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Review Article

Botanicals: A Promising Control Strategy Against Highly Zoonotic Foodborne Trichinosis

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Abstract

Trichinosis is a highly zoonotic disease caused by *Trichinella spiralis* in humans. The disease is majorly transmitted through the consumption of raw or undercooked meat. About 10000 people get infected with *T. spiralis* every year. Signs and symptoms of the disease may vary from mild to severe infection, depending on the worm load. Albendazole and mebendazole are two main anthelmintics that have been in use to treat trichinosis for a long time. Albendazole is a very effective drug in the early stages of the *T. spiralis* infection, but resistance has been reported multiple times. Drug resistance is the most prevailing issue that needs to be addressed as early as possible. Scientists have focused on the development of novel drugs for the treatment of *T. spiralis* infection. Botanical compounds have multiple medicinal and therapeutic properties which make them able to treat multiple parasitic diseases. Botanical compounds are considered the best alternatives for the development of new drugs for the treatment and control of trichinosis. This review covers the detailed mechanism of action of multiple botanical compounds for a better understanding of the formulation of new drugs. Saponins, tannins, and phenolic acids have potent anthelminthic activity against trichinosis.

Keywords: Botanical compounds, Food borne, Food safety, Meat, Mechanisms, Trichinosis, *T. spiralis*, Zoonosis

Introduction

Trichinosis, also known as Tricinellosis, is a parasitic disease that spreads through the consumption of undercooked contaminated meat [1]. Pork meat is the major source of the transmission of the disease while other carnivores also spread the disease to humans [2]. Trichinosis is among the 10 most common food-borne zoonotic diseases of humans [3]. Trichinosis can also be caused by the consumption of beef meat when it becomes contaminated with a pathogenic agent [4]. The etiological agents of trichinosis are multiple *Trichinella* spp., among all *Trichinella spiralis* is the most common cause of the infection [5]. *T. spiralis* is the smallest known nematode (2nd largest phylum in the animal kingdom) which belongs to the family Trichinellidae [6]. The average size of male nematodes is 1.4-1.6 mm while females are relatively larger up to 1.4-4 mm [7]. *T. spiralis* is a ubiquitous roundworm that infects about ten thousand people annually around the globe [8]. The infection rate is higher in the countries where swine (pork) meat is consumed commonly [9]. The mortality rate of people infected with trichinosis is quite low (about 0.2%) globally [10]. The fatality ratio may increase when the infection becomes severe and untreated [11]. However, the signs and symptoms of the disease may vary from mild to severe [4]. The signs and symptoms of the infection depend on the phase of the infection [12]. There are 2 phases of the infection: enteral (affecting the intestine of the patient) and parenteral (affecting the organs and parts outside of the intestine) [13]. During the initial stage of the infection, *T. spiralis* invades the intestine of the patient and causes mild to severe symptoms including vomiting, abdominal pain, dyspepsia, heartburn, and diarrhea [14-16]. These signs and symptoms usually vary with the worm load, small burdens cause mild or no signs and symptoms of the disease while large worm burdens cause severe expression of the infection [17]. When *T. spiralis* migrates from the intestine to the other parts of the body, the severity of the infection increases [18,19]. Periorbital edema, vasculitis, muscle pain, fever, weakness, and splinter hemorrhage in nails are some common signs and symptoms in the case of the parenteral phase of the infection [12,20]. To cure the disease, multiple anthelmintics have been in use for a long time [21].

Albendazole and mebendazole are two main anthelmintics that have been in use to treat trichinosis for a long time [22].

Both drugs are the first line of treatment for trichinosis and are effective in the early stage of the infection [23,24]. However, some other drugs including flubendazole and benzimidazole-2-carbonate derivatives can also be used to treat human trichinosis [25,26]. Albendazole and mebendazole expel *T. spiralis* from the intestinal tract of the infected patient by disrupting the formation of microtubules [27]. They bind with the colchicine site of β-tubulin which results in the inhibition of the polymerization of the microtubule and cell division [28]. Disruption in the microtubule formation directly affects glucose absorption [29]. The mobility of *T. spiralis* is retarded due to the absence of an adequate glucose level to move in the body [30]. This will ultimately kill the parasite and flush it out of the intestine of the body *(Fig. 1)*. However, resistance against antiparasitic drugs is a major problem to be solved.

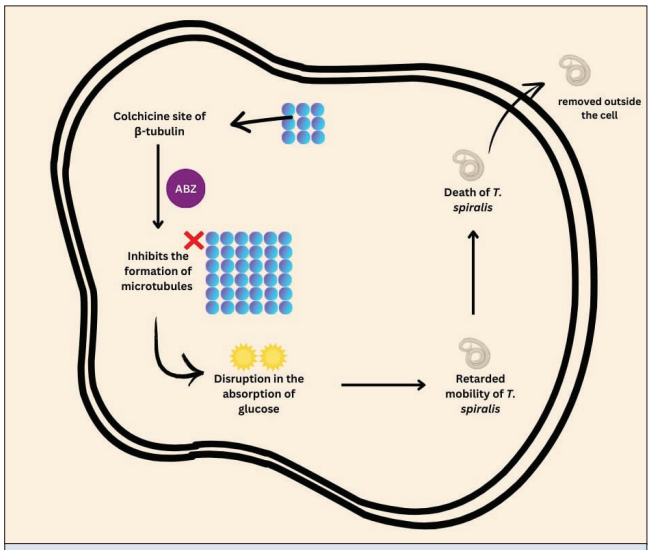

Fig 1. Basic mechanism of action of Albendazole against *T. spiralis*.ABZ: Albendazole

Multiple reports have been seen regarding the drug resistance and chemical drug residues of anthelmintics [31-33]. Drug resistance is the major emerging threat to the lives of humans as well as animals [34]. However, researchers are struggling hard to combat the resistance issue [35]. Because of the rising issue of drug resistance, scientists have focused on the development of alternative medicine therapy to cure the disease [36]. Alternative medicine strategies for the treatment of various diseases and infections include probiotics, prebiotics, herbal medicine, vaccines, immunogens, vitamins, peptides, etc. [37-41]. Among all the alternative medicine therapies, plant-based products are the best option because of their wide range of therapeutic effects [42-45]. Botanical compounds are safer and have more potent results against multiple parasitic diseases [46]. Plant-based compounds are well known for their various therapeutic and medicinal properties including antioxidative, anti-inflammatory, anti-infectious, immunomodulatory, and antimicrobial properties [47-50]. Some botanical compounds can be used directly as antimicrobial agents as they have a higher safety index and low side effects [51]. However, some compounds of plants are toxic to the vertebrates as well [52].

In this review, we will briefly discuss the control strategies for *T. spiralis* with the help of plant-based compounds. This review covers different botanical compounds that are derived from multiple plants including phenolics, tannins, and saponins. Detailed mechanisms of action of different botanical compounds will also be discussed. For a proper understanding of the control strategies and mechanism of action of botanical compounds, we must know the normal life cycle of *T. spiralis* and its pathogenesis.

Methodology

This review used Google Scholar *(www.scolar.google.com)* as the primary source of information. Furthermore, ResearchGate *(www.researchgate.com),* PubMed *(https://pubmed.ncbi.nlm.nih.gov/),* and ScienceDirect *(http://www.sciencedirect.com/)* were used as secondary search engines. Keywords used are "Botanicals", "*Trichinella spiralis*", "Food-borne zoonotic diseases", "Trichinosis", "*T. spiralis*", "Use of Botanical compounds for the control of *Trichinella spiralis*", and "Plants used for the control of *T. spiralis*". This is a qualitative review, so no statistical comparison was made, and the results are not quantified.

Life Cycle and Pathogenesis

The life cycle of *T. spiralis* contains 3 life stages which include newborn larvae, adult, and infective muscle larvae [53]. The primary hosts of *T. spiralis* are pigs and wild boars while the intermediate hosts include humans and other mammals [54,55]. The primary route of the transmission of the disease is through the consumption of contaminated meat [56,57]. However, vertical transmission of the disease is also reported in some cases [58]. After the ingestion of the meat contaminated with *T. spiralis,* the encysted larvae of the pathogen are released into the stomach and small intestine [57,59]. The larvae are released from their cysts after exposure to digestive enzymes (pepsin and gastric acid) [60]. The released larvae then invade into the small bowl mucosa. They take nutrients from the small intestine and become adults (male = 1.4-1.6 mm; female = 1.4-4 mm) [61,62]. The adult larvae in the small intestine reside there for about 4 weeks for mating and laying eggs [63]. Female nematode lays eggs after 1 week in the mucosal layer of the small intestine which then hatch into the larvae [60]. The hatched larvae enter the bloodstream and lymphatic system by penetrating the walls of the small intestine [64]. The newborn larvae enter the skeletal muscle cells from the bloodstream where they form nurse cell-

larvae complex [65]. Nurse cells protect the larvae from the host immune system and nourish them [66-68]. The larvae migrate to the striated muscles where they encyst *(Fig. 2)*. The encysted larvae remain dormant and may remain infectious for years [69].

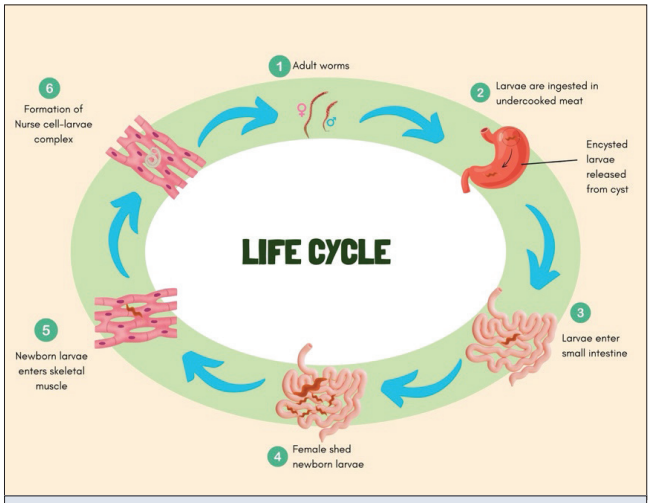

Fig 2. Life cycle of *T. spiralis*

Botanical Control

Naturally occurring plant-based compounds have been tested and proven effective for the treatment and control of multiple parasitic diseases [46,70-75]. Scientists are now focusing on the specific compounds of the plants instead of using whole parts (leaves, roots, bark, stem, and flowers) [76,77]. Botanical compounds have diverse medicinal and therapeutic properties including anti-inflammatory, antioxidant, anticancer, antimicrobial, immunomodulatory, antimutagenic, cardioprotective, neuroprotective, and antidiabetic activities [78-83]. This wide range of biological properties enables them to be used for the effective treatment and control of multiple diseases [84]. Botanical compounds are of very great concern to researchers because of the emerging threat of antimicrobial resistance [85]. Scientists are focused on the development of new drugs that have potent activity as an alternative to the previously existing antimicrobial drugs [86]. In this review, we will further discuss the activity of naturally occurring botanical compounds for the control of *T. spiralis*. For the development of new potent drugs from the botanical compounds, we are required to understand the mechanism of action of the botanical compounds. Details of multiple plant-based botanical compounds are given below:

Saponins

Saponin is a Latin word which is named because of the foamy/soapy quality when they are agitated with water [87]. Saponins are glycosides that have at least one glycosidic linkage between a glycone (sugar chain) and aglycone (non-sugar organic molecule) [88,89]. Saponins are most commonly used for the formation of soap, drug adjuvants, and for the synthesis of steroids [90]. Research has been conducted by multiple researchers to check the activity of saponins against *T. spiralis* [91]. Scientists have found potent results in the inhibition of *T. spiralis* by using saponin compounds [92-100].

Saponins have great potential to boost the immune response by stimulating chemokines and cytokines [101]. Saponins are responsible for regulating the recruitment of immune cells and intracellular signaling mechanisms [102]. They also aid in the proliferation and differentiation of T-cells into CD4+ T-helper cells [103]. Saponins have the ability to destroy *T. spiralis* due to their anti-inflammatory potential *(Fig. 3)*. TNF-α is a cytokine that is responsible for the regulation of other inflammatory cytokines [104]. Saponins depress the activity of TNF-α expression which will destroy *T. spiralis* [105]. However, severe destruction of newborns and adults of *T. spiralis* has been seen along with vesicular areas, loss of annulations, blebs, and marked swelling of the cuticle [91].

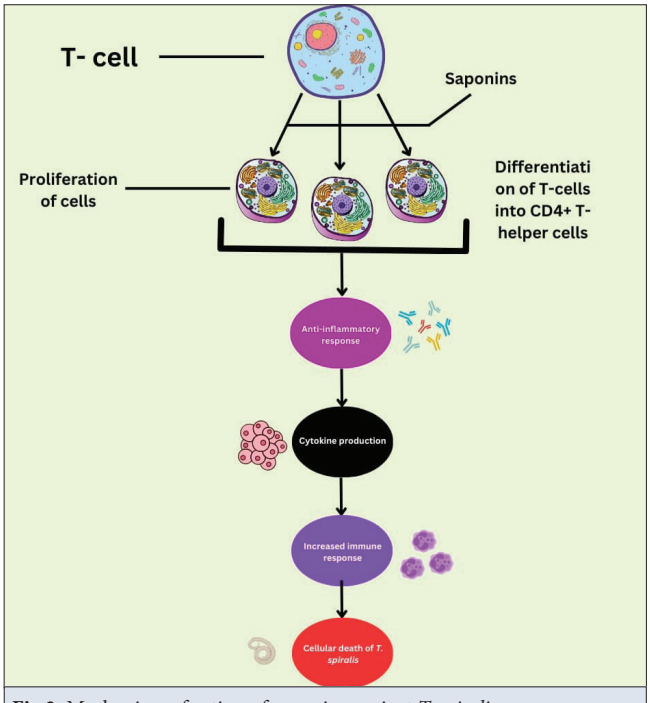

Fig 3. Mechanism of action of saponins against *T. spiralis*

Another mechanism of saponin has also been reported that saponins have the ability to alter the membrane permeability which will result in their cytostatic and cytotoxic activities [106-109]. This specific property of the saponins makes them able to destroy the pathogenic

Table 1. *Botanical Compounds used against T. spiralis and their mechanism of actions*

Sr. no.	Class of Compound	Compound Used	Plant Source	Investigation Medium	Organism Used	Mechanism of Action	Results	References
1	Saponins	Oleanolic-type triterpenoidal saponins	*Bassia indica*	*In vivo* & *in vitro*	Mice	Saponins act as an anti-inflammatory agent by depressing the TNF-α expression in the tissues of muscle	Severe destruction of *T. spiralis* newborn larvae and adult worms	[92]
		Triterpenoidal saponins	*Luffa aegyptiaca*	-do-	-do-	Increase the permeability of the cell membrane causing cytostatic and cytotoxic effects	Death of *T. spiralis* has been reported due to the altered membrane permeability and cytotoxic activity of the saponins	[91]
		Extract of *Holothuria polii* containing various saponin compounds	*Holothuria polii*	*In vivo*	-do-	-	The warm load has been decreased by 96.76% when *H. polii* extract was treated with albendazole	[93]
		Aqueous and alcoholic leaf extracts of *Tetradenia riparia* containing compounds of saponins	*Tetradenia riparia*	*In vitro*		-	The anthelminthic effect of saponins has been seen *in vitro* testing	[157]
2	Phenolics	Multiple phenolic compounds	*Bassia indica*	*In vitro* & *in vivo*	Mice	Anti-inflammatory response has been seen with the depression of TNF-α expression, resulting in the death of the parasite	Severe destruction of *T. spiralis* newborn larvae and adult worms	[92]
		Resveratrol	-	*In vitro*		-	Significant activity against newborn and adult *T. spiralis* has been seen. However, there is no activity seen against the muscle stage of the *T. spiralis*	[116]
		Ellagic acid (EA)	-	*In vivo*	Mice	EA increases catalase activities and decreases the nitric oxide in the muscular and intestinal tissues which will result in improving oxidative stress	EA has more potent results against the intestinal phase of *T. spiralis* as compared to the muscle phase	[117]
		Gallic acid	-	*In vivo*	Male mice	Gallic acid is a vigorous antioxidant, and it produces large amounts of free radicals which results in the lipid peroxidation process. Lipid peroxidation favors the condition to damage the cellular membranes of the parasite	When gallic acid is administered orally at the dose rate of 30 mg kg^{1}, the infection rate of *T. spiralis* is reduced at a noticeable amount as compared to the results of groups of mice treated with Albendazole	[118]

Table 1. *Botanical Compounds used against T. spiralis and their mechanism of actions (continued)*

Sr. no.	Class of Compound	Compound Used	Plant Source	Investigation Medium	Organism Used	Mechanism of Action	Results	References
3	Tannins	Extract containing various tannin compounds	*Luffa aegyptiaca*	*In vitro* & *in vivo*	Mice	Tannins increase the cellular permeability and reduce the CD34 expression[1]	Worms load has been reduced due to the altered cell membrane and reduction in the CD34 expression	[91]
		Tannic acid	-	*In vivo*	Swiss albino mice	-	Synergistic effects of tannic acid with Albendazole exhibit maximum activity against *T. spiralis.* However, tannic acid with a dose rate of 2000µg/ml has the same antiparasitic effects as Albendazole	[158]
		Dietary tannin compounds	Quebracho	*In vivo* & *in vitro*	Rats	-	Dietary quebracho tannins exhibited potent activity *in vitro.* However, no significant effects were seen in the small intestines of the rats	[159]
		Clove oil enriched with tannin compounds	-	*In Vitro*		Clove oil disrupts the folic acid cycle[2] or DNA synthesis of the parasite. However, clove oil significantly disrupts the metabolism of the parasite which results in the death or morphological changes in the parasite that hinders parasitic growth or multiplication	100% death of the adult worms was reported at the concentration of 50 µg/mL of clove oil. However, a significant change in the morphology of *T. spiralis* was reported at the concentration of 50 µg/mL	[160]
4	Others	Methanolic extract of *R. chalepensis*	*R. chalepensis*	*In vitro*		-	The n-hexane partition of the methanolic extract showed potent activity against *T. spiralis* with anLC_{50} value of 147.6 µg/mL	[119]
		Artemisinin	*Artemisia annua*	-	-	When the infected mice are treated with *A. annua* extract, the normal flora of the intestine is restored and the TGF-β expression was reduced	Adult worm count has been reduced in the intestinal tract of the infected animal	[120]

[1]*CD34 have the ability to inhibit cellular differentiation.*
[2]*Folic acid cycle plays a key role in the DNA synthesis of parasites. The folic acid cycle refers to the conversion of folic acid into tetrahydrofolate and dihydrofolate*

organism, i.e., *T. spiralis,* and can be used as a therapeutic agent [110]. However, to make new potent and safer drugs, more research must be conducted on the safety index of saponins.

Phenolics

Phenolics are the class of the compounds which have at least one hydroxyl group attached to the aromatic group of the hydrocarbons [111]. Phenolic compounds are classified on the basis of phenol units attached to the molecule [112, 113]. Phenolic compounds have multiple biological activities including enzyme inhibition, antioxidant, anti-cancer, and anti-inflammatory properties [114]. These properties make them able to be used as the cure for multiple diseases and conditions [115].

Phenolics have been reported effective in the control of *T. spiralis* by alleviating the inflammatory response [92,116-120].

The inflammatory response was increased because of the downregulation of IL-6 and increased expression of IL-10 in the tissues [121]. Phenolics can increase catalase activities and decrease the nitric oxide in the muscular and intestinal tissues which will result in improved oxidative stress [122,123]. Oxidative stress refers to an imbalance in the antioxidants and the free radicals which cause cellular death [124]. When the number of free radicals increases, it causes lipid peroxidation in the cell [125]. Lipid peroxidation provides a favorable environment to destroy the cellular membrane [126]. Oxidative stress plays a significant role in combating parasitic diseases by damaging the cells of the parasite [127]. In this condition, hosts use a large amount of reactive oxygen species, especially H_2O_2 to fight the developing parasitic disease [128]. However, some other mechanisms of action of phenolics are to disrupt the cell membrane and alter its permeability, denaturation of protein, and ion chelation (reaction between complexing agents which creates a ring structure and metal ions) [129-133].

Synthesis of novel drugs for the control and treatment of *T. spiralis* infection can be done with the help of understanding the mechanism of action of the commercial drugs and the comparative mechanism of the phenolic compounds [134]. However, there is still a need to do further research on the safety index of plant-based phenolic compounds.

Tannins

The term tannin refers to the abundance of oak bark compounds that were used for tanning the hides of the animals [135]. Tannins are large polyphenolic compounds that contain hydroxyls, carboxyl, or other suitable groups and make strong complex compounds with other macromolecules [136,137]. Tannins have a wide range of biological activities including anticancer, antioxidant, and anti-inflammatory properties [138-141]. However, multiple research projects have been made to check the effectiveness of tannin compounds for the control or treatment of various parasitic diseases [46,142-144].

Multiple research projects claimed the potent activity of tannins against *T. spiralis* [110,117,119,145]. Tannins have the ability to bind with the free available protein causing nutritional deficiency for *T. spiralis* [146]. The nutritional deficiency reportedly kills the larvae and adults of *T. spiralis* [147,148]. The activity of tannins against the cuticle of *T. spiralis* has more potent results because the cuticular structures are vital for the defense and nutritional role of the parasite [98,148-151]. However, another mechanism of action of tannins is also reported that tannin compounds disrupt the cellular metabolism of the parasite [152,153]. Tannins can cause disruption in the synthesis of DNA or the folic acid cycle [154-156]. In order to make new potent drugs for the treatment and control of *T. spiralis,* we must do further research on the safe administration of these compounds.

Conclusion

Trichinosis is a highly zoonotic disease of humans caused by *T. spiralis*. The disease is majorly transmitted through the consumption of raw or undercooked meat. Albendazole and mebendazole are two main anthelmintics that have been in use to treat trichinosis for a long time. Albendazole is a very effective drug in the early stages of the *T. spiralis* infection, but resistance has been reported multiple times. Drug resistance is the most prevailing issue that needs to be addressed as early as possible. Scientists have focused on the development of novel drugs for the treatment of *T. spiralis* infection. Botanical compounds have multiple medicinal and therapeutic properties which make them able to treat multiple parasitic diseases. Botanical compounds are considered as the best alternatives for the development of new drugs for the treatment and control of trichinosis. Multiple compounds have been tested against the newborn, larvae, and muscle stage of *T. spiralis*. Saponins, tannins, and phenolic acids have potent anthelminthic activity against trichinosis. However, synergistic activities of these botanical compounds with Albendazole have the highest activity in the reduction of the worm load. This review suggests further research on the safety index of effective botanical compounds to formulate novel drugs for the treatment and control of highly zoonotic trichinosis.

Declarations

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Research Article

Effect of X-Ray Exposure on Oxidative Stress in Liver and Kidney in Rats in Early Life: An Experimental Study

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Abstract

The aim of this study was to investigate the levels of oxidative stress and antioxidants in the liver and kidney tissue of baby rats exposed to whole-body x-ray by creating a newborn rat model. In this study, 60 baby rats obtained from 15 pregnant rats were used. Pregnant rats were randomly divided into five groups. The control group (Group I) was not subjected to X-ray. The 2nd and 3rd groups were subjected to both intrauterine and postnatal X-ray, and the 4th and 5th groups were subjected to only postnatal X-ray. At the end of the 4-week study period, oxidative stress markers were studied in the liver and kidney tissue. In all groups that received X-ray, an increase in the amounts of malondialdehyde (MDA) and advanced oxidation protein products (AOPP), a decrease in the amount of glutathione (GSH) and catalase (CAT) activity were detected in liver tissues ($P<0.05$), and an increase in the activities of MDA, AOPP and CAT, and a decrease in the amount of GSH were detected in kidney tissues ($P<0.05$). These findings indicate that X-ray exposure in early life disrupts the antioxidant defense system by inducing oxidative stress in liver and kidney tissues, highlighting the necessity of minimizing unnecessary radiation exposure in clinical practice.

Keywords: Antioxidant enzymes; New-born; Oxidative stress; X-ray

Introduction

Although ionizing radiation has tremendous diagnostic and therapeutic benefits for humans, it also has serious harmful effects [1]. Ionizing radiation causes serious damage to living systems by transferring high energy directly to macromolecules or by hydrolysing water. As a result of high energy exposure, it causes excessive production of reactive oxygen species (ROS) such as superoxide radicals, hydroxyl radicals, and so on [2]. These Reactive Oxygen Species react with biomolecules and cause oxidative damage to cells. The magnitude of the damage from ionizing radiation varies greatly depending on the absorbed dose, the duration of radiation exposure, the time between exposures, and the sensitivity of the tissues to radiation [3]. Ionizing radiation damage to biomolecules occurs either through the direct effects of radiation or through the attack of short-lived reactive oxygen species resulting from radiolysis [4].

The reduction of oxygen by aerobic cells produces small amounts of various highly reactive molecules known as reactive oxygen species [5]. As a result of normal oxygen respiration, a certain amount of ROS is constantly formed in the cell. And it is necessary for the redox regulation of various functions. For example, hydrogen peroxide is of great importance in signalling and regulatory processes in the cell. Cells have an enzymatic antioxidant system against excessive free radicals. This system cleans various radiolysis products that are soluble in water and fat. In addition, this system eliminates both long-lived ROS and hydrogen peroxide. When the amount of ROS exceeds the neutralizing capacity of the antioxidant defence system, it causes oxidative stress, which damages biological molecules and leads to the need to replace them [6-8]. In addition to the production of short-lived free radicals, ionizing radiation also produces long-lived radicals in mammalian cells because it can easily oxidize proteins [9]. In the presence of oxygen, ionizing radiation damages proteins by forming oxidized protein products; some of these oxidized proteins may have half-lives of several hours or longer [9].

Çibuk et al.[10] in a study they conducted, they revealed that X-ray application in newborn rats could disrupt Caspase signalling pathways and cause infertility. The wavelength of X-rays is small and the energy is high, so it has high penetration power. Therefore, exposure to X-rays can cause the formation of free radicals. The free radicals formed can attack biological molecules in the cell, causing cellular lipid peroxidation and Deoxyribose nucleic acid (DNA) damage [11].

With the increase in free radical concentration, cells produce endogenous antioxidants (such as glutathione, catalase) to minimize damage or eliminate free radicals. With the increase in the level of exposure to ionizing radiation, the expression of antioxidant enzymes increases [12].

The liver is considered to be a highly sensitive organ to radiation, and its damage when exposed to radiation can have profound deleterious effects due to its involvement in numerous metabolic functions [13]. Studies have shown that kidney tissue is moderately radiosensitive, and damage caused by radiation exposure is eliminated by regeneration [14,15]. However, some studies have revealed that the kidneys are one of the most radiosensitive organs of the abdominal system [16]. The harmful effects of ionizing radiation on various tissues resulting from excessive ROS production are well documented [17].

Diagnostic radiology is increasingly used in the evaluation and treatment of newborns requiring intensive care. Multiple radiographic examinations are often required, depending on the baby's birth weight, gestational age, and medical problems [18].

The aim of this study was to investigate the levels of oxidative stress and antioxidants in the liver and kidney tissue of baby rats exposed to whole-body x-ray by creating a newborn rat model.

Material and Methods

Ethical Statement

This study was conducted with the permission of Van Yüzüncü Yıl University Animal Experiments Local Ethics Committee, dated 28.12.2023 and numbered 2023/14-04.

Animals and Experimental Groups

The rats used in the study were obtained from Van Yüzüncü Yıl University Experimental Animals Unit. Fifteen Wistar Albino pregnant rats weighing 250-300 g were housed in cages with 12 h of light/dark at a temperature of 22±2°C, with food and fresh water in front of them during the trial.

In this study with a trial period of 4 weeks, rats were randomly divided into 5 groups.

Group I (control) (3 pregnant rats; the study was continued with six male and six female infant rats after birth): Pregnant rats were fed as standard, after birth, young rats were fed exclusively with breast milk for three weeks.

Group II (3 pregnant rats; the study was continued with six male and six female infant rats after birth): From the 12th day of gestation until birth, non-lethal (diagnostic 8 Grays (Gy)) dose of X-ray was applied to rats once a day [19]. After the birth, a non-lethal dose was administered to the cubs once a day for three weeks.

Group III (3 pregnant rats; the study was continued with six male and six female infant rats after birth): From the 12th day of gestation, a non-lethal (diagnostic) dose of X-ray was applied to rats every day. After birth, a non-lethal dose of X-ray was administered to the infant rats once a day for a week. After the birth, the young rats were continued to be fed with breast milk for three weeks.

Group IV (3 pregnant rats; the study was continued with six male and six female infant rats after birth): No application was applied to animals until birth, after birth, non-lethal dose of X-ray was given to the cubs every day for three weeks.

Group V (3 pregnant rats): No application was applied to the animal until birth, after birth, non-lethal dose X-ray was applied to the cubs once a day for a week.

Sample Collection and Biochemical Analysis

After the experimental application (at the end of the 4th week), 90 mg/kg ketamine HCl (Ketalar®, Pfizer, Brooklyn, New York, USA) and 10 mg/kg xylazine HCl (Rompun®, Bayer, Leverkusen, Germany) intraperitoneal was given to all rats, lying on the table in the dorso-ventral position, opening the thorax with a vertical incision in the midline, the heart was directly cannulated, and 5 mL blood samples were taken into anticoagulant and non-anticoagulant tubes. Blood samples without anticoagulant were separated by centrifugation at 4000 rpm for 5 min.

Rat livers and kidneys were homogenized in phosphate buffer (0.1 mol/L, pH=7.4). The samples were centrifuged at 10000 rpm for 20 min and the supernatant was stored at -80°C until the working day. GSH, MDA [20] concentrations and catalase activity [21] were measured spectrometrically in liver and kidney tissues on the study day.

Statistical Analysis

The "SPSS Statistic 20" package program was used in the analysis of the data. All data were given as mean (±) and standard error (SE). Analysis of variance (ANOVA) followed by Duncan's test was performed to determine if there were significant differences between the groups. Independent sample t-test was used for pairwise comparisons. It is possible to say that there is a statistically significant difference between the groups in the results where the P (sign) values are less than 0.05.

Table 1. MDA, CAT, AOPP and GSH results of liver tissue						
Parameters	**Group I (Control)**	**Group II**	**Group III**	**Group IV**	**Group V**	**P Value**
MDA (mmol/g tissue)	0.71±0.18^{b}	1.14±0.46^{a}	1.04±0.12^{a}	0.82±0.12^{ab}	1.11±0.39^{a}	0.019
CAT (U/L)	154.03±25.16^{a}	119.68±18.95^{b}	125.9±17.35^{b}	130.37±27.27^{b}	130.05±30.04^{b}	0.033
AOPP (mmol/g tissue)	82.59±8.92^{b}	96.26±7.18^{a}	96.55±10.36^{a}	95.88±8.75^{a}	95.40±10.45^{a}	0.005
GSH (µmol/g tissue)	1.44±0.33^{a}	0.92±0.3^{c}	1.1±0.19^{bc}	1.28±0.36^{ab}	1.3±0.27^{ab}	0.004

P<0.05 shows statistical significance
Different letters in the same column indicate statistical significance

Table 2. MDA, CAT, AOPP and GSH results of kidney tissue						
Parameters	**Group I (Control)**	**Group II**	**Group III**	**Group IV**	**Group V**	**P Value**
MDA (mmol/g tissue)	0.78±0.15^{bc}	1.13±0.24^{a}	0.98±0.24^{ab}	0.89±0.12^{bc}	0.76±0.25^{c}	0.002
CAT (U/L)	155.17±23.29^{a}	188.52±51.94^{a}	175.59±34.94^{a}	176.75±37.48^{a}	169.01±26.39^{a}	0.35
AOPP (mmol/g tissue)	65.45±10.79^{b}	89.4±17.22^{a}	73.29±13.43^{b}	72.7±15.05^{b}	74.29±11.29^{b}	0.007
GSH (µmol/g tissue)	1.44±0.26^{a}	1.07±0.24^{b}	1.04±0.3^{b}	1.03±0.25^{b}	1.1±0.44^{b}	0.025

P<0.05 shows statistical significance
Different letters in the same column indicate statistical significance

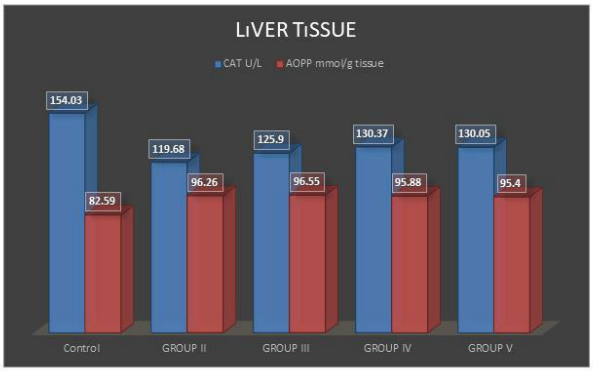

Fig 1. CAT and AOPP results of liver tissue

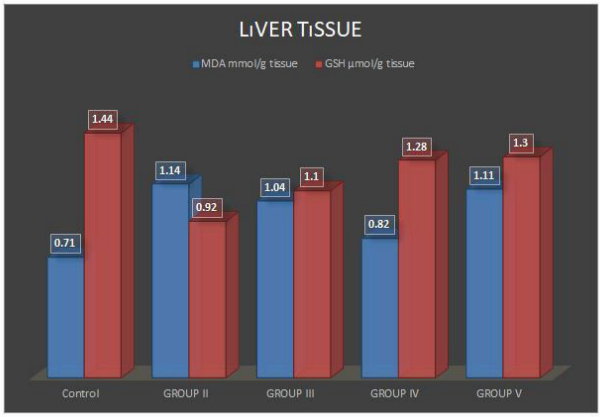

Fig 2. MDA and GSH results of liver tissue

Results

The obtained results are given in *Table1* and *Table 2*. When the results obtained from the liver tissue were examined, it was observed that the MDA level increased in all groups compared to the control group. And this increase is statistically significant (P<0.05). In addition, it was observed that the AOPP level increased significantly in the X-ray applied groups (P<0.05). Compared to the control group, a significant decrease in CAT activity and GSH amount was observed in the X-ray treated groups (P<0.05) *(Table 1, Fig. 1, Fig. 2)*.

It is observed that the amount of MDA and AOPP in the kidney tissue increased significantly in the X-ray treated groups compared to the control group. This increase is statistically significant (P<0.05). There was an increase in CAT activity in the X-ray treated groups compared to the control group, but no statistical significance was found in this increase (P>005). The amount of GSH decreased in all groups when compared with the control group. This decrease is also statistically significant (P<0.05) *(Table 2, Fig. 3, Fig. 4)*.

When liver and kidney tissue were compared, it was seen that the amount of AOPP in kidney tissue was significantly lower in control 3rd, 4th and 5th groups (P<0.05) *(Table 3)*. Although catalase levels were similar in kidney and liver tissues in the control group, it was seen that it decreased significantly in kidney tissue compared to liver tissue in experimental groups (P<0.05) *(Table 3)*. MDA levels were significantly higher in liver tissue compared to kidney

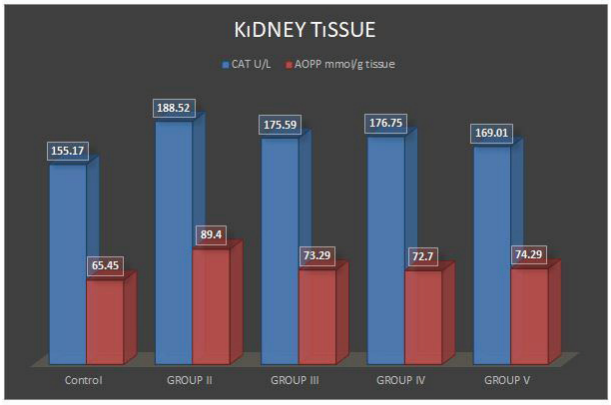

Fig 3. CAT and AOPP results of kidney tissue

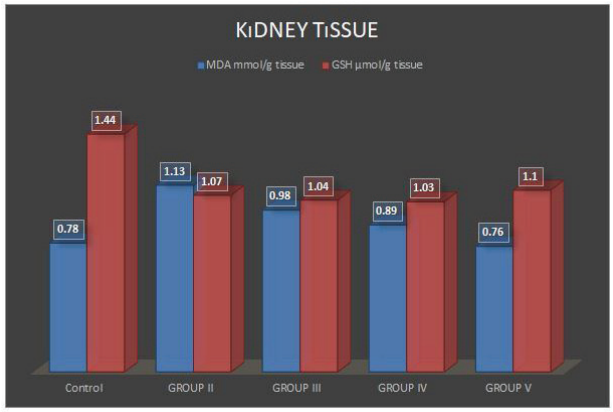

Fig 4. MDA and GSH results of kidney tissue

Table 3. *MDA, CAT, AOPP and GSH results pairwise comparison of liver and kidney tissues*

Tissues and P Value	MDAmmol/g tissue	CAT U/L	AOPP mmol/g tissue	GSH µmol/g tissue
Liver Control	0.71±0.18	154.03±25.16	82.59±8.92	1.44±0.33
Kidney Control	0.78±0.15	155.17±23.29	65.45±10.79	1.44±0.26
P Value	0.341	0.97	0.001	0.99
Liver Group II	1.14±0.46	119.68±18.95	96.26±7.18	0.92±0.3
Kidney Group II	1.13±0.24	188.52±51.94	89.4±17.22	1.07±0.24
P Value	0.97	0.002	0.27	0.24
Liver Group III	1.04±0.12	125.9±17.35	96.55±10.36	1.1±0.19
Kidney Group III	0.98±0.24	175.59±34.94	73.29±13.43	1.04±0.3
P Value	0.63	0.001	0.001	0.57
Liver Group IV	0.82±0.12	130.37±27.27	95.88±8.75	1.28±0.36
Kidney Group IV	0.89±0.12	176.75±37.48	72.7±15.05	1.03±0.25
P Value	0.21	0.005	0.001	0.096
Liver Group V	1.11±0.39	130.05±30.04	95.40±10.45	1.3±0.27
Kidney Group V	0.76±0.25	169.01±26.39	74.29±11.29	1.1±0.44
P Value	0.001	0.006	0.001	0.22

tissue in Group V ($P<0.05$) *(Table 3)*. GSH levels were significantly lower in kidney tissue compared to liver tissue in Group IV ($P<0.05$) *(Table 3)*.

Discussion

The aim of the current study was to examine oxidative stress and antioxidant levels in rats exposed to x-rays. X-ray application caused oxidative stress by increasing the levels of MDA and AOPP, which are oxidative stress parameters in the liver and kidney. At the same time, GSH levels also decreased. While liver tissue catalase activity decreased in the X-ray applied groups, it increased in kidney tissue. These findings suggest that X-ray radiation used for medical imaging may cause cellular damage in tissues in rats, increase oxidative stress, and affect the antioxidant defence system. Consistent with our study, studies in the literature reveal that repeated X-ray examinations of animals in veterinary clinics may pose potential health risks, such as acute harm and an increased cancer risk due to DNA damage [22,23].

Oxygen radicals react with polyunsaturated fatty acid (PUFA) residues in phospholipids, resulting in end products that are mostly reactive towards proteins and DNA. MDA is particularly known to play a role as a marker of oxidative stress, and its concentration is directly proportional to the cellular damage caused by free radicals [24,25]. It has been reported that low-dose X-ray may cause lipid peroxidation and cause an increase in MDA levels in rat lung and liver tissue [5]. In their study on rats, Salehi et al.[26] reported that the level of MDA increased in the serum of rats exposed to 7 Gy of x-ray for 30 days. In another study, it was shown that the level of lipid peroxidation (LPO) increased in the heart tissue of mice treated with 2 Gy X-ray for 4 days [17]. Bala et al.[27] showed that ROS

and LPO levels increased in the liver and kidney tissues of mice exposed to 2 Gy X-ray. In a study, it was revealed that ionizing radiation application affected apoptotic and oxidative stress regulatory genes in the hFOB 1.19 osteoblast cell line in a time and dose-dependent manner and that the harmful effects in this cell line might be due to mitochondrial pathway activation [28]. In a study conducted with cell culture, it was shown that gamma radiation increased ROS levels and caused apoptosis and DNA damage [29]. Ionizing radiation results in excessive ROS production due to high oxygen consumption and metabolic rate [30]. X-ray exposure results in cellular damage, either directly or indirectly, via the water radio dialysis mechanism, leading to the formation of ROS [31]. ROS affect various cellular functions by causing nucleic acid damage, oxidized proteins and lipid peroxidation [32]. In the presented study, it was found that X-ray application before and after birth (Group II) significantly increased the MDA level in liver and kidney tissue compared to the control group. Compared to the control group in Group III, liver and kidney tissue MDA levels were higher, but only liver tissue MDA levels were found to be significantly higher. In parallel with the above-mentioned studies, X-ray application increased oxidative stress and caused MDA levels to increase in liver and kidney tissue.

AOPP is a safe marker used to evaluate oxidative modification of proteins. AOPP is a marker of the severity of oxidative stress and oxidatively mediated protein damage in inflammation and is often produced during oxidative stress [33]. In the presented study, liver tissue AOPP levels in all groups treated with X-ray were found to be significantly higher than the control group. Kidney tissue AOPP level was found to be higher than the control group in the X-ray applied groups, but only Group II kidney tissue AOPP level was found to be significantly higher than the control group.

GSH is a tripeptide and non-enzymatic antioxidant produced in the body that plays a pivotal role in maintaining cellular redox balance [34]. It has been reported that GSH plays a protective role against oxidative stress by directly detoxifying H_2O_2 and lipid peroxides by scavenging hydroxyl radical and singlet oxygen, and also returns vitamins C and E, which are important antioxidants, to their active forms [35]. Decreased GSH level in tissues not only impairs cellular defence but also causes increased oxidative damage [36]. GSH deficiency is often an indicator of the presence of oxidative stress [24]. High doses of radiation lead to a decrease in GSH levels. This is thought to be due to the production of reactive oxygen and nitrogen species produced by short-lived ionizing radiation, which are then neutralized by reduced glutathione producing oxidized glutathione [24]. It was reported that the GSH content in the cardiac tissue of rats treated with 2 Gy X-ray for 4 days did not change when compared with the control group, but it decreased significantly in the lung tissue [17]. Bala et al.[27] showed that GSH levels were significantly reduced in the liver and kidney tissue of mice exposed to 2 Gy X-ray for 4 days compared to the control group. The decreased GSH activity in hepatic and renal tissues in X-ray-exposed animals may be due to its increased use in an attempt to detoxify ROS produced by ionizing radiation [27]. Decrease in GSH may cause an increase in hydroxyl radicals that attack lipid membranes [34]. In the presented study, GSH levels in the liver and kidney tissues of all groups exposed to X-ray were found to be lower than the control group. Especially liver and kidney tissue GSH levels of rats exposed to X-ray during pregnancy were found to be significantly lower compared to the control group. As the exposure time increased, the decrease in tissue GSH levels became greater. In Group IV and Group V, liver and kidney tissue GSH levels were found to be lower compared to the control group, but only kidney tissue GSH levels were found to be significant ($P<0.05$). The decrease in GSH level can be explained by the fact that X-ray exposure increases oxidative stress, which in turn decreases antioxidant capacity.

Catalase converts two molecules of hydrogen peroxide into molecular oxygen and two molecules of water. It has been shown that catalase activity in the testicular tissue of mice exposed to X-ray increased compared to the control group [34]. Another study reported that catalase activity increased in the liver and kidney tissue of mice exposed to X-ray [27]. Bala et al.[1] The increase in catalase activity in tissues may have occurred to scavenge excess ROS production due to X-ray exposure [27]. In the presented study, liver tissue catalase activity in all groups exposed to X-ray was found to be significantly lower than the control group. Especially Group II catalase activity was found to be lower than all other groups. In contrast to the liver tissue catalase activity, the X-ray exposed groups had higher catalase activity compared to the control group, but these elevations were not significant. This difference in catalase activity between tissues may depend on the tissue's response to exposure, the absorbed dose, and the sensitivity of the tissues. Additionally, the kidney is a moderately radiosensitive organ and has been reported to have the ability to regenerate after radiation-induced cytotoxic injuries [14,37]. The results obtained from this study showed that AOPP and MDA were generally higher in liver tissue *(Table 3)*. Catalase activity was higher in kidney tissue *(Table 3)*. When liver and kidney tissue were compared, it could be said that kidney tissue was more resistant to ionizing radiation than liver tissue.

In conclusion, X-ray exposure induces cellular damage in liver and kidney tissues by increasing oxidative stress and impairing the antioxidant defense system. These

findings suggest that repeated X-ray exposure in medical and veterinary settings may exacerbate cellular injury and contribute to severe health consequences, including carcinogenesis. Therefore, it is crucial to carefully evaluate the potential risks associated with X-ray exposure and minimize unnecessary imaging procedures to prevent long-term adverse effects.

Declarations

Availability of Data and Materials: The data used in this article will be provided by correspondin author (S. Ç.) upon request.

Financial Support: This study was not financially supported by any person or institution.

Ethical Statement

This study was conducted with the permission of Van Yüzüncü Yıl University Animal Experiments Local Ethics Committee, dated 28.12.2023 and numbered 2023/14-04.

Conflict of Interest: The authors declare that they have no conflict of interest.

Declaration of Generative Artificial Intelligence (AI): The tables and figures used in this article were not created by artificial intelligence.

Author Contributions: Forming the hypothesis and planning the study: S.Ç.; Carrying out the experimental phase: A.A. & S.Ç.; Obtaining data and writing the article: A.A. & S.Ç.

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RESEARCH ARTICLE

A Simple Colorimetric Detection of *Haemophilus parasuis* Based on Aptamer-Functionalized Gold Nanoparticles

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Abstract

Porcine *Haemophilus parasuis* disease, also known as Graze's disease, is the leading bacterial infectious disease caused by *H. parasuis* in modern pig farms. Due to the high nutritional requirement of *H. parasuis,* it usually gets a false positive result through the traditional methods of plate culture. Therefore, it is necessary to establish a simple and fast detection method for this pathogen identification. This work aimed to develop a colorimetric probe using aptamer-functionalized gold nanoparticles for identifying *H. parasuis*. The detection mechanism is based on the color change of gold nanoparticles (AuNPs) from red to blue-purple through NaCl induction after bacteria incubation and aptamer-target binding. First, aptamer-functionalized gold nanoparticles were synthesized and characterized. Then, the best concentration of aptamers and NaCl for detection was optimized at 80 pM and 0.25 M. Under the above optimized conditions, a good linear relationship between the absorbance ratio A678/521 and *H. parasuis* over the range from 10^3 to 10^9 CFU/mL. The limit of detection (LOD) is 12 CFU/mL. This method is simple and rapid, results in high sensitivity and specificity, and can be used to detect actual samples.

Keywords: Aptamers, Gold nanoparticles, Rapid detection, Colorimetric aptasensor, *Haemophilus parasuis,* Pig

INTRODUCTION

Haemophilus parasuis is a member of the *Pasteurellaceae*, and belongs to the Gram-negative microbiota with diverse forms. *H. parasuis* usually settles on the upper respiratory tract in pigs. Under specific conditions, the virulent strains invade the mucosal layer of the respiratory tract and colonized, destroy epithelial tissue, enter the bloodstream and quickly spread to lung tissue, causing damage and pneumonia symptoms, or invade other organs, leading to multiple fibrous pleurisy, peritonitis, and arthritis. With the worldwide prevalence of *H. parasuis* and the harm and economic losses it brings to the pig industry, early diagnosis and measures to treat *H. parasuis* disease are of great significance [1,2].

In recent years, with the rapid development of molecular biotechnology, various detection methods have been established according to the *H. parasuis* genome sequence, such as conventional PCR, real-time fluorescence quantitative PCR, digital PCR, and loop mediated isothermal amplification technology [3-7]. The application of these rapid molecular detection methods depend on expensive instruments. At the same time, immunoserological detection methods including enzyme-linked immunosorbent assay (ELISA), indirect hemagglutination assay (IHA), and complement binding assay (CF) for *H. parasuis* antibody investigation has been established [8-10]. However, due to the large number of *H. parasuis* serotypes and the lack of specific antibodies, the specificity of currently established serology methods is not high, making them unsuitable for large-scale promotion. These methods based on molecular biology, immune serology and other fields have opened up new ideas for the detection of *H. parasuis* [11,12]. But these above methods have more or less drawbacks, which is not appropriate for routine clinical detection and epidemiological

investigation. Therefore, there is an urgent need to develop simple and fast *H. parasuis* detection methods.

Aptamers, also called chemical antibody are a recognition molecule that has emerged in recent years, and can specifically bind to target molecules. Compared with traditional antibodies, they have advantages such as a wide range of target molecules, vitro preparation, stable performance, and ease of labeling and development of various detection methods [13]. In recent years, various detection methods have been developed based on aptamers, including colorimetry, electrochemistry, chemiluminescence, fluorescence, etc [14-16]. Nanogold (AuNP) is a popularly used nanomaterial for colorimetric detection. AuNP is easy to prepare and to adsorb biomolecules, has a high extinction coefficient and strong particle spacing optical effect. It appears red in a dispersed state and turns blue after condensation. Conversely, it can also change from blue to red. It has been reported that the sensitivity of colorimetric based on AuNPs can reach that of fluorescence method [17-19]. The rapid detection methods of aptamer AuNPs combined colorimetric technology have developed rapidly attributed to its advantages of simple, fast, sensitive, results visible to the naked eye, and ease of on-site use. It was demonstrated that the limit of detection (LOD) with the naked eye was 10^4 CFU/mL for identifying *P. aeruginosa* based on colorimetric biosensor using aptamer-functionalized gold nanoparticles [20].

In the present work, we report a simple visual and sensitive colorimetric method for detection of *H. parasuis* employing aptamer-functionalized gold nanoparticles. In this assay, the aptamers were used as a specific recognition probe, and AuNPs as a signal detector. Determine the presence of a target by observing the color changes of the solution with the naked eye. At the same time, conditions of temperature, incubation time, the concentration of aptamer and NaCl are optimized.

Material and Methods

Ethical Approval

The experimentation with animals was approved by the Experimental Animal Management Methods of Xinxiang Medical University (Approval number: 201206078) and followed Henan Authority's Experimental Animal Regulations.

Bacterial Strains

Staphylococcus aureus (ATCC 25923), *Escherichia coli* (ATCC 25922), *Listeria monocytogenes* (ATCC 19111), *Pasteurella multocida* (ATCC 19427), *Actinobacillus leuropneumoniae* (ATCC27088), *Vibrio parahaemolyticus* (ATCC33847), and *Salmonella enterica* (ATCC 14028) were obtained from the China General Microbiological Culture Collection Center (Beijing, China). *S. aureus, E. coli, S. enterica* and *V. parahaemolyticus* were cultured in nutrient broth medium (V.p containing 3.5% NaCl). *Haemophillus paragallinarum* and *Actinobacillus pleuropneumoniae* was cultured in trypticase soy broth medium containing NAD (10 µg/mL) and 5% (v/v) FCS, while *L. monocytogenes* and *Streptococcus suis* were cultured in brain-heart infusion (BHI) broth respectively at 37°C with 180 rpm shake for 18-24 h. The cultured bacteria were then serially diluted and surface plated on the agar dish containing the appropriate culture medium.

Preparation of AuNPs

The AuNPs was synthesized by citrate reduction of $HAuCl_4$ method according to AuNPs synthesis methods with some modification reported previously [21]. First, 1 mL $HAuCl_4 \cdot 4H_2O$ (1%, w/w) and 99 mL ultrapure water were added. The mixture was heated to boil, 10 mL sodium citrate (1%, w/w) was rapidly injected and reacted for 10 min under magnetic stirring. The solution changed color from pale yellow to purplish red and then to wine red. The obtained red wine solution was AuNPs. The AuNPs were purified for three times with a centrifugation (10.000 rpm, 25 min) and re-dispersed in 40 mL ultrapure and filtered through a 0.22-µm cellulose membrane and stored at 4°C. The AuNPs were analyzed using UV-Vis spectra, TEM and Dynamic Light Scattering (DLS). The AuNPs solution was stored at 4°C for further use.

Preparation of Aptamer-AuNPs probe and Aptamer Concentration Optimization

The *H. parasuis* aptamer discovered following an X-aptamer screening kit (Sangon Biotech, 1004728-0000) (5'-TATGGCGGCGTCACCCGACGGGGACTTGACATTATG ACAG-3') was dissolved in DEPC solution with a series concentration (10, 20, 30, 40, 50, 60, 70, 80, 90 pM) [22]. 100 µL aptamer with different concentration was taken to 500 µL AuNPs solution and mixed it respectively. The above mixture was co-cultured in 37°C shaker for 30 h. Lastly, 60 µL 200 mM NaCl solution was added into the above mixture. The color change of the solution was observed, and characterized using a UV spectrophotometer in the wavelength range at 400 and 700 nm after incubated in a 37°C shaker for 30 min.

After that, 400 µL supernatant were discarded after 10.000 rpm centrifuged for 10 min at 4°C. The aptamer-AuNPs probe was stored at 4°C in dark for further use.

Detection of *H. parasuis*

90 µL aptamer-AuNP probe was added to the 96-well plate, then 100 µL *H. parasuis* solution was added with a concentration of 10^6 CFU/mL to the well. Then 10 µL 200 mM NaCl was added to the well and mixed it. The color change of the solution was observed, and characterized using a UV spectrophotometer in the wavelength range

at 400 and 700 nm after incubated in a 37°C shaker for 30 min.

Optimization of NaCl Concentration

A volume of 20 µL NaCl in different concentrations (0, 1, 2, 2.5, 5, 10, 20, 25, 30, 40 M) was mixed with 180 µL of aptamer-AuNPs in a 96-well microplate. The color change was observed and characterized using a UV spectrophotometer in the wavelength range at 400 nm and 700 nm.

Specificity of the Colorimetric Method

100 µL *H. paragallinarum*, *S. enterica*, *P. multocida*, *A. pleuropneumoniae*, *V. parahaemolyticus*, *L. monocytogenes*, *S. aureus*, *S. suis* and *E. coli* bacterial solutions were added that contain same amount to *H. parasuis* (10^5 CFU/mL) and were tested in accordance with the optimal procedure to detect the specificity of this method.

Results

Principle of the Strategy

Firstly, single stranded DNA of *H. parasuis* specific aptamers can absorb onto the surface of AuNPs forming aptamer-AuNPs probe through electrostatic forces, protecting AuNP from aggregation in high salt concentration solutions; after adding *H. parasuis* to the aptamer-AuNPs probe solution, the aptamer has high affinity to *H. parasuis* that dissociates from the surface of AuNPs and forming a composite structure of aptamer-*H. parasuis*. Finally, adding a certain concentration of salt solution to the reaction system can cause unprotected AuNPs to aggregate under the action of salt ions, turning the solution purple or blue, and the degree of discoloration of the AuNPs solution is positively correlated with the *H. parasuis* concentration *(Fig. 1)*. Thus, a rapid detection technology for *H. parasuis* based on aptamer AuNPs colorimetric probe was established.

Characterization of Aptamer Functionalized AuNPs Probe

A variety of validation methods was conducted to characterize the formation of the aptamer-AuNPs probe complex. The transmission electron microscopy displayed that the AuNPs had a uniform particle size about 18 nm in diameter and with a slight aggregation. After they were coated with aptamer, the AuNPs demonstrated no change in particle size but exhibited better dispersion *(Fig. 2-A)*. The spherical gold nanoparticles were dispersed well attributed to the electrostatic repulsion produced by the negative charges of citrate anions coated on the AuNP surface. The UV-vis absorption spectrum assay demonstrated that the maximum absorption peak of AuNPs is at 520 nm *(Fig. 2-C)*, indicating that the AuNPs were well dispersed in the solution, no change was seen on the absorption peak when the aptamer were added, however, according to the results of dynamic light scattering, the hydrated particle size of the AuNPs increased from 24.62±0.39 nm to 28.92±0.46 nm after they were coated with the aptamer *(Fig. 2-B)*, indicating that aptamer was bound to the surface of the AuNPs.

Optimization of Incubation Time and Reaction Temperature for Aptamer Modified AuNPs

According to Li and Rothberg's research in 2004, single stranded DNA exhibits an irregular curling shape in solution and can be adsorbed on the surface of AuNPs through electrostatic forces, van der Waals forces, and water transport forces [23]. And the degree of adsorption is dependent on temperature. Therefore, this experiment optimized the incubation time and reaction temperature for the adsorption of the aptamer onto AuNPs. By measuring the absorbance values of the centrifuge supernatant at 260 nm at different incubation times under three reaction temperatures (4ºC, 20ºC, and 37ºC), the ratio of the absorbance values to the absorbance values

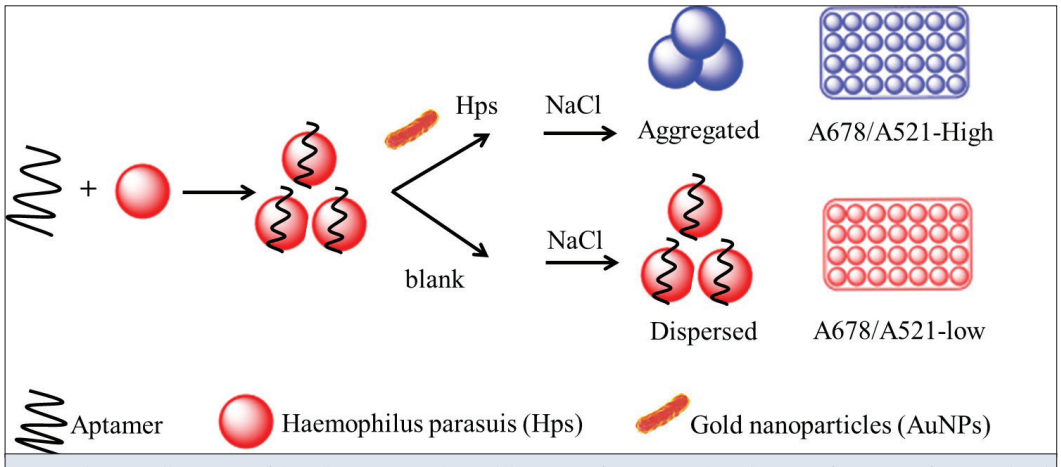

Fig 1. Schematic illustration of the colorimetric and visual detection of *H. parasuis* based on specific aptamer functionalized AuNPs probe

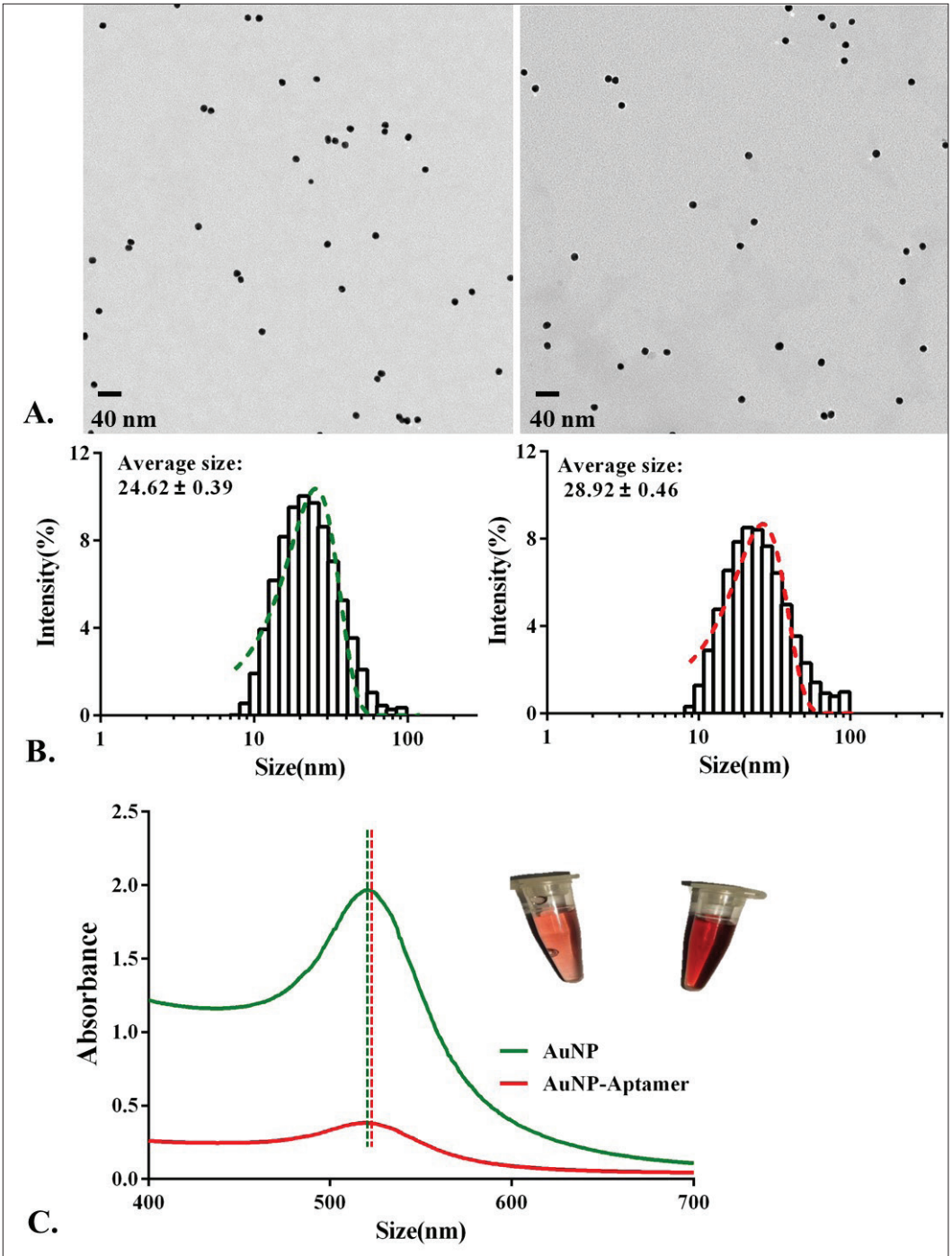

Fig 2. Characterization of AuNPs and aptamer-AuNPs complex. (A) Transmission electron microscopy (TEM) images; (B) Dynamic light scattering (DLS) measurements of the average hydrodynamic diameters; (C) UV-vis spectra

of the initially added adapter solution at 260 nm is the binding rate. The results are shown in *Fig. 3*. At the same temperature, as the incubation time increases, the binding rate continuously increases, reaching its maximum value at 30 h. Afterwards, as time increases, the binding rate no longer changes. As the reaction temperature continues to rise, the binding rate decreases continuously at the same incubation time. When the reaction temperature is 4ºC, the binding rate has reached over 50%. Considering that if the temperature further decreases, the requirements for the reaction equipment will increase, and at the same time, low temperature may accompany with inconvenient operation, the optimal incubation time is selected as 30 h, and the optimal reaction temperature is 4ºC.

