditional poultry slaughterhouses in Morocco. Foodborne pathogens, such as *Salmonella* spp. and *S. aureus*, were isolated from poultry meat using standard methods and then confirmed by biochemical tests (coagulase, catalase, oxidase, motility and API 20E for further biochemical identification) and an immunological test (serotyping test). The antibiogram of the isolates was determined using the agar diffusion method and interpreted according to the criteria of performance standards for antimicrobial susceptibility testing of the Clinical and Laboratory Standards Institute, Wayne, Pennsylvania, USA. A total of 540 poultry meat samples were collected and treated (360 poultry meat samples from traditional slaughterhouses and 180 poultry meat samples from modern slaughterhouses), out of which 15.92% were *S. aureus* positive and 7.40% were *Salmonella* spp. positive. In traditional poultry slaughterhouses, the prevalence rates of *Salmonella* spp. and *S. aureus* were 11.11% and 20.55%, respectively. In contrast, *Salmonella* spp. was not detected in poultry samples of modern poultry slaughterhouses, and the prevalence of *S. aureus* was 6.66%. All *S. aureus* and 97% of *Salmonella* spp. isolates were found resistant to at least one antibiotic, while 86% of *S. aureus* and 30% of *Salmonella* spp. showed resistance to more than three antibiotics. The obtained results of the present study confirmed that broiler chicken meat purchased from traditional poultry slaughterhouses was mainly contaminated by *Salmonella* spp. and *S. aureus*, indicating a major public health risk in Morocco. Therefore, considerable efforts should be made to apply appropriate hygiene practices.

**Keywords:** Antimicrobial resistance, Broiler chicken meat, Modern slaughterhouses, *Salmonella*, Prevalence, *Staphylococcus aureus*, Traditional slaughterhouses

## INTRODUCTION

Foodborne diseases (FBD) are pathological cases caused by the ingestion of food containing biological, chemical, or physical hazards. They affect both developed and developing countries and have an impact on public health and economy (Akbar and Anal, 2013). Several pathogenic bacteria can cause FBD, among which *Salmonella* spp. and *Staphylococcus aureus* (*S. aureus*) are the most common pathogenic bacteria in animal-source foods (Rortana et al., 2021).

Non-typhoidal Salmonella is a significant contributor to severe invasive illnesses in adults, children, and immunocompromised individuals in Africa (El-Tayeb et al., 2017). The invasive *Salmonella* spp. infections can affect several organs, such as the digestive tract, endothelial surfaces, pericardium, meninges, lungs, joints, bones, genitourinary tract, resulting in bacteremia, meningitis, osteomyelitis, or septic arthritis and sometimes even death (WHO, 2015). Of *Salmonella* spp. pathogens, *Salmonella enteritidis* and *typhimurium* are the two most commonly reported serotypes of human foodborne in most parts of the world (WHO, 2015). On the other hand, *S. aureus* infections can also range from minor skin problems to severe infections (MDH, 2010). It produces many toxins, including staphylococcal enterotoxins, which can cause FBD (ANSES, 2022).

In Morocco, there has been a progressive rise in collective food poisoning cases in recent years, indicating that cases have almost doubled in 9 years (from 866 in 2008 to 1631 in 2017, FISA, 2022a). Agents responsible for this issue have only been identified in 20% of cases, with 20% of *Salmonella* and 10% of *S. aureus* (DELM, 2018). Poultry meat is



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among the vectors responsible for these foodborne infections (Lundén et al., 2003; Prakash et al., 2005). As reported recently, consumption of poultry meat in the Moroccan kingdom has increased significantly over the last two decades, from 8.9 kg/year in 2000 to 19.3 kg/year in 2021, an increase of 117% (FISA, 2022a). Thus, poultry meat production, particularly broiler chicken meat in the formal sector, has experienced a progressive growth of 162% from 2000 (200,000 tons) to 2021 (525,000 tons, FISA, 2022a).

Another serious challenge facing humanity is antibiotic resistance. The prevalence of antibiotic-resistant foodborne pathogens is also increasing due to their excessive use in human and animal treatments (Akbar and Anal, 2013). Moreover, the determinants of antibiotic resistance can be transferred to other pathogenic bacteria, which can compromise the treatment of serious bacterial infections, and thus, constitutes a major threat to public health (Adesiji et al., 2011). This is the case with *Salmonella* strains resistant to antibiotics in most countries (Duc et al., 2019). Effectively, it has been reported in developed countries that this increased resistance in *Salmonella* spp. has a zoonotic origin, and the bacteria present in food animals acquire resistance before being transmitted to humans through the food chain (Threlfall, 2002; Andoh et al., 2016). Moreover, in poultry production, antibiotics are commonly used as Growth promoters at sub-therapeutic doses and obviously as treatment (FISA, 2022b). Therefore, chickens and chicken meat may contain antibiotic-resistant strains and serve as a vehicle for disseminating antibiotic-resistant strains to humans and the environment (Duc et al., 2019).

Notably, broiler chicken slaughter is carried out in formal and informal sectors in Morocco. The formal sector concerns approved industrial poultry slaughterhouses (Modern slaughterhouses), while the informal one represents the traditional slaughterhouses in the neighborhoods commonly known as "RYACHATE", which covers 80% of the Moroccan market (FISA, 2022b). However, the deplorable hygiene conditions of most traditional slaughterhouses present a threat to public health (FISA, 2022b). Therefore, in recent years, considerable efforts have been made to rationalize this sector and replace these traditional slaughterhouses with local, low-capacity poultry slaughterhouses that can meet the required hygiene standards by improving the legal framework. Given these conditions, the main objective of the present study was to evaluate the prevalence of *Salmonella* spp. and *S. aureus* contaminations in broiler chicken meat (neck skin, breast, and thigh) purchased from modern and traditional poultry slaughterhouses. This current study will help determine the prevalence and antibiotic resistance of the above-mentioned bacteria and discuss the major causes of bacterial contaminations during slaughtering.

## MATERIALS AND METHODS

### Ethical Committee Approval

All animal procedures in the present study were carried out following the Hassan II Agronomic and Veterinary Institute of Rabat 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

This study was carried out from July 2020 to February 2021 and concerned broiler chicken meat samples (the neck skin, breast, and thigh) from traditional and modern poultry slaughterhouses. Samples were randomly taken from the cities in Morocco, namely Casablanca, Mohammedia, Benslimane, Bouznika, and Rabat.

Regarding the informal sector, a total of 120 broiler chickens (Cobb 500) were purchased and slaughtered at four traditional slaughterhouses per city (Casablanca, Mohammedia, Benslimane, Bouznika, and Rabat) during the summer and winter seasons of the study period. While for the formal sector, a total of 60 broiler chickens in sealed and labeled trays from supermarkets (from modern poultry slaughterhouses) were purchased during the summer period. Once the samples were purchased, they were put in sterile collection bags and transferred to the laboratory of avian pathology at the Hassan II Agronomy and Veterinary Medicine Institute in Rabat, Morocco, in an isothermal box at 4°C. After that, chickens were sampled aseptically from the neck skin, thigh, and breast. Finally, 360 samples from the informal sector (Two seasons of Summer and Winter), and 180 samples from the formal sector (Summer season only) were collected.

### Isolation and identification of bacteria

The samples were analyzed separately for each bacterium. *Salmonella* spp. was isolated according to the International Standard ISO 6579, 2002. The 10 g of chicken samples (neck skin, thigh, breast) were transferred to water peptone buffer (CM 0509 Oxoid, Oxoid LTD, Basingstoke, Hampshire, England) and incubated at 37°C for 18-24 hours. Thereafter, 0.1 ml of the pre-inoculated water peptone buffer was transferred to 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 agar (BK058HA Biokar diagnostics, Zac de Ther, France) and incubated at 37°C for 24-48 hours (ISO, 2002).



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All the isolated bacteria were identified based on their morphology, color, shape, and color change of culture media. They were also strained using Gram stains and examined with a light microscope x100 (OPTIKA B-151, ITALY) using oil immersion. Furthermore, biochemical tests of coagulase test (6BR0020, Biokar diagnostics, Zac de Ther, France), catalase test (1870 SOLVAPUR), oxidase test (MICROBAT Oxoid LTD, United Kingdom), and motility test were carried out on each isolate. This diagnosis was also confirmed by API 20E and serotyping test according to the Kauffmann-White scheme using a slide agglutination test with *Salmonella* polyvalent O and H antisera according to Diagnostic Pasteur, Paris, France, for *Salmonella* detection (WHO, 2007).

Regarding staphylococcal detection, the ISO 6888-1: 1999 standard was used. The 25 g from each sample of neck skin, breast, and thigh was placed in a sterile bag in 225 mL of water peptone buffer. The suspension was then homogenized using the Stomacher to obtain a stock suspension titrated at 1/10. A series of dilutions down to $10^{-5}$ was carried out from the stock solution at $10^{-1}$ by taking 1 mL each time added to 9 mL of distilled water in a test tube (ISO, 1999).

The prepared Petri dishes were inoculated with 0.1 mL of different dilutions with a sterile glass rake in Baird Parker's selective medium (BK055HA Biokar diagnostics, Zac de Ther, France) with egg yolk and potassium tellurite (3554205Bio-Rad Marnes-la-Coquette, France). The final preparations were incubated at 37°C for 24 to 48 hours. At the end of the incubation period, the suspect colonies of the *S. aureus* were black and shiny, surrounded by a halo of lightening of the medium, and were confirmed using two tests, including the DNAse test (CM0321 Oxoid, Oxoid LTD., Basingstoke, Hampshire, England) and the coagulase test (6BR0020, Biokar diagnostics, Zac de Ther, France, Papanicolas et al., 2014).

**Antimicrobial susceptibility test**

Antimicrobial susceptibility of *Salmonella* spp. and *S. aureus* tests were determined by Kirby-Bauer diffusion method using Mueller-Hinton agar and seven antibiotics discs for *Salmonella*: nalidixic acid (30 µg), gentamicin (15 µg), trimethoprim/sulfamethoxazole (1,25/23.75 µg), cefoxitin (30 µg), kanamycin (30 µg), ciprofloxacin (5 µg), tetracycline (30 µg), and 6 antibiotics discs for *S. aureus*, namely erythromycin (15 µg), trimethoprim/sulfamethoxazole (1,25/23.75 µg), tetracycline (30 µg), kanamycin (30 µg), streptomycin (25 µg), ampicillin (10 µg). All the discs were purchased from Oxoid LTD, England.

The pre-incubated (24 hours) cultures of *Salmonella* spp. and *S. aureus* were diluted in sterile normal saline to McFarland standards of 0.5 and then were inoculated onto the Mueller-Hinton agar surface agar (Bk048HA Biokar diagnostics zac de ther BEAUVAIS-France), where the antibiotic discs were placed, then the isolates were incubated at 37°C for 18-24 hours. The clear area around each antibiotic disc was measured in millimeters then the results were interpreted according to performance standard criteria for antimicrobial susceptibility testing by the Clinical and Laboratory Standards Institute, Wayne, Pennsylvania, USA (CLSI, WEINSTEIN, 2018).

**Statistical analysis**

In order to analyze the results, three statistical methods were used. The chi-square ($\chi 2$) test was used to treat the descriptive analysis part as the prevalence study. The $p < 0.05$ was considered statistically significant. Multiple Correspondence Analysis (MCA) was employed to analyze the choice of exposure to the different tested risk factors. These methods aim to reduce the dimensions of the data tables to represent associations between individuals and between variables in small dimensions. The Cross-sectional study was used to answer the research questions of this experiment and therefore attempted to determine whether there was a relationship between various risk factors (season, sector type) and bacterial contaminations.

The ($\chi 2$) and MCA tests were realized by IBM SPSS version 24.0.0.0 statistical software. The Cross-sectional study was done by EPI INFO version 7.2.5.

## RESULTS

**Descriptive analysis**

Of 540 collected samples, the findings revealed that 86 were positive for *S. aureus* (15.92%) and 40 were positive for *Salmonella* spp. (7.40%), of which 27/40 (67.5%) were identified as *Salmonella enteritidis* while 13/40 (32.5%) were *Salmonella* Pullorum *and Salmonella* Gallinarum. In the traditional slaughterhouses, the prevalence of *Salmonella* spp. was 40/360 (11.11%), while the prevalence of *S. aureus* was 74/360 (20.55%), (Table 1).

Given that the effect of season on the prevalence of both *Salmonella* spp. and *S. aureus* were insignificant ($p > 0.05$), the data related to both seasons in the analysis of the effect of sector type on the prevalence of bacteria were pooled. In the formal sector, no *Salmonella* spp. was detected in poultry samples, and 12/180 (6.66%) of *S. aureus* was identified (Table 1).



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In the informal sector, it was found that out of all contaminated samples (114 samples), 8.7% of the broiler chicken meat samples were positive for both *Salmonella* spp. and *S. aureus.* The prevalence of *Salmonella* spp. was 19/180 (10.56%) during the winter, and 21/180 (11.67%) during the summer, while *S. aureus* 30/180 (16.67%) were positive during the winter and 44/180 (24.44%) during the summer (Table 2). In this study, the prevalence of *S. aureus* was significantly high in the neck skin, compared to the thigh and the breast ($p < 0.05$). However, the prevalence of *Salmonella* spp. was significantly higher in the thigh samples than in the neck skin and breast ($p < 0.05$, Table 3).

According to the Chi-2 test, the sample types and the prevalence of the bacteria studied were significantly related ($p < 0.05$). Concerning antibiotic resistance susceptibility, among all 40 *Salmonella* spp. isolates, 80% showed resistance to tetracycline, 57.50% to ciprofloxacin, 27.50% to kanamycin, 25% to nalidixic acid, and 5% to trimethoprim-sulfamethoxazole, while the lowest resistance of the isolates (2.50%) was against cefoxitin and no resistance to gentamycin (Table 4).

Regarding the antimicrobial susceptibility and resistance profiles of all *S. aureus* isolates, out of a total of 86 isolates, 47 showed resistance to erythromycin and 44 to streptomycin representing 54.65% and 51.16% of the total isolates, respectively. In contrast, only 1.16% showed resistance to trimethoprim-sulfamethoxazole. The highest resistance rate was against ampicillin, tetracycline, and kanamycin (100%, 81.40%, and 74.42%, respectively, Table 5).

The results revealed that 97.5 % of *Salmonella* spp. isolates showed resistance to at least one antibiotic, while 30% showed resistance to more than three antibiotics. However, all *S. aureus* isolates showed resistance to at least one antibiotic, and 86% showed resistance to more than three antibiotics (Graph 1, Table 6).

**Table 1.** Prevalence of *Staphylococcus aureus* and *Salmonella* spp. in informal and formal sectors

| | | Tested samples | Positive samples (%) | | p-value | |
|---|---|---|---|---|---|---|
| | | | ***Salm*[1]** | ***Staph*[2]** | ***Salm*** | ***Staph*** |
| Sector | Formal | 180 | 0 | 12 (6.66) | $p < 0.05^*$ | $p < 0.05^*$ |
| | Informal | 360 | 40 (11.11) | 74 (20.55) | | |

* Signifiant effet ($p < 0.05$). [1] *Salmonella* spp. [2] *Staphylococcus aureus*

**Table 2.** Prevalence of *Staphylococcus aureus* and *Salmonella* spp. in the informal sector

| | | Tested samples | Prevalence (%) | | p-value | |
|---|---|---|---|---|---|---|
| | | | ***Salm*[1]** | ***Staph*[2]** | ***Salm*[1]** | ***Staph*[2]** |
| Period | Summer | 180 | 21 (11.67) | 44 (24.44) | $p > 0.05$ | $p > 0.05$ |
| | Winter | 180 | 19 (10.56) | 30 (16.67) | | |
| Sample type | Breast | 120 | 5 (4.17) | 5 (4.17) | $p < 0.05$ | $p < 0.05$ |
| | Thigh | 120 | 23 (19.17) | 29 (24.17) | | |
| | Neck skin | 120 | 12 (10.00) | 40 (33.33) | | |
| City | Benslimane | 72 | 1 (1.39) | 3 (4.17) | $p < 0.05$ | $p < 0.05$ |
| | Bouznika | 72 | 9 (12.50) | 36 (50.00) | | |
| | Casablanca | 72 | 5 (6.94) | 18 (25.00) | | |
| | Mohammedia | 72 | 15 (20.83) | 13 (18.06) | | |
| | Rabat | 72 | 10 (13.89) | 4 (5.56) | | |

[1] *Salmonella* spp, [2] *Staphylococcus aureus*

**Table 3.** Distribution of *Staphylococcus aureus* and *Salmonella* spp. per sample type in the formal and informal sector

| Sample types | Breast | | | Thigh | | | Neck skin | | |
|---|---|---|---|---|---|---|---|---|---|
| | Positive | Negative | Total | Positive | Negative | Total | Positive | Negative | Total |
| *Staphylococcus aureus* | 6 | 174 | 180 | 29 | 151 | 180 | 51 | 129 | 180 |
| *Salmonella* spp. | 5 | 175 | 180 | 23 | 157 | 180 | 12 | 168 | 180 |

**Table 4.** Antimicrobial susceptibility of *Salmonella* spp. isolated from broiler chicken meat samples in the traditional slaughterhouses

| ***Salmonella* spp. positive samples** / **Antibiotics** | Resistant (%) | | Intermediate (%) | | Sensitive (%) | |
|---|---|---|---|---|---|---|
| Tetracycline | 32/40 | (80.0) | 6/40 | (15.0) | 2/40 | (5.0) |
| Ciprofloxacin | 23/40 | (57.5) | 7/40 | (17.5) | 10/40 | (25.0) |
| Kanamycin | 11/40 | (27.5) | 18/40 | (45.0) | 11/40 | (27.5) |
| Nalidixic acid | 10/40 | (25.0) | 10/40 | (25.0) | 20/40 | (50.0) |
| Trimethoprim-sulfamethoxazole | 2/40 | (5.0) | 2/40 | (5.0) | 36/40 | (90.0) |
| Cefoxitin | 1/40 | (2.5) | 0/40 | (0.0) | 39/40 | (97.5) |
| Gentamycin | 0/40 | (0.0) | 2/40 | (5.0) | 38/40 | (95.0) |



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**Table 5.** Antimicrobial susceptibility of *Staphylococcus aureus* isolated from broiler chicken meat samples in the traditional and modern slaughterhouses

| *Staphylococcus aureus* positive samples / Antibiotics | Resistant (%) | | Intermediate (%) | | Sensitive (%) | |
|---|---|---|---|---|---|---|
| Ampicillin | 86/86 | (100.00) | 0/86 | (0.00) | 0/86 | (0.00) |
| Tetracycline | 70/86 | (81.40) | 14/86 | (16.28) | 2/86 | (2.32) |
| Kanamycin | 64/86 | (74.42) | 0/86 | (0.00) | 22/86 | (25.58) |
| Erythromycin | 47/86 | (54.65) | 38/86 | (44.19) | 1/86 | (1.16) |
| Streptomycin | 44/86 | (51.16) | 0/86 | (0.00) | 42/86 | (48.84) |
| Trimethoprim-sulfamethoxazole | 1/86 | (1.16) | 4/86 | (4.65) | 81/86 | (94.19) |

**Table 6.** Comparison of the number of *Salmonella* spp. and *Staphylococcus aureus* resistant to up to 5 antibiotics at the same time.

| Number of Antibiotics | Resistant isolates of *Salmonella* spp. | | Resistant isolates of *Staphylococcus aureus* | |
|---|---|---|---|---|
| | Total samples | Percentage | Total samples | Percentage |
| **0** | 1 | 2.5% | 0 | 0% |
| **1** | 12 | 30% | 1 | 1% |
| **2** | 15 | 37.5% | 11 | 13% |
| **3** | 10 | 25% | 22 | 26% |
| **4** | 2 | 5% | 37 | 43% |
| **5** | 0 | 0% | 15 | 17% |
| **Total** | **40** | **100%** | **86** | **100%** |

The antibiotics used for *Salmonella* spp. contained Nalidixic acid, Gentamicin, Trimethoprim/sulfamethoxazole, Cefoxitin, Kanamycin, Ciprofloxacin, Tetracycline; For *Staphylococcus aureus*: Erythromycin, Trimethoprim/sulfamethoxazole, Tetracycline, Kanamycin, Streptomycin, Ampicillin.

