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Review

The Role of Bee Products in the Control of Antimicrobial Resistance and Biofilm Formation

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Abstract

The discovery of antibiotics saved many lives. Infections were not as deadly a problem for clinicians as they once were. However, due to inappropriate and excessive use of antibiotics, antibiotic resistance has increased dramatically worldwide. Infectious diseases are becoming more challenging to control, and they cause increased morbidity and mortality. Also, a significant risk to human health is posed by infections associated with biofilms. To combat these drug-resistant microorganisms, several novel and alternative strategies have been identified. Bee products such as honey, bee pollen, propolis, royal jelly, bee venom, bee wax, and bee bread have the potential of being used as antimicrobial or antibiofilm agents in various industrial and medical applications. Although these products have some restrictions such as their varying and complex composition, they possess significant potential in the field of medical practices as viable alternatives to antibiotics. They offer a potential solution to the issue of antibiotic resistance. The objective of this review was to offer a comprehensive analysis and evaluation of strategies based on bee products that are currently employed or have been suggested against antimicrobial resistance.

Keywords: Antimicrobial resistance, Antibiotic alternatives, Biofilms, Bee products

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Introduction

Antibiotics have revolutionized medical care by preventing life-threatening bacterial infections. However, misuse and overuse of antibiotics caused antimicrobial resistance which makes them insufficient or ineffective against bacterial infections. Antibiotic resistance refers to microorganisms' ability to counteract the effects of antimicrobial drugs, rendering them ineffective against bacterial growth [1,2]. Antibiotic misuse is an important issue regarding the development of resistance through two mechanisms: firstly, by suppressing susceptible bacteria and enabling the survival of resistant bacteria, and secondly, by triggering dormant resistance genes within bacteria due to the pressure exerted by antibiotics. The transmission of resistance genes among bacterial strains, influenced by antibiotic usage, can take place within individual hosts as well as between different hosts and communities. Therefore, the judicious administration of antibiotics in agriculture and healthcare is critical to prevent of multidrug-resistant bacterial infections. However, the emergence of antibiotic resistance has hindered progress in the clinical sector and threatened life expectancy, and food safety. The limited availability of new antibiotics due to declining progress and commercialization since the 1990s has compounded the problem [3,4]. This phenomenon poses a silent and dangerous threat to public health, particularly as antibiotics are being increasingly prescribed to treat infections that occur as a result of a primary ones [4,5]. Antibiotic resistance has become a global issue due to the escalating utilization of antibiotics in both medical practices and agriculture. The extensive utilization of antibiotics represents a significant threat to human health and has transformed into an urgent public health emergency. As per the assessment of the World Health Organization (WHO), antimicrobial resistance could lead to ten million deaths by the year 2050 [4,6,7].

Biofilms are conglomerates of bacterial colonies that attach to surfaces and are enveloped by a matrix of extracellular polymeric substances (EPS) manufactured by the cells themselves. These matrices have the capacity to harbor either single-species or multi-species communities of microorganisms, and the physical attributes of the matrix can differ among various bacterial strains. EPS consists of an assortment of biopolymers, encompassing proteins, polysaccharides, lipids, extracellular RNA, and extracellular DNA. They provide structural support and stability to biofilms, facilitate their adhesion to surfaces, safeguard biofilm cells from external forces, and establish a framework for the interconnected, immobilized three-dimensional structure of cells [8-12].

To assess the potential of bee products against antimicrobial resistance and biofilm formation, a descriptive review was conducted. Different bee products (such as propolis, honey, pollen, bee venom, royal jelly, beeswax and bee bread) were critically evaluated on the basis of their effects as documented in experimental studies. ScienceDirect, Web of Science, PubMed, and Google Scholar databases were used to collect bibliographic material. Articles that met the desired selection criteria were screened by evaluating their titles and then their abstracts.

This review aims to comprehensively examine the role of bee products on antimicrobial resistance and biofilm control.

Antimicrobial and Antibiofilm Effects of Bee Products

Honey

Apiculture is the art of rearing 'apis' or bees for the extraction of bee products, especially honey and beeswax. Honey, being high in nutrients, has been consumed worldwide since ancient times. The therapeutic properties of honey can be traced back to ancient times wherein Aristotle (384-322 BC) described honey to be "good as a salve for sore eyes and wounds". Honey is a powerful antioxidant, antimicrobial and antiproliferative agent and has been used traditional since long times [13-15]. It is extremely effective in promoting gastric health and is recommended for peptic ulcers and gastritis [16]. Recently, its anti-inflammatory, antihyperlipidemic, antidiabetic, and anticancer properties have been discovered [17,18]. According to the Codex Alimentarius, honey was described as "the natural sweet substance produced by honey bees from the nectar of plants or secretions of living parts of plants or excretions of plant-sucking insects on the living parts of plants" [19]. European Council Directive 2001/110/EC prohibits the addition to this natural product any food additives or other addition other than honey [20]. Honey is a saturated sugar (~80% v/v) solution whose composition depends on a varying range of factors including but not limited to the floral source, type of bee, and environmental and processing factors. These factors contribute to the texture, consistency, odour, color, and other physicochemical properties of honey. Currently, there are more than 300 types of honey, however, the core components of these honey stay more or less the same. A diagrammatic representation of major components in honey, as described by USDA, has been represented in *Fig. 1*. Approximately 20 different types of carbohydrates have been identified in honey with the principal carbohydrates being fructose and glucose. Honey also contains several disaccharides and trisaccharides at concentrations of 5% and 1% respectively [21]. Honeybees regurgitate the pollen of flowers and a small fraction of amino acids and proteins are retained in the honey. However, bee-derived

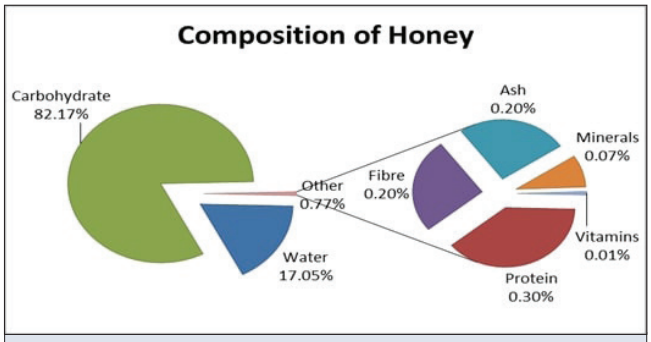

Fig 1. Pie diagram representing the constituents present in honey

and pollen proteins could theoretically be attributed to allergic reactions. Especially glandular proteins that bees produce and pollen from sunflower, ragweed, and sagebrush cause honey allergy. Symptoms of patients allergic to honey including local and systemic reactions. Local symptoms may assume itching in the mouth or gastrointestinal symptoms. Among systemic symptoms are bronchial asthma or generalized urticaria. Sometimes, anaphylaxis reaction is possible. Proline stands out as the most abundant amino acid, accounting for 50% of the total amino acid content in honey. Lipids are used up in the formation of beeswax and hence the amount of lipids in honey is almost negligible (0.002%) [22-24].

Antimicrobial Properties of Honey and Antibiotic Resistance

Honey has been called as supersaturated-sugar syrup containing almost 80% sugar in its total weight. High sugar concentration of honey implies to lower water activity (a_w). The optimum a_w values for bacteria, yeast and moulds are 0.9, 0.7 and 0.8, respectively [25] while the a_w value for honey ranges from 0.5-0.65 [26]. French et al.[27] demonstrated the use of pasture and manuka honey for inhibition of Coagulase-negative *Staphylococcus aureus* growth with MIC values of 3.6±0.7 v/v and 3.4±0.5 v/v respectively. The study showed that the osmotic effect of the sugar content of honey was 5.5 to 11.7 times greater than the antibacterial activity of natural honeys.

Honeybees collect sucrose from flowers and further break it down into glucose and fructose. This glucose is further oxidized and disintegrated into gluconolactone/gluconic acid and H_2O_2 by glucose oxidase enzyme (GOx) secreted by the bee's hypopharyngeal glands. The role of H_2O_2 in the antimicrobial effect of honey has been described by Adcock et al.[28] wherein the activity was diminished when H_2O_2 was decomposed by addition of enzymes such as catalase. Dustmann et al.[29] first demonstrated the antimicrobial activity of honey using a variety of organisms. In the research, it was found that *Pseudomonas*, *Proteus*, *Salmonella* spp., and *Streptococcus* sp. were less affected while more inhibition was observed in *Sarcina lute* and *S. aureus*. Furthermore, it was found that H_2O_2 concentration played an active role in contributing to antimicrobial activity against *Escherichia coli* K-12. A low concentration of H_2O_2 (1-2 mM) was enough to kill bacteria via DNA damage wherein H_2O_2 triggers Fenton reaction thereby leading to DNA strand breaks and generation of active forms of hydroxyl radicals [30]. When honey was added to bacterial cultures along with H_2O_2 supplementation, it was observed that honey reduced MIC of H_2O_2 from 2.5 mM, resulting in DNA damage in the bacterial cultures [31].

Preceding the knowledge of H_2O_2 production and sugar content, it was considered that the antimicrobial activity of honey was attributed to its low pH which ranged from 3.4 to 6.1 depending on several factors during its production [32]. Production of gluconolactone/gluconic acid during glucose breakdown is one of the key factors in its low pH. Most bacteria have an optimum pH at a neutral range and cannot tolerate lower pH. For example, *Salmonella* spp., *E. coli*, *Pseudomonas aeruginosa*, and *Streptococcus pyogenes* can tolerate pH up to 4.0, 4.3, 4.4, and 4.5 respectively, and thus honey has been proven beneficial for their inhibition [33,34]. To demonstrate the non-peroxide effect of honey, Tan et al.[35] used catalase along with tualang and manuka honey and demonstrated the bactericidal effect of honey against 13 bacterial isolates.

The flower source predominantly determines polyphenols present in honey. A wide array of flavonoids and phenolic acids have been discovered in honey and act as a biomarker for their authenticity [36]. Several phenolic acids including 4-hydroxybenzoic acid, caffeic acid, vanillic acid, and ferulic acid, and flavonoids such as apigenin, acacetin, kaempferol, chrysin, pinobanksin, naringenin, pinocembrin, and quercetin possessing antimicrobial activity have been discovered in honey [37].

Defensin-1 or def-1 is an antibacterial peptide chain found in honey and royal jelly. Kwakman et al.[38] demonstrated the presence of def-1 in the antimicrobial activity of honey wherein the H_2O_2 activity of honey was neutralized. The study found that 10-20% v/v of honey could effectively inhibit the growth of *Bacillus subtilis*, extended-spectrum ß-lactamase producing vancomycin-resistant *Enterococcus faecium*, *E. coli*, methicillin-resistant *S. aureus*, and ciprofloxacin-resistant *Pseudomonas aeruginosa*.

The presence of Methylglyoxal (MG) in honey is dependent on its storage temperature and flower source. It was first discovered in honey by Weigel et al.[39] in honey prepared by Leptospermum flowers wherein the bees convert the dihydroxyacetone into MG by the nonenzymatic reaction. Honey containing MG at a concentration of 1.1mM could effectively inhibit the growth of *E. coli* and *S. aureus* with a MIC value of 15% v/v [40,41]. The factors that contribute to the antimicrobial effect of honey have been presented in *Fig. 2*.

Fig 2. Factors contributing to the antimicrobial effect of honey

Use of Honey in Biofilm Control

A biofilm may be defined as aggregates or a syntrophic consortium of microbes embedded in a self-produced matrix. Bacteria existing in a biofilm can cause most chronic wound infections as compared to free-living or planktonic bacteria. Biofilms are highly recalcitrant to antibacterial agents and thus lead to a significant delay in wound healing. Combination therapy exhibiting synergistic effects is considered in such cases. Two types of assays are performed *in vitro* to determine the effectiveness of a product against biofilm, namely, biofilm eradication assay and prevention of formation of biofilms [42,43]. The roles of honey in antimicrobial resistance and biofilm formation were presented in *Table 1*.

S. aureus is the most notorious microorganism accounting for most of wound infections [44]. Liu et al.[45] used medical grade manuka honey with four conventional antibiotics to determine its response against *S. aureus* NCTC 8325 biofilms. The study found that rifampicin and fusidic acid showed enhancement in the treatment of established biofilms when combined with honey. Rifampicin prevents adherence of cells to the surface thereby preventing biofilm formation. This, in turn, increases the number of planktonic cells and makes them more susceptible to antibiotics [46]. This could be one of the major reasons for rifampicin exhibiting synergism with honey [45] and other antibiotics such as cefazolin, fusidic acid, gentamicin, etc. [46]. Fusidic acid, on the other hand, works by binding with prokaryotic elongation factor G (EF-G) inhibiting its protein synthesis which eventually results in anomalies and/or inhibition of translocation of peptide and disassembly of ribosomes [47].

Streptococcus pyogenes is one of the other commonly found bacteria forming biofilms on open wounds. It constitutes the normal flora of a healthy individual's nasopharynx and skin and acts opportunistically during infections and injury. *S. pyogenes* binding to fibronectin is inhibited by honey thereby preventing its biofilm formation [48,49]. Sojka et al.[50] showed the presence of def-1 in honeydew and manuka honey by electrophoresis. It was observed that 0.7 and 2.0 µg def-1 per g honey in manuka and honeydew could inhibit the growth of biofilms of *P. aeruginosa, S. aureus*, and *S. agalactiae*.

Table 1. *The role of honey in antimicrobial resistance and biofilm formation*

Honey Types	Targeted Microrganisms	Mode of Action	Efficacy	Main Outcome of Study	References
Pasture honey	Coagulase-negative *S. aureus*	Osmosis causes the dehydration of bacterial cells	MIC: 3.6±0.7 v/v	The increased sugar concentration exerts osmotic pressure on the bacterial cells. This causes dehydration and ultimately cell death	[27]
Manuka honey			MIC: 3.4±0.5 v/v		
Honey + H_2O_2	*E. coli*	DNA damage by Fenton Reaction	MIC 90 value of H_2O_2 decreased from 2.5 mM to 1.25 mM with addition of honey (1:1)	*E. coli* growth is sensitive to the oxidative action of honey H_2O_2	[31]
Tualang and manuka honey	*Stenotrophomonas maltophilia*	Bactericidal effect of honey	MBC: 25% (w/v) for Tualang and 11.25% (w/v) for Manuka	Non peroxide effect of honey was established by the addition of catalase. Similar growth inhibition patterns for most bacteria	[35]
	S. pyogenes		MBC: 25% (w/v) for both		
	Coagulase-negative *Staphylococci; Enterobacter cloacae; Proteus mirabilis,* and *Shigella flexneri*		MBC: >25% (w/v) for both		
Medihoney + Rifampicin	*S. aureus NCTC* 8325 biofilms	Bacteriostatic effect of Rifampicin + antimicrobial activity of both	MIC: Medihoney: Rifampicin = 8 w/v: 0.02 µg/mL	The combination showed strong synergism	[45]
Medihoney + Fusidic acid	*S. aureus NCTC* 8325 biofilms		MIC: Medihoney: Fusidic acid = 8 w/v: 0.04 µg/mL	The combination showed mild synergism	[45]
Manuka honey	Biofilms of *S. aureus, S. agalactiae,* and *P. aeruginosa*	Prevention of bacterial adhesion to the surface and/or early inhibition of bacterial growth	0.7 µg Def-1 in per g honey	Presence of def-1 in manuka and honeydew	[50]
Honey Dew			2.0 µg Def-1 in per g honey		
Manuka honey	*S. pyogenes* MGAS6180	Facilitating cell death and dissociation of cells from biofilms	MIC: 20% (w/v) MBC: 45% (w/v)	Honey could inhibit adherence of *S. pyogenes* MGAS6180 to fibronectin	[49]

Propolis

Propolis, often referred to as "bee glue" is a sticky and resinous substance crafted by bees using plant materials, including leaves, flowers, and bud exudates. Bees transform these natural elements through a combination of their secretions and wax [51]. Honeybees, scientifically known as *Apis mellifera*, produce propolis by gathering resin from evergreen or coniferous trees. They blend this resin with beeswax and their own salivary secretions to create a viscous, dark green substance that serves the purpose of constructing and upkeeping their hives. Since ancient times, people have consumed and utilized propolis as a medication for overall health [52]. Several historical studies have described propolis as a material that may heal wounds, whether used alone or in combination with other medicines. Propolis has lately been widely used as a supplement in beverages to enhance human health and prevent illnesses due to its wide range of natural uses. Propolis is widely used thanks to its antibacterial, antiviral, antioxidant, antiinflammatory, anaesthetic, antimutagenic, antitumoural, antiprotozoal, anti-fungal, antiseptic and antihepatotoxic abilities in addition to its cytotoxic effect [53,54].

Propolis chemical activity is affected by factors such as climatic conditions, geographical location, and the period in which it was harvested [55]. The various types of propolis pose a significant challenge to quality control procedures. To evaluate and quantify phenolic compounds in propolis, the method combining ultra-high-performance liquid chromatography with photodiode array detection (UHPLC-PDA) and electrospray ionization tandem mass spectrometry (ESI-MS/MS) is recommended. This approach is ideal for quality control purposes and for establishing the chemical composition of propolis compounds. The HPLC-PDA-MS technique has demonstrated its effectiveness in evaluating phenolic compounds in propolis, providing a combination of brief retention times and exceptional resolution [56]. Quality represents a fundamental aspect of propolis, significantly influencing the taste, ability to dissolve, preservation longevity of the nectar, as well as its physical characteristics like density, consistency, and potential for crystallization. Propolis is a intricate resinous substance composed of the subsequent components: resins (50%), waxes (30%), pollen (10%), essential oils (5%), and other organic compounds (5%) [57]. Propolis is mainly comprised of a diverse range of constituents, encompassing aromatic acids including ferulic acid, caffeic acid, and cinnamic acid. Also, it contains aromatic compounds vanillin, aromatic esters (cinnamic acid and caffeic acid esters). In addition, in the composion of propolis volatile compounds (e.g. β-eudesmol, nerol, geraniol, and farnesol) are available. Hydrocarbons are also present in propolis including pentacosane, tricosane, eicosane, steroids like stigmasterol, cholinasterol, and fucosterol. Enzymes are also found to be in propolis including amylase. Flavonoids (e.g. pinobanksin, tectochrysin, chrysin, pinocembrin, galangin, kaempferol, and apigenin) and acids (e.g. cerotic acids, melissic acid, and palmitic acid) are also found to be in propolis. Essential oils, encompassing monoterpenes and sesquiterpenes, and micro- and macronutrients such as Al, Ba, Ca, Cl, Fe, Zn, K, Mg, Na, Mn, along with vitamins like B_6, B_2, B_1, C, and E are present in the propolis [53].

Antimicrobial Properties of Propolis and Antibiotic Resistance

As per earlier accounts, a range of naturally existing antibacterial elements within propolis and its derived products have demonstrated efficacy against multiple bacterial strains. They also increase the effectiveness of conventional antibiotics. Chemicals including caffeic acid, flavanol, ester flavonoids, pinocembrin, and galangin are thought to be responsible for their antibacterial properties [58]. These chemicals may also block bacterial RNA polymerase. Propolis has been shown in several tests to be non-toxic and to have no negative effects in either human trials or animal models. Several researchers studied the synergistic antimicrobial capabilities of propolis, and they found that in most of their *in vivo* and *in vitro* tests, there was a significant decrease in bacterial resistance to traditional antibacterial drugs [59,60]. Propolis' antibacterial action should be assessed on two levels. The first is connected to a direct impact on the microorganism, while the second is connected to immune system stimulation, which causes the organism's innate defenses to become active. Nucleic acid synthesis inhibition, adenosine triphosphate (ATP) synthesis, membrane permeability, disturbance of membrane potential, reduced affinity to the development of biofilms and decreased bacterial motility are all effects of propolis that may be deduced from an investigation of its processes [61] *(Fig. 3)*.

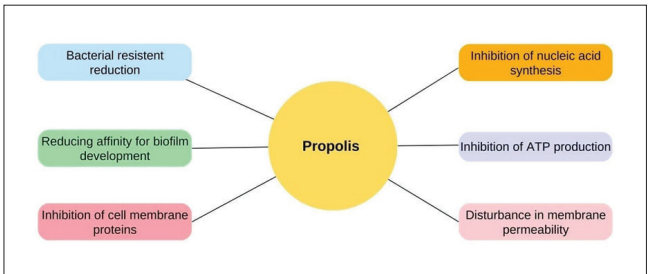

Fig 3. Mode of actions associated with the antibacterial activity of propolis

Afrouzan et al.[49], established the efficacy of poplar propolis against a wide range of bacteria, encompassing both Gram-positive and Gram-negative varieties, including the challenging multidrug-resistant MRSA. Furthermore,

Iranian and Brazilian propolis exhibit effectiveness against Gram-positive bacterial strains, albeit with less impact on Gram-negative counterparts. This disparity in effectiveness can be attributed to the comparatively simpler outer membrane structure of Gram-positive bacteria, rendering them more susceptible to the antibacterial components present in propolis [62]. It has been shown that the outer membrane structure of Gram-positive bacteria accounts for the increased antibacterial activity of propolis against those bacteria. In the cases of MRSA, *S. aureus*, and *S. epidermidis,* quercetin, and its various derivatives showed antibacterial effectiveness. One of the several phenolic mixtures found in propolis is artepillin C, which has effective antibacterial action against MRSA [63]. Similarly, Wojtyczka et al.[64] demonstrated that Polish propolis inhibited the growth of bacteria and altered the formation of biofilms.

Numerous studies have demonstrated that propolis and anti-infection medications work together synergistically. For instance, the combination of Brazilian honey and Brazilian red propolis with chloramphenicol showed synergism against *Salmonella typhi*, while the combination of Brazilian red propolis with fluconazole was beneficial against *Candida* spp. Investigated in Chilean propolis, other flavonoids such as apigenin and pinocembrin showed antibacterial efficacy against *Streptococcus mutans* [51]. The following Gram-negative bacteria, however, were successfully combated by apigenin: *Proteus mirabilis*, *Enterobacter aerogenes*, *Salmonella enterica* serotype Typhimurium, *Pseudomonas aeruginosa*, and *Klebsiella pneumoniae* [65]. Pinocembrin has also demonstrated antibacterial efficacy against *Listeria monocytogenes*, *Streptococcus sobrinus*, *E. faecalis*, *S. aureus*, *Pseudomonas aeruginosa*, and *Streptococcus mutans* [66].

According to the established research, synergistic anti-bacterial effects were shown when apigenin was added to the β-lactam to treat MRSA and when apigenin was combined with ceftazidime against *Enterobacter cloacae* [67]. Interestingly, cinnamic acid, which has demonstrated substantial activity against several bacteria, *Aeromonas* spp., *Bacillus* spp., *E. coli*, *Enterobacter cloacae*, *L. monocytogenes*, *Micrococcus flavus*, *Mycobacterium* TB, *Salmonella enterica* serotype, *Streptococcus pyogenes*, T*yphimurium*, *Vibrio* spp., and *Yersinia ruckeri* is abundant in propolis [68]. For instance, it is important to note that cinnamic acid inhibits bacterial binary fission, ATPase activity, and the formation of biofilms by breaking the bacterial cell membrane [69].