Optimization of NaCl Concentration

In general, by adding a high concentration salt solution to an unmodified AuNPs, the originally dispersed AuNPs

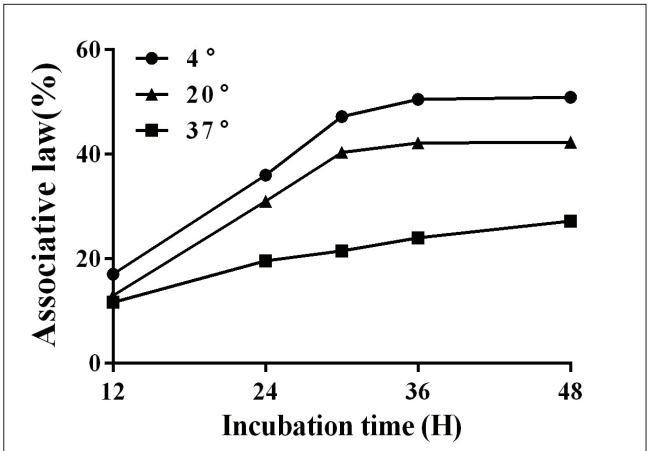

Fig 3. The influence of incubation time and reaction temperature for the binding rate of aptamers coated on AuNPs

will aggregate and forming an irregular network, make the color of the AuNPs change from red to blue. When the aptamer is modified on the surface of AuNPs and the salt concentration is within a certain threshold, due to the electrostatic forces of the aptamer ssDNA on salt ions, its negatively charged phosphate skeleton can effectively protect the AuNPs from aggregation and discoloration due to the presence of salt ions. Therefore, finding the maximum concentration of NaCl solution that can protect the AuNPs from discoloration is a key factor for the success of subsequent experiments. This experiment added 180 µL aptamer-AuNPs complex, then, a gradient increase concentration of 20 µL NaCl solution was added to observe color changes and characterized using a UV spectrophotometer. As shown in *Fig. 4-A*, when the concentration of NaCl solution added increased from 0.1 mol/L to 0.2 mol/L, the absorbance of the solution slightly decreased, and the color of the nanogold system remained red. When further increased to 0.25 mol/L, the color of the nanogold system became purple. The absorption spectrum visible through UV also showed a significant decrease in absorbance at 520 nm, a red shift in peak position, and an increase in absorbance at 678 nm, this indicates that the aggregation of gold nanoparticles occurs, and the concentration of NaCl exceeds the range within which the aptamer can protect the AuNPs from aggregation and discoloration. Therefore, in this experiment, 0.25 mol/L was chosen as the concentration for adding NaCl solution in subsequent experiments.

Optimization of the Ratio Between Aptamers and AuNPs

In order to explore the protective effect of aptamers on AuNPs in high concentration salt solutions, an appropriate amount of aptamers was explored. 100 µL aptamers was added under different dilution concentrations (10 pmol/L, 20 pmol/L, 30 pmol/L, 40 pmol/L, 50 pmol/L, 60 pmol/L, 70 pmol/L, 80 pmol/L) to 500 µL AuNPs reaction system and incubated at 37°C for 30 hours. Then certain amounts of NaCl solution was added to make it final concentration at 25 mM. The color changes were observed visually and characterized by UV spectrophotometer. As shown in *Fig. 4-B*, when the concentration of the aptamer increases from 10 pmol/L to 70 pmol/L, the protective effect of AuNPs becomes stronger, and it is less likely to change under the action of NaCl solution, and it approaches the UV visible

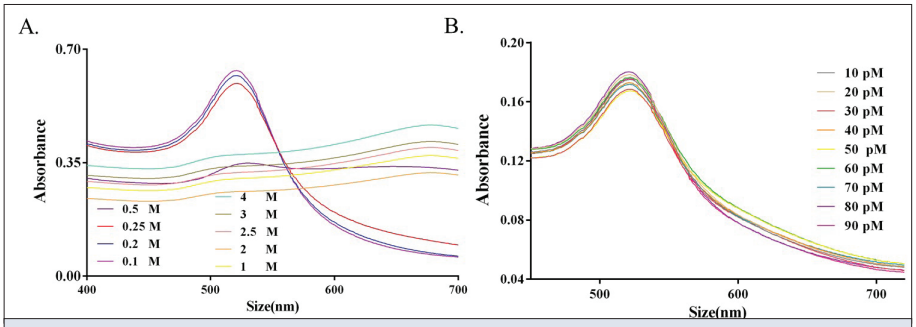

Fig 4. Optimization of the experiment conditions. (A) The UV-visible absorption spectra of AuNPs at different concentrations of NaCl solution (0.1 M, 0.2 M, 0.25 M 0.50 M, 1 M, 2 M, 2.5 M, 3 M, 4 M). (B) The UV-visible absorption spectra of AuNPs at different concentrations of aptamer solution (10 pM, 20 pM, 30 pM, 40 pM, 50 pM, 60 pM, 70 pM, 80 pM, 90 pM)

absorption peak of pure gold nanoparticles solution. When the amount of aptamer added further increased to 80 pmol/L, the UV visible absorption peak did not change significantly and almost overlapped, indicating that the amount of adapter added reached saturation. Therefore, 80 pmol/L was chosen as the optimal addition concentration for the adapter.

Linear Range and Detection Limitation of the Method

In order to explore the linear relationship between *H. parasuis* content and color reaction, the UV-vis absorption spectrum of different concentration of *H. parasuis* in reaction system was detected. When the addition of *H. parasuis* is 10 cfu/mL, no color change was observed and the UV-vis absorption spectrum almost overlapped with the results of the control group without the addition of *H. parasuis* bacteria. Due to low concentration of *H. parasuis*, the amount of AuNPs detached from the ligand protection is very small, and the NaCl solution is not enough to cause the aggregation of AuNPs make obvious color change of the solution. As the concentration of *H. parasuis* solution increases, color changes from red to purple obviously. And the absorption peak of AuNPs shifts red and the peak width gradually increase. The characteristic peak of AuNPs at 521 nm gradually decreases, while the absorption peak near 678 nm gradually increases. The values of A678/A521 also show regular changes. When the concentration of bacterial solution reaches 10^8 CFU/mL, the change is no longer obvious, possibly because all AuNPs have separated from the aptamer and the bacterial solution concentration has reached saturation. The concentration of *H. parasuis* solution ranges from 10^3 CFU/mL to 10^9 CFU/mL *(Fig.5-A)*, and the value of A678/A521 is linearly correlated with the number of colonies. The linear regression equation is Y=0.0817X+0.2075 (R^2=0.9923), with a LOD of 12 CFU/mL.

According to the above method, specificity studies were conducted using the same concentration (10^5 CFU/mL) of *H. paragallinarum*, *S. enterica, P. multocida, A. pleuropneumoniae, V. parahaemolyticus, L. monocytogenes, S. aureus, S. suis* and *E. coli* and *H. parasuis*. The color of these samples remains purple except of *H. parasuis* purple turn to blue. The ratio of A678/A521 in the UV vis absorption spectrum was shown in *Fig. 5-B*, demonstrating the good specificity of this detection method.

Detection of *H. parasuis* in Tissue Effusion from Pig

Several pericardial fluid and articular fluid that collect from different suspected cases was detected using the established detection method and plate counting method. And compare the results to obtain the recovery rate in *Table 1*. It can be concluded that the number of bacterial colonies measured by this method is basically consistent with the results obtained by traditional plate counting methods. The detection recovery rate of *H. parasuis* is good which resides between 91.7% and 129.2%. The results of the *H. parasuis* detection method established in this experiment are consistent with the traditional plate counting method indicating that can be applied to the detection of *H. parasuis* in actual samples.

In this study, a colorimetric strategy based on specific aptamer functionalized AuNPs probe was successfully developed for rapid detection of *H. parasuis*. According

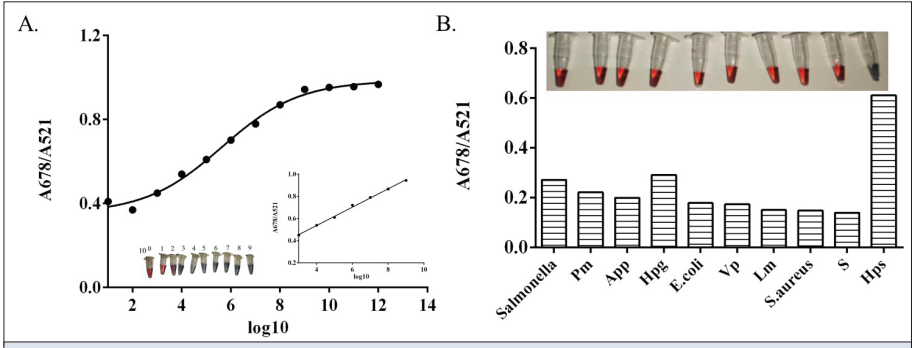

Fig 5. Standard curve and specificity of the detection method. (A) The linear relationship between A678/A521 and *H. parasuis* concentration from 10 to 10^{12} cfu/mL. (B) The specificity result for the detection of *H. parasuis, H. paragallinarum, S. enterica, P. multocida, A. pleuropneumoniae, V. parahaemolyticus, L. monocytogenes, S. aureus, S. suis* and *E. coli*

Table 1. *Comparison of the tissue sample results obtained from the colorimetric detection and classical plate counting method (all results were repeated three times and shown as average ± SD)*

Tissue Sample	Plate Counting	Colorimetric Method	Recovery Ration (%)
1	36±6	33±5	91.7
2	2.4x10^6±213	3.1x10^6±125	129.2
3	2674±89	2801±32	104.7

to the AuNPs probe, the positive sample change in color from red to blue was easily visualized with the naked eye. And, there was good linear relationship between the absorbance variation and *H. parasuis* in the range of 10^3 CFU/mL to 10^9 CFU/mL. Moreover, the aptamer functionalized AuNPs probe exhibited high selectivity to *H. parasuis* other than other bacteria. Therefore, we proposed colorimetric alternative method that offers a simple, rapid, and sensitive tool for *H. parasuis* detection.

Discussion

Recently, largely numbers of colorimetric biosensor for specific detection of pathogens or other molecular markers were developed by using DNA aptamer as recognition element and unmodified gold nanoparticles (AuNPs) as colorimetric indicator [20,24,25]. This research offers a significant breakthrough in the detection of *H. parasuis* by introducing an aptamer-AuNPs based colorimetric probe. This may provide new technical support for pathogen diagnosis according to these hard for culture due to its high nutritional requirement.

The electrostatic forces that shape the formation of the aptamer-AuNPs complex and its subsequent reactions to *H. parasuis* and salt ions form the core of this novel approach. The careful optimization of multiple parameters-incubation time, reaction temperature, NaCl concentration, and the ratio of aptamers to AuNPs is vital [26]. It not only boosts the performance and reliability of the detection system but also provides valuable insights into the underlying thermodynamic and kinetic processes. Transmission electron microscopy, UV-vis absorption spectroscopy, and dynamic light scattering offer an in-depth understanding of the properties of the aptamer-AuNPs probe, validating its formation and modification and reinforcing the detection mechanism. The establishment of a linear relationship between *H. parasuis* concentration and the absorbance ratio, along with a defined limit of detection, showcases the method's potential for sensitive and quantitative analysis. Specificity studies, revealing selective responses for *H. parasuis*, enhance confidence in its practical application. The successful application of the detection method in tissue fluid samples and comparable results to traditional plate counting methods highlights its potential for clinical and diagnostic purposes.

Nevertheless, further studies are necessary to overcome potential challenges such as matrix effects and to evaluate its performance in situations with low *H. parasuis* concentrations [27]. It displayed that the LOD of colorimetric detection method for *Vibrio parahaemolyticus* is 2.4 CFU/mL which higher sensitive than our method [28]. The sandwich structure based on magnetic nanoparticles (MNPs) and gold nanoparticles (AuNPs) proposes a high reference value. Future research could center on integrating the technology with portable devices for on-site detection and delving deeper into the molecular interactions for improved probe design.

Declarations

Availability of Data and Materials: Data and Materials are available from the corresponding author (J. Hu).

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Competing Interests: The authors declare no competing of interest.

Declaration of Generative Artificial Intelligence (AI): The article and tables and figures were not written/created by AI and AI-assisted technologies.

Authors Contributions: Conceptualization, S.Z.; methodology, S.Z. and M.Z.; software, K.L., and L.W.; resources, L.W.; writing - original draft preparation, S.Z.; writing - review and editing, J.H. and S.Z.; funding acquisition, S.Z., L.W. and J.H

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RESEARCH ARTICLE

Stanniocalcin-1 Regulates Ca^{2+}/Pi Uptake in Bovine Renal Tubule Epithelial Cells by Modulating Expression of Entry Channels *In vitro*

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Abstract

Stanniocalcin-1 (STC1), a glycoprotein, serves as an autocrine or paracrine factor in multiple processes in mammals, including the regulation of calcium/phosphorus (Ca^{2+}/Pi) transport. However, its underlying mechanisms are not fully elucidated. Here, we examined the intracellular Ca^{2+} ($[Ca^{2+}]_i$) and Pi concentrations ($[Pi]_i$) levels in primary bovine renal tubular epithelial cells (RTECs) using flow cytometry and phosphomolybdic spectrophotometry, respectively, following STC1 overexpression/inhibition, and treatments with vitamin D_3 receptor (VDR) agonist calcitriol or antagonist MeTC7. The expression of Ca^{2+}/Pi transporters (TRPV5, TRPV6, CB-D_{28K}, PMCA1b, NCX1, Npt2a, Npt2c) was measured by real-time qPCR and western blotting. The results revealed STC1 inhibition by STC1-shRNA promoted Ca^{2+} intake and inhibited Pi influx, whereas STC1 overexpression by pcDNA3.1/STC1 had the opposite effects. The calcitriol-induced increase in $[Ca^{2+}]_i$ was reversed by STC1 overexpression and MeTC7 treatment. Overexpression of STC1 reduced the expression of TRPV5, TRPV6, and VDR, while suppressing calcitriol-induced TRPV5 upregulation and enhancing Npt2a/Npt2c expression. STC1 had no effect on CB-D_{28K}, NCX1, or PMCA1b, which mediate Ca^{2+} diffusion and extrusion. In conclusion, our findings suggest STC1 inhibits Ca^{2+} transport and enhances Pi uptake in RTECs at least partly by regulating TRPV5/TRPV6 and Npt2a/Npt2c expression, respectively. Interference of 1,25 $(OH)_2D_3$/VDR axis may also contribute. The present findings provide new insights into the underlying mechanisms of STC1 and offer strategies to prevent mineral disorders in cattle.

Keywords: Ca^{2+}/Pi transport, TRPV5/6, Npt2a/2c, RTECs, Stanniocalcin-1

INTRODUCTION

Calcium (Ca^{2+}) and phosphorus (inorganic phosphate, Pi) are essential macrominerals that collaborate in various physiological processes, including bone formation, metabolic regulation, and milk production. Imbalances in Ca^{2+}/Pi levels in cattle, especially in the perinatal and lactating cows, can lead to severe metabolic disorders such as rickets, osteomalacia, and milk fever, which not only affect animal health but also result in substantial economic losses in the dairy and meat industries.Their balance is maintained through the coordinated actions of the gastrointestinal tract, bones, and kidneys. The kidneys play a key role by excreting an amount of Ca^{2+}/Pi equal to what is absorbed by gut, a process achieved via glomerular filtration and reabsorption in the renal tubules.

In mammals, reabsorption of Ca^{2+} by the renal tubules involves two distinct mechanisms: paracellular and transcellular pathways. Paracellular transport is a passive, non-saturated, and poorly regulated process. In contrast, active transcellular transport is a saturable and finely orchestrated process [1] comprising three steps. First, Ca^{2+} entry via epithelial Ca^{2+} channels in the apical membrane, such as transient receptor potential vanilloid receptor subtype 5 and 6 (TRPV5 and TRPV6) [2]. Second, Ca^{2+} binds to calbindin-D_{28k} (CB-D_{28k}), facilitating intracellular diffusion [3]. Third, Ca^{2+} efflux by the coordinated action of plasma membrane Ca^{2+} ATPase 1b (PMCA1b) and Na^+/Ca^{2+} exchanger 1 (NCX1) at the basolateral membrane [4]. 1,25-dihydroxyvitamin D_3 (1,25$(OH)_2D_3$, calcitriol) and its nuclear receptor, vitamin D_3 receptor (VDR), play

a central role in regulating the entire process of Ca^{2+} transport and regulation.

The renal absorption of Pi also occurs via two pathways: an active, transcellular sodium-dependent and a passive, paracellular pathway. Sodium-dependent phosphate co-transporter 2a and 2c (Npt2a and Npt2c) are exclusively expressed on the apical membrane of proximal RTECs and are responsible for transcellular Pi transport [5]. However, the mechanism by which Pi is extruded at the basolateral membrane of RTECs remains unclear.

Systemic Ca^{2+}/Pi homeostasis is mainly maintained through crosstalk among cells of the gastrointestinal tract, bone, kidney, and parathyroid gland. The coordinated action of transport proteins and regulatory factors, such as $1,25(OH)_2D_3$, parathyroid hormone, calcitonin [6], fibroblast growth factor 23, and αKlotho [7], constitute the key molecular mechanisms that ensure the maintenance of this homeostasis.

In addition to the aforementioned hormones and cytokines, STC1 appears to be another key player in this intricate regulatory network contributing to Ca^{2+}/Pi homeostasis in mammals, just similar to the function of its homolog (STC) in fish. Beyond its function in mineral homeostasis regulation, STC1 exhibits a wide range of effects, including promoting cell proliferation [8], anti-inflammatory and anti-oxidative activities [9-11], and mitigating nerve damage [12]. Furthermore, STC1 is expressed in almost all tissues [13, 14] but is typically absent from the circulation, suggesting that it may function as a paracrine/autocrine factor rather than as a classical endocrine hormone [15].

Although STC1 is expressed in multiple tissues and organs across various species and is involved in diverse biological and pathological processes, its regulatory effects on Ca^{2+}/Pi homeostasis are conserved. However, the precise molecular mechanism through which STC1 affects renal Ca^{2+}/Pi transport has not been thoroughly elucidated. This study will utilize a primary bovine renal tubule epithelial cell model, as these cells exhibit closer physiological similarities to the renal tubule of normal cattle, to provide more precise insights into the role of STC1 in the active transport of Ca^{2+}/Pi at cellular level, as well as its potential molecular mechanisms responsible for regulating bovine mineral metabolism.

Material and Methods

Ethical Statement

The experimental protocol was approved by the official Committee on the Ethics of Animal Experiments of Huaihua University [Approval no: 2024 (A01006)].

Primary Culture of Bovine RTECs

Primary bovine RTECs were isolated from the kidney cortex of the 1-day-old Chinese Holstein calves. The calves were humanely euthanized under anesthesia using an electric shock apparatus (Jianhua Co., Ltd, Qingdao, China). The kidney cortices were aseptically removed, dissected and minced into small pieces in pre-cooled D-Hank's buffer. The tissue was then ground using a 100-mesh steel wire sieve and filtered through a 150-mesh sieve. The retained cell clusters were collected and evenly dispersed by pipetting. After two rounds of centrifugation at 1200 rpm for 5 min each, the pellets were resuspended in 1 mL of 1 mg/mL collagenase I (Sigma, St. Louis, USA) and incubated at 37°C for 20 min with shaking. An equal volume of DMEM/F12 containing 10% FBS (Gibco, Carlsbad, USA) was then added to neutralize the enzyme. Following another round of centrifugation at 1200 rpm for 5 min, the pellet was resuspended in DMEM/F12 supplemented with 10% FBS, 100 U/mL penicillin, and 100 mg/L streptomycin and incubated at 37°C in a 5% CO_2 atmosphere until confluent. Immunocytochemistry staining was performed using mouse anti-PCK (Pan Cytokeratin, #BM0034, Boster, Wuhan, China), CK18 (Cytokeratin 18, BM4594, Boster), and vimentin monoclonal antibodies (BM0135, Boster) to distinguish epithelial cells from other cell types in the culture, as previously described [10]. The MDBK (Madin-Darby Bovine Kidney) cell line (GDC0290, CCTCC, Wuhan) served as a positive control.

Construction of STC-1 Expression Vector

Total RNA was extracted from calf kidney using Trizol reagent (Invitrogen, Carlsbad, USA) and converted to cDNA by a cDNA Synthesis Kit (TaKaRa, Dalian, China). Bovine full-CDS sequences of *STC1* were amplified using the primers listed in *Table 1*. The purified amplicons were digested with restriction enzymes *BamHI* and *XhoI* (TaKaRa) and ligated into the pcDNA3.1(+) expression vector (Invitrogen) using T4 DNA ligase (TaKaRa). The resulting recombinant plasmid, confirmed by DNA sequencing, was designated as pcDNA3.1/STC1. The extraction of endotoxin-free plasmid DNA from an overnight culture of *E. coli* DH5α (Invitrogen) harboring pcDNA3.1/STC1 was performed using a commercial kit (D6926-03, Omega, Doraville, USA).

Design of Annealed Oligonucleotides of Small Hairpin RNA (shRNA)

The shRNA oligonucleotide duplexes targeting bovine STC1 were designed online (Invitrogen Block-iT RNAi Designer) and synthesized by Huayu Gene Co., Ltd (Wuhan, China), the sequences were as follows: 5′-ccgg *ggatgtacgacatctgtaaat* ctcgag *atttacagatgtcgtacatcc* ttttg-3′ (Forward oligo) and 5′-aattcaaaaa *ggatgtacgacatctgtaaat* ctcgag *atttacagatgtcgtacatcc*-3′ (Reverse oligo). A functional non-targeted shRNA sequence (Addgene plasmid#1864, a gift from David Sabatini Lab) used as the negative control.

Table 1. *The primer sequences used in this study for gene clone and real-time qPCR analysis*

Gene	Genebank Accession No.	Primer (5'-3')	Product Length (bp)	Annealing Temperature (°C)
STC1 (for gene clone)	NM_176669.3	**GGATCC**CTCAGAGAATGCTCCAAAACTCA	744	60°C
		CTCGAGCTCCCCAGCTAGGCACT		
STC1(for qPCR)	NM_176669.3	GCTTCTGGTGCTGGTGAT	211	56°C
		GAAGGATTTACAGATGTCGTAC		
TRPV5	XM_010804626.3	GATTCGCCTCAGCGTTCT	148	56°C
		GGCAAGTCCACATCGTAGTT		
TRPV6	NM_001206189.1	CAATGAAACTGACCCCCG	195	56°C
		CCGAGTATGGTCTGTCCGA		
Npt2a	NM_001103223.1	AACGCCATCCTGTCCAAT	122	56°C
		AGAAGAGACCATGCTGACC		
Npt2c	XM_024999684.1	GTCATCAACGCCGACTTC	272	57°C
		AAGTGGATGAGAGCGACCT		
VDR	NM_001167932.2	ACAGTGAGGACGAGGGGAA	110	59°C
		CATTGTGTCTGGAGAGGAGGT		
NCX1	NM_176632.2	CTTAGATGGAGCCCTGGTT	191	56°C
		GAATACGGTAAAACGCTCG		
PMCA1	NM_174696.2	ATAGAACAGTGGCTATGGTCAA	152	56°C
		TCCGCTAACTCCTCCTCG		
CB-D_{28k}	NM_001076195.1	ACGGAAGTGGTTACCTGGA	88	56°C
		GATAACTCCAAACCAGCCTT		
GAPDH	NM_001034034.2	CACTCACTCTTCTACCTTCG	109	56°C
		CACCACCCTGTTGCTGT		

STC1, stanniocalcin-1; TRPV5 and 6, transient receptor potential vanilloid receptor subtype 5 and 6; Npt2a, sodium-dependent phosphate co-transporter 2b; Npt2c, sodium-dependent phosphate co-transporter 2c; VDR, vitamin D receptor; NCX1, sodium/calcium exchanger 1; PMCA1, plasma membraneCa^{2+}-ATPase 1; CB-D_{28k}, calbindin-D_{28K}; GAPDH, glyceraldehyde-3-phosphate dehydrogenase

The yielded double-stranded oligonucleotides were cloned into pLVX-Puro vector (TaKaRa) between the *Eco*RI and *Age*I (TaKaRa) restriction sites. The recombinant vectors designated STC1-shRNA and scrambled shRNA after sequencing, respectively.

Transfection and Treatment of RTECs

RTECs were detached using 0.25% trypsin (Gibco), and then seeded in a 6-well plate at a density of 2 x 10^5 cells/well. Cells were incubated in growth medium until 60%-70% confluence. After rinsing thoroughly with sterile PBS, lipofectamine™ 3000 (Invitrogen) was employed to transfect the cell with pcDNA3.1/STC1 (2.5 μg/well) or an equivalent amount of pcDNA3.1(+), STC1-shRNA, scrambled shRNA, or pLVX-puro plasmids. After a 48-h incubation in growth medium, total RNA, protein, and cells were harvested for further analysis.

To investigate whether STC1 regulates Ca^{2+} absorption through the 1,25$(OH)_2D_3$/VDR axis, approximately 2 x 10^5 RTECs were seeded in 60 mm plates, cultured in DMEM/F12 supplemented with 10% FBS, and treated with either vehicle (0.1% ethanol) or 200 nM calcitriol (HY-10002, MedChemExpress, Monmouth Junction,

Table 2. *The details of antibodies used in this study for western blotting*

Antibody Name	Host	Manufacturer	Art. No.	Dilution Ratio	Theoretical MW
STC1	Rabbit	Novus Biologicals, Littleton, USA	NBP1-59310	1:1,000	~28 kDa
TRPV5	Rabbit	Bioss, Beijing, China	bs-8534R	1:600	~90 kD
TRPV6	Rabbit	Bioss	bs-15506R	1:600	~67 kD
Npt2a	Rabbit	Novus Biologicals	NBP2-85748	1:1,000	~69 kD
Npt2c	Rabbit	Bioss	bs-20801	1:400	~63 kD
VDR	Rabbit	ABclonal, Woburn, USA	A2194	1:1,000	~60 kD
NCX1	Rabbit	Bioss	bs-1550R	1:400	~106 kD
PMCA1b	Rabbit	Bioss	bs-4978R	1:600	~138 kD
CB-D_{28k}	Rabbit	Bioss	bs-3758R	1:600	~29 kD
GAPDH	Rabbit	Bioswamp, Wuhan, China	AB36269	1:5.000	~36 kD
Anti-rabbit IgG-HRP	Goat	Servicebio, Wuhan	SAB43714	1:10.000	

STC1, stanniocalcin-1; TRPV5 and 6, transient receptor potential vanilloid receptor subtype 5 and 6; Npt2a, sodium-dependent phosphate co-transporter 2b; Npt2c, sodium-dependent phosphate co-transporter 2c; VDR, vitamin D_3 receptor; NCX1, sodium/calcium exchanger 1; PMCA1, plasma membrane Ca^{2+}-ATPase 1; CB-D_{28k}, calbindin-D_{28K}; GAPDH, glyceraldehyde-3-phosphate dehydrogenase

USA) for 48 h. The cells were then transfected with 4 µg pcDNA3.1(+) or pcDNA3.1/STC1, followed by incubation in growth medium containing 0.1% ethanol or 200 nM calcitriol for an additional 48 h. Two additional groups of cells were treated with 250 nM MeTC7 solubilized in dimethylsulfoxide for 12 h following a 48-h exposure to either 0.1% ethanol or 200 nM calcitriol [16, 17]. A separate group of cells was treated with 200 nM calcitriol alone for 60 h as a positive control. Subsequently, cells and proteins were harvested for further experiments.

Measurement of Intracellular Ca^{2+} Concentration

RTECs from each group were detached and collected by centrifugation. After three washes with PBS, cells were resuspended with 0.5 µM Fluo-3/AM (S1056, Beyotime, Shanghai, China) in PBS at 37°C for 30 min. Following two additional washes with PBS, the cells were then incubated in PBS at 37°C for 20 min. And then, the number of cells with fluorescence intensity above the baseline (positive cells) was measured using the NovoCyte™ flow cytometer (ACEA Bio, San Diego, USA) with a 1-min recording. NovoExpress software (ACEA) was used to collect and calculate the original data.

Measurement of Intracellular Pi Concentration

After treatments, about 5×10^6 cells from each group were detached and centrifuged at 1.200 × *g* for 5 min. The resulting pellets (20 µL of packed cells) were resuspended in 180 µL ddH_2O (about 10% cytocrit) and sonicated for 30 sec with 50% pulses. Following a 15-min centrifugation at 14.000 × *g*, the supernatants were collected and their Pi contents were measured using a phosphomolybdic acid kit (C006-1-1, Jiancheng, Nanjing, China). Briefly, 0.1 mL supernatant from the previous step was mixed with 0.4 mL of precipitant included in the kit, then spun at 2.200 x *g* for 10 min and the supernatant was sampled for testing. A 0.5 mM phosphorus served as standard solution. Then, 0.2 mL of sample, standard solution, and ddH_2O (blank control) were added to different test tubes. Afterward, 2 mL of a reagent mixture containing ammonium molybdate, antimony potassium tartrate, and ascorbic acid was added to each tube. Optical density (OD) values were measured at 660 nm and normalized using ddH_2O. The phosphate concentration in each tube was calculated using the formula:

$$\text{Phosphate concentration (mM)} = \frac{\text{OD(Test sample)} - \text{OD(Blank control)}}{\text{OD(Standard solution)} - \text{OD(Blank control)}} \times 0.5\ \text{mM} \times 5.$$

Real-time qPCR

The real-time qPCR reactions were performed on the CFX96 qPCR system (Bio-Rad, Hercules, USA), each reaction containing 1.0 µL cDNA, 10 µL SYBR Green qPCR Mix (KK4600, Kapa Biosystems, Wilmington, USA), 0.2 µM forward and reverse primers (Huayu gene) for each gene (*Table 1*), and DNase/RNase-free H_2O to reach a final volume of 20 µL. After a 5-min pre-denaturation at 94°C, the reactions proceeded with 40 cycles of amplification. Each cycle consists of a 5-sec denaturation at 94°C, a 10-sec annealing at 56°C-59°C (*Table 1*), and a 25-sec elongation at 72°C. A final extension step was performed at 72°C for 5 min. The data were normalized to that of *GAPDH* (data not shown).

Western Blotting

The ice-cold lysis buffer (P0013B, Beyotime) was used to prepare cell lysates. After centrifugation at 14.000 x *g* for 15 min, the supernatants were collected and standardized to 20 µg of protein per sample, then subjected to electrophoresis on a 12% SDS/PAGE gel. The separated proteins were transferred to PVDF membrane (Millipore, Bedford, USA). Following a 1-h blocking with

5% (w/v) non-fat milk, the membranes were incubated overnight with diluted rabbit polyclonal antibodies (as listed in *Table 2*). Immunoreactive bands were visualized by enhanced chemiluminescence substrate. Western blot band intensities were quantified with densitometry using an automatic analyzer (Tanon-5200, Shanghai, China).

Statistical Analysis

Data from triplicate or quadruplicate samples were subjected to one-way ANOVA using GraphPad Prism 9 software (San Diego, USA). All parameter values are expressed as the mean ± SEM. Statistical significance was defined as a P value <0.05.

Result

Microscopic Observations of Primary Cell Culture and Expression of STC1 in Transfected RTECs

After a 24-h incubation, microscopic observation showed digestion with collagenase I for 20 min with shaking yielded higher quality organoids and releasing abundant individual cells with a cobblestone-like morphology from disaggregated tubular fragments *(Fig. 1-A)*. Following a series of purification steps, the cells evenly dispersed on the T25 flasks, displaying polygonal or cuboidal shapes with distinct borders *(Fig. 1-B)*, and either dome-like or flattened appearances under phase-contrast microscopy *(Fig. 1-C)*. The morphological characteristics of the primary cultures closely resembled those of MDBK cells, which are typical epithelial cells *(Fig. 1-D)*. Moreover, both the primary cultures and MDBK cells exhibited obvious immunoreactivity for PCK *(Fig. 1-E2,F2)* and CK18 *(Fig. 1-E3,F3)* but not for vimentin *(Fig. 2-E4,F4)* or PBS *(Fig. 2-E1,F1)*, suggesting that the primary cultures predominantly consist of RTECs.

As *Fig. 1-G* shows the pcDNA3.1/STC1 plasmid significantly increased STC1 mRNA expression over 90-fold (***P<0.001) compared to control (normal cells grown in the same medium containing an equivalent volume of lipofectamine reagent) and empty vector groups, while STC1-shRNA effectively inhibited it (*P=0.0498 vs control), with no significant changes in the empty vector or scrambled shRNA groups (P>0.05). The western blotting *(Fig. 1-H,I)* further confirmed the successful up- and down-regulation of STC1 expression by pcDNA3.1/STC1 and STC1-shRNA, respectively.

Changes in the Intracellular Free Ca^{2+}/Pi Concentrations

As shown in *Fig. 2-A* and *2-B*, low STC1 expression group induced by STC1-shRNA transfection possessed higher levels of positive cells (*P=0.0189 vs control, *P=0.0123 vs scrambled shRNA group), while cells with high expression of STC1 via pcDNA3.1/STC1 transfection exhibited about half the positive signals compared to those observed in the control group (*P=0.0338) and the pcDNA3.1(+) transfection group (*P=0.0160), suggesting downregulation of STC1 expression augments cellular absorption of Ca^{2+}, while upregulation inhibits it.

In addition, cells with low STC1 expression had a lower Pi concentration in the diluted intracellular fluid (***P<0.001, ~ 2.11 mM) compared to the control and scrambled shRNA transfection groups (2.51 mM and 2.57 mM, respectively). In contrast, STC1 overexpression led to a higher Pi concentration in the cells (***P<0.001, ~ 3.51 mM). A relatively higher Pi concentration in the diluted

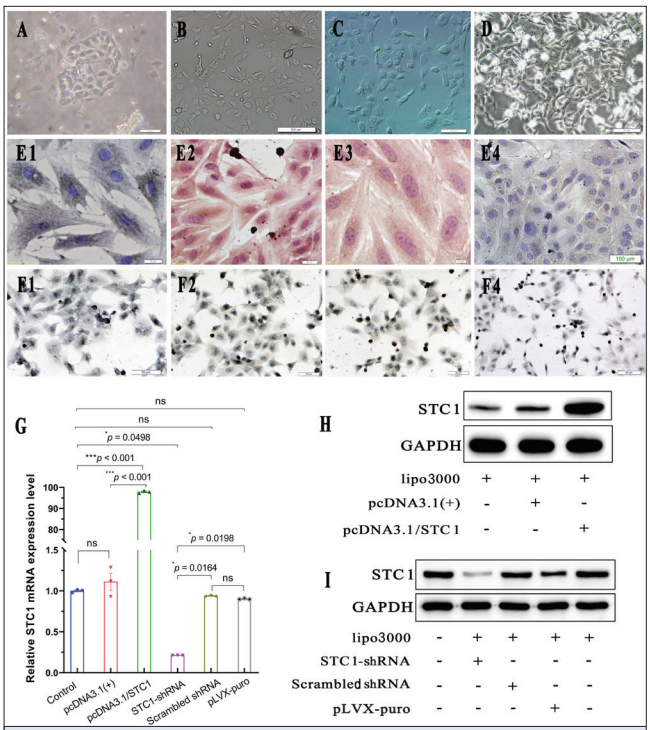

Fig 1. Characterization of morphology and immunocytochemistry in primary cultures, and expressions of STC1 in cells after transfection for 48 h. (A) Individual renal tubule epithelial cells displaying a cobblestone-like morphology, originating from adherent organoid structures in the primary cultures, scale bars=200 μm. (B) Following a series of purification steps, the cells evenly dispersed on the T25 flasks exhibiting an epithelioid cell morphology, scale bars =500μm. (C) The individual epithelial cell exhibited a dome-like, rounded morphology when observed under a phase contrast microscope, scale bars=200 μm. (D) Normal growth of MDBK cells displaying typical epithelioid cell morphology served as the positive control, scale bars=200 μm. (E1) - (E4) The cells in primary cultures stained with antibodies against PBS (as the negative control), PCK, CK18, or vimentin, respectively, scale bars=20 μm (E1), 50 μm (E2), 10 μm (E3), 100 μm (E4). (F1) - (F4) MDBK cells stained with antibodies against PBS, PCK, CK18, or vimentin, respectively, scale bars=50 μm (F1- F4). (G) The mRNA expressions of STC1 in RTECs analyzed by real-time qPCR. Each column represents a triplicate experiment (***P<0.001, **P<0.01, *P<0.05, stand for significant difference; ns stand for P>0.05, i.e., non-significant difference). (H) STC1 protein overexpression was assessed by western blotting at 48 h after transfection with pCDNA3.1(+) or pCDNA3.1/STC1. (I) Western blotting analysis to confirm the inhibition of STC1 protein expression in RTECs at 48 h post-transfection with STC1-shRNA, compared with the negative control (scrambled shRNA) and empty vector (pLVX-puro) transfected cells

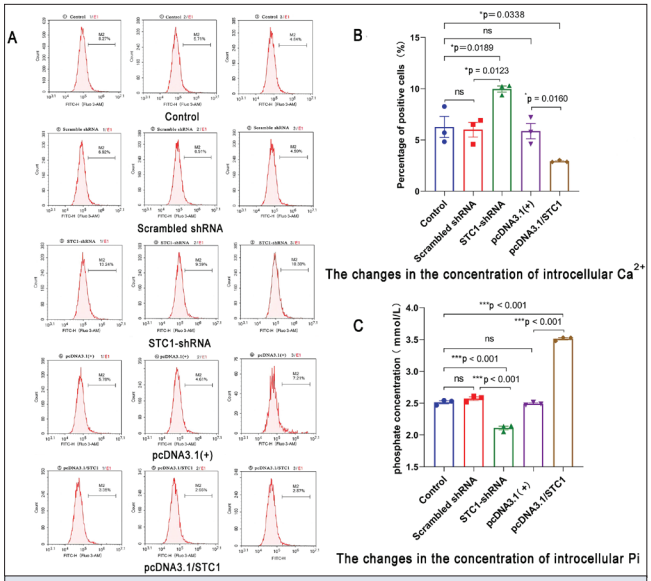

Fig 2. Changes in the intracellular free Ca^{2+} and Pi concentrations in RTECs. (A) Fluorescence histograms in the FITC-H channel from Fluo-3/AM-stained (positive for FITC-H) cells were analyzed by flow cytometry. The number of cells is displayed on the y axis and expressed as a percentage of M2, while the fluorescence intensity is shown on the x-axis. Each histogram is representative of a triplicate experiment. (B) Bar chart shows the percentages of Fluo-3/AM-positive cells, as calculated by flow cytometry. (C) Bar chart displays the intracellular Pi content in different treatment groups measured by the reduced phosphomolybdate photometric method. Note: Inhibition of STC1 by STC1-shRNA significantly increased Ca^{2+} intake (*P=0.0123) and markedly reduced Pi influx (***P<0.001), whereas STC1 overexpression via pcDNA3.1/STC1 resulted in opposite effects. Each column represents a triplicate experiment (***P<0.001, **P<0.01, *P<0.05, stand for significant difference; ns stand for P>0.05).

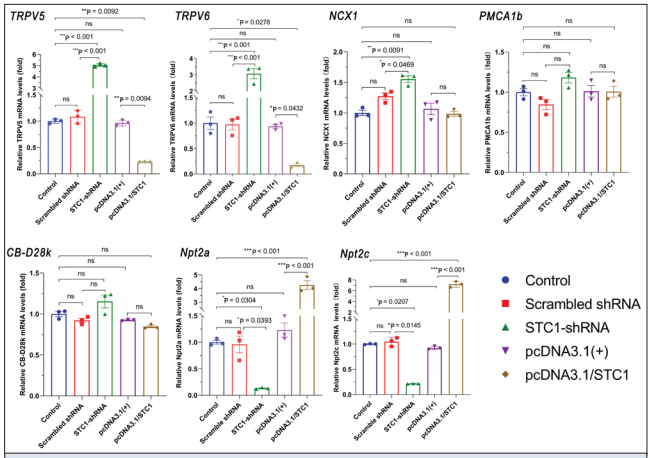

Fig 3. Bar chart showing the mRNA levels of Ca^{2+} and Pi transport proteins in RTECs cells were assessed by real-time qPCR after various treatments. Significant increases were observed in the expression levels of TRPV5, TRPV6, and NCX1 mRNA, and the levels of Npt2a and Npt2c were markedly inhibited, after 48 h transfection with STC1-shRNA. The mRNA expression levels of PMCA1b and CB-D_{28k} showed no significant changes after the inhibition of STC1. By contrast, following the overexpression of STC1 via transfection with pcDNA3.1/STC1 for 48 h, a notable decrease in the expression of TRPV5 and TRPV6 mRNA was observed, alongside a significant increase in the mRNA expression of Npt2a and Npt2c. The mRNA expression levels were corrected by *GAPDH* expression. Each column represents a triplicate experiment (***P<0.001, **P<0.01, *P<0.05, stand for significant difference; ns stand for P>0.05)

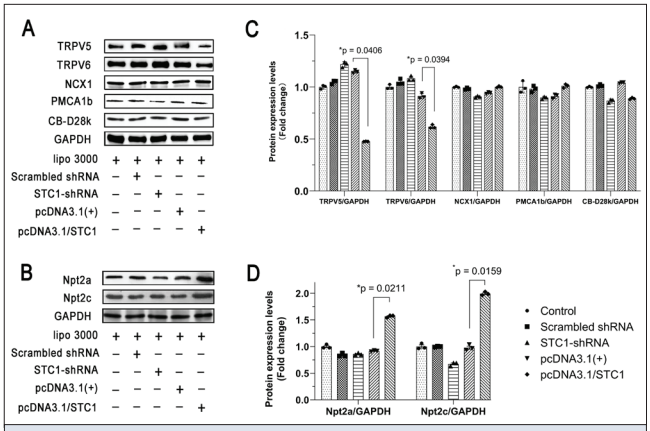

Fig 4. The protein expression levels of Ca^{2+} and Pi transport proteins in RTECs cells, as analyzed by western blotting after various treatments. (A) and (B) Specific protein bands of transcellular Ca^{2+} and Pi transport proteins. The results illustrated that the protein expression levels of TRPV5 and TRPV6 decreased significantly, and the expression levels of Npt2a and Npt2c promoted markedly, following transfection with pcDNA3.1/STC1, whereas those of NCX1, PMCA1b, and CB-D_{28k} appeared unchanged. (C) and (D) Bar charts show the densitometric analysis of the specific bands of the proteins listed in A and B, normalized to GAPDH and relative to the control group. Data are plotted as the mean ± SEM of three separate experiments, *P<0.05 *versus* the control, as determined by one-way ANOVA. The columns without marks represent a P>0.05

intracellular fluid indicates more Pi has been transported into cells, suggesting STC1 overexpression may stimulate Pi uptake by cells *(Fig. 2-C)*.

Effect of STC1 on the Expression of Ca^{2+} and Pi Transporters

After 48h transfection, real-time qPCR analysis *(Fig. 3)* showed significant increases in the mRNA expression of TRPV5 (***P<0.001), TRPV6 (***P<0.001), and NCX1 (**P=0.0091), and significant decreases in Npt2a (*P=0.0304) and Npt2c (*P=0.0207) mRNA expression, as observed in cells transfected with STC1-shRNA relative to control cells. By contrast, STC1 overexpression significantly inhibited TRPV5 (**P=0.0092) and TRPV6 (*P=0.0278) mRNA expressions, and markedly enhanced Npt2a (***P<0.001) and Npt2c (***P<0.001) mRNA expressions. However, PMCA1b, and CB-D_{28k} mRNA expressions appeared to be unaffected by the changes of STC1 expression (p>0.05).

Western blotting analysis *(Fig. 4-A,C)* further validates that STC1 overexpression significantly decreased the protein levels of TRPV5 and TRPV6, but not NCX1, PMCA1b or CB-D_{28k}. Conversely, it increased Npt2a and Npt2c levels *(Fig. 4-B,D)*. Inhibition of STC1 had no significant effect on these transporters. The densitometric analysis confirmed these findings *(Fig. 4-C,D)*.

The Interaction of STC1 with the $1,25(OH)_2D_3$/VDR Axis

After transfecting the cells with various plasmids and vectors, the results showed that STC1-shRNA transfection

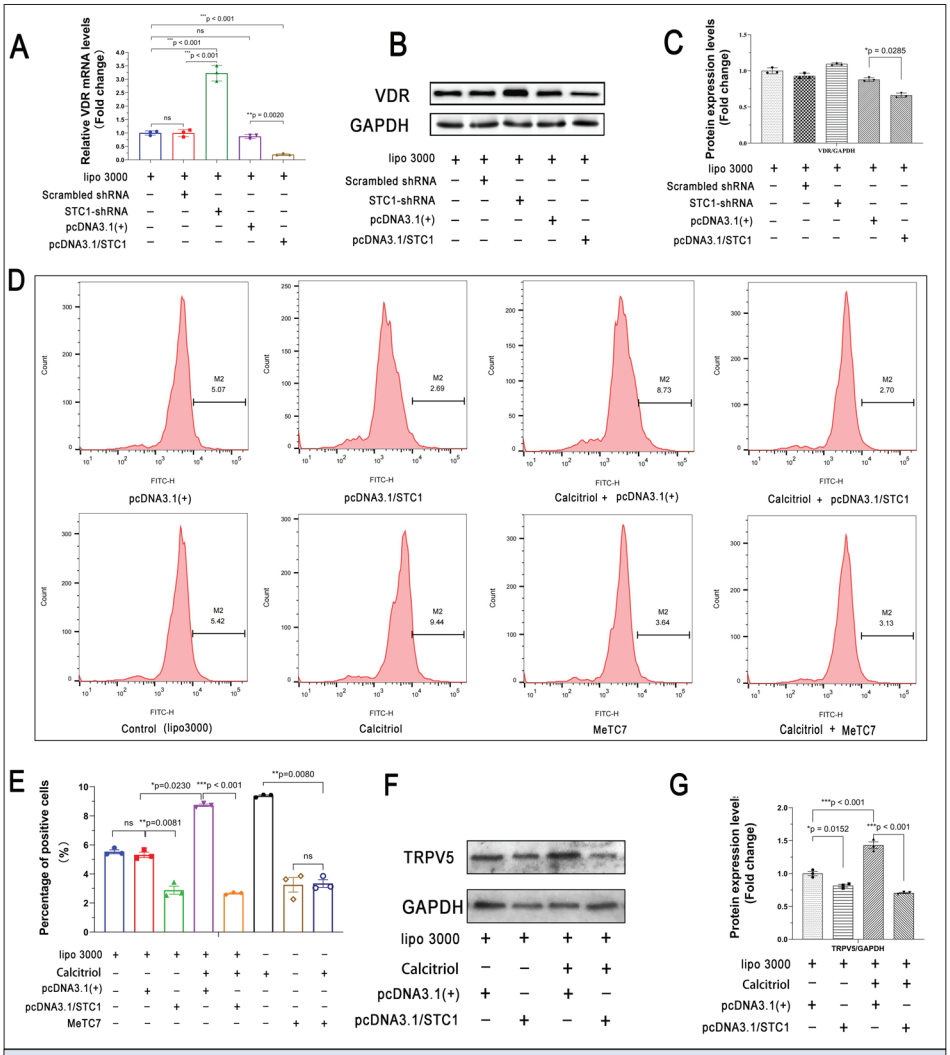

Fig 5. The interaction of STC1 with the $1,25(OH)_2D_3$/VDR axis was determined by real-time qPCR, western blotting, and flow cytometry in the RTECs. (A) Real-time qPCR analysis of VDR mRNA expressions. Transfection with STC1-shRNA for 48 h significantly enhanced VDR mRNA expression (***P<0.001), while transfection with the recombinant plasmid pcDNA3.1/STC1 notably inhibited its expression (**P=0.0020). (B) Western blotting analysis of VDR protein expression. (C) Densitometric analysis of the VDR protein bands in (B), normalized to GAPDH and expressed relative to the control group. The findings revealed that a 48-h transfection with pcDNA3.1/STC1 significantly suppressed VDR protein expression (*P=0.0285 vs. empty vector transfection group), while STC1-shRNA transfection had no significant effect on its expression (P>0.05). (D) The changes of $[Ca^{2+}]_i$) in RTECs were visualized using Fluo-3/AM staining and analyzed by flow cytometry. The cells were treated with 1% alcohol (control) or 200 nM calcitriol and then transfected with pcDNA3.1(+) or pcDNA3.1/STC1 as indicated. The results demonstrated that transfection with pcDNA3.1/STC1 significantly decreased the $[Ca^{2+}]_i$ in both untreated cells (**P=0.0081) and cells subjected to 200 nM calcitriol treatment (***P<0.001). Additionally, treatment with 250 nM MeTC7 for 12 h also significantly reduced the elevated $[Ca^{2+}]_i$ induced by 48-h calcitriol treatment (**P=0.0080). Each histogram represents the median of data from a single experiment (n=3) (F) and (G) TRPV5 expression in RTECs following 200 nM calcitriol treatment and plasmid transfection, as detected by western blotting and densitometry. The results indicated with transfection of the empty vector, 200 nM calcitriol increased TRPV5 expression following 2 days of treatment (***P<0.001). However, delivery of pcDNA3.1/STC1 to RTECs markedly weakened TRPV5 expression compared with pcDNA3.1(+) empty vector, even under 200 nM calcitriol treatment (***P<0.001)

significantly enhanced VDR mRNA expression (***P<0.001, *Fig. 5-A*). However, it did not significantly promote VDR protein expression (P>0.05, *Fig. 5-B,C*). In contrast, transfection with pcDNA3.1/STC1 remarkably suppressed both VDR mRNA (**P=0.0020, *Fig. 5-A*) and protein expression (**P=0.00285, *Fig. 5-B,C*).

To further investigate whether STC1 regulates cellular mineral absorption via $1,25(OH)_2D_3$/VDR axis, we enforced the expression of STC1 or treated cells with the VDR antagonist MeTC7 for 12 h in primary RTECs pre-treated with the VDR agonist calcitriol for 48 h. Flow cytometry results *(Fig. 5-D,E)* showed that transfection

with the control vector pcDNA3.1(+) had no influence on calcitriol-induced Ca^{2+} absorption enhancement (*P=0.0230, compared with the group transfected with the control vector only). However, overexpression of STC1, achieved via pcDNA3.1/STC1 transfection, significantly reduced the rate of detectable positive cells from an average of 8.87% (induced by 200 nM calcitriol) to ~2.67% (***P<0.001, compared with the calcitriol-treated and control vector-transfected group). In addition, after 48 h of calcitriol treatment followed by MeTC7 treatment, the detectable positive cell rate was notably reduced to an average of 3.34% from 9.39% (**P=0.0080, compared with the only calcitriol-treated group), achieving the same effect as STC1 overexpression.

To complement the above findings, the regulation of TRPV5 expression by STC1 and calcitriol was further investigated. Pre-treatment with pcDNA3.1/STC1, without calcitriol, evidently inhibited TRPV5 protein expression (*P=0.0152 vs. control vector-transfected group). Exposure of the cells to 200 nM calcitriol for 48 h followed by a transfection with the control vector up-regulated TRPV5 protein expression (***P<0.001 vs. control vector-transfected group). However, this trend was significantly reversed (***P<0.001 vs. calcitriol + pcDNA3.1/STC1 group) when cells were co-transfected with pcDNA3.1/STC1 *(Fig. 5-F, G)*.

Discussion

In dairy cattle, maintaining proper Ca^{2+}/Pi balance is vital for optimal milk production and preventing conditions like hypocalcemia, which is a common issue in post-partum cows. Elucidating the hormonal mechanisms governing Ca^{2+}/Pi regulation in dairy cows is essential for their homeostatic maintenance. Understanding these mechanisms can lead to better management practices and enhanced animal care in the dairy industry. STC1, a mineral-regulating hormone first identified in fish, is believed to play a crucial role in mammals as well.

Given the fact that numerous reports have indicated that STC1 appears to have a minor role in systemic Ca^{2+}/Pi homeostasis in mammals, it nevertheless exerts a crucial regulatory role in cellular-level Ca^{2+}/Pi transport [15]. However, the mechanism by which STC1 influences Ca^{2+}/Pi transport remains poorly understood. Our previous work demonstrated that STC1 upregulation in Caco2 cells suppressed the expression of TRPV5 and especially TRPV6 [18], and short-term exposure to low Pi and high Ca^{2+} concentration could stimulate the expression of STC-1 in RTECs, whereas its expression could be suppressed by prolonged exposure to either low or high concentrations of both Pi and Ca^{2+} (Data that has not been published in international journals). However, these data failed to reveal the interplay among STC1, channels, VDR, and Ca^{2+} absorption. In this study, we found that Ca^{2+} uptake decreased significantly after STC1 overexpression, while inhibiting STC1 had the corresponding opposite effect, suggesting that STC1 inhibits Ca^{2+} transport in bovine cells just similar to its function in intestinal tracts of swine and rats [19]. Our current research also demonstrates that STC1 influences cellular Ca^{2+}/Pi homeostasis, potentially by regulating the expression of TRPV5/6 channels, as suggested by our previous findings. These data further confirmed that ion transporters are the target of STC1, either directly or indirectly, regardless of the cell type, i.e., renal cells, intestinal cells [18], cardiomyocytes [20], zebrafish *(Danio rerio)* embryo cells [21], or gill cells [22]. Intriguingly, the downregulation of STC1 led to a notable upregulation of TRPV5/6 mRNA expression, whereas the corresponding protein levels remained unchanged. It is possible that the function of STC1 in mammalian mineral homeostasis regulation is minor or may have been compensated by alternative mechanisms or factors.

The present work found that both the mRNA and protein levels of CB-D_{28k} and PMCA1b were relatively unaffected by STC1 changes. Interestingly, the changes in STC1 expression only affect the expression of NCX1 mRNA markedly, not the protein, this was potentially associated with the fact that NCX1 serves as the primary extrusion mechanism in renal cells, where the role of PMCA1b may be of less importance [23]. Combining the previous findings [18,21], this finding further confirmed that STC1 does not influence ATP-driven Ca^{2+} efflux and Na^{+}/Ca^{2+} exchange, or cytosolic diffusion.

This study showed VDR protein levels in RTECs were markedly influenced by altering STC1 expression, specifically by STC1 overexpression. This suggests that, at least in RTECs, modulating the function of $1,25(OH)_2D_3$/VDR axis, a crucial pathway for calcium absorption [24], is a potential mechanism by which STC1 regulates the Ca^{2+} transcellular transport. Additionally, this work found that STC1 overexpression could enhance Pi absorption in RTECs, similar findings were observed in the duodenum of porcine and rats [19], as well as in the proximal tubules of fish [25]. Our findings further revealed that the enhancement of Pi absorption in RTECs was triggered by STC1 overexpression, probably achieved by upregulating the expression of Npt2a/2c, the channels mediating Pi entry.

This study supports the hypothesis that STC1 plays a conserved role in preventing Ca^{2+} transport and promoting Pi reabsorption in RTECs, specifically, were achieved by regulating Ca^{2+}/Pi influx processes mainly via impacting TRPV5/6 and Npt2a/2c expressions, respectively. STC1 appeared to have little significant effect on the Ca^{2+}/Pi intracellular diffusion and extrusion. Interestingly, the increase/inhibition in Ca^{2+}/Pi influx induced by the downregulation of STC1 seemed to be uncoupled to these channels, its downregulation possibly promotes

other mechanisms mediating Ca^{2+}/Pi absorption, which will be the focus of our future research. Given the fact that the broad distribution, diverse functions, minimal effects on body growth and development in $STC1^{(-/-)}$ mice [15,26], and the contradictory functions in multiple cancer cells [27], indicating a need for in-depth investigation into the molecular mechanisms of STC1 to determine the commonality of its effects.

This research can contribute to the development of better feeding strategies, enhanced disease prevention, and improved animal care, making it essential for both agricultural and biomedical fields. Additionally, understanding bovine Ca^{2+}/Pi regulation offers valuable insights into human health, particularly in the context of bone diseases and mineral metabolism.

Declarations

Availability of Data and Materials: The authors declare that the data and materials are available on request from the corresponding author (L. W).

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Research Article

Comparison of Virulence, Resistance Genes, and SCCmec Types in CoNS and *Staphylococcus aureus* Strains Isolated from Raw Cow Milk Samples

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Abstract

Although there has been extensive research on the presence of *Staphylococcus aureus (S. aureus)* in raw milk samples, new data on the prevalence and molecular characterization of *Coagulase-negative Staphylococci (CoNS)* are needed. This study aimed to compare the antimicrobial profiles, virulence genes, and *SCCmec* types distribution between *S. aureus* and *CoNS* isolated from one hundred and fifty raw cow milk samples in Istanbul. *Staphylococcus* isolates were identified using VITEK MS following classical culture methods. Phenotypic antibiotic susceptibility was determined using the disc diffusion method. In-house PCR was employed to detect resistance genes, while multiplex PCR and qPCR were utilized for *SCCmec* typing and virulence genes such as *SEs*, respectively. Out of the seventy-five contaminated samples (50%), 32% harbored *S. aureus*, and 68% were *CoNS*. Methicillin resistance was identified in 10.6% of *S. aureus* and 14.6% of *CoNS*. *SCCmec* type IV predominated in both MRSA (50%) and MRCoNS (54.5%). At least one toxin gene was present in 83.3% of *S. aureus* and 22.4% of *CoNS* isolates, with *sei* being the most frequently observed. None of the *S. aureus* isolates tested positive for the *sed, see,* and *pvl* genes. Similarly, the *sea, sed, see, tsst-1, and pvl* genes were not detected in any of the *CoNS* isolates. As a conclusion, *SCCmec* type IV and *sei* gene predominated and community-acquired resistance patterns were prominent in *Staphylococcus* strains, which carried various virulence and resistance genes. Beyond MRSA, the presence of MRCoNS should be considered and monitored as a significant public health concern in raw milk.

Keywords: CoNS, Raw milk, SCCmec types, *Staphylococcus aureus*, Virulence genes

Introduction

Staphylococcus aureus is a highly proficient opportunistic pathogen, implicated in various infectious diseases, ranging from minor skin and soft tissue infections to septicemia and toxic shock syndrome. Moreover, among the diverse diseases caused by *S. aureus*, staphylococcal food poisoning (SFP), a foodborne intoxication, results from the consumption of food contaminated with sufficient amounts of staphylococcal enterotoxins (*SEs*), primarily produced by specific *S. aureus* strains [1].

Coagulase-negative staphylococci *(CoNS)* play the role of opportunistic nosocomial pathogens, often linked to infections associated with foreign bodies and catheters, as well as conditions such as urinary tract infections and endocarditis, among others [2].