**Multiple correspondence analysis**

In MCA, the engaged factors were given two dimensions, which summarize the information given by the set investigated variables. Thus, Cronbach's Alpha value was 80%, indicating that all the variables measure the same construct. Figure 1 measures the trend of results for each variable. A grouping of variables neck skin and *S. aureus* was noticed, which confirmed a correlation by reflecting the high rate of *S. aureus* isolated in this sample. This goes to all correlations, resulting in a grouping represented in the graph.

Figure 2 is labeled by the identifier of each observation (sample). In the current study, cases were numbered from 1 to 540. On the graph, there was a grouping of all observations on one side except for 12 observations (all in the formal sector) and followed some different modalities. The two dimensions of the domain (dimension 1 with 26.5% of variance and dimension 2 with 11.68% of variance) depended on the projection model used. In this study, the choice was made on dimension 1, which best explained the direction of exposure between positives - negatives and the determining factors (Figures 1 and 2). Concerning Figure 3, it grouped correlating variables into four groupings, including *S. aureus* and neck skin, formal and informal sectors, *Salmonella* spp. and thighs, as well as summer and winter seasons. This was in accordance with the results obtained.

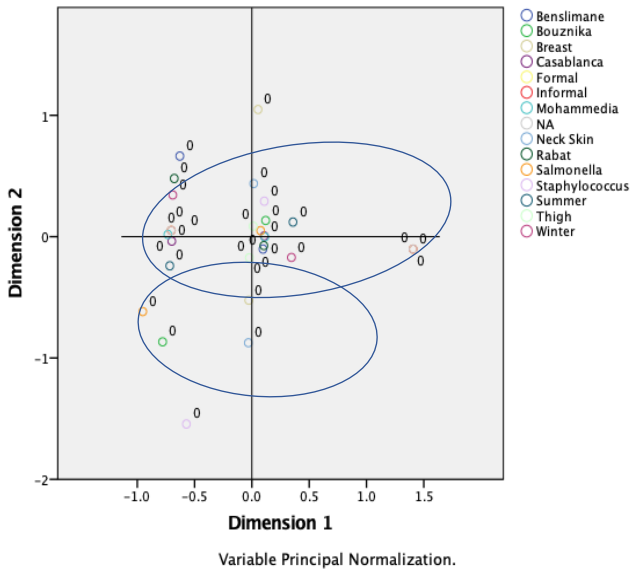


**Figure 1.** Projection of variables on the factorial axes

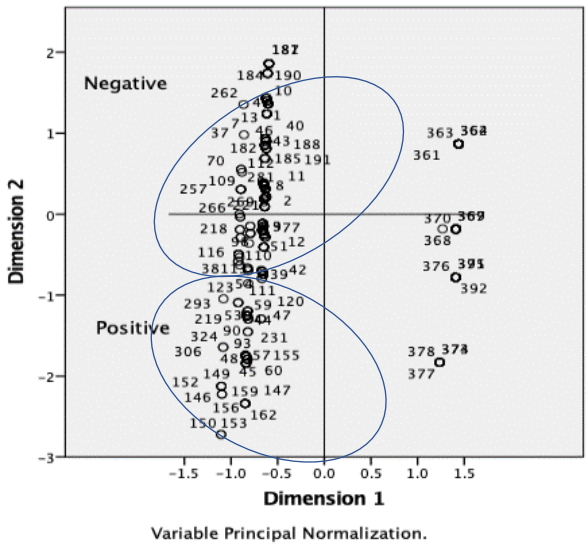


**Figure 2.** Projection of positives and negatives on the factorial axes



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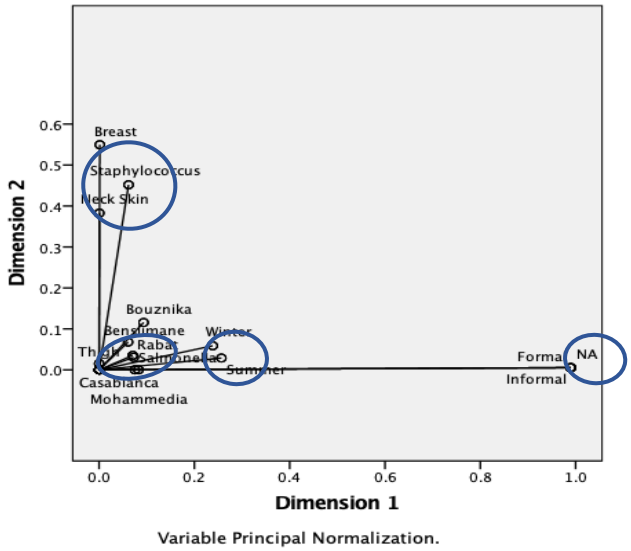


**Figure 3.** Variables differentiation analysis

**The cross-sectional study**

The present cross-sectional study included non-randomized comparison groups (exposed and unexposed) due to practical considerations in terms of time and data availability. The calculated sample size for an expected incidence in unexposed equaled 0.03, with a test power of 80% and a level of bilateral trust of 95%. To gain a risk ratio (RR) of 3, a total sample size of 486 samples was needed (243 per group, including exposed and unexposed).

**Comparison according to the sample type**

The *Salmonella* spp. and *S. aureus* loads in chicken meat were not the same regardless of the sampling type. There was strong evidence of an association between a specific sampling type and an overload of *Salmonella* spp. and *S. aureus* in chicken meat products. The RR estimate could be higher if sampling was on the neck skin and thigh than on the breast. For *Salmonella* spp., the RR estimate was about 3.5 (CI: 1.39-8.79, $p < 0.05$). The RR was significantly higher for *S. aureus*, with a RR of 6.66, compared to *Salmonella* spp. (CI: 2.96-14.9, ($p < 0.05$).

**Comparison according to the sector**

The risk of contamination of meat products with Salmonella spp. and S. aureus was significantly higher in the informal than in the formal sector. The RR estimate for *Salmonella* spp. and *S. aureus* was, respectively, 11.11 (CI: 7.86-14.35, $p < 0.05$) and 3.08 (CI: 1.72-5.52, $p < 0.05$).

**Comparison according to the size of the city**

The RR estimation was carried out only for the informal sector. It revealed a high risk of contamination by *S. aureus* in small-sized cities (Benslimane and Bouznika, RR = 1.77, CI: 1.10-2.83, $p < 0.05$). Exposure to small-sized cities determinant seems to be driving protection against *Salmonella* toxi-infection (RR < 1), but the RR estimate was not significant (RR = 0.66, CI: 0.30-1.43, $p > 0.05$).

**Comparison according to the seasonal pattern**

Regarding RR, meat handling and processing were subjected to contamination by *S. aureus* and *Salmonella* spp. in the summer period than in the winter with a RR of 1.46 for *S. aureus* (CI: 0.96-2.22, $p > 0.05$) and 1.10 for *Salmonella* spp. (CI: 0.61-1.98, $p > 0.05$). However, considering the p value, it was difficult to validate the significance of the association.

## DISCUSSION

The statistical analyses showed a significant difference between the prevalence of both investigated bacteria in the formal and informal sectors ($p < 0.05$). The prevalence of *S. aureus* and *Salmonella* spp. was higher in the informal sector compared to the formal sector, which might result from better control of the production chain and hygiene conditions in the formal sector. Taking into consideration that the informal sector is the main supplier of broiler chicken meat to the Moroccan market, it represents a higher risk of food contamination (FISA, 2022b) and remains a potential



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source of pathogens and a direct cause of enteric diseases worldwide, especially in developing countries (Adesiji et al., 2011; Akbar and Anal, 2013).

The results of this study revealed that 40/540 broiler chicken meat samples of both sectors (7.40%) were contaminated with *Salmonella*. This is in agreement with the previous studies, which found a prevalence of 7.30% and 6.67% of *Salmonella* spp. in broiler chicken meat purchased from poultry slaughterhouses in Thailand and Brazil, respectively (Chotinun et al., 2014; Panzenhagen et al., 2016). Moreover, Gu et al. (2020), Assèta et al. (2011), and Abba et al. (2017), respectively found the prevalence of 57% and 37% and 34.15% *Salmonella* spp. in broiler chicken meat samples from poultry slaughterhouses in China and open markets in Burkina Faso, and at points of sale in the markets of the city of N'Djaména, Tchad. In addition, the prevalence of *Salmonella enteritidis* in the present study was 67.5%. These findings are supported by a previous study that showed a prevalence of 55.7% of *Salmonella enteritidis* in turkey and broiler carcasses in Southern Brazil (Ruban et al., 2012).

The presence of *Salmonella* spp. in broiler chicken meat could be attributed to the lack of proper cold chains, inadequate power supply, and poor hygiene at retail outlets (Ruban et al., 2012). *Salmonella enteritidis* was also identified as the most common serotype in human cases; it was mainly found in broiler chicken meat and laying hens (Gu et al., 2020). Moreover, it was reported that *Salmonella* spp. contamination in broiler chicken meat decreases with the modernization of the slaughter process (Ruban et al., 2012).

This bacterium also colonizes a high percentage of broilers during fattening. The skin and meat of carcasses are frequently infected with the pathogen during slaughter and processing (FAO, 2022). several studies have reported *Salmonella* prevalence in broiler farms as 34.37%, 24%, 19.9%, in Algeria, Morocco, and Tunisia, respectively (Chaiba and Rhazi Filali, 2016; Djeffal et al., 2018; Oueslati et al., 2021). Therefore, it is important to consider farm contamination, which represents a critical stage in the development of *Salmonella*, both in terms of its impact on public health and the significant economic repercussions it can generate (Chaiba and Rhazi Filali, 2016).

Another study noted that 15.92% of broiler chicken meat samples from both sectors were contaminated with *S. aureus*. Similar rates were reported in Thailand and Morocco, with 18.18% and 16.66%, respectively (Akbar and Anal, 2013; Khallaf et al., 2014). A higher prevalence of *S. aureus* was reported in traditional slaughterhouses, compared to modern slaughterhouses (11.11% and 6.66%), which is in line with the results of Khallaf et al. (2014) at 27% and 8%, respectively. The results of the current study were indicative of the non-respect of good hygiene practices in traditional slaughterhouses, affecting the hygienic quality of broiler chicken meat.

The majority of the traditional slaughterhouses from which the samples were taken did not meet the minimal hygiene standards requirements, indicating the presence of *S. aureus*. In addition, the employers in these slaughterhouses did not undergo physical examinations, wore unclean and working uniforms, and worked in questionable hygienic conditions.

The use of modern slaughtering installations could considerably reduce the bacterial load in chicken meat, as was found in another study with a lower prevalence of *S. aureus* and absence of *Salmonella* spp. (Ruban et al., 2012). It was also found that there was a significant difference between the types of samples analyzed regarding *S. aureus*; a higher prevalence was found in the neck skin (59.3%), which could be explained by poor hygiene conditions and handling. In addition, a higher prevalence of *Salmonella* spp. was found in the thighs (57.5%), which can be due to their proximity to the point of evisceration and the maximum handling of the thigh region during dressing operations.

According to the results of MCA, the present study revealed positive samples (*Salmonella* spp. and *S. aureus*) when meat products were manipulated and processed in the informal sector, small cities, and during the summer season (Group I). Conversely, the negative samples (*Salmonella* spp. and *S. aureus*, group II) belonged to a context with best hygiene practices and efficient veterinary control (formal slaughterhouse factory, big-sized cities, winter period). To assess the effect of seasonality and size of the cities on the bacterial load in meat products, the study considered the primary measures, including refrigeration devices and rigorous good hygiene practices for the formal sector, which could considerably limit the bacterial load. Formal factories were not severely understaffed or suffered weaker links to the hygiene control process. Therefore, the season and city size factors were tested separately only for data from the informal sector. This compromised the sample size to 33% reduction and would limit the significant association, as mentioned above in the result section.

Regarding the *in-vitro* antibiotic sensitivity test results, several studies have focused on the antibiotic resistance of *S. aureus* and *Salmonella* spp. strains in poultry farms as well as in industrial or traditional poultry slaughterhouses. It was reported that high rates of *Salmonella* spp. resistance to tetracycline in chicken meat (Andoh et al., 2016; Abba et al., 2017). El Allaoui et al.(2017) found a high resistance level (79%) to tetracycline in Moroccan broiler turkey farms. On the other hand, Odoch et al. (2017) reported a relatively lower rate of tetracycline resistance (5.1%). The difference in the tetracycline resistance rates found could be explained by variability in the frequency and method of this molecule use.

In the current study, isolated *Salmonella* strains commonly showed resistance to ciprofloxacin and nalidixic acid and significant susceptibility to cefoxitin, gentamycin, and trimethoprim-sulfamethoxazole, similar to some other studies



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(Andoh et al., 2016; El Allaoui et al., 2017; Yang et al., 2010). Quinolones were part of the treatment of typhoidal salmonellosis. Although nalidixic acid has good activity *in vitro*, it was inefficient at the clinical practice level, probably due to its low bioavailability (Marchou and Meurisse, 1992), unlike fluoroquinolones, such as ciprofloxacin, having a higher oral bioavailability and have been the reference treatment for non-typhic salmonellosis for a decade (Marchou and Meurisse, 1992).

The wide use of antibiotics in veterinary and human medicine caused the emergence of *in vitro* resistance in certain serovars with failures to ciprofloxacin in some countries, such as the United Kingdom, India, Slovak Republic, and France (Piddock LJV, 1990; Weill et al., 2006). This could be related to the non-cautious use against other diseases, or illicit use of these molecules in food additives, or even acquired genes (Andoh et al., 2016). Moreover, *Salmonella* resistant to ciprofloxacin is usually resistant to several other antibiotics (Cui et al., 2008) and are associated with significant morbidity and mortality (El Allaoui et al., 2017). According to El Allaoui et al. (2017), the isolated *Salmonella* strains have a 27.50% resistance rate to kanamycin. However, high sensitivities were observed with cefoxitin (97.50%), trimethoprim-sulfamethoxazole (90%), and gentamycin (95%). This could be explained by the fact that aminoglycosides, especially gentamycin, have shown good activity against several human pathologies. Gentamycin is one of the most rarely recommended antibiotics in poultry farming because of its parenteral administration mode (Elared et al., 2001). According to the results of the present study, all *S. aureus* strains showed resistance against ampicillin, similar to same as a study conducted in Nigeria (Awogbemi et al., 2018). The high tetracycline resistance determined in previous studies was similar to that found in the present study (Kraushaar et al., 2017; Lika et al., 2021). Additionally, high antimicrobial resistance rates were demonstrated against kanamycin 74.41% in accordance with the results obtained in South Africa (Mkize et al., 2017). *Staphylococcus aureus* isolated from the chicken meat samples demonstrated moderate resistance to erythromycin and streptomycin with 54.65% and 51.16%, respectively, which was also reported in other studies (Mkize et al., 2017). However, 94.18% of *S. aureus* strains were susceptible to trimethoprim-sulfamethoxazole.

Based on the obtained results, it was observed that 97% of *Salmonella* spp. isolates were resistant to at least one antibiotic, while 30% were resistant to more than three antibiotics. All *S. aureus* isolates showed resistance to at least one antibiotic and a significant rate of 86% of resistance to more than three antibiotics. This high rate of multi-resistance to antibiotics is probably due to inappropriate treatment, either overtreatment or short treatment, or even inadequate treatment of these antibiotics. This situation can significantly impact the epidemiology of human salmonellosis, and thus considerably limit the choice of antibiotics for therapy (Elared et al., 2001). Therefore, regular and close monitoring and rationalization of poultry antibiotic use is recommended.

## CONCLUSION

Present results indicated that broiler chicken meat purchased from traditional poultry slaughterhouses of the study area (Ryachates) is mostly contaminated by *Salmonella* spp. and *S. aureus*. These Ryachates are mostly approached by Moroccan citizens, and directly threaten the consumers' health. Therefore, it is difficult to treat infected patients with antibiotic resistance. Therefore, to overcome this situation, a close collaboration of the staff in different sectors is required for attention to the hygiene standards and regulations in force. Therefore, future studies can be conducted to investigate a molecular study of the strains by sequencing and metagenomic analysis to determine the relationship between animal health and human health. Moreover, it is important to determine the minimum inhibitory concentrations (MIC) of the antibiotics used for virulence genes in each isolated bacterium.