Use of Propolis in the Biofilm Control

A multi-layered bacterial cluster called a biofilm is enclosed in an extracellular polysaccharide matrix. Persistent infections are aided by biofilms because they are known to boost bacteria's potential to colonize inert objects and protect them from the body's natural defences and antimicrobials. A biofilm is a mucilaginous matrix formed of polymeric extracellular components that is adherent to a surface and contains collections of both living and non-living microorganisms that assemble at the liquid-solid interface. The matrix shields the bacteria from harm by retaining nutrients and restricting access to biocides, oxidants, antibiotics, metallic cations, and poisons. Infections brought on by implanted medical devices like catheters and dental, cardiac, or urological prostheses are therefore greatly aided by biofilms [70].

Biofilms are strongly associated with chronic lung infections, a critical complication in people with cystic fibrosis. These infections are characterized by the presence of drug-resistant biofilms in bronchial mucus, together with regions of high reactive oxygen species levels, primarily due to neutrophil activity [71]. Many antibiofilm chemicals have been found in natural sources against this bacterium, some of which impede bacterial quorum sensing, such as garlic extract and a synthetic derivative of natural furanone. Additional natural antibiofilm compounds encompass ginseng aqueous extract and its component five ursine triterpenes, zingerone, asiatic acid, corosolic acid from *Diospyros dendo*, tannins sourced from *Commiphora leptophloeos, Myracrodruon urundeuva*, and *Anadenanthera colubrina* as well as bacterial products like 3-indolyl acetonitrile. Furthermore, plant extracts have been used to reduce the production of *P. aeruginosa* biofilms [72].

In a comparative examination of the effects of Brazilian red propolis' benzophenone-enriched fraction (BZP-BRP) on strains of *Candida glabrata* resistant to conventional antifungal drugs, Pippi et al.[73] discovered that the fluconazole-resistant bacteria displayed high sensitivity to propolis. Propolis alters gene expression, lowers bacterial viability, and prevents *S. epidermidis* from forming a biofilm, making it susceptible to further antibiotic treatment. It has been shown that Malaysian propolis and chitosan-propolis nanoparticles can inhibit the growth of *Enterococcus faecalis* biofilms [74]. Propolis is effective against *C. albicans* biofilms [75-79]. In situations of persistent infections that are challenging to treat because of the development of biofilm in the wound environment, propolis can minimize biofilm formation and speed up healing processes [80].

Findings indicate that propolis nanoparticles can enhance antibacterial activity and biofilm formation against *E. faecalis*, and when combined with other medications, they may have a synergistic impact that lowers the dosage of each treatment and the amount of time needed to kill germs [81-83]. In a different study, de Mélo Silva et al.[84] also demonstrated that red propolis polymeric nanoparticles

Table 2. *The role of propolis in antibacterial effect and synergestic effect with antibiotics*

Propolis Types	Targeted Organisms	Mode of Action	Efficacy (MIC)	Outcome of Study	References
Propolis ethanolic extract + Ampicillin	*S. typhi*	When compared to ampicillin alone, the combination produced a much larger zone of inhibition	8 µg/mL	Enhanced antibacterial activity	[91]
Propolis ethanolic extract + Cefixime	*S. enteric* in mice	Decreased bacterial load, increased lifespan, corrected hematological parameters, and shielded kidney, spleen, and liver from damage brought on by bacteria	2962 µg/mL	Enhanced antibacterial effect	[61]
Propolis ethanolic extract + Cefoxitin	*S. aureus* and MRSA	Greater inhibition diameter in comparison to each monotherapy	0.39 to 0.78 mg/mL	Enhanced anti-bacterial effect	[64]
Hydroalcoholic propolis extract mixed with carob in a proportion of (60/40, w/w) + Ceftriaxone	*E. coli*	When compared to utilizing ceftriaxone alone, propolis enhanced the effects of ceftriaxone and had a synergistic bactericidal effect	0.125 µg/mL	Synergistic effect	[92]
Hydroethanolic red propolis + Imipenem	*P. aeruginosa* and *S. aureus*	Using imipenem in combination with red propolis collected during the dry season resulted in a considerably decreased MIC value against *P. aeruginosa*; no benefit was shown against *S. aureus*	*P. aeruginosa*:512 µg/mL and *S. aureus*: 64 µg/mL to ≥1024 µg/mL.	*P. aeruginosa*: an enhanced anti-bacterial effect *S. aureus:* no interaction	[93]
Propolis ethanolic extract + Mupirocin	MRSA infected rabbits	Rats' nasal mucous membrane bacteria count and polymorphonuclear leukocyte levels were significantly reduced as compared to the respective monotherapy and combination treatments	-	Enhanced anti-bacterial effect	[94]
Propolis ethanolic extract + Vancomycin	MRSA, *E. faecalis*, *S. pyogenes*, *H. influenza* and *S. pneumonia*	Gram-positive bacteria grow more slowly than gram-negative bacteria due to a powerful synergistic relationship	0.3 to 2.5 mg/mL	Synergistic effect	[95]
Propolis ethanolic extract + Vancomycin	*S. aureus*	The Kirby, Bauer, and E-test methodologies identified synergism	1.5 µg/mL	Synergistic effect	[96]

have the capacity to suppress the growth of *S. aureus* and *Pseudomonas aeruginosa* biofilms. This could be because propolis nanoparticles penetrate the skin more effectively than propolis because of their smaller particle size and higher surface area-to-volume ratio.

According to a study, EEP affects probiotics' *in vitro* viability and capacity to build biofilms in concentration- and strain-dependent ways. In some circumstances, propolis can function as a prebiotic at low concentrations, but at higher concentrations, it may prevent the probiotics from growing in a planktonic state or from creating biofilms [85]. While Brazilian EEPs was extremely active against already-formed biofilms, European EEPs had the highest effect in delaying the creation of biofilms [78]. The Spanish ethanolic propolis extract (SEEP) showed effectiveness against *C. glabrata*. In addition to having antifungal properties, SEEP decreased this emerging opportunistic pathogen's ability to produce biofilms. It presents an intriguing therapeutic approach for the prevention and treatment of biodevice-associated infections due to its anti-biofilm action [86].

According to a different study, *Corynebacterium pseudotuberculosis* strains continue to exhibit great antibiotic sensitivity. There were differences in how the ethanolic and supercritical extracts of green, brown, and red propolis affected the *C. pseudotuberculosis* planktonic isolates. The strongest antibacterial activity among the isolates was demonstrated by the supercritical extract of red propolis and the ethanolic extract of green propolis, both of which were able to prevent the development of biofilm [87]. The ethanolic extract of propolis may inhibit the growth of biofilms and suppress the expression of *EFG1* in *C. albicans* biofilms [88].

To effectively combat *S. mutans*, propolis, essential oil (PEO) inhibits cell viability inside the biofilm, reduces the overall amount of biofilm biomass, and destroys the biofilm structure [89]. When compared to a well-researched antiseptic mouthwash with established outcomes, bee propolis demonstrated promising benefits in suppressing the growth of *S. mutans* produced in a biofilm [90]. Current studies in the literature on the antibacterial effect of propolis and its synergistic effect with antibiotics were reviewed and summarized in *Table 2* [61,64,91-96].

Bee Pollen

When a worker bee travels from one flower to another in search of nectar, pollen grains get attached to the bee's body. This pollen combines with nectar and salivary enzymes; and hardens to form a pellet known as bee pollen. The bees use bee pollen as a source of protein, lipids, micronutrients and minerals [97]. Pollen grains from various plants vary in size between 1.4 to 4 mm and weigh approximately 7.5 to 8 mg. They come in a variety of colours including creamy white, red, yellow, orange, grey, dark brown and green. The wide nutritional and medicinal properties of bee pollen make it a natural superfood. The bee pollen properties differ based on floral

source, geographical origin and seasonal variations [98]. This variation may contribute to the difference in the bee pollen's biological activities and thus its therapeutic effects [99]. Bee pollen may be divided into two groups: monofloral bee pollen and multifloral bee pollen. Bee pollen can be categorized into two primary types: monofloral and multifloral. Monofloral pollen consists of a minimum of 45% pollen grains from a single plant species and displays consistent organoleptic and biochemical characteristics. In contrast, multifloral pollen incorporates pollen grains from various plant sources without any one dominant plant species. The determination of the botanical source of bee pollen is accomplished through palynological analysis. Each individual component of the pollen grains is identified and examined under a microscope [100,101]. Bee pollen is an affordable nutraceutical that has great potential in the food industry [102].

The therapeutic effects of bee pollen are due to the presence of a vast range of secondary plant metabolites, which varies from one plant species to another [99]. Bee pollen is composed of proteins, carbohydrates, lipids, amino acids, enzymes, polyphenols, co-enzymes, minerals and vitamins [102]. According to a systemic review conducted by Thakur and Nanda involving over 100 studies, bee pollen on average contains 13-55% carbohydrates, 10-40% protein and 1-13% lipids [98]. In addition, bee pollen contains vital amino acids such as methionine, phenylalanine, valine, lysine, threonine, tryptophan, histidine, leucine and isoleucine. Lipids are found in the form of essential fatty acids, phytosterols and phospholipids. The total phenol content which includes polyphenols such as catechins, flavonoids (ex. quercetin, kaempferol and isorhamnetin), and phenolic acids make up an average of 30.59mg GAE/g [98,102]. Bee pollen is found to contain minerals such as Mg, Ca, Zn, Fe and Cu and vitamins such as provitamin A, niacin, biotin, thiamine, folic acid and vitamin E [99,103].

Antimicrobial Properties of Bee Pollen and Antibiotic Resistance

Antimicrobial resistance is a serious issue that is currently threatening global public health. Natural products can be used as an alternative method to control antibiotic-resistant pathogens [104]. Till now no microbe has developed resistance against bee pollen and hence it can be used along with traditional antibiotics against resistant microbes [105].

Bee pollen exhibits antimicrobial properties against various bacteria and fungi. Bee pollen from different regions such as Greece, Morocco, Portugal, Spain, Egypt, Turkey, Slovakia, Chile and Slovenia have been studied for its antimicrobial activity [106]. Some of the bacteria against which they have shown activity are Gram-positive bacteria: *S. aureus*, *Bacillus cereus*, *Staphylococcus epidermidis*, *Listeria monocytogenes*, *Clostridium butyricum*, *Streptococcus pyogenes,* and *Clostridium perfringens*, and Gram-negative bacteria: *Salmonella enterica, Campylobacter jejuni,* and *E. coli*. In addition, bee pollen has also been reported for antifungal activity against fungal species including *Candida tropicalis*, *Candida glabrata, Candida krusei*, *Zygosaccharomyces bailii, Aspergillus niger,* and *Aspergillus fumigatus* [106].

The presence of phenolic compounds is credited for the antimicrobial properties found in bee pollen [107]. The antibacterial effects of bee pollen might also involve the enzyme glucose oxidase, which is generated by honeybees [99]. Bee pollen acts against bacteria by breaking down their cytoplasmic membrane which causes the leakage of potassium ions. This triggers the autolysis of the bacterial cell leading to cell death [108]. Several studies have reported that Gram-negative bacteria are less sensitive to bee pollen compared to Gram-positive bacteria. A possible explanation could be that Gram-negative bacteria have a more chemically complex cell wall with a greater lipid content which renders them more resistant to pollen [105]. Antimicrobial resistance is often more pronounced in Gram-negative bacteria, owing to the presence of an outer membrane composed of lipopolysaccharides, which serves as an added layer of protection [109].

A study conducted for antimicrobial activity by Bakour et al.[110] investigated the effect of bee pollen from six different botanical origins *(Centaurium erythraea, Citrus aurantium, Coriandrum sativum, Quercus ilex*, *Punica granatum,* and *Ruta graveolens)* against six strains of human multidrug-resistant pathogens namely, *S. aureus*, *Pseudomonas aeruginosa*, *E. coli*, *Klebsiella pneumoniae, Acinetobacter baumannii*, *Enterobacter cloacae*. These microbes were tested for antibacterial susceptibility against 28 standard antibiotics and were found to be resistant to some of them. The agar disc diffusion method was used to study the antibacterial activity of the samples and the MIC and minimum bactericidal concentration (MBC) were estimated and summarised in *Table 3*. The findings of the study reported that the ethanolic pollen extracts of *Punica granatum* and *Quercus ilex* showed the best antimicrobial activity followed by *Ruta graveolens*, *Centaurium erythraea*, and *Coriandrum sativum* which showed intermediate activity. *Citrus aurantium* showed no antimicrobial activity. A positive correlation was observed between the antibacterial activity of the pollen extract and its antioxidant content. The difference in MIC and MBC values between different pollen extracts could be due to the variation in their chemical composition and the variability of the cell wall and membrane structure of the bacterial strain [110].

Another study by Pelka et al.[111] reported the antimicrobial resistance activities of bee pollen and bee bread from

different regions of Poland. Authors studied antimicrobial activity prepared extract of bee pollen in comparison to extract of bee bread. Three samples of bee pollen and three samples of bee bread, which were prepared separately showed high anti-staphylococcal potential, were tested against 3 clinical isolates of methicillin-resistant *S. aureus* (MRSA). Beebread showed better activity compared to bee pollen as bee pollen is converted to bee bread by the process of fermentation. This study suggests that the lactic acid bacteria involved in this process produce lactic acid and bacteriocin which are antimicrobial in nature. The lactic acid bacteria cause lipid hydrolysis which produces aliphatic acids which acting as an antimicrobial agent, and this may be the reason for the difference in antimicrobial activity between bee pollen and bee bread. The MIC value for bee pollen ranged between 5 to 10% (v/w) and the MBC value ranged between 5-10% (v/w) *(Table 3)* [111].

The antimicrobial compounds present in bee products work in a synergistic way which might be the reason why microbes are not resistant to bee pollen. Bee products are rich in flavonoids and polyphenols which are known to have the ability to counter bacterial resistance making them potential antimicrobial agents. Bioactive compounds present in bee pollen can be used in the combination with antibiotics to produce a synergistic antimicrobial effect [110]. A study reported that when kaempferol glycosides of plant origin were used along with hydrophilic fluoroquinolones against MRSA, the kaempferol helped to significantly reduce the MICs of the antibiotics used [112]. Another study revealed that the combination of rifampicin, quercetin, and kaempferol acted synergistically to inhibit the β-lactamase enzyme of clinical MRSA [110]. Another study tested the use of apigenin and other flavones along with a penicillin/streptomycin mix against two MRSA strains and coagulase-negative *Staphylococcus* (CNS). The apigenin and the other flavones were unable to improve the antimicrobial activity in this case [113]. The use of polyphenols along with antibiotics helps to increase the efficacy of the antibiotic, lowers its dose and therefore reduces the antibiotics' side effects [114].

Use of Bee Pollen in the Biofilm Control

Biofilm refers to bacterial communities that are encased within an exopolysaccharide matrix and adhere to a surface. It is the cause of many diseases including infective endocarditis, inflammatory bowel disease, impaired wound healing, cystic fibrosis, and pertussis [115].

Schuh et al.[116] studied the exosome-like vesicles (ELVs) present in various bee products for their bacteriostatic, bactericidal, and biofilm-inhibiting effects. These exosome-like vesicles are a part of the hypopharyngeal gland secretions produced by *Apis mellifera*. These ELVs were extracted from different bee products and their minimum biofilm inhibition concentration (MBIC) was estimated using a biofilm-forming strain, *S. aureus*. All the bee product-derived ELVs showed biofilm-inhibitory potential. The MBIC ratio of bee pollen was found to be 10:1 (vesicles: colony forming units). Bee pollen displayed 50% biofilm inhibition at a 1:1 ratio. The ELV fraction of bee pollen was compared with an exosome-depleted fraction. The findings reported that the exosome-depleted fraction showed antibacterial activity at 5% (v/v) concentration whereas the ELV fraction showed antibacterial activity at a lower concentration of 1%. Bee-derived ELVs could be potentially used in wound healing to treat wound-derived infections by preventing biofilm formation and by aiding migratory activity [116].

The ability of bee pollen extract to prevent the adherence of microbes to the inert substratum and cellular substrate (Hep-2 -human epithelioma cells) was studied [117]. The microbial strains used in assessing microbial adherence to an inert surface were standard strains of *S. aureus*, *E. faecalis, E. coli, P. aeruginosa, C. albicans* and clinically isolated strains of *E. cloacae*, *C. glabrata*, *C. famata*, *C. krusei*, *C. guillermondii* and *C. lusitaniae*. The effect of bee pollen in inhibiting microbial attachment to cellular substrates was carried out using *E. faecalis, S. aureus, C. albicans, P. aeruginosa, C. lusitaniae, E. cloacae, C. famata, C. guillermondii, C. glabrata, and C. krusei*. These findings revealed that *S. aureus, P. aeruginosa, and C. glabrata* were the most sensitive strains when it comes to adherence to inert surfaces. According to Ilie et al.[117], when compared to the control, the bee pollen extract exhibited reduced adherence capacity to the cellular surface in the case of the Gram-negative bacteria *E. cloacae and P. aeruginosa*, all yeast (except *C. famata*) and one Gram-positive bacteria *E. faecalis* [117].

Biofilm control is one of the mechanisms by which polyphenols exert antimicrobial properties. This was tested using plant-derived or synthetic compounds. Quercetin glycosides and kaempferol glycosides were found to have biofilm control over yeast and fungi whereas luteolin has antibiofilm properties against *S. aureus and L. monocytogenes* [106]. Plant phenolics prevent biofilm formation by interfering with the bacterial regulatory system such as quorum sensing [118]. It can be concluded from the above studies that the polyphenols present in bee pollen may be responsible for the antibiofilm activity.

Royal Jelly

Royal jelly (RJ) [119], is a creamy substance produced by the hypopharyngeal glands of the young nurse worker bees mainly to mature and maintain the queen bee. It is exclusively served to the queen bee throughout her life, whilst royal jelly is served to other sexually immature females for merely the first 2-3 days [120].

Table 3. *Role of bee pollen in antimicrobial-resistance*

Types of Bee Pollen	Targeted Microorganisms (Resistant Strain)	Mode of Action	Efficacy		Main Outcome of Study	References
			MIC	MBC		
Centaurium erythraea (botanical origin)	*S. aureus*	The presence of Polyphenols helps fight bacterial resistance	Ineffective	Ineffective	Intermediate antimicrobial activity	[110]
	P. aeruginosa		Ineffective	Ineffective		
	A. baumanii		Ineffective	Ineffective		
	E. coli		Ineffective	Ineffective		
	E. cloacae		2.5 mg/mL	2.5 mg/mL		
	K. pneumonia		Ineffective	Ineffective		
Citrus aurantium (botanical origin)	*S. aureus*	The presence of Polyphenols helps fight bacterial resistance	Ineffective	Ineffective	No antimicrobial activity	[110]
	P. aeruginosa		Ineffective	Ineffective		
	A. baumanii		Ineffective	Ineffective		
	E. coli		Ineffective	Ineffective		
	E. cloacae		Ineffective	Ineffective		
	K. pneumonia		Ineffective	Ineffective		
Coriandrum sativum (botanical origin)	*S. aureus*	The presence of Polyphenols helps fight bacterial resistance	2.5 mg/mL	2.5 mg/mL	Intermediate antimicrobial activity	[110]
	P. aeruginosa		Ineffective	Ineffective		
	A. baumanii		2.5 mg/mL	2.5 mg/mL		
	E. coli		Ineffective	Ineffective		
	E. cloacae		2.5 mg/mL	>2.5 mg/mL		
	K. pneumonia		2.5 mg/mL	>2.5 mg/mL		
Punica granatum (botanical origin)	*S. aureus*	The presence of Polyphenols helps fight bacterial resistance	0.62 mg/mL	0.62 mg/mL	Highest antimicrobial activity	[110]
	P. aeruginosa		2.5 mg/mL	2.5 mg/mL		
	A. baumanii		0.31 mg/mL	0.31 mg/mL		
	E. coli		2.5 mg/mL	2.5 mg/mL		
	E. cloacae		2.5 mg/mL	2.5 mg/mL		
	K. pneumonia		2.5 mg/mL	>2.5 mg/mL		
Quercus ilex (botanical origin)	*S. aureus*	The presence of Polyphenols helps fight bacterial resistance	0.62 mg/mL	0.62 mg/mL	Highest antimicrobial activity	[110]
	P. aeruginosa		2.5 mg/mL	2.5 mg/mL		
	A. baumanii		0.31 mg/mL	0.31 mg/mL		
	E. coli		1.25 mg/mL	2.5 mg/mL		
	E. cloacae		1.25 mg/mL	1.25 mg/mL		
	K. pneumonia		2.5 mg/mL	2.5 mg/mL		
Ruta graveolens (botanical origin)	*S. aureus*	The presence of Polyphenols helps fight bacterial resistance	1.25 mg/mL	1.25 mg/mL	Intermediate antimicrobial activity	[110]
	P. aeruginosa		Ineffective	Ineffective		
	A. baumanii		1.25 mg/mL	1.25 mg/mL		
	E. coli		2.5 mg/mL	2.5 mg/mL		
	E. cloacae		Ineffective	Ineffective		
	K. pneumonia		Ineffective	Ineffective		
Czarne, Poland (Geographical origin)	MRSA strain 1, 2,3(clinical isolate)	-	10% (v/w)	10% (v/w)	Lower anti-staphylococcal activity	[111]
Niżna Łąka, Poland (Geographical origin)	MRSA strain 1, 2,3(clinical isolate)	-	5% (v/w)	5% (v/w)	Higher anti-staphylococcal activity	[111]
Modzele, Poland (Geographical origin)	MRSA strain 1, 2,3(clinical isolate)	-	5% (v/w)	5% (v/w)	Higher anti-staphylococcal activity	[111]

MRSA- methicillin-resistant S. aureus; MBC- minimum bactericidal concentration; MIC- minimum inhibitory concentration.