S. aureus exhibits numerous virulence factors and a notable capacity to develop resistance to a broad spectrum of antibiotics. Methicillin resistance is particularly significant within *Staphylococcus* spp., especially in *S. aureus*. The methicillin resistance determinant, mecA, is an integral part of a mobile genetic element known as *SCCmec*. Although mecA is not exclusive to *S. aureus*, its presence has been documented in other Staphylococcal species originating from both human and animal sources. In addition to *S. aureus* and *S. sciuri*, reports indicate the occurrence of mecA in methicillin-resistant strains of *S. pseudintermedius, S. intermedius, S. vitulinus, S. epidermidis, S. haemolyticus, and S. saprophyticus* [3].

Methicillin resistance in *Staphylococci*, especially in *S. aureus* (MRSA), has a global presence, adapting to diverse environmental conditions and modulating its pathogenicity. The prevalence and epidemiology of MRSA, in particular, are continually escalating, attributed to the emergence of new MRSA clones in various geographical regions. Therefore, it is crucial to bear in mind that MRSA can continuously acquire new features and should be closely monitored [3,4].

Monitoring methicillin resistance in food is vital due to the ability of these bacteria to disseminate multiple antimicrobial resistance genes, transforming it into a significant public health concern [5]. While many studies on raw milk have focused on *S. aureus*, especially MRSA, several studies have started to emphasize the importance of CoNS [2,6].

This study aims to compare the antimicrobial profiles, virulence genes, and the distribution of *SCCmec* types between *S. aureus* and *CoNS* isolated from raw cow milk in what there is needed new data.

Material and Methods

Ethical Statement

This study does not require ethical permission.

Sampling and Storage

One hundred and fifty raw cow milk samples offered for sale in various districts of İstanbul were randomly collected between September 2018 to February 2020. None of the milk samples were packed. 500 mL of raw milk samples from each sample were collected in sterile containers and transported to the laboratory under appropriate transport conditions. All samples were kept at +4°C in a refrigerator and taken to the laboratory for processing.

Isolation of *S. aureus* and Other *Staphylococcus spp.*

A volume of 10 µL of each sample was performed on blood agar with 5% sheep blood and chocolate agar (Oxoid, Basingstoke, United Kingdom). Plates were examined after 24 h and 48 h. of aerobic incubation at 37°C. Typical *Staphylococcus* spp. colonies were tested. Gram stain and biochemical identification tests such as catalase test, novobiocin sensitivity, and mannitol fermentation were made for identification. The presence of beta hemolysis, clumping factor, tube coagulase activity, and DNase were also investigated [7].

The strains identified as *Staphylococcus* spp. were subcultured on tryptic soy agar and incubated at 37°C for 24-48 h. The VITEK MS (bioMérieux SA, Marcy l'Etoile, France) Matrix-Assisted Laser Desorption Ionization Time-of-Flight (MALDI-TOF) (bioMérieux SA, Marcy l'Etoile, France) system was employed for identification following the manufacturer's instructions. Subsequently, the isolates were individually stored in tryptic soy broth medium with 15% glycerol at −80°C for subsequent phenotypic and genotypic analyses [7].

Phenotypic Characterizations

Oxacillin disc diffusion testing: Methicillin resistance was detected by cefoxitin (30 µg) and oxacillin (1 µg) disks (Oxoid, Basingstoke, United Kingdom) according to the EUCAST criteria [7]. *S. aureus* ATCC 25923 (susceptible) and *S. aureus* ATCC 43300 (resistant) were used as control strains.

Antimicrobial susceptibility testing: Antimicrobial susceptibility testing was performed according to the disc diffusion method and the results were interpreted according to the EUCAST-2016 criteria (EUCAST. Version 6.1, 2016). The antimicrobial susceptibility of *Staphylococcus* was assessed for penicillin G, oxacillin, cefoxitin, erythromycin, gentamicin, amikacin, tetracycline, clindamycin, and vancomycin (Oxoid, Basingstoke, United Kingdom) on Mueller-Hinton agar. *S. aureus* ATCC 29213 served as a quality control strain. Isolates demonstrating resistance to at least three different antimicrobial classes were classified as multidrug-resistant [7].

Genotypic Characterisations

DNA Extraction: The High Pure PCR template preparation kit (Roche Diagnostics GmbH, Mannheim, Germany) was used to perform DNA isolation from the *Staphylococcus* strains according to the manufacturer's instructions.

SCCmec Typing in MRSA and MRCoNS via Multiplex PCR: SCCmec typing was performed via multiplex PCR using specific primer sets (IDT, Coralville, IA, USA) and same protocols used by Kondo et al.[8]. The *SCCmec* typing assignment of the isolates was conducted based on the *ccr* and *mec* gene complexes. Six different multiplex PCR sets were performed on T100 Thermal Cycler (Bio-Rad, Hercules, CA, USA) for *SCCmec* typing of each isolate. The protocol involves six M-PCR assays; M-PCR1 was identified the ccr type by targeting specific genes

within the ccr complex. M-PCR2 was determined the mec class by targeting specific regions within the mecA-mecI complex. M-PCR3 was identified the specific ORFs in the J1 region of type I and type IV SCCmec elements. M-PCR4 was identified the specific ORFs in the J1 region of type II, type III, and type V SCCmec elements. M-PCR5 was identified the J2 region of type II or type III SCCmec elements and the presence of ermA and cadB genes. M-PCR6 was identified the integrated plasmids in the J3 region, specifically targeting mecA, ant(4'), and tetK genes. By combining the results of these six M-PCR assays, can be classify *S. aureus* strains based on their SCCmec type. Except M-PCR6, Reaction mixture was included 10 ng DNA, 0.1 µM primers, 200 µM dNTPs, Taq buffer, 2.5U Taq polymerase, 3.2 mM $MgCl_2$, in 50 µL and PCR condition was denaturation for 94°C, 2 min, and 30 cycles annealing of 94°C at 2 min, 57°C at 1 min, 72°C at 2 min, and then final extension 72°C at 2 min. M-PCR1 annealing temperatures was 57°C at 1 min but M-PCR2 to 5 annealing temperatures was 60°C at 1 min. M-PCR6 was a long-range PCR and reaction mixture was included 10 ng DNA, 0.3 µM primers, 200 µM dNTPs, Expand High Fidelity buffer, 1.5 mM $MgCl_2$, 2.6U enzyme mix (Roche Diagnostics GmbH, Mannheim, Germany), in 50 µL. PCR condition of M-PCR6 was; denaturation (94°C, 2 min), 10 cycles (94°C, 15 s, 50°C, 30 s, 68°C, 8 min), 20 cycles (94°C, 15 s, 50°C, 30 s, 68°C, 12 min), and final extension (72°C, 7 min). Agarose gel electrophoresis was performed on mini-sub cell GT horizontal electrophoresis system (Bio-Rad, Hercules, CA) to analyze PCR products and gels were analysis using a Chemi-Doc MP Imaging System (Bio-Rad, Hercules, CA, USA) [8].

Detection of Antimicrobial Resistance Genes via In-house PCRs

Specific primers were used to determine the genotypical antimicrobial susceptibility via the previously described by Demir et al.[9] and Demirci et al.[7]. Fifteen genes were used to find the genotypical resistance. Oxacilline (mecA); penicillin *(blaZ);* aminoglycoside (gentamicin and amikacin) *(aac(6')-aph(2"), aph(3')-IIIa, ant(4')-Ia);* macrolide (erythromycin) *(ermA, ermB, ermC, and msrA),* tetracycline *(tetK, tetM)* [9] and vancomycin *(vanA, vanB, vanC1, and vanC2-C3)* specific genes were analyzed as described by Demirci et al.[7]. The 16S rDNA gene was used as an amplification control. The PCR amplifications were performed in a total of 25 µL volume on T100 Thermal Cycler (Bio-Rad, Hercules, CA, USA). Each reaction contained 5 µL of template DNA. Agarose gel electrophoresis were performed on mini-sub cell GT horizontal electrophoresis system (Bio-Rad, Hercules, CA) to analyze PCR products and gels were analysis using a Chemi-Doc MP Imaging System (Bio-Rad, Hercules, CA, USA) [9].

Detection of Staphylococcal SEs Genes, tsst -1 Gene, and pvl Gene via qPCR

qPCR was employed to detect virulence genes such as *Staphylococcal Enterotoxins-SEs*, *Panton-Valentine Leukocidin toxin-pvl*, and *toxic shock syndrome toxin 1- tsst-1* using specific primer sets (IDT, Coralville, IA, USA) as previously described by Demirci et al.[7]. The LightCycler 480 Sybr Green Master kit (Roche Diagnostics GmBH, Mannheim, Germany) was utilized with these primers on the LightCycler 480 II instrument (Roche Diagnostics GmBH, Mannheim, Germany) following the manufacturer's instructions.

Positive Controls Included *S. aureus* Strains

ATCC 14458 *(seb),* ATCC 13565 *(sea, sej),* ATCC 19095 *(sec, seh),* ATCC 27664 *(see),* ATCC 23235 *(sed, seg, sei),* ATCC 51650 *(tsst-1)* and, ATCC 25923 *(pvl),* while *S. aureus* ATCC 6538 served as the negative control.

Statistical Analysis

Descriptive statistics were presented with frequency and percentage values. Chi-square analysis was performed for proportional evaluations of *S. aureus* and *CoNS* groups. Bonferroni test was used to determine the groups with differences (a>b). P values less than 0.05 were considered significant in the study. SPSS v25 software (IBM) was used for the statistical analysis.

Results

One hundred and fifty (150) raw milk samples were included in our study. 50% were found to be contaminated with *Staphylococcus* species. *S. aureus* and *CoNS* were found 32% (24/75) and 68% (51/75), respectively *(Table 1).* We found phenotypically eight strains (10.6% - 8 out of 75) and ten strains (13.3% - 10 out of 75) identified as MRSA and *MRCoNS* respectively, but eleven strains of *CoNS* (14.6% - 11 out of 75) carried the *mec*A gene genotypically (One *S. sciuri* strain only carried mecA gene). In addition to *MRSA* and *MRCoNS*, 21.3% (16 out of 75 strains) *MSSA* and 54.6% (41 out of 75 strains) *MSCoNS* were isolated in raw milk samples.

The distribution of *CoNS isolates* was *Staphylococcus chromogenes* 33.3% (17/51), *S. sciuri* 23.5% (12/51), *S. haemolyticus* 13.7% (7/51), *S. saprophyticus* 5.9% (3/51), *S. vitilinus* 5.9% (3/51), *S. warneri* 5.9% (3/51), *S. cohnii*

Table 1. *Microbiological analysis of raw milk samples*

Results	Raw Milk Samples (n: 150)
Negative	75
Positive	75 *(S. aureus – CoNS)* (32% (24/75) – 68% (51/75))

spp. urealyticus 5.9% (3/51), *S. equorum* 3.9% (2/51) and *S. epidermidis* 1.9% (1/51).

Table 2. *The antimicrobial resistance patterns of the Staphylococcus spp. isolated from raw milk samples*

Antibiotics	***S. aureus* (n=24)**	**CoNS (n=51)**	**P****
	n (%)	**n (%)**	
Oxacillin	8 (33.3)[a]	10 (19.6)[b]	0.04*
Cefoxitin	8 (33.3)[a]	10 (19.6)[b]	
Penicillin	11 (45.8)	24 (47.9)	
Gentamicin	5 (20.8)	9 (17.6)	
Amikacin	3 (12.5)[a]	0 (0)[b]	
Tetracycline	8 (33.3)[a]	12 (23.5)[b]	
Erythromycin	5 (20.8)	13 (25.5)	
Clindamycin	4 (16.7)	12 (23.5)	
Multidrug-resistance	5 (20.8)	12 (23.5)	

*** Chi-square test was performed; * Significant difference at the 0.05 level, a>b*

Susceptibility testing was performed using seven different antibiotics for *Staphylococci* isolated from raw milk. The highest phenotypic resistance rate for *S. aureus* was detected in penicillin (45.8%) and tetracycline (33.3%), followed by gentamicin 20.8%, erythromycin 20.8%, and clindamycin 16.7%. The least resistance was observed against amikacin, with a rate of 12.5%. All of the *S. aureus* isolates were susceptible to vancomycin. The highest phenotypic resistance rate for *CoNS* was found at 47.9% for penicillin, 25.5% for erythromycin, and 23.5% for tetracycline, followed by 17.6% for gentamicin. The lowest rate of resistance was found to be against clindamycin 23.5%. All of the *CoNS* isolates were susceptible to amikacin and vancomycin. Methicillin, amikacin, and tetracycline resistance were found to be significantly higher (P<0.05) in *S. aureus* isolates compared to *CoNS* isolates, and similar resistance rates were found in other antibiotics *(Table 2)*.

Distribution of antimicrobial resistance genes; In *S. aureus* isolates were found as *blaZ* 54.2%, *aac(6')-aph(2")* 37.5%, *aph(3')-IIIa* 16.7%, *ant(4')- Ia* 8.3%, *ermA* 4.2%, *ermB* 8.3%, *ermC* 12.5%, *msrA* 20.9%, *tetK* 16.7%, *tetM* 16.7% and *vanA* 0%; In CoNS isolates were found as *blaZ* 47%, aac(6')-aph(2") 21.6 %, *aph(3')-IIIa* 3.9%, *ant(4')- Ia* 1.9%, *ermA* 13.7%, *ermB* 5.9%, *ermC* 29.4%, *msrA* 7.8%, *tetK* 9.8%, *tetM* 19.6% and *vanA* 0%.

Although two *S. aureus* strains carried the *blaZ* gene, phenotypic penicillin resistance was not observed. The *ermC* gene was detected only in *MSSA* isolates. All of the *CoNS* isolates were carrying the *msrA* genes were methicillin-resistant *(MRCoNS)*. The *msrA* gene was

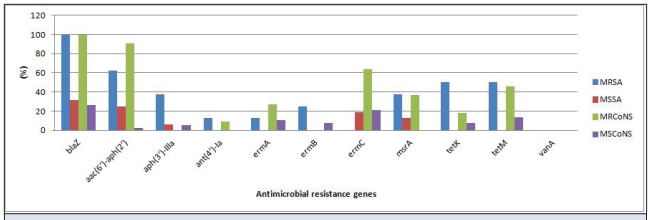

Fig 1. The distrubution of antimicrobial resistance genes between isolates

Table 3. *The distrubution of virulence genes in Staphylococcus spp. isolated from raw milk samples*

Status of Virulence Genes	***S. aureus* (n=24)**	**CoNS (n=51)**	**P****
	n (%)	**n (%)**	
tsst-1	1 (4.8)	0(0)	0,01*
Isolates carrying enterotoxin genome (SEs)	20 (83.3)[a]	11 (22.4)[b]	
Only one group of enterotoxin genome	6 (25.0)[a]	4 (8.2)[b]	
More than one group of enterotoxin genome	13 (54.2)[a]	7 (14.3)[b]	
Isolates non-carrying toxin genome	4 (16.7)[b]	40 (81.2)[a]	

*** Chi-square test was performed.* Significant difference at the 0.05 level, a>b*

significantly higher (P<0.05) in *S. aureus* isolates than in *CoNS* isolates. In addition, the *ermC* gene was significantly higher in *CoNS* isolates compared to *S. aureus* isolates (P<0.05). All phenotypic gentamicin-resistant isolates carried the *aph(3')-IIIa* gene. Although the two strains carried the *tetK* and *tetM* genes, they did not show phenotypic tetracycline resistance *(Fig. 1)*. In our study, a total of 5 (20.8%) isolates in *S. aureus* and a total of 12 (25.3%) isolates in *CoNS* were classified as MDR. *S. sciuri* (28.6%) was the most common species amongst the MDR *CoNS*.

The presence of at least one toxin-gene was found in 83.3% and 22.4% of the isolates for *S. aureus* and *CoNS* respectively. *S. aureus* isolates were carried a significantly higher proportion of toxin genes than *CoNS* (P<0.01) *(Table 3)*.

The sei gene was the most prevalent, and it was identified in 21.3% of all *Staphylococcus* isolates. When the distribution

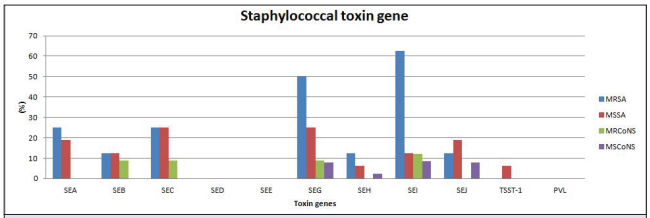

Fig 2. The distrubution of staphylococcal toxin genes between isolates

Table 4. The distrubution of SCCmec type, virulence genes, phenotypical resistance patterns and antimicrobial resistance genes in MRSA (8 strains)

SCCmec	SEs Genes	Phenotypical Resistance	Antimicrobial Resistance Genes
IV	seb+seh+sei	P+M+G+T+E	mecA+blaZ+aac(6')/aph(2") +ant(4')-Ia+ermA+tetK
IV	seg+sei	P+M+A+T+C	mecA+blaZ+aac(6')/aph(2")+msrA+tetK+tetM
IV	sea+seg+sei	P+M+E+T+C	mecA+blaZ+ aac(6')/aph(2")+ermB+msrA+tetK
IV	sea+seg+sei	P+M+A	mecA+blaZ+aac(6')/aph(2")+tetM
III	seg	P+M+T+E+C	mecA+blaZ+aph(3')-IIIa+ermB+tetM
II	ND	P+M+G	mecA+blaZ+aac(6')/aph(2") +ant(4')-Ia+tetK
I	sec+sei	P+M+G+T	mecA+blaZ+aph(3')-IIIa+tetM
NT	sec+sej	P+M	mecA+blaZ+aph(3')-IIIa+msr

P: penicillin; M: methicillin; G: gentamicin; A: amikacin; T: Tetracycline; E: erythromycin; V: vancomycin; MDR: multi drug resistant; NT: Nontypeable; ND: Not Determined SEs gene

Table 5. The distrubution of SCCmec type, virulence genes, phenotypical resistance patterns and antimicrobial resistance genes in MRCoNS (11 strains)

Species	SCCmec	SEs Genes	Phenotypical Resistance	Antimicrobial Resistance Genes
S. chromogenes	IV	seg + sei	P+M+G	mecA + blaz + aac(6')/aph(2")+ermC
S. epidermidis	IV	sei	P+M+G+C	mecA + blaZ + aac(6')/aph(2")+ermC+ tetM
S. haemolyticus	IV	seg +sei	P+M+G+C+T	mecA+blaZ+aac(6')/aph(2")+ermA+msrA+tetK
S. sciuri	IV	ND	P+M+G+E+C	mecA+blaZ+aac(6')/aph(2")+ermC+msrA+tetM
S. sciuri	IV	ND	P	mecA+blaZ+aac(6')/aph(2")
S. warneri	IV	ND	P+M+G+E+T+C	mecA+blaZ+aac(6')/aph(2")+ermC+msrA+tetM
S. chromogenes	II	ND	P+M+G+T	mecA+blaZ+aac(6')/aph(2")+ant(4')-Ia+ermA+tetK
S. haemolyticus	II	sec	P+M+G+T	mecA+blaZ+aac(6')/aph(2")+tetM
S. sciuri	II	ND	P+M+E	mecA+blaZ+ermC+tetK
S. saprophyticus	NT	seb + sej	P+M+G+E+T+C	mecA+blaZ+aac(6')/aph(2")+ermC+msrA+tetM
S. vitilinus	NT	ND	P+M+E+C	mecA+blaZ+aac(6')/aph(2")+ ermA+ermC

P: penicillin; M: methicillin; G: gentamicin; A: amikacin; T: Tetracycline; E: erythromycin; V: vancomycin; MDR: multi drug resistant; NT: Nontypeable; ND: Not Determined SEs gene

of toxin genes in *S. aureus* isolates was examined; the *seg* gene was the most frequent, which was detected in 33.3%, followed by *sei* in 29.2%, *sec* in 25%, *sea* in 20%, *sej* in 16.7%, *seb* in 12.5%, *seh* in 8.3% and *tsst*-1 in 4.6%. The *sed, see,* and *pvl* genes were not detected in any of the *S. aureus* isolates *(Fig. 2).* All MRSA isolates and 75% of MSSA isolates carried the toxin gene (P<0.05) and only one MSSA isolate harbored the *tsst-1* virulence gene. Distribution of toxin genes in CoNS strains; the *sei* gene was the most frequent, which was detected in 18.4% of the *CoNS* isolates, followed by *seg* in 8.2%, *sej* in 6%, *seb* in 2%, *sec* in 2% and, *seh* in 2%. The *sea, sed, see, tsst-1,* and *pvl* genes were not detected in any of the *CoNS* isolates. 45.5% of *MRCoNS* isolates and 18.4% of *MSCoNS* isolates were carried the *SEs* toxin gene (P<0.05).

SCCmec type distribution of *MRSA* isolates in our study; *type IV* (50%), *type I* (12.5%), *type II* (12.5%) and, *type III* (12.5%), *SCCmec type* of one isolate (12.5%) could not be determined (NT). *SCCmec type* distribution of *MRCoNS* isolates were determined as *type IV* (54.5%), and *type II* (27.3%), whereas *SCCmec* type of two isolates (18.2%) could not be determined *(NT). SCCmec type IV* was predominant in both *MRSA* and *MRCoNS* isolates. *SCCmec* type distribution of MRSA and MRCoNS strains are shown in *Table 4* and *Table 5* respectively.

Discussion

Although many studies with raw milk have focused on mainly *S. aureus* (especially MRSA), several studies have begun to highlight the importance of *CoNS* [2,6]. Therefore, in our study, we focused on determining both *S. aureus* and CoNS strains epidemiologically in raw milk samples. The contamination of raw milk by *S. aureus*, particularly harboring multiple antimicrobial resistance genes and carrying virulence genes such as enterotoxin, *tsst-1*, and *pvl*, continues to be a significant public health concern [10-12]. Incidents of outbreaks linked to the consumption of milk and dairy products contaminated with *S. aureus* have been reported in various locations [13]. When reviewing the studies conducted to ascertain the presence of *S. aureus* in

raw milk in Turkiye, the presence of *S. aureus* in the analyzed milk samples were observed to be 61.1% [14], 75% [15], which was quite higher than our results. Whereas, Can et al.[16] detected *S. aureus* in only 12.5% of the milk samples. When examining studies conducted in other countries; It has been reported that *S. aureus* was isolated from raw milk at a rate of 71% in Sweden [17], 46.2% in China [18], and 44% [19] in Egypt. The variation in the frequency of *S. aureus* recovered from milk and its products in different studies may be attributed to differences in sample sizes, origins, and geographic locations. It could reflect the effectiveness of applicable sanitary measures.

CoNS have been traditionally considered only as contaminants or minor pathogens. Nevertheless, their significance has grown as they have become the most frequently isolated group of species from bovine milk in numerous locations [2,19]. In this study, a high diversity of *Staphylococcus* spp among the *CoNS* emerged. Like our result amongst the *CoNS, S. chromogenes* was one of the most prevalent species in previous studies reported ranging between 23.3% and 78.8% of *CoNS* isolates [20-22]. *S. sciuri* is frequently isolated from the skin of humans and animals [22], but in addition to causing bovine mastitis, reported as an opportunistic pathogen [23]. In Australia, *CoNS* was isolated from milk samples, and the dominant strain was reported to be *S. sciuri* [24]. The monitoring of the methicillin resistance trait holds significance for public health and veterinary medicine [17,25,26].

The *mecA* gene conferring methicillin resistance is not only found in *S. aureus* but may be present in other staphylococcal species as well [3]. Limited data are available regarding the prevalence and molecular characterization of *MRCoNS* isolated from food. Some *MRCoNS* are now raising growing concerns in both human and veterinary medicine. A study conducted in Tunisia revealed that 29.4% of *CoNS* isolated from milk exhibited resistance to oxacillin, while 20.6% carried the *mecA* gene [2]. Methicillin resistance among CoNS was comparable to the rates reported in Poland (20%) [27] and China (17.1%) [28]. Khazandi et al.[24] indicated that *S. sciuri* and other *MRCoNS* could serve as a reservoir for gene cassettes containing *mecA* or *mecA* homologs in dairy cattle. Additionally, they may carry additional antimicrobial resistance genes, creating the potential for bidirectional transmission between humans and dairy cattle.

SCCmec is a molecular technique utilized to comprehend the epidemiology and clonal relationships of MRSA strains, particularly in the global occurrence of community-acquired MRSA infections [29]. While *SCCmec* types *I, II,* and *III* were predominant in earlier years, *SCCmec* type IV was initially identified in 2002 in two distinctive MRSA strains and has since become one of the most frequently isolated *SCCmec* types [3]. In the largest epidemiological survey in Türkiye, encompassing 397 MRSA strains collected from 12 hospitals across 11 cities in different geographical regions between 2006 and 2008, the study identified the presence of a hospital Turkish clone *SCCmec III* and a community clone *SCCmec IV* extensively disseminated in Türkiye [30]. Consistent with our findings, other studies conducted in Turkey have reported the dominance of *SCCmec* type IV and *SCCmec* type III clones [31-33].

Antibiotic-resistant staphylococci, which occur as a result of incorrect and unnecessary use of antibiotics in animals, can be transmitted with milk, thus spreading antibiotic-resistance genes, which constitutes an important public health problem [11,34].

When we examined the studies that conducted antimicrobial resistance of *S. aureus* in raw milk in Türkiye, it was seen that antimicrobial resistance is generally higher than our results [16,32,33]. In a study conducted in Switzerland, it was reported that antibiotic resistance rates were found to be quite low in *S. aureus* strains isolated from milk, as in our study [35]. In addition to antimicrobial resistance, the pathogenicity of *Staphylococcus* spp. is closely linked to their ability to produce staphylococcal enterotoxins and staphylococcal enterotoxin-like substances [36]. The presence of *SE* genes in *Staphylococcus* isolates from various food and milk products plays a significant and emerging role. These genes have been identified through screening in numerous surveillance studies, highlighting their potential as a serious public health hazard [5,13,37]. The presence of *SEs* genes were detected in 52.9% of MSSA strains isolated from raw milk in Northern Germany, and the most frequently occurring genes were *seg* and *sei* [38]. Studies showed that more than one *SE* gene encoding different enterotoxins can be found in *S. aureus* [5,37]. For a considerable period, *S. aureus* was regarded as the sole enterotoxigenic species within the genus, while *CoNS* were primarily categorized as contaminants. This classification persisted due to historical controversy surrounding the potential enterotoxigenicity of *CoNS* isolates [37]. Nevertheless, some studies have demonstrated that *CoNS* isolates can indeed harbor enterotoxin genes and, in certain instances, produce toxins at concentrations clinically significant [37,38].

The carriage of *tsst*-1 virulence genes by mobile genetic elements is important in terms of transferring them to people consuming milk and dairy products containing *tsst*-1 positive *S. aureus* [18]. Studies have shown that high *tsst-1* gene carrier rates range from 26.2% to 58.5% [39,40]. As with other virulence genes, the rates of *tsst*-1 gene in *S. aureus* isolates vary according to different geographical regions.

Although our study was not associated with fermented

foods, some *CoNS* species such as *S. carnosus, S. condimenti, S. equorum, S. piscifermentans, S. succinus,* and *S. xylosus* have been associated with fermented foods and have been proposed as very rare opportunistic pathogens [41]. These food-associated CoNS species were also not detected in our study. The CoNS species detected in our study were those considered to be pathogenic, similar to those in other studies [41].

In conclusion, the presence of different *Staphlococcus* species were found in unpackaged raw milk collected in Istanbul. It has been understood that attention should be paid to *MRCoNS* as well as *MRSA*. Most *MRSA* and *MRCoNS* bacteria harbored *SCCmec* type IV which may have assumed their possible community-acquired origins. *S. chromogenes* was the most prevalent species among *CoNS*. *CoNS* isolates also carried various virulence genes. *S. aureus* and *CoNS* isolated from raw milk should be considered a public health problem with both the transmission of resistant pathogens and the horizontal transmission of the carrying genes. In addition to routine control of bacteria in raw milk, these raw milk should be boiled before consumption.

Declarations

Availability of Data and Materials: The authors declare that data supporting the study findings are also available to the corresponding author (S. Ekici).

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Ethical Statement: This study does not require ethical permission.

Conflict of Interest: The authors declared that there is no conflict of interest

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Author Contributions: MMT, MD, SD, İK, AÖ, DP, DSE, BG, SA, OCA, SE and DD conceived and executed the idea, designed experiments, analyzed results and a deep revision of the manuscript. MMT, MD, SD, İK, AÖ, DP, DSE, BG, SA, OCA collected samples, performed experiments, contributed to tand implementation of the research. All authors listed have made a substantial, direct and intellectual contribution to the work and approved it for publication.

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Research Article

The Effects of Animal-Related and Environmental Factors on Feline Entropion: A Comprehensive Cohort Study of 272 Eyes

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Abstract

This study aims to provide a general perspective on entropion in cats, while offering a detailed assessment of the animal and environmental factors that may play a role in its occurrence. The study encompassed 170 cats diagnosed with entropion from a cohort of 648 cats that were presented with complaints of blepharospasm between 2020 and 2024. The signalment, history, and ophthalmic examinations were recorded. Eyelid deformities were evaluated based on the eye's location, position and the degree of the lid's rotation. A total of 170 cats were diagnosed with entropion in 272 eyes. The prevalence was significantly higher in brachycephalic cats (78.2%). Entropion was found to have a higher prevalence in male cats (90.58%). In the history, it was determined that 64.1% of the cases had been exposed to irritants. Entropions were located laterally (n=58), medially (n=1) and totally (n=213). Lid's rotation was graded as mild in 76 eyes, moderate in 35 eyes, and severe in 161 eyes. Our study findings emphasize that entropion remains a significant concern in brachycephalic cats. Environmental factors that may cause eye irritation and ocular conditions should be considered. The noticeable male predominance observed in the study also raises questions about whether orbicularis muscle laxity might be associated with gender.

Keywords: Brachycephalic, Cat, Environmental factors, Eyelid, Muscle laxity

Introduction

The eyelids comprise an outer dermal surface facing outward, a conjunctival surface facing the eye, and various muscle groups, serving to directly shield the eyes from external elements. They are anatomically specialized with a tarsus, providing a thick structure extending along the orbital septum. Orbicularis oculi, the major muscle in its structure, is responsible for the movement of the eyelids. The medial and lateral attachments of the orbicularis oculi muscle maintain the elliptical shape of the palpebral space and prevent it from becoming circular during contraction. An excessive increase in muscle tone that may occur for different reasons is called blepharospasm and is one of the important factors that can result in entropion [1].

Entropion is an eyelid deformation characterized by the inward rolling of the lower and/or upper eyelid margin towards the globe of the eye, encompassing either the lateral, medial, or complete extent of the eyelid [2]. It is more common in dogs and widely reported in the veterinary literature [3]. Nevertheless, it has been contended in previous studies that this conclusion cannot be unequivocally determined solely by examining the number of affected animals in the population and the prevalence of the disease. For instance, observations regarding entropion in cats have mostly been highlighted in comprehensive examinations of eye diseases [4]. It is noted that there is a considerable paucity of studies concerning the prevalence and incidence of entropion in cats. In these studies, the most affected cat breeds are Domestic Shorthair, Persians, Maine Coon, and British Blues/Shorthair [3,5,6].

Entropion is categorized into two main groups: primary (conformational) and secondary (spastic) entropion. The formation of primary entropion is influenced by factors such as the length of the palpebral aperture, structure of the skull, orbital morphology, gender, and skin laxity. Conversely, secondary entropion may result from severe and prolonged blepharospasm following painful ocular

conditions such as distichiasis, keratitis, corneal ulcers, and severe irritability conjunctivitis. Additionally, it may occur as a consequence of scar tissue formation following direct trauma to the eyelids or due to alterations in globe position or size (microphthalmos) [3,7,8]. This study aims to provide a general perspective on entropion in cats, while offering a detailed assessment of the animal and environmental factors that may play a role in its occurrence.

Material and Methods

Ethical Statement

The required ethics committee report for the study was obtained from Animal Experiments Local Ethics Committee of Ankara University (Approval No: 2024-08-65.). An "Informed Consent Form" was obtained from the animal owners before examination of animals.

Study Design

The study encompassed 170 cats diagnosed with entropion from a cohort of 648 cats of various breeds, ages, and genders that were presented to the Ankara University Faculty of Veterinary Medicine Animal Hospital Ophthalmology Clinic with complaints of blepharospasm between January 2020 and January 2024. After taking a detailed history and an ophthalmic examination involving direct ophthalmoscopy, slit-lamp biomicroscopy and fluorescein staining were performed on all cats.

Eyelid deformities were evaluated based on the position of the eyelid and the degree of rotation of the lid towards the eye. The evaluation of eyelid position was conducted in terms of the medial canthus, lateral canthus and entire eyelid. The degree of rotation was graded according to the severity of entropion: An inward rotation of approximately 45° on the lid was termed as mild entropion, while a rotation of 90° was classified as moderate entropion, and a rotation of 180° was characterized as severe entropion [9]. Simultaneously, a comprehensive examination of the eye and its surrounding tissues was performed.

Based on the anamnesis taken from the patient owners, it was suggested to eliminate the factors thought to cause eye irritation. In cases where another ocular condition coexisted with entropion, appropriate treatment for the concurrent disease was administered. Throughout the management process, all animals were prescribed Elizabethan collars and hyaluronic acid eye lubricants. In cases of severe corneal ulcers, analgesia was achieved by administering cyclopentolate eye drops two times daily. Owners were instructed to gently massage the folded eyelid downward with their thumb for this purpose. Surgical intervention was conducted in cases where entropion persisted beyond the first week.

General anesthesia was induced with propofol to perform oro-tracheal intubation and maintained with isoflurane via a circle breathing system. Animals were treated surgically with Hotz Celsus procedure used. The edge of the eyelid, which was inwardly folded towards the eye, was repositioned to its normal anatomical position by pulling it downward. After marking the tissue to be excised with forceps, the width of the tissue to be removed was determined, and the skin and a thin strip of the orbicularis muscle were carefully excised with Stevens tenotomy scissors. Single interrupted skin sutures were then used to close the Hotz-Celsus incisions with suture material 5/0 polyglactin 910 *(Fig. 1)*. In all animals, the sutures were removed on the 10th day, and the Elizabethan collar was removed on the 12th day.

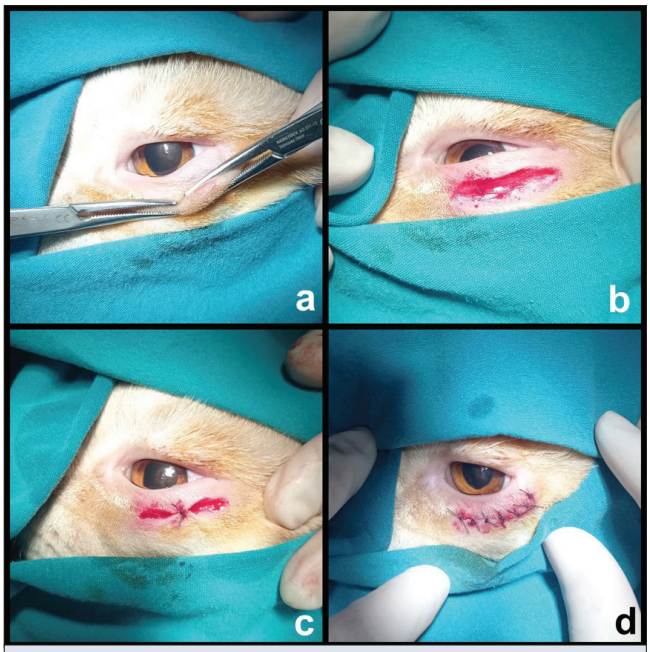

Fig 1. In the Hotz-Celsus procedure, the eyelid was repositioned to its normal anatomical position by pulling it downward. The tissue to be excised was marked with forceps, and the width of the tissue to be removed was determined (a). The skin and a thin strip of the orbicularis muscle were carefully excised using Stevens tenotomy scissors (b). Single interrupted skin sutures were then applied to close the incisions (c, d)

Statistical Analysis

The data were examined using the Pearson chi-square test to assess the statistical significance of differences in entropion incidence rates. A criterion of $P<0.05$ was used for all statistical comparisons. Data analysis was conducted using SPSS 27 software package.

Results

In the study, 648 cats complaining of blepharospasm were categorized as brachycephalic (n=253) and non-brachycephalic, with the latter group including other pedigree cats (n=38) and non-purebred cats (n=357) *(Table 1)*. Entropion was diagnosed in 26.2% (170/648)

Table 1. *Cat breeds presented with complaints of blepharospasm caused by various eye problems.*		
Cat Breeds		**Number of Animals**
Brachycephalic breeds	Bombay	1
	British Longhair	18
	British Shorthair	126
	Burmese	1
	Chinchilla Persian	1
	Exotic Shorthair	6
	Persian	26
	Scottish Fold	53
	Scottish Fold Longhair	5
	Scottish Fold Shorthair	14
	Selkirk Rex	2
Non-brachycephalic breeds	*Pedigree cats*	
	American Shorthair	2
	Turkish Angora	8
	Maine coon	1
	Norwegian Forest	1
	Russian Blue	2
	Siamese	9
	Siberian	1
	Somali	1
	Sphynx	5
	Turkish Van	9
	Non-purebreed cats	
	Mix	356
Total		648

Table 2. *Distribution of entropion in brachycephalic and non-brachycephalic cat breeds (P<0.001)*					
Cat Breeds	**Entropion (+) Group**		**Entropion (-) Group**		**Total**
	n	**%**	**n**	**%**	**n**
Brachycephalic	133	52.60	120	47.40	253
Non-Brachycephalic	37	9.40	358	90.60	395
Total	170	26.23	478	73.77	648

of the cats evaluated. Of the 170 cats diagnosed with entropion, 78.2% (133/170) were identified as being of brachycephalic breeds. Upon examination, the incidence rate of entropion in brachycephalic breeds was found to be statistically significantly higher than in non-brachycephalic breeds (P<0.001) *(Table 2)*.

Observations revealed that the mean age of entropion cases was 3.12±2.52 years old, with entropion exhibiting a higher prevalence (90.58%) in male cats during gender assessment. It was reported that 1.9% of the cats had been neutered before. Additionally, upon examining a total population of 648 cats presenting with blepharospasm, consisting of 176 males and 472 females, it was notably observed that 87.5% of the male cats were diagnosed with entropion.

All owners reported that their pets exhibited squinting for varying durations before the onset of entropion. In the history, it was determined that 64.1% of the cases had been exposed to irritants such as perfume, bleach, smoke, and cat litter. Entropion developed in 7 animals affected by viral conjunctivitis *(Table 3)*. In these animals, herpesvirus antigen test was detected positive in the samples taken from the tears. When the duration of complaints reported by animal owners and the potential causative factors influencing the severity of entropion were evaluated, no significant relationship was found.

Entropion evaluation was conducted on 272 eyes of the 170 cats included in the study. The animals had been diagnosed with primary unilateral (n=70) or bilateral (n=100) eyelid entropion. It was observed in the upper eyelids in 3 cats and in the lower eyelids in all other cats. Entropions were located laterally in 58 eyes, medially in 1 eye and along the totally margin in 213 eyes *(Fig. 2)*. According to the severity of entropion, it was graded as mild in 76 eyes,

Table 3. *Distribution of anamnesis and clinical examination findings in brachycephalic and non-brachycephalic cats with entropion. N/A: Complaint period unknown.*

Anamnesis and Clinical Findings	**Brachycephalic Breeds (n)**	**Non-brachycephalic Breeds (n)**
History		
Smoke	9	2
Parfume	13	4
Bleach	15	1
Litter	48	17
Idiopatic	39	11
FHV-1	6	1
Others (senil, palpebral deformity, postvaccination)	1	3
Duration		
up to 2 weeks	22	5
2-4 weeks	27	2
more than 4 weeks	25	10
N/A	59	20
Ophthalmological evaluation		
Corneal ulceration	93	7
Corneal necrosis	18	5
Corneal ulceration+necrosis	6	1
Corneal necrosis+eosinophilic keratitis	1	-
Conjunctivitis	55	9
Corneal scar	-	2
Blepharitis	4	-
Eosinophilic keratitis	1	-
Epiphora	45	19
Symblepharon	-	2
Third eyelid protrusion	2	-
Microphthalmia, palpebral deformation	-	2
Eyelid evaluation		
Location	Right: 29	Right: 17
	Left: 20	Left: 6
	Bilateral: 82	Bilateral: 16
Grade	Mild: 60	Mild: 16
	Moderate: 26	Moderate: 9
	Severe: 131	Severe: 30
Position of entropion	Laterally: 45	Laterally: 13
	Medially: 1	Medially: -
Total	188	25
Treatment		
Massage	53	23
Surgery	76	18

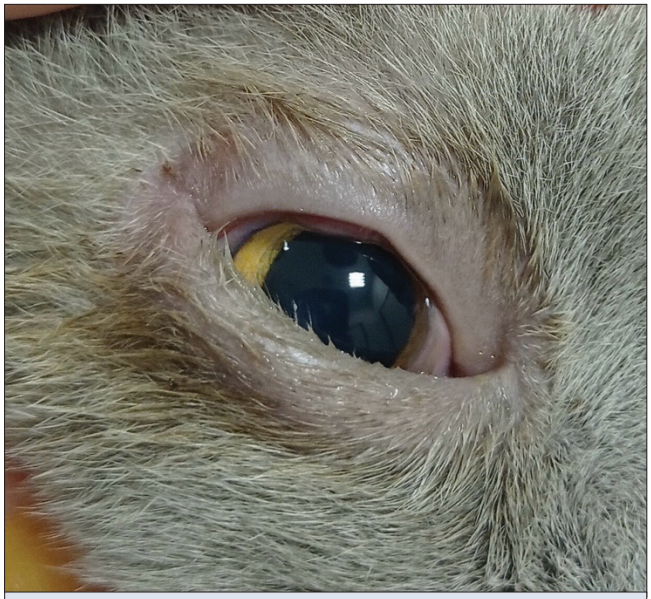

Fig 2. Entropion in the lower eyelid of the right eye in a 1-year-old male Scottish Fold cat. It was located totally

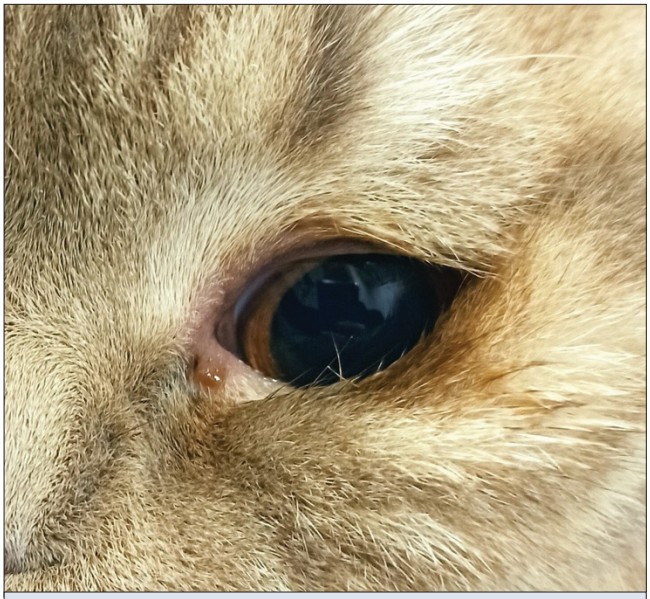

Fig 3. Severe entropion in the lateral lower eyelid with more than 180 degrees of rotation in a 1-year-old male British Shorthair

Fig 4. Severe keratitis and corneal ulceration accompanied by entropion were observed in a one-year-old British Shorthair cat. Central corneal necrosis was evident within the lesion *(arrow)*

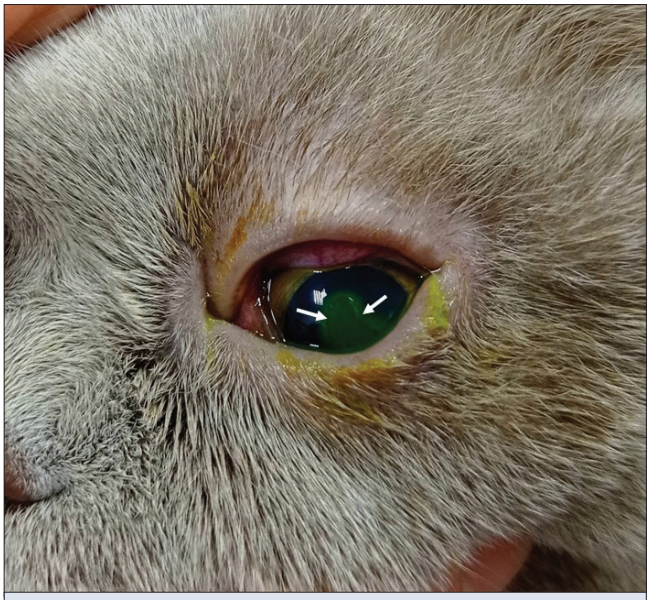

Fig 5. Based on the anamnesis, conjunctivitis secondary to entropion and a large, deep ulcer at the center of the cornea were noted. The ulcerated area tested positive with fluorescein staining *(arrows)*

moderate in 35 eyes, and severe in 161 eyes *(Fig. 3)*. In the ophthalmological examination, corneal ulceration was the most common finding, observed in 36.7% of cases. A positive fluorescein test was observed in all of these animals. Conjunctivitis and epiphora were the second most common findings, each occurring in 23.5% of cases *(Fig. 4, Fig. 5) (Table 3)*. All procedures were carried out by the same clinicians. While no complications occurred in animals that underwent surgical intervention, the average follow-up period was determined as 3 months.

Discussion

Cats are categorized into dolichocephalic, mesocephalic, and brachycephalic breeds based on the shape and structure of their skulls. Brachycephalic cat breeds have various malformations such as stenotic nares, shortening of the nasal bone and severe joint and bone deformities. They are characterized by their rounded, full skull and cheeks. The eyes are large and round, set wide apart [10]. Brachycephalic cats have been reported to be more

prone to eye-related problems such as keratitis, corneal ulceration [11], corneal sequestra [12,13] and entropion [6]. Among inherited and hereditary ocular abnormalities, entropion is highly prevalent in brachycephalic breeds [14]. According to Anagrius, the reason for this condition might be the nasal skin which is extending over the upper edge of the lower eyelid in brachycephalic breeds. The fact that all cases of entropion in their study results were formed in the medial part of the lower eyelid supports this condition [15]. However, upon reviewing our study results, it was notable that the number of eyes with entropion located just medially was only 1, with the majority being located totally (213/272). The current study findings revealed that the cat breed most affected by entropion is brachycephalic breeds. The observation of entropion being most prevalent in British Shorthair cats within this group suggested a potentially higher susceptibility to entropion in this breed. Although there are numerous entropion studies, it is noteworthy that Scottish Fold cats are rarely mentioned [3,5]. However, the second brachycephalic breed in which entropion was most commonly observed in the present study was the Scottish Fold. It is evident that the significant brachycephalic cat population in the study material was predisposed to entropion. However, it cannot be attributed solely to a single factor.

Spastic entropion is most commonly observed in cats. In situations that irritate the eye, the animal's persistent squinting due to pain leads to spasms in the orbicularis oculi muscle, resulting in spastic entropion [7]. When ocular conditions associated with entropion were examined in this study, the most notable were corneal ulceration (34.1%) and conjunctivitis (23.5%). However, it could not be definitively determined whether these spastic components were the cause of entropion or if the trauma to the cornea and conjunctiva was a result of the hairs on the inward-rolling eyelids and the animal's scratching due to irritation. This uncertainty stems from the anamnesis provided by the patient owners, which revealed that these animals had been exposed to eye-irritating substances for varying periods. Therefore, it was not possible to definitively identify the primary factor causing blepharospasm in these cases.

When assessing the impact of age on eyelid structures, particular attention should be drawn to the laxity that occurs in the lower eyelid. As age progresses, there is a decrease in the volume of the tissue surrounding the eyeball. The decrease in tissue volume, combined with the accelerated increase in laxity, ultimately disturbs the equilibrium between the surface of the eyeball and the eyelid, resulting in alterations to the normal anatomical positioning of the lid [16]. The vertical and horizontal laxity in the lower eyelid results in the preseptal orbicularis oculi muscle overriding the pretarsal orbicularis oculi muscle, leading to the formation of entropion in the lower eyelid [17]. This condition, associated with age in animals, is defined as senile entropion. However, upon evaluating the average age of the study group, it was deemed necessary to explore alternative etiologies for the cause of muscle laxity.

When the orbicularis oculi muscle contracts, it narrows the palpebral opening by pulling on the lateral and medial commissures horizontally. The levator palpebrae superioris muscle, Müller's muscle, which is closely associated with it, and the smaller levator anguli oculi medialis muscles raise the upper lid and malaris muscle depresses the lower eyelid to enlarge the eye opening [1]. In human, the most significant factor in the development of entropion is the difference in tension between the orbicularis oculi and malaris muscles. The malaris muscle spreads as a thin muscle to insert on the ventral orbicularis oculi muscle, and functions to move the lower eyelid downward. Excessive contraction of one muscle group while the other relaxes leads to the onset of entropion [18]. In this scenario, it is imperative to investigate the factors contributing to tension disparities in the eyelids and surrounding musculature, which can lead to entropion. The study observed that blepharospasm, manifested at varying severities, was a common clinical finding in all cats presenting with entropion. According to Lin, in human, horizontal laxity in the eyelid and laxity in the lower eyelid retractors, which pull the lower edge of the tarsus downward and backward, can cause a deterioration in the normal tension of the eyelids, leading them to turn inward toward the eye. The rotation of the skin and eyelashes, in conjunction with the movement of the eyelids, worsens blepharospasm, thereby aggravating entropion [19]. Although human and cat eyelid anatomy is not exactly similar, both species' retractor muscles of the upper eyelid, which possess a large surface area, are notably strong. Conversely, the lower eyelid features the weaker and more superficial retractor muscle. Considering this anatomical structure, the area most sensitive to the effects of blepharospasm is the lower eyelid in cats, just like in humans, due to its weaker structure. Indeed, the results of the study reflect this, as entropion occurred in only 3 eyes in the upper eyelid, while nearly all cases were observed in the lower eyelid.

Although muscle laxity appears to be linked to long-term blepharospasm, our study could not establish a direct and significant relationship between the duration and cause of the complaint and the severity of blepharospasm and entropion. This suggests the possibility of an alternative cause that triggers laxity in the orbicularis muscles, which may also influence the severity of entropion. It is noteworthy that 90.58% of the cats in this study were male. Similarly, a study by Bott et al.[5] found that entropion was most frequently observed in male cats,

though no statistically significant data were obtained. The relationship between hormones and muscles has been extensively studied in both human and veterinary medicine. Numerous studies have demonstrated that androgen receptors within the muscles play a significant role in the volume and functional alterations of various muscle groups. For instance, the pathogenesis of perianal hernias observed in intact male dogs has been attributed to the relaxin hormone secreted by the prostate gland, which induces laxity in the muscles of this area. Research results have shown that hypertrophy, especially in the prostate gland, increases relaxin release, causing local muscle atrophy and deterioration in connective tissue components. Relaxin hormone receptors were identified extensively in muscle samples obtained from dogs with perineal hernia [20]. Similarly, it has been known for years that estrogen and androgen receptors are effective in striated muscle functions. It achieves this effect by protecting myoblasts and suppressing atrophy pathways [21]. Experimental studies have demonstrated that a decrease in the presence of androgen and estrogen receptors leads to a reduction in the number of muscle fibers, particularly in tissues containing type II muscle fibers, and results in tissue atrophy [22,23]. In their clinical study involving patients with entropion, Cabuk et al.[24] proposed that the reduction in muscle fibers and consequent deformation observed in the orbicularis oculi muscles of these patients, which predominantly consist of type II muscle fibers, might be attributed to a decrease in androgen receptors. Estrogen receptors were entirely absent in male patients with blepharospasm, whereas they were present in the control group. Additionally, a decrease in both hormone receptors was observed in female patients with blepharospasm. When entropion was assessed in neutered cats in the present study, no clinical difference could be discerned regarding its localization on the eyelid or its severity. However, the excess male population in the study may suggest a potential association between eyelid deformities in animals and gender, possibly related to androgen hormones. This aspect could serve as an evaluative parameter for future studies.

The primary focus in the treatment of spastic entropion is to reduce or eliminate painful ocular conditions before the permanent surgical corrections [7]. In the study, it was observed that entropion regressed in many cases (44.7%) without surgical intervention by adding eyelid massage to the elimination of environmental irritants and medical treatment of eye conditions.

As a result, the increased popularity of brachycephalic cat breeds has led to a rise in the incidence and prevalence of numerous diseases and deformities specific to these animals. Our study findings emphasize that entropion remains a significant concern in brachycephalic cats. Potential predisposing factors for entropion have been examined; environmental factors that may cause eye irritation, as well as various ocular conditions, should be considered. The noticeable male predominance observed in the study also raises questions about whether orbicularis muscle laxity might be associated with gender. Further research on this topic is necessary.

Declarations

Availability of Data and Materials: The data that support the findings of this study are available on request from the corresponding author (O.O. Şenel).

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Authors' Contributions: İ.E.: Conceptualization, data curation, investigation, methodology, writing-original draft, writing-review and editing, project administration. S.S.: Data curation, investigation, writing-review and editing. A.U.: Data curation, writing-original draft. O.O.Ş.: Conceptualization, writing-review and editing, supervision.

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RESEARCH ARTICLE

The Use of Probiotic Footbaths for the Treatment of Ovine Interdigital Dermatitis

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Abstract

Ovine interdigital dermatitis (OID) is a mild infection of the skin that may lead to more serious infections, such as foot rot and foot abscess. The objective of this study was to investigate the efficacy of probiotic foot baths in controlling ovine interdigital dermatitis (OID). Prior to and following the administration of the treatment, swab samples were obtained from sheep exhibiting symptoms of OID. Each sheep underwent a single daily foot bath session for a period of five days, with each session lasting five minutes. The solution, comprising of *Lactobacillus acidophilus*, *Lacticaseibacillus rhamnosus*, *Lacticaseibacillus casei* and *Bifidobacterium bifidum*, was prepared with a probiotic microorganism concentration of 10^6 CFU per millilitre. The most prevalent bacterial strain was *Staphylococcus aureus* (36.62%), while *Fusobacterium necrophorum* (1.91%) was isolated and identified at the lowest frequency. Prior to the application of the probiotic solution, the total microbial load was 4.693±0.644 (TAMC), 3.969±0.625 (TC) and 3.612±0.644 (EC) log CFU/mL. Following the application of the probiotic foot bath, the corresponding values were 2.269±0.739, 1.823±0.783 and 1.538±0.742 ($P<0.05$). The results show that probiotic foot baths are effective for reducing pathogenic microbial loads in sheep feet. The study emphasises the importance of non-antibiotic strategies in the management of foot diseases and demonstrates the potential of probiotics as an alternative approach.

Keywords: Foot baths, Foot diseases, Ovine interdigital dermatitis, Probiotics

Introduction

Ovine interdigital dermatitis (OID), also referred to as 'scald', is a mild infection of the skin between the hooves of sheep [1,2]. Uncomplicated and non-progressive OID is caused by a superficial infection of the skin with *Fusobacterium necrophorum*. This can potentially facilitate the development of more severe infections, such as footrot and foot infection. It is inevitable that exposure to this organism will occur, given its ubiquity in soil and ruminant faeces [3]. The damage to the interdigital area renders sheep susceptible to colonisation by *F. necrophorum*. Subsequent infection with *Dichelobacter nodosus* is a probable consequence of OID. Footrot is known to develop following infection with *D. nodosus*. The global impact of footrot on the health and productivity of sheep is significant [2,4].

A variety of bacterial species are present on the feet of sheep, both in healthy individuals and those affected by footrot [4]. Bacteria such as *Staphylococcus* and *Streptococcus* are typically present in the interdigital area and on the surface of the skin, and these opportunistic bacteria have a secondary effect on the development of the disease [5]. In addition to *F. necrophorum* and *D. nodosus*, other bacteria, including *Bacteroides fragilis*, *Prevotella spp*. and *Treponema spp*., may also play a role in the pathogenesis of the disease. The involvement of these organisms, which have been isolated from footrot cases, is still a matter of ongoing debate [6].

The practice of footbathing has been demonstrated to be an efficacious and pragmatic method for the management of foot infections in sheep. However, these disinfectants have disadvantages, including adverse effects on both animal and human health, and problems with efficacy and solubility. The use of antibiotic solutions in foot baths can lead to increased antibiotic resistance and poses challenges for appropriate disposal, which is often not feasible [7].

In recent years, there has been a notable increase in the utilisation of probiotics in a multitude of physiological and

pathological cases. The outcomes have been favourable in a considerable number of instances, indicating the potential efficacy of this approach. This is largely attributed to their capacity to regulate the immune system at both local and systemic levels [8]. Probiotics are defined as live microorganisms that provide health benefits to the host when administered in sufficient amounts [9]. Extensive research has been conducted on probiotics in both clinical and experimental settings. These studies have documented the capacity of probiotics to exert a beneficial influence on not only intestinal function but also on skin health, due to their unique properties. A body of scientific evidence supports the hypothesis that specific probiotics can influence the cutaneous microflora, lipid barrier, and cutaneous immune system, thereby maintaining skin homeostasis. Topical probiotic formulations have been employed for the prevention and treatment of various dermatological conditions, including acne, yeast infections, bacterial infections, and dermatitis [8]. Despite the paucity of research in this area, the concept of utilising topical probiotics to prevent or treat dermatological conditions associated with altered microflora is gaining traction [10]. It is hypothesised that cutaneous dysbiosis may be a precursor to foot rot, suggesting that probiotic culture with established dermatological efficacy could be a promising topical treatment option [11].

The present study aimed to investigate the potential of probiotics with demonstrated efficacy in the treatment of skin diseases as a viable topical therapeutic option. The objective was to develop proposed treatment algorithms and assess their therapeutic potential, with a particular focus on the effect of probiotic foot baths on the healing process in cases of interdigital dermatitis.

Materials and Methods

Ethical Statement

The study was approved by the Balıkesir University Animal Experiments Local Ethics Committee (Balıkesir, Türkiye) on 24 May 2022 (decision number 2022/4-4).

Study Design

The study was conducted in autumn 2021 and winter 2022. Crossbred sheep in Balıkesir University Livestock Application and Research Centre and private sheep farms located in villages of central Balıkesir were examined for foot diseases. Sheep were housed in closed pens and allowed daily access to pasture. Visual and physical examinations were performed for lameness in all sheep. A total of 71 crossbred ewes (Karacabey Merino x Curly), aged between 2.5 and 4 years, showing signs of moisture, hyperaemia and inflammation in the interdigital space of one or more feet during clinical examination were included in the study. Each foot of every sheep was examined visually and scored according to the scoring system developed by Stewart and Claxton (1993). The severity of the lesions was quantified on a scale of 1 to 5, with 1 indicating minimal disease progression and 5 indicating severe disease progression and extensive hoof capsule involvement [12].

The day of clinical examination was considered as day 0. On this day, foot examinations, hoof trimming and lesion scoring were performed with the ewes lying on their side. To ensure consistency, the same person (GA) performed the scoring before and after the foot bath for each sheep. Although sampling, isolation and identification studies were carried out from a total of 71 animals, only two farms approved the 5-day probiotic bath application. Therefore, the number of animals that could be administered probiotics in the study was 19.

Sample Collection & Footbath Regime

To determine the microbiological load and infectious etiology on the feet of sheep, a piece of gauze was used to roughly clean the interdigital area of dirt and debris and swab samples were collected. Swab samples were collected from all four feet of each animal prior to and following bathing, and subsequently analysed for bacterial loads.

Swabs were coated with Amies Agar Gel with Charcoal Transport Swabs medium (Thermo Scientific™ TS0002A) and sent to the laboratory for analysis. A total of 76 swabs (one for each foot) were collected from 19 sheep before treatment (day 0). The sheep were divided into two groups of 9 and 10 sheep and housed in cleanly littered paddocks of approximately 25 m^2 each and fed ad libitum. The sheep were kept in these paddocks both during and after treatment until follow-up samples were collected (day 7) and received no treatment other than foot bathing. Two days after the completion of the 5-day treatment, foot examinations were performed again, lesion scoring was repeated and follow-up samples were collected before release to the flock on day 7.

Prior to the foot bath, the hooves were not cleaned; only before swab sampling a rough cleaning with a piece of gauze was performed to remove dirt and debris from the interdigital space. Each sheep (covering all 4 feet) underwent the foot bath treatment once daily for 5 days, with each session lasting 5 minutes. Foot baths were prepared again for each group. The bath pool was placed at the base of a restricter *(Fig. 1)*.