## DECLARATIONS

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### Authors' contribution

Sabrine Nacer and Sophia Derqaoui collected samples used in this study. Sabrine Nacer and Fatima Zahra El Ftouhy performed the analysis in the laboratories. Sabrine Nacer and Mounir Khayli contributed data analysis. Sabrine



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Nacer wrote the original draft. Mustapha Lkhider and Saadia Nassik 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**

The authors declare that there is no conflict of interest.

**Data availability**

The authors declare that they have all the necessary data and are available where appropriate or requested by the editor.

**Ethical considerations**

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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# Effects of Amphora Algae on Productive Performance and Immune Response of Broiler Chickens

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**ABSTRACT**

Microalgae, especially *Amphora coffeaeformis* (*A. coffeaeformis*), are introduced to poultry diets, mainly as a rich source of polyunsaturated fatty acids (PUFAs), α-linolenic acid, eicosapentaenoic (EPA) and docosahexaenoic (DHA). This study aimed to investigate the effect of dietary supplementation of *A. coffeaeformis* on broiler chickens' productive performance, physiological status, and immune response. A total of 180 (Ross 508) broiler chickens aged one day were wing banded and randomly divided into three treatments and a control group according to the form of *A. coffeaeformis*, with 45 chickens each. Each treatment had three replicates (15 chickens for each replicate). Chickens from the three treatments were fed a diet supplemented with *A. coffeaeformis* algae at levels of 0.15, 0.45, and 0.75% of the diet from the first week to the fifth weeks of age. The obtained results indicated a significant difference in live body weight (LBW), body weight gain (BWG), and growth rate (GR) at the different experimental periods due to the effects of *A. coffeaeformis* treatments compared to the control group. Chickens fed basal diet and diet with *A. coffeaeformis* at levels of 0.45%, and 0.75% significantly increased LBW, BWG, and GR% at all intervals (1-3), (3-5), and (1-5) weeks of age compared to *A. coffeaeformis* algae at levels of 0.15%. Chickens fed a diet supplemented with *A. coffeaeformis* 0.45% and AC 0.75% recorded higher plasma total protein insignificantly, albumin significantly, at five weeks of age compared to the other *A. coffeaeformis* treatments and control group. Moreover, the lower levels of plasma triglycerides, total cholesterol, LDL, and significantly higher levels of plasma HDL were found at a basal diet supplemented by *A. coffeaeformis* 0.15% and the control group. Also, AC 0.15% and *A. coffeaeformis* 0.45% recorded insignificantly lower plasma levels of Glutathione and Superproxedase (58.55 and 71.43 mg/l, respectively) when compared with other *A. coffeaeformis* treatments and control group. Dietary supplementation of chickens' feed with *A. coffeaeformis* microalgae can promote the proliferation of beneficial bacteria (microbiota).

**Keywords:** *Amphora coffeaeformis*, Antioxidative status, Broiler chickens, Blood parameter, Immune response, Microalgae

## INTRODUCTION

According to estimates, in 2025, the consumption of animal products will rise as an increasing population in the World, therefore, the consumption of proteins (FAO 2020). When the continued meat consumption is increased, the consumption of animal proteins will increase, which leads to overuse and subsequent limitation of traditional sources used for livestock and aquatic animal feed, such as corn, rice, soybeans, and fish meal which causes overexploitation (Cardinaletti et al., 2018; Valente et al., 2021). Due to the perfect nutritional structure, microalgae are hopeful of overcoming overuse of the consumption of animal products (Wild et al., 2019). Microalgae are a diverse category of photosynthetic organisms that live in freshwater and marine habitats and can be unicellular, multicellular, or eukaryotic (Bhuvana et al., 2019). Additionally, it gains a greater yield than conventional crops and does not involve using pesticides or causing land disputes with agricultural activities (Koyande et al., 2019; Wild et al., 2019). Substances necessary for feed during the growth of animals, including polysaccharides, polyunsaturated fatty acids (PUFAs), protein, essential amino acids, minerals, vitamins, lipids, phenolic, and antioxidant pigments are formed by Microalgae (Bhuvana et al., 2019; Santhakumaran et al., 2020). After being incorporated into animal feed, the microalgae biomass produces chemicals that are essentially required for the protein and energy for animal growth (de Tonnac et al., 2018, Kibria and Kim, 2019). Due to microalgae, biomass increases animal feed digestibility and immune response, improving meat's nutritional, technological, and sensory qualities (de Tonnac et al., 2018, Kibria and Kim, 2019).

Algae can manufacture powerful and advantageous natural substances (such as polyphenols, sterols, polyunsaturated fatty acids, proteins, sulfated polysaccharides, alkaloids, agonic acid, and carotenoids). For this reason, international pharmaceutical companies have recently been interested in using algae (Ayoub et al., 2019). *Amphora*



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*coffeaeformis* (*A. coffeaeformis*) is most frequently seen in brackish and alkaline freshwater environments (Bhosle et al., 1993). Microalgae are the source of Long-chain polyunsaturated fatty acids (LC-PUFAs); therefore, it is used as food in humans, animals, and aquaculture (Lee et al., 2019). As well as algae antioxidant capacity, especially AC revealed rich concentrations of PUFAs, particularly DHA, linoleic acid, and EPA (El-Bahr et al., 2020). So *A. coffeaeformis* dietary appeared to be more beneficial than *Chlorella vulgaris* and *Spirulina platensis* in antioxidant status, performance, and nutritional value (El-Bahr et al., 2020). This study aimed to investigate the effect of *A. coffeaeformis* supplement on broiler chickens' productive performance and physiological status and the impact on the immune response.

## MATERIAL AND METHODS

### Ethical approval

All samples were chosen per standard protocol without any animal stress or injury. Moreover, the study was done according to Cairo University Institutional Animal Care and Use Committee (CU- IACUC) Veterinary Medical and Agricultural Sciences Sector in Egypt, under the approval code # CU/II/F/19/21#.

### Study design

A total of 180 unsexed chicks aged one-day (Ross 508) broiler chickens were purchased from a local hatcher, a Gold Breeder Company. They were weighed individually (42 ± 1 g), given wing bands, and randomly assigned to three treatments and a control group according to the level of *A. coffeaeformis*, with 45 chickens each. Each treatment had three replicates (15 chickens in each replicate). Chickens from the three treatments were fed a diet with levels of *A. coffeaeformis* algae 0.15, 0.45, and 0. 75 %, respectively. The environment and hygienic conditions under which the chickens were kept in cages (1 m in length, 0.6 m in width, and 0.4 m in height) were similar. According to the vaccination program, the vaccinations were given to chickens against Newcastle, Gumboro diseases, and avian influenza. At 6, 10, and 14 days of age, we used the Hitchner B1 strain, H5N1, and Gumboro vaccines. Then, at 20 days of age, the chickens were revaccinated the samples against Newcastle virus disease (CEVA company, France). In the first three days of age, chickens were brood at 35°C inside Batteries with electric heaters and then decreased temperature of 2°C weekly till the end of the fourth week. The lighting schedule was 24 hours of light at three days, then reduced to 22 hours, and a 2-hour dark was applied from 6 to 35 days of age (the end of the experiment). Feed (as mash) and water were offered *ad libitum*. We fed starter and grower diets formulated as shown in Table 1.

**Table 1.** Composition and chemical analyses of starter and grower diets of broiler chickens (Ross 508)

| Ingredients (%) | Starter (1-14 days) | Grower (15-35 days) |
|---|---|---|
| Yellow corn | 56 | 59.89 |
| Soybean meal (46% protein) | 32 | 28.42 |
| Corn gluten | 6.05 | 4.95 |
| Soya oil | 1.5 | 2.53 |
| Mono-calcium phosphate | 1.55 | 1.38 |
| Limestone | 1.75 | 1.7 |
| Premix (Vitamin+Mineral)* | 0.2 | 0.2 |
| D.L. Methionine | 0.22 | 0.22 |
| L. Lysine Hcl | 0.25 | 0.25 |
| Salt | 0.40 | 0.40 |
| **Chemical analyses (%)** | | |
| Choline Chloride | 0.06 | 0.06 |
| Crude protein | 23 | 21 |
| Metabolizable energy (kcal/kg) | 3000 | 3100 |
| Calcium | 1.0 | 0.94 |
| Available phosphorus | 0.49 | 0.44 |
| Lysine | 1.4 | 1.3 |
| Methionine | 0.67 | 0.61 |
| Methionine + Cystine | 1.04 | 0.95 |
| Sodium | 0.18 | 1.8 |
| Total of diet | 100 | 100 |

*Every 2 grams of premix mixture contained: Vitamin A (trans-retinyl acetate), 9,000 IU; vitamin D3 (cholecalciferol), 2,600 IU; vitamin E (dl-α-tocopherol acetate), 16 mg; vitamin B1, 1.6 mg; vitamin B2, 6.5 mg; vitamin B6, 2.2 mg; vitamin B12 (cyanocobalamin), 0.015 mg; vitamin K3, 2.5mg; choline (choline chloride), 300 mg; nicotinic acid, 30 mg; pantothenic acid (d-calcium pantothenate), 10 mg; folic acid, 0.6 mg; d-biotin, 0.07 mg; manganese (MnO), 70 mg; zinc (ZnO), 60 mg; iron (FeSO4 H2O), 40 mg; copper (CuSO4 5H2O), 7 mg; iodine [Ca(IO3)2], 0.7 mg; selenium (Na2SeO3), 0.3 mg



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**Data collection**

**Body weight, weight gain, and growth rate**

Chickens were weighed individually at the first, third, and fifth weeks of age. Weight gain and growth rate were calculated separately with the formula reported by Broody **(**1949**).**

**Feed intake and Feed conversion ratio**

The experimental diets were provided regularly and measured daily. The feed intake was counted by subtracting the weighted given feed and remainder portion, further divided by the number of chickens for every experimental treatment, then expressed in grams per chicken at the period from (0-3), (3-5) and (0-5) weeks of age.

**Mortality rate**

The mortality rate percentage was calculated by subtracting the number of live chickens at the end of the experiment from the initial number.

**European production efficiency factor**

After the calculation of the Feed conversion ratio (FCR) and viability percentage, the European production efficiency factor (EPEF) was used to assess the growing process of broiler chickens, as found by Van (2003) and Marcu et al. (2013). European production efficiency factor was calculated according to Marcu et al. (2013) by Formula 1.

$$\text{EPEF} = \frac{\text{Viability (\%)} \times \text{BW(kg)}}{\text{Age (day)} \times \text{FCR (kg feed} \div \text{kg gain)}} \times 100 \qquad \text{Formula 1}$$

**Blood parameters**

After five weeks of age chickens, four blood samples were obtained randomly from each treatment from the wing vein for chemical analyses. Ten ml of blood samples were collected without anticoagulant into a clean centrifuge tube, and then heparinized blood samples were centrifuged at 2500 rpm for 15 minutes. Plasma samples were stored in the deep freezer at approximately -20°C until the time of chemical analyses. A commercial kit (Bio Diagnostic Company, Egypt) was used for the chemical analyses utilizing a colorimetric approach to determine the plasma protein fractions (albumin, total protein) and kidney function test (uric acid). The lipid profile includes triglycerides, total cholesterol, low-density lipoproteins (LDL), and high-density lipoproteins (HDL). The liver function had Aspartate aminotransferase (AST) and alanine aminotransferase, glucose, thyroid hormones (T3 and T4), measurements of antioxidant capacities of plasma (total antioxidants, glutathione peroxidase (GPx), and super peroxidase).

**Thyroid hormones**

The concentrations of T3 and T4 were calculated by Radioimmunoassay in plasma, as mentioned in previous studies by Huybrechts et al.(1989) and Darras et al. (1992). Intra assay of the variation of T3 and T4 at coefficients was 4.5 and 5.4%, respectively.

**Antioxidant capacities of plasma**

The samples were measured with GPx kits (Randox, Crumlin, UK). Total antioxidant status in plasma (Miller et al., 1993) and the activity of glutathione peroxidase in the blood (GPx, EC 1.11.1.9) was measured based on the technique by Wang et al. (2011).

**Immunity response**

The measurement of anti-Newcastle diseases vaccine antibody titers was done during weeks third and fifth to measure the humoral immunity by using a method described by Swayne (1998) that six serum samples that were subjected to hemagglutination inhibition.

**Bacteriological examination**

Ten samples of 5 grams of broiler intestine 2 cm long were isolated and cut-opened within complete sterilization conditions. After that, they were weighed and transmitted into Falcon tubes 50 ml which were sterile after adding 30 ml of saline solution (NaCl: 0.85%). At maximum speed, the samples were mixed for one minute by vortexing, and then tenfold serial dilutions from each sample by the same saline solution were prepared. Finally, the dilutions were used to detect and list various groups of bacteria. One ml from each of the previous dilutions was added into two individual aseptic Petri dishes, then added sterile plate agar that was melted and cooled. After mixing, counting total bacterial per gm, which incubated the inoculated plates at 30°C for 48 hours, and counting each plate that contained 30-300 colonies.

In two individual aseptic Petri-dishes that added one ml from each of the previous dilutions, sterile Eosin methylene blue agar (EMB) mediums were added that were melted and cooled. After mixing, the inoculated plates were incubated at 37°C for 48 hours. Based on the differential counts of green, colorless, and pink colonies, the *Escherichia*



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*coli* (*E. coli*), *Proteus* species., and *Enterobacter* species were counted per gram (Downes and Ito, 2001). The Xylose-lysine-Deoxycholate medium used to detect *Salmonella* as red colonies with the black center was recorded as positive for *Salmonella*.

### Statistical analysis

One-way analysis of variance was used to determine the effect of different levels of *A. coffeaeformis*: 0.15%, 0.45%, and 0.75% on the performance of growth, some blood analysis, and evaluation of humoral immunity. Data were statistically analyzed by the general linear model procedure of the SAS software (SAS 2004). The comparison of mean values was made by Duncan's multiple range test (Duncan 1955), and significant differences appeared to be ($p < 0.05$).

## RESULTS AND DISCUSSION

### Productive traits

Body weight, body weight gains, and growth rate as influenced by levels of *A. coffeaeformis* on broiler performance are shown in Table 2. Both *A. coffeaeformis* 0.45% and *A. coffeaeformis* 0.75% supplementation significantly improved broiler performance throughout the experiment period than *A. coffeaeformis* 0.15% and the control group. At weeks 3 and 5, *A. coffeaeformis* 0.45% and 0.75% had significant ($p < 0.05$) body weights (824.17 and 1955.61 g, respectively). Body weight gain and growth rate in all groups were similar at (1-5) weeks, and all treatments of *A. coffeaeformis* recorded significantly ($p < 0.05$) low values during the same periods obtained in Table 2.

This result may be due to *A. coffeaeformis* significantly improved appetite, which gave rise to higher feed intake and progressed growth (Ayoub et al., 2019). The absorption of minerals and vitamins was improved (Gružauskas et al., 2004, Mariey et al., 2012) as Kaoud (2012) reported dietary *Spirulina platensis* ($p < 0.05$) raised Life body weight. These results agreed with those discovered by Zhao et al. (2004), who indicated that chickens fed with *Amphora* showed a higher average daily gain. The growth rate improved due to the efficiency of *A. coffeaeformis* in getting better immune status and serum composition (Abudabos et al., 2013) and reducing microbial load in the gastrointestinal tract (Costa et al., 2022). The results obtained agree with those found by Kang et al. (2013), Khan et al. (2021), and Long et al. (2018), who reported that in broiler chickens, the growth parameters were improved by adding microalgae in feed.

**Table 2.** The effect of different levels of *Amorpha coffeaeformis* on body weight, body weight gain, and growth rate of broiler chickens (Ross 508) at first, third, and fifth weeks of age

| Treatment | Body weight (g) | | | Body weight gain (g) | | | Growth rate (%) | | |
|---|---|---|---|---|---|---|---|---|---|
| | 1 Weeks | 3 Weeks | 5 Weeks | 1-3 Weeks | 3-5 Weeks | 1-5 Weeks | 1-3 Weeks | 3-5 Weeks | 1-5 Weeks |
| Control | 184.2 ± 1.74$^{a}$ | 798.0 ± 15.17$^{a}$ | 1888.53 ± 22.70$^{b}$ | 613.59 ± 4.80$^{c}$ | 1087.15 ± 10.74$^{b}$ | 1700.74 ± 12.77$^{b}$ | 124.95 ± 0.44$^{b}$ | 81.02 ± 0.50$^{b}$ | 164.36 ± 0.24$^{c}$ |
| AC 0.15% | 172.90 ± 1.74$^{b}$ | 805.6 ± 13.84$^{a}$ | 1886.63 ± 20.93$^{b}$ | 632.69 ± 4.80$^{b}$ | 1080.41 ± 10.74$^{b}$ | 1713.10 ± 12.77$^{b}$ | 129.32 ± 0.44$^{a}$ | 80.28 ± 0.50$^{b}$ | 166.40 ± 0.24$^{b}$ |
| AC 0.45% | 176.45 ± 1.74$^{b}$ | 824.1 ± 13.84$^{a}$ | 1929.05 ± 20.42$^{ab}$ | 647.71 ± 4.80$^{a}$ | 1104.88 ± 10.74$^{b}$ | 1752.60 ± 12.77$^{a}$ | 129.46 ± 0.44$^{a}$ | 80.26 ± 0.5$^{b}$ | 166.48 ± 0.24$^{b}$ |
| AC 0.75% | 173.52 ± 1.74$^{b}$ | 749.5 ± 14.01$^{b}$ | 1955.61 ± 20.67$^{a}$ | 575.38 ± 4.80$^{d}$ | 1206.21 ± 10.74$^{a}$ | 1781.58 ± 12.77$^{a}$ | 124.72 ± 0.44$^{b}$ | 89.23 ± 0.50$^{a}$ | 167.39 ± 0.24$^{a}$ |
| p-value | 0.0001 | 0.0019 | 0.0613 | 0.0001 | 0.0001 | 0.0008 | <.0001 | <.0001 | <.0001 |

$^{abcd}$ Means different superscript letters in each column express significant differences ($p < 0.05$). AC: *Amorpha coffeaeformis*

Table 3 shows the effect of *A. coffeaeformis* supplementation levels (%) on feed intake, FCR, Mortality rate, and European broiler efficiency index. Broiler chickens fed the diet supplemented with *A. coffeaeformis* 0.75% and *A. coffeaeformis* 0.15 % were found to be consumed a lower average feed intake throughout the experiment period than 0.45% and the control group. Broiler chickens fed the diet supplemented with *A. coffeaeformis* at a level of 0.15% and *A. coffeaeformis* at 0.45% improved FCR insignificantly ($p > 0.05$) throughout the experiment than 0.75% and the control group. Broiler chickens fed the diet supplemented with *A. coffeaeformis* 0.45% recorded a significantly lower mortality rate ($p < 0.05$) during the experimental period than other treatments. Broiler chickens fed the diet supplemented with *A. coffeaeformis* 0.75% and *A. coffeaeformis* 0.45% showed significantly ($p < 0.05$) higher average EPEF% during the whole experimental period than other groups. The *A. coffeaeformis* 0.15% and the control group recorded the significantly highest average of MR% and EPEF% ($p < 0.05$), respectively, compared to the other *A. coffeaeformis* treatments.