Royal jelly has a complex chemical composition. It consists of water, minerals such as potassium, magnesium, calcium, etc., proteins (about 50% of the dry mass), lipids (primarily short-chain fatty acids including 10-hydroxy-2-decenoic acid), carbohydrates (about 30% of the dry matter), at least 17 amino acids, and vitamins [121]. The chemical makeup, color, and flavor of royal jelly are influenced by the bees' diet. One type of diet comprises solely of the bees' natural food sources, like nectar, pollen, and honey. The second type of diet involves the addition

Table 4. *Antimicrobial Activity of Royal Jelly*

Origins of the Royal Jellys	Targeted Microorganisms	Mean of Inhibition	Effect Same Antimicrobial	References
Chinese royal jelly 15 mg/mL	*Aspergillus niger* *Aspergillus fumigatus* *Candida albicans* *S. aureus* *Bacillis subtilis* *Escherichia coli*	13 to 15 12 to 13 9 to 10 13 to 14 16 to 17 16 to 17	< Clotrimazole < Clotrimazole < Clotrimazole < Penicillin g < Penicillin g < Penicillin g	[124]
Egyptian royal jelly 15 mg/mL	*Aspergillus niger* *Aspergillus fumigatus* *Candida albicans* *S. aureus* *Bacillis subtilis* *Escherichia coli*	20 to 21 24 to 25 19 to 20 23 to 24 24 to 25 10 to 11	= Clotrimazole = Clotrimazole > Clotrimazole = Penicillin g < Penicillin g < Penicillin g	[124]
Southern Córdoba (Argentina) royal jelly 7.1 and 14.5 mg/mL	*S. aureus* *Staphylococcus epidermidis* *Micrococcus luteus* *Streptococcus uberis* *Enterococcus faecalis* *Pseudomonas aeruginosa* *Escherichia coli* *Klebsiella pneumoniae*	15 to 21 13 to 14 15 to 16 11 10 to 12 5 to 6 9 to 10 ----------	---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------	[123]
Singapore royal jelly 100 µg/mL	*Aggregatibacter* *actinomycetemcomitans,* *Porphyromonas gingivalis,* *Prevotella intermedia,* *Fusobacterium nucleatum,*	No growth No growth No growth No growth No growth	---------- ---------- ---------- ---------- ----------	[125]
Chinese royal jelly	*S. aureus* *E. coli* *Klebsiella oxytoca* *Klebsiella pneumoniae* *Pseudomonas aeruginosa* *Methicillin* resistant *S. aureus* *Salmonella typhimurium* *Salmonella paratyphi* *Proteus vulgaris* *Enterobacter aerogenes*	1 to 1.5 1 to 1.5 0.5 to 1 1 to 1.5 1 to 1.5 1 to 1.5 2 to 2.5 1 to 1.5 1 to 1.5 1 to 1.5	= Penicillin Significant difference with Penicillin Significant difference with Penicillin Significant difference with Penicillin Significant difference with Penicillin Significant difference with Penicillin Significant difference with Penicillin Significant difference with Penicillin Significant difference with Penicillin Significant difference with Penicillin	[126]
Jordanian royal jelly	*S. aureus* *E. coli* *Klebsiella oxytoca* *Klebsiella pneumoniae* *Pseudomonas aeruginosa* *Methicillin* resistant *S. aureus* *Salmonella typhimurium* *Salmonella paratyphi* *Proteus vulgaris* *Enterobacter aerogenes*	1.75 to 2 2 to 2.5 2.40 to 2.75 1.40 to 1.75 1.5 to 1.75 0.75 to 1 0.75 to 1 1.5 to 1.75 1.4 to 1.5 1.5 to 1.75	= Penicillin Significant difference with Penicillin Significant difference with Penicillin Significant difference with Penicillin Significant difference with Penicillin Significant difference with Penicillin Significant difference with Penicillin Significant difference with Penicillin Significant difference with Penicillin Significant difference with Penicillin	[126]

of other nutrients, such as proteins and carbohydrates, to their natural food [122].

Antimicrobial Properties of Royal Jelly and Antibiotic Resistance

The results obtained in the study of García et al.[123] showed that 2 RJ specimens, acquired from different regions of Argentina, both inhibited the growth of Gram-negative and Gram-positive bacteria strains capable of infecting cutaneous wounds *Staphylococcus epidermidis*, *S. aureus*, *Micrococcus luteus, Streptococcus uberis, Enterococcus faecalis, Pseudomonas aeruginosa, S. aureus, E. coli,* and *Klebsiella pneumoniae* being the most sensitive strain of either RJ sample tested. Moselhy et al.[124] indicated that RJ obtained from various regions in Egypt and China possesed bacteriostatic effects on Gram-positive and Gram-negative bacteria at different levels. The effect of royal jelly on the growth of periodontopathic bacteria were examined in the study conducted by Coutinho et al.[125] and the results suggested that RJ samples were active against the growth of the tested periodontal pathogens. Al-Abbadi [126] showed that the effects of royal jelly acquired from Jordan and China against bacteria and fungi pathogenic to humans. The results of this study indicated that both RJ types prepared in different ways were effective against Gram-positive and/or Gram-negative bacteria *(Table 4)*.

Royal jelly's ability to combat microbes can be attributed to specific elements, including Major royal jelly proteins (MRJPs 2-5, 7), antimicrobial peptides (Jelleines I, II, and III, as well as royalisin), and fatty acids like 10-HAD [127,128]. The antibacterial activity of MRJPs against Gram-negative *E. coli* depends on their interaction with bacterial cell walls. Furthermore, several studies have shown the wide scope of antibacterial effects of MRJP4 and MRJP2 against fungi, yeasts and both Gram-positive and negative

bacteria. The proteins act as antimicrobial peptide (AMP)-like proteins because they can attach to the cell walls of bacteria and deconstruct its structure [129]. On the other hand antibacterial peptides are also positively charged which allows them to collapse the cell membrane through interacting with its anionic phospholipids [130]. Jelleines I, II and III, royalisin and 10-HDA seem to be effective against Gram-positive and negative bacteria with the latter two being also effective against fungi [127]. determined that both intramolecular disulfide linkages and the presence of 11 amino acids at the C-terminus in royalisin are crucial for its antimicrobial efficacy [131]. Moselhy et al.[124] showed that while the antimicrobial capabilities of royal jelly are evident, there doesn't seem to be any one definite cause, suggesting that the effect may be of a combined or synergistic nature [124].

In antoher study Mierzejewski et al.[132] examined the antimicrobial effects of three honeybee products including honey, royal jelly and propolis in comparison to three antibiotics including kanamycin, penicillin and tetracycline on bacteria strains *Bacillus cereus*, *Staphylococcus epidermidis*, *E. coli,* and *S. aureus***.** The results showed that kanamycin and tetracycline were the most effective antimicrobial agents in general; nonetheless, royal jelly was more effective against *Staphylococcus epidermidis*, and *E. coli*. The study concluded that honey, propolis, and royal jelly contain antibacterial components that can be used as standard first-line treatments for mild bacterial infections. The study also suggested that commercial products containing these components can be manufactured to increase effectiveness, which makes them also potentially preventative against infectious diseases [132]. Another study compared the antimicrobial activity of royal jelly with gentamicin, doxycycline, and the combination of the two antibiotics against *E. coli*. This study concluded that RJ shows significant activity against *E. coli* growth [133].

Use of Royal Jelly in Biofilm Control

A study was conducted both *in vitro* and *in vivo* using a rat model to investigate the effect of a 50% concentration of Royal Jelly (RJ). The results showed a highly statistically significant reduction in the adherence of MRSA bacteria in all samples tested ($P<0.01$) [134].

According to Shuch et al.[116] study, bee-derived exosome-like vesicles (ELVs) exhibit the capacity to impede the formation of biofilms in *S. aureus* strains in a laboratory setting. This finding indicates a potential role for ELVs in the prevention and management of wound infections. Notably, when the ratio of royal jelly was less than 1 vesicle per viable bacterial cell, it led to the inhibition of *S. aureus* growth and a significant reduction in biofilm formation, approximately by half [116]. In another study, it was showed that RJ extracellular vesicles (EVs) incorporated into collagen gels significantly reduced biofilm formation [135].

In a different research investigation, it was observed that royal jelly concentrations at 25% or higher effectively suppress bacterial growth. However, when the concentrations were subinhibitory, they were found to promote pyocyanin production and enhance biofilm formation in *P. aeruginosa* [136].

A recent study concluded that Sub-MICs of 10-HDA could be effective in inhibiting biofilm formation and eliminating the mature biofilms of *S. aureus*, as verified by significant reductions in biofilm biomass and cell viability. Furthermore, the biofilm structure was noticeably damaged after treatment with 10-HAD [137].

Bee Venom

Bee venom (BV), also called apitoxin, is an acidic transparent and odorless liquid secreted by bees [21]. BV is synthesized by bee workers and queens and used for the defence of the colony or of the individual [22,138]. BV discovery was attributed to ancient Egyptian. It had been used as a therapeutic product starting from the second century BC. BV was first used in Eastern Asia [20,21].

BV is a mixture of several active compounds. It is a complex mixture of polypeptides with melittin being the major constituents (40-60% of dry weight) [22]. Additional peptides like apamin, adolapin, and mast cell degranulating peptide can be found within BV. Phospholipase A2 ranks as the predominant enzyme in BV, closely followed by hyaluronidase. Other molecules are also present in BV composition such as: sugars, minerals, amino acids and catecholamines [23,24]. Volatiles compounds are also among BV components. However, due to their volatile properties they are lost easily during collection [19].

Bee venom is using in an alternative medicine for the treatment of some diseases, such as rheumatism arthritis, Parkinson's disease (PD), Alzheimer's disease (AD), and amyotrophic lateral sclerosis (ALS) [139].

Nonetheless, the necessity for standardizing it as a reliable and safe medicinal product is imperative. In this regard, the method for apitoxin standardization proposed in Korea appears to be a valuable approach. This method involves the purification of bee venom, followed by the application of a stepped-gradient open column (ODS-A; 120 Å, 150 mesh). Consequently, this process results in an increased yield of melittin while simultaneously eliminating allergenic proteins. The purified bee venom is analyzed by HPLC, and concentration of mellitin is examined. Finally, bee venom is diluted to required concentration and proper dose of apitoxin may be applied using injection water in pharmacopuncture [140].

Antimicrobial Properties of Bee Venom and Antibiotic Resistance

Bee venom has been largely investigated for its antimicrobial properties and many research papers and reviews have been published in the last recent years. BV was found to inhibit several bacterial strains including pathogenic bacteria *(Table 5)* [141-145].

Maitip et. al.[145] investigated the antimicrobial properties of crude BV of four bee species in Thailand: *A. cerena*, *A. meliffera*, *A. florea* and *A. dorsata* and compared its effectiveness with synthetic melittins derived from the four studied bee species. All BV and synthetized melittins were more active against Gram-positive bacteria. BV showed lower MIC values regarding all tested bacterial strains with the lowest values for *A. cerena* crude BV compared to melittin. Melittin inhibited Gram-positive bacteria in the following order: *S. epidermidis* (MIC=12.5-50 µg/mL) > *M. luteus* (MIC=25-50 µg/mL) > *S. aureus* (MIC=50-200 µg/mL) > MRSA (MIC=100-400 µg/mL) > *B. subtilis* (MIC >400 µg/mL). Differences between the studied BV was related to differences in their chemical composition [145].

The effectiveness of the tested peptide was pointed to be influenced by their seize, sequence, charge, structure, hydrophobicity and amphipathicity [146]. *A. florea* and *A. dorsata* melittins exhibited a higher activity when compared to the extracted BV. For *A. cerena* and *A. meliffera*, results of BV and melittins were similar [145]. Alia et al.[147] also reported an interesting effect of melittin on *L. monocytogenes* (ATCC 19111) and *S. aureus* (ATCC 11632) with MIC value of of 12.5 µg/mL and 25 µg/mL respectively. While melittin were less active on the tested Gram-negative bacteria *S. enterica* and *Y. kristensenii* with MIC values of of 100 µg/mL and 200 µg/mL respectively.

The emergence of antimicrobial resistance stands out as a highly concerning issue within the domain of public health. The spread at which bacteria develop resistance far exceeds the ability to treat it or at least to stop this evolution. The return to nature for research for new antimicrobial molecules is a key point in favor of bee products. Many investigations focusing on strains of increasing resistance have proved the effectiveness of BV and its synthetized or extracted polypeptides [148]. BV and melittin showed very promising results on resistant bacterial strains.

Table 5. *BV antibacterial properties*

Bee Venom and Its Active Constituents	Targeted Microorganisms	Mode of Action (If Available)	Efficacy (e.g. IC50)	Main Outcome of Study	References
Natural and commercial apitoxin, melittin and phospholipase A_2	*S. salivarius* (ATCC 25975), *S. sanguinis* (ATCC 10556), *S. sobrinus* (ATCC 33478), *S. mutans* ATCC 25175), *S. mitis* (ATCC 49452), *L.. casei* ATCC 11578), *E. faecalis* (ATCC 4082)	ND	20-40 µg/mL (apitoxin) 2-40 µg/mL (melittin) >400 µg/mL (phospholipase A_2) 6-80 µg/mL (melittin + phospholipase A_2)	Melittin was found to be the most active component apitoxin melittin, were found to be very effective against oral pathogens.	[141]
Egyptian BV sac Apitox (Apitonoc services, CANADA) Vacsera (Egyptian vaccine and serum organization)	*S. aureus, S. pyogens, K. pneumonia, E. coli, P. aeruginosa*	ND	1.00-3.80 mg/mL	BV inhibits the Survival and the growth of and of the tested. In consequence, it can be used ascomplementary antimicrobial agent	[142]
BV, melittin (synthetized and purified)	*S. aureus* ISP4790 and MU50 (clinical isolates), MRSA (USA300 (LAC), Newman, MW2, MRSA1, MRSA2), *S. agalactiae* CNCTC 10/84, *S. epidermidis* RP62a, *S. pneumonia* TIGR4, *S. gordonii* M99 and *S. bovis* NEM760	ND	1.56-12.5 µg/mL	BV had negative effects when used as an anti-MRSA therapy. Melittin may have a therapeutic potential for the management of MRSA infections.	[143]
Anatolian BV	*E. faecalis* (ATCC 29212), *L. monocytogenes* (ATCC 11994), *S. pyogenes S. aureus* (ATCC 25923 and MRSA), , *B. subtilis* (ATCC 6633 and clinical strain, *B. cereus* (702 Roma), *M. smegmatis* (ATCC 607), *E. coli* (ATCC 25922), *K. pneumoniae* subsp. *pneumoniae* (ATCC, 18883), *Y. pseudotuberculosis* (ATCC 911), *Vibrio* sp. (Clinic strain), *A. hydrophila* (ATCC 7966), *A. sobria* (ATCC 43979), *P. aeruginosa* (ATCC 27853)	Synergetic effect of melittin and PLA2 (mecanism of action not clarified)	3.06-50.00 µg/mL	BV is an interesting alternative source of antimicrobial compounds, including resistant microorganisms. *In vivo* research are required to evaluate BV safety and effectiveness for future therapeutic uses.	[144]
Crude venom *from A. mellifera, A. cerana, A. dorsata*, and *A. florea* (Thailand) and melittin	*S. aureus, MRSA* *S. epidermidis, B. Subtilus, M. luteus,* *K. pneumonia,* *E. coli, S. thyphimurium*	ND	16.7-7.2 ≥400.0±0.0 µg/mL	*A. cerana* crude venom and melittin were most effective in against Gram-positive bacteria and MRSA. Crude venom is more effective than melittin. Both substances might be potential sources of antimicrobial agents against Gram-positive and antibiotic-resistant bacteria.	[145]

ND: not determined

They were reported to be active on methicillin-resistant *S. aureus* [141,144,145] and *B. burdorferi* [149]. Melittin was also reported to inhibit other antibiotic-resistant bacterial strains. Melittin was tested on 20 isolates of *A. baumannii*. MIC and MCB values were between 0.5 and 16 µg/mL for MIC and 0.5 and 32 µg/mL for MBC [150]. Gopal et al.[151] demonstrated the effectiveness of melittin against 32 isolates of antibiotic-resistant bacteria including *E. coli*, *S. thyphimurium, P. aeruginosa* and *S. aureus* suggesting the potential use of melittin to treat microbial infection. Khozani et al.[152] investigated the antibacterial activity of melittin against 33 *P. aeruginosa* strains isolated from patients with burns (third degree) and compared its effect to conventional antibiotic namely colistin, ceftazidime and doripenem. *In vitro* results indicated that melittin was the most active. Then, authors conducted an *in vivo* study using topical application in animal model such as mouse with infected burn and confirmed the results obtained *in vitro*.

S. aureus represents a significant pathogenic agent responsible for infections in both community and healthcare settings. The rise of MRSA has presented a substantial challenge to healthcare systems worldwide [153,154]. Choi et al.[143] examined *in vitro* the effect of BV and melittin on MRSA and sensitive *S. aureus*. In addition, other Gram-positive bacteria were also tested. *In vivo* experiments were performed on infected mice with *S. aureus*. MRSA tested strains were more sensitive to BV with MIC values ranging between 0.78 and 3.12 µg/mL. While *S. aureus* sensitive strains were less active and exhibited a higher MIC values (3.13-12.5 µg/mL). However, the i.p injection of BV into mice infected with HSA300 strain indicated that the use of 1.25 or 2.5 µg/kg BV at the time of infection did not show any protective effect. The same results were reported with the injection of BV one hour before the induction of infection. All mice died in the treated group. BV treatment seems to enhance the caused bacteremia. Moreover, no significant difference was observed between the control and the BV treated group in an induced *staphylococcal* skin infection model. Treatments were applied daily for 10 days. Melittin was administered alone and in combination with PLA2 at various concentrations. No significant effect was observed on the MRSA cell death. Purified and synthetized melittin at 5 mg/kg injected into infected mice one hour after bacterial injection did not kill all the treated mice and 50% survived.

Several mechanisms have been suggested to explain melittin antibacterial properties. One of the proposed mechanisms is related to alpha helical form and its effect on the surface membrane of the cells. This form is involved in the membrane disintegration caused by the insertion of the alpha helical form into the surface membrane causing the formation of pores affecting membrane permeability and leading to osmotic cytolysis [155,156]. According to several authors, melittin antimicrobial effect is similar to other antimicrobial peptides. Because its amphipathicity, melittin can integrate into the phospholipide bilayer when low concentrations are used. However, in higher concentration, melittin become homodimerized and can form pores, disrupt phospholipid and release Ca^{2+} [157-159].

B. burgdorferi is a spirochetal bacterial causing lime disease. Several factors can cause the transformation of *B. burgdorferi* in a defensive form. Unfortunately, the defensive forms were reported to possess a high resistance to the currently used treatment [160]. Melittin effects on the surface components of spirochetal organisms include the increasing bleeding of surface membrane and the modification of the outer envelope integrity. However, the DNA is not damaged, and analysis showed intact DNA. Melittin act also on *B. burgdorferi* motility at the beginning of the treatment [149,161].

In addition to melittin, BV antimicrobial activity was also attributed to PLA2. Both components act by producing pores in bacteria membrane causing their damage then their lyse [139,162,163]. The two compounds may form a complex by hetero-oligomerization, which indicate that the toxic effect may result on an enhanced activity due to the synergism effect of the two BV toxins. The formed complex is responsible of the rapid lyse of the bacteria. The combination of melittin to PLA2 can enhance its ability to bind to the outer membrane. This binding enables melittin oligomerization and pore formation on the bacterial membrane [164]. The described binding was explained by several authors [165,166] as a result of an electrostatic attraction between melittin basic amino acid residues and phosphate group of a key constituent the cell membrane, phosphatidylcholine.

Use of Bee Venom in the Biofilm Control

Antimicrobial peptides are a promising strategy to combat resistant strains [167, 168]. The development of antibiotic resistance within biofilms primarily results from the existence of dormant populations residing within the established film. Standard antibiotics cannot act actively on biofilm because they are effective on actively growing population. Therefore, the dormant population is not eliminated [169]. Antimicrobial peptides such as melittin can act on both active and dormant populations. They cause membrane disruption followed by the death of both populations. Recently, antimicrobial peptides were pointed to act using other mechanisms affecting protein synthesis and/or nucleic acid [170].

BV was evaluated for its antibacterial and antibiofilm effect on multidrug resistant bacteria (MDR) isolated from clinical specimens. Results indicated that MDR

Gram-positive bacteria were more sensitive to BV. BV sub-MICs values reduced biofilm formation of *S. aureus*, vancomycin resistant *S. aureus*, *S. hemolyticus*, *E. faecalis* and *P. aeruginosa* with reduction varying from 63.8% to 92% [171].

In the last recent years, biofilm was described for *Borrelia*. The aggregate forms were found to be highly resistant both *in vivo* of *in vitro* studies [172,173]. Sacarros et al.[161] investigated the effect of *A. meliffera* BV and melittin on the biofilm (surface attached and floating-aggregates) caused by *B. burgdorferi* and compared it to cefoperazone, doxycycline and daptomycin used alone or in combination. The tested antibiotics were recently reported to be effective against persistent forms. The treatment by cefoperazone, daptomycin and antibiotic combination was found to reduce viable and persister *spirochetes*. While doxycycline was not active on persisted forms. BV antibiofilm effect was tested at 100, 400 and 800 µg/mL. While melittin was tested at 50, 200 and 400 µg/mL. Stationary cultures were incubated for 7 days. Finally, viability was assessed through the utilization of the SYBR Green I/PI assay and direct counting methods. It was observed that BV led to a marked reduction in the number of viable cells across all tested concentrations when compared to daptomycin and the negative control. After 7 days incubation, BV above 400 µg/mL was found to be as effective as cefoperazone and the tested combination of antibiotics. Melittin significantly reduced *spirochetes* at all tested concentrations compared to doxycycline and negative control. Persistent forms were significantly reduced by melittin, which was more effective than BV and all the tested antibiotics alone or in combination [161].

BV and melittin were studied against planktonic and biofilm states of *S. aureus* methicillin-resistant MRSA [174]. Melittin was more effective compared to BV with MIC and MCB values of 6.7 and 26 µg/mL respectively. BV and melittin demonstrated a bactericidal synergism with oxacillin. BV and melittin did not affect MRSA enterotoxin production or release. They seem to cause cell distortion and disintegration associated to cytoplasm loss of content.

Melittin effect on the formation and viability of bacteria within biofilms was evaluated by several authors [151,175-177]. Melittin was effective against several strains such as *P. aeruginosa (P. aeruginosa* ATCC15442, *P. aeruginosa* ATCC27853, clinical strain and MDR *P. aeruginosa), E. coli* ATCC8739 and clinical strain, *K. pneumonia* and *A. baumannii*. Melittin was able to decrease biofilm formation in a dose and time-dependent manner [151,152,175-177]. In addition, melittin was more active compared to chloramphenicol, ampicillin and levofloxacin [151].

Beeswax and Beebread

Beeswax is a honeycomb raw material and a bee product that has an extensive usage area. It is a product used in many sectors such as coating agents in the food sector, health sector, cosmetics, soaps and creams, textiles, ornaments production, paint, and paper industry [178]. Beeswax is a functional natural substance preferred by manufacturers in recent years due to its rich nutrient content. Currently, beeswax is used in the food industry as a brightener, flavor retainer, and coating agent, in chewing gum, confectionery, chocolate, snacks, nuts, and fruit and vegetable products [179]. Regarding the studies on extending the shelf life of foods and investigating the antimicrobial resistance of beeswax, it has been seen that there are still insufficient numbers of studies using beeswax. These studies are shown in *Table 6* and *Table 7*.