Following the foot bath, the sheep were returned to the paddock (*Fig. 2*). A modified footbath pool was used to fully immerse the hooves. The bath solution, containing 10^6 colony-forming unit (CFU) of probiotic microorganisms per mL, was prepared with strains including *Lactobacillus acidophilus, Lacticaseibacillus*

Fig 1. The bath pool was placed at the base of a restrictor, allowing sheep to comfortably stand with all four feet in the bath

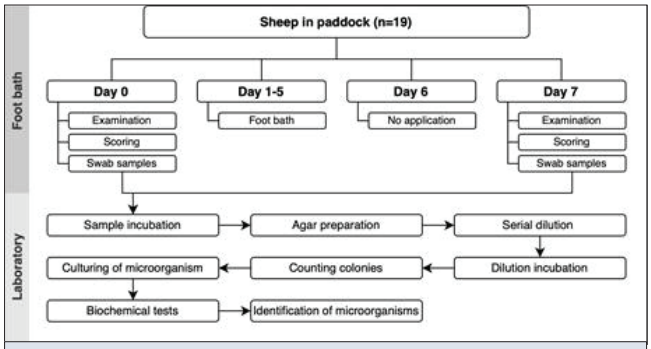

Fig 2. Experimental design

rhamnosus, Lacticaseibacillus casei, and *Bifidobacterium bifidum* [13]. This solution was formulated using packages containing $5x10^9$ CFU probiotic microorganisms: *Lactobacillus acidophilus*, *Lacticaseibacillus rhamnosus, Lacticaseibacillus casei,* and *Bifidobacterium bifidum* per 1 sachet (7 g) (Prolex, Ledapharma, Kocaeli, Türkiye). To achieve a concentration of 10^6 CFU/mL, the solution was prepared at a concentration of 0.14% (4 sachets per 20 L of water). Sheep were treated with the foot bath each day, and a fresh solution was prepared daily for treatment.

Laboratory Processing

Agent isolation and identification were made on the swabs to determine the microbial load. The swab samples collected from the animals were prepared and incubated for 1-7 days in appropriate media under both aerobic and anaerobic conditions. Samples were processed in the laboratory within 3 h of collection. The swabs were initially moistened in sterile brain heart infusion broth (Oxoid CM1135) and then placed in sterile 10 mL tubes containing 1 mL of same medium and vortexed for one minute. Serial dilutions were subsequently prepared using phosphate-buffered saline (PBS). These dilutions were then incubated on various media including MacConkey agar (Oxoid CM0007), blood agar (5% sheep blood) (Oxoid CM0055), and Wilkins-Chalgren agar (Oxoid CM0619) under both aerobic and anaerobic conditions at 37°C for 24-72 h. After the incubation, colonies were counted and CFU per mL (log CFU/mL) were calculated. For the ten-fold serial dilutions, 1 mL of homogenised swab sample was transferred into 9 mL of diluent, preparing dilutions in the range of 10^{-1} - 10^{-6} [14]. From these dilutions, a 0.1 mL aliquot was plated onto various media types for microbial counts [14,15] *(Fig. 2)*.

Total aerobic mesophilic bacteria count (TAMC): A 0.1 mL aliquot from the appropriate dilutions was spread onto plate count agar (Oxoid, CM0325). The inoculated plates were then incubated at 30°C for 48-72 h. Following incubation, media with 30 to 300 colonies were counted [16,17].

Total coliform count (TC): 0.1 mL from the previously prepared serial dilutions was spread on violet red bile agar (VRBA) plates and incubated at 37°C for 24 h. All suspicious purple colonies surrounded by purple halos were counted and recorded [16].

Total Enterobacter enumeration (TE): Enterobacteriaceae were counted on Mac Conkey agar and incubated at 37°C for 24 h [15,18].

Identification of Bacteria

Morphological features: The morphological characteristics of the developing colonies were evaluated by examining the shape, colour and surface of the colonies, their distinctive odour, texture, transparency, haemolysis characteristics on blood agar, and lactose fermentation on MacConkey agar.

Microscopic features: Gram staining was performed on the colonies. In addition, catalase, coagulase, oxidase, Tsu-Triple Sugar Iron Agar (TSI), urease, indole, Metil Red (MR), Voges Proskauer (VP), carbohydrate fermentation, and H_2S production tests were performed on the colonies.

Polymerase Chain Reaction (PCR)

Suspected anaerobic colonies were subjected to polymerase chain reaction (PCR) analysis. For *D. nodosus*, the 16S rRNA gene was amplified using the primers 5'-CGGGGTTTATGTAGCTTTGC-3' and 5'-TCGGTTACCGAGTATTTCTACCCAACACCT-3'. For *F. necrophorum*, the lktA gene was amplified using the primers 5'-AATCGGAGTAGTAGTAGGTTCTG-3' and 5'-CTTTGGTAACTGCCACTGC-3'. These agents are the most frequently isolated anaerobic bacteria from cases of ovine digital dermatitis. The HiGenoMB kit (HiMedia) was employed for the purposes of DNA isolation and extraction, which was conducted in accordance with the instructions provided by the manufacturer. The amplification process was conducted at a temperature of 94°C for a duration of 10 min, followed by 30 cycles of 30 sec at 94°C, 30 sec at 58°C, and 1 min at 72°C. Subsequently, the PCR products were subjected to electrophoresis in a 1.5% agarose gel, stained with OrisafeDNA (Sugenomics), and visualised under ultraviolet (UV) illumination. The temperature was maintained at 72°C for 5 min.

Statistical Analysis

All statistical analyses were performed using software (SPSS v20, IBM). The distribution of values was assessed for normality using the Shapiro-Wilk normality test. To compare the mean bacterial counts - total aerobic mesophilic bacteria count (TAMC), total coliform count (TC), and *Enterobacter* count (EC) in the samples collected from hooves before and after the probiotic foot bath treatment, the paired samples t-test was utilised. A p- value of less than 0.05 was considered statistically significant for all analyses.

Results

The study included 284 feet from 71 crossbred sheep. Deformities (slight overgrowth of the hoof wall covering of the sole) were observed in both the front and rear hooves of 30 sheep. During clinical examination, no increase in hoof temperature was observed in any sheep; claw hardness was normal and there was no imbibition (bleeding foci) on the sole. The lameness status, claw deformities and lesion scores of the sheep examined are given in *Table 1*.

Table 1. *Lameness status, hoof deformations and lesion scoring of the examined sheep*

Examined Sheep		**Sheep (n)**	**Ratio (%)**
Sheep with lameness		33	46.47
Claw deformation	No deformation	27	38.02
	Only on the forelegs	3	4.22
	Rear legs only	11	15.49
	Both front and rear legs	30	42.25
Lesion score	0	15	21.12
	1	22	30.98
	2	22	30.98
	3	6	8.45
	4	4	5.63
	5	2	2.81

Table 2. *Isolation results of the samples*

Total Number of Bacteria Isolated	**Microorganism Type**
115 (36.62%)	*Staphylococcus aureus*
92 (29.29%)	*Escherichia coli*
91 (28.98%)	*Bacillus* spp.
10 (3.18%)	*Trueperella pyogenes*
6 (1.91%)	*Fusobacterium necrophorum*

Table 3. *Microorganisms isolated according to assigned scores*

Score	**Isolated Microorganisms**
0	*Escherichia coli* *Bacillus* spp. *Staphylococcus aureus* *Yeast*
1	*Escherichia coli* *Bacillus* spp. *Staphylococcus aureus* *Yeast*
2	*Escherichia coli* *Bacillus* spp. *Staphylococcus aureus* *Yeast*
3	*Escherichia coli* *Bacillus spp.* *Staphylococcus aureus* *Trueperella pyogenes*
4	*Escherichia coli* *Bacillus* spp. *Staphylococcus aureus* *Trueperella pyogenes* *Fusobacterium necrophorum*
5	*Escherichia coli* *Staphylococcus aureus*

Isolations and identifications were made from samples taken from the fore and hind feet. In total, 314 bacterial strains were isolated. The most common bacterial strain was *Staphylococcus aureus* [115 (36.62%)], while *F. necrophorum* [6 (1.91%)] was isolated and identified with the lowest frequency. The table of isolated and

Table 4. *Microbial population density (Log CFU/mL) in the samples taken from hooves before (Day 0) and after the probiotic foot bath (Day 7). Data are expressed as mean ± SEM*

Microbial Population Density	TAMC	TC	EC
Before the probiotic foot bath (Day 0, n=19)	4.693±0.644	3.969±0.625	3.612±0.644
After the probiotic foot bath (Day 7, n=19)	2.269±0.739	1.823±0.783	1.538±0.742

TAMC: *Total Aerobic Mesophilic Bacteria Count;* ***TC:*** *Total Coliform;* ***EC:*** *Enterobacteriaceae Count*

Table 5. *The lesion scores of the 19 sheep before and after the treatment*

Sheep	Front Hooves (Score)		Hind Hooves (Score)	
	Before the Treatment	After the Treatment	Before the Treatment	After the Treatment
1	0	0	1	0
2	0	0	2	0
3	0	0	1	0
4	1	0	1	0
5	0	0	0	1
6	0	1	1	1
7	0	0	1	0
8	1	0	1	1
9	0	1	0	1
10	0	0	1	1
11	0	0	0	0
12	0	0	0	1
13	0	0	0	0
14	1	0	0	0
15	0	0	1	0
16	1	0	1	0
17	1	0	0	0
18	1	0	0	0
19	2	0	1	0

identified pathogens is presented below *(Table 2, Table 3)*.

In the bacterial isolation of the samples taken from 19 sheep that could be treated with probiotic foot bath, *S. aureus* was identified at the highest rate, while *F. necrophorum* and *D. nodosus* were not found. The *S. aureus* rate decreased significantly from 68.42% to 5.26% after the probiotic foot bath. In sheep where bacterial loads were compared before and after treatment, a statistically significant decrease in TAMC, TC and EC counts was observed ($P<0.05$). This was supported by the decrease in total microbial load and a lower score in the probiotic treated feet *(Table 4, Table 5)*.

Furthermore, a higher bacterial load was observed in feet with deformity and clinically high scores in the swab samples. This finding was found to be statistically significant.

Discussion

The use of probiotics to alter the gut microbiota has become an accepted concept for improving human gut health [19]. The effect of *Lactobacillaceae* on ovariectomy and lipopolysaccharide (OVX-LPS)-induced gut-bone dysbiosis in rats was investigated. Dairy products fermented with *Limosilactobacillus fermentum* MF27 and/or *L. casei* 393 were shown to selectively modulate the composition of the gut microbiota, improve gut barrier function, suppress osteoclastogenesis and thereby increase trabecular bone volume. These findings suggest that the gut-bone axis can be modulated not only by live *Lactobacillaceae* species, but also by *Lactobacillus*-fermented dairy products, which may contain metabolites and/or bioactive peptides [20]. Probiotics isolated from

Palmyra palm sugar, which can produce antimicrobial compounds against methicillin-resistant *Staph. aureus* (MRSA) and foodborne pathogens, have been found to be highly effective [21].

Recent scientific interest has focused on the topical application of specific probiotic microorganisms to assess their efficacy in preventing wound inflammation and accelerating the healing process. However, research into the effects of probiotics on the skin microbiome is still in its early stages [22]. There is considerable scientific interest in the role of skin microflora in the wound healing process. Probiotics reduce healing time by maintaining the balance of the microbiota [23]. In our study, the rate of *S. aureus* before the probiotic foot bath was 68.42%. It appears that *S. aureus* is a predominant pathogen in interdigital infections in sheep. Studies have demonstrated the antibacterial potential of specific probiotics *(L. acidophilus* and *L. casei)* against MRSA. Three different probiotics (e.g. *Limosilactobacillus reuteri*, *L. rhamnosus* and *Ligilactobacillus salivarius)* were tested against *S. aureus* infection in epidermal keratinocytes. Overall, it was found that *L. reuteri* and *L. rhamnosus* (but not *L. salivarius)* reduced the ability of the pathogen to induce keratinocyte cell death. Given that *S. aureus* adheres to epidermal keratinocytes via the alpha5β1 integrin, it has been suggested that both protective probiotics reduce keratinocyte cell death by competitively excluding the pathogen from the integrin binding sites on these skin cells [23]. There is evidence from recent studies that *Lactobacillaceae* bacteria and their topical application can help maintain a healthy skin microbiome [22]. In particular, *L. acidophilus* positively modulates the epidermal environment via cellular metabolites, antimicrobial peptides and the immune system [24,25]. *L. casei* has been shown to reduce skin inflammation either by inhibiting INF-γ or by mechanisms involving regulatory CD4+ T cells. In addition, the microorganism has also been shown to increase the production of IL-10, further supporting its specific mode of action against skin inflammation [23]. Consistent with the literature, the content of the commercial probiotic used in the present study, *Lactobacillaceae* bacteria, was found to contribute to the protection of skin health as a result of topical application. In particular, the use of *L. acidophilus*, *L. rhamnosus* and *L. casei* in the probiotic footbath resulted in a reduction of *S. aureus*, in line with literature data [26].

Another study investigating the foot skin microbiota in cattle with digital dermatitis lesions stated that studies similar to those on the use of probiotics on human skin microbiota may be successful in preventing the development of digital dermatitis lesions in cattle. It has been confirmed that these studies conducted for preventive treatment are promising and can potentially be carried out using a probiotic or prebiotic foot bath [26]. In the present study, the protective and therapeutic effects of probiotic footbath were demonstrated in line with these literature findings. In particular, there was a significant reduction in isolated *S. aureus* and an observable clinical improvement in the interdigital region following the probiotic foot bath. This highlights the potential of probiotic foot baths as an effective treatment strategy in the management of similar conditions.

There is a paucity of research evaluating the effect of topical probiotics on foot lesions in livestock. In one notable study, topical probiotic powder was found to be almost as effective as intramuscular oxytetracycline over a 28-day period for early stage interdigital necrobacillosis in dairy cows [13]. The use of powdered probiotics has been reported to have equivalent therapeutic properties to antibiotics. However, our results and the supporting literature suggest that the application of probiotics in a foot bath, rather than in powder form, is a more practical method of treating flocks. This approach allows for more practical and efficient administration of treatment on a flock-wide basis, as opposed to individual treatments.

In cases of foot rot, *F. necrophorum* and *D. nodosus* are the main causative agents and are reported to be present on the skin in the interdigital spaces of bovine feet [27]. In addition, *Porphyromonas levii*, *Porphyromonas asaccharolytica*, *Prevotella intermedia*, *Prevotella melaninogenica*, *S. aureus*, *E. coli* and *Trueperella pyogenes (T. pyogenes)* can also be isolated [28]. Nayakwadi et al.[29] showed that *F. necrophorum* was the major causative agent of foot rot in small ruminants, while *D. nodosus* was not detected in most cases. Conversely, another study identified both *D. nodosu*s and *F. necrophorum* as leading organisms causing foot rot, along with other Gram-negative and Gram-positive bacteria [28-30]. In our study, *E. coli*, *Bacillus spp*. and *S. aureus* were isolated from sheep with healthy/dry feet (score 0) and 1 and 2 scores according to culture results. In addition to these bacteria, *T. pyogenes* was isolated from feet with scores of 3 and 4. In cases with a lesion score of 4, *F. necrophorum* was isolated in addition to these bacteria. These findings are consistent with those of other studies. Eradication of *F. necrophorum* is challenging, particularly given its ability to persist in the environment through faecal shedding. *D. nodosus* was thought to persist in the environment for only a few hours to a few days. In contrast to *D. nodosus*, *F. necrophorum* is an opportunistic pathogen that causes necrotic lesions at various anatomical sites and in many host species. Furthermore, our study also gave positive results for the isolation of *F. necrophorum*.

In the present study, a probiotic foot bath solution with a concentration of 10^6 CFU/mL was used. This concentration is consistent with the range used in other

studies in various species, including cattle, horses, humans and laboratory rodents. Studies have investigated the effects of topical treatments of the *Lactobacillaceae* family on conditions such as interdigital necrobacillosis, limb wounds, diabetic leg ulcers and burn wounds, with concentrations varying between 10^5 and 10^8 CFU [13,27]. To illustrate the effect of probiotics on microbial load, it's worth noting that we observed a significant reduction in microbial load (log CFU/mL) with a total dose of 10^6 CFU. A dose of 10^6 CFU was therefore considered sufficient. The use of a probiotic foot bath did not cause any adverse reaction in sheep feet. The healing potential of probiotics observed in the current study is supported by the reduced total microbial load in the feet treated with the probiotic foot bath. These results led us to focus on evaluating the protective efficacy of a probiotic foot bath as a preventive application against foot rot, rather than as a treatment for foot rot.

It should be noted that this study is not without limitations. The study primarily focuses on short-term outcomes, without addressing long-term effects or follow-up. As a result, the duration of treatment efficacy and potential delayed adverse reactions remain unexplored. Moreover, the study does not investigate the potential for the development of resistance to probiotics, which is an emerging concern in microbial management. Additionally, the practicality and cost implications of implementing probiotic foot baths on a large scale have not been comprehensively evaluated, which is crucial for understanding the feasibility of this treatment approach in real-world settings. Further studies are necessary to address these limitations.

Declarations

Availability of Data and Materials: The corresponding author can provide the datasets of this research upon reasonable request.

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Ethical Statement: The study was approved by the Balıkesir University Animal Experiments Local Ethics Committee (Balıkesir, Türkiye) on 24 May 2022 (decision number 2022/4-4).

Conflict of Interests: The authors declare that they have no conflicts of interest.

Declaration of Generative Artificial Intelligence: The authors declare that the article and/or tables and figures were not written/created by AI and AI-assisted Technologies.

Author Contributions: G.A., the clinical examination on the animals, followed by scoring and sample collection. A.E.B., collected the samples and performed the laboratory analyses. E.T.A., participated in the fieldwork component of the study. Editing, all authors read and approved the final manuscript.

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Research Article

Survey of *Anaplasma* spp. in Ticks from Türkiye: First Molecular Evidence for *A. phagocytophilum*-like-1 and 2 Strains

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Abstract

In Türkiye, although there are several studies on *Anaplasma* spp. in vertebrate host, data on the occurrence of *Anaplasma* spp. in ticks are still lacking. This study aims to contribute to control strategies by providing new information on the epidemiology of *Anaplasma* species in Türkiye. A total of 2241 ticks divided into pools in which the presence of *A. ovis* and *A. phagocytophilum* was investigated by molecular methods. Maximum likelihood estimate (MLE) per 1000 ticks with 95% confidence intervals (CI) was used to compute infection rates. Overall MLE of infection rate was determined as 34.1 and 3.61 for *A. ovis* and *A. phagocytophilum*, respectively. While the infection rate for *A. ovis* varied from 9.21 (CI 2.81-21.3) in *Haemaphysalis parva* to 81 (CI 53.8-115) in *Dermacentor marginatus*, *A. phagocytophilum* varied from 2.21 (CI 0.11- 9.51) in *Rhipicephalus bursa* to 95.5 (CI 5.61-359) in *Hae. concinna*. *A. phagocytophilum*-like-1 and 2 strains in Turkish ticks were originally identified using sequencing and phylogenetic analysis based on the 16S rRNA gene. As a result, *A. ovis* and *A. phagocytophilum* could threat animal and human health in the region and strains of *A. phagocytophilum* should be taken into account when making a differential diagnosis for tick-borne diseases.

Keywords: *Anaplasma ovis*, *Anaplasma phagocytophilum* like 1 and 2, PCR, tick, Türkiye.

Introduction

Ticks are significant carriers of numerous diseases that affect both humans and animals, including Türkiye due to suitable climatic conditions and a large-variety animal population. Both domestic and wild animals are significantly impacted by tick-borne diseases (TBDs). Additionally, TBDs threaten human health especially in tropical and subtropical climatic regions including Türkiye [1]. Babesiosis, theileriosis, and anaplasmosis are the three most significant tick-borne illnesses that are known to be endemic in Türkiye [2,3]. Türkiye has a great potential for animal breeding and livestock population comprise about 17 million cattle, 171 thousand water buffalo, 45 million sheep and 12 million goats by year of 2023 according to the Turkish Statistical Institute *(http://www.tuik.gov.tr)*. However, the country's climate makes it a good place for many tick species to maintain their biological diversity [3]. The combination of a high tick species diversity, high livestock and wild animals' populations rise the frequency of TBDs in the country. In addition, the close relationship between animal and human habitats in some parts of Türkiye could increase the risk of human TBDs transmission [3-5].

An important group of tick-borne agents are *Anaplasma* species [6]. *Anaplasma phagocytophilum*, *A. centrale*, *A. marginale*, *A. bovis*, *A. ovis*, *A. platys*, and *A. capra* are all members of the genus *Anaplasma* [7,8]. *Anaplasma ovis* and *A. phagocytophilum* are well known *Anaplasma* species infecting small ruminants. Although *A. ovis* DNA has been detected in one symptomatic human patient in Cyprus [9] and in an asymptomatic person in Iran [10] it is not yet considered a zoonotic as *A. phagocytophilum*. Currently it is considered an important pathogen in small ruminants [11] that causes clinical signs in animals [12] due to some predisposing factors. Several tick species are reported to transmit *A. ovis* including *Rhipicephalus bursa*, *Haemaphysalis sulcata* [13] and *Dermacentor andersoni* [11]. *Anaplasma phagocytophilum* is a zoonotic gram-negative intracellular bacterium transmitted by *Ixodes* spp. ticks. Although it can infect a variety of domestic and wild species, only humans, domestic ruminants, horses, cats, and dogs have been shown to develop clinical infection

named tick-borne fever. The primary risk factors are age, host resistance, and tick contact of the susceptible host after it has left a tick-free area. Main characteristic symptoms are fever, anorexia and loss of weight and yield [7]. In recent years, studies on *A. phagocytophilum* have focused on the genetic diversity of the agent and *A. phagocytophilum*-like 1 and *A. phagocytophilum*-like 2 strains were described in cattle [14,15] small ruminants [4,5,16-20] and ticks [21].

Since TBDs cause significant health and management problems of domestic livestock it is important that an accurate diagnosis and an effective treatment should be performed. Several diagnostic methods can be used to detect and identify *Anaplasma* species in both vectors and hosts. Molecular-based methods have a higher sensitivity and specificity when compared to serological technics and microscopic evaluation of blood smears [14,15].

Several studies have already investigated the prevalence of both *A. ovis* and *A. phagocytophilum* in small ruminants in Türkiye [4,17,18,22]. Recently some molecular studies were conducted to determine *A. phagocytophilum* strains in small ruminants in Türkiye [17,18]. In these studies, *A. phagocytophilum* like-1 isolates were found frequently. Since climatic conditions and animal diversity is suitable for ticks, Black Sea Region was chosen as sampling area. Through species-specific PCR and sequence analysis, this study was conducted to examine *A. ovis* and *A. phagocytophilum* strains in ixodid ticks obtained from sheep and goats in the Black Sea Region of Türkiye.

Materials and Methods

Ethical Statement

Tick samples were collected during a project supported by TUBITAK between 2010-2012 and ethics committee approval was received from the "Firat University Animal Experiments Ethics Committee" (Document No: 16-78, 04.12.2008).

Study Area and Sampling

Black Sea Region of Türkiye constitutes 18% of Türkiye's surface area and represents two different climatic conditions. In general, the region has a humid climate with rainy and a close annual range of temperature in every season. Summers are cool and winters are warm. The mountains in the region prevent passing the humid air to coastal areas. In addition, terrestrial climate features are observed in the interior due to the decrease in the amount of precipitation and the decrease in temperature. Since suitable climatic features, high animal density, managed of animals in the traditional manner and high risk of tick-borne diseases, Black Sea region was selected as sampling area.

Ticks were taken from small ruminants in the Black Sea region of Türkiye's Bolu, Kastamonu, Çorum, Samsun, Tokat, Giresun, and Bayburt provinces over a three-year period *(Fig. 1)* [23]. Out of the 53 locations, a total of 2608 small ruminants (2161 sheep and 447 goats) were screened for the presence of ticks. At least 20-25 animals from each herd were examined for the presence of ticks in the areas under the tail, perineum, scrotum, udder, preputium, inside the ear, under the neck and on the sternum. Ticks were examined under a stereo microscope (Olympus SZX16) and identified according to their morphological characteristics [24].

DNA Extraction and Amplification

The 2241 ixodid ticks were divided into 310 pools *(Table 1)*. The ticks were pooled based on their sex, host, species, province, and degree of blood sucking. Tick counts ranged from 1 to 32 per pool. Each tick pool's total DNA was extracted using a commercial extraction kit in accordance with the manufacturer's instructions (QIAamp DNA Mini Kit, 51306).

The 181 bp region of the 60kDA chaperonin gene (cpn60 or hsp60) was amplified using JH0011

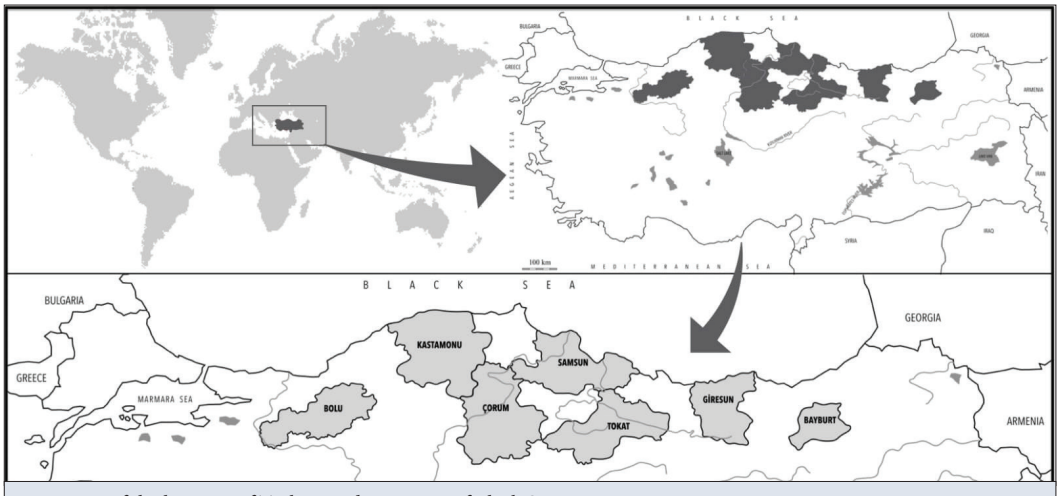

Fig 1. Map of the location of Türkiye and provinces of Black Sea Region

Table 1. *Infection rates for A. ovis and A. phagocytophilum in adult ixodid ticks removed from sheep and goats*

Tick Species	NAT/NAP		Province							Total
			Bolu	Kastamonu	Çorum	Samsun	Tokat	Giresun	Bayburt	NPP, MLE, 95% CI
R. bursa	468/49		1/0/0	99[a]/95[b]/10[c]	24/19/3	187/178/20	68/45/4	132/131/12	0/0/0	9, 20.7, 9.91-37.2
		Ao	-	1[d], 10.6[e], 0.61-46[f]	1, 54.1, 3.11-218	2, 11.5, 1.91-35.1	2, 57.2, 9.61-171	2, 17, 2.81-51.9	-	8, 18.2, 8.31-33.7
		Ap	-	-	-	-	-	1, 7.91, 0.41-34.6	-	1, 2.21, 0.11-9.51
R. turanicus	650/70		94/85/10	1/0/0	337/285/29	178/165/17	113/45/6	78/70/8	0/0/0	30, 58.6, 40.2-81.7
		Ao	2, 26.1, 4.31-78.9	-	15, 71.3, 41.2-113	6, 43.9, 17.5-87.8	2, 52.8, 8.91-157	3, 50.7, 12.7-128	-	28, 53.6, 36.3-75.6
		Ap	-	-	2, 7.11, 1.11-21.9	-	-	1, 15.8, 0.81-68.3	-	3, 4.71, 1.11-12.2
R. sanguineus s.l.	77/6		0/0/0	0/0/0	90/75/5	3/2/1	0/0/0	0/0/0	0/0/0	3, 52.5, 13.1-135
		Ao	-	-	2, 32.3, 5.31-98.1	-	-	-	-	2, 31.2, 5.21-95
		Ap	-	-	1, 14.4, 0.81-62.3	-	-	-	-	1, 14, 0.71-60.5
D. marginatus	367/73		99/83/15	82/77/18	71/63/11	103/93/17	40/16/5	11/6/2	57/30/6	25, 81, 53.8-115
		Ao	1, 12.1, 0.61-52.3	8, 141, 66-251	2, 33.7, 5.61-101	9, 135, 66-236	-	-	5, 339, 123-673	25, 81, 53.8-115
		Ap	-	-	-	-	-	-	-	-
Hae. parva	458/47		153/126/13	6/4/1	73/58/7	156/155/12	54/41/7	0/0/0	190/74/7	4, 9.21, 2.81-21.3
		Ao	-	-	-	4, 30.6, 9.51-70.2	-	-	-	4, 9.21, 2.81-21.3
		Ap	-	-	-	-	-	-	-	-
Hae. punctata	55/13		45/40/8	9/8/3	0/0/0	7/5/1	4/2/1	1/0/0	0/0/0	1, 18.9, 1.01-80.6
		Ao	-	-	-	1, NC	-	-	-	1, 18.9, 1.01-80.6
		Ap	-	-	-	-	-	-	-	-
Hae. sulcata	26/7		13/12/3	2/0/0	0/0/0	4/2/1	16/12/3	4/0/0	0/0/0	1, 41.8, 2.41-173
		Ao	-	-	-	-	-	-	-	-
		Ap	1, 102, 6.01-390	-	-	-	-	-	-	1, 41.8, 2.41-173
Hae. concinna	11/4		5/5/1	1/1/1	2/2/1	4/3/1	0/0/0	1/0/0	2/0/0	1, 95.5, 5.61-359
		Ao	-	-	-	-	-	-	-	-
		Ap	-	-	1, NC	-	-	-	-	1, 95.5, 5.61-359
Hy. marginatum	38/14		10/8/3	16/12/6	10/6/2	1/0/0	12/4/1	13/10/2	0/0/0	-
		Ao	-	-	-	-	-	-	-	-
		Ap	-	-	-	-	-	-	-	-
Hy. excavatum	24/7		0/0/0	2/2/2	25/17/4	0/0/0	5/5/1	1/0/0	0/0/0	1, 41.7, 2.41-171
		Ao	-	-	-	-	-	-	-	-
		Ap	-	1, 500, 38-962	-	-	-	-	-	1, 41.7, 2.41-171
Hy. scupense	9/6		3/3/3	1/1/1	0/0/0	1/0/0	8/5/2	0/0/0	0/0/0	
		Ao	-	-	-	-	-	-	-	-
		Ap	-	-	-	-	-	-	-	-
I. ricinus	58/14		7/6/3	3/2/1	0/0/0	3/1/1	0/0/0	56/49/9	0/0/0	-
		Ao	-	-	-	-	-	-	-	-
		Ap	-	-	-	-	-	-	-	-
Total	2241/310		430/368/59	222/202/42	632/525/62	647/604/71	320/175/30	297/266/33	249/104/13	75, 38.1, 30.1-47.2
		Ao	3, 8.31, 2.01-21.5	9, 49.3, 23.9-87.3	20, 46.1, 28.8-68.8	22, 42.2, 27-62	4, 25.2, 7.81-57.8	5, 20.7, 7.41-44	5, 53.9, 19.6-113	68, 34.1, 26.7-42.8
		Ap	1, 2.71, 0.11-12	1, 5.01, 0.21-21.8	4, 7.81, 2.41-18.1	-	-	2, 7.91, 1.31-24.1	-	8, 3.61, 1.61-6.71

NAT: Number of analyzed tick, NAP: Number of analyzed pool, NPP: Number of positive pool, MLE: Maximum likelihood estimation, CI: Confidence intervals, Ao: Anaplasma ovis, Ap: Anaplasma phagocytophilum, [a] Number of collected tick, [b] Number of analyzed tick, [c] Number of analyzed pool, [d] Number of positive pool, [e] MLE value, [f] CI value, NC: Not calculated (if all pools are positive)

(5'-TAAAAGCCAAGGAGGCTGTG-3') and JH0012 (5'-TTGCTCTCCTCGACCGTTAT-3') primers in order to identify *A. ovis* DNA in ticks [25]. A segment of 492-498 bp in the hypervariable V1 region of the 16SrRNA gene was amplified using primers 16S8FE (5'-GGAATTCAGAGTTGGATCMTGGYTCAG-3')andBGA1B-new (5'-CGGGATCCCGAGTTTGCCGGGRTTYTTCT-3') in order to analyze the sequencing of *A. ovis* [26].

A nested PCR was performed for amplification of *A. phagocytophilum* 16SrRNA gene. Primers EC12A (5′-TGATCCTGGCTCAGAACGAACG-3′) and EC9 (5′-TACCTTGTTACGACTT-3′) were utilized for the initial amplification, which amplifies 1462 bp for all *Anaplasma* and *Ehrlichia* species. Additionally, SSAP2f (5′-GCTGAATGTGGGGGGATAATTTAT-3′) and SSAP2r (5′-ATGGCTGCTTCCTTTCGGTTA-3′) were utilized to amplify 641 bp of *A. phagocytophilum* [27].

Sequencing and Phylogenetic Analyses

Sequence analysing was performed (Macrogen, South Korea) after purification of PCR products by a commercial kit (Qiagen, Hilden, Germany, 28004). The *A. phagocytophilum* (MH636805, MH643970, MH715976, and MT498084-MT498088) and *A. ovis* (MH636802) partial 16S rRNA gene sequences found in this investigation have been added to GenBank. To compare each sequence to the other sequences in the NCBI database *(http://www.ncbi.nlm.nih.gov/nuccore),* BLAST similarity searches were performed. The MAFFT Multiple Sequence Alignment Software Version 7 used the neighbor-joining approach to create a phylogenetic tree from the sequences of the 186 rRNA genes of *Anaplasma* species [28].

Calculation of Infection Rates in Tick Pools

Using the algorithm MLE_IR by Gu et al.[29], the ratio of infected tick numbers in positive tick pools was estimated by calculating the maximum likelihood estimation (MLE) of infection rates with 95% confidence intervals (CI) per 1000 ticks. Because it requires no additional data and produces more accurate results in small pool sizes, the MLE_IR algorithm was chosen.

Results

Prevalence and Distribution of *Anaplasma* spp. by PCR

In all, 2241 ixodid ticks from 12 species and 5 genera were gathered and split up into 310 pools. *Table 1* shows the prevalence of *A. ovis* and *A. phagocytophilum* infections in ixodid ticks by tick species and province. Out of the 310 pools, 75 (23.25%) were found positive to *A. ovis* and/or *A. phagocytophilum*, and the overall MLE of infection rate was 38.1 (CI 30.1-47.2).

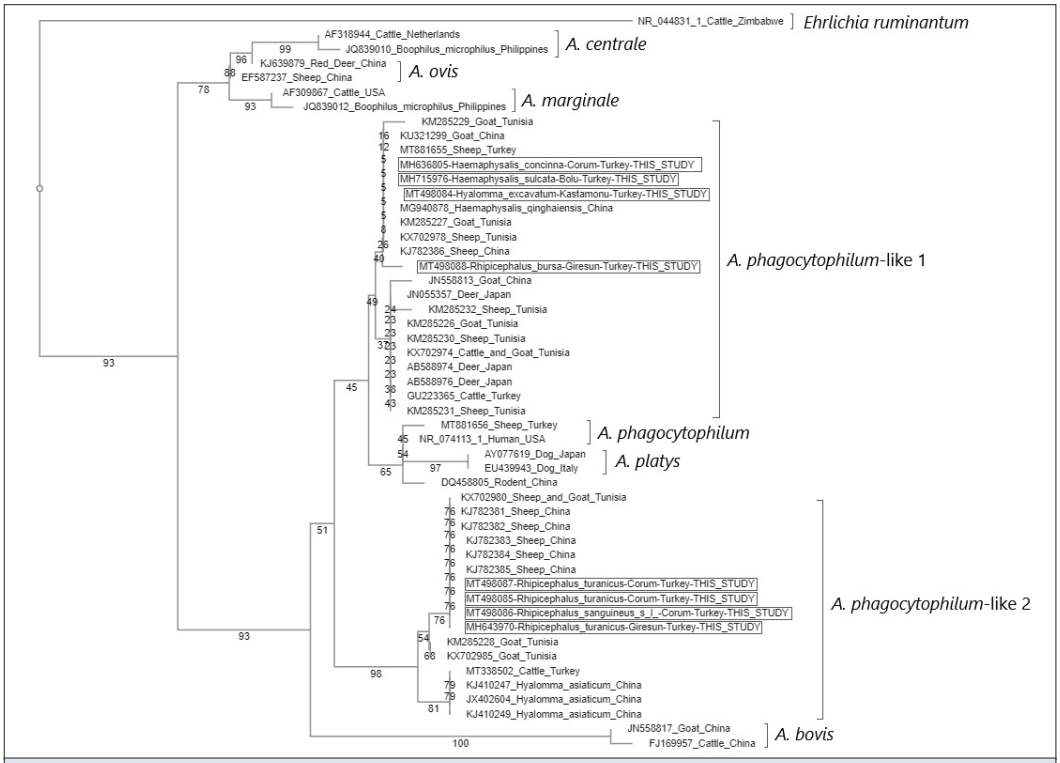

Fig 2. Neighbor-joining analysis of the 16S rRNA gene of the *Anaplasma* species were determined in this study and those present in the GenBank database. Numbers above the branch demonstrate bootstrap support from 1000 replications. The tree was constructed by using the MAFFT Multiple Sequence Alignment Software Version 7. The sequences were given as GenBank accession number, the strain or isolate name, host or vector and country. Sequences described in this study are with outer frame

Table 2. *Homology and genetic distance (%) between 16S rRNA sequences (598–599 bp) from Anaplasma phagocytophilum-like 1 and 2 detected in ticks from Türkiye (*) and other Anaplasma phagocytophilum-like 1 and 2 isolates found in GenBank. The numbers represent the nucleotide identity rates found between the sequences*

Genotypes	**Host or vector**	**Genetic variant**	**Country**	**GenBank**		**Genotype Row**															
						1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**
A. phagocytophilum-like 1	*Hae. concinna*	H107	Türkiye	MH636805*	**1**	100	100	100	99.82	99.82	100	100	99.65	98.57	98.57	98.59	98.59	98.59	98.74	98.56	98.41
A. phagocytophilum-like 1	*Hae. sulcata*	H5	Türkiye	MH715976*	**2**		100	100	99.82	99.83	100	100	99.66	98.57	98.57	98.27	98.30	98.32	98.61	98.61	98.47
A. phagocytophilum-like 1	*Hy. excavatum*	H101	Türkiye	MT498084*	**3**			100	99.82	99.82	100	100	99.64	98.57	98.57	98.57	98.57	98.57	98.74	98.56	98.56
A. phagocytophilum-like 1	*R. bursa*	H256	Türkiye	MT498088*	**4**				100	99.64	99.82	99.82	99.47	98.39	98.39	98.40	98.40	98.40	98.56	98.38	98.23
A. phagocytophilum-like 1	Goat	ApGGo4	Tunisia	KM285229	**5**					100	99.67	99.83	99.50	98.38	98.38	98.38	98.41	98.33	98.33	98.33	98.16
A. phagocytophilum-like 1	Sheep	Akseki26	Türkiye	MT881655	**6**						100	99.83	99.50	98.56	98.56	98.56	98.59	98.33	98.33	98.33	98.16
A. phagocytophilum-like 1	*Hae. qinghaiensis*	13QHF23	China	MG940878	**7**							100	99.67	98.56	98.56	98.56	98.59	98.50	98.50	98.50	98.33
A. phagocytophilum-like 1	Cattle	GS29	Türkiye	GU223365	**8**								100	98.57	98.57	98.59	98.62	98.58	98.50	98.50	98.03
A. phagocytophilum-like 2	*R. turanicus*	H141	Türkiye	MT498087*	**9**									100	100	100	100	100	99.82	99.64	99.64
A. phagocytophilum-like 2	*R. turanicus*	H102	Türkiye	MT498085*	**10**										100	100	100	100	99.82	99.64	99.64
A. phagocytophilum-like 2	*R. sanguineus*	H134	Türkiye	MT498086*	**11**											100	100	100	99.82	99.64	99.31
A. phagocytophilum-like 2	*R. turanicus*	H243	Türkiye	MH643970*	**12**												100	100	99.65	99.65	99.32
A. phagocytophilum-like 2	Sheep	YC38	China	KJ782381	**13**													100	99.67	99.67	98.91
A. phagocytophilum-like 2	Goat	ApGGo3	Tunisia	KM285228	**14**														100	99.33	99.17
A. phagocytophilum-like 2	Cattle	S9	Türkiye	MT338502	**15**															100	99.83
A. phagocytophilum-like 2	*Hy. asiaticum*	BL099-6	China	KJ410247	**16**																100

Anaplasma ovis was detected in 68 out of the 310 tick pools (21.93%) from all cities belonging to six tick species *(R. bursa*, *R. turanicus*, *R. sanguineus* s.l., *D. marginatus*, *Hae. parva* and *Hae. punctata*) with an overall MLE of infection rate of 34.1. While highest MLE of infection rate for *A. ovis* was in *D. marginatus* and in Bayburt city, lowest values were detected in *Hae. parva* and in Bolu city. *Anaplasma ovis* DNA has not been detected in *Hyalomma marginatum*, *Hy. scupense* and *I. ricinus*. Under accession number MH636802, one typical sample sequence for *A. ovis* may be found in the GenBank, EMBL, and DDBJ databases.

Anaplasma phagocytophilum was detected in the six tick species *R. bursa, R turanicus, R. sanguineus* s.l., *Hae. Sulcata, Hae. concinna* and *Hy. excavatum*, from Bolu, Kastamonu, Çorum and Giresun cities. *Anaplasma phagocytophilum* DNA was detected in eight of the 310 (2.58%) tick pools, resulting in an overall MLE of infection rate of 3.61. While MLE of infection rate was highest in Giresun city, lowest value was recorded in Bolu city. The MLE of infection rate for *A. phagocytophilum* differed by tick species, ranging from 2.21 in *R. bursa* to 95.5 in *Hae. concinna*. The bacterium DNA has not been detected in *D. marginatus, Hae. parva, Hae. punctata, Hy. marginatum, Hy. scupense* and *I. ricinus* ticks nor from Samsun, Tokat and Bayburt cities. Nucleotide sequences of all positive samples for *A. phagocytophilum* are available under accession numbers of, MH636805, MH643970, MH715976 and MT498084 - MT498088.

Molecular and Phylogenetic Analyses

All PCR positive samples in terms of *A. phagocytophilum* were sequenced to validate PCR results and to determine the variants. Phylogenetic analysis revealed that *A. phagocytophilum* variants determined with this study clustered in two different clades. The *Hae. concinna*, *Hae. sulcate*, *Hy. excavatum*, and *R. bursa* variations H107, H5, H101, and H256, respectively, formed a unique group with the *A. phagocytophilum*-like 1 cluster seen in ruminants and *Haemaphysalis qinghaiensis*. But the H141, H102, H134, and H243 variations from *R. turanicus* and *R. sanguineus* grouped together with the *A. phagocytophilum*-like 2 cluster found in ruminants and *Hyalomma asiaticum (Fig. 2)*. This study's isolates of *Anaplasma phagocytophilum*-like 1 and 2 shared 99.47-100% and 99.31-100% identity with other isolates of *A. phagocytophilum*-like 1 and 2 that are listed in GenBank *(Table 2)*.

Discussion

Rhipicephalus bursa is the main vector for *A. ovis*. However, it was reported that other tick species including *Dermacentor* spp., *Rhipicephalus* spp. and *Hyalomma* spp. can transmit *A. ovis* [11]. In this study, *A. ovis* DNA was detected in *R. bursa*, *R. turanicus*, *R. sanguineus* s.l., *D. marginatus*, *Hae. parva* and *Hae. punctata*. However, the detection of DNA of a pathogen in a tick species is not enough to consider it as a competent vector in the transmission of this pathogen to a host [30]. *Anaplasma ovis* was detected in 68 out of the 310 tick pools (MLE 34.1, CI 26.7-42.8) from all the surveyed cities. Our findings are consistent with earlier research showing that *A. ovis* is highly prevalent in different Turkish locations. Furthermore, a recent study showed that *A. ovis* has low prevalence (0.41% CI 0.02-2.01) in *R. bursa* ticks collected from humans in Türkiye [31]. *Anaplasma ovis* DNA was detected in *R. sanguineus* s.l. [32], *Hae. punctata* [33], *R. bursa* [31] and *D. marginatus* [32] with this study and our findings agree with those from previous studies. *Anaplasma ovis* is a prevalent tick-borne agent in small ruminants globally and although it produces generally mild infections, some cases have severe pathology. In this study we describe high tick infestations with *A. ovis*, therefore it can result with high animal infection rates in the region. Our results indicate that *A. ovis* is present in which is in accordance with several previous studies and further emphasize the possible high risk of *A. ovis* transmission to both animals and humans in the region.

Anaplasma phagocytophilum, a tick-borne rickettsial microorganism, causes granulocytic anaplasmosis or tick-borne fever in horses, dogs and other animals. Furthermore, it is the agent of human granulocytic anaplasmosis (HGA) [6]. Its presence was already shown in domestic animals [17,18,22] and ticks [31] in Türkiye. Infection rates of *A. phagocytophilum* were very low in ticks when compared to *A. ovis*. Similarly, other Turkish and foreign studies found lowest prevalence of *A. phagocytophilum* in hosts [22] and ticks [34,36] compared to *A. ovis*. Although *Ixodes* spp. is considered the main vector for *A. phagocytophilum* [37], there is a speculation about other potential tick vectors of *A. phagocytophilum* [38]. In the present study *A. phagocytophilum* was detected in *R. bursa*, *R. turanicus*, *R. sanguineus* s.l., *Hae. sulcata*, *Hae. concinna* and *Hy. excavatum*. Previous studies have shown the presence of *A. phagocytophilum* DNA in *I. ricinus* [4], *Hy. marginatum, Hy. excavatum* [40] and in *Hae. sulcata* [31] in Türkiye. Furthermore, *A. phagocytophilum* DNA has also been amplified from *I. ricinus* [36,41], *Ixodes persulcatus* [38], *Hy. marginatum* and *Hyalomma lusitanicum* [34], *Hyalomma anatolicum* [35], *Hae. concinna* [36,38], *Hae. longicornis* [42], *Hae. punctata* [33], *Dermacentor* spp. [36,38] and *R. sanguineus* s.l. [43-45]. Interestingly, *A. phagocytophilum* has not been detected in any of the *I. ricinus* ticks in the present study. This may be due to low sample number of *I. ricinus* from the studied area. Furthermore, this species is frequently found along the Black Sea coast, and while the ticks sampled in this study were representative of the Black Sea region, they

were primarily from inland. Primers SSAP2F and SSAP2r are commonly used for the detection of *A. phagocytopilum* in ruminants [19,22] and ixodid ticks [39]. However, they are also known to detect other strains genetically related to *A. phagocytophilum* such as *Anaplasma* sp. Japan [46] and *Anaplasma* sp. China [19], finally designed as *A. phagocytophilum*-like 1 and *A. phagocytophilum*-like 2 [16,47]. Therefore, all the positive samples were sequenced, and it has been shown that *A. phagocytophilum*-like 1 and 2 variants circulate in ticks in the region. In the previous studies carried out in Türkiye, *A. phagocytophilum*-like 1 and *A. phagocytophilum*-like 2 strains were detected in cattle [14] and small ruminants [4,17,18]. On the other hand, this is the first account of *A. phagocytophilum* – like 1 and 2 strains in ticks in the nation. Similar to our study, *A. phagocytophilum*-like 2 strain was detected in *R. turanicus* collected from small ruminants in Tunisia [48]. It is concluded that there may be other main vectors for *A. phagocytophilum* except *I. ricinus* and we suggest futher studies about *A. phagocytophilum* variants in ixodid ticks. Even though the zoonotic potential of these variants has not yet been established, we think that the distinction between *A. phagocytophilum*-like 1 and 2 in human and animal cases would be helpful in this respect.

In the studies on *A. phagocytophilum* variants in Türkiye, the presence and distribution of *A. phagocytophilum* - like 1 and 2 were investigated in cattle, sheep, goats and buffaloes [14,17,18,49]. This study provided important data on the presence of these variants in ticks in Türkiye. The data obtained in this study suggest that these variants should be taken into consideration in the differential diagnosis of tick-borne infections in the Black Sea region, where tick contact is intensively seen in humans and animals. Presence and distribution of *Anaplasma* species is influenced by several factors including climate condition and tick diversity of the region [50]. In this investigation, *A. phagocytophilum* was found in tick vectors in both humid and terrestrial climates, despite *I. ricinus* being the predominant species in humid regions. This is also similar to other findings from the region [31]. This could support the theory that *A. phagocytophilum*'s ecology and epidemiology, particularly its transmission to animal host species, may be significantly influenced by other tick species.

In conclusion, *A. ovis* and *A. phagocytophilum* were investigated in a large geographic area of the Black Sea Region of Türkiye. *Anaplasma ovis* was detected with high prevalence (MLE 34.1) in six tick species *(R. bursa, R. turanicus, R. sanguineus* s.l., *D. marginatus, Hae. parva and Hae. punctata*) and in all investigated provinces. As the first record in Türkiye, *A. phagocytophilum*-like 1 and *A. phagocytophilum*-like 2 strains were also found in tick species *(A. phagocytophilum*-like 1 in *Hae. concinna, Hae. sulcate*, *Hy. excavatum* and *R. bursa*; *A. phagocytophilum*-like 2 in *R. turanicus* and *R. sanguineus* s.l.,) but not in its main European vector, *I. ricinus*. Additionally, this bacterium was detected only in four provinces (Bolu, Kastamonu, Çorum and Giresun) and with a lowest MLE of infection rate (3.61%) when compared to *A. ovis*. These findings showed that *A. ovis* and *A. phagocytophilum* are common in the area and pose a serious risk to the health of people and animals. We hope these data will help to sensitize for the implementation of anaplasmosis control methods in the region.

Declarations

Availability of Data and Materials: When needed, the corresponding author (M.F. Aydın) can provide the data and materials used in this study.

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Research Article

Anatomy of the Knee Joint in Cattle (*Bos taurus*): Gross, Radiographic and Computed Tomographic Insights

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Abstract

Limb joint diseases significantly reduce domestic animals' productive and reproductive capabilities. The present study was conducted to examine the anatomical features of the knee joint in cattle. Four naturally dead cattle of 2-3 years were collected and used in this study to record gross anatomical, radiographical characteristics and computed tomographic (CT) scans of the knee joints. Radiographs and CT scan images provided 2D and 3D visualization of the knee joint that was made up of articulatio antebrachiocarpea, articulatio intercarpea, and articulatio carpometacarpea. The synovial sacs of the intercarpal and carpometacarpal articulations were interconnected. The interosseous ligament connected the depression on the posterior margin of the distal radius with the intermediate and ulnar carpal bones. In the intercarpal articulation, one interosseous ligament was observed between the non-articular area on the distal surface of the intermediate carpal and the non-articular area of the fourth carpal. In the carpometacarpal joint, an interosseous ligament was observed connecting the non-articular area on the lateral surface of the fused second and third carpals to the non-articular area at the proximal end of the large metacarpal. The fibers of this ligament were blended with the interosseous ligament that connects the two carpal bones in the distal row. This study can help as an analytic tool for definitive diagnosis, prognosis, and research of the cattle knee joint.

Keywords: Carpal bones, Cattle, Computed tomography, Knee joint, Ligament, Radiography

Introduction

The conditions affecting the joints of animal limbs significantly impair their productive and reproductive capabilities [1]. The carpal joint is referred to as a common cause of forelimb lameness in cattle [2,3]. The carpal region is a complex thoracic limb part comprising various structures, including the knee joint, ligaments, tendons, and muscles [2-5].

The carpal joint consists of three main joints: the antebrachiocarpal joint, intercarpal joints, and carpometacarpal joints, among all the carpal bones [6-12]. The knee joint may experience pathological changes affecting the joint structures, ligaments, and tendons in the carpal region [13-16]. Furthermore, bovine carpal joints provide a cost-effective, safe, and easily reproducible model for educating on basic ligament studies and their repair techniques before patient interaction [17].

Diagnostic imaging techniques, such as radiography and CT scans, can enhance the chances of a definitive diagnosis, potentially improving the prognosis and treatment outcomes in affected cattle [18]. CT scans have shown significant utility in diagnosing various musculoskeletal conditions in veterinary medicine [19]. Their primary advantages over radiography and ultrasonography include three-dimensional imaging and the ability to simultaneously visualize bone and soft tissue structures without overlapping. CT scan offers advantages such as improved bone contrast and reduced examination time. Additionally, there is increasing interest in utilizing CT scans and MRI for bovine orthopedic applications [20]. Comprehensive information exists regarding the anatomy of joints in horses, dogs, pigs, and ox, as outlined in standard textbooks on veterinary anatomy [5,21-23]. The data generated from this study can enhance diagnostic accuracy by establishing a baseline for normal radiographic and CT findings. This baseline aids in distinguishing between normal variations and pathological changes in cattle limbs. Furthermore, the information can facilitate the

early detection of conditions such as osteochondrosis, arthritis, or fractures. Comprehensive anatomical and imaging insights allow for better planning of minimally invasive surgical procedures, including arthrotomy or joint stabilization techniques, guiding arthroscopy. This approach can reduce recovery times and improve overall outcomes.

This study aims to thoroughly characterize the anatomy of cattle knee joints through gross observation, radiography, and computed tomography. The nomenclature from the Nomina Anatomica Veterinaria was used in this article to describe the anatomical terminologies with respect to cattle knee joints [24].

Materials and Methods

Ethical Statement

Institutional animal ethics approval is not necessary for this study because the samples were collected from cattle that died naturally.

Animals

The present study was conducted on the eight knee joints (right and left forelimbs from each animal) of the cattle. The duration of the study was January 2024 to September 2024. Four naturally dead cattle were obtained from a Gaushala near the College of Veterinary Science, Guru Angad Dev Veterinary and Animal Sciences University, Rampura Phul, Punjab, as well as from the Teaching Veterinary Clinical Complex (TVCC) of the college. After postmortem examination, the samples were processed using standard anatomical techniques for this study.

Processing of Samples for Gross Anatomy

After deskinning the collected cattle, the internal viscera/organs were removed carefully, preserved in a 10% buffered formalin solution, and used for undergraduate student teaching. For this study, the knee joints were cut by the electric saw and macerated with a hot water maceration technique to remove the skin and fascia over the knee joint. The gross photographs of the cattle knee joints were captured by the Apple iPhone 14 Pro in the department and labeled accordingly with the help of Adobe Photoshop version 24.0.

Radiography and CT Scan of the Knee Joints

The radiographs were taken from the X-ray machine (Toshiba ROTANODE™-Toshiba X-Ray Tube E7869X) from the Department of Veterinary Surgery and Radiology, College of Veterinary Science, Guru Angad Dev Veterinary and Animal Sciences University, Rampura Phul, Punjab. CT scans of the knee joint were taken from the dorsal, ventral and lateral aspects using a CT scan machine (General Electronic company, Japan: GE Prospeed F2) with the following custom: X-ray tube potential: 120 kV, tube current: 80 mA, slice thickness: 3 mm. Image analysis was conducted on a desktop computer using specialized software (RadiAnt DICOM Viewer 2024.2) for multiplanar 3D reconstructions. The images were assessed to visualize and identify bone, cartilage, and soft-tissue structures of the cattle knee joint.

Results

The gross anatomical, radiographical and CT scan anatomy of the knee joint of cattle was demonstrated in *Fig. 1*, *Fig. 2*, *Fig. 3*, *Fig. 4*, *Fig. 5*, and *Fig. 6*. Two- and three-dimensional (2D and 3D) images produced by radiography and CT scans were used to analyze the cattle knee joint *(Fig. 3*, *Fig. 4*, *Fig. 5*, and *Fig. 6)*. The carpal or knee joint in cattle was a composite diarthrodial joint, often referred to as the knee joint in animals. It comprises three main articulations: the articulatio antebrachiocarpea (radio-carpal joint), formed by the distal extremity of the radius and ulna (styloid process), the proximal carpal row (radial, intermediate, ulnar, and accessory carpal); articulatio intercarpea (the intercarpal joint), situated between the proximal and

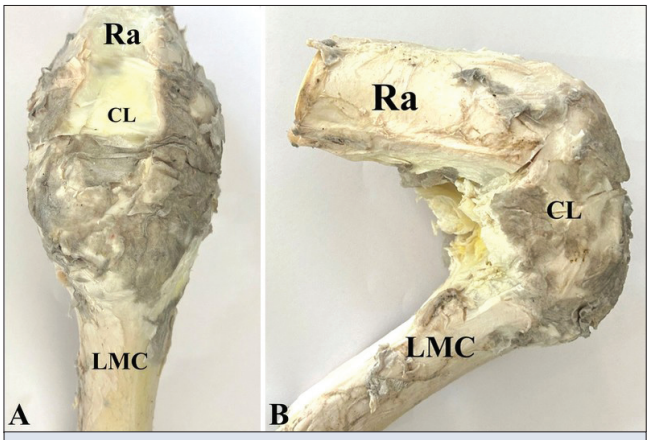

Fig 1. Dissected knee joint (A, B) of cattle (left limb) showing radius (Ra), capsular ligament (CL) and large metacarpal bone (LMC)

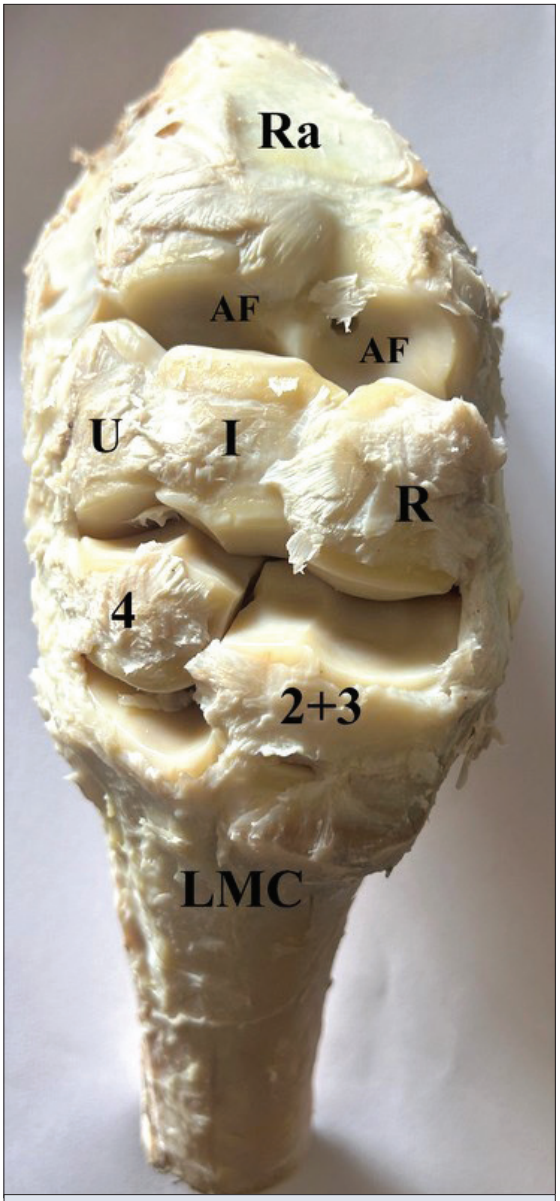

Fig 2. Dissected knee joint of cattle (right limb) showing radius (Ra) articular facets of radius (AF), carpal bones of proximal and distal row (R, I, U, 2+3, 4), and large metacarpal bone (III)

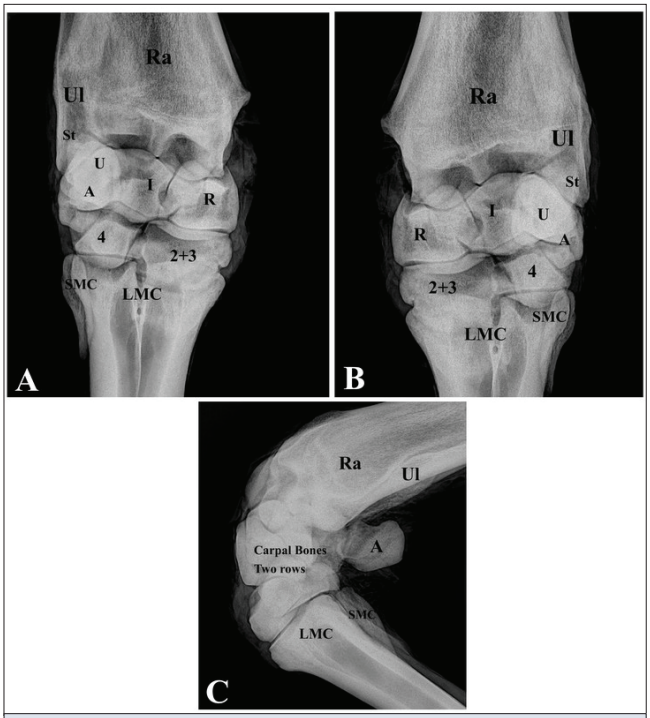

Fig 3. Radiographs (A, B, C) of the knee joint (left limb) of cattle showing radius (Ra), ulna (Ul), radial, intermediate, ulnar and accessory carpal bones (R, I, U, A) in the proximal row; 2+3, 4th in the distal row, fused third and fourth large metacarpal (LMC-III+IV) and fifth small metacarpal (SMC-V)

distal rows (second-third fused and fourth carpal) of carpal bones; and the articulatio carpometacarpea (carpometacarpal joint), which connects the distal carpal row to the proximal end of the large metacarpal bone (III+IV) and the structures adjacent to these osseous components.