The results obtained agree with those reported by Kharde et al. (2012), which indicated that adding microalgae *Spirulina platensis* to broiler chicken diets significantly boosted FCR compared to the control diet. This enhancement



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could be attributed to a healthy microbial community in the gastrointestinal system, which improves the absorption of dietary vitamins and minerals and plays a critical part in the health of broilers (Belay et al., 1996; Kharde et al., 2012). The results obtained may be attributed to *A. coffeaeformis* has various elements, including vitamins and minerals that may help to promote growth, improvement in the FCR (Belay et al., 1996), and getting better in the digestibility of nutrients which were in agreement with Zhao et al. (2004). It is possible that Amphora's bioactive chemicals, which include antibacterial, antiviral, anti-inflammatory, and antioxidant activities, are responsible for the favorable results that have been concluded by (Rajput and Mishra 2012, Salahuddin et al., 2017). The increased EPEF and decreased mortality rate of chickens fed on supplemented diets could be strengthened the usefulness of dietary additives (microalgae) on nutrients and feed efficiency as well as being antibacterial and pathogens (Alwaleed et al., 2021). The results agree with those reported by Abdel-Moneim et al. (2022), which indicated that in correlation with the amounts and mixtures of the dietary supplements of microalgae, the EPEF increased significantly.

**Table 3.** The effect of different levels of *Amorpha coffeaeformis* on feed intake, feed conversion, ratio, mortality rate and European broiler efficiency index of broiler chickens (Ross 508) at first, third, and fifth weeks of age

| Treatment | Feed intake (g) | | | Feed conversion ratio (g) | | | Mortality rate (%) | EBI (%) |
|---|---|---|---|---|---|---|---|---|
| | 1-3 Weeks | 3-5 Weeks | 1-5 Weeks | 1-3 Weeks | 3-5 Weeks | 1-5 Weeks | 1-5 Weeks | 1-5 Weeks |
| Control | 830.39± 17.58 | 1740.39± 36.28$^{b}$ | 2591.78± 83.02 | 1.35 ± 0.03 | 1.60 ± 0.02$^{b}$ | 1.53 ± 0.05 | 19.05 ± 1.84a | 295.15 ± 11.70$^{b}$ |
| AC 0.15% | 849.38± 17.58 | 1628.21± 36.28$^{c}$ | 2539.12± 83.02 | 1.34 ± 0.03 | 1.5 0± 0.02$^{c}$ | 1.48 ± 0.05 | 4.76 ± 1.84b | 346.31 ± 11.70$^{b}$ |
| AC 0.45% | 870.71± 17.58 | 1858.33± 36.28$^{a}$ | 2750.99± 83.02 | 1.34 ± 0.03 | 1.68 ± 0.02$^{a}$ | 1.57 ± 0.05 | 0.00±0.00c | 351.37 ± 11.70$^{a}$ |
| AC 0.75% | 826.43± 17.58 | 1837.90± 36.28$^{ab}$ | 2686.58± 83.02 | 1.44 ± 0.03 | 1.52 ± 0.02$^{c}$ | 1.51 ± 0.05 | 2.38 ± 1.84b | 363.63 ± 11.70$^{a}$ |
| p-value | 0.2917 | 0.0008 | 0.3006 | 0.1500 | 0.0002 | 0.7354 | <.0001 | 0.0027 |

$^{abc}$ Means different superscript letters in each column express significant differences ($p < 0.05$). AC: *Amorpha coffeaeformis*

**Blood parameters**

The impact of different levels of *A. coffeaeformis* supplement on blood plasma constituents at three weeks is shown in tables 4 and 5. The *A. coffeaeformis* 0.15% caused increased Total protein ($p > 0.05$), Albumin ($p > 0.05$), AST ($p < 0.005$), ALT ($p > 0.05$), T3, T4 total antioxidant insignificantly ($p > 0.05$), respectively compared to the other *A. coffeaeformis* treatments and control group. Also decreased insignificantly GPX ($p > 0.05$) and Superoxidase (SPX, $p > 0.05$), same as *A. coffeaeformis* 0.45% when compared with other *A. coffeaeformis* treatments and control group. However, *A. coffeaeformis* 0.45% decreased total plasma cholesterol insignificantly ($p > 0.05$) and LDL ($p < 0.05$). They also significantly increased plasma triglycerides ($p < 0.05$) and insignificantly HDL ($p > 0.05$) compared to the other *A. coffeaeformis* treatments and control group. Furthermore, *A. coffeaeformis* 0.75% insignificantly increased blood glucose ($p > 0.05$). However, the control group was insignificantly ($p > 0.05$) lower in plasma uric acid levels at three weeks than those in the *A. coffeaeformis* treatments, and also increased AST ($p < 0.05$) showed in the control group.

The effect of different levels of *A. coffeaeformis* supplement on blood plasma constituents at five weeks are shown in tables 6 and 7. Plasma albumin levels were affected significantly ($p > 0.05$) by experimental treatments only at five weeks of age at all levels of *A. coffeaeformis* treatment. The *A. coffeaeformis* 0.45% significantly increased TP ($p < 0.05$) compared to the other *A. coffeaeformis* treatments and control group. The *A. coffeaeformis* of levels 0.45% and 0.75% were significant decreases of GPX ($p < 0.05$) and SPX and insignificant increases of antioxidants ($p < 0.05$). However, *A. coffeaeformis* 0.75% increased blood glucose ($p > 0.05$), T3, and T4 ($p > 0.05$) and also increased AST ($p < 0.05$) compared to the other *A. coffeaeformis* treatments and control group. Although *A. coffeaeformis* 0.15% decreased plasma Triglycerides ($p < 0.05$), Total cholesterol ($p < 0.05$) and LDL ($p < 0.05$) also increased ALT ($p > 0.05$) compared to the other *A. coffeaeformis* treatments and control group. Furthermore, the control group was significantly ($p < 0.05$) lower in uric acid levels at five weeks. Those in the *A. coffeaeformis* treatments also significantly increased HDL in the control group ($p < 0.05$).

These results agree with Long et al. (2018), who discovered that the addition of microalgae (MA) to broiler chicken's diet led to higher levels of plasma albumin/globulin ratio, lower levels of plasma total cholesterol and LDL compared to the control group, and higher levels of plasma glucose. Brown and Cline (1974) reported that the microalgae reduced plasma uric acid, exciting microalgae-assisted chickens for more efficient nitrogen utilization. These results did not agree with those reported by Sugiharto et al. (2018), who noticed that the serum biochemical parameters such as AST and AST were not significantly different ($p > 0.05$) across the microalgae treatments.



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**Table 4.** The influence of different levels of *Amorpha coffeaeformis* on plasma total protein, albumin, total cholesterol, triglycerides, low-density lipoprotein, high-density lipoprotein, aspartate aminotransferase and alanine aminotransferase in broiler chickens (Ross 508) aged three weeks

| Treatment | T.P (g/dl) | Al (g/dl) | TCH (mg/dl) | TG (mg/dl) | LDL (mg/dl) | HDL (mg/dl) | AST (mg/dl) | ALT (mg/dl) |
|---|---|---|---|---|---|---|---|---|
| Control | 7.42 ± 0.80 | 3.97 ± 0.24 | 281.346 ± 16.4$^{a}$ | 63.42 ± 13.70$^{b}$ | 209.70 ± 18.3$^{a}$ | 58.97 ± 5.53 | 37.97 ± 2.00$^{a}$ | 8.45 ± 0.46 |
| AC 0.15% | 8.23 ± 0.80 | 3.68 ± 0.24 | 256.88 ± 16.4$^{ab}$ | 117.5 ± 13.70$^{a}$ | 158.99 ± 18.3$^{ab}$ | 74.37 ± 5.53 | 31.34 ± 2.00$^{b}$ | 8.57 ± 0.46 |
| AC 0.45% | 8.17 ± 0.80 | 3.71 ± 0.24 | 223.24 ± 16.4$^{b}$ | 125.0 ± 13.70$^{a}$ | 123.30 ± 18.3$^{b}$ | 74.94 ± 5.53 | 30.49 ± 2.00$^{b}$ | 7.26 ± 0.46 |
| AC 0.75% | 7.33 ± 0.80 | 3.74 ± 0.24 | 245.87 ± 16.4$^{ab}$ | 98.15 ± 13.70$^{ab}$ | 160.03 ± 18.3$^{ab}$ | 66.21 ± 5.53 | 26.92 ± 2.00$^{b}$ | 7.32 ± 0.46 |
| p-value | 0.7837 | 0.8292 | 0.1262 | 0.0214 | 0.0272 | 0.1667 | 0.0075 | 0.0975 |

$^{ab}$ Means different superscript letters in each column express significant differences ($p \leq 0.05$). AC: *Amorpha coffeaeformis*, TP: Total protein, AL: Albumin, TCH: Total cholesterol, TG: Triglycerides, LDL: Low-density lipoprotein, HDL: High-density lipoprotein, AST: Aspartate aminotransferase, ALT: Alanine aminotransferase.

**Table 5.** The effect of different levels of *Amorpha coffeaeformis* on plasma triiodothyronine, thyroxine, glutathione, superoxidase, total antioxidant, glucose and uric acid in broiler chickens (Ross 508) aged three weeks

| Treatment | T3 (mg/dl) | T4 (mg/dl) | GPx (mg/dl) | SPX (mg/dl) | TAX (mg/dl) | Glucose (mg/dl) | UA (mg/dl) |
|---|---|---|---|---|---|---|---|
| Control | 5.67 ± 0.37 | 2.52 ± 0.03 | 91.08 ± 11.45 | 103.97 ± 8.80$^{a}$ | 0.620 ± 0.07 | 95.10 ± 6.24 | 3.95 ± 0.44 |
| AC 0.15% | 6.00 ± 0.37 | 2.63 ± 0.03 | 58.55 ± 11.45 | 76.98 ± 8.80$^{ab}$ | 0.637 ± 0.07 | 85.29 ± 6.24 | 4.07 ± 0.44 |
| AC 0.45% | 6.47 ± 0.37 | 2.62 ± 0.03 | 58.55 ± 11.45 | 71.43 ± 8.80$^{b}$ | 0.603 ± 0.07 | 89.71 ± 6.24 | 4.77 ± 0.44 |
| AC 0.75% | 5.93 ± 0.37 | 2.62 ± 0.03 | 78.06 ± 11.45 | 84.43 ± 8.80$^{ab}$ | 0.557 ± 0.07 | 103.68 ± 6.24 | 4.75 ± 0.44 |
| p-value | 0.5101 | 0.0648 | 0.1564 | 0.0800 | 0.898 | 0.2192 | 0.4153 |

$^{ab}$ Means different superscript letters in each column express significant differences ($p \leq 0.05$). AC: *Amorpha coffeaeformis*, T3: Triiodothyronine, GPx: Glutathione, SPX: Superoxidase, TAX: Total antioxidant, T4: Thyroxine, UA: Uric acid, MSE: Mean standard error

**Table 6.** The effect of different levels of *Amorpha coffeaeformis* on plasma total protein, total cholesterol, triglycerides, low-density lipoprotein, high-density lipoprotein, aspartate aminotransferase, alanine aminotransferase, and Albumin in broiler chickens (Ross 508) at five weeks of age

| Treatment | TP (g/dl) | Al (g/dl) | TCH (mg/dl) | TG (mg/dl) | LDL (mg/dl) | HDL (mg/dl) | AST (mg/dl) | ALT (mg/dl) |
|---|---|---|---|---|---|---|---|---|
| Control | 4.33 ± 0.37 | 3.87 ± 0.16$^{b}$ | 304.59 ± 4.50$^{a}$ | 130.55 ± 11.76$^{ab}$ | 233.64 ± 5.73$^{a}$ | 56.66 ± 2.31$^{a}$ | 31.34 ± 1.99$^{a}$ | 10.58 ± 0.64 |
| AC 0.15% | 4.58 ± 0.37 | 4.5 ± 0.16$^{a}$ | 242.20 ± 4.50$^{b}$ | 64.35 ± 11.76$^{c}$ | 181.63 ± 5.73$^{b}$ | 47.70 ± 2.31$^{b}$ | 21.15 ± 1.99$^{b}$ | 11.89 ± 0.64 |
| AC 0.45% | 5.50 ± 0.37 | 3.94 ± 0.16$^{b}$ | 300.92 ± 4.50$^{a}$ | 108.80 ± 11.76$^{b}$ | 223.64 ± 5.73$^{a}$ | 55.52 ± 2.31$^{a}$ | 33.04 ± 1.99$^{a}$ | 11.76 ± 0.64 |
| AC 0.75% | 5.29 ± 0.37 | 3.53 ± 0.16$^{b}$ | 296.64 ± 4.50$^{a}$ | 151.85 ± 11.76$^{b}$ | 224.02 ± 5.73$^{a}$ | 51.49 ± 2.31$^{ab}$ | 35.08 ± 1.99$^{a}$ | 10.83 ± 0.64 |
| p-value | 0.1142 | 0.0018 | <.0001 | 0.0003 | <.0001 | 0.0492 | 0.0004 | 0.3912 |

$^{ab}$ Means different superscript letters in each column express significant differences ($p \leq 0.05$). AC: *Amorpha coffeaeformis*, TP: Total protein, AL: Albumin, TCH: Total cholesterol, TG: Triglyceridesö LDL: low-density lipoprotein, HDL: high-density lipoprotein, AST: aspartate aminotransferase and ALT: alanine aminotransferase.

**Table 7.** The effect of different levels of *Amorpha coffeaeformis* on plasma triiodothyronine, thyroxine, glutathione, superoxidase, total antioxidant, glucose and uric acid in broiler chickens aged five weeks

| Treatment | T3 (mg/dl) | T4 (mg/dl) | GPx (mg/dl) | SPX (mg/dl) | TAX (mg/dl) | Glucose (mg/dl) | UA (mg/dl) |
|---|---|---|---|---|---|---|---|
| Control | 7.07 ± 0.27 | 2.59 ± 0.03 | 84.57 ± 5.75$^{a}$ | 76.99 ± 3.69 | 0.597 ± 0.04 | 94.12 ± 5.59$^{b}$ | 2.8 ± 0.26$^{b}$ |
| AC 0.15% | 7.00 ± 0.27 | 2.59 ± 0.03 | 58.55 ± 5.75$^{b}$ | 70.24 ± 3.69 | 0.473 ± 0.04 | 100.73 ± 5.59$^{ab}$ | 4.91 ± 0.26$^{a}$ |
| AC 0.45% | 6.07 ± 0.27 | 2.58 ± 0.03 | 50.43 ± 5.75$^{b}$ | 77.86 ± 3.69 | 0.490 ± 0.04 | 101.96 ± 5.59$^{ab}$ | 4.57 ± 0.26$^{a}$ |
| AC 0.75% | 6.70 ± 0.27 | 2.60 ± 0.03 | 58.55 ± 5.75$^{b}$ | 68.25 ± 3.69 | 0.553 ± 0.04 | 116.42 ± 5.59$^{a}$ | 5.04 ± 0.26$^{a}$ |
| p-value | 0.0675 | 0.9545 | 0.0026 | 0.2009 | 0.1974 | 0.0646 | <.0001 |

$^{ab}$ Means different superscript letters in each column express significant differences ($p \leq 0.05$). AC: *Amorpha coffeaeformis*, T3: Triiodothyronine, GPx: Glutathione, SPX: Superoxidase, TAX: Total antioxidant, T4: Thyroxine, UA: Uric acid.



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**Response of humoral immune**

The effect of different dietary levels of *A. coffeaeformis* supplementation (%) on the antibody titers against NDV is shown in Table 8. At 23 days of age, *A. coffeaeformis* 0.75% gained the highest titer (7.67) significantly compared with other *A. coffeaeformis* treatments and the control group. The results obtained may be referred to *A. coffeaeformis*, which was abundant in several pigments and polyphenolic chemicals, including Catechin, Gallic acid, and P-coumaric acid, which led to this immune system activation (El-Sayed et al., 2018). In addition, Jaswir et al. (2011) demonstrated the attendance of -carotene and fucoxanthin in *A. coffeaeformis*, which were frequently utilized as food additives in addition to the many nutraceuticals uses including pro-vitamin A, antioxidant, anticancer, and anti-obesity, made the plant a powerful radical scavenger. The results obtained disagree with those reported by Sugiharto et al. (2018), who noticed that there was no significant difference between the algae treatments that are found in the serum biochemical parameters and antibody titer against NDV.

**Table 8.** The influence of different levels of *Amorpha coffeaeformis* on antibody titer against Newcastle disease virus in Broiler Chickens (Ross 508)

| ND titer (Log2) / Treatment | Day 18 | Day 23 | Day 26 | Day 28 |
|---|---|---|---|---|
| Control | 5.00 ± 0.65 | 5.00 ± 0.70$^{b}$ | 10.00 ± 0.66$^{a}$ | 9.33 ± 0.63$^{a}$ |
| AC 0.15% | 4.67 ± 0.65 | 5.00 ± 0.70$^{b}$ | 8.67 ± 0.66$^{ab}$ | 9.33 ± 0.63$^{a}$ |
| AC 0.45% | 3.67 ± 0.65 | 5.67 ± 0.70$^{ab}$ | 8.33 ± 0.66$^{ab}$ | 6.33 ± 0.63$^{b}$ |
| AC 0.75% | 4.00 ± 0.65 | 7.67 ± 0.70$^{a}$ | 6.67 ± 0.66$^{b}$ | 4.00 ± 0.63$^{c}$ |
| p-value | 0.4695 | 0.0431 | 0.0165 | <.0001 |

$^{abc}$ Means different superscript letters in each column express significant differences ($p < 0.05$).AC: *Amorpha coffeaeformis*, HI: Humoral immunity, ND: Newcastle disease.