Bee bread plays a critical role in human nutrition and is a valuable source of polyunsaturated fatty acids, which the human body cannot produce naturally. The ratio of chemical and nutritional components of bee bread; contains 5.91% water, 7.79% total lipid content, approximately 20% complete protein, 24-35% total carbohydrates, and various vitamins, minerals, and enzymes [180]. Bee bread is one of the bee products with high nutritional content. It is produced by anaerobic fermentation from bee pollen by means of several *Lactobacillus* spp. It possesses water, carbohydrates, free amino acids, proteins, lipids, fatty acids, vitamins, and bioactive compounds. Bee bread serves as the primary source of nutrition for both queen bees and young bees. The young bees that emerge from the pupa is fed with bee bread for the first 5 days [181,182]. Bee bread is a functional natural ingredient that has been preferred by producers recently with its rich nutritional content and bioactive properties. However, when we look at the studies on extending the shelf life of foods, it has been seen that there are very few studies using bee bread [183]. These studies were given in *Table 6* and *Table 7*.

Bee bread is created through the collection of pollen by bees, which they then process by adding honey and enzymes before storing it in honeycombs [184] *(Fig. 4)*.

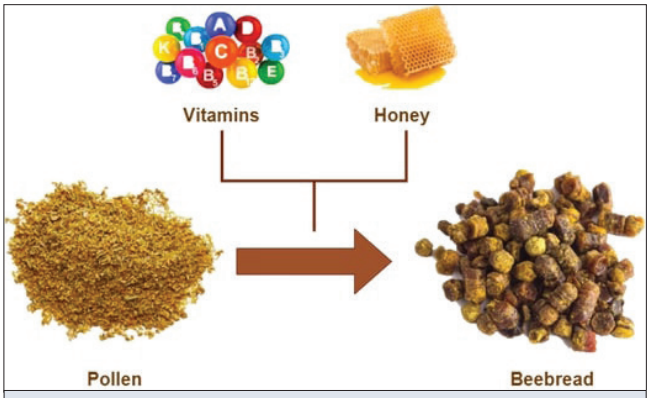

Fig 4. Bee bread production

Antimicrobial Properties of Beeswax, Bee Bread and Antibiotic Resistance

Ochoa et al.[185] evaluated the physicochemical and antimicrobial effects of microemulsified wax-based edible films. Beeswax was applied to the modified starch films as a 1% microemulsion, creating homogeneity in the edible films, and no change in thickness and opacity. The water vapor permeability, elasticity, and tensile strength exhibited a decline. In edible films treated with 1% wax, the activity of the combination of natural antimicrobials *(Rhizopus stolonifer*, *Salmonella saintpaul*, *Botrytis cinerea*, and *Colletotrichum gloeosporioides)* was inhibited. In the data obtained, it has been seen that beeswax can be used as a coating to increase the shelf life of fresh products without being felt by the consumers. In edible films, it offered a homogeneous surface, less water vapor permeability, and improved mechanical properties without a thickness or opacity that could negatively affect the preference for coated foods.

Meindrawan et al.[186] conducted an investigation to evaluate the extension of the shelf life of salak fruit *(Salacca zalacca)* using an edible film coating composed of glucomannan-wax-chitosan polymer. This edible coating, which consisted of glucomannan-wax-chitosan, led to a 10% reduction in the activity of *E. coli* and *S. aureus* compared to the control group. Additionally, it effectively curbed the rate of water vapor transfer and decreased the weight loss of salak fruit by 27% when compared to the control group. Salak fruit treated with glucomannan-wax-chitosan did not show any mold growth for 3 days at room temperature. Judging by the results in this test, the glucomannan-wax-chitosan edible coating successfully demonstrated antimicrobial activity on the lowest processed salak fruit, suggesting that similar results could probably be obtained from other fruits as well.

Disayathanoowat et al.[187] studied both bacterial and fungal viability in bee bread collected in the hive of two different honeybees, *Apis cerana*, and *Apis mellifera* in China. While a pH decrease occurred in the bee bread collected in the pack, it was noted in the data that while the bacterial population decreased, the fungal population did not change. In a concise time, there was a serious decrease in the number of bacteria in the bee bread accumulated in the hive. However, the amount of *Acinetobacter* was high. In the amount of fungus, Cladosporium genus was found in a large amount in bee bread. In addition, *Cladosporium* and various other filamentous fungi that survived in bee bread stored in the hive stimulated honeybees to conserve pollen by releasing organic acids. In this study, the microbial interactions of two honeybees in the food source were examined. It was determined that bee bread showed an antimicrobial resistance against bacteria but could not show an effect against fungi.

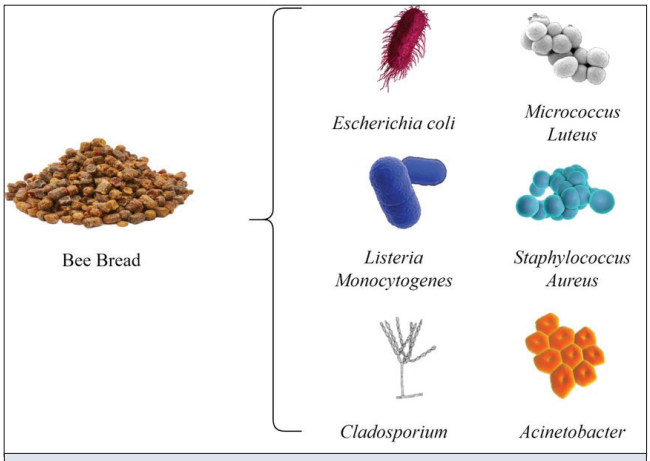

Fig 5. Antimicrobial properties of bee bread against some pathogens

In the study conducted by Kaya et al.[181], extracts of bee bread were prepared using three different solvents: methanol, distilled water, and a mixture of methanol and pure water. These extracts were then tested for their antibacterial effects against *L. monocytogenes, E. coli, M. luteus*, and *S. aureus*. The results indicated that all bee bread samples dissolved in methanol displayed effectiveness against *E. coli*. Similarly, bee bread samples dissolved in methanol and a methanol: pure water mixture were found to be effective against *L. monocytogenes*, except for two samples from the methanol-dissolved group, which did not show efficacy against *M. luteus.* It was established that all other samples, as well as those of bee bread dissolved in methanol, exhibited antibacterial activity against *S. aureus (Fig. 5)*.

In the study by Kowalski and Makarewicz [188] the functional properties of honey enriched with bee bread and propolis were investigated. The effects of enriched honey on total phenolic content, growth of microorganisms, and antioxidant activity were observed. It has been observed that honey enriched with bee products has antibacterial solid activity and exhibits strong antioxidant properties, especially against *E. coli*, but not all tested honey shows the same effect against *Micrococcus luteus*. It was stated that bee bread has the most important effect on antioxidant activity and the addition of 1% propolis has antibacterial activity. Studies have shown that fortifying honey with both bee bread and propolis is highly beneficial for its antioxidant and antimicrobial properties. Consequently, bee bread and propolis have the potential to serve as natural dietary supplements with robust antibacterial activity, particularly against *E. coli*, and high antioxidant properties.

Use of Beeswax and Bee Bread in Biofilm Control

Consumption of foodstuffs shortly after their production is generally not possible. Therefore, food should be packaged to preserve its quality and nutritional value

Table 6. *Antibacterial properties of the beeswax and bee bread*

Beeswax/Bee Bread Type	Applied Product	Targeted Microbe (Fungal/Bacterial)	Outcomes	Reference
Beeswax	Modified corn starches	*R. stolonifer, C. gloeosporioides, B. cinerea,* and *S. Saintpaul*	It has been observed that beeswax can be used as a coating	[185]
Glucomannan, chitosan, and beeswax	*Salacca zalacca*	*S. aureus, Escherichia coli*	Successfully demonstrated antimicrobial activity on beeswax-treated salak fruit	[186]
Apis mellifera and *Apis cerana* bee breads	-	*Acinetobacter, Cladosporium, Oxalis sp.* and *Coreopsis sp.*	It was determined that bee bread showed antimicrobial resistance against bacteria, but it was not effective against fungi	[187]
Bee Bread	-	*E.coli, L.monocytogenes, M.luteus,* and *S.aureus*	It was determined that bee bread showed antibacterial activity against all the factors mentioned	[181]
Bee Bread and Propolis	Honey	*E.coli* and *M.luteus*	Studies have shown that enriching bee bread and propolis with its antioxidant and antimicrobial properties is beneficial for honey	[188]

throughout its shelf life [189-191]. The most critical tasks of edible films and coatings; keeping oxygen, carbon dioxide, and lipid transfer in balance, delaying the loss of taste and aroma, and preserving antioxidants, antimicrobial substances, pigments, ions, and vitamins that inhibit browning reactions in the food, while prolonging the quality and shelf life [72].

Hromiš et al.[192] tested the antibacterial properties of chitosan and the antimicrobial effects of combining beeswax with cumin essential oil were examined, using *E. coli* and *S. aureus* as the targeted bacteria. The introduction of essential oil and beeswax led to alterations in the visual and sensory attributes of the pure chitosan film. The application of wax to the chitosan film expanded its coverage area by diminishing its susceptibility to ambient humidity, resistance to swelling at different pH levels, and water solubility. Moreover, the inclusion of wax into the chitosan film also resulted in a reduction in the water vapor transmission rate. In the group with the highest wax content, there was a noteworthy 7-fold reduction in water vapor permeability. These modified films demonstrated successful antimicrobial and antioxidant properties, which are highly advantageous characteristics for packaging applications.

Oliveira et al.[193] aimed to create biopolymer films that offer the best water vapor transmission rate with a biopolymeric coating hydrophobized with wax for postharvest storage of guavas. Biopolymeric coatings are highly efficient in maintaining the chemical and sensory qualities of fruits and vegetables, as they play a crucial part in preserving a multitude of nutrients during storage. In this context, beeswax was incorporated into the polymeric matrix as a hydrophobic agent at different proportions relative to the dry weight of the biopolymer. Among the tested biofilm variations, the one containing 10% wax yielded the most favorable outcomes in terms of the water vapor transmission rate. It was also very effective in delaying the loss of chlorophyll. An increase of 80% in elasticity values and a decrease of 15% in solubility indicated that its resistance to adverse environmental conditions increased. In the physicochemical analysis, the application of beeswax minimized weight loss and gave the fruits sufficient ripening opportunity for 15 days. Sensory analyzes performed at the end of the storage period of coated and uncoated guavas showed that fruits stored with wax-treated films achieved greater acceptability. Looking at all these data shows that the potential of the wax applied coating is quite high.

Hromiš et al.[194] beeswax in various proportions, together with chitosan, was applied to the collagen casings used in the production of sausages. FTIR spectra, Film thickness, and water vapor barrier properties were measured. As a result of the addition of beeswax at various rates to the chitosan layer, the film thickness increased to 112 µm in the sheath with 5 g of beeswax, 225 µm with 25 g of beeswax, and 83 µm in the collagen sheath in the control group. The thickness of the films resulting from the beeswax added in various proportions increased by approximately 25% to 63%. The water vapor barrier performance improved in parallel with the increasing amount of beeswax in the chitosan layer. It was measured as 130.71 g/m^2/24 h in the 5 g beeswax applied to film, 66.96 g/m^2/24 h in the 25 g beeswax applied to film, and 290.64 g/m^2/24 h in the control group. Beeswax application has shown that the laminated collagen-chitosan film has a significant potential to increase the water vapor barrier performance. With the beeswax application, the water vapor permeability rate decreased by up to 77%.

Wultańska et al.[195] study tested the antibacterial and antibiotic activity of bee bread against *Clostridioides*. Biofilm was cultured in titration plates. The MIC values of bee bread for *Clostridioides* were adjusted as 50 mg/L, 100 mg/L, and 200 mg/L respectively. Bee bread affected biofilm formation at 200 mg/L and 100 mg/L concentrations. At the same time, bee bread increased the adhesion of *Clostridioides*. Bee bread did not affect biomass formation. In the data obtained, it was observed that bee bread was active against the *Clostridioides* strains.

Table 7. *Beeswax and bee bread anti-biofilm properties*

Beeswax/bee bread type	Applied product	Targeted effect	Outcomes	References
Chitosan, caraway essential oil and beeswax	Petri dishes	Antimicrobial activity against *E. coli* and *S. aureus*	The combined use of chitosan, essential oil, and beeswax gave positive results in the use of biofilm	[192]
Brasil beeswax	'Paluma' variety guava	Creating biopolymer films that offer the best water vapor transmission rate by applying wax	A positive effect was obtained on the water vapor transmission rate by using beeswax	[193]
Chitosan, caraway essential oil and beeswax	Sausage coating	Film thickness and water vapor barrier properties were determined	Positive results were obtained. There was a decrease in water vapor transmission	[194]
Bee bread (a combination of flower pollen, honey, royal jelly, and bee enzymes)	Biofilm plates	*Clostridioides*	Bee bread was found to be active against *Clostridioides* strains	[195]

Conclusion and Future Perspectives

As a result of this review, it is seen that bee products have a significant potential for biofilm control along with their antimicrobial properties. Bee products such as propolis, royal jelly, and honey contain antimicrobial compounds that control pathogenic microorganisms. In addition, these products can prevent biofilm formation. Biofilm is a structure formed due to microorganisms attaching to surfaces and creating a film. Biofilm formation can increase antimicrobial resistance and make infections more difficult to treat. However, bee products can inhibit biofilm formation and therefore help control microorganisms.

In summary, bee products represent a significant alternative approach for addressing antimicrobial resistance and managing biofilms. These products can play a crucial role in controlling pathogenic microorganisms and mitigating resistance. Nevertheless, additional research is warranted to ascertain their efficacy and determine appropriate dosages. Notably, bee products exhibit fewer adverse effects and are environmentally sustainable due to their natural origins. Thus, the utilization of bee products in the battle against antimicrobial resistance stands to offer substantial benefits for human and animal health alike.

Highlight Keypoints

· Bee products can offer natural alternatives to control drug-resistant infections.

· Compounds obtained from bee products have the potential to inhibit biofilm formation in bacterial infections.

· Natural bee products can serve as a resource to support innovative treatments to combat antimicrobial resistance.

· More research is needed to explore the full health potential of bee products.

· One Health approach, involving collaboration between human and veterinary medicine, can optimize the use of bee products in combating biofilm-related challenges.

Declarations

Competing Interests: There is no conflict of interest.

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References

1. Beceiro A, Tomás M, Bou G: Antimicrobial resistance and virulence: A successful or deleterious association in the bacterial world? *Clin Microbiol Rev,* 26 (2): 185-230, 2013. DOI: 10.1128/CMR.00059-12

2. Nadeem SF, Gohar UF, Tahir SF, Mukhtar H, Pornpukdeewattana S, Nukthamna P, Moula Ali AM, Bavisetty SCB, Massa S: Antimicrobial resistance: More than 70 years of war between humans and bacteria. *Crit Rev Microbiol,* 46 (5): 578-599, 2020. DOI: 10.1080/1040841X.2020.1813687

3. Singer AC, Kirchhelle C, Roberts AP: (Inter)nationalising the antibiotic research and development pipeline. *Lancet Infect Dis,* 20 (2):e54-e62, 2020. DOI: 10.1016/S1473-3099(19)30552-3

4. Pulingam T, Parumasivam T, Gazzali AM, Sulaiman AM, Chee JY, Lakshmanan M, Chin CF, Sudesh K: Antimicrobial resistance: Prevalence, economic burden, mechanisms of resistance and strategies to overcome. *Eur J Pharm Sci,* 170:106103, 2022. DOI: 10.1016/j.ejps.2021.106103

5. Frieri M, Kumar K, Boutin A: Antibiotic resistance. *J Infect Public Health,* 10 (4): 369-378, 2017. DOI: 10.1016/j.jiph.2016.08.007

6. de Kraker MEA, Stewardson AJ, Harbarth S: Will 10 million people die a year due to antimicrobial resistance by 2050? *PLoS Med,* 13:e1002184, 2016. DOI: 10.1371/journal.pmed.1002184

7. Health Organization W: Antimicrobial resistance: Global report on surveillance. 2014. https://www.who.int/publications/i/item/9789241564748, *Accessed:* 02.06.2023

8. Abe K, Nomura N, Suzuki S: Biofilms: Hot spots of horizontal gene transfer (HGT) in aquatic environments, with a focus on a new HGT mechanism. *FEMS Microbiol Ecol,* 96 (5): 1-12, 2020. DOI: 10.1093/femsec/fiaa031

9. Condinho M, Carvalho B, Cruz A, Pinto SN, Arraiano CM, Pobre V: The role of RNA regulators, quorum sensing and c-di-GMP in bacterial biofilm formation. *FEBS Open Bio*, 13 (6): 975-991, 2022. DOI: 10.1002/2211-5463.13389

10. Kilic T, Bali EB: Biofilm control strategies in the light of biofilm-forming microorganisms. *World J Microbiol Biotechnol,* 39 (5): 131, 2023. DOI: 10.1007/s11274-023-03584-6

11. Asma ST, Imre K, Morar A, Imre M, Acaroz U, Shah SRA, Hussain SZ, Arslan-Acaroz D, Istanbullugil FR, Madani K, Athanassiou C, Atanasoff A, Morar D, Herman V, Zhu K: Natural strategies as potential weapons against bacterial biofilms. *Life,* 12 (10):1618, 2022. DOI: 10.3390/life12101618

12. Asma ST, Imre K, Morar A, Herman V, Acaroz U, Mukhtar H, Arslan-Acaroz D, Shah SRA, Gerlach R: An overview of biofilm formation–combating strategies and mechanisms of action of antibiofilm agents. *Life,* 12 (8):1110, 2022. DOI: 10.3390/

life12081110

13. Olaitan PB, Adeleke OE, Ola IO: Honey: A reservoir for microorganisms and an inhibitory agent for microbes. *Afr Health Sci,* 7 (3): 159-165, 2007.

14. İstanbullugil FR, Taş N, Acaröz U, Arslan-Acaroz D, Çakmak Ö, Evrenkaya S, Gürler Z: A review on the antimicrobial effect of honey on *Salmonella* and *Listeria monocytogenes*: Recent studies. *MJAVL,* 13 (2): 210-225, 2023. DOI: 10.53518/mjavl.1379465

15. Çakmak Ö, Taş N, Acaröz U, Arslan-Acaröz D, İstanbullugil FR, Evrenkaya S, Gürler Z: Inhibitory effects of honey on *Staphylococcus aureus* and *Escherichia coli*: A current review. *Bull Vet Pharmacol Toxicol Assoc,* 14 (3): 133-148, 2023. DOI: 10.38137/vftd.1385968

16. Nzeako BC, Al-Namaani F: The antibacterial activity of honey on *Helicobacter pylori*. *Sultan Qaboos Univ Med J,* 6 (2): 71-76, 2006.

17. Rao PV, Krishnan KT, Salleh N, Gan SH: Biological and therapeutic effects of honey produced by honey bees and stingless bees: A comparative review. *Rev Bras Farmacogn,* 26 (5): 657-664, 2016. DOI: 10.1016/j.bjp.2016.01.012

18. Nobar RS, Teli AAS, Shahryar HA, Abadi HRKS: Impact of various carbohydrate sources on functional attributes, colony population, feed intake, and quality of honey produced by the honeybee. *Kafkas Univ Vet Fak Derg,* 24 (4): 503-510, 2018. DOI: 10.9775/kvfd.2018.19445

19. FAO; WHO: Codex Alimentarius-International Food Standards: Standard for honey CXS 12-1981. Adopted in 1981, amended in 2019; FAO: Rome, Italy; WHO: Genewa, Switzerland, 2019.

20. Anonymus: https://www.eumonitor.eu/9353000/1/j4nvhdlglbmvdzx_j9vvik7m1c3gyxp/vj36rp89slx9; *Accessed:* 15.05.2023.

21. Ranneh Y, Akim AM, Hamid HA, Khazaai H, Fadel A, Zakaria ZA, Albujja M, Bakar MFA: Honey and its nutritional and anti-inflammatory value. *BMC Complement Med Ther,* 21 (1):30, 2021. DOI: 10.1186/s12906-020-03170-5

22. Alvarez-Suarez JM, Tulipani S, Romandini S, Bertoli E, Battino M: Contribution of honey in nutrition and human health: A review. *Med J Nutrition Metab,* 3 (1): 15-23, 2010. DOI: 10.1007/s12349-009-0051-6

23. Bauer L, Kohlich A, Hirschwehr R, Siemann U, Ebner H, Scheiner O, Kraft D, Ebner C: Food allergy to honey: Pollen or bee products? Characterization of allergenic proteins in honey by means of immunoblotting. *J Allergy Clin Immunol,* 97 (1): 65-73, 1996. DOI: 10.1016/s0091-6749(96)70284-1

24. Karakaya G, Kalyoncu AF: Honey allergy in adult allergy practice. *Allergol Immunopathol,* 27 (5): 271-272, 1999.

25. Gleiter RA, Horn H, Isengard HD: Influence of type and state of crystallisation on the water activity of honey. *Food Chem,* 96 (3): 441-445, 2006. DOI: 10.1016/j.foodchem.2005.03.051

26. Chen C: Relationship between water activity and moisture content in floral honey. *Foods,* 8 (1):30, 2019. DOI: 10.3390/foods8010030

27. French VM, Cooper RA, Molan PC: The antibacterial activity of honey against coagulase-negative staphylococci. *J Antimicrob Chemother,* 56 (1): 228-231, 2005. DOI: 10.1093/jac/dki193

28. Adcock D: The effect of catalase on the inhibine and peroxide values of various honeys. *J Apic Res,* 1 (1): 38-40, 1962. DOI: 10.1080/00218839.1962.11100047

29. Dustmann JH: Antibacterial effect of honey. *Apiacta,* 14 (1): 7-11, 1979.

30. Imlay JA, Linn S: Bimodal pattern of killing of DNA-repair-defective or anoxically grown *Escherichia coli* by hydrogen peroxide. *J Bacteriol,* 166 (2): 519-527, 1986. DOI: 10.1128/jb.166.2.519-527.1986

31. Brudzynski K, Abubaker K, St-Martin L, Castle A: Re-examining the role of hydrogen peroxide in bacteriostatic and bactericidal activities of honey. *Front Microbiol,* 2:213, 2011. DOI: 10.3389/fmicb.2011.00213

32. White JW: Composition of American honeys. 124, U.S. Department of Agriculture, 1962.

33. Molan PC: The antibacterial activity of honey. *Bee World,* 73 (1): 5-28, 1992. DOI: 10.1080/0005772x.1992.11099109

34. Yupanqui Mieles J, Vyas C, Aslan E, Humphreys G, Diver C, Bartolo P: Honey: An advanced antimicrobial and wound healing biomaterial for tissue engineering applications. *Pharmaceutics,* 14 (8):1663, 2022. DOI: 10.3390/pharmaceutics14081663

35. Tan HT, Rahman RA, Gan SH, Halim AS, Hassan SA, Sulaiman SA, Kirnpal-Kaur B: The antibacterial properties of Malaysian tualang honey against wound and enteric microorganisms in comparison to manuka honey. *BMC Complement Altern Med,* 9:34, 2009. DOI: 10.1186/1472-6882-9-34