The radiographs and CT images clearly show the two rows of carpal bones, which were distinctly separated by the radiocarpal, intercarpal, and carpometacarpal joints *(Fig. 3, Fig. 5)*. To better understand the cattle knee joint in three dimensions, 3D reconstruction was created and demonstrated *(Fig. 6)*.

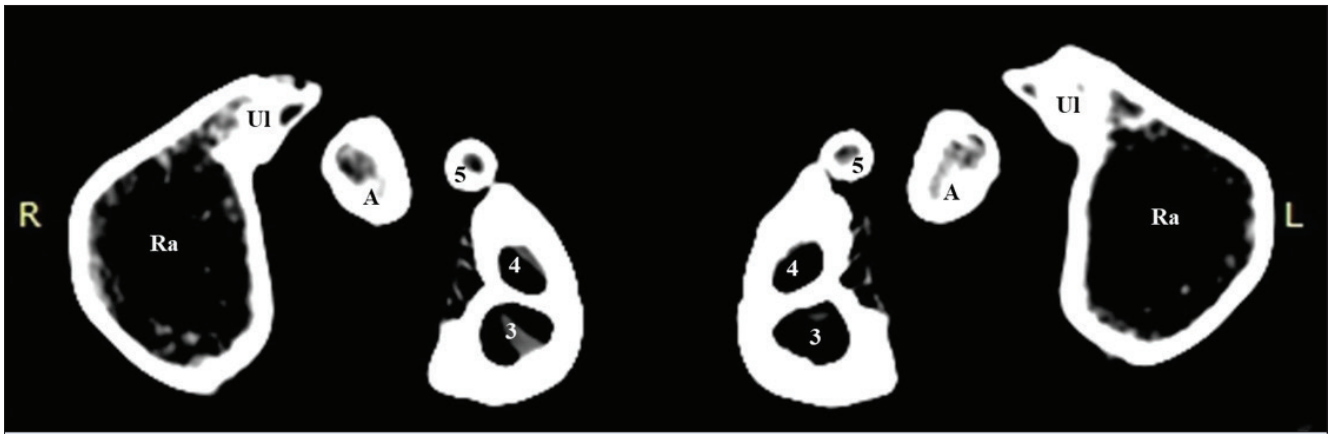

Fig 4. Cross-sectional computed tomographic (CT) image of the knee joint of right (R) and left (L) side of cattle showing radius (Ra), ulna (Ul), and accessory carpal bones (A), two medullary cavities of large metacarpal (III+IV) and small metacarpal (V)

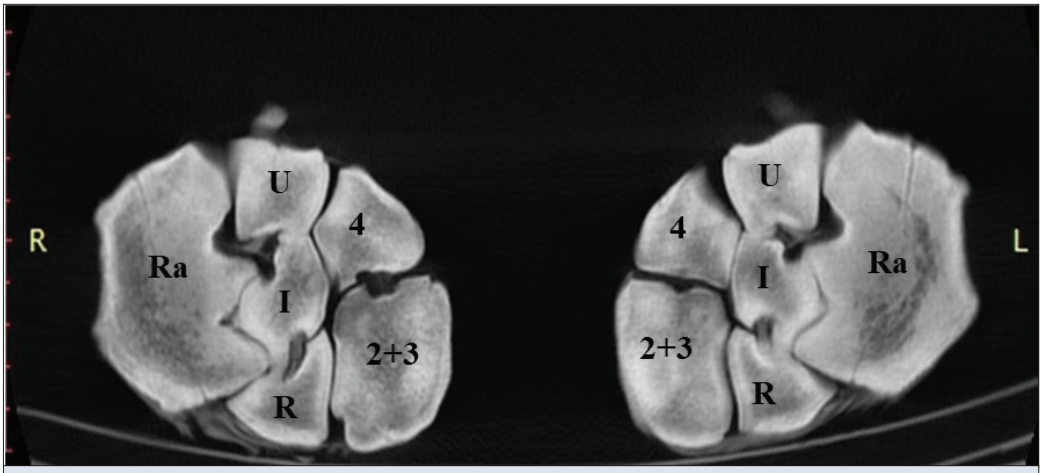

Fig 5. Transverse CT scans of the cattle knee joint of the right (R) and left (L) side showing radius (Ra), radial, intermediate and ulnar (R, I, U, A) in the proximal row, 2+3, 4th in the distal row

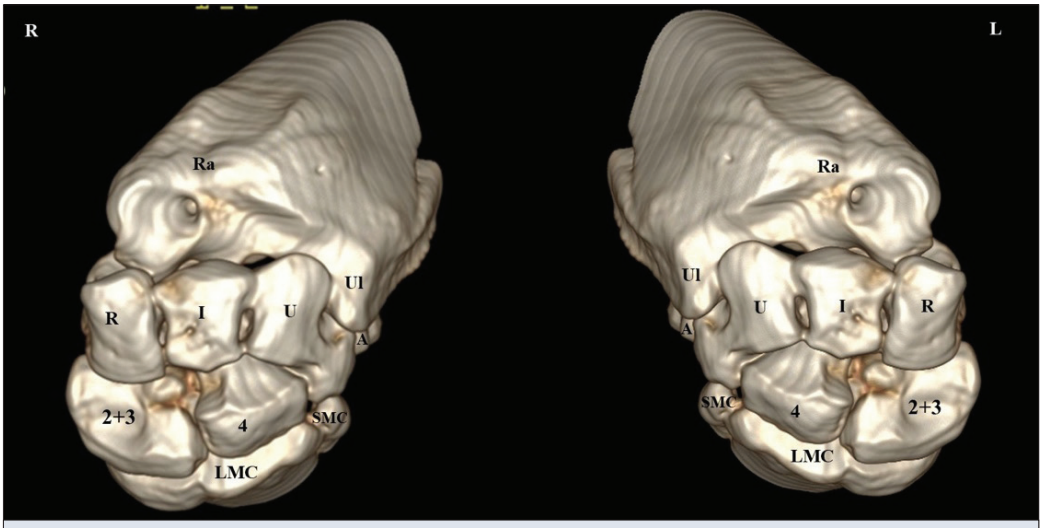

Fig 6. 3D computed tomographic (CT) images of the cattle knee joint of the right (R) and left (L) side showing radius (Ra), ulna (Ul), radial, intermediate, ulnar and accessory carpal bones (R, I, U, A) in the proximal row, 2+3, 4th in the distal row, large metacarpal (LMC) and small metacarpal (SMC)

The results showed that the anatomical features of the cattle knee joint resembled small and large ruminants, and few differences were noticed among other domestic animals, as discussed in this study.

Discussion

The gross, radiographic and CT scan morphology of cattle knee joint was composed of a diarthrodial joint formed by the articulatio antebrachiocarpea, articulatio intercarpea and the articulatio carpometacarpea, as reported earlier in buffalo calves, bovines, cattle and ox [1,2,21,25]. In contrast to this finding, the distal end of the ulna was not involved in the formation of the carpal joint in donkeys [12]. This is because the ulna does not extend to the distal end of the radius and is limited to the distal third of the radius. The radiographs and CT images visualize the two rows of carpal bones, which were distinctly separated by the radiocarpal, intercarpal, and carpometacarpal joints. Also, the metacarpus in cattle comprised a large metacarpal bone and a smaller external metacarpal bone. The large metacarpal bone (III+IV) is formed by the fusion of the third and fourth metacarpal bones during fetal development, with remnants of this dual origin still observable in adulthood. The smaller metacarpal bone (V) is positioned along the upper outer edge of the large metacarpal bone and does not contribute to the articulation with the carpus as mentioned in bovines and ox [2,5]. The knee joint capsule of cattle was comprised of a common outer fibrous capsular ligament and three inner synovial pouches, one for each joint (radiocarpal, intercarpal, and carpometacarpal joint) as described previously in buffalo calves [1].

In the present study, the dorsal ligament of the knee joint was membranous, with its superior border attached to the radius and its inferior border attached to the proximal extremity of the large metacarpal, as reported earlier

in buffalo calves [1]. The right and left borders were united with the medial and lateral collateral ligaments of the knee joint, as reported in buffalo calves [1]. It was strengthened dorsally by the retinaculum extensorium, which surrounded the extensor tendons of the extensor muscles of the forearm region.

The volar carpal ligament was thick and strong, extending from the distal articular surface of the radius to the proximal end of the large metacarpal. The retinaculum flexorium arising from the accessory carpal to bridge the sulcus carpi was attached to the bones of the carpus and the metacarpus region in the present study, as confirms the findings of [1] in buffalo calves.

The lateral collateral ligament of the knee joint is a flat band attached proximally to the distal end of the radius and the styloid process of the ulna, as reported in ox [21]. Distally, it connected to the proximal ends of the large and small metacarpals, as observed earlier in buffalo calves [1]. Small and deeply placed, the proximal, middle, and distal limbs of the lateral collateral ligament connected the ulna to the ulnar carpal, the ulnar carpal to the fourth carpal, and the fourth carpal to the large metacarpal as reported in buffalo calves [1,26]. The previous study described the two deep branches as extending from the styloid process of the ulna, with one limb going to the ulnar carpal and the other to the fourth carpal [22].

The medial collateral ligament was thick, wide, and strong and extended between the styloid process of the radius and proximal extremity of the large metacarpal, as also reported in buffalo calves [1]. The small, short, deeply placed proximal, middle, and distal limbs connected the distal end of the radius and the radial carpal, radial carpal, and the fused second and third carpal and fused second and third carpal and large metacarpal, respectively. The medial collateral ligament was stronger in the cattle knee joint due to the axis of the limb having a medial deviation at the carpus, as earlier reported in buffalo calves [1].

The radio-ulnar-carpal articulation (articulatio antebrachiocarpea) is a ginglymus joint formed by the radius, ulna, and the proximal articular surfaces of the first row of carpal bones, as noted in buffalo calves and bovines [1,2]. The articular surface of the radius was elongated and featured three oblique facets for the radial, intermediate, and ulnar carpals. In contrast, the ulna (specifically the styloid process) had a single articular facet for the ulnar carpal. Additionally, the accessory carpal was articulated with the ulnar carpal at the posterior aspect through a single articular facet.

The articulatio antebrachiocarpea consisted of radio-carpal (articulatio radiocarpea) and ulnar-carpal (articulatio ulnocarpea). This joint is supported by one oblique ligament, four posterior ligaments, and one interosseous ligament. The oblique ligament, known as the ligamentum radiocarpium dorsale, extended from the dorsal aspect of the distal end of the radius to the ulnar carpal. On the volar aspect, four ligaments were extended from the radius to the radial carpal, from the radius to the intermediate carpal in two bands, from the radius to the ulnar carpal, and from the ulna to the ulnar carpal. The interosseous ligament connects the depression located in the middle of the posterior margin of the distal end of the radius to the non-articular areas on the lateral surface of the intermediate carpal and the medial surface of the ulnar carpal. This has also been reported in buffalo calves [1], where the authors described the ligament as 'λ' shaped. Additionally, two oblique and three posterior ligaments were identified in ox [21] and yaks [27].

The composite hinge joint between the proximal and distal row of carpal bones was articulatio mediocarpea, as reported in buffalo calves [1]. Two oblique anterior ligaments, four posterior ligaments, and one interosseous ligament were observed. One of the two oblique ligaments extended between the radial and fourth carpal, while the other connected the ulnar carpal to the fourth. On the volar aspect, the ligaments connected the radial carpal to the fused second and third carpals, the intermediate carpal to the fourth carpal, the ulnar carpal to the fourth carpal, and the accessory carpal to the fourth carpal. Additionally, one interosseous ligament was found between the non-articular area on the distal aspect of the intermediate carpal and the non-articular area of the fourth carpal. Four posterior ligaments were noted in the ox [21,23].

The articulatio intercarpea (intercarpal articulations) was formed between the carpal bones of the same row of the proximal and distal rows. These articulations were characterized by well-defined articular surfaces, ensuring stability and functionality within the cattle knee joint, as reported previously in buffalo calves [1]. The articulatio intercarpea proximale were plane (arthrodial) type and consisted of three ligaments intercarpea dorsalia (dorsal internal carpal ligament) and two ligaments intercarpea volaria (volar intercarpal ligament). On the dorsal aspect, the dorsal intercarpal ligaments connect the radial carpal to the intermediate carpal, the intermediate carpal to the ulnar carpal, and the ulnar carpal to the accessory carpal, as reported previously in buffalo calves [1]. On the volar aspect, the volar intercarpal ligaments connect the intermediate carpal to the ulnar carpal and the ulnar carpal to the accessory carpal. Three interosseous ligaments connected the non-articular areas of the adjacent carpal bones. The articulatio intercarpea distale was plane (arthrodial) type and had one dorsal, one volar, and an interosseous ligament between the fused second-third carpal and fourth carpal. The interosseous ligament connected the non-articular areas of the lateral surface of

the fused second and third carpals to the medial surface of the fourth carpal, as reported previously in buffalo calves [1].

The articulatio carpometacarpea (carpometacarpal articulation) was an amphiarthrodial joint formed by flattened articular surfaces of carpal bones of the distal row and those of the large metacarpal as reported in buffalo calves [1]. Two dorsal and two volar ligaments connected fused second & third carpal and fourth carpal separately to the large metacarpal. An interosseous ligament was present, connecting the non-articular area of the lateral surface of the fused second and third carpals to the non-articular area at the proximal end of the large metacarpal. The fibers of this ligament blended with the interosseous ligament that connects the two carpal bones of the distal row, as noted in buffalo calves [1], but this blending was not observed in the white cattle by the same authors. The presence of two anterior ligaments, two posterior ligaments, and two interosseous ligaments was described earlier in ox [19]. The radiocarpal (articulatio antebrachiocarpalis) and midcarpal joints (articulatio mediocarpalis) allowed for flexion, extension, adduction, and abduction. The intercarpal joints (articulatio intercarpalis) exhibited slight gliding movements, whereas the carpometacarpal joints (articulatio carpometacarpalis) showed no movement. The individual carpal bones featured well-developed articular facets. Additionally, the interosseous and collateral ligaments were robust structures that limited joint movement, as noted in buffalo calves [1]. The present study provided a detailed gross and radiographic anatomy of the cattle's knee joint. The structures of cattle's knee joints were similar to those of small and large ruminants; however, they differed from those of other domestic animals.

Several technological advancements in veterinary anatomy have been revolutionizing this discipline. However, the need for gross specimens in veterinary anatomy education cannot be replaced by artificial intelligence applications, as dissections provide valuable insight into anatomical structures [28-31]. This study conducted a detailed examination of the knee joint anatomy of cattle and suggested that dissected specimens are more valuable than online veterinary anatomy content and AI applications.

Detecting fractures in the complex carpal joints of domestic animals can be challenging with radiography due to overlapping bone structures. In such cases, CT scans help identify fractures and determine their location and configuration [12,32,33]. The use of cross-sectional imaging techniques in CT has significantly enhanced the ability of radiologists to make proper diagnoses. The application of CT in bovine orthopedics is constrained by factors such as cost, limited availability, and the requirement for general anesthesia [34]. However, despite these costs, CT scans should be considered valuable diagnostic tools, particularly for economically significant cattle. Early diagnosis using these imaging techniques can help prevent financial losses associated with delayed identification of conditions and their prognosis [18].

In summary, the anatomical and imaging of the knee joint of cattle resembled that of other ruminants. The present study provided an in-depth analysis of cattle knee joints that can be used in pathological conditions related to the joint. Cross-sectional imaging provides a solution for assessing complex structures when radiographic interpretation proves challenging, as it spatially separates overlapping structures seen in standard radiographs. This enables precise evaluation of the number and anatomical details of the bones. This study also offers valuable anatomical insights into the cattle knee joint and can add new data to the available literature, benefiting clinical practice and research.

Declarations

Availability of Data and Materials: The data that support the findings of this study are available from the corresponding author (O.P. Choudhary) upon reasonable request.

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Research Article

Evaluation of Gum Arabic Extract for Enhancing Growth Performance, Blood Parameters, Oxidative Status, Gut Microbiota, and Organ Histology in Heat-Stressed Rabbits

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Abstract

The study investigated the bioactive properties and effects of gum arabic (GA) on various biological parameters, including antioxidant, antimicrobial, anticancer, and growth-promoting activities, as well as its impact on blood biochemistry, oxidative status, immunity, and gut microbiota in heat-stressed rabbits. The main compounds in the GC/MS profile of GA were saccharides, i.e., arabinose, galactose, and rhamnose accounted for 41%, followed by phenolic acids with 36.1%. GA extract (10 %) contains 66 mg/g phenolic compounds and 24 mg/g flavonoids, demonstrating a significant % antioxidant activity of 91% and potent antimicrobial activity against pathogenic microorganisms; it also showed promising anticancer activity, reducing MCF-7 cancer cell viability by 88%. In growth performance studies, GA supplementation significantly enhanced the body weight gain, growth rate, feed conversion ratio, and performance index by 19, 18, 10, and 30% compared to the control. GA improved red blood cell production, liver function, and lipid metabolism. GA treatments significantly increased red blood cell count, hemoglobin, hematocrit, mean corpuscular hemoglobin, mean corpuscular volume, and mean corpuscular hemoglobin concentration by 5-15% compared to the control. Albumin, total protein, and globulin levels increased by 10-20%, indicating improved protein synthesis and liver function. Liver and kidney parameters, such as ALT, AST, creatinine, urea, triglycerides, glucose, total cholesterol, and LDL, decreased by 20-40%, while HDL cholesterol increased by 15-25%, suggesting enhanced lipid metabolism and reduced oxidative stress. GA supplementation also enhanced antioxidant defense systems, increasing total antioxidant capacity, superoxide dismutase and glutathione peroxidase (GSH-Px) by 20-30% while reducing malondialdehyde (MDA) levels by 40-50%. Immunoglobulin A, G, and M levels increased by 25-35%, indicating improved immunity. GA 10% concentration significantly reduced proinflammatory cytokines (IL-1β by 78% and TNF-α by 82%) and precancerous markers (BAX and Casp-3 by 75-80%) in heat-stressed rabbits while increasing heat stress proteins (HSP70 by 291% and HSP90 by 130-210%). Histological examinations revealed that GA preserved liver and kidney integrity under heat stress, with 6% and 10% concentrations showing near-normal tissue architecture compared to heat-stressed rabbits displayed cuboidal epithelial cell necrosis and exfoliation of the brush border. Additionally, GA positively modulated gut microbiota, reducing harmful bacteria like *E. coli* and Salmonella by 25-40% while increasing beneficial lactic acid bacteria by 45%. It concluded that GA supplementation enhanced the growth, blood biochemistry, and immunity of heat-stressed rabbits.

Keywords: Antioxidants, Blood, Cancer, Gum arabic, Heat stress, Rabbit

Introduction

Heat stress is a significant challenge in rabbit farming, particularly in regions with high ambient temperatures [1]. Rabbits are susceptible to heat due to their limited ability to dissipate body heat, as they lack sweat glands and rely primarily on panting and ear vasodilation for thermoregulation [2]. When environmental temperatures exceed their thermoneutral zone (15-25°C), rabbits experience heat stress, leading to a cascade of physiological,

metabolic, and immunological disruptions. This problem is exacerbated by high humidity, poor ventilation, and overcrowding in farming systems. Heat stress not only compromises the health and welfare of rabbits but also reduces productivity, making it a critical issue for the rabbit industry [3].

Heat stress, including physiological, metabolic, and immunological functions, negatively impacts rabbits, disrupting thermoregulation and leading to elevated body temperature, increased respiratory rate, and dehydration. These changes strain the cardiovascular system, reducing blood flow to vital organs and impairing nutrient and oxygen delivery [4]. Prolonged heat stress can cause heatstroke, organ failure, and even mortality. Heat-stressed rabbits exhibit reduced feed intake and altered metabolism [5]. To cope with heat, they shift energy utilization from growth to maintenance, leading to weight loss, poor growth performance, and reduced feed efficiency.

Additionally, heat stress disrupts lipid and glucose metabolism, increasing the risk of metabolic disorders. Also, it suppresses the immune system, making rabbits more susceptible to infections and diseases. It reduces the production of immunoglobulins and antioxidants, impairing the body's ability to combat oxidative stress and inflammation. This weakened immunity increases mortality rates and reduces overall productivity [6,7]. The reproductive performance reduced in both male and female rabbits. In males, it reduces sperm quality and quantity, while in females, it disrupts estrus cycles, decreases conception rates, and increases embryonic mortality. These effects lead to lower breeding efficiency and reduced litter sizes. The heat-stressed rabbits exhibit behavioral changes such as lethargy, reduced activity, and altered feeding patterns. These changes further exacerbate the negative impacts on growth and reproduction [8].

The conventional solutions focus on environmental modifications and management practices, such as providing well-ventilated housing with proper insulation to help maintain a cooler environment. Fans, misting systems, and evaporative coolers are commonly used to reduce ambient temperature and humidity. Installing shade structures and cooling pads in rabbit housing can help lower temperatures [9,10]. Access to cool, clean water is also essential to prevent dehydration. Feeding rabbits during cooler days and providing high-energy, easily digestible diets can help mitigate the effects of reduced feed intake during heat stress. Also, breeding heat-tolerant rabbit breeds is a long-term strategy to improve resilience to high temperatures [9,10]. Conventional methods are somewhat effective but often involve high costs, energy consumption, and infrastructure requirements, making them less accessible to small-scale farmers. Additionally, they may not fully address the physiological and metabolic challenges heat stress poses.

In recent years, alternative solutions, particularly nutritional interventions, and natural supplements, have gained attention due to their potential to mitigate heat stress in rabbits. These cost-effective and sustainable approaches address the root causes of heat stress at the cellular and metabolic levels [9,10].

Heat stress generates excessive reactive oxygen species (ROS), leading to oxidative damage. Supplementing diets with antioxidants such as vitamin C, E, and selenium can neutralize ROS, reduce oxidative stress, and improve overall health [11,12]. For example, studies have shown that vitamin E supplementation enhances immune function and reduces mortality in heat-stressed rabbits. Also, probiotics (beneficial bacteria) and prebiotics (dietary fibers that promote probiotic growth) improve gut health and nutrient absorption. They enhance the gut microbiota's resilience to heat stress, reduce inflammation, and boost immunity. Probiotic supplementation has been shown to improve growth performance and reduce the incidence of diarrhea in heat-stressed rabbits [13].

On the other hand, herbal extracts such as ginger, turmeric, and moringa possess anti-inflammatory, antioxidant, and immunomodulatory properties. These natural compounds can mitigate the adverse effects of heat stress by enhancing antioxidant capacity, reducing inflammation, and improving metabolic efficiency [14]. Supplementing diets with omega-3 fatty acids, found in flaxseed and fish oil, can reduce inflammation and strengthen thermoregulation in heat-stressed rabbits. These fatty acids enhance cell membrane integrity and support immune function. Providing electrolyte solutions helps maintain hydration and electrolyte balance, which is critical during heat stress. Electrolytes such as sodium, potassium, and magnesium support cardiovascular function and nutrient transport [15].

Arabic gum (GA) is a natural, complex substance derived from the sap of Acacia trees, primarily Acacia Senegal and Acacia seyal. This branched-chain hydrocolloid consists mainly of arabinose and galactose sugars, proteins, and minerals like calcium, magnesium, and potassium. With a neutral or slightly acidic nature, GA was employed as an adhesive for mummification and in mineral paints for hieroglyphs [16]. Today, GA finds widespread application in various industries, including food, pharmaceuticals, and many others. The main medicinal uses of GA include respiratory disorders, gastrointestinal issues, liver and kidney health, and skin and inflammatory disorders. Additionally, GA extract has been used to treat chronic hepatitis and has therapeutic benefits against various viruses [16-18].

Studies have found that GA extract exhibits potent antibacterial activity against standard *S. aureus* and MRSA

strains by reducing lipase activity and inhibiting virulence gene expression [19]. Arabic gum has the potential to accelerate burn wound healing through the following properties: anti-inflammatory effects, antimicrobial properties, antioxidant activity, wound contraction and shrinkage, reduced pain and discomfort, faster epithelialization, and enhanced angiogenesis [20]. Abd El-Azeem et al.[21] demonstrated that supplementing diets with turmeric, MOS, and Biostrong significantly improved doe weight, milk yield, birth size, antioxidant capacity (TAC, GPx, SOD), and protein levels while reducing oxidative stress (MDA), highlighting their effectiveness as natural antioxidants in mitigating heat stress during pregnancy and lactation in rabbits. To the best of our knowledge, this is the first study to investigate the effects of GA extract on heat stress in rabbits; therefore, in this study, the active compounds in GA extract were detected using GC-MS, then examined its antioxidant, anticancer, and antimicrobial activity, elucidating the effect of GA extract on the heat stressor, monitoring the biochemical, molecular and histological changes.

Materials and Methods

Ethical Approval

The Unit of Biomedical Ethics has approved the animal study, Research Ethics Committee (REC HA-02-J-008, King Abdul Aziz's University). The accommodation and administration of the animals and the experimental protocols were conducted per the principles delineated in the Guide for the Care & Use of Lab Animals following the National Committee of Bioethics NCBE (2023). The Ethical code number 511-89.

Preparation of Gum Arabic Extract

Aqueous extracts of GA powders were prepared following the method of Todorović et al.[22] with modifications. Briefly, 20 grams of GA powder were mixed with 180 mL of deionized water and homogenized; the mixture was then agitated at 200 rpm for 1 h at room temperature. The extraction was repeated twice, and the filtrates were combined and concentrated using a rotary evaporator at 55°C under reduced pressure.

Active Compounds Profile of GA Extract Using GC-MS

GC-MS analysis was conducted on a methanol extract of GA using a Shimadzu QP2010PLUS GC-MS system. The system employed a capillary column and utilized split-less injections with a purification time of 0.1 min. Helium was the carrier gas at a 1 mL/min flow rate. The column temperature was programmed as follows: 50°C for 3 min, followed by a ramp of 5°C/min to 80°C, and then 10°C/min to 340°C. The inner temperature of the detector was set at 250°C and 340°C, respectively, with a solvent delay of 4 min. Peak identification was achieved by comparing the obtained mass spectra with entries in the National Institute of Standards and Technology library (NIST 08 and NIST 08S) and referencing previously published data [23].

Biological Activities of GA Extract

DPPH-scavenging Activity: The antioxidant activity of GA was evaluated as follows, 1 mL of GA (2, 4, 6, 8, and 10%) mixed with 3 mL of DPPH, maintained for 60 min, and the developed color was measured at 517 nm using a colorimeter [24]. The OD is recorded in the equation 1.

$$\%\ Antioxidant\ activity = \frac{OD\ DPPH - OD\ sample}{OD\ DPPH} \times 100$$

Antibacterial and Antifungal Activity: The bactericidal activity of GA was evaluated using a disc assay [25]. The PCA plates were inoculated with 100 µL of *Bacillus cereus* (BC), *Streptococcus aereus* (SA), *Escherichia coli* (EC), and *Klebsiella pneumoniae* (KP) cultures, then 8 mm discs saturated with GA concentrations were placed on the PCA surface. The plates were kept at 37°C for 24 h, and then the IZDs were recorded (mm) [26,27]. The MIC was evaluated following Saad et al.[28].

The antifungal potential of GA concentrations (2-10%) was tested against pathogenic fungi *Aspergillus niger, Fusarium oxysporuim, Penicillium chrysogenum, Alternaria alternata, Pythium aphanidermatum,* and *Podosphaera xanthii*. These pathogens were isolated from spoiled feed and confirmed through genetic analysis using 16S rRNA gene sequencing. A disc diffusion assay was employed to assess the antimicrobial activity of GA. The fungal suspensions were inoculated onto PDA plates. Sterile paper discs impregnated with various GA concentrations were placed on the inoculated plates 25°C for 7 days. After incubation, the diameter of the inhibition zones was measured (mm) [25].

Anticancer Activity: Breast cancer cell lines sourced from the American Type Culture Collection (ATCC) were initially grown as a single layer. These cells were then enzymatically detached using trypsin and subsequently counted. The cell count was adjusted to a concentration of 100.000 cells per milliliter using a growth medium (FBS-enriched DMEM). Subsequently, 100 µL of this diluted cell suspension, containing approximately 10.000 cells, was carefully introduced into each well of a 96-well microtiter plate. The plate was then placed in a CO_2 incubator maintained at a constant temperature of 37°C for 24 h. The culture medium was then replaced with fresh medium supplemented with varying concentrations of FBS and GA (2-10%), followed by an additional 48 hours of incubation at 37°C. Cells were detached from their culture surface using a trypsin-EDTA solution. Trypan blue staining was then used to differentiate between live and dead cells.

The number of viable cells was counted. The results were recorded as % inhibition of cell viability [29].

Experimental Layout

One hundred and sixty weaned male New Zealand White rabbits (age: ~35 days, average weight: 669 g) were randomly allocated to four experimental groups, each consisting of 40 rabbits and 10 replicates. The housing environment maintained a 12-h light-dark cycle, temp (36-38°C), % RH (71-75%), and THI of 36.5 *(Table 1)*. All experimental groups were under heat stress: control group fed a basal under heat stress 36.8°C; G1, rabbits treated with GA 4% in drinking water; G2, rabbits treated with GA 6% for a month; G3, rabbits treated with GA 10% for a month under heat stress.

Growth Performance

At the end of the experiment (35 days), the rabbits were euthanized by cervical dislocation. Subsequently, the heart, brain, liver, kidneys, lungs, and spleen were dissected. After removing any excess fat, the relative weight of each organ was determined as a percentage of total body weight [30].

Blood Biochemistry

Liver function: Liver enzymes, AST, and ALT were measured using kits from Spectrum, Sigma, UK. Kidney function: Urea and Creatinine levels were also determined using Spectrum kits [31,32]. Oxidative stress markers, MDA, and defense system CAT, POD, and SOD were measured using recommended kits [33,34]. Hormonal analysis: Sexual hormones were measured following the methods described by Steyn et al.[35].

Heat Stress, Proinflammatory, and Precancerous Markers

Total RNA was extracted following the method described by Dong et al.[36]. The isolated RNA was subsequently used for quantitative real-time PCR (qRT-PCR) analysis. All qRT-PCR reactions were performed using SYBR Green chemistry on a real-time PCR system. Melting curve analysis was conducted to ensure PCR product specificity. Gene-specific primers for Hsp70, hsp90. Bax, Caspase 3, and IL-6 *(Table 2)* were employed for qRT-PCR. The 2-ΔΔCt method determined relative gene expression [37].

Table 1. *Monthly values of ambient temperature, relative humidity, and temperature-humidity index throughout the study period*

Item	July	August	Overall
AT (°C)	38±0.2	37±0.1	39.5±0.3
RH (%)	71.33±0.6	75.65±0.9	73±0.8
THI	37±0.8	36±0.7	36.5±0.2

n=3, Data are presented as mean ±SE. AT: ambient temperature, RH: relative humidity, THI: temperature-humidity index

Table 2. *Forward and reverse primers sequence for Hsp70, hsp90, Bax, Caspase 3, IL-6 genes*

Gene	Primer 5-3	MW
Hsp70	F: ATCACCATCACCAACGAC R: ACTTGTCCAGCACCTTCTT	70-kDa
hsp90	F: ATCACTGGTGAGAGCAAGAAGGC R: TTAGTCGACCTCCTCCATCTTGCT-3	90 kD
Bax	F: ACACCTGAGCTGACCTTG, R: GCCCATGATGGTTCTGATC	21.1 kDa
Caspase 3	F: CTGAACTTCGGGGTGATCG, R: GCT TGG TGG TTT GCT ACG AC	17 kDa
IL-6	F: CCACTTCACAAGTCGGAGGCTTA R: CCAGTTTGGTAGCATCCATCATTTC	25 kDa

Histology

Liver and Kidney tissue samples were fixed in 10% buffered formalin saline for histopathological analysis. Sections (0.5 cm thick) were prepared using standard paraffin-embedding techniques. Subsequently, paraffin specimens (5-µm in thick) were stained with H&E stain to assess kidney damage. After GA and cisplatin treatments, acute tubular necrosis was evaluated in the outer medulla and cortex of the kidney [38].

Statistical Analysis

A one-way ANOVA test was conducted using SPSS (version 22, USA). The triplicate data means were compared using LSD to indicate the differences were considered statistically significant at $P<0.05$.

Results

The Chemistry of Gum Arabic Extract

Active Compounds Profile: GC-MS analysis of a GA extract identified diverse bioactive compounds. *Table 3* summarizes these compounds' retention time (RT), molecular weight, peak area, and reported biological activities. The GC/MS profile of GA showed a variety of active compounds; the main compounds in the GC/MS profile were saccharides, i.e., arabinose, galactose, and rhamnose accounted for 41%, followed by phenolic acids with 36.1% (ferulic acid, caffeic acid, p-coumaric acid, syringic acid, gallic acid, ellagic acid, tannic acid, cinnamic acid, and chlorogenic acid, also essential fatty acids and flavonoids were detected in the profile.

Antioxidant Activity: Gum arabic extract, containing 66 mg/mL phenolic compounds and 24 mg/mL flavonoids, demonstrated significant DPPH free radical scavenging activity comparable with ascorbic acid (AsA) with a value of 92% *(Fig. 1)*. Notably, a 10% (w/v) concentration of GA exhibited a potent scavenging effect, neutralizing 91% of DPPH radicals with an IC50 of 2%

Table 3. GC-MS profile of active compounds in gum arabic extract

RT (min)	Compound	Molecular Weight (g/mol)	Chemical Formula	% Area
5.23	Arabinose	150.13	$C_5H_{10}O_5$	12.5±0.9
7.45	Galactose	180.16	$C_6H_{12}O_6$	18.3±0.8
9.12	Rhamnose	164.16	$C_6H_{12}O_5$	8.7±0.2
11.34	Glucuronic Acid	194.14	$C_6H_{10}O_7$	10.2±0.1
13.56	4-Hydroxybenzoic Acid	138.12	$C_7H_6O_3$	5.6±0.3
15.78	Catechol	110.11	$C_6H_6O_2$	3.4±0.1
17.89	Vanillin	152.15	$C_8H_8O_3$	7.8±0.6
20.12	Ferulic Acid	194.18	$C_{10}H_{10}O_4$	9.1±0.2
22.34	Quercetin	302.24	$C_{15}H_{10}O_7$	6.3±0.3
24.56	β-Sitosterol	414.72	$C_{29}H_{50}O$	4.5±0.1
26.78	Oleanolic Acid	456.70	$C_{30}H_{48}O_3$	3.2±0.2
28.90	Linoleic Acid	280.45	$C_{18}H_{32}O_2$	2.8±0.3
30.12	Palmitic Acid	256.43	$C_{16}H_{32}O_2$	4.1±0.2
32.34	Stearic Acid	284.48	$C_{18}H_{36}O_2$	3.7±0.6
34.56	Caffeic Acid	180.16	$C_9H_8O_4$	5.9±0.3
36.78	p-Coumaric Acid	164.16	$C_9H_8O_3$	4.8±0.2
38.90	Syringic Acid	198.17	$C_9H_{10}O_5$	3.5±0.3
40.12	Luteolin	286.24	$C_{15}H_{10}O_6$	2.9±0.1
42.34	Apigenin	270.24	$C_{15}H_{10}O_5$	2.6±0.2
44.56	Kaempferol	286.24	$C_{15}H_{10}O_6$	3.1±0.3
46.78	Myricetin	318.24	$C_{15}H_{10}O_8$	2.4±0.2
48.90	Gallic Acid	170.12	$C_7H_6O_5$	4.3±0.1
50.12	Ellagic Acid	302.19	$C_{14}H_6O_8$	3.8±0.2
52.34	Tannic Acid	1701.20	$C_{76}H_{52}O_{46}$	1.9±0.2
54.56	Cinnamic Acid	148.16	$C_9H_8O_2$	2.7±0.1
56.78	Chlorogenic Acid	354.31	$C_{16}H_{18}O_9$	3.6±0.2
58.90	Rutin	610.52	$C_{27}H_{30}O_{16}$	2.1±0.3
60.12	Hesperidin	610.56	$C_{28}H_{34}O_{15}$	1.8±0.4

n=3, Data are presented as mean ±SE. retention time (RT)

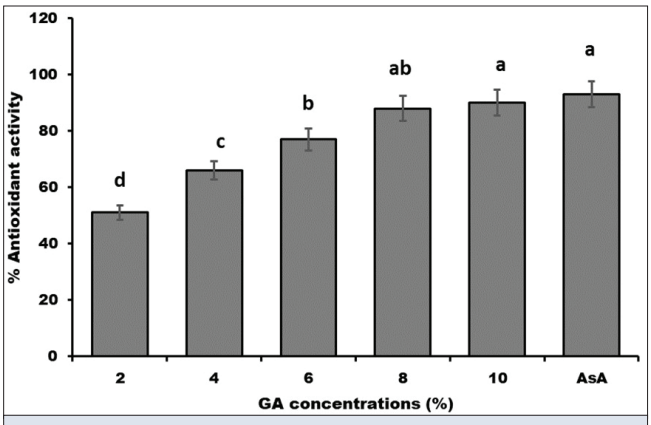

Fig 1. Antioxidant activity of gum arabic extract against DPPH radicals. *n*=3, Data are presented as mean ±SE. Lowercase letters above columns indicate significant differences (P<0.05)

Antimicrobial Activity: *Table 4* shows the antibacterial activity of GA at different concentrations, where GA 10% reduced the growth of pathogenic bacteria with IZDs of 12-30 mm; s*taphylococcus aureus* was the most vulnerable bacteria to GA; however, *Klebsiella pneumonia was the most* resistant. This study evaluated the antifungal efficacy of GA against six pathogenic microorganisms. Inhibition zone diameters (IZDs) around GA-treated discs increased proportionally with GA concentration, ranging from 13 to 31 mm *(Table 4, Fig 2)*. The 10% GA concentration exhibited the strongest antimicrobial activity. Among the tested fungi, *P. chrysogenum, F. oxysporuim, A. alternata,* and *B. cinerea* showed the highest resistance, displaying smaller inhibition zones. At the highest GA concentration, IZDs ranged between 27 and 31 mm

Table 4. *Antifungal activity of gum arabic extract (2-10 %) against pathogenic microorganisms*

Microrganisms		GA (%)/IZD (mm)				
		2	4	6	8	10
Pathogenic bacteria	*Bacillus cereus*	13±0.2b	16±0.6b	19±0.5b	21±0.6b	27±0.5b
	Staphylococcus aureus	16±0.3a	20±0.2a	24±0.3a	26±0.5a	30±0.7a
	Escherichia coli	12±0.8bc	15±0.5bc	18±0.5bc	22±0.8bc	25±0.2c
	Klebsiella pneumonia	10±0.2c	14±0.6c	16±0.8c	17±0.2c	21±0.3d
Pathogenic fungi	*F. oxysporuim*	14±0.2b	21±0.5b	25±0.3b	28±0.3b	32±0.2b
	A. niger	13±0.3c	18±0.4c	22±0.2c	26±0.3bc	28±0.8c
	P. chrysogenum	16±0.3b	18±0.3c	24±0.3b	27±0.2bc	32±0.3bc
	Alternaria alternata	19±0.5a	23±0.1a	27±0.1a	30±0.3a	35±0.5a
	P. aphanidermatum	15±0.6bc	16±0.3d	21±0.5d	22±0.1c	27±0.7c
	Podosphaera xanthii	14±0.5bc	18±0.6c	22±0.6c	25±0.5bc	28±0.9bc

n=3, Data are presented as mean ±SE. Different lowercase letters in the same column indicate significant differences (P<0.05)

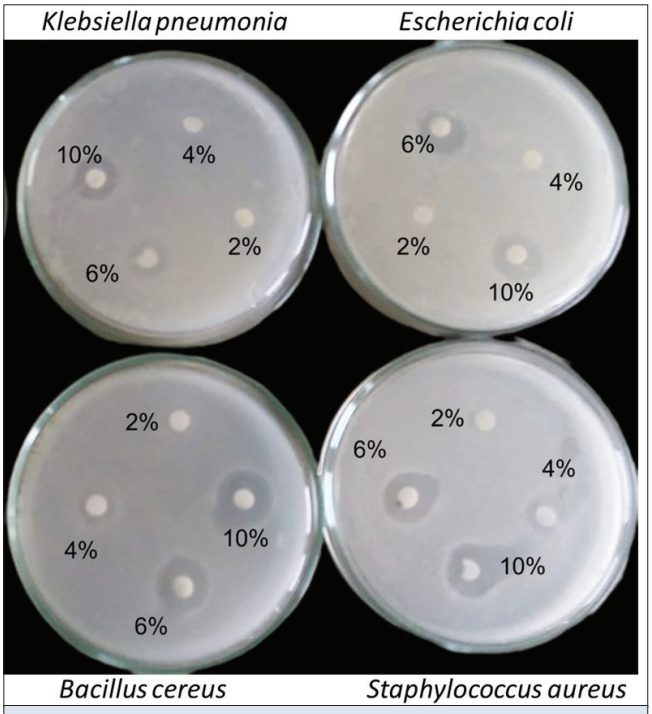

Fig 2. Antimicrobial activity of gum arabic against selected bacterial strains (disc assay)

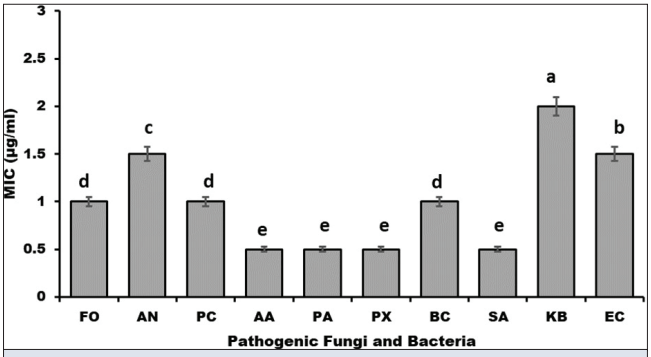

Fig 3. MIC values of gum arabic extract against pathogenic bacteria and fungi. *n*=3, Data are presented as mean ±SE. Lowercase letters above columns indicate significant differences (P<0.05). *Fusarium oxysporium* (FO), *Aspergillus niger* (AN), *P. chrysogenum* (PC), *Alternara alternara* (AA), *P. aphanidermatum* (PA), *Podosphaera xanthii* (PX), *Bacillus cereus* (BC), *Staphylococcus aereus* (SA), *Klebsila pneumonia* (KP), *Esherchia coli* (EC)

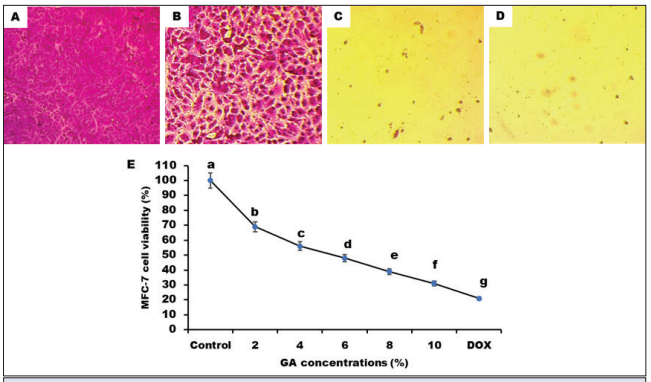

Fig 4. (A) Microscopic images of the cytotoxicity effect of MCF-7 viability GA on the. (B) photograph of the cytotoxicity effect of GA 2% on the MCF-7 viability, (C) the cytotoxicity effect of GA 10% on the MCF-7 viability compared to doxorubicin (DOX, 300 µg/mL) (D). (E) Histogram of the effect of GA concentrations on the viability of MCF-7 compared to DOX. *n*=3, Data are presented as mean ±SE. The Lowercase letters above columns indicate significant differences (P<0.05)

(Table 4). GA demonstrated significant antifungal activity, effectively inhibiting microbial growth within a 0.5-2% concentration range. *Fusarium oxysporium* and *Botrytis cinerea* exhibited the highest resistance to GA with a minimum inhibitory concentration (MIC) of 2%, while *Aspergillus niger* and *Rhizoctonia solani* were the most sensitive with an MIC of 0.5% *(Fig. 3)*.

Anticancer Activity: *Fig. 4* shows that GA concentrations had considerable anticancer activity against MCF-7 cancer cell viability. GA 10% reduced 78% of cancerous cell progress compared to doxorubicin, which inhibited MCF-7 by 81%.

Growth Performance

Table 5 presents the growth performance of rabbits under different treatments, including a control group and three groups receiving GA at varying concentrations (4%, 6%, and 10%). The parameters measured include: Live body weight (LBW) at 5 weeks, body weight gain (BWG) from 5 to 13 weeks, feed intake (FI) from 5 to 13 weeks, feed conversion ratio (FCR) from 5 to 13 weeks, growth rate (GR) from 5 to 13 weeks, and performance index (PI). The GA treatments, particularly at higher concentrations (6% and 10%), showed significant improvements in growth parameters compared to the control group.

The results showed that GA 6% and 10% groups exhibited significantly higher BWG compared to the control. GA 6% showed a 12.2% increase, and GA 10% showed a 19.6% increase. Similar to BWG, GA 6% and 10% groups had significantly higher GR than the control. GA 6% showed a 11.4% increase, and GA 10% showed a 18.8% increase. Also, the 6% and 10% groups displayed significantly better FCR than the control, indicating improved feed efficiency. GA 6% showed a 7.1% improvement, and GA 10% showed a 9.7% improvement. The performance index of GA 6% and 10% groups had significantly higher PI values than the control, suggesting better overall performance. GA 6% showed a 10.6% increase, and GA 10% showed a 14.3% increase.

The improved growth performance in the GA groups could be attributed to various factors, i.e., improved feed digestibility and nutrient absorption, where GA might enhance the digestibility of nutrients in the feed, leading to better nutrient utilization and growth. Reduced oxidative stress, where GA has antioxidant properties that could help mitigate the negative effects of heat stress on oxidative stress, leading to improved growth and improved gut health, where GA might have beneficial effects on gut microbiota, promoting better nutrient absorption and overall health.

The results suggest that incorporating GA into the diet of rabbits under heat stress can significantly enhance their growth performance. The higher concentrations (6% and 10%) of GA appear more effective in improving BWG, GR, FCR, and PI than the control.

Blood Biochemistry

Table 6 shows that GA treatments significantly increased the blood parameters, RBCs, Hemoglobin, Hematocrit, MCH, MCV, MCHC compared to the control group ($P<0.05$). This suggests that GA supplementation improved red blood cell production and oxygen-carrying capacity, which benefit overall health and performance. Additionally, GA treatments significantly enhanced the Albumin, Total protein, and Globulin content compared to the control group ($P<0.05$) under heat stress. This indicates that GA supplementation may have improved protein synthesis and liver function under heat stress.

The liver and kidney parameters, ALT, AST, Creatinine, Urea, Triglycerides, Glucose, TC, LDL were significantly improved by GA treatments, where these parameters decreased compared to the control group ($P<0.0001$). This suggests that GA supplementation may have improved liver function, reduced oxidative stress, and improved lipid metabolism. On the other hand, GA treatments significantly increased HDL cholesterol compared to the control group ($P<0.0001$). This is beneficial as HDL cholesterol helps remove excess cholesterol from the bloodstream.

Based on the overall improvements in blood parameters, the GA 10% group appears to be the most beneficial. It consistently showed significant improvements in hematology and serum metabolites compared to the control group. However, it is important to note that further research is needed to determine the optimal GA concentration and supplementation strategy for different breeds and environmental conditions. GA 6% and GA 4% groups also showed significant improvements in most parameters compared to the control group. However, the magnitude of improvement was generally lower than in the GA 10% group. In some cases, the GA 10% group showed greater improvements than the GA 6% and GA

Table 5. *The effect of dietary GA with different concentrations on the growth performance of heat-stressed rabbits*

Treatments (mg/kg)	LBW (g)		BWG (g)	FI (g)	FCR	GR	PI
	5w	13 w	5w-13w	5w-13w	5w-13w	5w-13w	5w-13w
Control	670.14±11.3	1950±3.6c	1279.86±11.2d	4508±11.2c	3.52±0.2a	171.33±1.1d	363.36±10.3d
GA 4 %	668.33±10.3	2055±1.2b	1386.67±10.3c	4585±12.5c	3.31±0.3b	185.65±1.5c	419.37±9.5c
GA 6 %	671.36±12.5	2096±3.5b	1424.64±9.5b	4606±9.2b	3.23±0.2b	191.22±2.1b	440.64±8.2b
GA 10 %	672.58±11.6	2185±5.4a	1512.42±8.9a	4809±9.4a	3.18±0.4c	203.54±2.3a	475.65±7.7a
p-value	0.669	0.00012	0.0011	0.0013	0.00	0.0036	0.0002

n=3, Data are presented as mean ±SE. The row's lowercase letters indicate significant differences (P<0.05). live body weight (LBW), body weight gain (BWG), feed intake (FI), feed conversion ratio (FCR), growth rate (GR), performance index (PI)

4% groups, suggesting a possible dose-dependent effect of GA. The results suggest that GA supplementation, particularly at 10% concentration, can significantly enhance rabbits' blood hematology and chemistry under heat stress by improving red blood cell production, liver function, lipid metabolism, and antioxidant status.

The results of a study investigating the effect of different GA concentrations (0%, 4%, 6%, and 10%) on rabbits' oxidative status, liver oxidative status, and immunity parameters under heat stress are presented in *Table 6*. GA treatments 6 and 10% significantly increased TAC, SOD, and GSH-Px in serum compared to the control group (P<0.05). This suggests that GA supplementation enhanced the antioxidant defense system in rabbits while decreasing the MDA levels compared to the control group (P<0.0001). This indicates that GA supplementation reduced lipid peroxidation and oxidative damage. Similarly, the 6 and 10% GA treatments significantly increased liver TAC compared to the control group (P<0.00236). This suggests that GA supplementation improved the liver's antioxidant capacity. However, liver MDA levels were decreased compared to the control group (P<0.0001). This indicates that GA supplementation reduced oxidative damage in the liver.

The GA treatments 6 and 10% significantly increased all three immunoglobulin levels compared to the control

Table 6. *The effect of dietary GA with different concentrations on blood hematology and chemistry of heat-stressed rabbits*

Blood Parameters			**Control**	**GA 4 %**	**GA 6 %**	**GA 10 %**	**P-value**
Haematology	Hemoglobin (g/dL)		10.93±0.2c	11.69±0.5b	12.24±0.8a	11.88±0.5b	0.0122
	RBCs (x 10^6/mm3)		4.687±0.1c	5.96±0.6b	6.51±0.7a	6.15±0.5a	0.036
	WBCs (x 10^3/mm3)		5.657±0.3c	6.87±0.5b	7.42±0.6a	7.11±0.2a	0.024
	Platelet (x 10^3/mm3)		281.45±11.3	287.73±1.4	288.28±1.5	287.11±10.3	0.78
	Hematocrit (%)		28.95±0.6b	29.55±0.9ab	30.1±0.3a	29.55±0.6ab	0.041
	MCH (pg)		23.97±0.6b	25.51±1.7ab	26.06±0.8a	25.70±0.5a	0.0356
	MCV (µm^3)		67.37±0.9a	65.47±0.5b	66.02±0.6ab	65.66±0.2b	0.0123
	MCHC (g/dl)		34.22±0.8b	34.98±0.8b	35.53±0.5a	35.17±0.6a	0.023
Serum metabolites	Albumin, g/dL		3.5±0.2b	4.06±0.2ab	4.39±0.4a	4.3±0.2a	0.02
	Total protein, g/dL		5.97±0.5c	6.87±0.3b	7.33±0.1a	7.24±0.6a	0.00253
	Globulin, g/dL		2.72±0.1c	2.97±0.1a	2.97±0.2a	2.88±0.2b	0.77
	ALT, IU/L		62.63±1.3a	45.42±0.3b	39.6±0.4c	41.3±0.6b	0.0001
	AST, IU/L		32.3±0.8a	27.7±0.5b	22.3±0.5c	21.3±0.8c	0.0001
	Creatinine, mg/dL		1.25±0.2a	0.99±0.9b	1.01±0.1ab	0.92±0.01b	0.036
	Urea, mg/dL		40.01±0.5a	39.22±0.4a	36.55±0.2b	36.46±0.5b	0.01
	LDL, mg/dL		34.69±0.6a	24.46±0.3b	21.33±0.5c	22.3±0.2c	<0.0001
	Triglycerides, mg/dL		86.68±1.1a	68.41±0.1b	55.36±0.3d	59.33±0.6c	<0.0001
	Glucose, mg/dL		84.18±1.2a	83.53±0.6a	79.66±0.8b	79.57±0.6b	0.032
	TC, mg/dL		91.01±0.9	77.95±1.2b	72.08±0.9b	73.66±0.3b	0.012
	HDL, mg/dL		37.34±0.5b	39.07±0.8b	41.13±0.5a	41.04±0.5a	0.029
Oxidative status	Serum	TAC, ng/mL	0.28±0.02c	0.46±0.05b	0.63±0.02a	0.59±0.01ab	0.0112
		SOD, U/mL	0.36±0.01c	0.52±0.06a	0.54±0.03a	0.49±0.06b	0.041
		Glutathione, mg/dL	14.14±0.2	14.97±0.5	14.81±1.2	14.69±0.8	0.89
		GSH-Px, mg/dL	3.03±0.3c	5.23±0.3a	4.89±0.3b	4.77±0.2b	0.011
		MDA, nmol/mL	13.42±0.6a	8.32±0.5b	8.26±0.5b	8.14±0.3b	0.033
	Liver	TAC, nmol/mL	0.86±0.02b	1±0.1a	1.15±0.2a	1.05±0.2a	0.0236
		MDA, nmol/mL	6.22±0.6a	4.79±0.b6	3.42±0.6c	3.5±0.2c	<0.0001
Immunity	IgG, mg/dL		489.33±12.3b	523.99±11.3a	529.19±11.6a	525.3±13.8a	0.0223
	IgA, mg/dL		134.75±14.3b	162.43±10.3a	163.75±6.3a	161.3±12.6a	0.0132
	IgM, mg/dL		115.87±10.3c	140.62±14.5ab	144.1±9.2a	139.5±11.7b	<0.0001

n=3, Data are presented as mean ±SE. The row's lowercase letters indicate significant differences (P<0.05). Red blood cells (RBCs), white blood cells (WBCs), Mean corpuscular Hemoglobin (MCH), Mean corpuscular volume (MCV), Mean corpuscular Hemoglobin concentration (MCHC), Alanine aminotransferase (ALT), Aspartate aminotransferase (AST), total antioxidant content (TAC), superoxide dismutase (SOD), glutathione peroxidase (GSH-Px), malondialdehyde (MDA), immunoglobin G (IgG), *immunoglobin A* (IgA), *immunoglobin M* (IgM)

group (P<0.0001). This suggests that GA supplementation enhanced the immune response in rabbits. The results suggest that GA supplementation at 6 or 10% can significantly improve rabbits' oxidative status, liver oxidative status, and immune function under heat stress.

The Expression of Inflammatory, Heat Stress Protein and Cancer Markers

Table 7 shows the gene expression levels of various markers in rats exposed to heat stress and fed different concentrations of GA extract (0%, 4%, 6%, and 10%). Proinflammatory Cytokines: GA 6% showed the lowest expression in IL-1β (1.4d), followed by GA 10% (2.1c) and GA 4% (4.0b). The control group had the highest expression (9.7a), with a relative decrease of 85% compared to the control. GA 10% showed a 78% reduction, and GA 4% showed a 59% reduction. Also, the expression of TNF-α reduced in GA 6% showed a decrease of 87% compared to the control. GA 10% showed an 82% reduction, and GA 4% showed a 64% reduction.

The heat stress proteins: All GA groups showed significantly higher expression of HSP70 and HSP90 than the control. GA 6% had the highest expression (45a), followed by GA 10% (44a) and GA 4% (41a). Relative increases of 273% in GA 4%, 309% in GA 6%, and GA 10% showed a 291% increase. The increases in HSP90 expression ranged between 130-210%.

Precancerous markers, BAX and Casp-3, showed the lowest expression (1.3d, 1.3c) in GA 6%, followed by GA 10% (2.2c, 1.5c) and GA 4% (5.0b, 3.2b). The control group had the highest expression (10a, 7.2a).

GA supplementation, especially at lower concentrations (6% and 10%), significantly reduced the expression of proinflammatory cytokines (IL-1β and TNF-α) and precancerous markers (BAX and Casp-3) in heat-stressed rabbits while considerably increasing the expression of heat stress proteins (HSP70 and HSP90), which are essential for cellular protection against stress.

Histology of Liver and Kidney of Heat-Stressed Rabbits

Fig. 5-I showed the histological examination of the liver revealed the following: (A) Control heat-stressed rabbits exhibited hepatic damage characterized by necrosis, inflammation, bile duct proliferation, and cholestasis. (B) In rabbits treated with 4% GA, signs of regeneration were observed in the hepatocytes with mild inflammation. (C) The 6% GA group displayed normal liver architecture with proper arrangement of hepatocytes, bile ducts, and sinusoids. (D) The 10% GA group also exhibited a normal arrangement of hepatocytes with normal sinusoidal dilation.

Histological examination of the kidney presented in *Fig. 5-II* where (A) in control heat-stressed rabbits, proximal convoluted tubules displayed cuboidal epithelial cell necrosis and exfoliation of the brush border. (B) In the GA 4% group, kidney sections showed necrosis, epithelial cell swelling, and tubules with exfoliated brush borders. (C) The 6% GA group under heat stress exhibited normal kidney parenchyma with no significant changes. (D) Histological examination of the 10% GA group revealed normal parenchyma with intact glomeruli and tubules.

Gut Microbiota

Fig. 6 presents the gut microbial count (CFU/g) of different bacterial groups in the digestive tract of heat-stressed rabbits treated with various GA concentrations. The results suggest that GA supplementation, particularly at higher concentrations (6% and 10%), significantly reduced the microbial counts of most bacterial groups compared to the control group. The treatments GA 6% and GA 10% showed a significant decrease (25-40%) in TBC, coliform, *E. coli*, *Salmonella* compared to the control. GA 4% showed a moderate decrease. While GA 6% and GA 10% showed a significant increase (45%) in LAB counts compared to the control. GA 4% showed a moderate increase.

The reduction in TBC, *E. coli*, Coliform, and *Salmonella* counts suggests that GA supplementation may help

Table 7. *The effect of dietary treatments GA extract on proinflammatory cytokines, precancerous markers, and heat stress protein in heat stressed rats*

Genes	Proinflammatory Markers		Heat Stress Protein		Precancerous Markers	
	IL-1β	TNF-α	HSP70	HSP90	BAX	Casp-3
Control	9.7±0.3a	9.6±0.6a	11±0.6b	10±0.3c	10±0.3a	7.2±0.6a
GA 4%	4.0±0.2b	3.5±0.5b	44±0.9a	31±0.5a	5.0±0.4b	3.2±0.2b
GA 6%	1.4±0.1d	1.2±0.2c	41±1.1a	23±0.8b	1.3±0.5d	1.3±0.1c
GA 10%	2.1±0.6c	1.7±0.1c	45±1.2a	29±0.5ab	2.2±0.7c	1.5±0.2c
P value	<0.0001	<0.0001	0.0021	<0.0001	<0.0001	<0.0001

n=3, Data are presented as mean ±SE. The column's lowercase letters indicate significant differences (P<0.05)

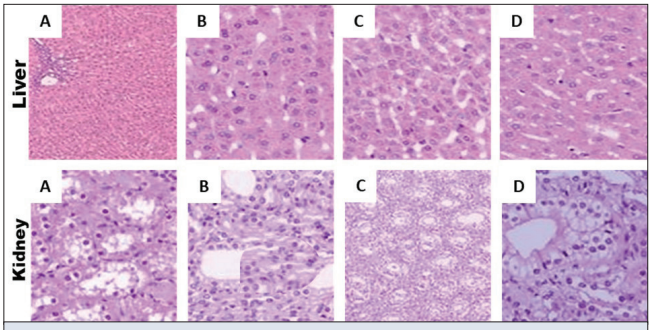

Fig 5. Histopathological evaluation of liver and kidney sections in paracetamol-induced hepatotoxicity in rabbits treated with GA. (H&E staining, 40x, scale bar = 100 µm, n = 6 rabbits/group). A) untreated control under heat stress, B) GA 4%-treated group under heat stress, C) GA 6%-treated group

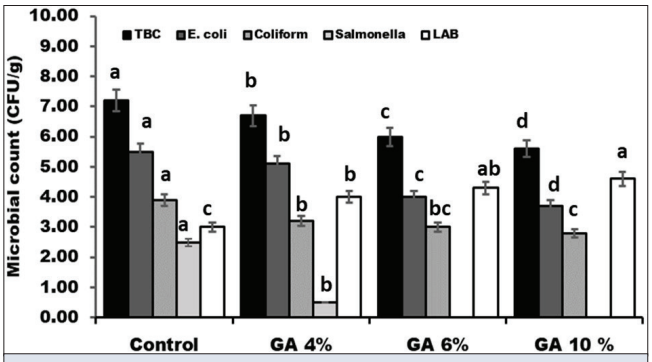

Fig 6. The effect of dietary GA on the gut microbiota (total bacterial count, *E. coli* count, Coliform, Salmonella, and Lactic acid bacteria count) of heat-stressed rabbits. *n*=3, Data are presented as mean ±SE. Lowercase letters above columns indicate significant differences (P<0.05)

to improve gut health and reduce the risk of bacterial infections in heat-stressed rabbits. The increase in LAB counts is beneficial as LAB are beneficial bacteria that contribute to gut health and digestion.