**Intestinal bacteriological counts**

The effect of various dietary levels of *A. coffeaeformis*% supplementation on the intestinal bacterial count is shown in Table 9. Compared to the control group, the results found a highly significant ($p < 0.05$) effect due to *A. coffeaeformis* treatments on E. coli. Broiler chickens fed the diet supplemented with *A. coffeaeformis* 0.45% and *A. coffeaeformis* 0.75 recorded the highest beneficial bacteria, absent *E. coli*, and the lowest count of *Proteus* species. as well as *Enterobacter* species which mounted 8.82, -negative 6.62 and 6.20, respectively when other levels of *A. coffeaeformis* treatments and were compared with the control group. The results obtained agree with those reported by Mariey et al. (2012) and Jamil et al. (2015), which showed that *A. coffeaeformis* activated the hens' immune systems and made them resistant to harmful microorganisms, including *E. coli*, *Enterobacter*, and *Proteus* proliferation.

**Table 9.** The influence of different levels of *Amorpha coffeaeformis* on intestinal bacterial counts in Broiler Chickens (Ross 508)

| Treatment | Beneficial Bacteria (CFU/ml) | *Escherichia coli* (CFU/ml) | *Proteus* species (CFU/ml) | *Enterobacter* species (CFU/ml) |
|---|---|---|---|---|
| Control | 8.620 ± 0.161$^{ab}$ | 5.136 ± 0.158$^{b}$ | 7.156 ± 0.242 | 7.156 ± 0.242 |
| AC 0.15 % | 8.210 ± 0.161$^{b}$ | 6.360 ± 0.158$^{a}$ | 6.706 ± 0.242 | 6.706 ± 0.242 |
| AC 0.45 % | 8.826 ± 0.161$^{a}$ | -ve | 6.736 ± 0.242 | 6.736 ± 0.242 |
| AC 0.75 % | 8.806 ± 0.228$^{a}$ | -ve | 6.620 ± 0.242 | 6.620 ± 0.242 |
| p-Value | 0.0001 | 0.0003 | 0.4219 | 0.4219 |

$^{ab}$ Means different superscript letters in each column express significant differences ($p < 0.05$). AC: *Amorpha* coffeaeformis, -ve: Negative

## CONCLUSION

From the productive and physiological point of view, it could be recommended that *A. coffeaeformis* microalgae at levels of 0.45% and 0.75% of diet did not have harmful effects on broiler chicken's health. In addition, these levels indicated the best product performance and immunological status, biochemical parameters, as well as suppressed *E. coli*, *Enterobacter*, and *Proteus* proliferation, at the same time increased beneficial bacteria (microbiota) proliferation in the intestine. Further investigations should be carried out on supplementing different levels of A. coffeaeformis to improve our knowledge of these microorganism's properties and evaluate their other effects on the broiler chicken's health and quality products.



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## DECLARATIONS


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### Authors' contribution

Dr. Ahmed El-Kaiaty designed this study, and Yasmina Mokhtar carried out the experiment. Hany Ramadan Contributed to the design of the study and performing the experiments. The supervisor in writing this article is Ahmed El-Kaiaty. Hamada Okasha analyzed the data under the guidance of Ahmed El-Kaiaty. All authors checked and confirmed the final analyzed data and the final draft of the manuscript.

### Competing interests

All research authors agree to publish this research and do not have any conflict of interest.

### Ethical considerations

This research was truthful and did not plagiarize or pattern any other papers or ideas. Any fabrication or falsification did not find in this research. This article or any scientific results did not submit to any journals except World`s veterinary Journal.

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# Semen Characteristics and Blood Metabolites of Hi-Plus Buck Rabbits Fed on Microalgae *Nannochloropsis oculata* Meal during the Summer Season

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**ABSTRACT**

Feeding tiny amounts of micro-algae meal to animals enhances animal physiology by improving immune response, disease resistance, and gut function, as well as enhancing anti-inflammatory and antibacterial protection, reproductive performance, feed conversion ratio, and weight gain. The purpose of this study was to identify the impact of dietary microalgae meal (*Nannochloropsis oculata*) on physical semen quality, serum biochemical parameters, and oxidative status of Hi-Plus buck rabbits for 12 weeks during the summer. A total of 45, Hi-Plus buck rabbits aged 20-24 weeks were divided into three equally comparable experimental groups. Bucks in the first, second, and third groups were daily supplemented in their diets with 0% (control), 0.50% (T1), and 1.0% (T2) microalgae meal, respectively. Semen and blood samples were collected to evaluate semen quality traits and some serum biochemical constituents, and oxidative status, as well as serum triiodothyronine ($T_3$) and testosterone (Ts) hormones concentrations. The obtained data revealed that dietary supplementation of *Nannochloropsis oculata* meal significantly improved most physical semen characteristics, including ejaculate volume, progressive sperm motility, semen pH value, sperm cell concentration, total sperm output, live sperm, and semen quality factor. Blood serum glucose, total proteins, and their fractions increased significantly in T1 and T2, compared with the control group, while total serum cholesterol and hepatic enzymes concentrations recorded a significant decrease in bucks supplemented with T1 and T2, compared with the control group. The total antioxidant capacity of serum significantly increased in both two levels of microalgae, compared with the control group. Serum $T_3$ concentration significantly increased in both levels of dietary microalgae compared with the control group. In conclusion, dietary supplementation with *Nannochloropsis oculata* meal (1.0%) was advised to improve semen quality, serum constituents, and antioxidative status without any adverse effects on the liver and kidney functions of rabbits.

**Keywords:** Bucks rabbits, Microalgae, Semen quality, Serum metabolite

## INTRODUCTION

Microalgae is a superfood with various impacts on growth, antioxidant systems, health, and livability (Nasirian et al., 2017), rendering it important for cell regeneration and growth. *Spirulina* algae, commonly referred to as blue-green algae, is a highly nutritious feed source for various essential animal species (Holman and Malau-Aduli, 2013). The microalgae have significant substances, such as a high protein content (60-70% dry matter) and amino acids (Jung et al., 2019), vitamins ($B_{12}$ and β-carotene), poly-unsaturated fatty acids (γ-linolenic acid), and minerals (Ca, Cr, K, Mg, Cu, Fe, Na, P, Mn, Zn and Se (Hoseini et al., 2013). Microalgae contain numerous substances has biological activities and serve as antioxidant factors (Kurd and Samavati, 2015), anti-inflammatory (Vide et al., 2015), antiviral, and immune-modulatory (Sahan et al., 2015). Microalgae improve animal welfare, health, and physiological responses, which potentially enhances the reproductive performance and fertility of farm animals, including rabbits (Abd El-Hamid et al., 2022). As a result, the positive effects of various amounts of microalgae supplement on productive performance, physiological responses, and health status of various farm animals have previously been reported (Bonos et al., 2016; Mirzaie et al., 2018).

Buck's reproductive efficiency is important in the rabbit economy, and using semen with high traits avoid the loss of valuable genotypes (Vizzarri et al., 2019). Under oxidative stress, reactive oxygen species (ROS) generation enhances the normal physiological process in animal tissue and organs, including the testes. Rabbit's spermatozoa have high metabolic activity and are abundant in poly-unsaturated fatty acids, which increases lipid peroxidation (Attia et al., 2017) and makes them vulnerable to ROS attacks (Castellini et al., 2006). In roosters, increased lipid peroxidation reduces motility, fragments DNA, and reduces sperm fertilization capacity (Opuwari and Henkel, 2016; Attia et al., 2019; Okab et



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al., 2013). Practically, the safe and cost-effective administration of numerous natural antioxidant resources could help to diminish the detrimental effects of oxidative stress on rabbit buck reproductive efficiency by reducing the negative effects of oxidative stress on sperm parameters (El-Desoky et al., 2017).

Rabbit's reproductive success is affected by the semen quality of buck's rabbit, the climate changes, and the physiological state of the does (Ahmed et al., 2006 and Elnagar, 2010). Summer temperatures in Egypt can exceed more than 40°C. In addition, relative humidity (RH) and metabolic heat are also causatives of heat stress. Cold or heat stress, wind, ventilation, moisture, light, and solar radiation can deleteriously impact male fertility by causing oxidative stress (Córdova-Izquierdo et al., 2014). This stressor induces an increase in free radical accumulation, which damages spermatogenic cells (El-Desoky et al., 2013). A low amount of -ROS is required for normal sperm activity (El-Tohamy and El-Nattat, 2010). However, oxidative stress in the sperm occurs when ROS- levels exceed the total antioxidant capacity, which reduces fertility (El-Tohamy and El-Nattat, 2010). Antioxidants protect cellular components from damage caused by cellular free radicals and ROS. Damage can occur when antioxidants are absent, at a sub-optimal amount, or not accessible at the precise location within the cell where free radicals develop (El-Tohamy et al., 2012). Natural active ingredients such as microalgae might enhance animal reproductive performance (Kistanova et al., 2009). It is well known that the positive effects of microalgae, such as *Spirulina* in buck's rabbit, depend on treatment methods, pelleted diet, drinking water, and oral administration (Bashandy et al., 2016).

Abd El-Hamid et al. (2022) found that under heat stress conditions, supplementation of marine microalgae *Nannochloropsis oculata* at a level of 0.5 or 1 % to the doe rabbit's diets might improve serum progesterone and triiodothyronine profiles, some blood metabolites, oxidative status, and reproductive and productive performances. Therefore, the present study was designed to examine the effect of microalgae *Nannochloropsis oculata* on semen quality, some blood serum constituents, and total antioxidant capacity of Hi-Plus buck rabbits during the summer.

## MATERIALS AND METHODS

### Source of animals

The field portion of this study was conducted in a privet rabbits farm (Latitude 31° 29′ N; Longitude 32° 34′ E), North Sinai governorate, Egypt, during the summer season (from June to August 2020). Laboratory analyses were carried out at the Animal and Poultry Physiology Laboratory, Animal and Poultry Production Division, Desert Research Center, Ministry of Agriculture and Reclamation, Cairo, Egypt.

### Ethical approval

This experiment was performed according to all ethics and animal rights (Desert Research Center). This work considered all rules and regulations in conformity with the European Union directive for the protection of experimental animals (2010/63/EU).

### Experimental design and management

#### *Experimental animals*

Forty-five of Hi-Plus buck's rabbits at 5 months of age with an average initial live body weight (LBW) of 2686.0 ± 37.09 g were used in this study. Bucks were randomly distributed into three homogeneous groups (15 in each) based on the similarity of their LBW. Bucks were individually housed in galvanized wire mesh cages provided with feeders and automatic stainless steel nipple drinkers. All bucks were fed *ad libitum* on a commercial complete pelleted diet throughout the experimental period (3 months).

#### *Diet and experimental design*

The basal diet contained 24.60% Barley grain, 31.00% alfalfa hay, 13.25% soybean meal, 28.00% wheat brain, 1.60% dicalcium phosphate, 0.95% limestone, 0.30% sodium chloride, and 0.30% minerals-vitamins premix. The nutrient composition of the basal diet (% on dry matter basis) included 17.08% crude protein, 2.20% ether extract, 12.55% crude fiber, and 2416 digestible energy (DE, kcal/kg diet), and it was manually offered twice daily. The calculated analysis of the basal diet was done according to the feed composition tables for rabbits' feedstuffs used by De Blas and Wiseman (2010) and Villamide et al. (2010). The requirements of DE (kcal/kg diet) and crude protein (CP) were provided according to FEDIAF (2013). In this study, two levels of microalgae meal produced by the National Research Center, Dokki, Cairo, Egypt, were used. Microalgae were prudently added to the experimental basal diets while mixing the diet ingredients.

Bucks were fed on the experimental basal diet without supplementation in the first group and served as a control group. However, in the second and third groups, bucks were supplemented with a basal diet containing 0.50% (5g/kg diet) and 1.0 % (10g/ kg diet) of microalgae meal, respectively.

All rabbits were kept under the same experimental conditions. The composition of *Nannochloropsis oculata* as a fraction of dry weight (DW) biomass is presented in Table 1.



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**Table 1.** The composition of *Nanochloropsis oculata* constituents by Gass Chromatography mass

| **The composition (g/100g) of microalgae (*Nannochloropsis oculata*)** | |
|---|---|
| Moisture | 7.15 |
| Crude protein | 55.78 |
| Fat | 6.61 |
| Ash | 12.29 |
| Total carbohydrates | 18.17 |
| **Quantitative constituents of minerals profile (mg/100g) in microalgae (*Nannochloropsis oculate*)** | |
| Fe | 29.35 |
| Zn | 1.02 |
| Sodium | 1862.70 |
| Calcium | 229.00 |
| Potassium | 798.00 |
| Magnesium | 173.00 |
| **Quantitative constituents of amino acids profile (mg/g) in microalgae (*Nannochloropsis oculate*)** | |
| Methionine | 69.52 |
| Cystine | 17.30 |
| Phenylanlanine | 16.24 |
| Lysine | 15.20 |
| Isoleucine | 55.95 |
| Leucine | 65.11 |
| Aspartic acid | 30.16 |
| Glutamic acid | 15.07 |
| Histidine | 13.22 |
| Tyrosine | 87.69 |
| Threonine | 39.21 |
| Valine | 50.36 |
| Serine | 11.64 |
| Glycine | 9.98 |
| Proline | 31.52 |
| Alanine | 20.24 |
| Arginine | 8.56 |

Source: Abd El-Hamid et al. (2022)

**Table 2.** Overall means of indoor ambient temperature, relative humidity, and temperature humidity index throughout the experimental period, North Sinai, Egypt (according to Abd El-Hamid et al., 2022).

| **Month** | **AT ($^{o}$C)** | | **RH (%)** | | **THI** | |
|---|---|---|---|---|---|---|
| | **Minimum** | **Maximum** | **Minimum** | **Maximum** | **Minimum** | **Maximum** |
| **June** | 28.7 | 32.8 | 42.2 | 54.0 | 26.7 | 29.5 |
| **July** | 28.2 | 33.6 | 43.0 | 63.8 | 26.7 | 30.2 |
| **August** | 28.9 | 34.1 | 42.2 | 58.6 | 27.0 | 30.6 |
| **Overall** | 28.6 | 33.5 | 42.4 | 58.8 | 26.7 | 30.1 |

AT: Ambient temperature RH: Relative humidity, THI: Temperature humidity index

**Climatic conditions**

Ambient temperature (°C) and RH, were measured in percentage three days/week between 12 pm to 2 pm using automatic thermos-hygrometer (HANNA Instrument, Italy). Temperature Humidity Index (THI) was calculated using the following equation:

THI= db°C – [(0.31 - 0.31 × RH) × (db°C - 14.4)] according to Marai et al. (2001).

Where, db°C is dry bulb temperature in centigrade. The THI values were classified as the absence of heat stress (< 27.8), moderate heat stress (27.8-28.8), severe heat stress (28.9-29.9), and very severe heat stress (> 30.0). The overall means of monthly climatic conditions are found in Table 2.

**Growth indices**

Bucks were individually weighed to the nearest ±1.0 g by a digital weighing scale at the beginning of June and at the end of August 2020 as initial and final weights, respectively. The total weight gain per animal in grams was calculated individually by subtracting the final from the initial weights using the following equation:

Total weight gain (g/buck) = ($LBW_F$–$LBW_I$)

While the relative growth rate (GR, %) was calculated using the following equations:

Growth rate (GR, %) = $LBW_F$ – $LBW_I$ / $LBW_I$ × 100

Where, $LBW_F$ is inal buck weight (g) and $LBW_I$ denotes initial buck weight (g)



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**Blood collection and serum biochemical parameters**

At the end of the experimental period, individual de-coagulated blood samples (approximately 5 ml) were collected from the lateral ear vein. Blood samples were centrifuged at 4000 rpm for 20 minutes to separate the clear serum in Eppendorf tubes, and then stored in a deep freezer at -20℃ till biochemical analyses. The determined biochemical parameters of serum samples included total protein (TP), albumin (ALB), total cholesterol (CHO), glucose (GLU), liver enzymes activity (ALT and AST), calcium (CA), and phosphorous (P) which were calorimetrically analyzed using commercial kits (produced by Bio-diagnostic, Egypt), according to the procedure outlined by the manufacturers. Serum globulin (GLO) was calculated by subtracting the values of ALB concentration from the corresponding values of TP, and then the albumin/globulin (A/G) ratio was calculated.

**Serum oxidative capacity**

The total antioxidant capacity (TAC, µmol/mL) as lipid peroxidation biomarker was assayed in serum samples using commercially available kits (Bio Diagnostic Research, Erel, 2004).

**Serum hormones**

Serum testosterone (Ts) concentration was determined by immunoassay (Biosource-Europe S.A. 8, rue de L'Lndustrie. B-1400 Nivelles. Belgium). Moreover, serum triiodothyronine ($T_3$) concentration was determined with enzyme immunoassay using commercial kits obtained from immunotech crop, Boston, MA 02134**.**

**Semen collection and evaluation of its physical characteristics**

An individual semen sample was collected (Three times during the experiment) using an artificial vagina maintained at 42-45°C and a teaser doe. The reaction time (RT, sec.) was estimated as the time elapsed from introducing a teaser doe to the buck till to complete ejaculation of the artificial vagina. Immediately after semen collection, ejaculates were kept at 37 °C in the water bath and transferred to the laboratory. The ejaculated semen sample from each rabbit buck was evaluated for ejaculate volume (EV) without gel mass and for pH value using a pH paper (Spezial-Indikatorpapier pH 5.5-9.0, MACHEREY-NAGEL. Germany). In addition, the percentages of progressive sperm motility (PSM), live sperm (LS), and abnormal sperm (AS) were determined. The sperm cell concentration (SCC) was estimated using Neubauer hemocytometer slide. The total sperm output (TSO) was calculated by multiplying semen EV (ml) by SCC/ml; motile sperm output (MSO) was calculated by multiplying PSM (%) by TSO, and sperm quality function (SQF) was calculated by multiplying SCC by EV and by LS/100. The percentages of LS, dead sperm (DS), and (AS) were determined using stains that penetrated cells with damaged membranes. Normal LS excluded the eosin stain and appeared pinkish in color because of loss of membrane and integrity. Normal sperm showed an oval head with a long tail, while abnormal sperm showed head, mid-piece, or tail defects, such as a large or misshapen head or a crooked or double tail (Correa and Zavos, 1994).