36. Estevinho L, Pereira AP, Moreira L, Dias LG, Pereira E: Antioxidant and antimicrobial effects of phenolic compounds extracts of Northeast Portugal honey. *Food Chem Toxicol,* 46 (12): 3774-3779, 2008. DOI: 10.1016/j.fct.2008.09.062

37. Wahdan HA: Causes of the antimicrobial activity of honey. *Infection,* 26 (1): 26-31, 1998. DOI: 10.1007/BF02768748

38. Kwakman PHS, Velde AAt, de Boer L, Speijer D, Christina Vandenbroucke-Grauls MJ, Zaat SAJ: How honey kills bacteria. *FASEB J,* 24 (7): 2576-2582, 2010. DOI: 10.1096/fj.09-150789

39. Weigel KU, Opitz T, Henle T: Studies on the occurrence and formation of 1,2-dicarbonyls in honey. *Eur Food Res Technol,* 218 (2): 147-151, 2004. DOI: 10.1007/s00217-003-0814-0

40. Suortti T, Mälkki Y: Antimicrobial activities of heated glucose and fructose solutions and their elucidation by high performance liquid chromatography. *Food Chem,* 15 (3): 165-173, 1984. DOI: 10.1016/0308-8146(84)90001-3

41. Mavric E, Wittmann S, Barth G, Henle T: Identification and quantification of methylglyoxal as the dominant antibacterial constituent of Manuka (*Leptospermum scoparium*) honeys from New Zealand. *Mol Nutr Food Res,* 52 (4): 483-489, 2008. DOI: 10.1002/mnfr.200700282

42. Percival SL, Thomas J, Linton S, Okel T, Corum L, Slone W: The antimicrobial efficacy of silver on antibiotic-resistant bacteria isolated from burn wounds. *Int Wound J,* 9 (5): 488-493, 2012. DOI: 10.1111/j.1742-481X.2011.00903.x

43. Percival SL, McCarty SM: Silver and alginates: Role in wound healing and biofilm control. *Adv Wound Care,* 4 (7): 407-414, 2015. DOI: 10.1089/wound.2014.0541

44. Bowler PG, Duerden BI, Armstrong DG: Wound microbiology and associated approaches to wound management. *Clin Microbiol Rev,* 14, 244-269, 2001. DOI: 10.1128/CMR.14.2.244-269.2001

45. Liu MY, Cokcetin NN, Lu J, Turnbull L, Carter DA, Whitchurch CB, Harry EJ: Rifampicin-manuka honey combinations are superior to other antibiotic-manuka honey combinations in eradicating *Staphylococcus aureus* biofilms. *Front Microbiol,* 8:2653, 2017. DOI: 10.3389/fmicb.2017.02653

46. Saginur R, Stdenis M, Ferris W, Aaron SD, Chan F, Lee C, Ramotar K: Multiple combination bactericidal testing of staphylococcal biofilms from implant-associated infections. *Antimicrob Agents Chemother,* 50 (1): 55-61, 2006. DOI: 10.1128/AAC.50.1.55-61.2006

47. Fernandes P: Fusidic acid: A bacterial elongation factor inhibitor for the oral treatment of acute and chronic staphylococcal infections. *Cold Spring Harb Perspect Med,* 6 (1):a025437, 2016. DOI: 10.1101/cshperspect.a025437

48. Kreikemeyer B, Oehmcke S, Nakata M, Hoffrogge R, Podbielski A: *Streptococcus pyogenes* fibronectin-binding protein F2: Expression profile, binding characteristics, and impact on eukaryotic cell interactions. *J Biol Chem,* 279 (16): 15850-15859, 2004. DOI: 10.1074/jbc.M313613200

49. Maddocks SE, Lopez MS, Rowlands RS, Cooper RA: Manuka honey inhibits the development of *Streptococcus pyogenes* biofilms and causes reduced expression of two fibronectin binding proteins. *Microbiology,* 158 (3): 781-790, 2012. DOI: 10.1099/mic.0.053959-0

50. Sojka M, Valachova I, Bucekova M, Majtan J: Antibiofilm efficacy of honey and bee-derived defensin-1 on multispecies wound biofilm. *J Med Microbiol,* 65 (4): 337-344, 2016. DOI: 10.1099/jmm.0.000227

51. Almuhayawi MS: Propolis as a novel antibacterial agent. *Saudi J Biol Sci,* 27 (11): 3079-3086, 2020. DOI: 10.1016/j.sjbs.2020.09.016

52. Castaldo S, Capasso F: Propolis, an old remedy used in modern medicine. *Fitoterapia,* 73 (Suppl.-1): S1-S6, 2002. DOI: 10.1016/s0367-326x(02)00185-5

53. Anjum SI, Ullah A, Khan KA, Attaullah M, Khan H, Ali H, Bashir MA, Tahir M, Ansari MJ, Ghramh HA, Adgaba N, Dash CK: Composition and functional properties of propolis (bee glue): A review. *Saudi J Biol Sci,* 26 (7): 1695-1703, 2019. DOI: 10.1016/j.sjbs.2018.08.013

54. Segueni N, Boutaghane N, Asma ST, Tas N, Acaroz U, Arslan-Acaroz D, Shah SRA, Abdellatieff HA, Akkal S, Peñalver R, Nieto G: Review on propolis applications in food preservation and active packaging. *Plants,* 12 (8):1654,, 2023. DOI: 10.3390/plants12081654

55. Huang S, Zhang CP, Wang K, Li GQ, Hu FL: Recent advances in the chemical composition of propolis. *Molecules,* 19 (12): 19610-19632, 2014. DOI: 10.3390/molecules191219610

56. Contieri LS, de Souza Mesquita LM, Sanches VL, Viganó J, Martinez J, da Cunha

DT, Rostagno MA: Standardization proposal to quality control of propolis extracts commercialized in Brazil: A fingerprinting methodology using a UHPLC-PDA-MS/MS approach. *Food Res Int,* 161:111846, 2022. DOI: 10.1016/j.foodres.2022.111846

57. Wagh VD: Propolis: A wonder bees product and its pharmacological potentials. *Adv Pharmacol Sci,* 2013:308249, 2013. DOI: 10.1155/2013/308249

58. Uzel A, Sorkun K, Onçağ O, Cogülu D, Gençay O, Salih B: Chemical compositions and antimicrobial activities of four different Anatolian propolis samples. *Microbiol Res,* 160 (2): 189-195, 2005. DOI: 10.1016/j.micres.2005.01.002

59. Pobiega K, Kraśniewska K, Przybył JL, Bączek K, Żubernik J, Witrowa-Rajchert D, Gniewosz M: Growth biocontrol of foodborne pathogens and spoilage microorganisms of food by Polish propolis extracts. *Molecules,* 24 (16):2965, 2019. DOI: 10.3390/molecules24162965

60. Burgut A: Effects of propolis extracts on biogenic amine production by foodborne pathogens. *Kafkas Univ Vet Fak Derg*, 26 (2): 193-200, 2019. DOI: 10.9775/kvfd.2019.22718

61. Przybyłek I, Karpiński TM: Antibacterial properties of propolis. *Molecules,* 24 (11):2047, 2019. DOI: 10.3390/molecules24112047

62. Afrouzan H, Tahghighi A, Zakeri S, Es-haghi A: Chemical composition and antimicrobial activities of Iranian propolis. *Iran Biomed J,* 22 (1): 50-65, 2018.

63. Veiga RS, De Mendonça S, Mendes PB, Paulino N, Mimica MJ, Lagareiro Netto AA, Lira IS, López BGC, Negrão V, Marcucci MC: Artepillin C and phenolic compounds responsible for antimicrobial and antioxidant activity of green propolis and *Baccharis dracunculifolia* DC. *J Appl Microbiol,* 122 (4): 911-920, 2017. DOI: 10.1111/jam.13400

64. Wojtyczka RD, Dziedzic A, Idzik D, Kępa M, Kubina R, Kabała-Dzik A, Smoleń-Dzirba J, Stojko J, Sajewicz M, Wąsik TJ: Susceptibility of *Staphylococcus aureus* clinical isolates to propolis extract alone or in combination with antimicrobial drugs. *Molecules,* 18 (8): 9623-9640, 2013. DOI: 10.3390/molecules18089623

65. Nayaka HB, Londonkar RL, Umesh MK, Tukappa A: Antibacterial attributes of apigenin, isolated from *Portulaca oleracea* L. *Int J Bacteriol,* 2014:175851, 2014. DOI: 10.1155/2014/175851

66. Kharsany K, Viljoen A, Leonard C, van Vuuren S: The new buzz: Investigating the antimicrobial interactions between bioactive compounds found in South African propolis. *J Ethnopharmacol,* 238:111867, 2019. DOI: 10.1016/j.jep.2019.111867

67. Eumkeb G, Chukrathok S: Synergistic activity and mechanism of action of ceftazidime and apigenin combination against ceftazidime-resistant *Enterobacter cloacae*. *Phytomedicine,* 20 (3-4): 262-269, 2013. DOI: 10.1016/j.phymed.2012.10.008

68. Guzman JD: Natural cinnamic acids, synthetic derivatives and hybrids with antimicrobial activity. *Molecules,* 19 (12): 19292-19349, 2014. DOI: 10.3390/molecules191219292

69. Vasconcelos NG, Croda J, Simionatto S: Antibacterial mechanisms of cinnamon and its constituents: A review. *Microb Pathog,* 120, 198-203, 2018. DOI: 10.1016/j.micpath.2018.04.036

70. Chew SC, Yang L: Biofilms. Encyclopedia of Food and Healthed., 407-415, Elsevier, 2016.

71. De Marco S, Piccioni M, Pagiotti R, Pietrella D: Antibiofilm and antioxidant activity of propolis and bud poplar resins versus *Pseudomonas aeruginosa*. *Evid Based Complement Alternat Med,* 2017:5163575, 2017. DOI: 10.1155/2017/5163575

72. Song X, Xia YX, He ZD, Zhang HJ: A review of natural products with anti-biofilm activity. *Curr Org Chem,* 22 (8): 789-817, 2018. DOI: 10.2174/1385272821666170620110041

73. Pippi B, Lana AJD, Moraes RC, Güez CM, Machado M, de Oliveira LFS, Lino von Poser G, Fuentefria AM: *In vitro* evaluation of the acquisition of resistance, antifungal activity and synergism of Brazilian red propolis with antifungal drugs on *Candida* spp. *J Appl Microbiol,* 118 (4): 839-850, 2015. DOI: 10.1111/jam.12746

74. Ong TH, Chitra E, Ramamurthy S, Siddalingam RP, Yuen KH, Ambu SP, Davamani F: Correction: Chitosan-propolis nanoparticle formulation demonstrates anti-bacterial activity against *Enterococcus faecalis* biofilms. *PLoS One,* 12 (4):e0176629, 2017. DOI: 10.1371/journal.pone.0176629

75. Capoci IRG, Bonfim-Mendonça PdS, Arita GS, Pereira RRdA, Consolaro MEL, Bruschi ML, Negri M, Svidzinski TIE: Propolis is an efficient fungicide and inhibitor of biofilm production by vaginal *Candida albicans*. *Evid Based Complement Alternat Med,* 2015:287693, 2015. DOI: 10.1155/2015/287693

76. Tobaldini-Valerio FK, Bonfim-Mendonça PS, Rosseto HC, Bruschi ML, Henriques M, Negri M, Silva S, Svidzinski TI: Propolis: A potential natural product to fight *Candida* species infections. *Future Microbiol,* 11:1035-1046, 2016. DOI: 10.2217/fmb-2015-0016

77. Ożarowski M, Karpiński TM, Alam R, Łochyńska M: Antifungal properties of chemically defined propolis from various geographical regions. *Microorganisms,* 10 (2):364, 2022. DOI: 10.3390/microorganisms10020364

78. Stähli A, Schröter H, Bullitta S, Serralutzu F, Dore A, Nietzsche S, Milia E, Sculean A, Eick S: *In vitro* activity of propolis on oral microorganisms and biofilms. *Antibiotics (Basel),* 10 (9):1045, 2021. DOI: 10.3390/antibiotics10091045

79. Barros ILE, Veiga FF, de Castro-Hoshino LV, Souza M, Malacrida AM, Diniz BV, Dos Santos RS, Bruschi ML, Baesso ML, Negri M, Svidzinski TIE: Performance of two extracts derived from propolis on mature biofilm produced by *Candida albicans*. *Antibiotics (Basel),* 12 (1):72, 2022. DOI: 10.3390/antibiotics12010072

80. Oryan A, Alemzadeh E, Moshiri A: Potential role of propolis in wound healing: Biological properties and therapeutic activities. *Biomed Pharmacother,* 98, 469-483, 2018. DOI: 10.1016/j.biopha.2017.12.069

81. Kazemi F, Divsalar A, Saboury AA, Seyedarabi A: Propolis nanoparticles prevent structural changes in human hemoglobin during glycation and fructation. *Colloids Surf B Biointerfaces,* 177, 188-195, 2019. DOI: 10.1016/j.colsurfb.2019.01.046

82. Afrasiabi S, Pourhajibagher M, Chiniforush N, Bahador A: Propolis nanoparticle enhances the potency of antimicrobial photodynamic therapy against *Streptococcus* mutans in a synergistic manner. *Sci Rep,* 10 (1):15560, 2020. DOI: 10.1038/s41598-020-72119-y

83. Moukarab D: Evaluation of antimicrobial activity of manually agitate (nano-chitosan and nano- propolis) against *Enterococcus faecalis* in comparison with sodium hypochlorite: An *in-vitro* study. *Egypt Dent J,* 66 (1): 587-596, 2020. DOI: 10.21608/edj.2020.79132

84. de Mélo Silva IS, do Amorim Costa Gaspar LM, Rocha AMO, da Costa LP, Tada DB, Franceschi E, Padilha FF: Encapsulation of red propolis in polymer nanoparticles for the destruction of pathogenic biofilms. *AAPS PharmSciTech,* 21 (2):49, 2020. DOI: 10.1208/s12249-019-1576-8

85. Alfarrayeh I, Fekete C, Gazdag Z, Papp G: Propolis ethanolic extract has double-face *in vitro* effect on the planktonic growth and biofilm formation of some commercial probiotics. *Saudi J Biol Sci,* 28 (1): 1033-1039, 2021. DOI: 10.1016/j.sjbs.2020.11.047

86. Fernández-Calderón MC, Hernández-González L, Gómez-Navia C, Blanco-Blanco MT, Sánchez-Silos R, Lucio L, Pérez-Giraldo C: Antifungal and anti-biofilm activity of a new Spanish extract of propolis against *Candida glabrata*. *BMC Complement Med Ther,* 21 (1):147, 2021. DOI: 10.1186/s12906-021-03323-0

87. Santos LM, Rodrigues DM, Kalil MA, Azevedo V, Meyer R, Umsza-Guez MA, Machado BA, Seyffert N, Portela RW: Activity of ethanolic and supercritical propolis extracts in *Corynebacterium pseudotuberculosis* and its associated biofilm. *Front Vet Sci,* 8:700030, 2021. DOI: 10.3389/fvets.2021.700030

88. Prawiro SR, Dewi AAS, Diniayuningrum A, Maghfiroh DO, Nurdiana N, Endharti AT, Rahardjo B: Propolis ethanolic extract influenced biofilm formation and gene expression level of EFG1 in *Candida albicans*. *AIP Conf Proc,* 2353 (1): 1-6, 2021. DOI: 10.1063/5.0052928

89. Yuan J, Yuan W, Guo Y, Wu Q, Wang F, Xuan H: Anti-biofilm activities of Chinese poplar propolis essential oil against *Streptococcus* mutans. *Nutrients,* 14 (16):3290, 2022. DOI: 10.3390/nu14163290

90. Irvine P: Can propolis mouthwash inhibit the growth of *Streptococcus* mutans in a biofilm? *BDJ Team,* 10 (1): 14-15, 2023. DOI: 10.1038/s41407-023-1707-7

91. Al-safi SM: Synergistic effect of propolis and antibiotics on the *Salmonella* Typhi TY21. *KJVS,* 4 (1): 118-127, 2013.

92. Lavigne J-P, Ranfaing J, Dunyach-Rémy C, Sotto A: Synergistic effect of propolis and antibiotics on uropathogenic *Escherichia coli*. *Antibiotics (Basel),* 9 (11):739, 2020. DOI: 10.3390/antibiotics9110739

93. Regueira MS, Tintino SR, da Silva ARP, Costa MdS, Boligon AA, Matias EFF, de Queiroz Balbino V, Menezes IRA, Melo Coutinho HD: Seasonal variation of Brazilian red propolis: Antibacterial activity, synergistic effect and phytochemical screening. *Food Chem Toxicol,* 107, 572-580, 2017. DOI: 10.1016/j.fct.2017.03.052

94. Onlen Y, Tamer C, Oksuz H, Duran N, Altug ME, Yakan S: Comparative trial of different anti-bacterial combinations with propolis and ciprofloxacin on *Pseudomonas* keratitis in rabbits. *Microbiol Res,* 162 (1): 62-68, 2007. DOI: 10.1016/j.micres.2006.07.004

95. Al-Ani I, Zimmermann S, Reichling J, Wink M: Antimicrobial activities of european propolis collected from various geographic origins alone and in combination

with antibiotics. *Medicines (Basel),* 5 (1):2, 2018. DOI: 10.3390/medicines5010002

96. Fernandes Júnior A, Balestrin EC, Betoni JEC, Orsi RdO, da Cunha MdLRdS, Montelli AC: Propolis: Anti-*Staphylococcus aureus* activity and synergism with antimicrobial drugs. *Mem Inst Oswaldo Cruz,* 100 (5): 563-566, 2005. DOI: 10.1590/s0074-02762005000500018

97. Ghosh S, Jeon H, Jung C: Foraging behaviour and preference of pollen sources by honey bee *(Apis mellifera)* relative to protein contents. *J. Ecol. Environ.* 44 (1):4, 2020. DOI: 10.1186/s41610-020-0149-9

98. Thakur M, Nanda V: Composition and functionality of bee pollen: A review. *Trends Food Sci Technol,* 98, 82-106, 2020. DOI: 10.1016/j.tifs.2020.02.001

99. Denisow B, Denisow-Pietrzyk M: Biological and therapeutic properties of bee pollen: A review. *J Sci Food Agric,* 96 (13): 4303-4309, 2016. DOI: 10.1002/jsfa.7729

100. Alimoglu G, Guzelmeric E, Yuksel PI, Celik C, Deniz I, Yesilada E: Monofloral and polyfloral bee pollens: Comparative evaluation of their phenolics and bioactivity profiles. *LWT,* 142:110973, 2021. DOI: 10.1016/j.lwt.2021.110973

101. Komosinska-Vassev K, Olczyk P, Kaźmierczak J, Mencner L, Olczyk K: Bee pollen: Chemical composition and therapeutic application. *Evid Based Complement Alternat Med,* 2015:297425, 2015. DOI: 10.1155/2015/297425

102. Khalifa SAM, Elashal MH, Yosri N, Du M, Musharraf SG, Nahar L, Sarker SD, Guo Z, Cao W, Zou X, Abd El-Wahed AA, Xiao J, Omar HA, Hegazy MF, El-Seedi HR: Bee pollen: Current status and therapeutic potential. *Nutrients,* 13 (6):1876, 2021. DOI: 10.3390/nu13061876

103. Ali A, Paramanya A, Poojari P, Arslan-Acaroz D, Acaroz U, Kostić AŽ: The utilization of bee products as a holistic approach to managing polycystic ovarian syndrome-related infertility. *Nutrients,* 15 (5):1165, 2023. DOI: 10.3390/nu15051165

104. Elmaidomy AH, Shady NH, Abdeljawad KM, Elzamkan MB, Helmy HH, Tarshan EA, Adly AN, Hussien YH, Sayed NG, Zayed A, Abdelmohsen UR: Antimicrobial potentials of natural products against multidrug resistance pathogens: A comprehensive review. *RSC Adv,* 12 (45): 29078-29102, 2022. DOI: 10.1039/d2ra04884a

105. Soares de Arruda VA, Vieria dos Santos A, Figueiredo Sampaio D, da Silva Araújo E, de Castro Peixoto AL, Estevinho LM, de Almeida-Muradian LB: Brazilian bee pollen: Phenolic content, antioxidant properties and antimicrobial activity. *J Apic Res,* 60 (5): 775-783, 2021. DOI: 10.1080/00218839.2020.1840854

106. Didaras NA, Karatasou K, Dimitriou TG, Amoutzias GD, Mossialos D: Antimicrobial activity of bee-collected pollen and beebread: State of the art and future perspectives. *Antibiotics (Basel),* 9 (11):811, 2020. DOI: 10.3390/antibiotics9110811

107. Elsalam EA, Foda HS, Abdel-Aziz MS, Abd El-Hady FK: Antioxidant and antimicrobial activities of Egyptian bee pollen. *Middle East J Appl Sci,* 8 (4): 1248-1255, 2018.