Discussion

The detrimental effects of heat stress on rabbit production are a growing concern, demanding innovative and sustainable solutions. Gum arabic, a natural polysaccharide with multifaceted biological activities, has emerged as a promising feed additive to mitigate these challenges [39]. The efficacy of GA in alleviating heat stress is attributed to its complex composition and diverse mechanisms of action. GA's high molecular weight and branched structure contribute to its prebiotic properties, fostering the growth of beneficial gut bacteria [40]. Its phenolic compounds and polysaccharides act as potent antioxidants, scavenging free radicals and enhancing antioxidant enzyme activity [41]. Furthermore, GA can modulate immune responses and reduce inflammation, contributing to overall health and resilience [42].

These mechanisms are interconnected, creating a synergistic effect that enhances the overall well-being of heat-stressed rabbits. For instance, the prebiotic effect of GA promotes a healthy gut microbiota, which in turn improves nutrient absorption and reduces inflammation. This improved gut health contributes to enhanced growth performance and reduced oxidative stress. Similarly, the antioxidant properties of GA protect cellular components from oxidative damage, preserving organ function and improving blood parameters [43,44].

Heat stress induces a cascade of physiological changes that negatively impact performance of animal growth [45]. Reduced feed intake is a primary consequence of hormonal imbalances and increased energy expenditure for thermoregulation [46]. GA can counteract this by modulating gut hormones such as ghrelin and leptin, potentially stimulating appetite and improving feed intake. Moreover, GA enhances fiber digestion and nutrient absorption by promoting the growth of cellulolytic and saccharolytic bacteria in the gut. This improved nutrient utilization translates to better feed conversion efficiency and enhanced growth rate. Furthermore, GA can reduce the inflammatory responses associated with heat stress. Chronic inflammation can impair nutrient utilization and growth performance. GA helps maintain a healthy gut environment and optimize nutrient utilization by modulating immune responses and reducing the production of pro-inflammatory cytokines [47]. El-Sabrout et al.[48] demonstrated that GA supplementation at 1.5% of the diet significantly improved body weight gain and feed conversion ratio in heat-stressed rabbits. The study attributed these effects to enhanced gut health and nutrient absorption.

Heat stress disrupts the delicate balance of blood parameters, leading to alterations in hematological and biochemical profiles [49]. Elevated cortisol levels, a hallmark of stress, can impair immune function and metabolic processes [50]. GA has demonstrated the ability to reduce cortisol levels, mitigating stress responses. This reduction in cortisol may be mediated by the modulation of the hypothalamic-pituitary-adrenal (HPA) axis.

Changes in lipid profiles, such as increased cholesterol and triglyceride levels, are also observed in heat-stressed animals. GA can improve lipid profiles by binding bile acids and promoting their excretion, thereby reducing cholesterol and triglyceride levels [51]. Furthermore, GA can enhance glucose metabolism by improving insulin sensitivity and reducing insulin resistance [52]. Hematological parameters, such as red blood cell count, hemoglobin concentration, and white blood cell count, are also affected by heat stress [53]. GA can positively influence these parameters, reflecting improved overall health status. This improvement may be attributed to the antioxidant and immunomodulatory properties of GA, which protect blood cells from oxidative damage and

enhance immune function. GA reduces serum levels of stress markers such as cortisol and heat shock proteins (HSPs) while improving liver and kidney function markers (e.g., ALT, AST, creatinine) [54].

Heat stress induces a surge in ROS production, overwhelming the antioxidant defense system and leading to oxidative stress [55]. This imbalance can damage cellular components, including lipids, proteins, and DNA, impairing physiological functions. GA's potent antioxidant properties are crucial in mitigating this oxidative damage. GA's phenolic compounds and polysaccharides act as free radical scavengers, neutralizing ROS and preventing cellular damage [56]. Furthermore, GA can enhance the activity of antioxidant enzymes such as SOD, CAT, and GPx, bolstering the antioxidant defense system [57]. These enzymes play crucial roles in detoxifying ROS and maintaining cellular redox balance. Furthermore, GA protects cellular membranes and other vital components from oxidative damage by reducing MDA levels, a marker of lipid peroxidation, and enhancing antioxidant enzyme activity [51]. This improvement in oxidative status contributes to heat-stressed rabbits' overall health and well-being.

The gut microbiota is critical in nutrient digestion, immune modulation, and overall health [58]. Heat stress can disrupt the delicate balance of gut microbiota, leading to dysbiosis and impaired gut function [59]. GA acts as a prebiotic, selectively promoting the growth of beneficial bacteria such as *Lactobacillus* and *Bifidobacterium* [60]. These bacteria produce short-chain fatty acids (SCFAs), which serve as energy sources for intestinal epithelial cells, enhance gut barrier function, and modulate immune responses. By inhibiting the growth of pathogenic bacteria, GA helps maintain a healthy gut environment and prevent gut dysbiosis. This modulation of gut microbiota enhances gut barrier function, improving nutrient absorption and reducing inflammation. A healthy gut barrier prevents the leakage of endotoxins and other harmful substances into the bloodstream, reducing systemic inflammation.

Heat stress can induce pathological changes in various organs, including the liver, kidneys, and intestines. GA has demonstrated protective effects on organ histology by mitigating the adverse effects of heat stress. GA can reduce hepatic lipid accumulation in the liver, a common consequence of heat stress. This reduction may be attributed to the ability of GA to modulate lipid metabolism and enhance antioxidant defense. GA can improve tubular integrity in the kidneys, protecting against heat stress-induced renal damage. The antioxidant and anti-inflammatory properties of GA may mediate this protective effect. GA can enhance villus height and crypt depth in the intestines, improving nutrient absorption and gut barrier function. This improvement may be attributed to the prebiotic effect of GA, which promotes the growth of beneficial bacteria and enhances gut health. While the existing research highlights the potential of GA as a feed additive for heat-stressed rabbits, further studies are needed to optimize its application and explore its long-term effects. Future research should focus on determining the optimal dosage of GA for different breeds and ages of rabbits under varying heat stress conditions, investigating the mechanisms by which GA exerts its beneficial effects, including its interactions with gut hormones, immune cells, and signaling pathways. Also, assessing the long-term effects of GA supplementation on growth performance, reproductive performance, and overall health of rabbits. Exploring the synergistic effects of GA with other feed additives, such as probiotics, prebiotics, and antioxidants. Evaluating the economic feasibility of GA supplementation in rabbit production. Utilizing advanced omics technologies to understand the changes produced by GA comprehensively. Furthermore, it is essential to consider the source and quality of GA, as variations in composition and purity can affect its efficacy. Standardization of GA production and quality control are crucial for ensuring consistent results. In conclusion, GA holds excellent promise as a natural feed additive for mitigating the adverse effects of heat stress in rabbits. Its multifaceted biological activities, including prebiotic, antioxidant, and immunomodulatory properties, enhance growth performance, improve blood parameters, reduce oxidative stress, modulate gut microbiota, and protect organ histology. Further research is warranted to optimize its application and explore its full potential in rabbit production.

Declaration

Availability of Data and Materials: The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

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Ethical Approval: The animal study has been reviewed and approved by ZU-IACUC committee. was performed in accordance with the guidelines of the Egyptian Research Ethics Committee and the guidelines specified in the Guide for the Care and Use of Laboratory

Animals (2022). Ethical code number ZU-IACUC/2/F/394/2022. Written informed consent was obtained from the owners for the participation of their animals in this study.

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Research Article

Efficacy of Pyocyanin Isolated from *Pseudomonas aeruginosa* and *Lactobacillus plantarum* Against Methicillin Resistant *Staphylococcus* aureus Caused Bovine Mastitis

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Abstract

Staphylococcus aureus is an important pathogen causing mastitis in dairy cows and methicillin-resistant *Staphylococcus aureus* (MRSA) is found on farms globally. The risk of foodborne zoonotic infection from bovine MRSA poses significant challenges to veterinary medicine. These challenges are compounded by limited treatment options, particularly non-beta-lactam antibiotics such as tetracyclines, fluoroquinolones, etc. Innovative treatment strategies aiming to replace antibiotics in mastitis management are being investigated. The activity of pyocyanin and *Lactobacillus plantarum* was evaluated by Kirby-Bauer disc diffusion method, minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC), antibiofilm activity and fractional inhibitory concentration method. Cytotoxic effects were also analyzed by infecting a cell line with MRSA. The results indicated that pyocyanin produced a 15 mm inhibition zone, while *Lactobacillus plantarum* yielded a 10 mm zone; their combination resulted in an 18 mm zone, suggesting a synergistic effect. The MIC values were determined to be 64 µg/mL for pyocyanin and 128 µg/mL for *Lactobacillus plantarum,* both requiring an MBC of 512 µg/mL. Furthermore, pyocyanin exhibited a 58.6% antibiofilm effect, whereas *Lactobacillus plantarum* demonstrated a 50.3% effect. Gene expression analysis revealed that *Lactobacillus plantarum* was more effective in reducing MRSA DNA levels, underscoring its potential for use in combination therapy against MRSA infections.

Keywords: *Lactobacillus plantarum*, Mastitis, Pyocyanin, *Pseudomonas aeruginosa*

Introduction

Bovine mastitis is an inflammation of the mammary gland in dairy cattle. It mainly results in infections from the bacteria *Staphylococcus aureus*, *Escherichia coli*, and *Streptococcus* spp. [1]. These bacteria causing bovine mastitis primarily enter the mammary duct and reach the mammary gland. The invasion of these bacteria is facilitated by weakened defence mechanisms of the udder, inadequate hygiene conditions and inappropriate environmental factors (e.g. damp and dirty housing environment, inappropriate sanitation practices). This leads to rapid multiplication of bacteria and initiation of inflammatory processes, resulting in increased severity of mastitis. In addition, incorrect or incomplete veterinary interventions can also complicate the treatment of bacterial infection, leading to additional complications [2]. Mastitis is classified into clinical and subclinical forms. Clinical mastitis presents with visible symptoms such as swelling, redness and heat. The milk may appear abnormal, it may contain clots, flakes, or blood. The affected cows show systemic symptoms such as fever, appetite loss and drowsiness. Subclinical mastitis may not show significant symptoms, but is detected by high somatic cell count in milk [3]. Livestock-derived Methicillin Resistance *S. aureus* (MRSA) has become increasingly important in cases of bovine mastitis in recent years. MRSA, clonal complex 398 (CC398), is considered to be the main cause of bovine

mastitis. It is associated with farm animals and has been found in various dairy cows around the world, indicating that it is widespread in the dairy industry [4]. The presence of MRSA in dairy cows with mastitis leads to a decrease in the quality and quantity of milk. The infected cows are experiencing significant productivity losses, creating economic difficulties for dairy farmers. Livestock-derived MRSA can be transmitted between cows and humans, raising public health concerns. Bacteria can contaminate milk and dairy products and cause potential infections in people who consume these products or in contact with infected animals [5]. Milking hygiene is of great importance to prevent MRSA transmission in dairy cattle. One of the most common mistakes is not cleaning and disinfecting the milking equipment regularly. Milk residues left in the milking machines provide a favourable breeding environment for MRSA, facilitating animal-to-animal transmission. In addition, dirty or inadequately disinfected milking cloths and hands cause the bacteria to be carried directly to the teat. In addition, inappropriate use of bedding also increases the risk of infection. Bedding materials that are damp and contaminated with urine or faeces are a breeding ground for prolonged survival and spread of MRSA. Failure to change bedding regularly and ensure proper disinfection increases the risk of transmission from infected animals to healthy individuals. In addition, failure to detect and isolate infected cows early accelerates the spread of MRSA within the herd. Therefore, within the scope of effective hygiene practices, it is of great importance to clean teats with antiseptic solutions before and after milking, clean milking equipment with appropriate disinfectants after each use, change bedding materials regularly and keep infected animals separate. Maintaining cleanliness and appropriate maintenance techniques are essential to control cases of mastitis and prevent the spread of MRSA among dairy cattle [4]. MRSA strains are resistant to widely used antibiotics, which complicates treatment options for mastitis in cattle. This resistance leads to prolonged infections and increased costs of veterinary care, and further affects the dairy industry. Parallel to antibiotic resistance, MRSA is a critical factor in the pathogenicity and continuity of infections [5]. The ica genes (icaA, icaB, IcaC, and icaD) are part of the intercellular adhesion (ica) locus responsible for the synthesis of polysaccharide adhesive (PIA), which is necessary for biofilm formation in *S. aureus*. Biofilms provide a protective environment for bacteria, making them more resistant to antibiotics and host immune response [6]. Studies have shown a high prevalence of icaABCD genes in MRSA isolates. For example, one study that all MRSA strains tested were positive for these genes, which suggests biofilm formation is a common feature among these resistant strains. This suggests that MRSA's biofilming ability contributes significantly to virulence and persistence in clinical settings [7]. Relationships also extend to other genes that play a role in adhesion and biofilm development, such as clfA (adhesion factor A) and clfB (adhesion factor B), which are surface proteins that facilitate adherence to host tissues. The presence of these genes in conjunction with the root genes complicates treatment options by increasing the ability of MRSA to create infection and biofilm [8,9]. That's why researchers have turned to searching for alternative treatments depending on the growing antibiotic resistance. Pyocyanin is a blue-green pigment produced by the gram-negative bacterium *Pseudomonas aeruginosa*. It is a toxic secondary metabolite with a defined chemical structure, which exhibits antibiotic activity against Gram-positive bacteria in particular [10]. It causes oxidative stress and cell damage in MRSA cells by producing reactive oxygen species (ROS). Furthermore, it inhibits ATP production by disrupting the energy metabolism of the bacterium and suppresses biofilm formation, making it difficult to adhere to surfaces. Due to these properties, pyocyanin is being investigated as a potential antimicrobial agent against antibiotic-resistant bacteria, but its clinical use is limited due to toxicity. Studies have that psoriasis is effective against MRSA, with minimum inhibitor concentration (MIC) values ranging from 8 to 400 µg/mL [11]. *Lactobacillus plantarum* is a genus of probiotic bacteria known for its beneficial effects on human health. *L. plantarum* develops in the human gastrointestinal system. It can effectively resist low pH levels and high bile concentrations, which allows it to colonize and show its beneficial effects. Research has shown that *L. plantarum* has antibacterial activity against MRSA [12]. Studies have shown that the cell-less superfluid may inhibit the growth of MRSA and have dose-dependent effects on bacterial vitality and biofilm formation. This suggests that *L. plantarum* can effectively reduce MRSA populations in various environments, including wound infections [13]. In the light of this information, in this study, we evaluated the antibacterial and antibiotic activity of pyocyanin isolated from *P. aeruginosa* and *L. plantarum* against MRSA, which is a major factor in mastitis, and its effect on cell vitality and cytotoxicity, and on virulence genes.

Material and Methods

Ethical Approval

Since the *S. aureus* ATTC 23235 bacterial strain and *L. plantarum* ATCC 8014 bacterial strain used in our study were the reference strains, ethical approval is not required.

Isolation of Methicillin Resistant *S. aureus* and *L. plantarum*

Commercially purchased *S. aureus* ATTC 23235 and *L. plantarum* ATCC 8014 were grown on blood agar, nutrient

agar and mannitol saline agar plates (Oxoid, Hampshire, UK). Bacteria inoculated by line inoculation method were then incubated at 37°C for 24-48 h. For further analyses, they were stored at -80°C in medium containing 10% glycerol (v/v).

Production of Pyocyanin

Pyocyanin isolated from *P. aeruginosa* (CAS No. 85-66-5, Sigma, Aldrich) was purchased commercially. Confirmed by High Performance Liquid Chromatography (HPLC) with a rate of ≥98%. It was prepared as a stock solution of 1024 µg/mL of pyocyanin dissolved in double distilled water in 1:1 ratio.

Antimicrobial Susceptibility Testing of Methicillin Resistant *S. aureus*

Antimicrobial susceptibility test was performed using disc diffusion technique. The isolated strains were tested for susceptibility to cefoxitin (Cef; MRSA indicator), penicillin (Pen), ampicillin-sulbactam (Amp-Sul), amoxicillin-clavulanic acid (AmoCla), tetracycline (Tet), cefotaxime (Ceft) and erythromycin (Ery) (Oxoid). In addition, inhibition zone diameters were evaluated after 20 µL impregnation of pyocyanin and *L. plantarum* on 6 mm discs. The selected antimicrobials represent drugs used in human and animal industry and were selected according to the National Antimicrobial Resistance Monitoring System (NARMS) registry. The test was performed on Mueller Hinton agar medium (MH, Oxoid) and incubated at 37°C for 24 h. The inhibition zones obtained were determined with digital callipers and compared with the specific zone diameters for resistance or susceptibility based on European Committee on Antimicrobial Susceptibility Testing (EUCAST)/Clinical Laboratory Standards Institute (CLSI) standards.

Determination of Minimum Inhibitory Concentration (MIC), Minimum Bactericidal Concentration (MBC) and Fractional Inhibitory Concentration (FIC)

Minimum Inhibitor Concentration was determined by liquid microdilution test on Mueller Hinton Agar liquid medium. Briefly, 100 µL Mueller Hinton Broth (MHB) (MilliporeSigma) medium and 50 µL pyocyanin at concentrations ranging from 1024-16 µg/mL and *L. plantarum* were added to the wells by dilution technique. Then, the prepared bacterial colonies were inoculated 50 µL into all wells according to McFarland 0.5 scale (10^8CFU/mL) and incubated at 37°C for 24 h. After incubation, the wells in which bacterial growth stopped were determined. Then, the minimum bactericidal concentration was determined in the medium in which up to 5 colonies were observed to grow by inoculation from the wells with higher concentrations than the well in which bacterial growth stopped. Fractional inhibitor concentration was determined at pyocyanin 1024, 512, 256, 128, 16 µg/mL and *L. plantarum* 1024, 512, 256, 128, 16 µg/mL concentrations and synergistic, additive and antagonistic activity in the wells were evaluated using the Fractional Inhibitory Concentration (FIC) index. FIC index ≤0.5 was considered as synergistic, 0.5-1 as additive, 1-4 as indifferent and ≥4 as antagonistic. The dilution steps for *L. plantarum* were performed using the double dilution method, and two-fold dilutions were performed consecutively from the initial concentration. Thus, the antibacterial effects of L. plantarum in combination with pyocyanin at different concentrations were analysed [14].

Determination of Antibiofilm Activity

After determining the MIC, a 0.1% solution was used for 15 min to determine the presence of biofilm in the wells. The wells were stained with crystal violet and then washed with phosphate-buffered saline (PBS). Remaining biofilm layer 33% (volume/volume) after dissolution with glacial acetic acid, microplate, spectrophotometric measurements at 595 nm using a microplate reader (Sunrise™, TECAN, Switzerland). Eradication biofilm inhibition rate with the formula [(OD (control)-OD (test)/OD (control))x100] to obtain the percentage evaluated [15].

Cell Culture

Cytotoxicity Study: The cytotoxic effect of the given substances in healthy cells was investigated in the healthy cell line dermal fibroblast. Cells were grown and maintained in DMEM medium containing 10% fetal bovine serum (FBS). The grown cells were seeded in 96 well plates and incubated at 37°C for 24 h at 5%CO_2. At the end of the incubation, the substances given at a concentration of 200-6.25 µM were prepared from the master stock and given to the cells and incubated at 37°C for 24 h at 5%CO_2. At the end of incubation, WST-8 (Water-Soluble Tetrazolium-8) assay was performed according to the manufacturer's kit protocol to evaluate cell viability and metabolic activity. The WST-8 assay contains a tetrazolium salt that is converted into water-soluble orange coloured formazan by mitochondrial dehydrogenase enzymes. WST-8 reagent was added to the cell culture at the end of the specified incubation period and incubated at 37°C for a specified time (usually 1-4 h). Cell viability was then determined by measuring the colour change spectrophotometrically at a wavelength of 450 nm. The colour intensity is directly proportional to the number of metabolically active cells and the result of the test was evaluated according to this principle [16].

Determination of Resistance Gene Expression

Extraction and Homogenisation of Pellets: MRSA, MRSA+ Pyocyanin, MRSA+ *L. plantarum* and MRSA+ Pyocyanin + *L. plantarum* in polystrene tubes (Eppendorf,

USA) were centrifuged at 500 xg x10 m at 4ºC to obtain pellets. The supernatant was collected in another tube and 20 micro glass balls were placed in these tubes on the pellet and these tubes were placed in the Tissue lyser LT system (Qiagen, USA) device whose header was previously cooled in -80°C deep freezer and lysed for 1 min. The lysed samples were spun in a centrifuge to collect the lysate on the edge of the tube to the bottom and these samples were kept in -80°C deep freezer for 5 min. Then they were put back into the Tissue lyser LT and lysed for 1 min. This digestion process was repeated 3 times until the samples were completely homogenised. When the pellet was completely homogenised, the samples were removed and the RNA isolation stage was started [17].

RNA Isolation and cDNA Synthesis: RNA isolation from the samples was performed according to the kit protocol using the PureLink RNA mini kit (Thermo Fisher Scientific, USA) "Protocol for purification of RNA from bacterial cells" protocol. In this context, fresh lysozyme solution was prepared first. For this solution, 10 mM Tris-HCl (pH 8.0), 0.1 mM EDTA, and 10 mg/mL lysozyme were mixed in ultrapure water. This solution was used as 100 µL for each 1x10^9 bacterial cell population. After this step, Lysis Buffer was prepared with 10 µL 2-mercaptoethanol in 1 mL with the materials available in the kit. 10% SDS solution was also prepared in ultrapure water. After these solutions were prepared, 100 µL of lysozyme solution and 500 µL of 10% SDS solution were added into the tubes homogenised above and these tubes were incubated at room temperature for 5 min using heating block. Then 350 µL Lysis Buffer was added to this mixture. The lysate in the tubes was inverted and vortexed at intervals until completely homogenised. The lysate was then homogenised using a DAIHAN homogeniser with a fine blade at 26000 rpm. The homogenate was centrifuged at 2600xg for 5 min at room conditions and the particles were allowed to settle to the bottom. The supernatant was taken into clean tubes and 1.5 vol of 100% ethanol (Merck, USA) was added to these tubes. Then vortexed until completely dissolved. This mixture was loaded onto the columns in the Purelink RNA mini kit (Thermo Fisher Scientific, USA) at a rate of 700 µL each time. The columns were centrifuged sequentially at 12.000 g for 30 sec and the RNAs were loaded onto the columns and then these columns were washed first with washing solution 1 and then twice with washing solution 2 by centrifugation at 12.000 g for 30 sec at each stage. After washing, the columns were centrifuged 1 time at 12.000 g for 3 min to dry the columns, then the columns were placed in sterile new 1.5 mL polystrene tubes (Eppendorf, USA), 60 µL of the solution given in the kit was pipetted into the centre of the membrane in the column, and these columns were centrifuged at 12.000 g for 1 min and pure RNAs were collected in the polystrene tube. The purity of the collected RNAs was determined by Optizen NanoQ Lite micro-volume spectrophotometer (Mecasys, South Korea) and the RNA quantity obtained are given in *Table 1*.

Table 1. *RNA quantity and purity values obtained from the samples*

Samples	Quantity (ng/ul)	Purity
MRSA	3.70	1.87
MRSA+ Pyocyanin	5.48	1.89
MRSA+ *L. plantarum*	2.65	1.96
MRSA+ Pyocyanin + *L. plantarum*	7.22	2.16

The obtained RNAs were equilibrated with DNAse and RNAse free ultrapure water (Sigma, USA) to 25 ng/10 µL for the next step. After the equilibration process, complementary DNA was synthesised to ensure that the obtained RNAs could be amplified by PCR. At this stage, Pathwayscanner cDNA Reverse Transcription Kit (Micromolecules, Türkiye) was used, and according to the kit protocol, the enzyme, dNTP mix and random primers in the kit were mixed and pipetted into PCR tubes as 10 µL. Afterwards, total RNA, which was equalised to 25 ng/10 µL in the previous section, was put into the same tubes. These tubes were synthesised cDNA in Applied Biosystems® ProFlex™ PCR System thermal cycler using step 1, 25°C, 10 min; step 2 37°C, 120 min; step 3 85°C, 5 min cycles. The cDNAs obtained were stored at -20°C.

Quantitative Real-time PCR Study, Determination of Gene Expression: In the study, the expression levels of resistance genes in control and treatment groups of examples were analysed by qRT-PCR method. The primers used to investigate the changes in the expression of these genes are given below in 5'-3' order. The genes and primers were given in *Table 2*. In gene expression studies, cDNAs were used. These cDNAs were analysed in qRT-PCR according to the Pathwayscanner cyber green qPCR MasterMix (Micromolecules, Türkiye) protocol. The reaction mix contained Hot Start Taq DNA polymerase (enzyme), SYBR Green I dye, dNTPs (0.2 mM each), $MgCl_2$ (3 mM), and stabilizers in a proprietary buffer. In the study, cDNAs were amplified using Applied Biosystems QuantStudio 5 Real-Time PCR device. Step 1 for qRT-PCR reaction to take place: Enzyme activation: 95ºC-3 min; 2nd step: Denaturation: 95ºC-15 sec; Primer binding-chain elongation: 60ºC-1 min; Step 3: Melting curve: 95ºC-15 sec, 60ºC-1 min, 95ºC-15 sec. Ct values of the peaks obtained during the replication process were used to determine gene expression and gene expression was calculated by 2-ΔΔΔCt method. Endogenous control 16 S rRNA expression was used as calibration and correction factor.

Table 2. Genes and primers				
Gene		**Primer (5'-3')**	**Size bp**	**Reference**
icaA (intercellular adhesion)	F	GAGGTAAAGCCAACGCACTC	151	[18]
	R	CCTGTAACCGCACCAAGTTT		
icaB (intercellular adhesion)	F	ATACCGGCGACTGGGTTTAT	140	
	R	TTGCAAATCGTGGGTATGTGT		
icaC (intercellular adhesion)	F	CTTGGGTATTTGCACGCATT	209	
	R	GCAATATCATGCCGACACCT		
icaD (intercellular adhesion)	F	ACCCAACGCTAAAATCATCG	211	
	R	GCGAAAATGCCCATAGTTTC		
nbA (fibronectin binding proteinA)	F	AAATTGGGAGCAGCATCAGT	121	
	R	GCAGCTGAATTCCCATTTTC		
nbB (fibronectin binding proteinB)	F	ACGCTCAAGGCGACGGCAAAG	197	
	R	ACCTTCTGCATGACCTTCTGCACCT		
clfA (clumping factorA)	F	ACCCAGGTTCAGATTCTGGCAGCG	165	
	R	TCGCTGAGTCGGAATCGCTTGCT		
clfB (clumping factorB)	F	AACTCCAGGGCCGCCGGTTG	159	
	R	CCTGAGTCGCTGTCTGAGCCTGAG		
16S rRNA	F	GGGACCCGCACAAGCGGTGG	191	
	R			

Table 3. Percent inhibition (%) of biofilm formation of MRSA at different concentrations						
Agents	**Concentrations (µg/mL)**					
	1024	**512**	**128**	**64**	**32**	**16**
Pyocyanin	58.6	48.6	40.6	31.1	17.3	15.1
L. plantarum	50.3	50	40.1	21.9	12.7	0
Pyocyanin+ *L. plantarum*	17.2	8.6	3.9	2.7	0.5	0
MRSA: *Methicillin Resistance Staphylococcus aureus*						

Results

Antimicrobial Susceptibility Testing of MRSA Results

Disc diffusion zone diameters of Pyocyanin, *L. plantarum* and Pyocyanin+*L. plantarum* against MRSA. The MRSA suspension has an inhibition area of 15 mm in the test with pyocyanin. The inhibition area in the test with *L. plantarum* was 10 mm. In a combination of pyocyanin and *L. plantarum*, the inhibition area of MRSA was measured at 18 mm. This suggests that when the two agents were used together, they create a synergistic effect, inhibiting the growth of MRSA more effectively.

Minimum Inhibitory Concentration (MIC), Minimum Bactericidal Concentration (MBC) and Fractional Inhibitory Concentration (FIC) Results

The MIC and MBC values of pyocyanin and *L. plantarum* versus MRSA and the FIC index for pyocyanin+*L. plantarum*. MIC, Pyocyanin: 64 µg/mL *L. plantarum*: 128 µg/mL. These results indicate that Pyocyanin has a lower MIC value over MRSA. Pyocyanin needs less concentration to inhibit the growth of MRSA. *L. plantarum* shows a higher MIC, which indicates that its effect is weaker. MBC, Pyocyanin: 512 µg/mL, *L. plantarum*: 512 µg/mL. MBC values indicate that both agents need a similar concentration to kill MRSA. FIC showed synergistic effect with the FIC index of 0.36 at pyocyanin+*L. plantarum* 128 µg/mL.

Antibiofilm Activity Results

Antibiofilm activity of pyocyanin, *L. plantarum* and pyocyanin+*L. plantarum* on MRSA was given as % in *Table 3*. At the highest concentration (1024 µg/mL), pyocyanin provides a biofilm inhibition of 58.6%. As the concentration decreases, the inhibition percentage was also decreasing, with values of 48.6% at 512 µg/mL and 40.6% at 128 µg / mL. This indicates that pyocyanin effectively inhibits biofilm formation. *L. plantarum* produces an inhibition of 50.3% at 1024 µg/mL, while

at lower concentrations its effect is reduced. It drops to 12.7% at 32 µg/mL and 0% at 16 µg/mL. This suggests that *L. plantarum* was effective at higher concentrations, but its effect at lower concentrations remains limited. In a combination of pyocyanin and *L. plantarum*, the inhibition percentage was relatively low at all concentrations. Values such as 8.6% were obtained at the highest concentration (1024 µg/mL) at 17.2% and 512 µg/mL.

Cell Culture Results

Cell culture cytotoxicity results were given in *Fig. 1*. In the control group, proliferation was accepted as 100%. In 200 µM pyocyanin application, proliferation was around 140%. In 100 µM pyocyanin application, proliferation reached 160%. When *L. plantarum* was applied alone, proliferation decreased to around 80% at 50 µM. In the combination of pyocyanin and *L. plantarum*, proliferation decreased to 60% at 50 µM. The lowest proliferation, around 40%, was observed in the combination of 25 µM pyocyanin + *L. plantarum*.

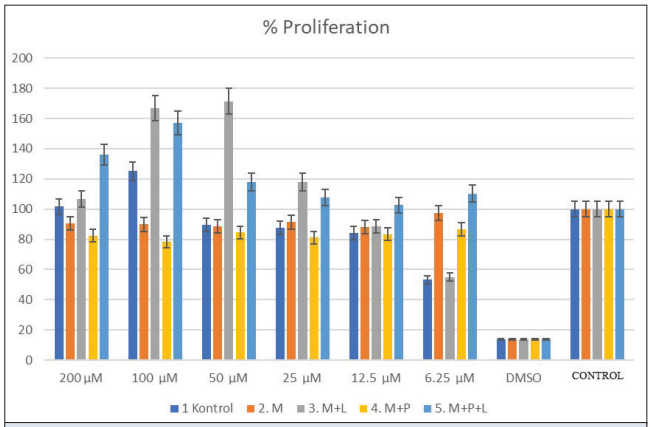

Fig 1. Cell culture results. M: MRSA, L: *L. plantarum,* P: Pyocyanin

Genes Expression Results

icaA and icaB Expression: The icaA gene expression of pyocyanin and *L. plantarum* on MRSA was shown in *Fig. 2*. Target, repeat number and Cq (Cycle quantification) values were determined for each sample. MRSA and MRSA+ Pyocyanin Cq values, i.e. DNA quantity, are very close to each other (21.193 and 21.22). MRSA+ *L. plantarum* Cq value (27.812) was higher than the other samples. This means that the DNA amount of this sample was less. MRSA+ Pyocyanin + *L. plantarum* Cq value (21.351) was slightly higher than MRSA and MRSA+ Pyocyanin but still lower than MRSA+ *L. plantarum*. MRSA+*L. plantarum* gave the most significant result by reducing the quantity of DNA compared to the other sample groups *(Fig. 2)*. The icaB gene expression of pyocyanin and *L. plantarum* on MRSA and MRSA was shown in *Fig. 2*. MRSA and MRSA+ Pyocyanin, Cq values (19.796 and 19.821) were very close to each other, indicating that the DNA quantity of these samples was similar. MRSA+ *L. plantarum* Cq value (16.591) was significantly lower than the other samples. This indicates that MRSA+ *L. plantarum* has a higher amount of DNA. MRSA+ Pyocyanin + *L. plantarum* Cq value (19.626) was slightly higher compared to MRSA and MRSA+ Pyocyanin, but still lower than MRSA+ *L. plantarum*. icaB expression was most suppressed in MRSA+ Pyocyanin *(Fig. 2)*.

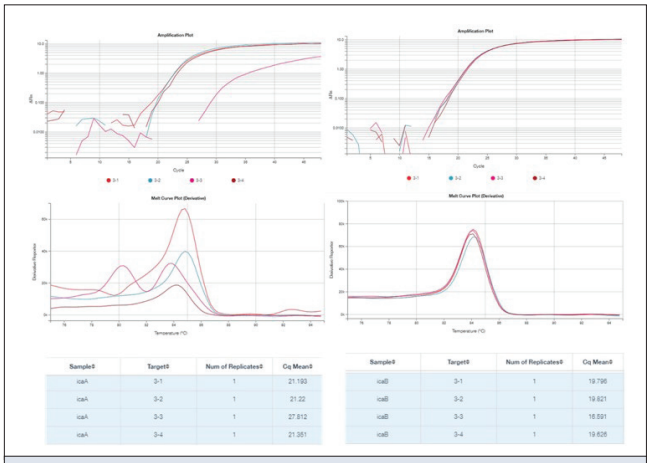

Fig 2. icaA and icaB expression levels. 3-1: MRSA, 3-2: MRSA+ Pyocyanin, 3-3: MRSA+ *L. plantarum,* 3-4: MRSA+ Pyocyanin + *L. plantarum*

icaC and icaD Expression: The icaC gene expression of pyocyanin and *L. plantarum* on MRSA and MRSA was shown in *Fig. 3*. MRSA and MRSA+ Pyocyanin Cq values (29.454 and 29.908) were close to each other, indicating that the DNA quantity of these samples was similar. MRSA+ *L. plantarum*, Cq value (27.511) was lower than the other samples. This indicates that the quantity of DNA of MRSA+ *L. plantarum* was higher. MRSA+ Pyocyanin + *L. plantarum* Cq value (29.919) was slightly higher compared to MRSA and MRSA+ Pyocyanin but still lower than MRSA+ *L. plantarum (Fig. 3)*. The icaD gene expression

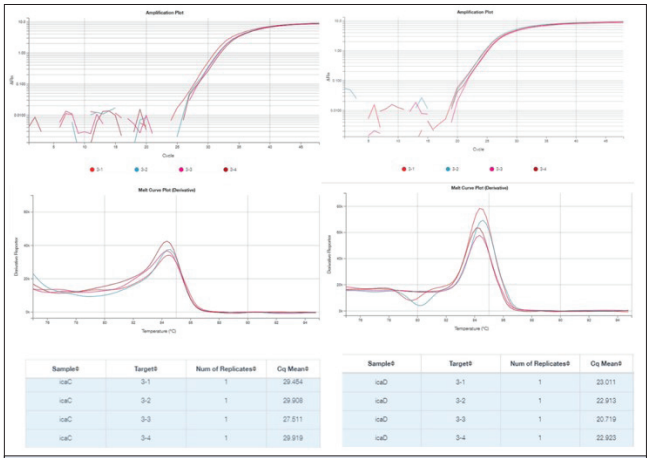

Fig 3. icaC and icaD expression levels. 3-1: MRSA,3-2: MRSA+ Pyocyanin, 3-3: MRSA+ *L. plantarum,* 3-4: MRSA+ Pyocyanin + *L. plantarum*

of pyocyanin and *L. plantarum* on MRSA was shown in *Fig. 3*. MRSA and MRSA+ Pyocyanin Cq values (23.011 and 22.913) were very close to each other, indicating that the DNA quantity of these samples was similar. MRSA+ *L. plantarum*, Cq value (20.719) was significantly lower than the other samples. This indicates that the amount of DNA was higher in MRSA+ *L. plantarum*. MRSA+ Pyocyanin + *L. plantarum* Cq value (22.923) was slightly higher compared to MRSA and MRSA+ Pyocyanin but still lower than MRSA+ *L. plantarum (Fig. 3)*.

nbA and nbB Expression: The nbA gene expression of pyocyanin and *L. plantarum* on MRSA was shown in *Fig. 4*. MRSA and MRSA+ Pyocyanin Cq values (20.591 and 20.266) were close to each other, indicating that the DNA quantity of these samples was similar. MRSA+ *L. plantarum*, Cq value (17.59) was lower than the other samples. This indicates that the quantity of MRSA+ *Lactobacillus plantarum* DNA was higher. MRSA+ Pyocyanin + *L. plantarum* Cq value (19.953) was higher compared to MRSA and MRSA+ Pyocyanin but still lower than MRSA+ *L. plantarum (Fig. 4)*. The nbB gene expression of pyocyanin and *L. plantarum* on MRSA was shown in *Fig.4*. MRSA+ *L. plantarum* has the lowest Cq value (25.784), indicating that MRSA+ *L. plantarum* has the highest quantity of DNA/RNA. The Cq values of MRSA, MRSA+ Pyocyanin and MRSA+ Pyocyanin + *L. plantarum* were close to each other (28.134, 28.553 and 28.463), but MRSA+ Pyocyanin has the highest Cq value, indicating the lowest quantity of DNA/RNA *(Fig. 4)*.

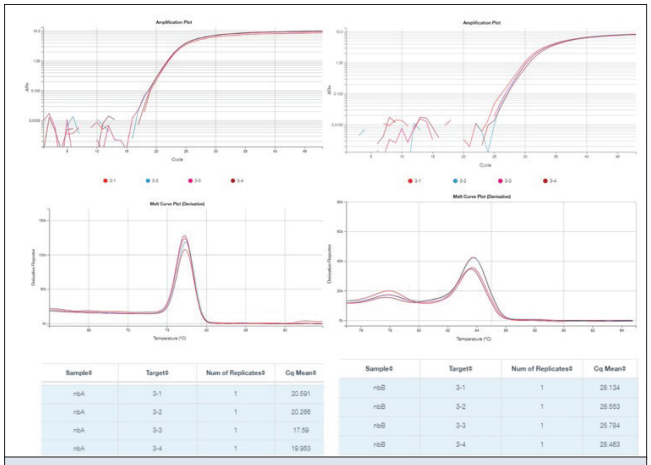

Fig 4. nbA and nbB expression levels. 3-1: MRSA, 3-2: MRSA+ Pyocyanin, 3-3: MRSA+ *L. plantarum,* 3-4: MRSA+ Pyocyanin + *L. plantarum*

ClfA and ClfB Expression: The ClfA gene expression of pyocyanin and *L. plantarum* on MRSA was shown in *Fig. 5*. MRSA+ *L. plantarum* has the lowest Cq value (25.577), indicating the highest amount of DNA/RNA in this sample. MRSA+ Pyocyanin has the second lowest Cq value (28.314). MRSA+ Pyocyanin + *L. plantarum*

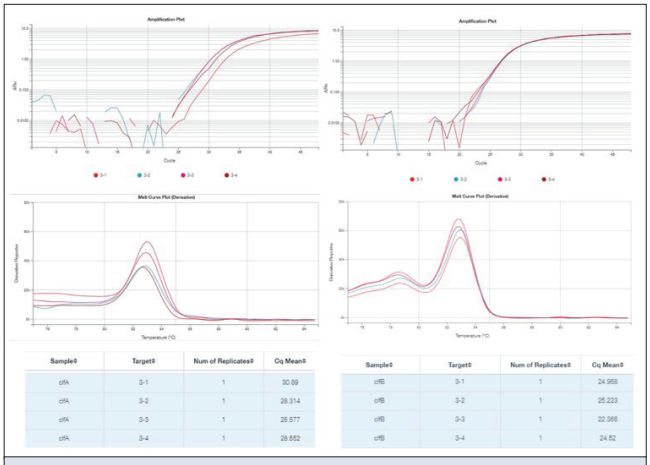

Fig 5. ClfA and ClfB expression levels. 3-1: MRSA,3-2: MRSA+ Pyocyanin, 3-3: MRSA+ *L. plantarum,* 3-4: MRSA+ Pyocyanin + *L. plantarum*

Cq value 28.682 was slightly higher than MRSA+ *L. plantarum*. MRSA has the highest Cq value (30.89), indicating the lowest concentration of genetic material *(Fig. 5)*. The ClfB gene expression of pyocyanin and *L. plantarum* on MRSA and MRSA was shown in *Fig.5*. MRSA+ *L. plantarum* has the lowest Cq value (22.366), indicating the highest amount of DNA/RNA in MRSA+ *L. plantarum*. MRSA has the second lowest Cq value (24.968). MRSA+ Pyocyanin + *L. plantarum*, Cq value 24.52, was slightly lower than MRSA. MRSA+ Pyocyanin has the highest Cq value (25.223), indicating the lowest concentration of genetic material *(Fig. 5)*.

Relative Quantification Results

RQ (Relative Quantification) shows the gene expression level of each sample *(Fig. 6)*. clFA; MRSA+ Pyocyanin, highest RQ value, *L. plantarum* and MRSA+ Pyocyanin + *L. plantarum* lower RQ values, MRSA+ Pyocyanin + *L. plantarum* RQ value was close but lower than MRSA+ *L. plantarum*. clFB; MRSA+ Pyocyanin, higher RQ value, MRSA+ *L. plantarum* lower RQ value, MRSA+ Pyocyanin + *L. plantarum* lowest RQ value, showing a significant decrease compared to MRSA+ Pyocyanin and MRSA+ *L. plantarum*. icaA, icaC, icaD, nbA, nbB; In general, RQ values are higher in MRSA+ Pyocyanin. Between MRSA+ *L. plantarum* and MRSA+ Pyocyanin + *L. plantarum*, they remain at similar levels for some genes, but MRSA+ *L. plantarum* is generally higher *(Fig. 6)*.

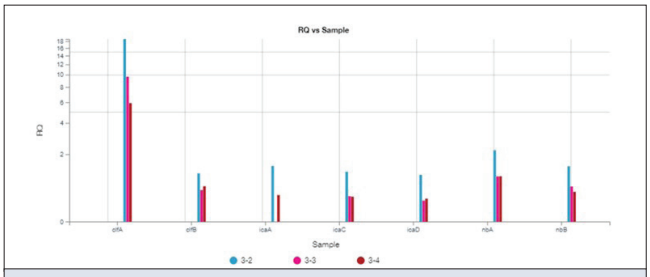

Fig 6. Relative quantification results. 3-2: MRSA+ Pyocyanin, 3-3: MRSA+ *L. plantarum,* 3-4: MRSA+ Pyocyanin + *L. plantarum*

Discussion

The relationship between bovine mastitis and MRSA is significant, particularly concerning the implications for animal health, milk quality, and public health. Antibiotic resistance developed by MRSA and biofilm therapy make it more difficult to prevent infection. In this percentage, we evaluated the therapeutic activity of pyocyanin and *L. plantarum in vitro* as alternative therapies in this study. Lactic acid bacteria inhibit the growth of pathogens through the secretion of bacteriocins, H_2O_2 or other antimicrobial compounds, as well as the adhesion properties of epithelial cells that inhibit pathogen adhesion through specific competition or steric hindrance [19]. The ability of *L. casei* to prevent bMEC invasion against two bovine strains of *S. aureus* RF122 and Newbould305 causing acute and moderate mastitis was evaluated. *Lactobacillus casei* strains affected the adhesion and/or internalisation of *S. aureus* in a strain-dependent manner. *L. casei* CIRM-BIA 667 reduced *S. aureus* Newbould305 and RF122 internalisation by 60% to 80% [20]. Several *Lactobacillus* strains, such as *L. plantarum* and *Lactobacillus brevis*, have demonstrated the ability to inhibit common mastitis-causing pathogens such as *S. aureus* and *E. coli*. For example, *L. plantarum* KLDS 1.0344 reduced bacterial growth and the expression of proinflammatory cytokines such as IL-6 and TNF-α in response to lipopolysaccharide (LPS) stimulation in both *in vitro* and *in vivo* studies [21]. In another study, the potential of *L. lactis* LMG 7930 strain to be used as an antibiotic alternative in the treatment of mastitis in dairy ruminants was investigated. In particular, the probiotic properties of this strain against pathogens causing mastitis were investigated. *L. lactis* LMG 7930, a nisin-producing strain, is widely used in the food industry and is considered safe for human consumption. *L. lactis* showed antagonistic properties against various pathogens in *in vitro* tests. In particular, it has a broad spectrum of inhibitory activity against Gram-positive bacteria. However, it could not inhibit the growth of *E. coli*. The strain was able to ferment various carbohydrates such as galactose, glucose and fructose. It also fermented eight of the 14 carbohydrates metabolised by some mastitis isolates. Surface hydrophobicity of *L. lactis* was found to be moderate and electron donor and acceptor properties were found to be low. Auto-aggregation and co-aggregation abilities were found to be low. However, the ability of *L. lactis* to adhere to bovine mammary epithelial cells was found at a certain level. *L. lactis* tended to reduce the internalisation of some pathogens, but this effect was not statistically significant [22]. Forty lactobacilli, including *L. plantarum*, were isolated from healthy bovine milk samples and selected isolates were evaluated for their probiotic properties on microorganisms [23]. However, no gene expression was evaluated in this study. Among the lactobacilli isolates, varying levels of activity (9 to 19 mm) against the bovine mastitogens *S. aureus, E. coli* and *Streptococcus dysgalactiae* were observed in a well diffusion assay. These isolates showed auto-aggregation (ranging from 14.29±0.96% to 62.11±1.09%) and co-aggregation (ranging from 9.21±0.14% to 55.74±0.74%) with mastitogens after 2 h. *L. plantarum* CM49 showed sensitivity to most antibiotics tested and showed strong inhibitory effects in co-culture experiments with an average $\log_{10}$ reduction of 3.46 for *S. aureus*, 2.82 for *E. coli* and 1.45 for *S. dysgalactiae*. Moreover, *L. plantarum* CM49 significantly reduced the adhesion rate of mastitogens in bovine mammary cell line and mouse model, demonstrating its potential efficacy in preventing mastitis [23]. Pyocyanin is a pigment produced by *P. aeruginosa* that exhibits notable antibacterial properties against a wide range of pathogens, including *S. aureus*. The mechanism of action of pyocyanin is primarily attributed to its ability to generate reactive oxygen species (ROS), such as superoxide radicals and hydrogen peroxide, through redox cycling. These ROS cause oxidative stress within the bacterial cells, leading to damage to cellular components, including proteins, lipids, and DNA. Pyocyanin also disrupts bacterial energy metabolism by interfering with mitochondrial function and ATP production. Furthermore, it has been shown to inhibit biofilm formation by *S. aureus*, which is a critical factor in its pathogenicity and resistance to antibiotics. Overall, the bactericidal effect of pyocyanin is due to its ability to induce cellular damage through oxidative stress, energy depletion, and biofilm disruption, making it a promising candidate for combating antibiotic-resistant infections [24]. Pyocyanin shows strong antibacterial activity primarily against Gram-positive bacteria. Studies have shown that it is effective against organisms such as *S. aureus* and *Bacillus* species, but its activity against Gram-negative bacteria such as *E. coli* is relatively lower. One study reported that pyocyanin produced inhibition zones of 24 mm against *S. aureus* and 26 mm against *S. typhi*, indicating strong antibacterial effects against these pathogens. However, no studies have been performed against *S. aureus*, the causative agent of mastitis [25]. In the disc diffusion test, pyocyanin 15 mm, *L. plantarum* 10 mm and their combinations 18 mm zone diameter were determined. Minimum inhibitory concentration was determined as 64 µg/mL for pyocyanin and 128 µg/mL for *L. plantarum*. Both agents require a minimum bactericidal concentration of 512 µg/mL. In the antibiofilm activity test, pyocyanin showed 58.6% inhibitory effect and *L. plantarum* showed 50.3% inhibitory effect. In gene expression analyses, *L. plantarum* was more effective in reducing the amount of DNA of MRSA.

Declarations

Availability of Data and Materials

The data that support the findings of this study are available from the corresponding author (D. Çelebi) on reasonable request.

Funding Support

The authors declared that this study has received no financial support.

Ethical Approval

Ethical approval is not required for this study.

Competing Interests

The authors have no conflicts of interest to declare.

Author Contributions

D.C., O.C., S.B., B.A., S.E.K., A.K.: Concept, Design, Supervision, Resources, Materials Data, Collection and/or Processing, Analysis and/or Interpretation, Literature Search, Writing and Critical Reviews.

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Research Article

In Vitro Effect of Boron Compounds in Combination with Photobiomodulation Therapy by 905 nm on the Viability of Human Gingival Fibroblasts

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Abstract

This study aimed to evaluate the effect of Low-level laser therapy (LLLT) and Boric acid (BA) or Borax decahydrate (BD) on the cell survival of gingival fibroblasts. Fibroblast planted plates were divided into 10 groups: Control group, BD 100 µg/mL (BD 100), BD 200 µg/mL (BD200), BA 100 µg/mL (BA100), BA 200 µg/mL (BA200), LLLT, LLLT+BD100 µg/mL (LLLT+BD100), LLLT+BD200 µg/mL (LLLT+BD200), LLLT+BA100 µg/mL (LLLT+BA100) and LLLT+BA 200 µg/mL (LLLT+BA200) groups. LLLT was performed at a dose of 4 J/cm^2 for 160 sec. Following LLLT, MTT analysis was performed after the cells were kept in the incubator for 24 h. The LLLT+BA 200 group exhibited the highest cell density. In MTT analysis, significantly higher cell numbers were observed in the BA200, LLLT+BA100, and LLLT+BA200 groups compared to the control group ($P<0.05$). Then, the 3-(4,5-dimethyl thiazolyl-2)-2, 5-diphenyltetrazolium bromide assay and 8-hydroxy-2'-deoxyguanosine and Bcl-2 Associated X-Protein were analyzed. The 8-hydroxy-2'-deoxyguanosine and Bcl-2 Associated X-Protein levels were decreased in the LLLT+BA200 group compared to the control group ($P<0.05$). This study suggests that BA200, LLLT+BA100, and LLLT+BA200 increase the survival of fibroblast cells.

Keywords: Borax decahydrate, Boric acid, MTT, Photobiomodulation, Photobiostimulation

Introduction

Boric acid (BA) and borax decahydrate (BD) are recognized for their diverse biological effects, which include antimicrobial [1], anti-inflammatory [2], and antioxidant properties [3]. These compounds have garnered attention across various disciplines, particularly in periodontology, for their potential therapeutic applications. Notably, boron, present in both BA and BD, is frequently used in dentistry due to its antimicrobial and immunomodulating properties [4,5].

Low-level laser therapy (LLLT), also known as photobiomodulation, has emerged as a promising therapeutic avenue for various medical conditions, including wound healing, fracture healing, and pain management [6-11]. Despite its demonstrated efficacy in diverse applications, the combination of LLLT with BA remains relatively unexplored. A previous study [6] employing a combination of laser and a chemical compound concluded that concurrent application of the compound and laser accelerates bone formation.

Fibroblasts, integral to wound healing and tissue repair, play a crucial role in extracellular matrix production and maintenance [12,13]. Gingival fibroblasts, specifically, are pivotal for gum wound healing and exhibit rapid adaptability to the oral cavity environment [14,15]. Apoptosis, regulated by a balance of antiapoptotic and proapoptotic proteins, is fundamental to cellular homeostasis. Bcl-2 family proteins, including BAX, serve as key regulators of apoptosis, with BAX predominance promoting apoptosis [16,17]. Additionally, oxidative stress, characterized by elevated reactive oxygen species levels, can induce DNA damage, as evidenced by the marker 8-hydroxy-2'-deoxyguanosine (8-OHdG) [18,19].

This study aims to investigate 8-OHdG and BAX positivity in gingival fibroblasts treated with BA and BD alongside LLLT, examining their relationship and potential effects on fibroblast viability. We hypothesize that BA and BD in combination with LLLT will enhance fibroblast survival.

Material and Methods

Ethical Approval

The Atatürk University Animal Experiments Local Ethics Committee approved the study protocol of this study (Decision No: 17/2022).

Chemical and Reagent

BA and BD were obtained from Eti Boron Mining Institute. The Dulbecco's Modified Eagle's Medium Nutrient Mixture F-12 (DMEM-F12), Fetal Bovine Serum (FBS), and Antibiotic (penicillin, and streptomycin) were obtained from Sigma Aldrich (Missouri, USA). The flask 25 cm^2 and plates were purchased from Corning (Arizona, USA).

Cell Cultures

Primary Gingival Fibroblast Normal; Human, Adult (HGF) was purchased at ATCC (CRL-2014, Virginia, USA). The cell was resuspended by fresh medium Dulbecco's Modified Eagle's Medium nutrient mixture F-12 (DMEM-F12), 10% Fetal Bovine Serum, and antibiotic 1% (penicillin, amphotericin B, and streptomycin) (Corning, USA). After the cells reached 80% coverage, they were removed with the help of 0.25% trypsin-0.02% ethylenediaminetetraacetic acid and inoculated on 96 and 24-well plates with the use of new medium. The planted plates were kept in the incubator (5% CO_2 and 37°C) until the start of the experiment.

Experimental Design

Fibroblast planted plates were divided into 10 groups: Control group (Neither treated with BD/BA nor LLLT), BD 100 µg/mL (BD 100), BD 200 µg/mL (BD200), BA 100 µg/mL (BA100), BA 200 µg/mL (BA200), LLLT, LLLT+BD100 µg/mL (LLLT+BD100), LLLT+BD200 µg/mL (LLLT+BD200), and LLLT+BA100 µg/mL (LLLT+BA100) and LLLT+BA 200 µg/mL (LLLT+BA200) groups.

Laser Irradiation

A GaAs (gallium arsenide) laser device (Lasermed 2200, Eme Phsio, Italy) was set at λ = 905 µm, 10000 Hz, 25 mW, and peak power 25 W in continuous mode used for laser therapy. The device was calibrated before the experiments. LLLT was applied by concentric circular motions of the optical fiber at a distance of 10 mm from the surface of the cell culture to irradiate the entire well-containing cells. By inserting the probe from the bottom through a hole in a specific shelf that was made especially for this purpose, culture plates in the research groups were subjected to laser treatment. To provide an even distribution of pulse energy across the whole surface area of the plate where the culture plate was exposed to radiation, a distance of 3 cm between the cell culture plate and probe lens (divergent lens component) was adjusted. It was done at ambient temperature and in the dark. Laser treatment (power/beam field) was calculated according to the formula (power/beam field)×time= J/cm^2. 30 min after drug addition, laser application was performed at 4 J/cm^2 for 160 sec. All LLLT procedures were carried out under aseptic conditions in a Biological Safety Cabinet (ESCO, Esco Micro Pte. Ltd, Singapore).

MTT Assay

Following the LLLT, the cells were kept in the incubator for 24 h. Then, 10 µL (5 mg/mL) of MTT solution was added to the wells and 100 µL of DMSO solution was added to each well to dissolve the purple-colored formazan crystals formed after 4 h. The absorbance of the purple color formed was measured with an ELISA plate reader at 570 nm (µQuant, Bad Friedrichshall, Biotek), and the values obtained were expressed as % cell viability versus control.

The 8-hydroxy-2'-deoxyguanosine (8-OHdG) and Bcl-2 Associated X-Protein (BAX) Analysis

Cultured cells were incubated in a paraformaldehyde solution for 30 min. The cells were then incubated in 3% H_2O_2 for 5 min. 0.1% Triton-X solution was dripped onto the cells washed with PBS and left for 15 min. After the incubation period, protein blocks were dripped onto the cells and kept in the dark for 5 min. Then, the primary antibody (8-OHdG Cat no: sc-66036, Dilution Ratio:1/100 US) was dropped and incubated according to the instructions for use. An immunofluorescence secondary antibody was used as a secondary marker (FITC Cat No: ab6785 Diluent Ratio: 1/500, UK) and kept in the dark for 45 min. Then, the second primary antibody (BAX Cat No: 7480, Dilution Ratio: 1/100, US) was

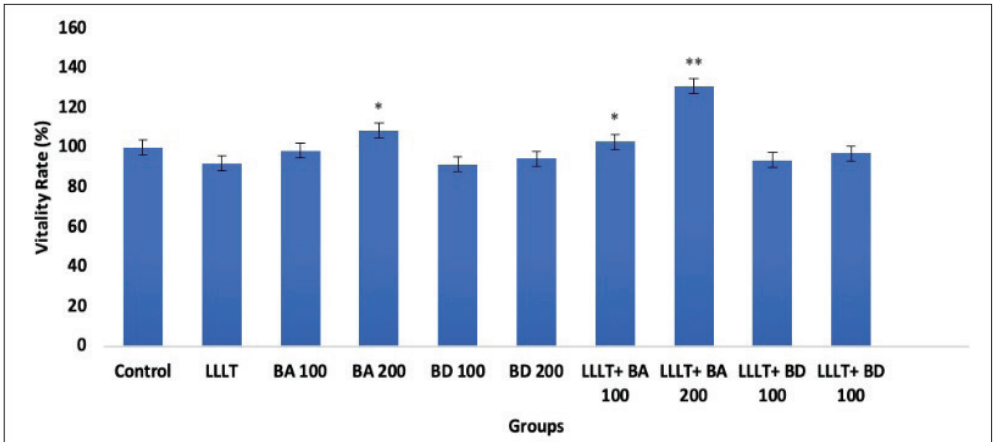

Fig 1. Cell viability was measured with an MTT assay (n = 6). Effects of Boric acid (BA) and Borax decahydrate (BD) with and without Low Level Laser Therapy (LLLT) on vitality rate (%) of gingival fibroblasts for 24 h. The results are presented as the average of three separate experiments. Statistical significance: *P<0.05; **P<0.001

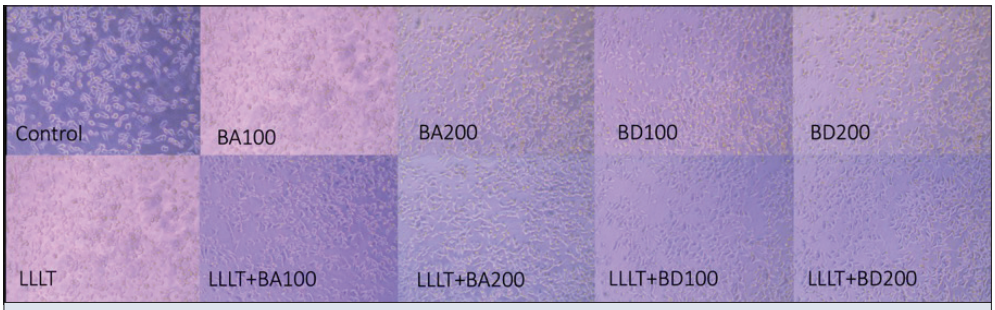

Fig 2. Light microscope images (×40) of gingival fibroblasts cells treated with different concentrations of boric acid (BA), borax decahydrate (BD) and Low Level Laser Therapy (LLLT) (4 J/cm^2) for 24 h

dripped onto the tissues and incubated by the instructions for use. An immunofluorescence secondary antibody was used as a secondary marker (Texas Red Cat No: ab6787 Diluent Ratio: 1/1000 UK) and kept in the dark for 45 min. Afterward, DAPI with mounting medium (Cat no: D1306 Dilution Ratio: 1/200 UK) was dripped onto the sections and kept in the dark for 5 min, and the sections were closed with a coverslip. In order to determine the intensity of positive staining from the pictures obtained as a result of immunofluorescent staining; 5 random areas were selected from each image and evaluated in the ZEISS Zen Imaging Software program.