**Statistical analysis**

All numerical data were statistically analyzed using General Linear Model's procedure of the SAS (2009) program. A one-way ANOVA design was used to investigate the effect of different levels of dietary SA on the tested parameters by using the following model:

$Y_{ij} = \mu + T_i + e_{ij}$

Where, $Y_{ij}$ is an observation, µ denotes the overall mean, $T_i$ signifies the effect of treatment (i: control, 0.5%, and 1.0% microalga, respectively), and $e_{ij}$ refers to random error. Differences between means among all treatments were subjected to Duncan´s Multiple Range-test (Duncan, 1955).

## RESULTS AND DISCUSSION

**Growth indices**

Table 3 shows the positive effects of microalgae administration on the LBW, total gain, and growth rate of bucks supplemented with 0.50% or 1.0% microalgae meal, compared to the control group. Numerically, the final body weight, total gain, and growth rate were of the highest values for bucks fed 1.0% microalgae (2964 g, 276 g, and 10.27%), followed by bucks fed 0.5% microalgae (2950 g, 270 g, and 10.07%). However, the lowest values of LBW and total gain were recorded in the control group bucks (2944 g, 263 g, and 9.80%). As seen in Table 3, results showed that the total gain and growth rate values did not differ fundamentally between 0.50% and 1.0% microalgae groups after 12 weeks of treatment. In accordance, El-Ratel (2017) found that the final body weight of doe rabbits was higher ($p < 0.05$) in the group that received oral 300 mg of *Spirulina platensis*/doe in drinking water than both of the doe rabbits that received oral 600 mg of *Spirulina platensis*/doe in drinking water and control does for 4 weeks period. Similarly, it was found that the dietary addition of *Spirulina platensis* increased the growth performance and feed intake of growing rabbits



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(Gerencser et al., 2012; El-Desoky et al., 2013). The positive effect *of Spirulina platensis* on growth indices may reflect the nutritive value of algae, which contains essential amino and fatty acids, photosynthetic pigments, vitamins, minerals, carotenoids, chlorophyll, pigments, and essential poly-unsaturated fatty acids in amounts ranging from 50 to 70% (on DM basis, Hoseini et al., 2013; Jung et al., 2019).

**Table 3.** Effect of dietary microalgae meal (*Nannochloropsis oculata)* on growth indices of Hi-Plus buck rabbits during the summer season, North Sinai, Egypt

| Experimental groups / Growth indices | Control group | Level of dietary microalgae meal | | ± SE |
|---|---|---|---|---|
| | | 0.5 % | 1.0 % | |
| Initial body weight (g) | 2681 | 2680 | 2688 | 66.57 |
| Final body weight (g) | 2944 | 2950 | 2964 | 82.87 |
| Total gain (g) | 263 | 270 | 276 | 45.60 |
| Growth rate % | 9.80 | 10.07 | 10.27 | 2.32 |

SE: Standard error

**Plasma biochemical parameters**

***Plasma proteins responses***

As can be seen in Table 4, bucks fed 0.5% and 1.0% microalgae meal in their pelleted diets for 12 weeks period significantly showed an increase in serum TP, ALB, GLO, and GLU concentrations, compared with bucks in the control group ($p < 0.05$). However, there was a significant decrease in total CHO concentration as compared to bucks in the control group ($p < 0.05$). Similar results were reported regarding the concentrations of serum TP and ALB in rabbits and fed diet containing microalgae (Abd El-Hamid et al. (2022). The recorded increase in serum TP, ALB, and GLU concentrations may be related to high contents of protein, essential amino acids, vitamins, minerals, phospholipids, and antioxidants in microalgae meal (Jung et al., 2019). Similarly, El-Ratel and Gabr (2020) found a significant increase in plasm TP as a result of the rise in A/G concentrations for buck rabbits treated with 150 or 300 mg *Spirulina platensis*/liter drinking water, respectively. This result reflects a similar A/G ratio in treated groups compared with the control group. According to Moor et al. (2017), these results indicated that algal extracts could activate the enzyme lecithin cholesterol acyltransferase, which inhibits cholesterol biosynthesis and may play a role in the transverse cholesterol pathway when cells are unable to metabolize cholesterol. Previous studies performed by Hamed et al. (2015) revealed that marine *Spirulina* spp. acted as biological material by the dietary treatment for decreasing blood lipid concentrations.

**Table 4.** Effect of dietary microalgae meal (*Nannochloropsis oculata)* on blood serum metabolites of Hi-Plus buck rabbits during the summer season, North Sinai, Egypt

| Experimental groups / Blood Serum constituents | Control group | Level of dietary microalgae meal | | ± SE |
|---|---|---|---|---|
| | | 0.5 % | 1.0 % | |
| Total proteins (g/dl) | $6.38^{b}$ | $7.22^{a}$ | $7.10^{a}$ | 0.119 |
| Albumin (g/dl) | $4.39^{a}$ | $4.19^{b}$ | $4.15^{b}$ | 0.075 |
| Globulin (g/dl) | $1.99^{b}$ | $3.03^{a}$ | $2.95^{a}$ | 0.166 |
| A/G ratio | $2.32^{a}$ | $1.50^{b}$ | $1.50^{b}$ | 0.164 |
| Glucose (mg/dl) | $70.82^{b}$ | $88.34^{a}$ | $85.10^{a}$ | 2.59 |
| Total cholesterol (mg/dl) | $137.94^{a}$ | $114.23^{b}$ | $114.56^{b}$ | 2.22 |
| ALT (IU/L) | $27.37^{a}$ | $23.59^{b}$ | $23.38^{b}$ | 0.460 |
| AST (IU/L) | $91.11^{a}$ | $88.73^{b}$ | $89.03^{b}$ | 0.568 |
| $T_3$ (ng/ml) | $0.678^{b}$ | $0.744^{a}$ | $0.722^{a}$ | 0.01 |
| Ts (ng/ml) | $7.69^{b}$ | $9.88^{a}$ | $9.55^{a}$ | 0.27 |
| Calcium (mg/dl) | $9.89^{b}$ | $11.68^{a}$ | $11.00^{a}$ | 0.197 |
| Phosphor (mg/dl) | $4.63^{b}$ | $5.56^{a}$ | $6.44^{a}$ | 0.204 |
| TAC (µmol/L) | $0.697^{b}$ | $0.866^{a}$ | $0.860^{a}$ | 0.017 |

ALT: Alanine amino transaminase, AST: Aspartic amino transaminase, $T_3$: Triiodothyronine, TAC: Total antioxidant capacity, A//G: albumin/globulin, Ts: Total testosterone, SE: Standard error, $^{a,b}$: Different superscript letters on the same row indicates significant differences ($p < 0.05$).

Regarding the effect of *Nannochloropsis oculata* meal on blood serum GLU concentration, the results could be found in Table 4. The results indicated that dietary supplementation with *Nannochloropsis oculata* significantly increased the GLU concentrations to 70.82, 88.34, and 85.10 mg/dl for control and 0.50, and 1.0 % microalgae-treated groups, respectively. This result may be due to the cell wall of *Nannochloropsis* being rich in several polysaccharides, and can interfere with the solubilization and digestion of the cell compounds. However, the cell wall polysaccharides of



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*Nannochloropsis oculata* contained almost 68% glucose along with about 4-8% rhamnose, mannose, ribose, xylose, fructose, and galactose (Brown, 1991).

**Liver and kidney functions**

***Alanine amino transaminase and aspartate amino transferase enzymes***

Regarding the effect of microalgae on ALT and AST, the results presented in Table 4 indicated that bucks fed 0.50% or 1.0% microalgae in their pelleted diets recorded a significant decrease ($p < 0.05$) in ALT (23.59 and 23.38IU/L) and AST (88.73 and 89.03IU/L) enzymes activities, compared with a control group (27.37 and 91.11 IU/L). These results may indicate that microalgae had a positive effect on protein metabolism, lipid profile, and liver functions of treated buck rabbits, and consequently, better health status compared with the control group. In accordance, Abd El-Hamid et al. (2022) demonstrated a significant increase in serum AST and ALT concentrations of doe rabbits supplemented with 0.5 or 1.0% microalga meal in their diets during the summer season. Bhattacharyya and Mehta (2012) mentioned that microalga might play a protective role against liver dysfunctions. Thus, our results suggest that dietary microalgae to 1.0% did not trigger liver impairment but had a protective effect on the biological functions of liver cells.

**Thyroid hormone (tri-iodothyronine) response**

The means of blood circulating concentrations of $T_3$ and total Ts in buck rabbits supplemented with two levels of microalgae meal for three months during the summer season are seen in Table 4. The obtained results revealed that serum $T_3$ significantly increased ($p < 0.05$) in both treatment levels (0.774 and 0.772 ng/ml), compared to the control group (0.678 ng/ml). The recorded rates were 9.73 and 6.45% for 0.50 and 1.0% of microalgae meal, respectively as compared with the control group. In accordance, Abd El-Hamid et al. (2022) reported that blood ($T_3$) and thyroxine ($T_4$) hormones increased significantly as the increased dietary supplementation ratio of sea woods (*Sargassum* meal) of Leghorn layers or in doe rabbits supplemented with 0.5 or 1.0% *microalga* meal in their diets during the summer season. It is well known that thyroid hormones affect spermatogenesis (Zarifkar et al., 2007). Moreover, the thyroid hormone receptor expresses in the germ cells from spermatogonia to primary spermatocytes (Buzzard et al., 2000).

**Blood mineral absorption**

The means of serum C and P concentrations are listed in Table 4. The obtained results indicated that dietary microalgae significantly increased serum concentrations of C and P values ($p < 0.05$). The recorded values for C were 9.89, 11.68, and 11.0 mg/dl for the control, 0.5, and 1.0% groups, respectively, while the corresponding values for P were 4.65, 5.56, and 6.44 mg/dl, respectively. Similarly, Recently, Abd El-Hamid et al. (2022) reported a significant increase in serum C and P concentrations in doe rabbits supplemented with 0.5 or 1.0% of microalgae meal in their diets during the summer season.

**Antioxidant capacity status**

According to Table 4, dietary microalgae significantly increased the total antioxidant capacity in both treatment levels, compared to the control group ($p < 0.05$). The results of TAC showed that bucks supplemented with microalgae showed a significant increase in TAC with a similar value (0.82 µmol/l) for both two levels compared with non-treated bucks (0.69 µmol/l). This result is in accordance with previous results indicating that the increase of serum TAC values in bucks treated microalgae meal may be due to their richness in natural biological substances, which may contribute to mitigating oxidative stress via enhancing enzymes and non-enzymes antioxidants (Abdelnour et al., 2020a; Abdelnour et al., 2020b; Abd El-Hamid et al., 2022).

Typically, the body's metabolism generates oxygen free radicals in a dynamic balance controlled by the antioxidant system. However, this balance can be disrupted by a rise in oxygen free radicals or deterioration of the antioxidant mechanism, resulting in oxidative damage to cells and lipid peroxidation (Xu and Pan, 2013). Thus, the antioxidant enzymes revealed the condition of the body's antioxidant mechanism, which reflects the body's capacity to metabolize oxygen free radicals and protect animal tissues from oxidative stress. Some biological functions are related to sugar complexes, such as glucose, a variety of mannose, galactose, rhamnose, N-acetylglucosamine, N-acetylgalactosamine, and arabinose residues, which are described by immune activity (1, 3-glucan) in all microalga species. The polysaccharides from *Spirulina platensis* had strong scavenging activities on hydroxyl radicals (Kurd and Samavati, 2015), in addition; they reduce blood lipid levels, such as triglycerides and cholesterol (Hamed et al., 2015).

**Physical semen characteristics**

Regarding physical semen trait responses, Figure 1 reveals that dietary supplementation of microalgae meal significantly improved most physical semen characteristics, including EV, PSM, semen pH value, SCC, TSO, LS, and SQF ($p < 0.05$). These results indicated a linear relationship between the level of microalgae meal and these traits. The best significant improvement of these traits was recorded for buck rabbits on 1.0% of microalgae meal supplementation.



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In contrast, each of the DS and AS percentages decreased significantly on both 0.5 and 1.0% levels, compared to the control group ($p < 0.05$), as this decrease was classified as a complementary part of the improvement of semen quality traits. The semen quality of bucks is the main factor in determining the reproductive efficiency of rabbit does (Attia et al., 2017). The obtained results indicated a higher fertilizing ability of spermatozoa of bucks treated with *Nannochloropsis oculata* as an antioxidant. This improvement was associated with a pronounced elevation in the ejaculate volume and sperm concentration and a reduction in DS and AS percentages, which resulted in enhancement in physical semen traits. Similar results were reported by Calogero et al. (2017), Fouda and Ismail (2017), and El-Ratel and Gabr (2020). Increased pH value in the semen of treated groups was associated with elevated levels of sperm cell concentration and semen volume. The increase in semen volume may be attributed to an increase in testosterone in treatment groups. The antioxidant components of *Spirulina platensis* may be responsible for the improved semen characteristics of treated bucks (Rezvanfar et al., 2008). *Spirulina platensis* can prevent cell damage through antioxidative defense systems that counteract the effects of ROS and protect cellular functions from damage under stress conditions (El-Tohamy et al., 2012).

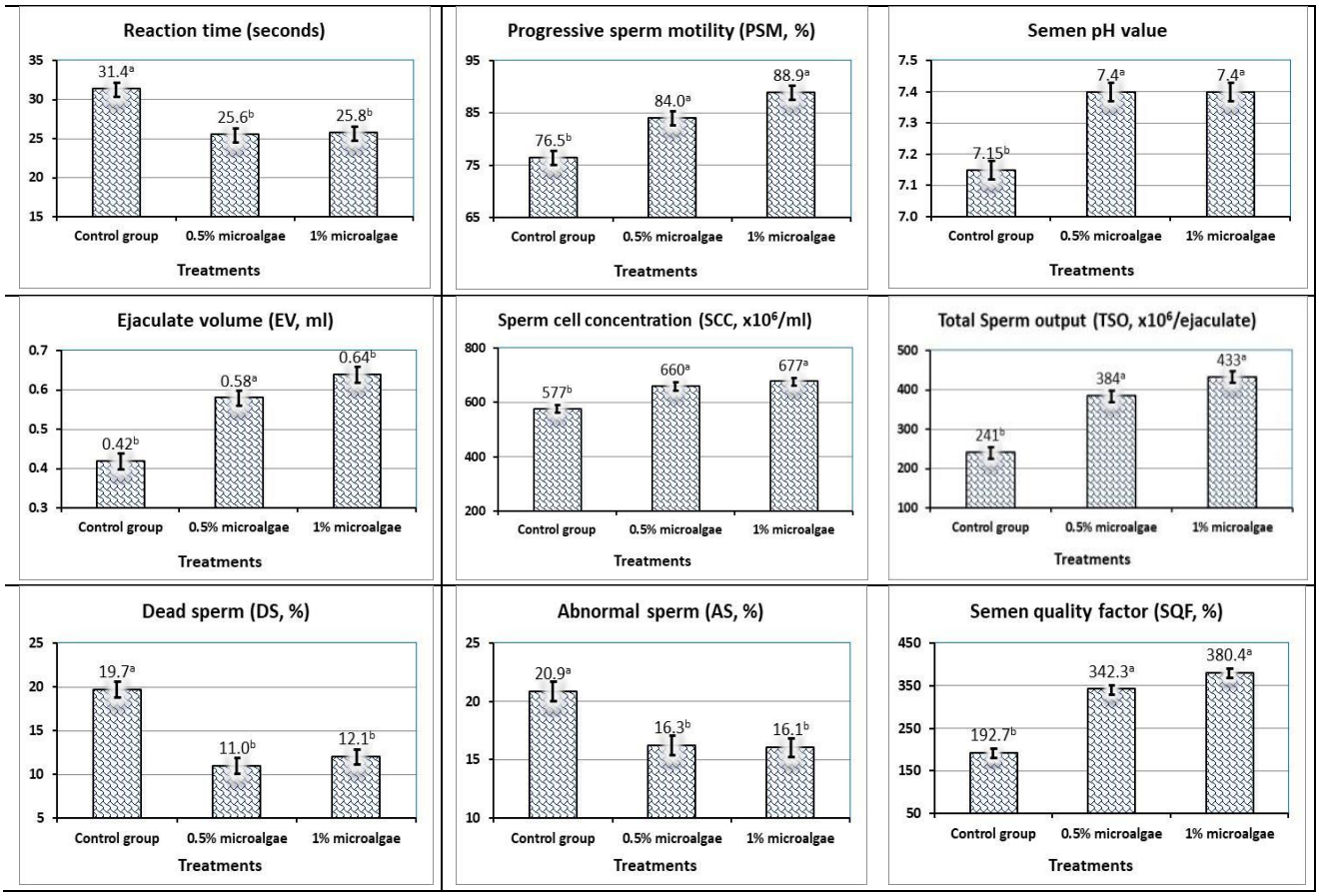


**Figure 1.** Effects of dietary microalgae meal (*Nannochloropsis oculata*) on physical semen characteristics and sperm output of Hi-Plus buck rabbits during the summer season, North Sinai, Egypt. [a,b]: Different superscript letters on the same row indicates significant differences ($p < 0.05$).