108. Suriyatem R, Auras RA, Intipunya P, Rachtanapun P: Predictive mathematical modeling for EC50 calculation of antioxidant activity and antibacterial ability of Thai bee products. *J Basic Appl Pharm Sci*, 7 (9): 127-133, 2017. DOI: 10.7324/japs.2017.70917

109. Epand RM, Walker C, Epand RF, Magarvey NA: Molecular mechanisms of membrane targeting antibiotics. *Biochim Biophys Acta,* 1858, 980-987, 2016. DOI: 10.1016/j.bbamem.2015.10.018

110. Bakour M, Laaroussi H, Ousaaid D, Oumokhtar B, Lyoussi B: Antioxidant and antibacterial effects of pollen extracts on human multidrug-resistant pathogenic bacteria. *J Food Qual,* 2021: 5560182, 2021. DOI: 10.1155/2021/5560182

111. Pełka K, Otłowska O, Worobo RW, Szweda P: Bee bread exhibits higher antimicrobial potential compared to bee pollen. *Antibiotics (Basel),* 10 (2):125, 2021. DOI: 10.3390/antibiotics10020125

112. Liu M-H, Otsuka N, Noyori K, Shiota S, Ogawa W, Kuroda T, Hatano T, Tsuchiya T: Synergistic effect of kaempferol glycosides purified from *Laurus nobilis* and fluoroquinolones on methicillin-resistant *Staphylococcus aureus*. *Biol Pharm Bull,* 32 (3): 489-492, 2009. DOI: 10.1248/bpb.32.489

113. Hariri BM, McMahon DB, Chen B, Adappa ND, Palmer JN, Kennedy DW, Lee RJ: Plant flavones enhance antimicrobial activity of respiratory epithelial cell secretions against *Pseudomonas aeruginosa*. *PLoS One,* 12 (9):e0185203, 2017. DOI: 10.1371/journal.pone.0185203

114. Daglia M: Polyphenols as antimicrobial agents. *Curr Opin Biotechnol,* 23 (2): 174-181, 2012. DOI: 10.1016/j.copbio.2011.08.007

115. Vestby LK, Grønseth T, Simm R, Nesse LL: Bacterial biofilm and its role in the pathogenesis of disease. *Antibiotics (Basel),* 9 (2):59, 2020. DOI: 10.3390/antibiotics9020059

116. Schuh CMAP, Aguayo S, Zavala G, Khoury M: Exosome-like vesicles in *Apis mellifera* bee pollen, honey and royal jelly contribute to their antibacterial and pro-regenerative activity. *J Exp Biol,* 222 (20):jeb208702, 2019. DOI: 10.1242/jeb.208702

117. Ilie CI, Oprea E, Geana EI, Spoiala A, Buleandra M, Gradisteanu Pircalabioru G, Badea IA, Ficai D, Andronescu E, Ficai A, Ditu LM: Bee pollen extracts: Chemical composition, antioxidant properties, and effect on the growth of selected probiotic and pathogenic bacteria. *Antioxidants (Basel),* 11 (5):959, 2022. DOI: 10.3390/antiox11050959

118. Slobodníková L, Fialová S, Rendeková K, Kováč J, Mučaji P: Antibiofilm activity of plant polyphenols. *Molecules,* 21 (12):1717, 2016. DOI: 10.3390/molecules21121717

119. LiverTox: Clinical and research information on drug-Induced liver Injury [Internet]. Bethesda (MD): National Institute of Diabetes and Digestive and Kidney Diseases; 2012-. Royal Jelly. [Updated 2022 May 1]. https://www.ncbi.nlm.nih.gov/books/NBK580274. *Accessed: 25.05.2023*

120. Andrade MOD, Lima UdA: Variação estacional da composição centesimal do peixe de água doce, Pimelodus darias Bloch (Mandi). *An Esc Super Agric Luiz de Queiroz,* 32, 575-587, 1975. DOI: 10.1590/S0071-12761975000100050

121. Bărnuțiu LI, Mărghitaș LA, Dezmirean DS, Mihai CM, Bobiș O: Chemical composition and antimicrobial activity of Royal Jelly-Review. *Anim Sci Biotechnol,* 44 (2): 67-72, 2011.

122. ISO 12824:2016 - Royal jelly - Specifications, https://www.iso.org/obp/ui/#iso:std:iso:12824:ed-1:v1:en. *Accessed:* 05.06.2023.

123. García MC, Finola MS, Marioli JM: Antibacterial activity of royal jelly against bacteria capable of infecting cutaneous wounds. *J Apiproduct Apimedical Sci,* 2 (3): 93-99, 2010. DOI: 10.3896/ibra.4.02.3.02

124. Moselhy WA, Fawzy AM, Kamel AA: An evaluation of the potent antimicrobial effects and unsaponifiable matter analysis of the royal jelly. *Life Sci J,* 10 (2): 290-296, 2013.

125. Coutinho D, Karibasappa SN, Mehta DS: Royal jelly antimicrobial activity against periodontopathic bacteria. *J Interdiscip Dent,* 8 (1): 18, 18-22, 2018. DOI: 10.4103/jid.jid_72_17

126. Al-Abbadi AA: The antimicrobial potential of royal jelly against some pathogenic bacteria and fungi. *JJBS,* 12 (4): 445-451, 2019.

127. Ahmad S, Campos MG, Fratini F, Altaye SZ, Li J: New insights into the biological and pharmaceutical properties of royal jelly. *Int J Mol Sci,* 21 (2):382, 2020. DOI: 10.3390/ijms21020382

128. Bagameri L, Baci G-M, Dezmirean DS: Royal jelly as a nutraceutical natural product with a focus on its antibacterial activity. *Pharmaceutics,* 14 (6):1142, 2022. DOI: 10.3390/pharmaceutics14061142

129. Mureșan CI, Dezmirean DS, Marc BD, Suharoschi R, Pop OL, Buttstedt A: Biological properties and activities of major royal jelly proteins and their derived peptides. *J Funct Foods,* 98:105286, 2022. DOI: 10.1016/j.jff.2022.105286

130. Splith K, Neundorf I: Antimicrobial peptides with cell-penetrating peptide properties and vice versa. *Eur Biophys J,* 40 (4): 387-397, 2011. DOI: 10.1007/s00249-011-0682-7

131. Bílikova K, Huang SC, Lin IP, Šimuth J, Peng CC: Structure and antimicrobial activity relationship of royalisin, an antimicrobial peptide from royal jelly of *Apis mellifera*. *Peptides,* 68, 190-196, 2015. DOI: 10.1016/j.peptides.2015.03.001

132. Mierzejewski M: The antimicrobial effects of royal jelly, propolis and honey against bacteria of clinical significance in comparison to three antibiotics. *Coll Arts Sci Biol,* 2014:2014.

133. Hasan AA, Hillawi SSH, Khudhur HR: Antimicrobial activity of royal jelly against *E. Coli* in comparison with three selective antibiotics. *Ann Trop Med Public Health,* 23 (14): 231-414, 2020. DOI: 10.36295/ASRO.2020.231414

134. El-Gayar MH, Aboshanab KM, Aboulwafa MM, Hassouna NA: Antivirulence and wound healing effects of royal jelly and garlic extract for the control of MRSA skin infections. *Wound Medicine,* 13, 18-27, 2016. DOI: 10.1016/j.wndm.2016.05.004

135. Ramírez OJ, Alvarez S, Contreras-Kallens P, Barrera NP, Aguayo S, Schuh CMAP: Type I collagen hydrogels as a delivery matrix for royal jelly derived extracellular vesicles. *Drug Deliv,* 27 (1): 1308-1318, 2020. DOI: 10.1080/10717544.2020.1818880

136. Amly DA, Hajardhini P, Jonarta AL, Yulianto HDK, Susilowati H: Enhancement of pyocyanin production by subinhibitory concentration of royal jelly in *Pseudomonas aeruginosa*. *F1000Res,* 10:14, 2021. DOI: 10.12688/f1000research.27915.4

137. Gao K, Su B, Dai J, Li P, Wang R, Yang X: Anti-biofilm and anti-hemolysis activities of 10-hydroxy-2-decenoic acid against *Staphylococcus aureus*. *Molecules,* 27

(5):1485, 2022. DOI: 10.3390/molecules27051485

138. Celebi D, Celebi O, Baser S, Taghizadehghalehjoughi A: Evaluation of antimicrobial and antibiofilm efficacy of bee venom and exosome against *Escherichia coli* K99 strain. *Kafkas Univ Vet Fak Derg,* 29 (3): 239-246, 2023. DOI: 10.9775/kvfd.2023.29132

139. Wehbe R, Frangieh J, Rima M, El Obeid D, Sabatier J-M, Fajloun Z: Bee venom: Overview of main compounds and bioactivities for therapeutic interests. *Molecules,* 24 (16):2997, 2019. DOI: 10.3390/molecules24162997

140. Lee Y, Kim SG, Kim IS, Lee HD: Standardization of the manufacturing process of bee venom pharmacopuncture containing melittin as the active ingredient. *Evid Based Complement Alternat Med,* 2018:2353280, 2018. DOI: 10.1155/2018/2353280

141. Leandro LF, Mendes CA, Casemiro LA, Vinholis AHC, Cunha WR, de Almeida R, Martins CHG: Antimicrobial activity of apitoxin, melittin and phospholipase A_2 of honey bee *(Apis mellifera)* venom against oral pathogens. *An Acad Bras Cienc,* 87 (1): 147-155, 2015. DOI: 10.1590/0001-3765201520130511

142. Hegazi A, Abdou AM, El-Moez S, Allah FA: Evaluation of the antibacterial activity of bee venom from different sources. *WASJ,* 30 (3): 266-270, 2014.

143. Choi JH, Jang AY, Lin S, Lim S, Kim D, Park K, Han SM, Yeo JH, Seo HS: Melittin, a honeybee venom-derived antimicrobial peptide, may target methicillin-resistant *Staphylococcus aureus*. *Mol Med Rep,* 12 (5): 6483-6490, 2015. DOI: 10.3892/mmr.2015.4275

144. Sonmez E, Kekecoglu M, Bozdeveci A, Karaoglu SA: Chemical profiling and antimicrobial effect of Anatolian honey bee venom. *Toxicon,* 213, 1-6, 2022. DOI: 10.1016/j.toxicon.2022.04.006

145. Maitip J, Mookhploy W, Khorndork S, Chantawannakul P: Comparative study of antimicrobial properties of bee venom extracts and melittins of honey bees. *Antibiotics (Basel),* 10 (12):1503, 2021. DOI: 10.3390/antibiotics10121503

146. Tossi A, Sandri L, Giangaspero A: Amphipathic, alpha-helical antimicrobial peptides. *Biopolymers,* 55 (1): 4-30, 2000. DOI: 10.1002/1097-0282(2000)55:1<4::AID-BIP30>3.0.CO;2-M

147. Alia O, Laila M, Antonious A: Antimicrobial effect of melittin isolated from Syrian honeybee *(Apis mellifera)* venom and its wound healing potential. *Int J Pharm Sci Rev Res*, 21, 318-324, 2013.

148. Yacoub T, Rima M, Karam M, Fajloun JSAZ: Antimicrobials from venomous animals: An overview. *Molecules,* 25 (10):2402, 2020. DOI: 10.3390/molecules25102402

149. Lubke LL, Garon CF: The antimicrobial agent melittin exhibits powerful *in vitro* inhibitory effects on the Lyme disease spirochete. *Clin Infect Dis,* 25 (Suppl.-1): S48-S51, 1997. DOI: 10.1086/516165

150. Giacometti A, Cirioni O, Kamysz W, D'Amato G, Silvestri C, Del Prete MS, Łukasiak J, Scalise G: Comparative activities of cecropin A, melittin, and cecropin A-melittin peptide CA(1-7)M(2-9)NH2 against multidrug-resistant nosocomial isolates of *Acinetobacter baumannii*. *Peptides,* 24 (9): 1315-1318, 2003. DOI: 10.1016/j.peptides.2003.08.003

151. Gopal R, Lee JH, Kim YG, Kim M-S, Seo CH, Park Y: Anti-microbial, anti-biofilm activities and cell selectivity of the NRC-16 peptide derived from witch flounder, *Glyptocephalus cynoglossus*. *Mar Drugs,* 11 (6): 1836-1852, 2013. DOI: 10.3390/md11061836

152. Shams Khozani R, Shahbazzadeh D, Harzandi N, Feizabadi MM, Pooshang Bagheri K: Kinetics study of antimicrobial peptide, melittin, in simultaneous biofilm degradation and eradication of potent biofilm producing MDR *Pseudomonas aeruginosa* isolates. *Int J Pept Res Ther,* 25 (1): 329-338, 2019. DOI: 10.1007/s10989-018-9675-z

153. Hebert C, Weber SG: Common approaches to the control of multidrug-resistant organisms other than methicillin-resistant *Staphylococcus aureus* (MRSA). *Infect Dis Clin North Am,* 25 (1): 181-200, 2011. DOI: 10.1016/j.idc.2010.11.006

154. Rasigade J-P, Vandenesch F: *Staphylococcus aureus*: A pathogen with still unresolved issues. *Infect Genet Evol,* 21, 510-514, 2014. DOI: 10.1016/j.meegid.2013.08.018

155. Midoux P, Mayer R, Monsigny M: Membrane permeabilization by alpha-helical peptides: A flow cytometry study. *Biochim Biophys Acta,* 1239, 249-256, 1995. DOI: 10.1016/0005-2736(95)00163-w

156. Pawlak M, Meseth U, Dhanapal B, Mutter M, Vogel H: Template-assembled melittin: Structural and functional characterization of a designed, synthetic channel-forming protein. *Protein Sci,* 3 (10): 1788-1805, 1994. DOI: 10.1002/pro.5560031019

157. Brogden KA: Antimicrobial peptides: Pore formers or metabolic inhibitors in bacteria? *Nat Rev Microbiol,* 3 (3): 238-250, 2005. DOI: 10.1038/nrmicro1098

158. Andersson M, Ulmschneider JP, Ulmschneider MB, White SH: Conformational states of melittin at a bilayer interface. *Biophys J,* 104 (6): L12-14, 2013. DOI: 10.1016/j.bpj.2013.02.006

159. Takahashi T, Nomura F, Yokoyama Y, Tanaka-Takiguchi Y, Homma M, Takiguchi K: Multiple membrane interactions and versatile vesicle deformations elicited by melittin. *Toxins,* 5 (4): 637-664, 2013. DOI: 10.3390/toxins5040637

160. Miklossy J, Kasas S, Zurn AD, McCall S, Yu S, McGeer PL: Persisting atypical and cystic forms of *Borrelia burgdorferi* and local inflammation in Lyme neuroborreliosis. *J Neuroinflammation,* 5:40, 2008. DOI: 10.1186/1742-2094-5-40

161. Socarras K, Theophilus P, Torres J, Gupta K, Sapi E: Antimicrobial activity of bee venom and melittin against *Borrelia burgdorferi*. *Antibiotics (Basel),* 6 (4):31, 2017. DOI: 10.3390/antibiotics6040031

162. Carpena M, Nuñez-Estevez B, Soria-Lopez A, Simal-Gandara J: Bee venom: An updating review of its bioactive molecules and its health applications. *Nutrients,* 12 (11):3360, 2020. DOI: 10.3390/nu12113360

163. Khalil A, Elesawy BH, Ali TM, Ahmed OM: Bee venom: From venom to drug. *Molecules,* 26 (16):441, 2021. DOI: 10.3390/molecules26164941

164. Pucca MB, Ahmadi S, Cerni FA, Ledsgaard L, Sørensen CV, McGeoghan FTS, Stewart T, Schoof E, Lomonte B, Auf dem Keller U, Arantes EC, Çalışkan F, Laustsen AH: Unity makes strength: Exploring intraspecies and interspecies toxin synergism between phospholipases A2 and cytotoxins. *Front Pharmacol,* 11:611, 2020. DOI: 10.3389/fphar.2020.00611

165. Gajski G, Garaj-Vrhovac V: Melittin: A lytic peptide with anticancer properties. *Environ Toxicol Pharmacol,* 36 (2): 697-705, 2013. DOI: 10.1016/j.etap.2013.06.009

166. Yang L, Harroun TA, Weiss TM, Ding L, Huang HW: Barrel-stave model or toroidal model? A case study on melittin pores. *Biophys J,* 81 (3): 1475-1485, 2001. DOI: 10.1016/S0006-3495(01)75802-X

167. Batoni G, Maisetta G, Brancatisano FL, Esin S, Campa M: Use of antimicrobial peptides against microbial biofilms: Advantages and limits. *Curr Med Chem,* 18 (2): 256-279, 2011. DOI: 10.2174/092986711794088399

168. Strempel N, Strehmel J, Overhage J: Potential application of antimicrobial peptides in the treatment of bacterial biofilm infections. *Curr Pharm Des,* 21 (1): 67-84, 2014. DOI: 10.2174/1381612820666140905124312

169. Lewis K: Riddle of biofilm resistance. *Antimicrob Agents Chemother,* 45 (4): 999-1007, 2001. DOI: 10.1128/AAC.45.4.999-1007.2001

170. Le CF, Fang CM, Sekaran SD: Intracellular targeting mechanisms by antimicrobial peptides. *Antimicrob Agents Chemother,* 61 (4):1128, 2017. DOI: 10.1128/AAC.02340-16

171. Fadl AE-W: Antibacterial and antibiofilm effects of bee venom from *(Apis mellifera)* on multidrug-resistant bacteria (mdrb). *Al-Azhar J Pharm Sci,* 58 (2): 60-80, 2018. DOI: 10.21608/ajps.2018.46641

172. Sapi E, Balasubramanian K, Poruri A, Maghsoudlou JS, Socarras KM, Timmaraju AV, Filush KR, Gupta K, Shaikh S, Theophilus PAS, Luecke DF, MacDonald A, Zelger B: Evidence of *in vivo* existence of *Borrelia* biofilm in *Borrelial lymphocytomas*. *Eur J Microbiol Immunol,* 6 (1): 9-24, 2016. DOI: 10.1556/1886.2015.00049

173. Sapi E, Bastian SL, Mpoy CM, Scott S, Rattelle A, Pabbati N, Poruri A, Burugu D, Theophilus PAS, Pham TV, Datar A, Dhaliwal NK, MacDonald A, Rossi MJ, Sinha SK, Luecke DF: Characterization of biofilm formation by *Borrelia burgdorferi in vitro*. *PLoS One,* 7 (10):e48277, 2012. DOI: 10.1371/journal.pone.0048277

174. Marques Pereira AF, Albano M, Bérgamo Alves FC, Murbach Teles Andrade BF, Furlanetto A, Mores Rall VL, Delazari dos Santos L, de Oliveira Orsi R, Fernandes Júnior A: Influence of apitoxin and melittin from *Apis mellifera* bee on *Staphylococcus aureus* strains. *Microb Pathog,* 141:104011, 2020. DOI: 10.1016/j.micpath.2020.104011

175. Dosler S, Karaaslan E, Alev Gerceker A: Antibacterial and anti-biofilm activities of melittin and colistin, alone and in combination with antibiotics against gram-negative bacteria. *J Chemother,* 28 (2): 95-103, 2016. DOI: 10.1179/1973947815Y.0000000004

176. Picoli T, Peter CM, Zani JL, Waller SB, Lopes MG, Boesche KN, Vargas GDá, Hübner SdO, Fischer G: Melittin and its potential in the destruction and inhibition of the biofilm formation by *Staphylococcus aureus, Escherichia coli* and *Pseudomonas aeruginosa* isolated from bovine milk. *Microb Pathog,* 112, 57-62, 2017. DOI: 10.1016/j.micpath.2017.09.046

177. Bardbari AM, Arabestani MR, Karami M, Keramat F, Aghazadeh H, Alikhani MY, Bagheri KP: Highly synergistic activity of melittin with imipenem and colistin

in biofilm inhibition against multidrug-resistant strong biofilm producer strains of *Acinetobacter baumannii*. *Eur J Clin Microbiol Infect Dis,* 37 (3): 443-454, 2018. DOI: 10.1007/s10096-018-3189-7

178. Topal E, Ceylan Ö, Kösoğlu M, Mărgăoan R, Cipcigan MC: Bal mumunun yapısı, kullanım alanları ve bazı temel sorunları. *Uludağ Arıcılık Dergisi,* 20 (2): 209-220, 2020. DOI: 10.31467/uluaricilik.781259

179. Food: Direct food substances affirmed as generally recognized as safe. *FDA Med Bull*, 184, 417-418, 2015.

180. Smati N: Bee bread preservation methods: Physical, chemical and microbial stability throughout storage [M.Sc.]. Portugal: Instituto Politecnico de Braganca (Portugal); 2021. https://www.proquest.com/dissertations-theses/bee-bread-preservation-methods-physical-chemical/docview/2748386573/se-2?accountid=15333. *Accessed:* 10.06.2023.

181. Kaya İ: Arı ekmeğinin besinsel kalitesi, biyoaktif ve antimikrobiyal özelliklerinin belirlenmesi 2022. http://earsiv.odu.edu.tr/xmlui/handle/11489/8. *Accessed:* 10.06.2023.

182. Asma ST, Bobiş O, Bonta V, Acaroz U, Shah SRA, Istanbullugil FR, Arslan-Acaroz D: General nutritional profile of bee products and their potential antiviral properties against mammalian viruses. *Nutrients,* 14 (17):3579, 2022. DOI: 10.3390/nu14173579

183. Nurko E, Nakilcioğlu-Taş E: Kovandaki gizli mucize: Arı poleni ve arı ekmeği ile gıdaların zenginleştirilmesi. *Gıda,* 47 (4): 604-615, 2022. DOI: 10.15237/gida.GD22044

184. Podrižnik B, Božič J: Maturation and stratification of antibacterial activity and total phenolic content of bee bread in honey comb cells. *J Apic Res,* 54 (2): 81-92, 2015. DOI: 10.1080/00218839.2015.1090774

185. Ochoa TA, Almendárez BEG, Reyes AA, Pastrana DMR, López GFG, Belloso OM, González CR: Design and characterization of corn starch edible films including beeswax and natural antimicrobials. *Food Bioprocess Technol,* 10 (1): 103-114, 2017. DOI: 10.1007/s11947-016-1800-4

186. Meindrawan B, Ofe O, Susanto CS, Ayman A, Mangindaan D, Kasih TP: Glucomannan-beeswax-chitosan antimicrobial edible coating to maintain the storage quality of Salak fruit *(Salacca zalacca)*. *Macromol Symp,* 391 (1):1900164, 2020. DOI: 10.1002/masy.201900164

187. Disayathanoowat T, Li H, Supapimon N, Suwannarach N, Lumyong S, Chantawannakul P, Guo J: Different dynamics of bacterial and fungal communities in hive-stored bee bread and their possible roles: A case study from two commercial honey bees in China. *Microorganisms,* 8 (2):264, 2020. DOI: 10.3390/microorganisms8020264

188. Kowalski S, Makarewicz M: Functional properties of honey supplemented with bee bread and propolis. *Nat Prod Res,* 31 (22): 2680-2683, 2017. DOI: 10.1080/14786419.2017.1286481

189. Gennadios A, Hanna MA, Kurth LB: Application of edible coatings on meats, poultry and seafoods: A review. *LWT - Food Sci Technol,* 30 (4): 337-350, 1997. DOI: 10.1006/fstl.1996.0202

190. Perez-Perez C, Regalado-González C, Rodríguez-Rodríguez CA, Barbosa-Rodríguez JR, Villaseñor-Ortega F, Guevara-González RG, Torres-Pacheco I: Incorporation of antimicrobial agents in food packaging films and coatings. **In,** Guevara-González RG; Torres-Pacheco I (Eds): Advances in Agricultural and food Biotechnology. 193-216, Research Signpost, 2006.