Statistical Analysis

Statistical comparisons between groups in MTT analysis were calculated using one-way ANOVA and Tukey's HSD method. One-way ANOVA followed by Tukey's test was also performed to compare positive immunoreactive cells and immunopositive stained areas with healthy controls. All calculations were performed using SPSS 20 software for statistical analysis, and a P<0.05 was considered a statistically significant difference in all tests. Data were presented as mean and standard deviation (mean ± SD).

Results

MTT Assay

The MTT assay results are shown in *Fig. 1.* No significant difference was observed between the control and LLLT, BA100, BD100, BD200, LLLT+BD100, and LLLT+BD200 groups in regards to MTT analysis (P>0.05). A significantly higher cell count was observed in the BA200, LLLT+BA100 and LLLT+BA200 groups than in the control group (P<0.05). There was a proliferative peak in the LLLT+BA 200 group (P<0.001). Light microscope results are shown in *Fig. 2.* The microscope and MTT results show a positive correlation. The highest cell density was seen in the LLLT+BA 200 group.

The 8-hydroxy-2'-deoxyguanosine (8-OHdG) and Bcl-2 Associated X-Protein (BAX) Analysis

The 8-hydroxy-2'-deoxyguanosine (8-OHdG) and Bcl-2 Associated X-Protein (BAX) expressions in cell lines were very mild in the LLLT+BA 200 group, mild in the BA200, LLLT+BA100, LLLT+BD100, LLLT+BD200 groups, moderate in the control, BA100, BD100, and

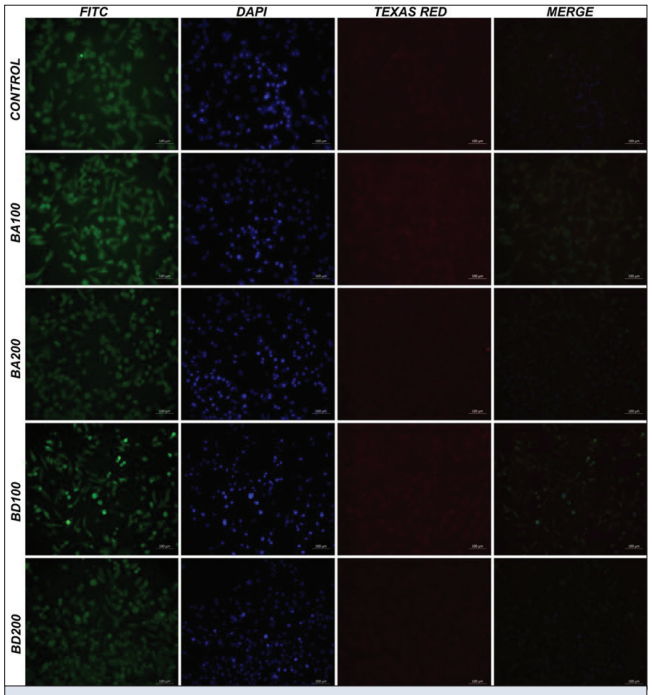

Fig 3. Fibroblast line, 8-OHdG expressions (FITC), BAX expressions (Texas Red), IF, Bar: 100 µm

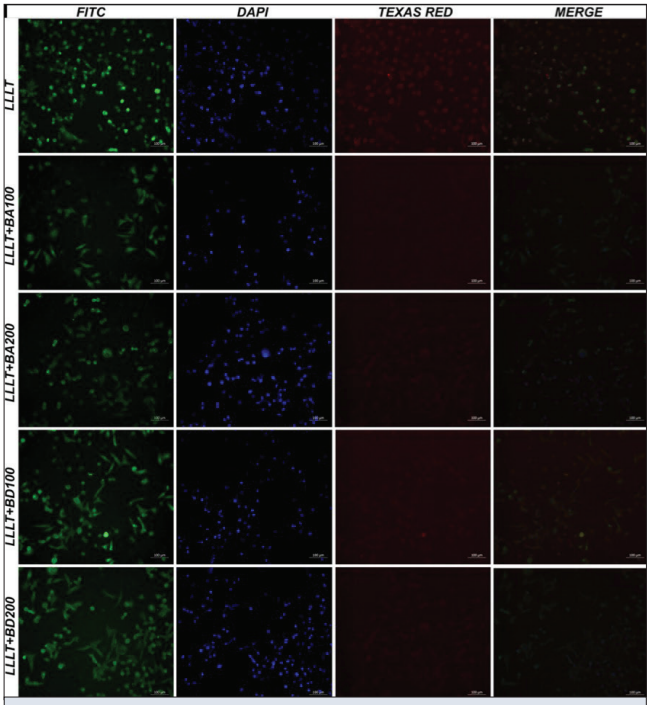

Fig 4. Fibroblast line, 8-OHdG expressions (FITC), BAX expressions (Texas Red), IF, Bar: 100 µm

Table 1. *Detection of 8-OHdG and BAX expression levels in gingival fibroblasts after treated with control, BA100, BA200, BD100, BD200 with and without Low Level Laser Therapy (LLLT)*

Group	8 OHdG	BAX
Control	42.19±2.18[a]	35.43±1.02[a]
BA100	39.15±3.85[a]	33.42±1.75[a]
BA200	28.13±2.57[b]	22.19±2.99[b]
BD100	35.42±2.74[a]	36.18±2.08[a]
BD200	39.74±1.94[a]	34.18±2.26[a]
LLLT	52.48±1.68[c]	51.67±1.23[c]
LLLT+BA100	18.43±2.06[b]	20.36±0.75[b]
LLLT+BA200	14.26±3.06[d]	16.43±1.07[d]
LLLT+BD100	20.12±2.18[b]	19.35±0.73[b]
LLLT+BD200	19.85±2.98[b]	19.52±1.68[b]

Different lowercase letters (a, b, c, d) in the same column represent a statistically significant difference
BA100: Boric acid 100 µg/mL, BA200: Boric acid 200 µg/mL, BD100: Borax decahydrate 100 µg/mL, BD200: Borax decahydrate 200 µg/mL, LLLT: low-level laser therapy

BD200 groups, and severe in the LLLT group *(Fig. 3, Fig. 4)*. 8-OHdG and BAX levels were decreased in the LLLT+BA200 group compared to the control group (P<0.05) *(Table 1)*.

Discussion

This *in-vitro* experimental study aimed to investigate the effects of administering BA and BD in combination with LLLT on gingival fibroblasts. In line with the experimental hypothesis, viability significantly increased in the LLLT+BA 200 group compared to the control group, while 8-OHdG and BAX levels decreased.

Co-administration of BA with LLLT in gingival fibroblasts resulted in biostimulation of cell cultures and increased cell viability. The molecular absorption of laser light is a prerequisite for cellular action. The effects of laser on cells are considered to be wavelength and dose-dependent [20]. Overall, LLLT exposure induced positive and negative effects on gingival fibroblasts depending on the range dose. Previous studies have shown that laser irradiation has stimulating effects at energy densities up to 4 J/cm^2, while at energy densities greater than 4 J/cm^2 it has inhibitory properties of fibroblast proliferation [20-22]. The laser dose was determined as 4.0 J/cm^2 by reference to a previous report [22], which suggests that the dose accelerates fibroblast proliferation.

In this study, we detect that boric acid (BA) has supportive effects when used with laser at doses of 100 and 200 µg/mL. The cell viability data of our study shows that 200 µg/mL BA applied with laser is greater than 100 µg/mL BA. There are many *in vitro* studies on boric acid. A study by Bunning et al.[23] found that 100 µg/mL boron caused no toxicity in normal cells and also eliminated cancer cells

with over 90% killing, demonstrating an outstanding therapeutic index. Ozansoy et al.[24] showed that both sodium borate decahydrate and boric acid had viability-promoting effects in the amyloid-beta toxicity model when used at doses of 200 µg/mL. Cell viability data in the same study showed that 10 µg/mL BA was more toxic than other concentrations (5-15-20-50-100-200 µg/mL). In this study, 100 and 200 µg/mL boron was applied to cell cultures. A similar dose of boron had previously been applied to cell cultures [23,24].

At all times of cellular life, the DNA molecule is subject to oxidative damage caused by reactive oxygen species. Cells provide control of DNA damage by endogenous antioxidant mechanisms and DNA repair systems. Moreover, if the rate of DNA damage exceeds the cell's repair capacity, the accumulation of errors can overwhelm the cell. These cumulative DNA damages can lead to mutations and potentially cancer [22,25]. To control the negative effects of DNA damage, cells present an apoptosis mechanism based on the detection of DNA damage by the p53 protein. Bcl-2 and BAX molecules belonging to the Bcl-2 family are key regulators of the caspase activation pathway initiated by mitochondria during apoptosis [26]. As an antiapoptotic gene, Bcl-2 maintains mitochondrial membrane integrity and prevents the release of internal calcium stores into the cytoplasm [27]. In contrast, as a proapoptotic gene, BAX moves from other cellular compartments to mitochondria in response to apoptotic stimulus and promotes cytochrome c release [28]. Subsequently, activation of the caspase family triggers a series of apoptosis-related events [29]. Apoptosis is triggered by the downregulation of Bcl-2 and overexpression of BAX c, which triggers the change in the mitochondrial membrane that carries cytochrome c from this organelle to the cytoplasm. An increase in cytochrome c levels can trigger the caspase apoptotic pathway, which includes a regulator protein CASP 8 and its executive proteins CASP3 and CASP1 [30]. Therefore, cellular boric acid exposure can trigger apoptotic events in fibroblast cells [31]. Therefore, in this study examined the effects of LLLT and BA on CASP modulation. The reason why 8 OHdG and BAX expressions were moderate in the control, BA100, BD100 and BD200 groups, and severe in the LLLT group was thought to be DNA damage and apoptosis.

Previous studies have shown that laser radiation has stimulatory effects at energy densities up to 4 J/cm^2 [22] while it has highly inhibitory properties at higher energy densities [22,32]. Therefore, a dose of 4 J/cm^2 was used in this study.

The limitation of the study is that different energy densities are not used combine with boron compounds in this study. Determining the optimum dose is extremely important, as the biological response to LLLT stimulation is dependent on wavelength, irradiance, time, pulse, light consistency, polarization, and many other parameters [22]. Therefore, it is extremely important to determine the best LLLT exposure dose that may have some beneficial effects in reversing the changes induced by fibroblast exposure to boron compounds.

The main limitation of this study is that the efficacy of different doses of LLLT on the proliferation of BA and BD was not evaluated. Different energy densities have been tested in most *in vitro* studies [20,33]. Previous studies have shown that an energy density of 2-4 J/cm^2 is most effective in improving cell growth [34,35]. Power density is also important in achieving improved cell proliferation [33].

In conclusion, our study has revealed that the combined application of boric acid and 905 nm LLLT at a dose of 4 J/cm^2 caused a statistically significant increase in the survival of gingival fibroblasts. Clinically, the combination of boric acid and LLLT may be useful in periodontal diseases, wound healing, and obtaining an optimal number of fibroblasts for soft tissue regeneration. These laboratory results will guide future studies on laser tissue irradiation, its effects on tissue healing *in vivo* and the clinical use of boron compounds and LLLT. Further studies are required to elucidate the effects of different power densities and different BA doses on gingival fibroblasts.

Declarations

Availability of Data and Materials: The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

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Research Article

Phenolic Composition and Antioxidant Properties of Bee Pollens Belonging to *Drimia* spp. and *Castanea sativa* L.

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Abstract

Bee pollen is a nutrient-dense food and dietary supplement whose nutritional and bioactive properties are largely influenced by its botanical source. Its composition reflects the diversity of the plants from which they are collected, resulting in a rich array of essential nutrients, phenolic compounds, and other beneficial components that contribute to its value as a functional food. In this study, the biologically active molecules and antioxidant properties of two different bee pollen species from the Aydın and Kastamonu regions were investigated. Antioxidant markers, including total phenolic compounds, total flavonoids, ferric reducing antioxidant power (FRAP), and 2,2-diphenyl-1-picrylhydrazyl ($DPPH^{\cdot}$) free radical scavenging activity, were measured. Additionally, 25 phenolic compounds were analysed using reversed-phase high-performance liquid chromatography (RP-HPLC-PDA). The total phenolic content was determined to be 27.3 mg GAE/g in chestnut (*Castanea sativa* L.) pollen and 8.5 mg GAE/g in Morca pollen *(Drimia* spp.). The findings revealed significant differences in both phenolic composition and antioxidant capacity between the two pollen species. The chestnut bee pollen *(Castanea sativa* L.) exhibited the FRAP, which is likely attributed to its high polyphenol content. Like chestnut honey and propolis, the strong FRAP of chestnut pollen confirms its high potential for use in apitherapeutic and nutraceutical applications.

Keywords: *Antioxidant, Bee pollen, Castanea sativa L., $DPPH^{\cdot}$, Drimia spp.*

Introduction

Bee pollen is the male gametophyte of angiosperms, collected by honeybees during foraging activities on floral structures. It adheres to the bees' hind legs, where it is mixed with nectar and digestive enzymes, forming granules that are then stored within the hive. This nutrient-dense substance supplies honeybees with vital nutrients for their growth and development, including proteins, lipids, sterols, vitamins, and minerals essential during the post-larval stage [1-3]. Bee pollen is also a highly nutritious food for humans, containing a range of macronutrients, such as proteins (10-40%), lipids (1-12%), carbohydrates (13-55%), and fibers (0.3-20%). It is rich in essential amino acids, fatty acids, B vitamins, carotenoids, and polyphenols, as well as sugars like fructose, glucose, and sucrose. Given its balanced nutrient profile and health-supportive properties, bee pollen is often used as a dietary supplement [4-6]. Studies have shown its benefits for immune function, potential cancer prevention, and protection against allergies, skin conditions, liver damage, and more. Furthermore, bee pollen intake has shown positive effects on reproductive health, neuroinflammation, and other conditions, making it a valued supplement for a wide range of health benefits [5,6].

The *Drimia* genus, belonging to the *Asparagaceae* family comprises several species known for their medicinal and ecological significance. Native primarily to the Mediterranean region, Africa, and parts of Asia, *Drimia* species thrive in arid and semi-arid climates. These plants are often characterized by bulbous structures and long, narrow leaves [7]. Many *Drimia* species, such as *Drimia maritima* (also known as sea squill), are rich in bioactive compounds like phenols and flavonoids, which have shown potenFt antioxidant and antimicrobial properties. Traditionally, *Drimia* spp. has been used in folk medicine, is locally called Morca (purple plant) in the Aegean region, and has purple pollen. This pollen, bee pollen, has non-toxic properties and is especially preferred for boosting the immune system. The bioactive composition of *Drimia* spp. is highly influenced by geographic and climatic factors, contributing to their diverse applications in pharmacology and nutraceuticals [7-9].

Castanea sativa, also known as the sweet chestnut, is a deciduous tree native to southern Europe and Asia Minor. Belonging to the *Fagaceae* family, this species is highly valued for its edible nuts, commonly known as chestnuts, which are rich in carbohydrates, vitamins, and minerals. *Castanea sativa* thrives in temperate climates and is often found in well-drained, slightly acidic soils across Europe, including regions of Türkiye, Italy, and France. Besides its nutritional value, sweet chestnut wood is also prized for its durability and is widely used in construction and furniture making. The tree's flowers provide an important nectar source for bees, resulting in honey with a distinctive, robust flavor, and the nuts themselves are enjoyed both as a staple food and in traditional culinary applications across Europe and Asia [10,11]. The chestnut tree *(C. sativa)* is highly valued for its diverse range of products, including its durable wood, edible nuts, and its medicinally significant honey, pollen, and propolis. Chestnut honey, in particular, is noted for its dark color and resistance to crystallization. It is distinguished by exceptionally high polyphenol levels, making it one of the most polyphenol-rich honeys worldwide. This rich polyphenolic content contributes to its potent antioxidant and antimicrobial properties, which have garnered attention in apitherapy therapeutic field utilizing bee products for health benefits. As a result, chestnut honey is not only a valued nutritional supplement but also a potential functional food with therapeutic applications [11].

Polyphenols are a diverse group of naturally occurring compounds known for their antioxidant properties, which help neutralize free radicals and protect against cellular damage. In bee products such as honey, propolis, pollen, and royal jelly, polyphenols contribute significantly to their health-promoting qualities. These compounds, especially flavonoids and phenolic acids, are associated with a range of biological activities, including anti-inflammatory, antimicrobial, and even anticancer effects [12]. Bee pollen is a natural product with a high protein content and is considered a functional food due to its significant phenolic compound composition. In addition to serving as a source of proteins, minerals, vitamins, and antioxidants for worker bees, it is also a valuable natural product with potential applications in apitherapy. Although bee pollen is rich in polyphenols, their composition varies depending on their botanical origin. Therefore, identifying bee products with high phenolic content, particularly bee pollens, could contribute to the discovery of novel sources for complementary medicine applications [13,14].

The objective of this study is to analyze and compare the phenolic profile and antioxidant properties of two distinct species of bee pollen. By establishing this comparative analysis, the study aims to provide a foundation for future research into the biologically active compounds and potential health benefits inherent to these two types of bee pollen.

Material and Methods

Samples and Extraction

The chestnut bee pollen used in this study was sourced in 2023 from experienced beekeepers in Kastamonu, Türkiye, while the Morca bee pollen was obtained from Aydın, Türkiye. The pollen samples, collected in dried form, were stored at +4°C to maintain their quality.

Ethanolic extracts of the bee pollen samples were prepared for total phenolic content (TPC) analysis. Approximately 3 g of bee pollen was mixed with 30 mL of 70% ethanol in a 50 mL falcon tube and stirred continuously at room temperature for 24 h using a shaker (Heidolph Promax 2020, Schwabach, Germany). The mixture was filtered first through coarse filter paper, then with a fine Whatman® membrane filter (Merck, Germany). The filtered extract was stored at -20°C for analysis, with one portion reserved for antioxidant testing and the other for phenolic profile analysis using liquid-liquid extraction [15].

Botanical Analyses

Microscopic analysis was conducted to verify the botanical origin of the bee pollen samples. This method was based on identifying pollen morphologies, following established protocols in the literature [16].

Determination of Total Phenolic Content

The TPC of the samples was assessed using the Folin-Ciocalteu method [17]. In brief, 20 µL of each extract was combined with 400 µL of 0.2N Folin-Ciocalteu reagent in a test tube and then diluted to 680 µL with distilled water. After a 3-min incubation, 400 µL of 7.5% sodium

carbonate (Na_2CO_3) was added to the mixture, followed by a 2-hour incubation at room temperature. Absorbance was subsequently measured at 760 nm using a UV-VIS spectrophotometer (Thermo Scientific Evolution TM 201, USA). TPC was calculated as mg gallic acid equivalents (GAE) per gram of sample based on a standard curve prepared with various gallic acid concentrations (0.031-1.0 mg GAE/mL).

Determination of Total Flavonoid Content

The TFC of the samples was determined following the method by Fukumoto and Mazza [18]. In this procedure, 0.25 µL of the sample extract was mixed with 50 µL of 10% aluminum nitrate ($Al(NO_3)_3$ and 50 µL of 1M ammonium acetate (NH_4CH_3COO). The mixture was diluted to a total volume of 3 mL with 99% methanol and incubated at room temperature for 40 minutes. Absorbance was then recorded at 415 nm against a blank. TFC was calculated as mg quercetin equivalent (QUE) per gram of sample using a standard curve, which was prepared with various concentrations of quercetin standards (0.031-0.5 mg QUE/mL).

Ferric Reducing Antioxidant Power (FRAP)

The FRAP of the samples were assessed using the ferric reducing antioxidant power (FRAP) assay, following the method of Benzie and Szeto [19]. For this, freshly prepared FRAP reagent was made by combining ferric tripyridyltriazine (Fe-III-TPTZ), iron(III) chloride ($FeCl_3$), and acetate buffer in a 40 mM HCl solution, along with 2.5 mL of a 20 mM iron(III) chloride hexahydrate ($FeCl_3.6H_2O$) solution. The sample was prepared by mixing 3 mL of the FRAP reagent with 0.1 mL of the extract in a test tube, then incubating at 37°C for 4 min. Following incubation, absorbance was measured at 595 nm against a blank containing distilled water. A standard curve was created using various concentrations of iron (II) sulfate heptahydrate ($FeSO_4.7H_2O$) ranging from 1000 to 31.25 mmol/mL.

Determination of Radical Scavenging Capacity (DPPH· Assay)

The radical scavenging capacity was determined by a simple method of DPPH [20]. The DPPH· radical was measured with the SC_{50} (mg sample/mL). For this, a 0.75 mL ethanolic extract was mixed with 0.75 mL of DPPH· radical. This mixture was incubated for 50 min in the dark at room temperature. After the incubation, absorbance was measured at 517 nm. The DPPH· capacity was measured using Trolox as a standard, and the results were expressed as SC_{50} (mg sample per mL).

Determination of Phenolic Profiles

Phenolic component analysis via RP-HPLC-PDA was performed following an enrichment step through liquid-liquid extraction. Initially, 10 mL of bee pollen extract was subjected to solvent evaporation at 40°C using a rotary evaporator. The dried extract was then redissolved in 10 mL of purified water, and the pH was adjusted to 2 using concentrated HCl. Organic phases from three successive extractions with diethyl ether and ethyl acetate were pooled. After complete removal of the solvents, the residue was dissolved in 2 mL of methanol, filtered through a 0.45 µm RC membrane filter, and injected into the HPLC system for phenolic analysis [15].

The phenolic profile of the samples was analyzed using a high-performance liquid chromatography (HPLC) system (Shimadzu LC-20AT, Shimadzu Corporation) equipped with a photodiode array detector (PDA) and a C18 column (250 mm × 4.6 mm, 5 µm; GL Sciences). Standard calibration curves for 26 phenolic compounds were constructed based on absorbance readings at a wavelength of 250 nm. The mobile phase utilized was a mixture of 2% acetic acid in water (Phase A) and acetonitrile: water (70:30) (Phase B). Each sample and standard were injected at a volume of 20 µL, with the column maintained at 30°C and a flow rate set to 1.0 mL/min.

Statistical Analysis

The findings were calculated in Excel (Microsoft Corporation) using arithmetic means and standard deviation values from three repeated analyses. Since a limited number of data were used, no further statistical analysis was performed.

Results

Through this study, photographs of the bee pollen samples from two different botanical sources are presented in *Fig. 1*. The total phenolic and flavonoid content of the ethanolic extracts of the dried pollen samples are shown in *Fig. 2* and *Fig. 3*. The average total phenolic content (TPC) of the chestnut and Morca bee pollen was determined to be 27.3 mg GAE/g and 8.5 mg GAE/g, respectively.

It was observed that chestnut bee pollen has approximately three times higher phenolic compound contained than the Morca bee pollen. Similarly, when examining the total

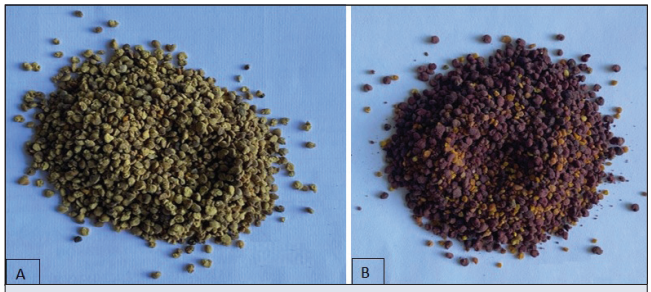

Fig 1. The *Castanea sativa* (A) and *Drimia* spp. (B) bee pollen samples

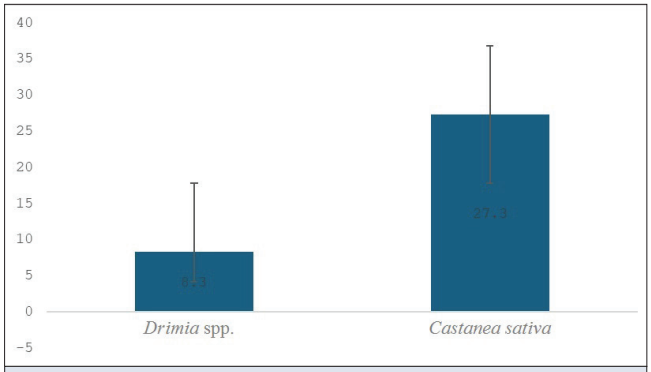

Fig 2. Total phenolic content (TPC) of the bee pollen samples

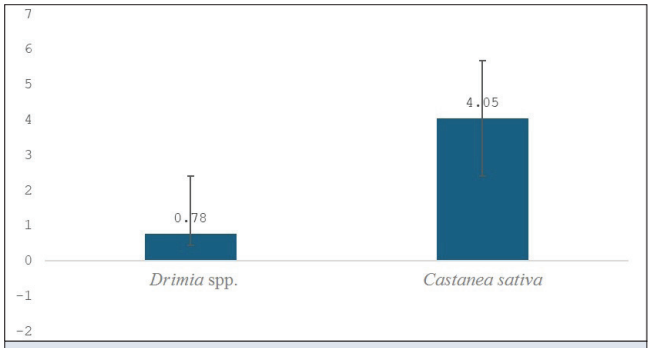

Fig 3. Total flavonoid content (TFC) of the bee pollen samples

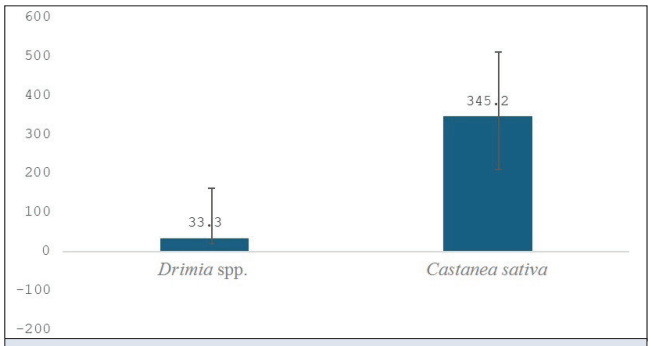

Fig 4. Ferric reducing antioxidant power (FRAP) of the bee pollen samples

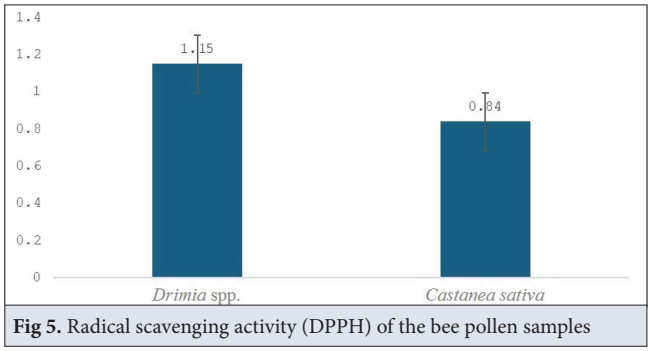

Fig 5. Radical scavenging activity (DPPH) of the bee pollen samples

flavonoid content, the average values were determined to be 4.05 mg QUE/g for chestnut bee pollen and 0.78 mg GAE/g for *Drimia* spp. It was found that chestnut pollen contains approximately five times higher levels of flavonoid compounds.

Table 1. *The phenolic composition of the bee pollen samples using HPLC-PDA*

Bioactive Compounds	**Phenolic Species**	***Drimia spp.***	***Castanea sativa* L.**
Phenolic acids (µg/100 g)	Gallic acid	-	-
	Protocatechuic acid	1095	-
	Chlorogenic acid	-	-
	p-OH Benzoic acid	404	1240
	Caffeic acid	-	-
	Vanillic acid	-	-
	Syringic acid		-
	p-Coumaric acid	10008	5420
	Ellagic acid	-	-
	Ferulic acid	-	-
	t-Cinnamic acid	3486	1250
Flavonoids (µg/100 g)	Catechin hydrate	-	-
	Epicatechin	-	-
	Rutin	-	640
	Myricetin	-	-
	Daidzein	-	-
	Luteolin	-	-
	Quercetin	-	-
	Naringenin	-	-
	Apigenin	-	-
	Hesperidin	-	-
	Rhamnetin	-	-
	Chrysin	6570	8480
	Pinocembrin	7160	3450
	CAPE	-	588
	Galangin	-	-

The current study analyzes the phenolic compositions of two pollen samples that were analyzed using RP-HPLC-PDA. The investigation was conducted based on 26 phenolic standards, previously validated for phenolic analysis. The data obtained are summarized in *Table 1*. The similarities and differences in common and unique components between the two bee pollen samples are summarized in the table. Protocatechuic acid was detected in significant amounts in the Morca bee pollen, while it was found to be below the detection limits in chestnut pollen. Similarly, rutin and CAPE were identified in chestnut pollen but were below the detection limits in bee pollen. On the other hand, p-hydroxybenzoic acid, *p*-coumaric acid, trans-cinnamic acid, chrysin, and pinocembrin were identified as common components in both pollen samples.

The antioxidant capacities of these two bee pollen samples were evaluated using two different methods. The results

are given in the *Fig. 4* and *Fig. 5*. The antioxidant capacities of these two bee pollen samples were evaluated using two different methods. In the FRAP (Ferric Reducing Antioxidant Power) assay, the capacity is measured by the pollen's ability to reduce the Fe (III)-TPTZ complex. A higher FRAP value indicates greater antioxidant capacity, with chestnut pollen exhibiting approximately ten times higher total antioxidant capacity than the other pollen sample.

Discussion

Bee pollen, a complex natural product obtained from the male gametophytes of flowering plants and collected by honeybees, plays a critical role in fulfilling the nutritional requirements of the colony and ensuring its survival. The phenolic profile, in particular, is considered a pivotal determinant of its biological activity and a reliable marker for the authentication of its botanical origin. Although widely utilized, inconsistencies in quality standards, along with insufficient knowledge regarding its bioavailability and long-term impacts, underscore the necessity for comprehensive scientific research to validate its potential applications in nutrition and medicine [1,5].

The biological quality parameters of honey, pollen and propolis are assessed based on their biologically active properties, with the total phenolic and flavonoid content measured as key chemical markers [21]. In this study, the total phenolic and flavonoid content in chestnut pollen was found to be approximately three times higher. A review of the literature indicates that chestnut pollen possesses significantly higher phenolic content compared to approximately nine other botanically sourced bee pollens [1]. Although the TPC value of Morca bee pollen was lower than that of chestnut pollen, it was found to have higher phenolic content than several other pollen types and excluding it from *Fagus* spp., *Rosa* spp., *Helix* spp., and *Rhododendron* spp. Given that chestnut pollen is distinct from all other pollen types, excluding it from comparison, it can be concluded that Morca bee pollen also contains a significant number of phenolic compounds. Comparing the TPC values of these two bee pollen types with reported values for bee bread in the literature, it was found that the phenolic compound content in bee pollen is approximately 3 to 5 times higher than that in bee bread [2,22]. Furthermore, when the phenolic content of these pollen samples was compared with that of strawberries and figs, it was observed that both pollen types contain higher polyphenol levels than either fruit [13,23].

The total antioxidant capacities of these samples were measured using ferric reducing antioxidant power (FRAP). In the assay, the capacity is measured by the pollen's ability to reduce the Fe(III)-TPTZ complex. A higher FRAP value indicates greater antioxidant capacity, with chestnut pollen exhibiting approximately ten times higher total antioxidant capacity compared to the other pollen sample.

The other antioxidant test was DPPH· radical scavenging activity, that the values were found related with FRAP results. The results, expressed as SC_{50} values, represent the amount of extract needed to scavenge 50% of the DPPH· synthetic radical. A lower SC_{50} value indicates higher radical scavenging activity, with chestnut pollen exhibiting a lower SC_{50} value, demonstrating greater activity. The antioxidant properties of pollens are largely attributed to their polyphenol content, making this outcome expected. However, while chestnut pollen showed an FRAP value approximately ten times higher, its DPPH· was only about twice as high, suggesting that polyphenols alone may not fully explain this result. The high DPPH· capacity in Morca pollen is likely due to other antioxidant compounds present, potentially including non-polyphenolic antioxidants [24]. A study reported that extracts obtained from *Drimia maritima* flowers exhibit strong antioxidant, anti-inflammatory, and photoprotective activities [25].

In Morca bee pollen, *p*-coumaric acid was identified as the major phenolic acid component, along with the presence of protocatechuic acid, *p*-hydroxybenzoic acid, *p*-coumaric acid, and t-cinnamic acid. Among the flavonoid compounds analyzed, only chrysin and pinocembrin were detected in Morca pollen. In chestnut pollen, chrysin, pinocembrin, and *p*-coumaric acid were found at major levels, while p-hydroxybenzoic acid, cinnamic acid, rutin, and CAPE was present in smaller quantities. Rutin, CAPE, and epicatechin were exclusively detected in chestnut pollen. In a study similar to ours, chestnut pollen was reported to be rich in p-hydroxybenzoic acid, rutin, and cinnamic acid [1,2]. Extracts obtained from *Drimia maritima* flowers have been shown to be rich in coumaric acid, ferulic acid, cinnamic acid, gallic acid, *p*-coumaric acid, chlorogenic acid, and salicylic acid [25].

As with chestnut bee pollen, chestnut honey and chestnut propolis, the unique biological value of chestnut-derived products is well-documented, and their significance as apitherapeutic products is widely acknowledged. Research conducted on chestnut flowers has demonstrated their abundance in chestnut pollen, and that aqueous extracts of these flowers exhibit elevated antioxidant and antimicrobial properties [26,27].

In conclusion, this study found that bee pollen contains significant amounts of polyphenols, with the quantity of these compounds varying depending on the botanical origin of the pollen. Chestnut pollen was identified as a product with remarkably high biological activity. However, it was also determined that Morca pollen *(Drimias* spp.), while not as potent as chestnut pollen, possesses a

noteworthy antioxidant capacity. Conversely, research on chestnut pollen has been conducted; however, this study is the inaugural one to present Morca bee pollen *(Drimia* spp.) in the scientific literature.

Declarations

Availability of Data and Materials: The data that support the findings of this study are available from the corresponding author (M. Maharramov) upon reasonable request.

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Conflict of Interest: There is no conflict interest.

Declaration of Generative Artificial Intelligence: I declare that the article, tables and figures were not written by artificial intelligence and artificial intelligence-supported technologies.

Author Contributions: CB: Methodology and HPLC analyses, FYK: Antioxidant analysis, EA: Designing an article and botanical analyses, BM: Statistical analysis, MM: Designing the article. All authors have read and agreed to the published version of the manuscript.

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Research Article

Outcomes of Treatment of Cats with Effusive Feline Infectious Peritonitis Using Parenterally Administered Remdesivir with Two Different Maintenance Dose Concentrations

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Abstract

The emergence of antiviral drugs against human coronavirus offers a promising approach for treating progressive and fatal feline infectious peritonitis (FIP). This study aims to evaluate the effectiveness of remdesivir in treating effusive FIP and to compare the lower and upper maintenance doses. Sixteen cats suffering from effusive FIP were randomly assigned to two treatment groups, eight cats in each group. Both groups (A) and (B) were treated with the same initial dose of 10-12 mg/kg body weight by IV route for the first 3 days, while for maintenance dose, group (A) was treated with a lower limit of 5-6 mg/kg body weight and group (B) was treated with upper limit 10-12 mg/kg body weight by SC route till day 84. The overall survival rate was 87.5%. Two cats (one from each group) died within the first 48 h of the treatment protocol. The recurrence rate excluding the two dead cats was 14.28% for group A; one case had disease recurrence, while there was no case recurrence in group B; no significant difference was observed between the two treatment groups in recurrence rate ($P<0.05$). At the end of the treatment period, all 14 surviving cats had normalized clinicopathological findings and disease remission. The lower maintenance dose of remdesivir is as effective as the upper dose.

Keywords: Antiviral, Effusive FIP, Feline coronavirus, Survival rate, Treatment doses

Introduction

FIPV (feline infectious peritonitis virus) is a virulent biotype of feline coronavirus (FCoV) that causes feline infectious peritonitis (FIP), a highly fatal disease with a worldwide distribution affecting both wild and domestic felines of different ages, usually at the age of 3 months to 2 years [1,2].

FIP has two well-recognized clinical forms: effusive (wet) and non-effusive (dry). The main clinical presentation of the effusive form is abdominal distension with fluid, and the accumulated fluid is more likely yellow in color with high protein content; the fluid also accumulates in the pleura resulting in dyspnea. The non-effusive form is less common and chronic form; it causes granulomatous lesions in internal organs such as the liver, kidney, intestine, and lymph nodes. Ocular and central nervous symptoms such as ataxia and coordination are more likely in cats suffering from the non-effusive form [3-5].

Diagnosis of FIP remains a challenge in veterinary practices; clinical signs combined with various diagnostic techniques may be helpful for the diagnosis of FIP in living animals. Ante-mortem definitive diagnosis is particularly significant following the development of effective antiviral drugs against FIP [6]. Nonspecific clinical signs make the diagnosis of FIP embarrassing especially in case of dry form; while in wet form presence of typical effusions ascites and pleural effusions improve the diagnostic procedure and some simple helpful and rapid test as Rivalta's test can be done on effusive fluids [7].

The distinctive histopathological changes and the immunohistochemical technique of the affected tissues remain the gold standard methods for diagnosis of FIP, but it's usually performed after post-mortem examination [6,7]. Many laboratory diagnostic techniques can not differentiate between the two pathotypes of FCoV: feline enteric coronavirus (FECV) and FIPV; however, there is a great difference in virulence between the two pathotypes. FECV is a highly prevalent contagious infection, even though it's usually asymptomatic or may only cause mild diarrhea in some cases [5,6]. FIPV emerged as a result of, different mutations that occurred in the avirulent biotype (FECV); mutations lead to changes in the viral tropism from enterocytes to monocyte immune cells, causing severe disseminated systemic disease [2,3,8].

FIP was considered a highly fatal disease the median survival period without treatment is only eight to nine days instead of using some immunomodulatory agents; these drugs only increase the survival time of diseased cats without complete recovery [4,9]. Recently, the development of some antiviral drugs as "GS 441524" an antiviral medication that acts as a nucleoside analog, and its prodrug remdesivir (GS-5734), which were developed to treat human corona viruses SARS-CoV-1 and SARS-CoV-2 which causes COVID-19 pandemic, have given highly encouraging results with high recovery rates in experimentally and naturally infected cats with FIP [10-13].

Remdesivir (GS-5734) is a broad-spectrum antiviral drug with a small molecule that acts particularly against RNA viruses, including *Coronaviridae;* it interferes with the viral genome replication process [11]. Remdesivir is evaluated in many studies to treat different forms of FIP [1,13]. The recommended dose of remdesivir for treatment of FIP varies significantly between different studies from 5-30 mg/kg body weight depending on the treatment phase (initial or maintenance), FIP form (effusive or dry), presence of ocular or nervous signs, route of administration parenteral method by IV or SC routes or orally (low bioavailability requires high doses), and the study protocol [1,7,11-13].

There are some studies investigating FIP in Africa and the Middle East countries, including Egypt [14-16]. Our study was conducted to spotlight on the common clinical and clinicopathological findings of effusive FIP, evaluate the efficacy of remdesivir in effusive FIP treatment, and compare the efficacy of two different maintenance dose concentrations of remdesivir in effusive FIP treatment.

Material and Methods

Ethical Approval

The Institutional Animal Care and Use Committee of the Faculty of Veterinary Medicine Cairo University (Vet. CU. IACUC) with code number (Vet CU131020241022) approved all the methods of animal handling and sampling included in this study.

Study Design and Inclusion Criteria

Cats with a highly suspected diagnosis of effusive FIP were recruited for this single-centered prospective randomized treatment trial. All of the included diseased cats in treatment procedures were client-owned cats; the study was established without control untreated or placebo group as the untreated group is unethical because untreated FIP is a fatal disease.

Suspected animals were admitted to the small animal clinic of referral teaching veterinary hospital at the Faculty of Veterinary Medicine, Cairo University. Cats that suffered from effusive FIP form were identified from January 2023 to March 2024.

Case data and physical examination: patient signalment, vital parameters (body temperature, respiratory rate, and heart heart) and clinical signs (general health conditions, presence of body cavities effusions, and other clinical signs) were recorded for each case.

Sample collection and analysis: blood sample collection during the animal examination was performed on the plain tube for blood biochemistry and EDTA tube for CBC at zero weeks and 12 weeks of treatment. Body cavity centesis and collection of effusive fluid in sterile containers as a sample for Rivalta's test, detection of protein concentration, and other biochemical analyses were performed during the physical examination of suspected cases.

Imaging: X-rays and ultrasonography were performed on each examined animal for detection and assessment of different body cavity effusions.

Inclusion criteria for treatment procedures:

Inclusion criteria used for diagnosis of FIP cases to be subjected to treatment procedure were defined as highly suspected cases of FIP [13,17] by the following criteria:

1. Clinical signs: typical effusions (ascites +/- pleural and pericardial effusions).

2. Characteristic high proteinaceous effusive fluid (>35 g/L) with positive Rivalta's test [17,18].

3. Decrease in albumin globulin ratio (A/G ratio) with cut of point <0.6 [1,8].

4. Presence of ≥3 other clinicopathological findings such as pyrexia, anemia, lymphopenia, neutrophilia, hyperbilirubinemia, hypoalbuminemia, and hypergammaglobulinemia [1,8,12].

5. Negative test results for feline immunodeficiency virus (FIV) and feline leukemia virus (FeLV) infection. A SNAP

Combo FeLV Ag/FIV Antibody rapid immunoassay (Product Code 502A.02, IDEXX laboratories) was performed for each serum sample according to the manufacturer's instructions to detect feline leukemia virus (FeLV) antigen and feline immunodeficiency virus (FIV) antibodies in feline serum [1,8].

Treatment Procedures and Protocol

The drug used: remdesivir - Eva Pharma Egypt (20 mL vial containing 100 mg remdesivir) for parenteral administration (I/V or S/C).

Treatment regimen: all diagnosed cats with FIP were initially treated for the first three days of therapy at a dose of remdesivir (10-12 mg/kg diluted to 10 mL with saline and given over 10 minutes) by intravenous route as loading dose to speed up the antiviral efficacy. The treatment regime was then changed to the subcutaneous route with a maintenance doses [12] illustrated in *(Table 1)*. The treated cats were randomly selected for either group (A) or group (B); the treatment period lasted for 84 days.

Table 1. *Treatment regime: doses concentration, method of administration, and treatment groups*

Groups	Treatment Regime and Dose Concentrations	
	Initial Dose (every 24 h for 3 days, IV)	**Maintenance Doses (every 24 h from day 4 to day 84, SC)**
Group (A)	10-12 mg/kg body weight	5-6 mg/kg body weight
Group (B)		10-12 mg/kg body weight

Treated cases follow-up: veterinarians examined and evaluated cats weekly for the first month of treatment, then monthly until the end of the treatment period, and for another three months of follow-up of treated cases. Clinical follow-ups of cases and monitoring animal's body weight to adjust the drug dose were done by patient owners, caretakers, and veterinarians.

Study Outcomes and Statistical Analysis

Study outcomes were evaluated as primary outcomes, including survival rate, disease remission (complete resolution of clinical signs such as pyrexia, lethargy, and body cavity effusions) [19], and recurrence rate during 84 days of treatment and 3 months of follow-up and the progression and normalization of clinical and clinicopathological findings as a secondary outcome. For evaluation of liver and kidney function ALT, ALP, and creatinine were measured pre and post-treatment.

Pearson's Chi-squared test was performed to test whether there is a statistically significant difference in the recurrence rate between the two treatment groups (A and B) ($P<0.05$).

An independent sample T-test was used to test for the difference between both groups before treatment and the difference between both groups after the treatment among each studied hematological and serum parameter. A one-way analysis of variance (ANOVA) was used to test the effect of the two treatment protocols (groups A and B) on the studied hematological and serum parameters. The Shapiro-Wilk test was utilized for normality analysis of the variables, and Levene's test was used to evaluate the homogeneity of variance. Parametric statistical tests were used for analyzing data with a normal distribution, and if there were significant differences, the least significant difference (LSD) test was used for post-hoc analysis. Otherwise, the non-parametric tests were used for the data that was not distributed normally. Data were presented as the mean ± standard error (SE). Statistical significance was set at $P<0.05$. All analyses were performed with SPSS® version 20.

Results

Animals

Of 31 suspected effusive cases suffering from ascites and other body cavity effusions on physical examination and imaging, sixteen cases (16/31) are considered highly suspected cases of FIP (n=16) according to the study inclusions criteria and owners' approval to be included in the treatment procedures.

Demographic character of included animals: according to sex (10 males and six females), according to age (14 cats <2 years and two cats >2 years), and according to breed (8 Persian, 4 mixed, and four domestic short hair). The different recorded clinical and clinicopathological findings of the 16 included cats are illustrated in *(Table 2)*, body cavities effusions (mainly ascites *(Fig. 1-A,B)*, lethargy, inappetence, and pyrexia were the most common clinical findings in examined cases. All included cats are Rivalta's test positive *(Fig. 1-C,D)* and feline immunodeficiency virus (FIV) and feline leukemia virus (FeLV) rapid commercial test negative. X-Ray imaging and ultrasonographic examination reveals different body cavities effusions *(Fig. 2, Fig. 3-A,C)*.

Primary Outcome of the Study

The survival rate from the beginning till the end of the treatment protocol (84 days) and 3 months of follow-up is 87.5%; one cat from each group A and B died within 48 h from the beginning of the treatment regime. The survival rate after 48 h from the beginning of the treatment regime reaches 100%. All the surviving 14 cats had disease remission by the end of the treatment period. In group (A) receiving the lower dose of remdesivir 5-6 mg/kg body weight as a maintenance dose, one case had disease recurrence after ending the treatment regime by

Table 2. *Number and percent of included cats with different clinical and clinicopathological findings*	
Clinicopathological Finding	**Number and Percent of Cats**
Lethargy	15/16 (93.75%)
Pyrexia	13/16 (51.25%)
Labored breathing and tachypnea	10/16 (62.5%)
Tachycardia and cardiac arrhythmia	5/16 (31.25%)
Inappetence	15/16 (93.75%)
Abdominal distension	16/16 (100%)
Peritoneal effusions	16/16 (100%)
Thoracic effusions	4/16 (25%)
Pericardial effusions	2/16 (12.5%)
Anemia	8/16 (50%)
Lymphopenia	8/16 (50%)
Neutrophilia	9/16 (56.25%)
Hyperbilirubinemia	8/16 (50%)
Hypoalbuminemia	9/16 (56.25%)
Hypergammaglobulinemia	11/16 (68.75%)
A/G ratio (< 0.6)	16/16 (100%)
ALT	3/16 (18.75%)
ALP	1/16 (6.25%)
Creatinine	2/16 (12.5%)
Protein content (>35g/L) of effusive fluid	16/16 (100%)

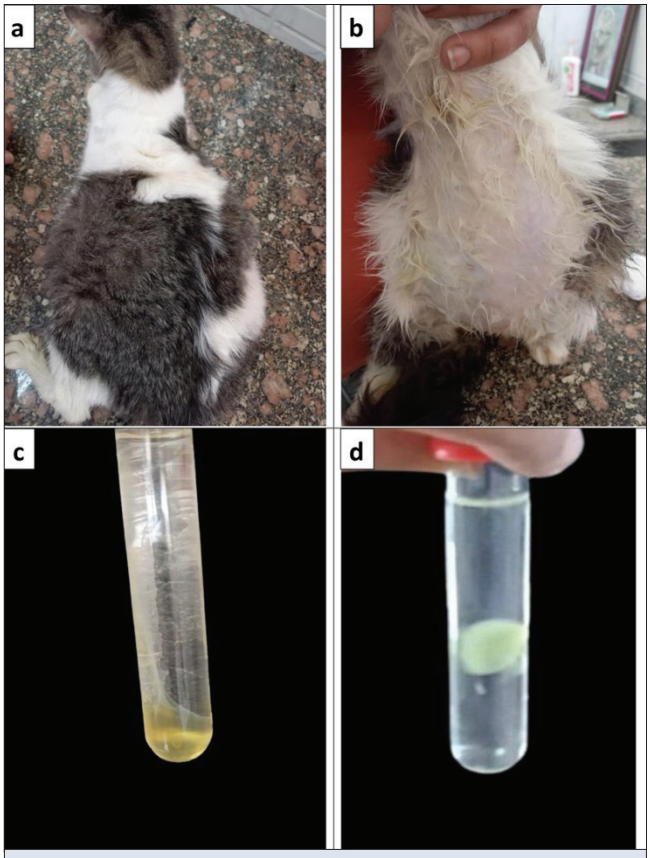

Fig 1. a, b- five months old cat suffering from abdominal distension (ascites); **c, d**- positive Rivalta's test, note the drops of the examined fluids retain its shape

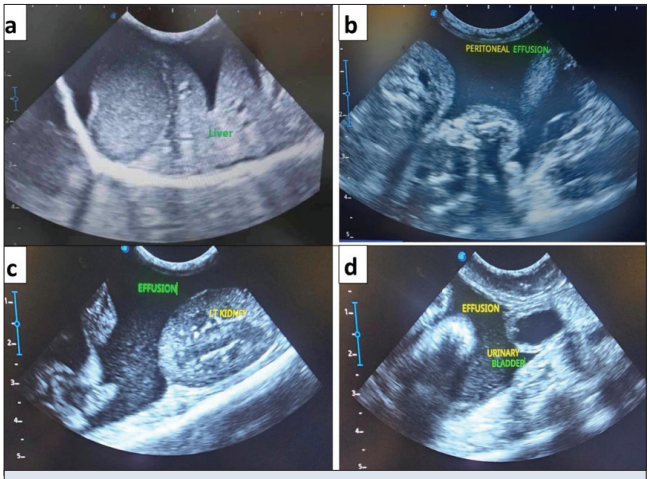

Fig 2. Ultrasonic imaging of cats suffering from effusive FIP; **a**- one years old Persian cat suffering from peritoneal perihepatic effusions; **b, c, d**- one and half years old DSH cat suffer from abdominal effusions, **b**- peritoneal effusions, **c**- peritoneal effusions around kidney, **d**- effusions in pelvis cavity around the urinary bladder

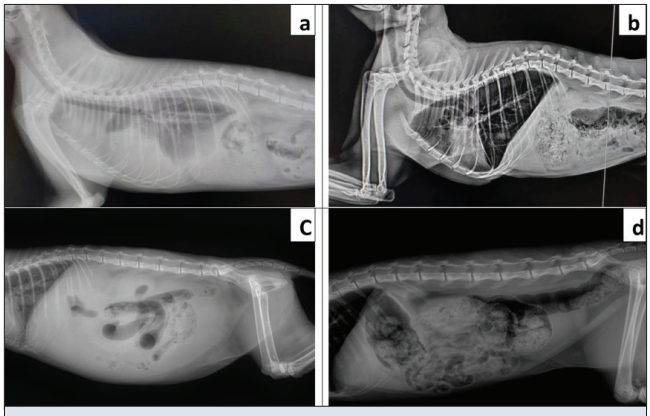

Fig 3. X-Ray imaging of cats suffering from effusive FIP; **a, b**- one year old Persian cat suffer from pleural and peritoneal effusions a- pre-treatment, **b**- 15 days post-treatment; **c, d**- eight months old DSH suffer from severe peritoneal effusions; **c**- pre-treatment, **d**- 15 days post treatment

10 days with the appearance of few amounts of peritoneal effusions and pyrexia.

The overall recurrence rate for surviving cats was 7.14% (1/14); the recurrence rate was 14.29% (1/7) in group (A) while, in group (B), no detected recurrence cases (0/7). No significant difference in disease recurrence between the two groups was detected at $P<0.05$. No another recurrent cases were reported until the date of paper submission.

Secondary Outcome of the Study

Obvious clinical improvement was observed in all of the 14 cats survived during the study, the. The main clinical signs improved within short period after starting the

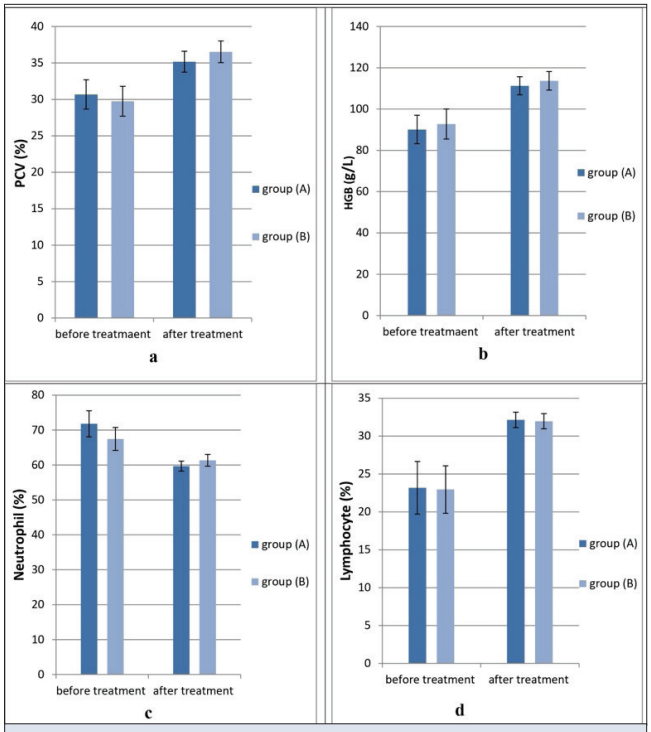

Fig 4. Hematology findings of group A and group B before and after treatment; bars represent the values in mean and bar-lines represent the standard error, P value at <0.05. **a**- PCV, **b**- HGB, **c**- neutrophils, **d**- lymphocyte

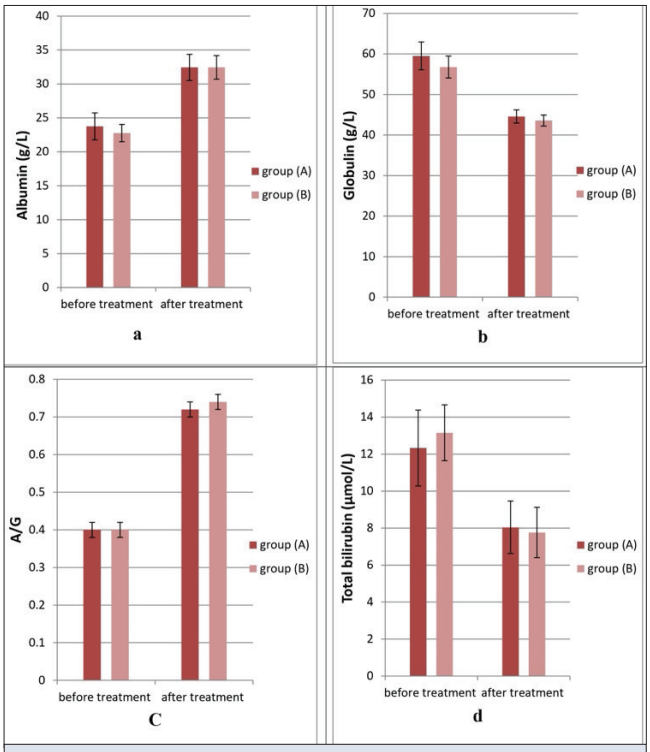

Fig 5. Biochemical findings of group A and group B before and after treatment; bars represent the values in mean and bar-lines represent the standard error; **a**- Albumin, **b**- Globulin, **c**- A/G ratio **d**- Total bilirubin

treatment protocol; pyrexia resolved in a mean of 8 days, effusions resolved in a mean of 14 days *(Fig. 3-B,D)*, and improvement of lethargy and return to normal activity occurred in a mean of 20 days.

Clinicopathological finding results analyses using one-way ANOVA of both groups before and after treatment were illustrated in *(Fig. 4, Fig. 5)*. A non-significant difference between both groups before and after treatment for almost all studied variables was detected at P<0.05. For hematological findings, PCV and HGB increased markedly after treatment in group B compared to groups A and B before treatment. Neutrophils decreased significantly in both groups after treatment compared to before treatment, while lymphocytes showed a significant increase after treatment in both groups. Regarding blood biochemistry findings, albumin and A/G ratio showed a significant increase after treatment in groups A and B; however, globulin revealed a marked decrease after treatment in groups A and B. Total bilirubin decreased obviously in group B after treatment compared to before treatment. All of the evaluated clinicopathological findings were improved toward their standard values at the end of the treatment period.

Clinicopathological finding related to liver and kidney function test such as ALT, ALP and creatinine were also improved toward their standard value at the end of treatment period.

Discussion

FIP was considered a progressive fatal disease with unfavorable prognosis [20]; the development of antiviral drugs for the treatment of human corona viruses gives a new hope for effective FIP treatment and lifesaving. In many countries, treatment of FIP is still limited; many veterinarians still use unlicensed and unregistered antiviral drugs in their countries for the treatment of FIP [22-24]. Remdesivir, a widely used drug for COVID-19, was evaluated in many studies for treating FIP in cats. The effusive form is the common clinical form of FIP; the recommended maintenance dose of remdesivir for effusive FIP form in cats ranged from 5-6 mg/kg body weight to 12 mg/kg body weight [12,24].

The main primary outcome of this treatment study using parenteral remdesivir is the survival rate; the survival rate in this randomized treatment study was 87.5% overall from the beginning of the treatment regime and reached 100% after 48 h from the beginning of the treatment regime, our finding is similar to previous study investigated the treatment of FIP in Sydney [12] with parenteral administration of remdesivir with or without transition administration of GS-441524, the overall survival rate from the beginning of treatment regime was 86% and after 48 h from start of treatment regime reach 96% and also agree with a retrospective study performed from 2020-2022 [13] illustrated that at completion of initial treatment

period by injectable remdesivir or oral GS-441524, 88.6% of treated cats were alive.

Our finding is not similar to a previous blinded study to treat effusive FIP, which indicated that the survival rate of oral remdesivir from the start of the treatment protocol to the end of the study was 77% [1], the authors attributed the lower survival rate to the medical condition of the included animal, which may be severely compromised at the start of antiviral treatment. Moreover, it may be related to dose adjustment of oral remdesivir and its bioavailability after oral administration.

Another important primary outcome of the study is disease recurrence and relapsing of the clinical signs recorded in treating FIP with various antiviral drugs. The overall relapsing rate in this study was 1/14 (7.14%) after excluding the two dead cats; this finding is nearly similar to the previous study [13], which recorded the recurrence rate of FIP after treatment by remdesivir or GS-441524 as (6.6%) and disagreed with the previous study [12], which recorded recurrence rate of the remdesivir treatment as 25%, the authors attributed the higher recurrence to unwilling remdesivir dose drop due to the increase in the animal's body weight during the treatment period and also disagree with Cosaro et al.[1] who recorded no recurrence rate however, one of the nine survived cats showed seizures after disease remission and was suspected to had the nervous manifestation of FIP but not confirmed after euthanization.

The secondary outcome of the treatment study is the clinical and clinicopathological changes that occur with antiviral treatment of FIP; the disease remission depends on the resolution of clinical signs and normalization of clinicopathological findings. The main clinical signs of effusive FIP in this study are pyrexia, body cavity effusions, and lethargy resolving in a mean period of 8, 14, and 20 days, respectively; this finding is consistent with a previous study [12] that reported quick and clear improvement of clinical signs and the median time for resolution of pyrexia and effusions by 7 and 9.5 days respectively. These findings disagree with the prior study [1], which reported that the effusions take 6 weeks to resolve in all surviving cats treated with oral remdesivir, and this is attributed to the study protocol of the treated animals as they only evaluated at three visits (0, 6,16 weeks).

Clinicopathological findings mostly take longer than clinical signs to normalize again and reach normal reference values; even though the average time for normalization of these values can not be detected in this study due to the absence of follow-up sampling and testing. One limitation of this study is that the evaluation of hematology and blood biochemistry was only done twice during the study: once before starting the treatment and once at the end of the treatment course on day 84.

All clinicopathological findings were adjusted toward their normal values after 84 days of treatment by parenteral remdesivir. One of the most clinicopathological findings used for the diagnosis of FIP is the A/G ratio; hyperglobulinemia with or without hypoalbuminemia leads to a decrease in the A/G ratio [25,26]. Our findings showed normalization in the value of albumin by an increase in the mean value by 9 g/L, reduction in the mean value of globulin by 14 g/L, and an increase in the mean value of A/G ratio by 0.3; these findings are nearly consistent with other studies [12,13] that recorded normalization in values of albumin, globulin and A/G ratio after treatment with remdesivir or GS-441524 or both, even though Cosaro et al.[1] reported that one cat still had elevated globulin level at 16 weeks of the treatment protocol, but they attributed this to other concurrent infections with gingivostomatitis and upper respiratory infection.

The selection of a cutoff point <0.6 for the A/G ratio in this study is to reach adequate specificity (87%), sensitivity (75%) and positive predictive value (95%), while selection of lower cutoff point will adversely lower the sensitivity reaching 50% [18].

In this randomized prospective centric study, we compare the efficacy of a low recommended maintenance dose (5-6 mg/kg body weight) of remdesivir in group (A) to higher maintenance dose (12 mg/kg body weight) of remdesivir in group (B) to treat effusive FIP.