**Sexual desire response**

Regarding the effect of dietary *Nannochloropsis oculata* on sexual desire response, the obtained results in Table 4 and Figure 1 indicated that bucks fed a diet supplemented with microalgae showed a significant decrease ($p < 0.05$) in their RT and serum (Ts) concentration as indicators of sexual desire in bucks under both two levels of microalgae meal supplementation. With respect to RT, the results indicated lower values in both treated groups (25.6 and 25.8 sec) compared with the control group (31.4 sec). In contrast, the serum $T_3$ concentration indicated a significant ($p < 0.05$) increase in both treated groups (9.88 and 9.55 ng/ml), compared to the control group (7.69 ng/ml). Testosterone is the main male sex hormone, which plays a crucial role in the suitable development of reproductive organs and the maintenance of male sexual characteristics. These obtained results indicated that microalgae meal treatment for 12 weeks during summer months improved the libido of buck rabbits and subsequently markedly enhanced the sexual desire response of treated bucks compared with control ones. Similar results were reported in bucks treated orally with *Spirulina platensis* (750 mg/buck/day) for five weeks pre-semen collection (Fouda and Ismail, 2017) or with (200 and 400 grams of red algae per ton diet for 3 months (Ali and Mervat, 2013). Recently, El-Ratel and Gabr (2020) reported



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that increasing semen's pH value was associated with increased sperm cell concentration and ejaculate volume of buck rabbits fed microalgae (*Spirulina)*. However, increasing ejaculate volume may be attributed to an increase in the testosterone hormone of bucks fed *Spirulina*, which increases accessory sex glands activity.

## CONCLUSION

It could be concluded that the supplementation of marine microalgae *Nannochloropsis oculata* at a level of 1.0 % to the buck rabbit's diets improves semen quality and blood serum constituents during the summer season. Future research on the effect of microalgae on the reproductive organs (morphology and histology) is needed.

## DECLARATIONS


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### Competing interests

The authors declare that they have no conflict of interest with respect to the research, authorship, and/or publications of this article. The authors declare that they have no competing interests.

### Authors' contribution

All the authors were collaborated in work planning, experimental design, measurement of parameters, and writing of the manuscript. Dr. Ahmed Sobhy El-Hawy designed the experiment, article writing, and revision. Dr. Moharram Fouad El-Bassiony designed the experiment, statistical analysis, tabulation of experimental data, manuscript writing, commenting, and approval. Dr. Ibrahim Samir Abd El-Hamid designed the experiments, measured the parameters, statistically analyzed data, wrote and revised the manuscript. Dr. Hesham Attia Shedeed designed the experiments, measured the parameters, wrote and revised the manuscript. Dr. Wafaa Adel Fouda designed the experiments, measured the parameters, statistically analyzed data, wrote, and revised the manuscript. Dr. Safaa Ali Mostafa designed the experiments, measured the parameters, wrote, and revised the manuscript. Dr. Reda Abd-Ellattif Abd-Elazem designed the experiments, measured the parameters, wrote, and revised the manuscript. Dr. Ali Saber Morsy designed the experiments, collected the samples, performed the experiments, and wrote and revised the manuscript. Dr. Khamis Refaay Said Emam helped in the field study, data collection, tabulation of experimental data, and article writing and revision. All the authors read and approved the final manuscript.

### Data availability

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

### Ethical considerations

All authors admitted that they followed ethical issues concerning plagiarism, approval to publish, errors in fabrication, double publication, and submission.

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# Development of an Aggressive Treatment Protocol against Neonatal Calf Diarrhea: The Last Chance to Rescue Severely Infected Calves

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**ABSTRACT**

Despite many efforts to control and treat neonatal calf diarrhea (NCD), it remains the primary cause of calf mortality in dairy herds worldwide. The objective of this article was to develop and discuss an empirical therapeutic protocol to save newborn calves with severe diarrhea. The pathophysiology of diarrhea has been well described previously. However, there is a significant gap between scientific findings and practical implementations. Reducing the number of calves with failure of passive transfer, regular sanitation of the calf environment, and optimal dry cow nutrition and management are fundamental measures in controlling diarrhea in commercial settings. As such, optimizing colostrum feeding management and improving ambiance hygiene are among the most important management practices to prevent calf diarrhea. Nonetheless, the occurrence of NCD would be unavoidable due to its multifactorial nature and pathophysiology. According to the degree of dehydration and general appearance of ill calves (e.g., degree of sunken eye and loss of suck reflex), NCD can be classified into mild to severe cases. Early diagnosis and treatment of both mild and severe cases could reduce pathogens shedding into the calf environment. Notably, diarrhea treatment needs profound scientific farm education and mentoring regarding the physiology of NCD. Since a variety of organisms, such as bacteria, viruses, and protozoa, may be responsible for NCD, it is evident that reliable diagnosis requires optimal sampling and laboratory analysis. However, waiting for laboratory results may waste the golden time of treatment. Therefore, rapid and decisive treatment would be mandatory, especially in severely infected calves or sepsis cases. Accordingly, an effective aggressive treatment protocol was developed and discussed in this article as the last chance to keep diarrheic calves alive.

**Keywords:** Aggressive treatment, Calf diarrhea, Dairy calf, Farm Management, Prevention

## INTRODUCTION

Diarrhea and other digestive tract disorders account for the most important factors causing calf mortality worldwide (Zhang et al., 2019). The importance of calf health and well-being has been described over the last decade from short-term (calf loss and veterinary cost) and long-term (herd future productivity) perspectives (Lorenz, 2021). Enhancing the health of newborn calves by optimizing management factors such as colostrum feeding and welfare, and early diagnosis and treatment of ill calves can significantly enhance herd productivity and longevity. After birth, as newborn calves adapt to the new extra-uterine environment, they are exposed to a variety of harmful pathogens (Malmuthuge and Guan, 2017). Importantly, however, the immune system of neonatal calves is still not fully developed. Newborn calves depend almost totally on maternal colostrum ingestion to acquire immunity (Nikkhah and Alimirzaei, 2021). As such, the balance between host immunity and environmental contaminations determines calf susceptibility to infectious diseases such as diarrhea. Diarrheic calves and even those recovered from diarrhea shed infectious agents into the surroundings and are considered as main contaminators. Therefore, early diagnosis and effective treatment are vital for saving diarrheic calves.

As noted, many pathogenic organisms are responsible for severe diarrhea in neonatal calves. Bacteria including enterotoxigenic *E. coli* (ETEC), and *salmonella enterica*, *salmonella dublin*, *salmonella typhimurium;* viruses such as *rotavirus* and *coronavirus*; and protozoa such as *cryptosporidium parvum* are amongst the most important pathogenic organisms that can infect young calves and causes mortality (Cho and Yoon, 2014). Independent of the type of pathogens involved in the etiology of diarrhea, clinical symptoms are usually similar, thus making specific diagnoses difficult. Watery feces, lethargy, anorexia, dehydration, and loss of suckling behavior are common signs in all types of diarrheas. However, because of the importance of immediate interventions to save ill calves, the type of diarrhea should



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be known. The age at which calves are infected and the score of feces can help veterinarians and clinicians infer the principal cause of diarrhea. Hence, they can decide how to deal with an ill calf. For instance, ETEC can infect neonatal calves during 2-4 days of age and cause watery diarrhea. *Salmonella spp.* infection can also be diagnosed with mucoid-bloody diarrhea in calves younger than 3 weeks of age (Cho and Yoon, 2014). In case of viruses, in calves younger than 3 weeks of age, watery-pale-yellowish diarrhea can be considered as viral diarrhea (Gomez and Weese, 2017). As viruses invade mature intestinal villi, nutrient malabsorption results in prolonged diarrheic days and calf weakness (Cho and Yoon, 2014). It is important to note that calf excretions should be sampled carefully to avoid environmental contaminations and sent to the laboratory for accurate diagnosis. Laboratory testing is needed for making right management decisions and developing long-term preventive protocols.

As described previously (Nikkhah and Alimirzaei, 2021), mild cases of diarrhea (feces not very watery with calves being able to stand and suckle) can be treated successfully by the administration of oral fluid electrolytes and related appropriate therapies. However, in severe cases, the scenario is totally different. The severely infected calves lose considerable volumes of water and electrolytes (e.g., sodium, potassium, chloride, and bicarbonate), making calves highly dehydrated, known by sunken eyes. It appears that fluid loss during severe diarrhea would exceed the farm staff's imaginations and expectations. Water loss in calves with severe diarrhea can range from 13-18% (or even greater) of body weight daily (Berchtold, 2009). Knowing that approximately 75% of calf body weight consists of water, a diarrheic calf with 40 kg body weight may lose about 6 lit/d water (Naylor, 2009). Underestimating water and electrolyte requirements of diarrheic calves is one of the most important reasons for calf mortality in dairy herds. In addition, severely diarrheic calves may develop metabolic acidosis, which may lead to central nervous system malfunction, failure of suckling behavior, recumbency, coma, and death (Berchtold, 2009). Therefore, correcting metabolic acidosis is vital for calf survival. Since an immediate intervention is required for successful treatment, the following practical protocol is recommended for on-farm use.

## PROTOCOL PRESENTATION

Intravenous (IV) fluid therapy must be used in severe cases (recumbent cases with sunken eyes) to replace the lost water and electrolytes to restore extracellular and plasma fluid volumes (Naylor, 2009). The amount of fluid needed in the first injection is totally dependent on the dehydration rate and general wellness of the ill calf (Berchtold, 2009; Constable et al., 2021). However, as a practical guideline, 2-4 liters of dextrose-saline serum (5% dextrose, 0.9% saline) must be injected immediately. As noted above, in severe cases, water loss may be more than expected; thus, the second and third IV injections may be needed. Two liters of isotonic dextrose-saline serum is recommended every 5 or 6 hours. The isotonic serum is used to maintain the balance between blood sugar and electrolytes (Naylor, 2009). The IV injections should be stopped when the suckling reflex is recovered, and the calf is able to stand easily. After that, IV fluid therapy could be replaced by oral fluid therapy (Constable et al., 2021). Usually, systemic inflammation occurs in heavily infected calves or those with septicemia (Constable et al., 2021), leading to organ failure and death. Consequently, alleviating inflammatory responses should be considered the second step in treating severe cases of diarrhea. Hypertonic saline solution (7.2%) is useful for alleviating inflammatory responses and increases plasma volume and cardiac output (Constable et al., 2021). Thus, it should be administered for about 300-400 ml in the first injection combined with an isotonic dextrose-saline solution. According to the authors' extensive farm experience, adding 1-3 ml of anti-inflammatory drugs, such as dexamethasone, meloxicam, or flunixin meglumine into the first injection solution can contribute to mitigating unwanted severe anti-inflammatory responses and could help the calf resume optimal organ's function Moreover, eliminating the infectious agent, especially in sepsis cases would be essential. As such, IV administration of antibiotics accompanied by anti-inflammatory drugs is necessary for eliminating bacterial agents responsible for diarrhea (Berchtold, 2009). In severe cases, antibiotics use can be replicated every 12 hours. With regard to herd's veterinarian recommendation, antibiotics, such as ceftriaxone, gentamicin, or marbofloxacin must be added to the first injection solution. It is also important to note that serum solutions are usually prepared and presented in 1-liter containers; thus, anti-inflammatory drugs and antibiotics could be gradually added to the second container. Alongside drug administration, adding 500 ml of isotonic sodium bicarbonate or 100 ml of hypertonic sodium bicarbonate is needed for correcting acidosis. All the above procedures must be performed in the first injection time (immediately after diagnosing severe diarrhea). In the second or third injections, isotonic dextrose-saline solution would be enough for the rehydration of affected calves. Vitamins, including B-complex, would be highly recommended because calves with severe diarrhea are almost energy-deficit (Berchtold, 2009). Administration of B-group vitamins can fuel energy-releasing pathways and thereby help calves recover rapidly. Fat-soluble vitamins (A, $D_3$, and E) are also recommended because of their effects on repairing mucosal membranes during bacterial or viral invasion (Constable et al., 2021). The above drugs and materials should be administered 5 consecutive days to ensure bacterial elimination. Antibiotics and anti-inflammatory drugs can help prevent secondary bacterial infection in viral diarrhea cases.



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Given the above-mentioned fluid and antibiotics-drug therapies, optimal nursing is an important factor in determining calf survival. All in all, although aggressive treatment would be the last chance to save sepsis or heavily infected and diarrheic calves, it might not be effective in all cases because of individual pathophysiological differences among calves.

## CONCLUSION

Neonatal calf diarrhea can be fatal if the disease is not detected early enough and goes forward to induce sepsis. In severe cases, early intervention is vital for rescuing affected calves. Therefore, aggressive treatment would be needed to recover calves from deadly infection status. Administration of normal and hypertonic saline serum and sodium bicarbonate is fundamental to return lost water and electrolytes as well as to correct metabolic acidosis. Intravenous therapy should be continued with isotonic serum until calf recovery from the risk point. Aggressive treatment, as developed and presented here, would be the last chance to save highly infected calves. It is necessary to note that individual calf differences in resistance to disease is a determining factor in their full or partial recovery. Thus, aggressive treatment might not be necessarily successful in all cases.

## DECLARATIONS


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### Authors' contribution

The authors' contribution to this work was equal. The tasks included idea conceptualization, strategic development and contemplation, and manuscript writing and editing. The final draft of the manuscript was checked by all authors.

### Competing interests

None.

### Ethical considerations

The authors have made necessary ethical considerations (e.g., plagiarism, consent to publish, misconduct, data fabrication and/or falsification, double publication and/or submission, and redundancy).

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# Apoptosis in Bali Cattle Embryo Cells Produced *In Vitro*

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## ABSTRACT

*In vitro* production of Bali cattle embryos still needs in-depth investigations to produce embryos suitable for transfer. The current study aimed to examine the level of cell apoptosis in Bali cattle embryos produced *in vitro* and at three stage of oocyte maturation, fertilization, and embryo culture. A total of 107 pairs of ovaries derived from slaughterhouses of Indonesia were collected. The used oocytes were grades A and B (Grade A had compact cumulus oocyte complex (COC) cells surrounded by five or more layers of cumulus cells, and grade B had a non-compact COC and a dark cytoplasm with complements from the complete radiata corona but surrounded by no more than five layers of cumulus cells). Fertilization of oocytes was done using the semen of a Bali bull. Bali cattle semen was frozen in straw semen for 5 minutes at 1500 rpm twice, then the supernatant and spermatozoa were separated and equilibrated for 30 minutes. Fertilization lasted for 5-6 hours in the incubator. Then, oocyte culture was carried out using CR1aa media and evaluated at 48 hours post-insemination (hpi). The result of the current study showed that the development of Bali cattle embryos produced *in vitro* after 48 hours of culture included 2 cells (31.91%), 4 cells (32.97%), 8 cells (24.46%), and 16 cells (10.63%). The percentage of embryos containing at least one nucleus exhibiting Terminal dUTP nick-end labeling (TUNEL) characteristics of apoptosis entailed 28.33% (2 cells), 41.93% (4 cells), 43.48% (8 cells), and 50% (16 cells). The division ability of embryos aged 48 hpi consisted of 2, 4, 8, and 16 cells. In conclusion, apoptosis in Bali cattle began to be detected in the two-cells stage. The sooner a cell undergoes apoptosis, the lower the level of the cell's ability to develop further.

**Keywords:** Apoptosis, Bali cattle, Embryo, *In vitro*, cell cleavage

## INTRODUCTION

Bali cattle are local cattle native to Indonesia and are widely developed in community farms. Bali cattle have eminent traits including adaptability to high temperatures (24-35°C) and feed use efficiency (Baco et al., 2013; Putra et al., 2019). One way to maintain the quality and quantity of Bali cattle is by applying reproductive technology assisted by *in vitro* embryo production. *In vitro* embryo production technology can be applied by utilizing the ovaries of cattle livestock from slaughterhouses. The technology can provide information on livestock infertility (Karja et al., 2010). The challenge for in vitro production laboratories can increase the number of embryos produced with high-quality in each round of in vitro culture (Lonergan et al., 2004). The success of *in vitro* embryo production is usually determined by the number of embryos that reach the morula or blastocyst (Oliveira et al., 2019). Morula and blastocyst stages are resistant to freezing and can be of worthy transfer to the recipient (Bó and Mapletoft, 2013; Hansen, 2020). *In vitro* embryo production consists of three main stages, namely, maturation (IVM), *in vitro* fertilization (IVF), and *in vitro* culture (IVC). Each stage plays a crucial role in supporting the success of embryo production (Kharche et al., 2011). Some factors, such as the genotypic effect, the quality of oocytes, the microenvironment, the conditions of in vitro production, lipids, and other molecules, determine the embryo's competence (Marsico et al., 2019). Overall, the efficiency of this biotechnology process is still low because many processes can still lead to the failure of embryo production *in vitro*, including culture conditions that are not under *in vivo* conditions of female reproduction (Smith et al., 2012).

Fast-developing embryos are of higher quality than slow-developing embryos (Velker et al., 2012). During *in vitro* culture, stressful conditions affect the embryos' quality and survival ability (Ramos-Ibeas et al., 2020). The development of the zygote to the blastocyst stage varies greatly, and the individual outcome is uncertain (Leidenfrost et al., 2011). In embryonic development, mainly at the stages of morula and blastocysts, apoptosis mediates the elimination of certain cells (Ramos-Ibeas et al., 2020). Abnormal embryos produced *in vitro* trigger aging at the cellular level by entering the cessation of the cell cycle and showing active metabolism and high levels of reactive oxygen species (ROS, Nandi et al., 2019). The protective role of aging and apoptosis is to ensure that unhealthy cells and early embryos do not develop, avoiding long-term adverse effects (Galluzzi et al., 2018). Cell death during this process has been reported with unclear interpretations ranging from pathological phenomena to an integral part of normal blastocyst development (Betts and King, 2001). Preliminary studies on the embryonic development of Bali cattle cultured *in vitro* have different developments on the same culture day (Hasbi et al., 2020). In order to find out the differences, it is necessary to measure



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the fragmentation of DNA in embryonic cells. DNA fragmentation indicates the occurrence of apoptosis or cell death (Hadi, 2011). Studies on DNA fragmentation have been reported in zebu cows (*Bos Indicus*) (Garcia et al., 2015), buffalo oocytes (Gustina et al., 2019), and humans (Hardy et al., 2003). Apoptosis is considered the cause of death, resulting in the embryo's failure to undergo subsequent division. Given the importance of this issue, there is a dearth of research on apoptosis (DNA fragmentation) in Bali cattle. Therefore, this study was conducted to test the level of apoptosis of cattle embryos produced *in vitro*.

## MATERIALS AND METHODS

### Ethical approval

This study has been approved by the Animal Ethics Commission, Hasanuddin University, Makassar, Indonesia, number 404/UN4.6.4.5.31/PP36/2022.