191. Vermeiren L, Devlieghere F, van Beest M, de Kruijf N, Debevere J: Developments in the active packaging of foods. *Trends Food Sci Technol,* 10 (3): 77-86, 1999. DOI: 10.1016/s0924-2244(99)00032-1

192. Hromiš NM, Lazić VL, Markov SL, Vaštag ŽG, Popović SZ, Šuput DZ, Džinić NR, Velićanski AS, Popović LM: Optimization of chitosan biofilm properties by addition of caraway essential oil and beeswax. *J Food Eng,* 158, 86-93, 2015. DOI: 10.1016/j.jfoodeng.2015.01.001

193. Oliveira VRL, Santos FKG, Leite RHL, Aroucha EMM, Silva KNO: Use of biopolymeric coating hydrophobized with beeswax in post-harvest conservation of guavas. *Food Chem,* 259, 55-64, 2018. DOI: 10.1016/j.foodchem.2018.03.101

194. Hromiš NM, Lazić VL, Šuput DZ, Popović SZ, Tomović VM: Improvement of water vapor barrier properties of chitosan-collagen laminated casings using beeswax. *Anal Tech Szeged,* 9 (1): 31-38, 2015. DOI: 10.14232/analecta.2015.1.31-38

195. Wultańska D, Paterczyk B, Nowakowska J, Pituch H: The effect of selected bee products on adhesion and biofilm of *Clostridioides difficile* strains belonging to different ribotypes. *Molecules,* 27 (21):7385, 2022. DOI: 10.3390/molecules27217385

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RESEARCH ARTICLE

Determination of Subtypes, Serogroups, And Serotypes, Virulence, and/or Toxigenic Properties of *Escherichia coli* Isolated From Cattle, Sheep, and Goat Feces by Multiplex PCR [1]

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Abstract

In the study, rectal swabs taken from 300 ruminant animals including cattle (100), sheep (100), and goats (100) were inoculated into Mac Conkey Agar and incubated for 18 h at 37°C. *Escherichia coli* isolates were confirmed by biochemical tests and the BBL Crystal rapid diagnosis system. O26, O45, O103, O111, O121, O145, and O157 serotypes by PCR test following DNA isolation; ETEC *(elt, Stla);* EPEC *(eaeA,bfpA);* STEC *(stx1, stx2, eaeA);* EHEC *(EhlyA)*; EAEC *(CVD432)* tested for virulence and/or toxigenic genes. As a result of the isolation studies, 50 *E. coli* from cattle feces, 92 from sheep feces, and 80 from goat feces were isolated and identified. Apart from the first 5 serotypes frequently seen in studies (O157, O26, O103, O111, and O145), higher rates were found in serogroups such as O45 and O121, and subtypes such as STECs *(stx1* and *stx2),* EPEC (*eaeA* and *bfpA)* and EAEC *(CVD432)* types compared to other studies. The EAEC *(CVD432)* subtype was found to be very high in this study. It has been determined that serotypes and subtypes detected at high rates in cattle, sheep, and goat feces in our region may cause an increase in the incidence of some critical food-borne infections in humans. Within the framework of the concept of one health, taking the necessary precautions is important for public health.

Keywords: *E. coli*, Serotype, Subtype, Virulence, Toxigenic properties

INTRODUCTION

Shiga toxin-producing *Escherichia coli* (STEC), also known as verocytotoxigenic *E. coli* (VTEC), can have one or both of the Shiga toxin genes *(stx1* and *stx2)*. STECs are commensal in the gastrointestinal tract of ruminant livestock such as cattle, sheep, and goats and are the main source of transmission of STEC infections in humans [1,2]. During milking, slaughter, and removal of internal organs, foods of animal origin become contaminated with STECs present in feces and may pollute the environment. *E. coli* O157, O26, O103, O111, and O145 serogroups have been most commonly identified as the "top five" serogroups found to be associated with human infections. STEC infections in humans are asymptomatic or can cause the transition from non-bloody to bloody diarrhea; cause colitis, hemolytic uremic syndrome (HUS), and hemorrhagic diseases that can result in death [1,3]; cause outbreaks of diarrhea in children and the elderly [4]. O157, especially from STECs, is the main food-borne pathogen. For non-O157 STECs, cattle are considered to be the main reservoir [5]. According to the 2020 European Union One Health Zoonoses Report, the number of confirmed human cases of STEC infections was 4.446 [6].

Enterohemorrhagic *E. coli* (EHEC) is the most common foodborne pathogen. EHEC strains not only produce potent cytotoxins but have also acquired the ability to

adhere to the intestinal mucosa. There are also certain types of virulence factors. All strains produce hemolysin *(hlyA)* and at least one Shiga-like toxin *(stx1* and/or *stx2),* and most produce intimin, a 97 kDa binding and deletion protein *(eaeA)* [7]. In India, Vietnam, and the USA, EAEC has been reported to be the most common diarrheagenic *E. coli* in children with diarrhea. There are two types of EPEC: typical EPEC (Type I, *eae,* and *bfp* positive) and atypical EPEC (Type II, *eae* positive only). Typical EPEC, causes gastroenteritis in babies and is usually seen in babies under 2 years of age. Recent studies indicate an increase in the prevalence of atypical EPEC strains [8]. Enteroaggregative *E. coli* (EAggEC), even in the absence of Stx/VT, can cause diarrhea in children and adults. EAggEC is best known for causing persistent diarrhea (>14 days) in infants and developing children. In Mongolia, India, Brazil, Nigeria, Israel, Venezuela, Congo, and many other countries, EAggEC is the most common *E. coli* pathotype in infants. EAggEC is the nontoxic-secreting *E. coli* type of ETEC. It synthesizes aggregative adherence fimbriae with the *aggR* gene. EAggEC usually causes watery and often persistent diarrhea. Infection begins with the bacterium first adhering to the terminal ileum and colon of the aggregative adherence fimbriae. The damage/secretion phase occurs with the release of cytokines, mucosal toxicity, intestinal secretion, and induction of mucosal inflammation [9].

Carcasses to be contamined with intestinal contents during slaughterhouses. Therefore, bacteria present in the intestinal contents can contaminate the carcass and cause foodborne infections and toxicity. This study aimed to determine the subtypes, serotypes, virulence, and/or toxigenic properties of *E. coli*, which are at risk of causing disease in humans, especially from the feces of ruminant animals.

Material and Methods

Ethical Statement

The study has been approved by the Ethics Committee of Kırıkkale University (Approval No. E-60821397-619-177126).

Sample Collection

In this project, rectal swab samples were collected from 300 ruminants, such as healthy cattle (100), sheep (100), and goats (100). Rectal swabs from cattle were collected aseptically from slaughterhouses in Kırıkkale province, and the rectal swabs from sheep and goats were delivered to the laboratory in a transport medium with a cold chain at 4-8°C.

Isolation and Identification

The swabs were seeded on Mac Conkey Agar and incubated at 37°C for 18 h. Biochemical testing was performed on isolates that exhibited typical characteristics of *E. coli,* and the presence was confirmed using both conventional methods and the rapid diagnostic system BBL Crystal.

DNA Isolation

A 100 µL of sterile deionized water was added to each of five (n=5) *E. coli* colonies. For DNA isolation, they were boiled at 95°C for 10 min with a hot plate and centrifuged at 1000 g. for 10 min. The supernatant was transferred to sterile Eppendorf tubes, and the amount of DNA was measured with a Nanodrop spectrophotometer, and then the concentration was adjusted to 100 ng/µL [8].

PCR

For PCR: 12.5 mL of master mix, 1 µL of DNA, 5 µL of each primer, and 6.5 µL of sterile distilled water were added, and the total volume was adjusted to 25 µL. The thermocycling conditions were as follows: 1 min at 95°C; 35 cycles at 94°C for 1 min, 60°C for 1 min, 72°C for 1 min, and finally one cycle at 72°C for 5 min. *E. coli* serotypes and serogroups were screened for virulence and/or toxigenic genes by PCR assays: serotypes O26, O45, O103, O111, O121, O145, O157 [5] and subtypes ETEC *(elt, Stla),* EPEC *(eaeA, bfpA),* EHEC *(EhlyA),* EAEC *(CVD432)* [8] and STEC *(stx1, stx2, eaeA)* [7]. Amplicons (10 µL) generated by PCR were electrophoresed on a 2% agarose gel, in 1xTAE buffer, at 100 volts for approximately 1 h. The bands were evaluated under UV light [8].

Statistical Analysis

The KAPPA test was used to compare the analysis results of the methods used in the study for *E. coli* serogroups and serotypes. The results were evaluated according to Mc Hugh [8].

Results

Stool samples from slaughterhouses and farms in Kırıkkale were collected under sterile conditions and delivered to the laboratory as soon as possible. As a result of the isolation studies, 50 *E. coli* were isolated and identified from cattle feces, 92 from sheep feces, and 80 from goat feces.

PCR analysis of 92 *E. coli* isolates from sheep feces revealed the following serotypes and their ratios, ranking from highest to lowest: 26 of O26 (28.3%), 22 O157 (23.9%), 18 O103 (19.6%), 13 O121 (14.1%), 9 O45 (9.8%), 4 O111 (4.3%); O145 was not found. PCR analysis of 80 *E. coli* isolates from goat feces revealed the following serotypes and their ratios, ranking from highest to lowest: 20 of O45 (25%), 20 of O111 (25%), 16 O26 (20%), 12 O121 (15%), 8 O157 (10%), 4 O145 (5%); O103 was not found. PCR analysis of 50 *E. coli* isolates from bovine feces determined serotypes and their ratios as follows, ranking from highest

Table 1. *PCR analysis results of E. coli serotypes and ratios from all animal fecal samples*

Animals (Total Isolates)	**Serotypes Numbers (%)**						
	O26	**O45**	**O103**	**O111**	**O121**	**O145**	**O157**
Sheep Feces (92)	26 (28.3)	9 (9.8)	18 (19.6)	4 (4.3)	13 (14.1)	0 (0)	22 (23.9)
Goat Feces (80)	16 (20)	20 (25)	0 (0)	20 (25)	12 (15)	4 (5)	8 (10)
Cattle Feces (50)	5 (10)	11 (22)	5 (10)	7 (14)	7 (14)	2 (4)	21 (42)
Total (222)	47 (21.1)	40 (18)	23 (10.4)	31 (14)	32 (14.4)	6 (2.7)	51 (23)

Table 2. *PCR analysis results of E. coli subtypes and ratios from all animal fecal samples*

Animals (Total Isolates)	**Subtypes Numbers (%)**									
	ETEC			**EPEC**		**STEC**			**EHEC**	**EAEC**
Genes	*Elt*	*Stla*	*all*	*eaeA*	*bfpA*	*stx1*	*stx2*	*eaeA*	*EhlyA*	*CVD432*
Sheep (92)	32 (35)	9 (10)	18 (20)	5 (5)	37 (40)	32 (35)	41 (45)	32 (35)	23 (25)	**69 (75)**
Goat (80)	32 (40)	24 (30)	0 (0)	40 (50)	**52 (65)**	40 (50)	**68 (85)**	20 (25)	24 (30)	52 (65)
Cattle (50)	20 (39.1)	11 (21.7)	4 (8.7)	24 (47.8)	22 (43.5)	30 (60.9)	33 (65.2)	9 (17.4)	15 (30.4)	**46 (91.3)**
Total (222)	84 (37.8)	44 (19.8)	22 (9.9)	69 (31.1)	111 (50)	102 (45.9)	**142 (64)**	61 (27.5)	61 (27.5)	**164 (73.9)**

Table 3. *Kappa test results*

Animals and Serotypes		**K**									
		ETEC *(elt)*	**ETEC *(Stla)***	**EPEC *(eaeA)***	**EPEC *(bfpA)***	**STEC *(stx1)***	**STEC *(stx2)***	**STEC *(eaeA)***	**ETEC *(all)***	**EHEC *(EhlyA)***	**EAEC *(CVD432)***
Sheep	O45	0.146	0.002	-0.06	-1.005	0.175	0.117	0.174	0.368	-0.099	-3.294
	O157	0.434	0.62	0	0.49	1.61	0.352	0.519	-0.029	0.46	0.176
	O26	-0.048	-0.186	0.216	-0.117	0.462	-0.179	-0.514	-0.336	-0.146	-0.124
	O103	-0.193	0.497	-0.052	-0.185	-0.203	-0.08	-0.379	0.566	-0.072	-0.153
	O121	-0.19	-0.114	-0.016	0.41	0.602	-0.203	0.542	0.223	-0.174	0.06
Goat	O45	-0.019	0.068	-0.133	-0.043	-0.1	-0.037	-0.124	0.66	-0.125	0.272
	O157	-0.19	-0.171	-0.2	0.006	0.2921	-0.612	-0.16	0	-0.29	0.33
	O26	-0.136	0.21	0.2	0.22	-0.01	-0.12	-0.149	-1.3	-0.05	-0.126
	O103	0	0	0	0	0	0	0	0	0	0
	O121	0.52	0.604	0.01	0.183	0.7	0.1	0.076	0	1.09	3.568
	O145	0.146	-0.093	0.1	0.05	0.11	0.018	-0.9	0	-0.093	0.055
	O111	0	-0.125	0.288	-0.043	0.3	0	0.46	0	0.375	0.92
Bovine	O45	0.194	0.558	-0.46	0.186	0.158	0.158	0.601	0.33	-0.042	0.067
	O157	-0.131	-0.129	0.279	-0.012	-0.123	1.573	0.821	-0.19	0.029	-0.185
	O26	-0.131	-0.151	0.516	-0.174	-0.289	0.11	-0.123	-0.098	-0.098	0.02
	O103	-0.086	-0.074	0.99	-0.091	-0.082	0.061	-0.07	-0.059	-0.8	0.012
	O121	0.165	-0.201	1.041	-0.096	0.168	-3.25	0.15	-0.357	0.016	0.033
	O145	-0.126	-0.073	0.091	0.103	0.055	-0.08	0.309	0.996	0.197	-1.74
	O111	0.25	0.232	0.09	0.024	0.084	0.084	-0.07	0.581	0.194	-1.74

to lowest: 21 of O157 (42%), 11 of O45 (22%), 7 of O121 (14%), 7 of O111 (14%), 5 O26 (10%), 5 of O103 (10%), 2 O145 (4%). PCR analysis of 222 *E. coli* isolates from all animal fecal samples revealed the following serotypes and their ratios; 51 of O157 (23%), 47 of O26 (21.6%), 40 of O45 (18%), 32 of O121 (14.4%), 31 of O111 (14.4%), 23 of O103 (10.4%), 6 of O145 (2.7%). The most common serotypes of *E. coli* isolated and identified from various animal fecal samples are, respectively: In sheep: O26, O157, O103, 121, O45, O111; in goats: O45, O111, O26, O121, O157, O145; and in cattle: O157, O45, O121, O111, O26, O103, O145. PCR analysis results of *E. coli* serotypes and ratios from all animal fecal samples are shown in *Table 1*.

When evaluated as *E. coli* subtypes ratios from sheep fecal samples, ETEC *(elt)* are 35%, and ETEC *(Stla)* are 10%; EPEC *(eaeA)* are 5% and EPEC *(bfpA)* are 40%; STEC *(stx1)* are 35%, STEC *(stx2)* are 45%, STEC *(eaeA)* are 35%; ETEC *(all)* are 20%; EHEC *(EhlyA)* are 25% and EAEC *(CVD432)* are 75%.; *E. coli* subtypes ratios from goat fecal samples ETEC *(elt)* is 40%, and ETEC (Stla) is 30%; EPEC *(eaeA)* is 50% and EPEC *(bfpA)* is 65%; STEC *(stx1)* is 50%, STEC *(stx2)* is 85%, STEC *(eaeA)* is 25%; ETEC *(all)* is 0%; EHEC *(EhlyA)* is 30% and EAEC *(CVD432)* is 65%.; *E. coli* subtypes ratios from cattle fecal samples ETEC *(elt)* is 39.1%, and ETEC *(Stla)* is 21.7%, EPEC *(eaeA)* is 47.8% and EPEC *(bfpA)* is 43.5%; STEC *(stx1)* is 60.9%, STEC *(stx2)* is 65.2% STEC *(eaeA)* is17.4%; ETEC *(all)* is 8.7%; EHEC *(EhlyA)* is 30.4% and EAEC *(CVD432)* is 91.3%. PCR analysis results of *E. coli* subtypes and ratios from all animal fecal samples are shown in *Table 2*.

As a result, subspecies of *E. coli* isolated and identified from individuals most frequently seen in different animals are EAEC *(CVD432),* STEC *(stx2),* EPEC *(bfpA),* ETEC *(elt),* STEC *(stx1),* STEC *(eaeA),* EHEC *(EhlyA),* ETEC *(all),* ETEC *(Stla),* EPEC *(eaeA)* in sheep; in goats these were STEC *(stx2),* EAEC *(CVD432),* EPEC *(bfpA),* EPEC *(eaeA),* STEC *(stx1),* ETEC *(elt),* EHEC *(EhlyA),* ETEC *(Stla),* STEC *(eaeA).* In cattle, it was detected as EAEC *(CVD432),* STEC *(stx2),* STEC *(stx1),* EPEC *(eaeA),* EPEC (*bfpA*), ETEC *(elt),* EHEC *(EhlyA),* ETEC *(Stla),* STEC *(eaeA),* ETEC *(all).* The identified ones in all animals were EAEC *(CVD432),* STEC *(stx2),* EPEC *(bfpA),* STEC *(stx1),* ETEC *(elt),* EPEC *(eaeA),* EHEC *(EhlyA),* STEC *(eaeA),* ETEC *(Stla),* ETEC *(all).*

The results of the Kappa test performed were evaluated *(Table 3).* Remarkably, that there is an almost perfect correlation between the results of the sheep O517-STEC *(stx1),* goat O121-EHEC and EAEC, goat O111-EAEC, bovine O157-STEC *(stx2* and *eaeA),* bovine O121-EPEC *(eaeA),* and bovine O145-EIEC.

Discussion

Studies have reported that STECs transmitted from feces during slaughter in ruminants, especially cattle, are the main transmission source and primary reservoir of HUS infections in humans. In the studies conducted in the European Union country in the 2000s, the first 5 serotypes were O157, O26, O103, O111, and O145 [1]. In the study we conducted in Kırıkkale Region, O45 and O121 serotypes were also detected in cattle together with these 5 serotypes.

The prevalence of STEC in ready-to-eat foods detected in some countries is as follows: In Botswana, *E. coli* O157:H7 was detected at 3.8% in ground beef [10], and *E. coli* O157:H7 at 0.1% in ground beef [11]. *E. coli* O157:H7 was detected as 0.4% in ground beef in Italy. STEC was 33.1% in cattle feces in the USA, and O157 was 35.5% [12]. STECs in New Zealand were 17% in lamb and 12% in cattle [13]. In cattle feces in Italy, O157 was found at 6.3%, and O26 was found at 3.8% [14] and in addition to these literatures, STEC was detected at 3.9% in cattle and 5.1% in pig carcasses in Czechia, respectively [15].

In another study, relatively similar rates were found in cattle: *stx2* was 65.2%, *stx1* was 60.9%; less in sheep, *stx2* was 45%, *stx1* was 35%. In a study conducted in 2015, O157 was detected at a rate of 6.3%, and O26 was at 3.8% in Italy [13]. In our study, a high rate of O157 serotype was detected in cattle with a rate of 42% when compared to other countries. STEC *(stx1, stx2, eae, ehxA)* was 63.5% in cattle herds and 56.5% in sheep herds in Spain [16]. *stx2* (10%) and *hlyA* (35.9%) were the most common genes in ruminant animals in Australia [17]. 12.4% was *stx* positive in cattle and children in Poland [18]. In our study, *stx2* and *Ehly* were significantly higher than studies in Australia and Poland; it was found to be more compatible with the findings in Spain.

In a study conducted in Brazil, 52.8% *stx1* and 14.3% *stx2* were detected in sheep [19]. In another study conducted in China, 61.5% of STEC was detected in sheep and 12.9% in cattle; 69.1% of STECs in sheep were determined as *stx1*, 29.4% as *stx2*. It has been reported that STECs with *eae* gene and *stx2* gene are seen in 91.07% of HUS cases. In this study, STEC was found to be quite high; *stx1* was found to be less than this study with a rate of 32%, *stx2* was found to be higher with 41%, and *eaeA* was found to be 32% [20,21]. In another study conducted in China, 55.4% of 56 STECs isolated from cattle and sheep were found as *stx1*, which is in correlation with our study, and 3% as *stx2*, which is less than our study [22]. In another study conducted in India, 91 *E. coli* were isolated from a total of 120 infants (60) and calves (60) with diarrhea, and 30.76% *fimA* gene and 7.69% *aggR* gene were detected from EAEC genes by PCR [23]. In our study, the EAEC *(CVD432)* gene was detected at a very high rate of 73.9%. In a study conducted in France in 2023, they studied 500 fecal samples collected in a calf slaughterhouse in one year; they identified 30 STECs from

28 calves and reported that 2 of them were O103 and O26 (73%), followed by O145 and O157 [24]. In our study, O157 (42%), O26 (10%), and O45 (22%) were detected at a higher rate given in order, and O103 (10%) at a lower rate than other serotypes when compared.

Seker et al.[25], in their study on buffaloes in Afyon, Türkiye, detected *stx1* and *stx2* at 27.3%; *eaeA at* 9.1%; *ehlyA* at 72%. Kuyucuoglu et al.[26] conducted a study on cattle feces in Afyon; these rates were determined as *ehlyA* 92.8% and *eaeA* 57.1%. Yilmaz et al.[27], in their study conducted in Istanbul in 2006, found 70.3% *vt1*, *vt2,* and *eaeA*; 11.1% *eaeA* in cattle feces, carcass, and environmental samples. In Hatay, vtx was detected in 80%, *vtx1,* and *vtx2* in 3.9%, *eaeA* in 93.5%, and e*hlyA* in 96.1% in cattle rectal swabs [28]. In our study, STEC *stx2* was 65%, STEC *stx1* was 61%, EPEC *eaeA* was 48%, STEC *eaeA* was 17%, and EHEC *EhlyA* was 30%, which were found to be higher. Apart from the first 5 serotypes that are frequently seen in our study, the presence of serotypes such as O45 and O121 was also detected. STEC and *eaeA* were detected at higher rates than in other studies. In addition, the EAEC *(CVD432)* subtype was found to be very high in our study. It is seen as an important cause of diarrhea in epidemic and non-epidemic situations and causes irritable bowel syndrome. Also, STEC and EPEC genes were determined as common genes; *stx1, stx2, bfpA,* and *eaeA* had higher ratios than other genes. When considered among animals, it was found that EPECs and STECs were highest in goats and cattle. It was determined that *stx2* was highest in goat feces, and *stx1* was highest in cattle and goat feces.

E. coli serotypes and subtypes detected at high rates in cattle, sheep, and goat feces can pose a risk to human health by contaminating meat from carcasses. It can cause some important infections originating from red meat, especially in humans. Within the framework of the concept of one health, it will be beneficial to prevent *E. coli* serotypes and subtypes seen in ruminant feces such as cattle, sheep, and goats, especially in slaughterhouses, by taking necessary biosecurity measures to protect public health.

Declarations

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

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Ethical Statement: The study has been approved by the ethics committee of Kırıkkale University (Approval No. E-60821397-619-177126).

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

Author Contributions: SK conceived and executed the idea, designed experiments, analyzed results, and conducted a deep revision of the manuscript. FEA, AUÖ, MY, CÖG and EMÇ collected the samples, performed experiments, and contributed to the implementation of the research. All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

References

1. Food Safety Authority of Ireland (FSAI): Report of the scientific committee of the food safety authority of Ireland. Advice on Shiga toxin-producing *Escherichia coli* (STEC) detection in food. *Annual Report,* 2019. https://www.fsai.ie; *Accessed:* 05/01/2021.