In the consideration that the effusive FIP form without ocular or nervous signs is mostly less severe form; the study aims to determine whether the lower limit dose of remdesivir is as effective as the upper limit dose. Using the lower maintenance dose limit will significantly reduce the cost of treatment. The cost of the treatment regime of FIP, which usually takes 3 months or more, is still challenging to many patient owners, especially in developing countries as remdesivir is considered an expensive drug, particularly the available parenteral form.

In this study, the survival rates for both groups A and B were similar from the beginning of the study to 6 months including three months of follow-up (87.5%) and after 48 hrs. from the beginning of the treatment protocol (100%). Although the recurrence rate in group (A) receiving the lower dose was 1/7 (14.28%) and there were no recurrence cases in group (B), statistically no significant difference was detected between the two groups in recurrence rate.

There is no statistically significant difference in almost all clinicopathological findings between the two treatment groups, especially the A/G ratio, which is critical for the diagnosis of effusive FIP. Even though the

included animals in the study were randomly assigned to two treatment groups, no statistical differences were found in clinicopathological characteristics between the two groups. This may be related to the fact that all of the included animals were suffering from the same disease form of effusive FIP without ocular or nervous manifestation and were nearly similar in disease severity at the time of inclusion.

Another limitation of the study is that all included cats are considered highly suspected cases of effusive FIP due to the lack of confirmatory diagnostic tests, especially the gold standard test in effusive FIP in living animals' immunocytochemistry on effusive fluid instead of immunohistochemistry tissue biopsy [27,28], which requires special specific labs and professional technicians.

However, many previous treatment studies [13,29] for FIP include highly suspected cases of FIP in their study protocol and inclusion criteria. Furthermore, some studies [1,8,12] depend on the detection of FCoV RNA by PCR in body cavity effusions as a main test in the study inclusion criteria, which have variable sensitivity (72-100%) and specificity (83-100%) [6]. The combination of characteristic clinical signs of body effusions, distinctive effusive fluid with high protein content (positive Rivalta's test), low A/G ratio, and other clinicopathological findings without detecting any other relevant health disorders enhanced effusive FIP diagnosis, more over the response of most treated cases to remdesivir without any other specific treatment make the diagnosis more rationale.

The drug side effects in this study are related to the method of administration by subcutaneous injection as pain and discomfort; the same side effects reported previously [12,30,31]; registered and licensed oral remdesivir to be used in cats is highly recommended to overcome side effects related to the method of administration, be easily applied, and decrease the cost of therapy. However, no follow-up blood and biochemical analysis was performed in this study, and the clinicopathological findings evaluation depends on pre and post-treatment analyses; no clinically relevant adverse events showed by treated animals suspect liver or other organ insufficiency require further blood and biochemical analysis during treatment protocol or discontinued of treatment protocol.

In conclusion, remdesivir is effective in effusive FIP treatment without signs of nervous or eye manifestations with a high survival rate, especially for cats that survive the first 48 h of the treatment regime. Low-maintenance doses of remdesivir are as effective as high doses with a non-significant recurrence rate. Oral registered and licensed remdesivir is required to be used in animals with convenient concentration. It may be more cost-effective and easily administrated to avoid adverse side effects of injectable remdesivir. Additional future studies are essential to compare the efficacy of different antiviral drugs in FIP therapy. A rapid, reliable, and simple confirmatory test for FIP diagnosis is highly required.

Declarations

Availability of Data and Materials: The data illustrated in this study is available upon request from the corresponding author (E.S.)

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Ethical Approval: The Institutional Animal Care and Use Committee of the Faculty of Veterinary Medicine Cairo University (Vet. CU. IACUC) with code number (Vet CU131020241022) approved all the methods of animal handling and sampling included in this study.

Conflict of Interest: The authors declare that there is no conflict of interest

Declaration of Generative Artificial Intelligence (AI): The authors declare that the article and/or tables and figures were not written/created by AI and AI-assisted technologies.

Author's Contributions: Conceptualization, E.S. and A.Z.; methodology, E.S. and M. S.; investigation, A.Z. and E.S.; formal analysis M. S. and E.S.; writing-review & editing, all the authors. All authors have read and agreed to the published version of the manuscript.

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Review Article

Meta-Analysis of Performance and Carcass Values in Lamb Fattening in Türkiye

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Abstract

This study aimed to reach a common conclusion for different breeds, regions, years, ages and fattening periods by examining studies on lamb fattening in Türkiye between 2000-2024 years concerning performance and carcass characteristics with a meta-analysis. The analysis included 51 research results from 21 studies that met the study criteria consisting of being conducted after the year 2000, investigating variables such as initial live weight (ILW), slaughtered body weight (SBW), daily weight gain (DWG), feed conversion ratio (FCR), hot carcass weight (HCW) and dressing percentage (DP), cold carcass weight (CCW) and yield (CCY), with at least three studies on a breed, and being conducted in Türkiye. The highest values for ILW and SBW were detected in the Awassi breed and the Eastern Anatolia region. For DWG, significantly highest value was calculated in the Morkaraman breed (265.95 g; P<0.05). The best values for FCR were calculated in Akkaraman breed (5.10 kg); in Eastern Anatolia region (4.84 kg); <90 days (5.06 kg) according to fattening start age, and according to fattening period, ≥90 days (4.77 kg) (P<0.01). The Awassi breed had the highest values concerning HCW, DP, CCW, and CCY from the carcass data, whereas Marmara region had the lowest values among the regions. In conclusion, it can be speculated that the Awassi, Morkaraman, and Akkaraman breeds are preferred primarily, Eastern and Central Anatolia are more suitable regionally, and fattening lambs <90 days old and long-term fattening (≥90 days) are prominent both technically and economically.

Keywords: Carcass, Lamb, Meta-analysis, Performance, Türkiye

Introduction

Lamb fattening is the process of raising and feeding lambs after weaning for butchering. This process is carried out to ensure that the animals grow healthily, demonstrate optimum yield capabilities and obtain high amounts of quality meat. Red meat obtained from lamb farming is one of the leading sources of meeting animal protein needs [1]. At least 40-50% of the daily protein requirements of a balanced and healthy diet must be met by animal-based proteins [2].

The total red meat production of Türkiye in 2023 was calculated as 2.384.047 tons, of which 70.1% (1.670.606 tons) was beef, 23.9% (569.066 tons) was mutton, and the remaining 6% (144.375 tons) was goat and buffalo meat [3]. In sheep breeding in Türkiye, one of the primary sources of income is lamb meat, and lamb meat production holds particular importance due to the presence of 42 million sheep and the traditional consumption habits of the people [4]. Considering the negative effects of red meat production and the instability in prices in recent years, sheep breeding has become a good alternative that can be used to solve these problems.

The demand for animal products in Türkiye has been increasing every day due to rapid population growth, tourism, refugee migration, and socio-economic and cultural development. To meet this increasing demand, increasing the carcass and meat yield per unit of animal is more economically important than increasing the number of animals.

The fattening performance of lambs, which is affected by factors such as breed, sex, age, care-feeding method, feed amount and quality, can be listed as post-slaughter carcass weight, yield and quality [5]. Producers raising lambs aim to achieve maximum fattening performance and carcass yield at minimum cost by paying optimum attention to the existing elements. There are many individual studies on lamb fattening, conducted over many years on different breeds, regions, times, ages and fattening periods in Türkiye that were used in the present study.

The current article is a comprehensive study in which lamb fattening researches carried out after the year 2000 in Türkiye are handled together, analyzed and interpreted in a way to guide producers/stakeholders.

The results obtained from individual studies conducted independently on a particular subject can be very diverse and different. In the current study, meta-analysis appeared to be a good option for both evaluating and interpreting information from a large number of studies, obtaining new information, and reaching a more comprehensive and reliable common conclusion by eliminating heterogeneity between studies [6]. Meta analysis is the process of reaching a common conclusion by systematically combining the results of studies conducted independently on a certain subject using a specific method. With this analysis, the results of studies with small sample sizes can be combined with scientific methods, and parameter estimates with higher power and precision can be made with larger samples [7,8].

In the current study, it was aimed to reach a common conclusion for different breeds, regions, years, ages, and fattening periods by examining studies on lamb fattening in Türkiye between 2000-2024 regarding performance and carcass characteristics through a meta-analysis and to guide producers who will engage in lamb fattening in the future.

Material and Methods

The study material consisted of results from studies investigating the fattening performance and carcass yield characteristics of sheep in Türkiye conditions. This study was not subject to ethics committee approval because the research data were obtained from articles available in the open-access literature. The flow diagram for selecting eligible studies for meta-analysis is given in *Fig. 1*.

In the study, separate meta-analyses were performed for initial live weight (ILW), slaughtered body weight (SBW), daily weight gain (DWG), feed conversion ratio (FCR), hot carcass weight (HCW), dressing percentage (DP), cold carcass weight (CCW), and cold carcass yield (CCY) variables. In the meta-analysis, results that did not have mean and standard deviation values specified for the variables and did not form subgroups were eliminated. Therefore, the number of studies in the general meta-analysis and subgroup analysis differed according to variables. In this study, subgroups were determined as breed (Awassi, Akkaraman, Kıvırcık, Morkaraman, Tuj, Karayaka), geographical region (Southeast Anatolia, East Anatolia, Central Anatolia and Marmara), fattening start age (<90 days, 90-120 days and >120 days) and fattening duration [Short (≤60), Medium (61-89) and Long (≥90)].

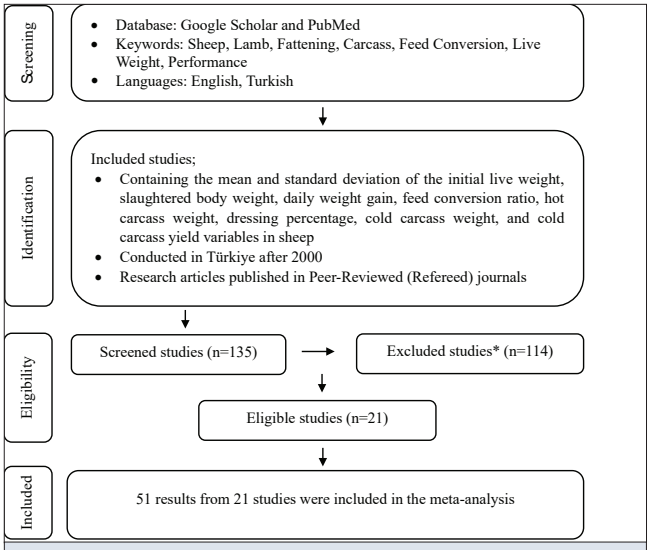

Fig 1. Flow diagram of studies included in the meta-analysis (* Studies that do not meet the inclusion criteria)

Heterogeneity among the study results included in the analysis was evaluated using Cochran's Q and I^2 statistics. Meta-analysis results were presented with a random effects model in studies with heterogeneity, and with a common effects model in studies with homogeneity. Egger's linear regression test was used to detect publication bias, and Duwal and Tweedie's trim and fill method was used to eliminate publication bias. Furthermore, effect sizes and weights (%) of the studies were showed with forest plot [9].

The statistical significance level was determined as $P<0.05$, for the difference between effect sizes and $P<0.10$ for Cochran's Q statistics. Analyses were performed with the "meta" package in RStudio (version 2024.04.22+764) software. Characteristics of the studies used in the meta-analysis are shown in *Table 1*.

Results

In this study, the analysis values of fattening performance (ILW, SBW, and DWG), carcass yield (HCW, DP, CCW, CCY), and other statistical information are presented in *Table 2*.

According to the meta-analysis results, it was observed that there was a high level of heterogeneity among the studies for all variables. The values calculated with the random effects model for some variables were found as

Table 1. Characteristics of studies used in meta-analysis	
No	**References**
1	**Adıgüzel Işık S, Sarı M, Muammer T, Önk K:** The effect of fattening time on fattening performance, slaughter and carcass characteristics in Tuj male lambs. *MAS JAPS,* 8 (2): 256-264, 2023.
2	**Altın T, Karaca O, Cemal İ, Yılmaz M, Yılmaz O:** Kıvırcık ve Karya kuzularda besi ve karkas özellikleri. *J Anim Prod,* 46 (1): 19-29, 2005.
3	**Balcı F, Karakaş E:** The effect of different slaughter weights on the fattening performance, slaughter and carcass characteristics of male Karayaka lambs. *Turk J Vet Anim Sci,* 31 (1): 25-31, 2007.
4	**Demir H, Kahraman R, Özcan M, Kaygısız FH, Ekiz B:** Kıvırcık kuzuların rasyonuna katılan zinc bacitracin'in besi performansına, bazı karkas özelliklerine ve kuzu maliyetine etkisi. *İstanbul Üniv Vet Fak Derg,* 28 (1): 185-198, 2002.
5	**Esenbuga N, Macit M, Karaoglu M, Aksakal V, Aksu MI, Yoruk M A, Gül M:** Effect of breed on fattening performance, slaughter and meat quality characteristics of Awassi and Morkaraman lambs. *Livest Sci*, 123, 255–260, 2009.
6	**Gökdal Ö, Atay O, Eren V, Demircioğlu SK:** Fattening performance, carcass and meat quality characteristics of Kivircik male lambs. *Trop Anim Health Prod,* 44, 1491-1496, 2012.
7	**Gül M, Yörük MA, Macit M, Esenbuga N, Karaoglu M, Aksakal V, Aksu IM:** The effects of diets containing different levels of common vetch (Vicia sativa) seed on fattening performance, carcass and meat quality characteristics of Awassi male lambs. *J Sci Food Agric,* 85, 1439-1443, 2005.
8	**Gül S, Biçer O:** İvesi koyunlarında besi performansı ve EAAP metoduna göre karkaslarının değerlendirilmesi. *MKUJAS*, 25 (1):20-26, 2020.
9	**Gürbüz A, Akman N, Ankaralı B, Öztürk H:** Ile De France (If), Akkaraman (Ak) ve bunların melezi (F1 Ve G1) erkek kuzularda besi performansı. *Lalahan Hay Araşt Enst Derg,* 40 (2) 27-33, 2000.
10	**Karabacak A, Boztepe S:** Yağlı kuyruklu ve yağsız ince kuyruklu koyun ırklarının besi performanslarının karşılaştırılması. *Selcuk J Agric Food Sci,* 21 (42): 89-95, 2007.
11	**Koyuncu M:** Growth performance and carcass quality of fattening lambs of Kivircik and Karacabey Merino breeds. *LRRD,* 20 (12):197, 2008.
12	**Kul S, Şeker İ:** İvesi ve Tahirova x İvesi Melezi (F1) erkek kuzuların besi performansı, kesim ve karkas özellikleri. *F U Vet J Health Sci,* 16 (1): 57-64, 2002.
13	**Küçük M, Bayram D, Orhan Y:** Morkaraman ve Kıvırcık X Morkaraman (G1) melezi kuzularda büyüme, besi performans, kesim ve karkas özelliklerinin araştırılması. *Turk J Vet Anim Sci,* 26, 1321-1327, 2002.
14	**Macit M:** Growth and carcass characteristics of male lambs of the Morkaraman breed. *Small Ruminant Res,* 43, 191-194, 2002.
15	**Mis A, Öztürk Y:** Akkaraman toklularda besi performansı, kesim ve karkas özellikleri. *MAKÜ Sag Bil Enst Derg,* 6 (2): 72-83, 2018 .
16	**Önk K, Sarı M, Yüksel A, Muammer T, Tuncay T, İsa Y:** Effects of different fattening systems on fattening performance, slaughter and carcass characteristics of male tuj lambs. *Kafkas Univ Vet Fak Derg,* 23 (1): 109-115, 2017.
17	**Özbey O, Akcan A:** Morkaraman, Kıvırcık x Morkaraman (F1) ve Sakız x Morkaraman (F1) melez kuzulurda verim özellikleri II. Besi performansı, kesim ve karkas özellikleri. *YYÜ Vet Fak Derg,* 14 (2): 35-41, 2003.
18	**Şen U, Sirin E, Ulutas Z, Kuran M:** Fattening performance, slaughter, carcass and meat quality traits of Karayaka lambs. *Trop Anim Health Prod,* 43, 409-416, 2011.
19	**Şahin E H, Akmaz A:** Farklı kesim ağırlıklarında Akkaraman kuzuların besi performansı, kesim ve karkas özellikleri. *Vet Bil Derg,* 18 (3): 29-36, 2002.
20	**Tekel N, Şireli HD, Vural M E:** Besi süresinin İvesi erkek kuzuların besi performansı ve karkas özelliklerine etkisi. *JAS,* 13 (4): 372-378, 2007.
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follows; ILW 24.96 kg, SBW 43.18, DWG 244 kg, FCR 5.44, DP 48.80% and CCY 7.73% (P<0.001; *Table 2)*. Forest plot analysis variables are shown in *Fig. 2* and *Fig. 3*.

The publication bias values in this study are presented in *Table 3*.

According to Egger's linear regression test results, there was a publication bias among the studies in terms of the results of the ILW, FCR, and HCW variables (P<0.05). Duwal and Tweedy's trim and fill method was applied to eliminate publication bias, and the means were adjusted to 22.43 (95% CI 19.89-24.96), 4.27 (95% CI 3.48-5.07), and 26.74 (95% CI 24.76-28.71), respectively, by adding seven virtual studies to ILW, 10 to FCR, and 24 to HCW *(Table 3)*.

To determine the sources of heterogeneity in this study, subgroup analyses of data on performance (ILW, SBW, DWG, FCR and hot/cold carcass weight (HCW, CCW) and dressing percentage (DP) were performed according to breed, region, fattening start age and fattening duration, and the results are presented in *Table 4* and *Table 5*, respectively.

In the subgroup analysis of ILW, a statistically significant difference was found for all subgroups (P<0.05). The subgroups with the lowest and highest ILW values were

Table 2. *General meta-analysis results for performance and carcass data*

Variables	k	n	Mean (%95 CI)	Heterogeneity	
				Cochran's Q	I^2(%)
ILW, kg	51	808	24.96 (22.94-26.98)	Q=2592.47, Df=50, P<0.001	98.1
SBW, kg	47	753	43.18 (41.10-45.27)	Q=4757.91, Df=46, P<0.001	99.0
DWG, g	45	733	244.78 (233.71-255.86)	Q=8542076.00, Df= 44, P<0.001	100.0
FCR, kg	25	368	5.44 (4.80-6.07)	Q=1637.52, Df= 24, P<0.001	98.5
HCW, kg	47	766	21.26 (20.13-22.40)	Q=21941.00, Df=46, P<0.001	99.8
DP, %	36	631	48.80 (48.01-49.58)	Q= 169833.34, Df= 35, P<0.001	100.0
CCW, kg	44	727	20.90 (19.73-22.07)	Q= 5263.40, Df=43, P<0.001	99.2
CCY, %	37	642	47.73 (46.93-48.53)	Q=9126.42, Df= 36, P<0.001	99.6

k: *Number of studies;* ***n:*** *Number of animals;* ***CI:*** *Confidence interval;* ***I^2:*** *The proportion of total variation in study estimates that is due to heterogeneity;* ***Cochran's Q:*** *The estimation of a weighted average of effect in the overall population*

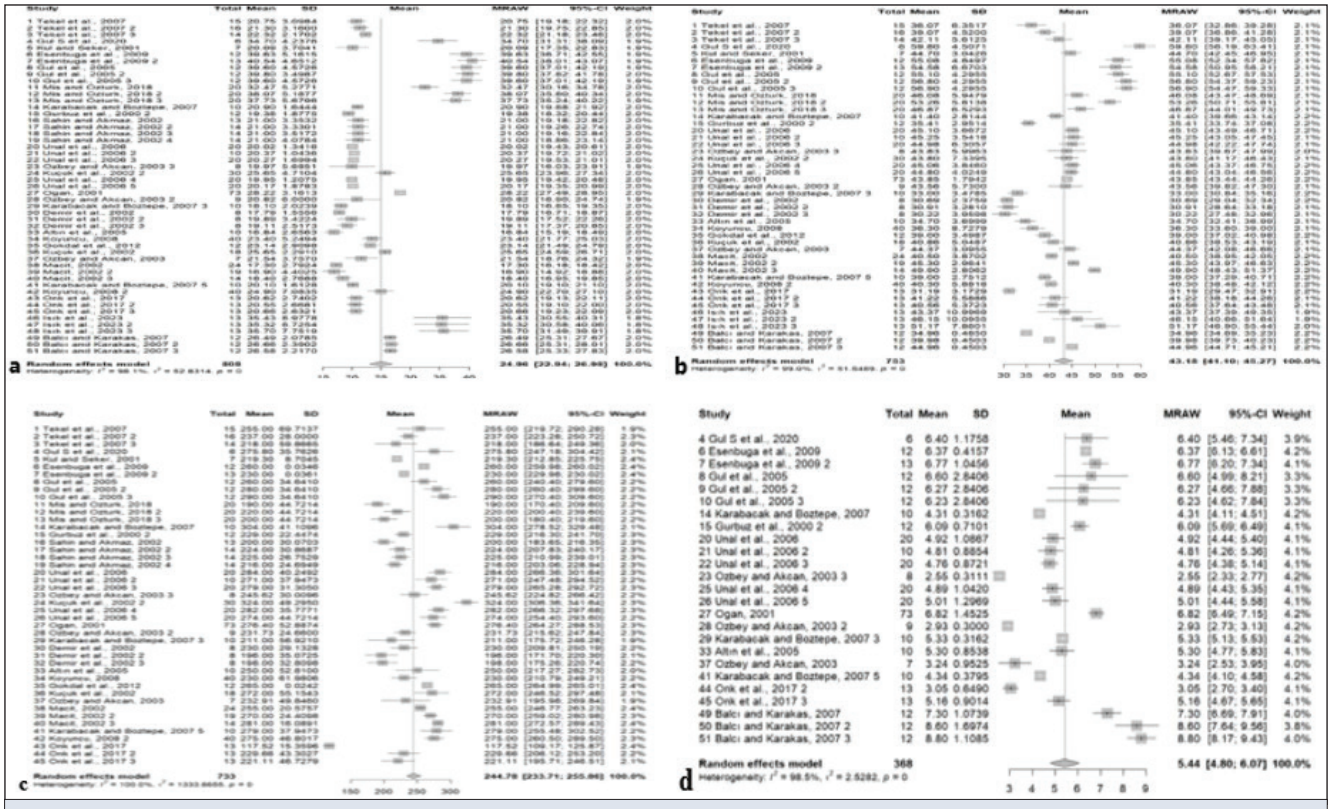

Fig 2. Forest plots. a-ILW, b-SBW, c-DWG, d-FCR

calculated in the following subgroups: Kıvırcık (19.73 kg) and Awassi, (31.79 kg) breeds; Central Anatolia (20.11 kg) and Eastern Anatolia (28.73 kg) regions; <90 days (20.02 kg) and >120 days (35.81 kg) fattening start age; and ≥90 days (21.92 kg) and ≤60 days (28.73 kg) fattening periods, respectively (P<0.05; *Table 4*).

In the subgroup analysis for SBW, there was a significant difference between the subgroups of breed, geographical region, and fattening start age (P<0.001), whereas no significant difference was found between the fattening duration subgroups (P>0.05). The subgroups with the lowest and highest values in terms of SBW were calculated in the following subgroups: Kıvırcık (33.55 kg) and Awassi, (50.01 kg) breeds; Marmara (36.99 kg) and Eastern Anatolia (46.75 kg) regions; and <90 days (39.52 kg) and >120 days (50.70 kg) fattening start age subgroups, respectively (P<0.001; *Table 4*).

The difference between breed subgroups for DWG was statistically significant (P<0.05), whereas no significant difference was found between geographical region, fattening start age and fattening duration subgroups (P>0.05). The subgroups with the lowest and highest

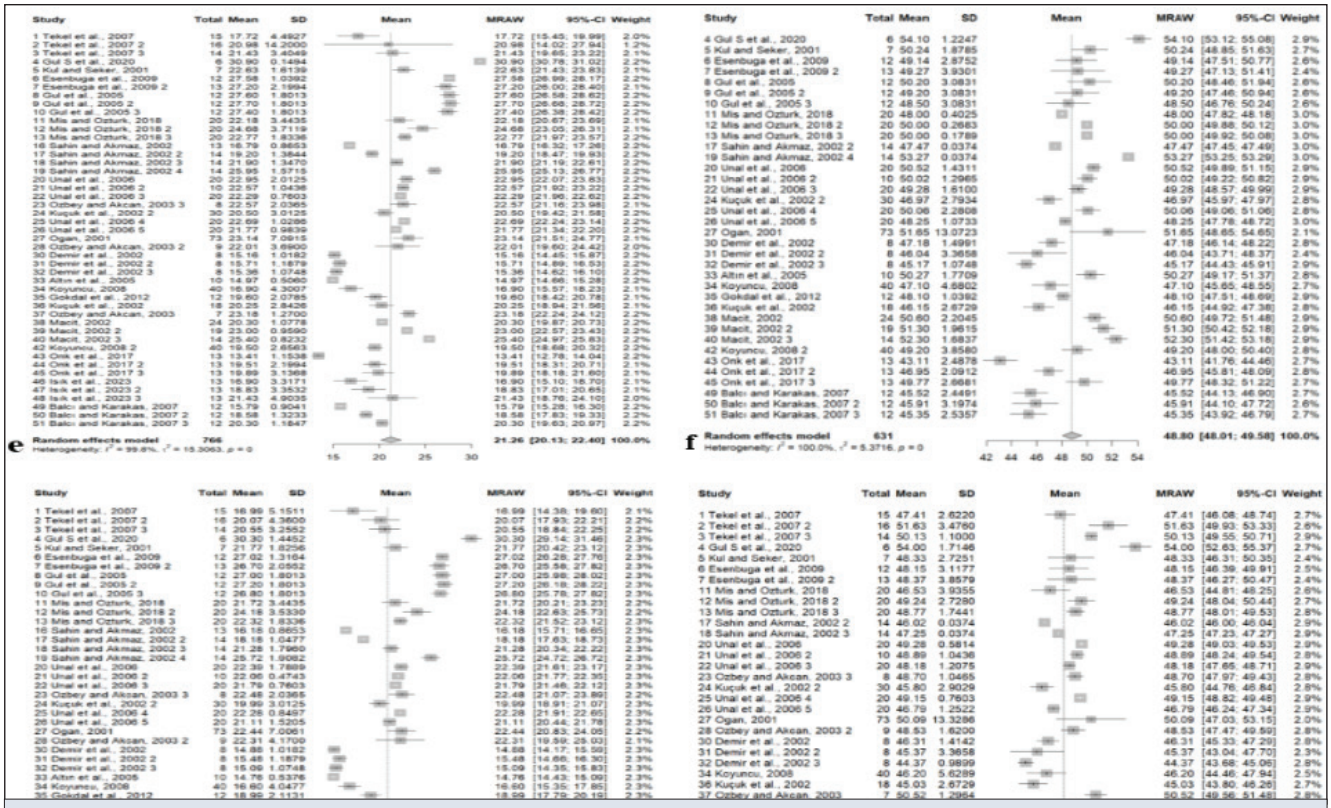

Fig 3. Forest plot e-HCW, c-DP, g-CCW, h-CCY

Table 3. *Publication bias results for performance and carcass data*

Variables	Egger's Linear Regression Test		
	t-statistic	Df	P-value
ILW, kg	3.59	49	**<0.001**
SBW, kg	1.21	45	0.231
DWG, g	-0.23	43	0.817
FCR, kg	2.19	23	**0.039**
HCW, kg	-4.35	45	**<0.001**
DP, %	-0.30	34	0.763
CCW, kg	1.04	42	0.306
CCY, %	1.11	35	0.273

DWG values were calculated in the Tuj (188.60 g) and Morkaraman (265.95 g) breeds, respectively (P<0.05; *Table 4*).

A statistically significant difference was found in all subgroups for FCR (P<0.01). The lowest and highest FCR values were calculated in the subgroups of Akkaraman (5.10 kg) and Karayaka (8.21 kg) according to breeds; Eastern Anatolia (4.84 kg) and Marmara (7.83 kg) according to geographical regions; <90 days (5.06 kg) and >120 days (6.58 kg) according to fattening start age and ≥90 days (4.77 kg) and ≤60 days (6.70 kg) according to fattening duration, respectively (P<0.01; *Table 4*).

There was a significant difference in all subgroups for HCW (P<0.01). The lowest and highest values for HCW were calculated in the subgroups of Kıvırcık (16.22 kg) and Awassi, (25.38 kg) according to breeds; Marmara (17.79 kg) and Eastern Anatolia (22.50 kg) according to geographical regions; 90-120 days (19.52 kg) and >120 days (25.01 kg) according to fattening start age and 61-89 days (20.12 kg) and ≥90 (25.53 kg) according to fattening period, respectively (P<0.01; *Table 5*).

In the subgroup analysis for DP, there was a significant difference between the subgroups in terms of breed, geographical region and fattening period (P<0.05), whereas no significant difference was found between the subgroups in terms of fattening start age (P>0.05). The

Table 4. *Subgroup analysis results for ILW, SBW, DWG and FCR data*

Variables	ILW (kg)			SBW (kg)			DWG (g)			FCR (kg)		
	Mean (%95 CI)	k	I^2	Mean (%95 CI)	k	I^2	Mean (%95 CI)	k	I^2	Mean (%95 CI)	k	I^2
Breed Subgroups												
Awassi	31.79 (25.97-37.62)	10	98.8	50.01 (44.68 (55.33)	10	97.3	251.85 (235.94-267.75)	10	100.0	6.42 (6.22-6.63)	6	0.0
Akkaraman	25.21 (20.50-29.92)	10	98.2	44.63 (39.85-49.41)	6	96.9	228.75 (206.69-250.80)	10	91.8	5.10 (4.07-6.13)	3	96.8
Kıvırcık	19.73 (17.79-21.66)	7	90.8	33.55 (31.09-36.01)	7	89.4	226.98 (206.55-247.41)	7	93.9	-	-	-
Morkaraman	19.96 (16.67-23.25)	5	97.2	44.23 (40.84-47.61)	5	95.4	265.95 (252.91-278.99)	5	82.1	-	-	-
Tuj	27.77 (21.25-34.28)	6	95.5	42.03 (36.50-47.56)	6	95.6	188.60 (117.15-260.05)	3	98.4	-	-	-
Karayaka	26.57 (25.84-27.29)	3	0.0	39.97 (34.31-45.62)	3	99.9	-	-	-	8.21 (7.25-9.17)	3	84.0
General	25.69 (23.25-28.12)	41	98.1	43.13 (40.49-45.77)	37	99.2	236.35 (224.03-248.67)	35	100.0	6.54 (5.77-7.31)	12	97.3
Significance	Cochran's Q (df=5) = 58.24, **P<0.001**			Cochran's Q (df=5) = 50.03, **P<0.001**			Cochran's Q (df=4) = 17.05, **P=0.002**			Cochran's Q (df=2) = 19.45, **P<0.001**		
Regional Subgroups												
Southeastern Anatolia	21.58 (20.61-22.56)	3	28.5	39.13 (35.88-42.38)	3	73.0	236.32 (224.49-248.16)	3	16.6	-	-	-
Eastern Anatolia	28.73 (25.03-32.43)	23	98.5	46.75 (44.06-49.43)	23	96.9	241.43 (221.95-260.92)	20	100.0	4.84 (3.73-5.94)	10	98.9
Central Anatolia	20.11 (19.87-20.35)	13	35.9	41.55 (38.47-44.62)	9	95.7	252.03 (233.21-270.86)	13	93.2	4.93 (4.57-5.29)	9	92.5
Marmara	23.71 (21.19-26.23)	9	97.6	36.99 (33.27-40.70)	9	99.8	235.26 (206.82-263.71)	6	93.5	7.83 (6.87-8.80)	4	91.8
General	24.97 (22.89-27.05)	48	98.1	43.10 (41.04-45.17)	44	99.1	243.47 (231.77-255.18)	42	100.0	5.40 (4.71-6.09)	23	98.6
Significance	Cochran's Q (df=3) = 35.79, **P<0.001**			Cochran's Q (df=3) = 22.10, **P<0.001**			Cochran's Q (df=3) = 2.06, P=0.560			Cochran's Q (df=2) = 30.82, **P<0.001**		
Feeding Start Age (Days) Subgroups												
<90	20.02 (18.69-21.35)	13	89.9	39.52 (36.97-42.07)	13	96.4	254.56 (240.11-269.01)	13	88.5	5.06 (4.40-5.72)	5	96.3
90-120	22.88 (20.80-24.96)	22	95.0	40.73 (37.75-43.72)	18	99.5	228.99 (207.55-250.43)	16	98.1	5.71 (4.56-6.85)	10	97.6
>120	35.81 (32.07-39.56)	10	98.0	50.70 (46.76-54.63)	10	97.9	247.41 (226.26-268.57)	10	100.0	6.58 (6.30-6.87)	6	10.8
General	24.99 (22.77-27.22)	45	98.2	42.78 (40.53-45.03)	41	99.1	241.91 (230.08-253.75)	39	100.0	5.76 (5.15-6.37)	21	97.3
Significance	Cochran's Q (df=2) = 61.56, **P<0.001**			Cochran's Q (df=2) = 23.07, **P<0.001**			Cochran's Q (df=2) = 3.76, P=0.153			Cochran's Q (df=2) = 18.27, **P=0.001**		
Feeding Duration (Days) Subgroups												
Short (≤60)	28.73 (24.77-32.68)	17	98.5	42.84 (38.29-47.39)	17	99.3	256.45 (239.28-273.63)	15	100.0	6.70 (6.38-7.02)	7	46.2
Medium (61-89)	23.68 (20.83-26.54)	22	97.2	41.86 (39.36-44.36)	20	96.1	235.91 (215.79-256.04)	19	98.1	5.11 (4.41-5.81)	12	95.9
Long (≥90)	21.92 (19.40-24.44)	12	93.7	46.34 (43.92-49.36)	10	91.9	244.33 (228.79-259.87)	11	94.8	4.77 (2.83-6.72)	6	98.8
General	24.96 (22.94-26.98)	51	98.1	43.18 (41.10-42.27)	47	99.0	244.78 (233.71-255.86)	45	100.0	5.44 (4.80-6.07)	25	98.5
Significance	Cochran's Q (df=2) = 8.10, **P=0.017**			Cochran's Q (df=2) = 5.12, P=0.077			Cochran's Q (df=2) = 2.43, P=0.297			Cochran's Q (df=2) = 19.26, **P<0.001**		

k: *Number of studies;* ***CI:*** *Confidence interval;* ***I^2:*** *The proportion of total variation in study estimates that is due to heterogeneity;* ***Cochran's Q:*** *The estimation of a weighted average of effect in the overall population*

Table 5. *Subgroup analysis results for HCW, DP, CCW and CCY data*

Variables	HCW (kg)			DP (%)			CCW (kg)			CCY (%)		
	Mean (%95 CI)	k	I^2	Mean (%95 CI)	k	I^2	Mean (%95 CI)	k	I^2	Mean (%95 CI)	k	I^2
Breed Subgroups												
Awassi	25.38 (22.86–27.91)	10	98.6	50.18 (48.68–51.69)	7	90.0	24.54 (21.96–27.12)	10	96.0	49.76 (47.98–51.54)	7	90.0
Akkaraman	22.02 (19.98–24.06)	8	98.7	49.87 (48.22–51.53)	6	100.0	21.46 (19.32–23.61)	8	98.7	47.86 (46.70–49.02)	6	99.9
Kıvırcık	16.22 (14.85–17.60)	6	91.9	47.36 (45.90–48.81)	6	92.6	15.90 (14.65–17.15)	6	90.2	45.48 (44.36–46.59)	4	74.5
Morkaraman	22.46 (20.54–24.37)	5	98.6	50.11 (47.48–52.74)	4	95.5	21.97 (20.01–23.93)	5	98.4	49.18 (47.08–51.28)	5	94.6
Tuj	18.21 (15.89–20.53)	6	96.5	46.60 (42.84–50.37)	3	95.5	17.06 (12.92–21.19)	3	98.2	45.25 (41.35–49.15)	3	96.4
Karayaka	18.22 (15.63–20.80)	3	98.3	45.55 (44.68–46.42)	3	0.0	17.67 (15.05–20.28)	3	98.8	44.08 (43.30–44.87)	3	0.0
General	21.11 (19.72–22.51)	38	99.8	48.68 (47.74–49.63)	29	100	20.75 (19.29–22.20)	35	99.3	47.57 (46.54–48.59)	28	99.7
Significance	Cochran's Q (df=5) = 59.82, **P<0.001**			Cochran's Q (df=5) = 43.28, **P<0.001**			Cochran's Q (df=5) = 56.85, **P<0.001**			Cochran's Q (df=5) = 59.80, **P<0.001**		
Regional Subgroups												
Southeastern Anatolia	19.84 (16.95-22.73)	3	68.8	-	-	-	19.38 (17.36–21.41)	3	61.6	49.70 (47.35-52.04)	3	88.9
Eastern Anatolia	22.50 (20.98-24.02)	23	98.8	48.94 (47.89–49.99)	17	97.6	22.53 (20.94–24.12)	20	98.8	47.90 (46.77–49.02)	17	94.6
Central Anatolia	21.78 (20.12-23.45)	9	98.7	49.84 (48.43–51.25)	7	100.0	21.21 (19.45–22.97)	9	98.9	47.93 (46.98–48.88)	7	99.9
Marmara	17.79 (16.03-19.54)	9	97.1	46.84 (45.64–48.03)	9	84.1	17.36 (15.68–19.04)	9	97.3	45.71 (44.50–46.92)	9	83.3
General	21.22 (20.13-22.32)	44	98.9	48.61 (47.83–49.40)	33	100.0	20.87 (19.74–22.00)	41	99.0	47.56 (46.81–48.30)	36	99.6
Significance	Cochran's Q (df=3) = 17.85, **P<0.001**			Cochran's Q (df=2) = 11.48, **P=0.003**			Cochran's Q (df=3) = 21.14, **P<0.001**			Cochran's Q (df=3) = 13.07, **P=0.005**		
Feeding Start Age (Days) Subgroups												
<90	19.99 (17.75–22.24)	9	99.6	50.19 (48.79–51.59)	6	88.8	19.48 (17.41–21.55)	9	99.4	49.28 (48.05–50.50)	8	87.3
90-120	19.52 (18.14–20.90)	22	99.0	47.94 (46.70-49.18)	17	100.0	19.07 (17.54–20.60)	19	99.2	46.41 (45.36–47.47)	17	99.8
>120	25.01 (23.19–26.83)	10	96.7	49.23 (48.55–49.90)	10	98.1	24.46 (22.64–26.28)	10	96.4	48.50 (47.71–49.29)	6	37.8
General	20.97 (19.77–22.17)	41	99.2	48.77 (48.00–49.54)	33	100.0	20.58 (19.33–21.82)	38	99.2	47.52 (46.71–48.34)	31	99.7
Significance	Cochran's Q (df=2) = 23.68, **P<0.001**			Cochran's Q (df=2) = 5.79, P=0.055			Cochran's Q (df=2) = 22.02, **P<0.001**			Cochran's Q (df=2) = 14.36, **P<0.001**		
Feeding Duration (Days) Subgroups												
Short (≤60)	20.83 (18.49–23.17)	17	99.3	47.81 (46.93–48.68)	15	84.9	20.58 (18.18–22.99)	16	99.2	46.41 (45.43–47.39)	12	83.3
Medium (61-89)	20.12 (18.71–21.53)	18	99.2	48.63 (47.55–49.72)	14	99.8	19.60 (18.09–21.11)	16	99.2	47.51 (46.24–48.79)	14	99.0
Long (≥90)	23.53 (21.91–25.16)	12	99.7	50.98 (48.88–53.08)	7	97.3	23.06 (21.46–24.66)	12	97.5	49.25 (47.80–50.69)	11	97.8
General	21.26 (20.13–22.40)	47	99.8	48.80 (48.01–49.58)	36	100.0	20.90 (19.73–22.07)	44	99.2	47.73 (46.93–48.53)	37	99.6
Significance	Cochran's Q (df=2) = 9.98, **P=0.007**			Cochran's Q (df=2) = 7.76, **P=0.021**			Cochran's Q (df=2) = 9.73, **P=0.008**			Cochran's Q (df=2) = 10.20, **P=0.006**		

k: *Number of studies;* ***CI:*** *Confidence interval;* ***I^2:*** *The proportion of total variation in study estimates that is due to heterogeneity;* ***Cochran's Q:*** *The estimation of a weighted average of effect in the overall population*

subgroups with the lowest and highest values for DP were determined as Karayaka (45.55%) and Awassi (50.18%) breeds; Marmara (46.84%) and Central Anatolia (49.84%) regions; and ≤60 days (47.81%) and ≥90 days (50.98%) fattening period, respectively (P<0.05; *Table 5*).

A significant difference was found for CCW in all subgroups (P<0.01). The lowest and highest values for CCW were calculated in the subgroups of Kıvırcık (15.90 kg) and Awassi (24.54 kg) according to breed; Marmara (17.36 kg) and Eastern Anatolia (22.53 kg) according to geographical regions; 90-120 days (19.07 kg) and >120 days (24.46 kg) according to fattening start age and 61-89 days (19.60 kg) and ≥90 days (23.06) according to fattening period, respectively (P<0.01; *Table 5*).

A statistically significant difference was found for CCY in all subgroups (P<0.01). The lowest and highest values for CCY were calculated in the following subgroups: Karayaka (44.08%) and Awassi (49.76 according to breed; Marmara (45.71%) and Southeastern Anatolia (49.70%) according to geographical regions; 90-120 days (46.41%) and <90 days (49.28%) according to fattening start age; and ≤60 days (46.41%) and ≥90 days (49.25%) according to fattening duration, respectively (P<0.01; *Table 5*).

Discussion

Lamb farming is very important for meeting the red meat demand in the country, reducing the foreign trade deficit, and strengthening the national economy. However, lamb fattening can be done profitably and sustainably by selecting appropriate breeds suitable for fattening or by crossbreeding between local and meat breeds. The fattening performance of animals can increase to the extent that their genetic capacity allows [10]. However, breed selection alone is not enough; the region where fattening is performed and the fattening period must also be well determined. In addition, it is beneficial to protect and improve existing pastures, which are important for lamb fattening.

In addition to the increasing population in Türkiye, immigration from abroad and tourism have increased the total demand for food and meat. As production was not adequately planned in response to the increasing demand, a supply deficit occurred, and the country started importing live animals and red meat [11].

Under normal conditions, in Türkiye, the demand for red meat is mostly met by beef, whereas 23.9% is met by sheep meat. To meet the demand for red meat and eliminate the foreign trade deficit, sheep meat, which is produced in smaller quantities than beef, must be increased as an alternative. The way to realize this is not only to increase the number of sheep but also to increase the productivity per unit of animal. To increase productivity, it is necessary to select suitable breeds for fattening and implement appropriate feeding programs.

In the current study, a meta-analysis was performed to determine the suitable breeds for lamb fattening. Thus, this study enabled the comparison of different breeds and provided decision support to producers regarding the selection of appropriate breeds. In terms of fattening performance, daily weight gain and feed conversion were evaluated together [12]. Therefore, in the current study, the breeds with higher/better fattening performance in Türkiye's conditions were Awassi, and Morkaraman in terms of DWG, whereas the Akkaraman breed was better for FCR.

This study showed that the best performance concerning starting age for fattening is achieved in the young age group (<90 days old), and that should not be late to start fattening for better results and development, and fattening should be started after weaning.

On the other hand, it was determined that the breed with the highest values concerning ILW, SBW, HCW, DP, CCW and CCY was Awassi. The breed with the lowest value concerning ILW, SBW, DWG, HCW and CCW was Kıvırcık, whereas the breed with the lowest value in terms of DP and CCY was Karayaka. Studies reporting the higher fattening performance of Awassi breed than other breeds support the results of the current study [10,13]. In addition, studies have shown that one of the factors affecting fattening performance is the season [14,15], and the fact that the Awassi breed is generally raised in provinces with high temperatures in the Southeastern Anatolia Region (Şanlıurfa, Gaziantep, etc.) is another reason why the fattening performance of this breed is better than that of other breeds.

Different lamb feeding methods are applied in every region of Türkiye. The Eastern Anatolia Region has more pastures than the other regions [16]. This causes lamb farming enterprises in the Eastern Anatolia Region to engage in extensive pasture-based farming. In Western Anatolia, the scarcity of pastures has directed breeders to intensive lamb farming. Extensive pasture-based lamb farming creates lower production costs compared to intensive lamb farming due to the lower feed costs. Therefore, producers in the Eastern Anatolia Region can continue lamb fattening for a longer period of time and achieve higher fattening performance compared to the other regions. Lamb producers in the Western Anatolia Region tend to slaughter lambs early because of the increasing costs of long-term fattening. It can be seen that production costs affect the differences in fattening performance between regions. Breeding different breeds in each region also causes differences in the fattening performance between regions. Sheep breeding has been

performed in the Eastern Anatolia Region for many years, and the producers here have more experience in sheep breeding, which is one of the factors that positively affect the fattening performance of the animals.

Different fattening periods are another factor affecting fattening performance. It was observed that breeders prefer lambs with higher IW in short fattening periods. Thus, lambs can reach the desired slaughter weight in a short time. In the long term, producers appear to prefer animals with lower weights. The reason for this may be to reduce the cost of purchasing the feeding material. Better FCR and higher carcass yields in long-term fattening (≥90 days) indicated that short-term fattening preferred for early lamb slaughter is not the right choice in economic terms.

Concerning carcass yields, the Awassi and Morkaraman breeds prominent with DP values above 50% and CCY values close to 50%. Regarding carcass values, it can be said that long-term feeding (≥90 days) is more advantageous. Although the Awassi and Morkaraman breeds had the best fattening performance among the existing lamb breeds in Türkiye, previous studies [17-19] reported that the fattening performance data of meat lamb breeds (Suffolk, German Meat Merino, Ile de France, etc.) were higher than the Awassi and Morkaraman breeds. This shows that concerning efficiency, the use of meat lamb breeds or their crossbreeds in lamb fattening can be considered in Türkiye. In conclusion, when both fattening performance and carcass values are evaluated together, according to the criteria determined in the current study for lamb fattening in Türkiye, it can be speculated that the Awassi, Morkaraman, and Akkaraman breeds are preferred primarily, Eastern Anatolia and Central Anatolia are more suitable regionally, and fattening lambs <90 days old and long-term fattening (≥90 days) are prominent both technically and economically.

Declarations

Availability of Data and Materials: The data and materials of this study are available from the corresponding author (S. Demir).

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Case Report

Multiparasitism in the Endangered Marbled Polecat (*Vormela peregusna*): A Case Report

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Abstract

The marbled polecat *(Vormela peregusna)* is classified as an endangered species within the small mammal group. In this report a young, injured male marbled polecat was brought to the Wildlife Protection, Rescue, Rehabilitation Application and Research Center of Kafkas University from the Sarıkamış district. Ectoparasitic examination revealed a flea sample identified as *Nosopsyllus fasciatus*. *Macracanthorhynchus* sp., belonging to the phylum *Acanthocephala*, and *Eimeria* sp. were detected in the examination of the fecal samples collected from the animal. This is the first documented report of these parasites observed in marbled polecat from Türkiye.

Keywords: *Eimeria* sp, *Macracanthorhynchus* sp, Marbled polecat, *Nosopsyllus fasciatus, Vormela peregusna*

Introduction

Vormela peregusna (Güldenstaedt, 1770), commonly known as the marbled polecat, is a small carnivore mammal belonging to the family Mustelidae [1-3], which has attracted scientific interest because of its ecological significance and conservation status. The International Union for Conservation of Nature (IUCN) has classified *Vormela peregusna* as vulnerable, with the species facing various threats, including habitat loss and anthropogenic pressures [3,4]. In addition to habitat loss, infections caused by various pathogens also contribute to population decline. Epidemiological data on marbled polecats, which serve as reservoirs for pathogens of medical and veterinary significance, are limited [5]. The geographic distribution of marbled polecats encompasses Central Asia, Europe, Mongolia, and Northern China [1,2].

Marbled polecats are known hosts for various pathogens, including *Rickettsia raoultii*, which has been detected in ticks infesting these animals [6]. Previous studies have reported various pathogens such as bacteria, helminths [7] and coccidian agents [8] in polecats; however, *Macracanthorhynchus*, a parasite of wild boars, has been detected in mustelids (European polecats) [7].

Fleas are common ectoparasites that can infest marbled polecats, potentially leading to discomfort and secondary infections. In a study focusing on flea surveillance in wild mammals, including marbled polecats, it was noted that these animals are often hosts for various flea species, which can affect their overall health and reproductive success [9]. Limited information is available regarding the marbled polecat, although phylogenetic and ecological considerations suggest susceptibility to a similar suite of diseases, and particular regional endemism [8].

This reports the first documented occurrence of *Nosopsyllus fasciatus*, *Macracanthorhynchus* sp., and *Eimeria* sp. in marbled polecat, contributing to the limited wildlife data for this species.

Case History

This report was prepared with the permission of the Republic of Türkiye Ministry of Agriculture and Forestry General Directorate of Nature Conservation and National Parks with the number E-21264211-288.04-16070652.

The material of the report was a severely injured young male marbled polecat, which was rescued from a canine attack by citizens in the Sarıkamış district of Kars and subsequently brought to the Wildlife Protection, Rescue, Rehabilitation Application, and Research Center of Kafkas University. Clinical examination revealed severe dehydration and a closed femoral fracture.

The flea sample was washed with distilled water and clarified in 10% potassium hydroxide (KOH) solution for 24 to 48 h. Subsequently, it was immersed in 70% and 99% alcohol series for 24 h, prepared with Canada balsam, and dried in an oven at 55ºC for approximately 15 days. The flea sample was examined under a light microscope at 4x and 10x magnification.

Macroscopic examination of the fecal sample revealed one helminth belonging to the phylum *Acanthocephala*, and the parasite sample was examined under a stereomicroscope. The fecal sample was subjected to flotation with zinc sulfate ($ZnSO_4$) and sedimentation with tap water, followed by microscopic examination at 10x and 40x magnification. Unsporulated oocysts were observed in the sample; therefore, potassium dichromate was added, and the oocysts were allowed to sporulate in an oven at 37ºC. Additionally, the sample was agitated three to four times daily to ensure oxygenation and sporulation.

Based on the morphological characteristics and measurements of the flea sample [10], it was identified as *Nosopsyllus fasciatus* (female), commonly known as the northern rat flea *(Fig. 1)*.

The helminth sample belonging to the phylum *Acanthocephala* was found to have a relatively damaged head and was identified as a male *Macracanthorhynchus*

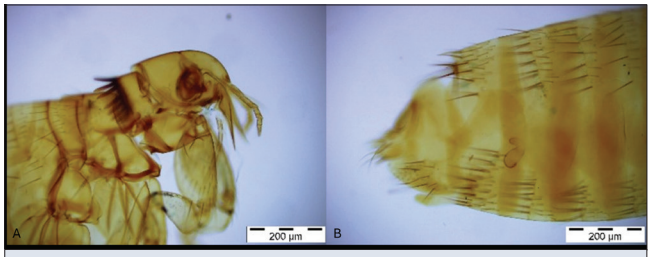

Fig 1. *Nosopsyllus fasciatus* fleas. **A**- Head, **B**- Abdomen

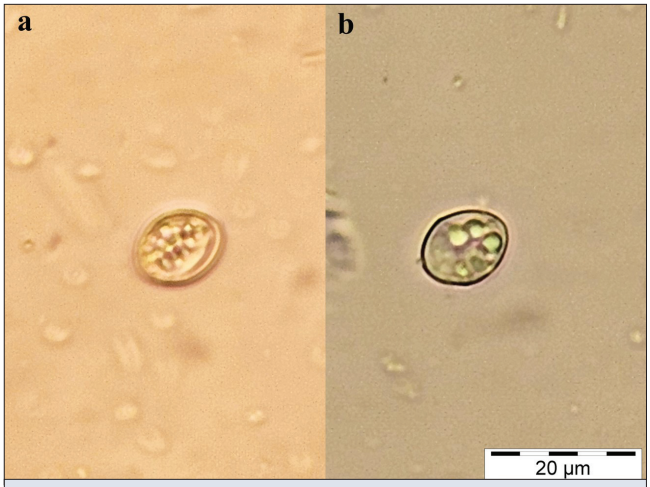

Fig 2. *Eimeria* sp. oocyst. **a**- (12.1x14.3 µ); Unsporulated oocyst, **b**- (11.4x13.2 µ); sporulated oocyst

sp. based on its morphological characteristics [11,12]. No eggs of this helminth were observed in the feces. The morphological features and structures of the sporulated oocysts were measured and identified [13] as *Eimeria* sp. *(Fig 2)*. The number of oocysts per gram of feces was determined using the Modified McMaster Technique, and the oocyst count was found to be <50 g/oocysts.

Discussion

Flea infestations have ecological implications that extend beyond individual health effects. Elevated flea burdens can potentially affect the overall fitness of marbled polecat, particularly its survival and reproductive success. This is especially relevant in the context of conservation efforts because maintaining healthy populations of marbled polecats is essential for ecosystem balance [9]. *Nosopsyllus fasciatus,* frequently observed in urban and rural environments, is renowned for its broad distribution and adaptability to areas where rodent hosts thrive [14].

This flea species is particularly significant in the context of zoonotic diseases, as it can serve as a vector for various pathogens. In a study conducted in China, four flea species, *Echidnophaga oschanini*, *Xenopsylla conformis conformis*, *X. gerbilli minax* and *Nosopsyllus laeviceps laeviceps*, were found to be dominant on marbled polecats and *Pulex irritans* was observed for the first time in marbled polecat. Furthermore, studies have demonstrated that *N. fasciatus* can transmit *Rickettsia* species, causing diseases in both animals and humans [6,15]. The presence of these pathogens in fleas collected from rodents indicates a risk of transmission to other species that may prey on or come into contact with infested rodents [5,6].

Coccidiosis caused by *Eimeria furonis*, *E. ictidea* [8], *Isospora eversmanni,* and *I. pavlovskyi* [16] in mustelids has been previously reported, but no data were found for

marbled polecat. The broader implications of *Eimeria* spp. as a pathogen in wildlife, due to its habitat conditions and host interactions, suggest that these parasites may pose a risk to the health of marbled polecats. Further research is needed to investigate the prevalence, ecological effects, and impact of *Eimeria* spp. in this species.

Macracanthorhynchus sp. is a thorny- or spiny-headed acanthocephalan parasite of swine worldwide. Adult worms can affect a wide range of hosts including canids, birds, and humans [11,17]. Its presence has been reported in foxes as wild carnivores, primarily in wild boars globally, and exclusively in wild boars in Türkiye [18,19]. However, this parasite has not yet been detected in mustelids or polecats.

According to the report data, multiparasitism was observed in marbled polecat, which was identified as a potential new host for each detected parasite. The findings also emphasize the importance of understanding the health and disease dynamics associated with marbled polecat, particularly in the context of wildlife conservation and management strategies.

Declarations

Availability of Data and Materials: The data of this case report are available from the correaponding author (N. Ölmez) upon reasonable request.

Conflict of Interest: The authors declared that there is no conflict of interest.

Declaration of Generative Artificial Intelligence (AI): The authors declare that the report and/or figures were not written/created by AI and AI-assisted technologies.

Author Contributions: NÖ investigated and designed the report. EEE and BB collected samples. NÖ and NA analysed the data. BS, YK, and GTT confirmed the data. NÖ wrote the original drafting. NÖ, BS, and YK edited and finalized the draft. All authors have read and approved the final version of the manuscript.

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ETHICAL PRINCIPLES AND PUBLICATION POLICY

Kafkas Universitesi Veteriner Fakultesi Dergisi follows and implements internationally accepted ethical standards to provide the necessary support to original scientific ideas and to publish high quality, reliable scientific articles in this direction. The journal's publication policy and ethical principles include the ethical standards of conduct that should be followed by author(s), journal editor(s), associate editors, subject editors, reviewers, and publishers who are the participants of this action.

The ethical statement of Kafkas Universitesi Veteriner Fakultesi Dergisi is based on the principles indicated in the "COPE Code of Conduct and Best Practice Guidelines for Journal Editors" (http://publicationethics.org/files/Code_of_conduct_for_journal_editors_Mar11.pdf) and "COPE Best Practice Guidelines for Journal Editors" (http://publicationethics.org/files/u2/Best_Practice.pdf).

GENERAL ETHICAL PRINCIPLES

• Objectivity and Independence

Editor-in-chief, editors, associate editors, and referees conduct the evaluation process of the manuscript sent to the journal objectively and in coordination within the framework of ethical principles. Editorial decisions are independent, and internal or external factors cannot influence these decisions. In accordance with the principle of impartiality, academics working in our institution are not deemed eligible to work as a section editor in Kafkas Universitesi Veteriner Fakultesi Dergisi, in order not to be effective in the evaluation of articles due to conflict of interest.

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The content of the articles and the personal information of the authors such as name, e-mail address, and telephone numbers that are sent to Kafkas Universitesi Veteriner Fakultesi Dergisi are used only for the scientific purposes of the journal and not for other purposes, and cannot be shared with third parties. Article evaluation processes are also carried out confidentially.

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The competence of the authors to the subject of the study is evaluated by the editor within the framework of deontological rules and the professional fields of each author.

The corresponding author of the article should declare the contributions of the authors to the work under the title of "Author contributions". The corresponding author is primarily responsible for the problems that may arise in this regard.

In multidisciplinary studies, 2 authors who are from different disciplines can be "equivalent first name authors" and up to most 3 authors who are also from different disciplines can be "equivalent second name authors".

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ARTICLE EVALUATION AND PUBLICATION PROCESS

• Initial Evaluation Process

Articles submitted to Kafkas Universitesi Veteriner Fakultesi Dergisi are primarily evaluated by the editors and associate editors. At this stage, articles not having suitable scope and aims, with low original research value, containing scientific and ethically important errors, having low potential to contribute to science and the journal, and having poor language and narration are rejected by the editor without peer-review process. Initial evaluation process takes up to most 2 weeks.

• Preliminary Evaluation Process

Articles that are deemed appropriate for editorial evaluation are sent to the subject editor related to the category of articles to be examined in terms of scientific competence and to the statistics editor for evaluation in terms of statistical methods. The suject editors examine the article in all aspects and report their decisions (rejection, revision or peer-review) to the chief editor. This stage takes about 1 month.

• Peer-review Process

Double-blind peer-review is applied to the articles that have completed preliminary evaluation process. Suggestions of subject editors are primarily considered in referee assignment. In addition, reviews can be requested from the referees registered in the journal's referee pool. At least 2 referees are assigned for peer-review. Opinion of more referees can be required depending on the evaluation process. At this stage, referees send their decision (reject, revision or accept) about the article to the editor-in-chief. If the rejection decision given by a referee reflects sufficient examination and evidence-based negativities or ethical problems about the scientific content and accuracy of the article, this decision is checked by the editor-in-chief and associate editors and submitted to the authors regardless of the other referees' decisions. The time given to referees to evaluate an article is ~4 weeks.

• Publication Process of an Article

Total evaluation period of an article, which is completed in the peer-review phase after completing the initial and preliminary evaluation process, takes 4-6 months. The articles that have completed the subject editorial and peer-review evaluation stages and accepted by the editorial are sent to the corresponding author for final checks and necessary final additions. After the acceptance, the article designed in the publication format of the journal is given an DOI number and published immediately on the Article in Press page. When it is time to publish the periodic edition of the journal, a selection is made from the articles kept on the Article in Press page, taking into account the submission date. The time it takes for the article to be published by taking the page number is 6-12 months.

NO PUBLICATION FEE

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The publisher undertakes to carry out an independent and fair decision-making mechanism for its editors and assistants in the article evaluation process and decisions.

The publisher undertakes to carry out an independent and fair decision-making mechanism for its editors and associate editors in the article evaluation process and decisions.

Editor-in-chief/editors/associate editors of Kafkas Universitesi Veteriner Fakultesi Dergisi evaluate the articles submitted to the journal regardless of their race, gender, religious belief, ethnicity, citizenship or political views. In addition, it undertakes not to give any information about the article except for the authors, subject editors and referees.

Kafkas Universitesi Veteriner Fakultesi Dergisi follows internationally accepted principles and criteria and takes the necessary decisions to apply in the journal.

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The editorial office of the Kafkas Üniversitesi Veteriner Fakültesi Dergisi and the publisher (Dean's Office of the Faculty of Veterinary Medicine, Kafkas University) keep all the articles (electronic and printed) published in the journal in their archives. All articles and their attachment files sent to the journal are kept securely in the archive. In light of the technological developments, the editorial office of the Kafkas Üniversitesi Veteriner Fakültesi Dergisi regularly performs electronic processes for the development and updating of materials in digital environment and presents them to its readers on condition of keeping in safe the original documents and information regarding the articles.

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Author information is kept confidential in articles sent to the subject editor for preliminary evaluation by the editor.

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