### Collection and selection of oocytes

The 107 pairs of ovaries of Bali cattle from the slaughterhouse in Makassar city, Indonesia, were taken to the laboratory *in vitro* production at Hasanuddin University, Makassar, by transport media (0.9% NaCl solution plus antibiotic gentamycin 100 µ/mL, Sigma-Aldrich, USA). The oocytes were collected using the slicing method (Hasbi et al., 2017). Oocytes grades A and B were selected using a microscope (Olympus, Japan). Grade A had compact cumulus oocyte complex (COC) cells surrounded by five or more layers of cumulus cells, and grade B had a non-compact COC and a dark cytoplasm with complements from the complete radiata corona but surrounded by no more than five layers of cumulus cells (Kakkassery et al., 2010; Bakri et al., 2016). Of 838 total oocytes, 505 were selected. All chemicals and reagents were purchased from Sigma-Aldrich Chemical Company (USA) unless otherwise indicated.

### Oocyte maturation

The selected oocytes were washed three times using collection media by phosphate buffered saline (PBS, Gibco by life technologies, USA) to which 0.2% bovine serum albumin (BSA, Sigma-Aldrich, USA) and 50 µg/mL gentamycin (Sigma-Aldrich, USA) were added. Then, they were matured in a maturation media consisting of M199 (Gibco by Life Technologies, USA) through the addition of 0.3% BSA, 10 IU/mL pregnant mare serum gonadotrophin (PMSG, Intergonan, Intervet Deutschland GmbH, Netherlands), 10 IU/mL human chorionic gonadotrophin (HCG, Chorulon, Intervet International BV Boxmeer-Holland, European Union), and 50 µg/mL gentamycin (Sigma-Aldrich, USA). Maturation was carried out in the form of a drop (80 µL/drop) with an oocyte count of 10-15/drop and covered with mineral oil (Sigma-Aldrich, USA). Maturation was performed in a 5% $CO_2$ incubator with a temperature of 38.5°C for 24 hours (Hasbi et al., 2017).

### *In vitro* fertilization

Bali cattle semen was frozen in straw by centrifuging the 0.25 mL semen for 5 minutes at 1500 rpm twice, then the supernatant and spermatozoa were separated (Hasbi et al., 2020). The semen was added with fertilization media (Suzuki et al., 2000) so that the final concentration of spermatozoa was 1.5 x $10^6$ cells/mL (Hasbi et al., 2020). Then, four drops (80 µL/drop) were placed into a Petri dish covered with mineral oil (Sigma-Aldrich, USA), and equilibrated for 30 minutes. The maturated oocytes were then put into the equilibrated drop and stored in the incubator for 5-6 hours.

### *In vitro* culture

After fertilization for 5-6 hours, oocytes were washed twice using CR1aa culture media, transferred into an 80-µL drop of CR1aa culture media following modification by Sagirkaya et al. (2006) and Somfai et al. (2010) with some modifications. In the next step, 5 mg/mL BSA and 2.5% FBS were added, oocytes were covered with mineral oil (Sigma Chemical Company, USA), and cultured in a 5% $CO_2$ incubator at the temperature of 38.5°C for 48 hours (Hasbi et al., 2020). On the second day of culture, classification was carried out based on the stages of division. The 2, 4, 8, and 16 cells of the embryo were then transferred to the culture media. Embryo evaluation and medium utilization were carried out every 48 hours and cultured for 96 hours.

### DNA fragmentation

The cell fragmentation of each division group was analyzed using a combined technique for nucleic staining and Terminal dUTP nick-end labeling (TUNEL, *in situ* cell death detection system, USA, procedure modified by Gustina et al., 2019). Embryos were fixed overnight at 4°C in 3.7% (weight/volume) paraformaldehyde diluted in PBS. After overnight fixation, the embryos were washed four times in PBS containing 3% (w/v) of polyvinyl alcohol (PVA) and permeabilized in 0.5% (v/v) Triton-X100 for 1 hour and then incubated in a blocking solution (PBS + 10 mg/mL BSA) overnight at 4°C. After washing them in PBS-PVA, the positive control and all treated embryos were incubated in


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fluorescein-conjugated dUTP and terminal deoxynucleotidyl transferase (TdT, TUNEL reagents) at 38.5 °C for 1 hour in dark. As a positive control, one to two embryos per TUNEL analysis were incubated in 1000 IU/mL of deoxyribonuclease I (DNase I, Sigma-aldrich, USA) for 20 minutes. Meanwhile, the negative controls were incubated in fluorescein dUTP without TdT. After TUNEL, embryos were washed three times in PBS-PVA, and later stained with 50 ug/mL propidium iodide (PI) for 20 minutes to label all nuclei. The embryo was extensively washed in the blocking solution, placed on a glass slide, and covered with a glass cover. The embryo was examined under a fluorescence microscope (Zeiss Axio Imager A2, Germany) using excitation at wavelengths of 488 nm and 568 nm to detect the TUNEL and PI reactions. The pictures were taken with a digital camera (Zeiss AxioCam HRc, Germany, Loo, 2011).

**Statistical analysis**

The data of cell division were analyzed descriptively. The Pearson correlation coefficient was run for the total number of cells and the apoptosis index using SPSS software (version 20). The significance was defined at $p \leq 0.05$.

## RESULTS AND DISCUSSION

The results showed that 48 hours post-insemination, 40.52% of embryos could be divided into 2, 4, 8, and 16 cells (Table 1). Meanwhile, the percentage of embryos which could develop into 2, 4, 8, and 16 cells were 31.91%, 32.97%, 24.46%, and 10.63%, respectively.

Cells showing signs of apoptosis can be seen in Figure 1. The percentages of embryos with at least one nucleus displaying the TUNEL characteristics of apoptosis were in 2, 4, 8, and 16 cells were 28.33%, 41.93%, 43.48%, and 50%, respectively (Figure 2).

The correlation between the total-number of cells and the apoptosis rate in Bali cattle embryos produced *in vitro* is indicated in Figure 3. There was no significant correlation between the apoptosis rate and the total-number of cells observed in embryos produced *in vitro* $R^2 = 0.1355$ and $p > 0.05$.

**Table 1.** Cell cleavage of Bali cattle embryos 48 hours post insemination

| Oocyte | Cultured | Cleaved | | Cell cleavage (%) | | | |
|---|---|---|---|---|---|---|---|
| Number | Number | Number | Percentage | 2 cells | 4 cells | 8 cells | 16 cells |
| 505 | 464 | 188 | 40.52 | 60 (31.91) | 62 (32.97) | 46 (24.46) | 20 (10.63) |

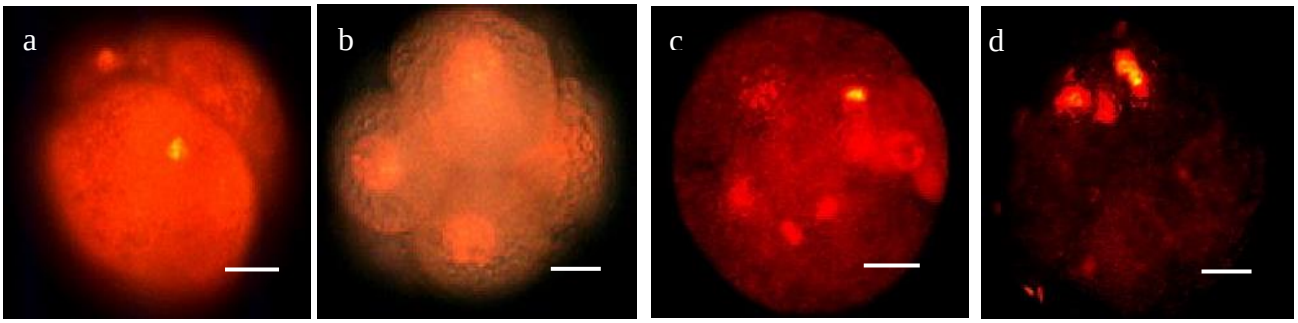


**Figure 1.** Detection of apoptotic and all nuclei in cattle embryos by TUNEL (fluorescein isothiocyanate-conjugated dUTP; green channel) and propidium iodide (red channel), **a:** 2 cells, **b:** 4 cells, **c:** 8 cells, **d:** 16 cells. Scale bars represent (abcd) 40 µm.

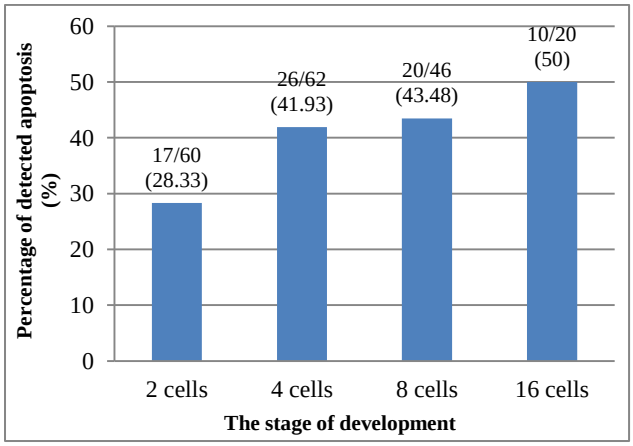


**Figure 2.** Percentage of embryos in Bali cattle containing at least one nucleus showing characteristics (TUNEL) of core apoptosis.

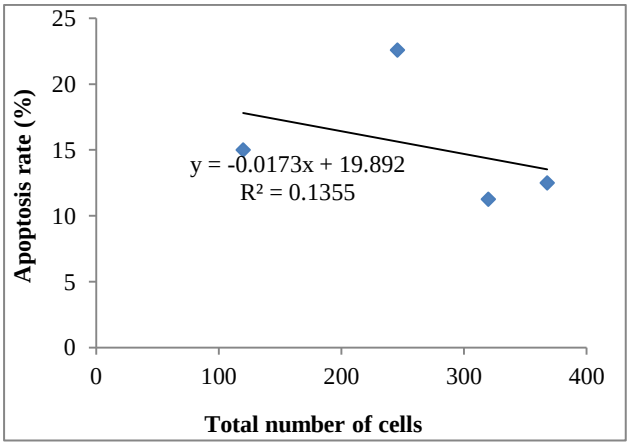


**Figure 3**. The relationship between the number of cells and apoptosis rate in Bali cattle embryos produced *in vitro*. R: the correlation coefficient for the observations made.



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Oocytes fertilized in the study were oocytes that experienced suitable cumulus expansion after maturation. The oocytes undergoing expansion have reached metaphase II. The quality of oocytes is an early predictor of the development of the embryo's potential (Goovaerts et al., 2010). The oocytes maturated *in vitro* will undergo cumulus cell expansion, and changes occur in the perivitelline space with the formation of the polar body I (Hassa et al., 2014). This indicates the meiosis stage and the success of the metaphase II (MII) stage (Lv et al., 2010; Zafar et al., 2021).

The embryo division ability after 48 hours post insemination (hpi) in Bali cattle *in vitro* reached up to 16 cell divisions. Several factors can cause fertilization failure, including imperfect maturation process of the nucleus and cytoplasm due to poor quality of oocytes (Swain and Pool, 2008), spermatozoa failure to carry out capacitation and acrosome reactions, disabling spermatozoa to fertilize oocytes (Fujihara et al., 2020), and spermatozoa failure to condense in the oocyte cytoplasm, leading to a failure in male pronucleus formation (Zafar et al., 2021). *In vitro* embryo production derived from oocytes surrounded by multiple cumulus cells or bare oocytes results in the lower formation of the blastocyst, compared to oocytes surrounded by a dense layer of cumulus cells (Merton et al., 2012). The selection of oocytes based on the COC was carried out on donors. Oocytes and embryos cultured separately per donor obtained an average blastocyst rate of 16-18% (Machado et al., 2006; Merton et al., 2012). Small-group embryos showed lower total cell number and higher apoptosis rate than large-group cultured embryos (24.17% vs. 12.14%, Cebrian-Serrano et al., 2013).

Bali cattle embryos produced *in vitro* exhibited signs of apoptosis, which was undergoing DNA fragmentation starting from stage 2 cells which continued to increase to the subsequent division (4, 8, and 16 cells). Apoptosis occurs during the pre-implantation development of bovine embryos produced *in vivo* and *in vitro* (Gjørret et al., 2003). Apoptosis has been confirmed from the stage of 6 cells *in vitro* and the stage of 21 cells *in vivo*. In case an error occurs in embryo production (both *in vitro* and *in vivo*), the failure of the first division will directly lead to the death of the embryo or cause the subsequent development to deviate (Burruel et al., 2014). The failure of such cleavages will be the primary source of heterogeneity development (Shi et al., 2015). At the blastosis stage, if there is substantial cell death in the inner cell mass then cell death develops faster (Morris et al., 2010). The main causes of cell death at the beginning of the development of bovine embryos are not mediated by caspase (Leidenfrost et al., 2011).

The first signs of apoptosis commonly occur in slow-cell groups than in fast-cell groups (Morris et al., 2010). Apoptotic cells detected at 48 hpi revealed a possible mechanism of programmatic cell death activation before genome activation occurred. Cell apoptosis observed in slow-developing embryos showed a link between the pro-cell-death and the kinetics of embryonic development in zebu *in vitro* produced embryos (Garcia et al., 2015). Brad et al. (2007) reported that failure of caspase-9 activation could be the cause of the resistance of the two-cells embryos to experience apoptosis. Somfai et al. (2010) used time-lapse cinematography, describing oocytes that underwent direct division from one cell into three or four blastomeres, a phenomenon associated with high frequencies of chromosomal abnormalities. Cells that experience apoptosis will round and shrink, fragmented chromatin nuclei and organelles containing cytoplasm will shrink (Voss and Strasser, 2020).

The TUNEL staining will detect all types of DNA damage and analyze the morphological features of apoptosis and necrosis observed in target cells (Rodríguez et al., 2006). It is used with other apoptosis-specific test combinations (Loo, 2011). The apoptosis rate and the total number of detected cells did not have a strong relationship in Bali cattle embryos produced *in vitro* (r=-0.368). Fragmentation of the core condensed by karyorrhexis is another important component of apoptosis likely affected by *in vitro* production (Betts and King, 2001). Apoptosis features were not observed before the morula stage *in vivo* but were more quickly observed in 9 to 16 cells *in vitro* (Gjørret et al., 2003). DNA fragmentation occurs on the second day of culture in human embryos, for which fragmentation above 25% is considered bad (Hardy et al., 2003). According to Gustina et al. (2019), DNA fragmentation in buffalo oocytes was around 15%, and using 0.05% sericin could reduce DNA fragmentation by up to about 7%.

## CONCLUSION

The division ability of an embryo aged 48 hours post-insemination varies by 2, 4, 8, and 16 cells. The incidence of apoptosis in Bali cattle happens in the two-cell stage. The sooner a cell experiences apoptosis, the lower the level of cell ability to develop further. It is, therefore, recommended to conduct future research on apoptosis in Bali cattle embryos *in vivo*.

## DECLARATIONS

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**Authors' contribution**

Erni Damayanti collected the sample, drafted the manuscript, and formatted it, Herry Sonjaya and Sudirman Baco were responsible for the data analysis, Hasbi Hasbi was responsible for designing the study. All authors approved the final manuscript

**Competing interests**

The authors declared that they did not have any conflict of interest.

**Ethical considerations**

The research had all credibility and trust and did not plagiarise or copy from any other papers or ideas. The present findings did not have any fabrication or falsification. The authors consent to publish only in World's Veterinary Journal and did not submit this article or any part of the present scientific results in any other journals.

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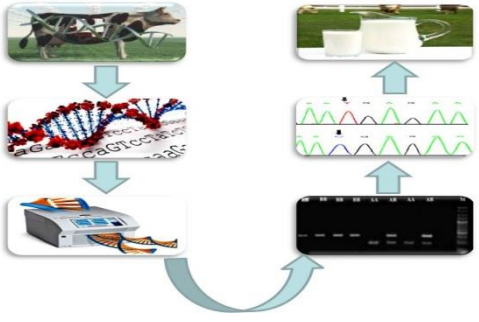

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Discussion and Conclusion can be presented jointly if preferred.

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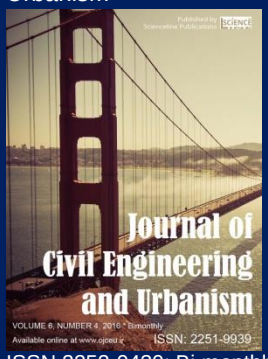


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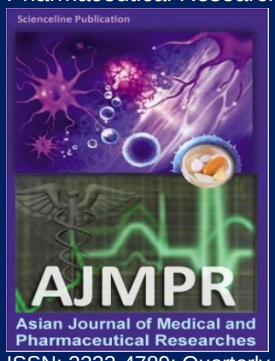


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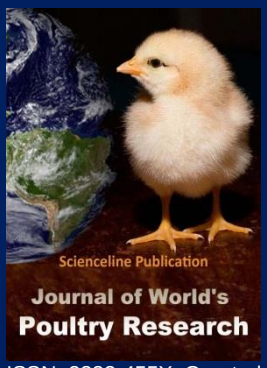


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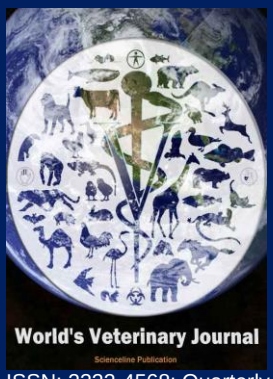


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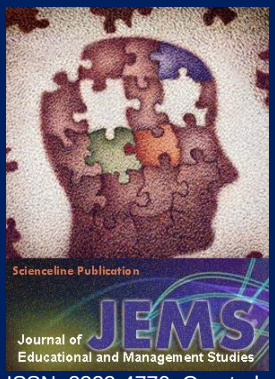


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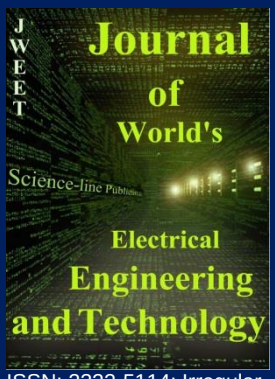


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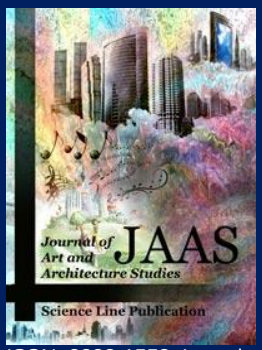


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