2. Newell DG, La Ragione RM: Enterohaemorrhagic and other Shiga toxin-producing *Escherichia coli* (STEC): Where are we now regarding diagnostics and control strategies? *Transbound Emerg Dis*, 65 (Suppl. 1): 49-71, 2018. DOI: 10.1111/tbed.12789

3. Ferhat L, Chahed A, Hamrouche S, Korichi-Ouar M, Hamdi TM: Research and molecular characteristic of Shiga toxin-producing *Escherichia coli* isolated from sheep carcasses. *Lett Appl Microbiol*, 68 (6): 546-552, 2019. DOI: 10.1111/lam.13142

4. Mühlen S, Dersch P: Treatment strategies for infections with Shiga toxin-producing *Escherichia coli. Front Cell Infect Microbiol*, 10:169, 2020. DOI: 10.3389/fcimb.2020.00169

5. Paddock Z, Shi Xiaorong Shi, Jianfa Bai, Nagaraji TG: Applicability of a multiplex PCR to detect O26, O45, O103, O111, O121, O145, and O157 serogroups of *E. coli* in cattle feces. *Vet Microbiol*, 156, 381-388, 2012. DOI: 10.1016/j.vetmic.2011.11.017

6. European Food Safety (EFSA): The European Union one health 2020 zoonoses report. *EFSA J*, 19 (12):6971, 2021. DOI: 10.2903/j.efsa.2021.6971

7. Fagan PK, Hornıtzky MA, Bettelheim KA, Djordjevic SP: Detection of Shiga-like toxin *(stx1* and *stx2),* intimin *(eaeA),* and enterohemorrhagic *E. coli* (EHEC) Hemolysin (EHEC *hlyA)* genes in animal feces by multiplex PCR. *Appl Environ Microbiol*, 65 (2): 868-872, 1999. DOI: 10.1128/AEM.65.2.868-872.1999

8. Hegde A, Ballal M, Shenoy S: Detection of diarrheagenic *E. coli* by multiplex PCR. *Indian J Med Microbiol,* 30 (3): 279-284, 2012. DOI: 10.4103/0255-0857.99485

9. Scheutz F, Nielsen EM, Moller JF, Boisen N, Morabito S, Tozzoli R, Nataro JP, Caprioli A: Characteristics of the enteroaggregative Shiga toxin/verotoxin-producing *Escherichia coli* O104:H4 strain causing the outbreak of haemolytic uraemic syndrome in Germany, May to June 2011. *Eurosurveillance,* 16 (24):pii=19889, 2011. DOI: 10.2807/ese.16.24.19889-en

10. Magwira CA, Gashe BA, Collison EK: Prevalence and antibiotic resistance profiles of *E. coli* O157:H7 in beef products from retail outlets in Gaborone, Botswana. *J Food Protect*, 68 (2): 403-406, 2005. DOI: 10.4315/0362-028X-68.2.403

11. Pradel N, Livrelli V, De Champs C, Palcoux JB, Reynaud A, Scheutz F, Sirot J, Joly B, Forestier C: Prevalence and characterization of Shiga toxin-producing *E. coli* isolated from cattle, food, and children during a one-year prospective study in France. *J Clin Microbiol*, 38 (3): 1023-1031, 2000. DOI: 10.1128/JCM.38.3.1023-1031.2000

12. Conedera G, Dalvit P, Martini M, Galiero G, Gramaglia M, Goffredo E, Loffredo G, Morabito S, Ottaviani D, Paterlini F, Pezzotti G, Pisanu M, Semprini P, Caprioli A: Verocytotoxin-producing *E. coli* O157 in minced beef and dairy products in Italy. *Int J Food Microbiol*, 96 (1): 67-73, 2004. DOI: 10.1016/j.ijfoodmicro.2004.03.010

13. Brooks JT, Sowers EG, Wells JG, Greene KD, Griffin PM, Hoekstra RM, Strockbine NA: Non-O157 Shiga toxin-producing *E. coli* infections in the United States, 1983-2002. *J Infect Diseases,* 192 (8): 1422-1429, 2005. DOI: 10.1086/466536

14. Bonardi S, Alpigiani I, Tozzoli R, Vismarra A, Zecca V, Greppi C, Bacci C, Bruini I, Brindani F: Shiga toxin-producing *E. coli* O157, O26 and O111 in cattle faeces and hides in Italy. *Vet Rec Open*, 2:e000061, 2015. DOI: 10.1136/vetreco-2014-000061

15. Skočková A, Koláčková I, Kubelová M, Karpíšková R: Shiga toxin-producing *E. coli* (STEC) in the Czech Republic: Characterization of pathogenic strains isolated from pig and cattle carcasses. *J Food Nutr Res,* 56 (4): 362-371, 2017.

16. Oporto B, Ocejo M, Alkorta M, Marimon JM, Montes M, Hurtado A: Zoonotic approach to Shiga toxin-producing *E. coli*: Integrated analysis of virulence and antimicrobial resistance in ruminants and humans. *Epidemiol Infection,* 147 (164): 1-9, 2019. DOI: 10.1017/S0950268820000357

17. Paton AW, Paton JC: Direct detection and characterization of Shiga Toxigenic *Escherichia coli* by multiplex PCR for stx_1, stx_2, *eae*, *ehxA*, and *saa*. *J Clin Microbiol,* 40, 271-274, 2002. DOI: 10.1128/JCM.40.1.271-274.2002

18. Osek J: Development of a multiplex PCR approach for the identification of Shiga toxin-producing *E. coli* strains and their major virulence factor genes. *J Appl Microbiol,* 95, 1217-1225, 2003. DOI: 10.1046/j.1365-2672.2003.02091.x

19. Martins FH, Guth BE, Piazza RM, Leao SC, Ludovico A, Ludovico MS, Dahbi G, Marzoa J, Mora A, Blanco J, Pelayo JS: Diversity of Shiga toxin-producing *Escherichia coli* in sheep flocks of Paraná State, southern Brazil. *Vet Microbiol*, 175 (1): 150-156, 2015. DOI: 10.1016/j.vetmic.2014.11.003

20. Han Y, Liu M, Han Y, Shi N, Wang Q, Cui T, Yang L, Zhang X, Zhu L, Qian H, Jin H, Dong C: Genetic and phylogenetic characterization of Shiga toxin-producing *Escherichia coli* and enteropathogenic *E. coli* from livestock in Jiangsu by using whole-genome sequencing. *J Appl Microbiol,* 132 (5): 3925-3936, 2022. DOI: 10.1111/jam.15494

21. Hua Y, Bai X, Zhang J, Jernberg C, Chromek M, Hansson S, Frykman A, Yang X, Xiong Y, Wan C, Matussek A: Molecular characteristics of eae-positive clinical Shiga toxin-producing *Escherichia coli* in Sweden. *Emer Microbes Infect,* 9 (1): 2562-2570, 2020. DOI: 10.1080/22221751.2020.1850182

22. Liu Y, Li H, Chen X, Tong P, Zhang Y, Zhu M, Su Z, Yao G, Li G, Cai W: Characterization of Shiga toxin-producing *Escherichia coli* isolated from cattle and sheep in Xinjiang province, China, using whole-genome sequencing. *Transbound Emerg Dis*, 69 (2): 413-422, 2022. DOI: 10.1111/tbed.13999

23. Beegum R, Karthikeyan A, Nambiar P, Pu M, Pratheesh MD: Molecular detection and characterization of Enteroaggregative *E. coli* (EAEC) from diarrhoeal samples of human infants and calves. *J Foodborne Zoonotic Dis*, 7 (2): 12-16, 2020.

24. Auvray F, Bience-Terrier C, Um MM, Dupouy V, Nzuzi N, David L, Allais L, Drouet M, Oswald E, Bibbal D, Brugere H: Prevalance and characterization of the seven major serotypes of Shiga toxin-producing *E. coli* (STEC) in veal calves slauhtered in France. *Vet Microbiol,* 282:109745, 2023. DOI: 10.1016/j.vetmic.2023.109754

25. Şeker E, Kuyucuoğlu Y, Sareyyüpoğlu B, Yardımcı H: PCR detection of Shiga toxins, enterohaemolysin and intimin virulence genes of *E. coli* O157:H7 strains isolated from faeces of Anatolian water buffaloes in Turkey. *Zoonoses Public Health,* 57 (7-8): e33-e37, 2010. DOI: 10.1111/J.1863-2378.2009.01285.x

26. Kuyucuoğlu Y, Şeker E, Sareyyüpoğlu B, Gürler Z: Detection of enterohemolysin and intimin genes in *E. coli* O157:H7 strains isolated from calves and cattle in Afyonkarahisar-Turkey. *Kafkas Univ Vet Fak Derg,* 17 (4): 663-666, 2011. DOI: 10.9775/kvfd.2011.4577

27. Yilmaz A, Gun H, Ugur M, Turan N, Yilmaz H: Detection and frequency of *VT1*, *VT2* and *eaeA* genes in *E. coli* O157 and O157:H7 strains isolated from cattle, cattle carcasses and abattoir environment in Istanbul. *Int J Food Microbiol*, 106, 213-217, 2006. DOI: 10.1016/j.ijfoodmicro.2005.05.018

28. Aslantaş Ö, Erdoğan S, Cantekin Z, Gülaçtı İ, Evrendilek GA: Isolation and characterization of verocytotoxin-producing *E. coli* O157 from Turkish cattle. *Int J Food Microbiol*, 106, 338-342, 2006. DOI: 10.1016/j.ijfoodmicro.2005.08.005

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Research Article

Prevalence and Molecular Characterization of Tick-Borne Pathogens in Dogs in Northeast Anatolia Region, Türkiye [1]

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Abstract

This study was carried out to determine the prevalence and molecular characterization of tick-borne pathogens (TBP) *(Babesia* spp., *Theileria* spp., and *Hepatozoon* spp.) in asymptomatic dogs in the northeast Anatolia region of Türkiye. Blood samples of 400 clinically healthy dogs were analyzed using PCR and RLB techniques. TBP prevalence and the relationship between habitat, gender, age, and breed of dogs were determined by using the R package prevalence (version 0.2.0.) and the Pearson chi-square statistics. *Babesia* spp., *Theileria* spp., *Hepatozoon* spp. DNAs were detected in 13% (52/400) of the dogs by PCR, while the prevalence of TBPs was 24.25% (CI: 20.24-28.62) by subsecuantial RLB assay. Sequence analysis of two *Theileria ovis*, two *Babesia canis* and two *Hepatozoon canis* isolates showed 100% identity with the corresponding reference isolates. In this study, *Theileria ovis* positivity was detected for the first time in dogs in the northeast Anatolia region of Türkiye.

Keywords: Dog, Molecular characterization, Prevalence, Tick-borne pathogens

Introduction

Ticks transmit a variety of pathogens to dogs, including *Hepatozoon* spp., *Babesia* spp., and *Theileria* spp., threatening animal health and causing severe economic losses [1-4].

Canine babesiosis is a disease transmitted by *Dermacentor, Rhipicephalus*, and *Haemaphysalis* ticks and caused by large (5 x 2.5 µm) *(Babesia canis, B. vogeli, B. rossi*, and *Babesia* sp.) and small (2 x 1.5 µm) *(B. gibsoni, B. conradae*, and *B. microti-like*) *Babesia* species. In studies conducted in different countries around the world, *Babesia* spp. infections in dogs have been reported with varying prevalence rates [1-3,5-12].

Theileriosis in dogs is manifested with clinical signs including fever, anemia, thrombocytopenia, immune-mediated syndrome, bleeding tendencies, lethargy, pale mucous membranes and corneal opacity [5,9,13]. Several *Theileria* species such as *Theileria annae*, *T. sable, T. luwenshuni, T. buffeli*, *T. orientalis, T. ovis, T. annulata,* and *T. equi* have been reported in dogs [4,5,8,9,13-19].

Hepatozoonosis is a protozoal infection in dogs caused by *Hepatozoon canis* and *H. americanum*. While *H. canis* is transmitted by *Rhipicephalus sanguineus s.l.* ticks, *H. americanum* is transmitted by *Amblyomma maculatum*. In studies conducted in Türkiye and different countries around the world, the prevalence of hepatozoonosis has been found to be at least 1% and at most 57.8%. Only *H. canis* species was found in dogs in Türkiye [1,3,20-29].

Epidemiologic data on hepatozoonosis, babesiosis, and theileriosis in dogs are limited for the northeast Anatolia region. In this study, it was aimed to determine the prevalence and molecular characterization of tick-borne pathogens (TBPs) (*Babesia* spp., *Theileria* spp., and *Hepatozoon* spp.) in dogs in this region of Türkiye.

Material and Methods

Ethical Statement

This study was approved by the Kafkas University Animal Experiments Local Ethics Committee (Approval no: KAÜ-HADYEK: 2020-121).

Study Area

Mountains, plateaus, plains, and rivers occupy a large place in Kars, Ardahan, and Iğdır provinces, which cover an area of approximately 20.000 km^2 in the northeast Anatolia region of Türkiye. The region is located in a strategically important geographical position in terms of bordering Armenia, Iran, Nakhchivan and Georgia. Its economy is mainly based on animal husbandry, especially pasture-based livestock production. Many dogs in the region are also bred to guard the herds. In Kars and Ardahan provinces, the autumn and winter months are cold, while the summer months are cool and rainy. The dry season is not very common because it can rain in all seasons. In Iğdır, there is a temperate climate due to the microclimate created by the earth formations. While the average annual temperature is 4.7°C in Kars and 3.7°C in Ardahan, it is around 12.2°C in Iğdır. In addition to parasitic diseases transmitted by ticks, the distribution of tick species that transmit pathogens also varies regionally and can be found at high rates in different regions of Türkiye [30].

Ardahan, Kars, and Iğdır provinces have large pastures and plateaus because of their geographical location in the northeastern Anatolia region of Türkiye. The economy of the region is primarily based on agriculture and livestock grazing. In the rural parts of the region, dogs are bred in almost every home mainly to guard the livestock. The urban/peri-urban population of dogs in the region is mostly consisted of stray animals. According to the Animal Protection Law (No: 5199) in Turkey, stray animals should be collected in shelters, sterilized, vaccinated, and left to the place where they were found, which are mostly in the streets or periurban areas of the cities. Unfortunately, such regulations do not fulfill the expectations to reduce the number of stray animals. On the contrary, they lead to an increase in the population of stray animals that lack health control.

This study was carried out in the rural and urban/peri-urban areas where dogs were previously diagnosed with parasitic tick-borne infection (records of Kafkas University Faculty of Veterinary Medicine Clinics) [31,32] in the provinces of Kars, Ardahan, and Iğdır in the northeast Anatolian region of Türkiye.

Collection of Blood Samples

Blood samples were taken from *Vena cephalica antebrachii* of 400 asymptomatic dogs from various habitats, ages, genders, and breeds, randomly selected from 22 locations (*Fig. 1*). The location, gender, breed, and age of each animal were recorded.

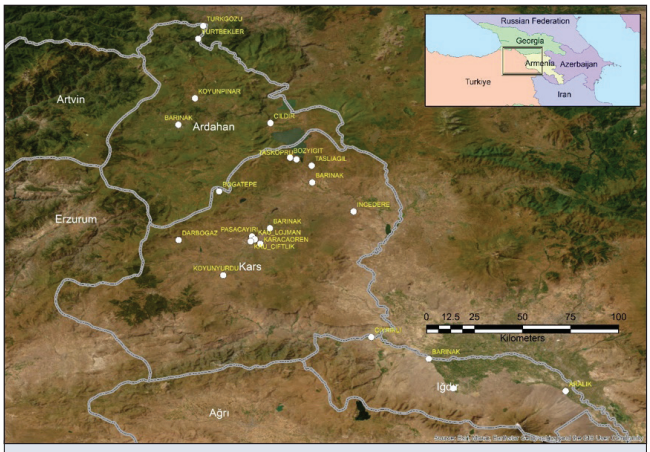

Fig 1. The map of study area

DNA Extraction and PCR Analysis of Blood Samples

DNA was extracted from blood samples using a commercial kit (EcoSpin Blood Genomic DNA Kit, Cat. No: EcoBGD-50x, Türkiye) according to the manufacturer's instructions. DNA samples were stored at -20°C until use. Touchdown PCR was performed on genomic DNA samples using specific primers for TBPs in a thermal cycler (Biometra, Analytik Jena, Germany). The RLBF2 (5'-GACACAGGGAGGTAGTGACAAG-3') and RLBR2 (biotin-5'-CTAAGAATTTCACCTCTGACAGT-3') primers, which amplify the 460-540 bp fragment of the V4 region of the *18SSSU rRNA* gene for *Theileria* spp. and *Babesia* spp., and Hep-F (5′-ATACATGAGCAAAATCTCAAC-3′) and Hep-R (biotin-5′-CTTATTATTCCATGCTGCAG-3′) primers, which amplify the 666 bp fragment of the *18S rRNA* gene for *Hepatozoon* spp., were used.

A total volume of 25 µL reaction mixture containing 8.5 µL nuclease-free water, 12.5 µL master mix (MyTaq, Bioline), 1 µL reverse primer, 1 µL forward primer, and 2 µL of template DNA was used. Positive and negative controls (nuclease-free water) were used to check for contamination [8,23,28,29,33-35]. The PCR products were analyzed on a 1.5% agarose gel using 0.5X TBE buffer and visualized by staining Ethidium bromide under ultraviolet light.

Reverse Line Blotting (RLB)

Species-specific probes were synthesized by a commercial company (Macrogen, Korea), and the RLB hybridization technique was performed for TBPs with some modifications [30,33]. Lyophilized probes were diluted with DNase- and RNase-free bi-distilled water at a concentration of 100 pmol/µL. The PCR products were perpendicularly bouned to the membrane in order to detect TBPs. The nucleotide sequences of the probes used in RLB were shown in *Table 1.*

Table 1. *Nucleotide sequences of probes used in RLB*

Probs	Sequences (5'-3')	References
Babesia catch all 1	ATT AGA GTG TTT CAA GCA GAC	[15]
Babesia catch all 2	ACT AGA GTG TTT CAA ACA GGC	[15]
Theileria annulata	CCT CTG GGG TCT GTG CA	[33]
Theileria/Babesia catchall	TAA TGG TTA ATA GGA (AG)C(AG) GTT G	[33]
Ehrlichia canis	TCT GGC TAT AGG AAA TTG TTA	[34]
Ehrlichia chaffeensis	ACC TTT TGG TTA TAA ATA ATT GTT	[34]
Babesia vogeli	AGC GTG TTC GAG TTT GCC	[36]
Babesia rossi	CGG TTT GTT GCC TTT GTG	[36]
Babesia canis	TGC GTT GAC CGT TTG AC	[36]
Babesia canis 2	TGG TTG GTT ATT TCG TTT TCG	[36]
Babesia canis canis	TGC GTT GAC GGT TTG AC	[36]
Theileria equi	TTC GTT GAC TGC GYT TGG	[36]
Hepatozoon catch all	GCT TTG TAA TTG GAA TGA TAG A	[36]
Ehrlichia ruminantium	AGT ATC TGT TAG TGG CAG	[36]
Anaplasma- Ehrlichia catch all	GGG GGA AAG ATT TAT CGC TA	[36]
Anaplasma marginale	GAC CGT ATA CGC AGC TTG	[36]
Anaplasma ovis	ACC GTA CGC GCA GCT TG	[36]
Anaplasma centrale	TCG AAC GGA CCA TAC GC	[36]
Anaplasma phagocytophilum V1	TTG CTA TAA AGA ATA ATT AGT GG	[36]
Anaplasma phagocytophilum V2	GAA CGG ATT ATT CTT TGT AGC	[36]
Theileria genus-specific	GTT GAA TTT CTG CT(A/G) CAT (C/T)GC	[37]
Theileria ovis	TTT TGC TCC TTT ACG AGT CTT TGC	[37]
Babesia ovis	GCG CGC GGC CTT TGC GTT TAC T	[37]
Babesia gibsoni	CTG CGT TGC CCG ACT CG	[38]
Babesia conradae	CGT TCC CTT CGG GGC	[38]
Theileria annae	CTT ATC ATT AAT TTC GCT TCC GAA CG	[38]
Hepatozoon canis	GCA TAT TCA GGA CTT TTA CTT TGA	[38]

Sequence Analysis

In order to confirm and characterize the species, DNA of the RLB positive samples were subjected to PCR (BTS18SF2/BTS18SR2 and HepF/HepR primers), and resulting products were purified (QIAquick® PCR Purification Kit, Qiagen) and then bidirectionally sequenced (Sanger Dideoxy Sequencing Method). Sequences were oriented, edited, and aligned using Geneious Prime software. The sequences were compared for similarity to sequences deposited in the GenBank database *(www.ncbi.nlm.nih.gov/BLAST).*

Statistical Analysis

Statistical evaluation was performed using Pearson chi-square, Fisher's exact test, and SPSS 20.0 software to determine the relationship between infections and locations. Prevalence values were estimated with R package prevalence (version 0.2.0.) developed by Devleesschauwer et al.[39]. R package apparent prevalence (AP) was calculated using Jeffreys confidence intervals as in the package. True prevalence (TP) was calculated with perfect test assumption and a uniform prior beta distribution in a Bayesian framework with the following model developed using the package: Model {x ~ dbin (AP, n) AP <- SE * TP + (1 - SP) * (1 - TP), SE <- 1, SP <- 1, TP ~ dbeta (1, 1) }.

Results

This study was carried out between April 2021-October 2021, and a total of 22 locations (4 from Iğdır, 8 from Ardahan, and 10 from Kars) were visited. Fifty-two (13%) of 400 PCR products were found positive for *Babesia, Theileria*, and *Hepatozoon* spp. by agarose gel electrophoresis, while this rate was 24.25% (97/400) after RLB hybridization assay. The apparent prevalence of the

tested pathogens was calculated as 3.5% for *T. ovis*, 13% for *B. canis*, and 7.75% for *H. canis*. Mixed infections were not detected in any of the samples tested in the northeastern Anatolia region of Türkiye *(Table 2)*.

In the study, the highest prevalence of *B. canis* was detected as 5.55% (CI: 1.90-12.66) in KafkasX, 6.66% (CI: 1.41-19.70) in the 2-year group, 6.66% (CI: 2.29-15.07) in females and 4.00% (CI: 1.13-10.28) in shelter dogs in Iğdır.

The highest prevalence of *B. canis* in Kars was detected as 39.24% (CI: 29.01-50.23) in KafkasX, 31.68% (CI: 23.22-41.17) in the 3-year and older group, 38.46% (CI: 27.34-50.58) in females, and 71.42% (CI: 57.82-82.58) in shelter dogs.

The prevalence of *B. canis* was highest as 10.34% (CI: 3-25.09) in KafkasX, 10% (CI: 2.13-28.38) in the 2-year group, 3.44% (CI: 0.72-10.60) in males, and 50.00% (CI: 16.68-83.31) in shelter dogs in Ardahan.

The highest prevalence of *H. canis* was detected as 20.83% (CI: 11.24-33.81) in KangalX, 20% (CI: 8.80-36.66) in the 2-year group, 20.77% (CI: 12.89-30.81) in males, and 17.33% (CI: 10.07-27.05) in shelter dogs in Iğdır.

The highest prevalence of *H. canis* in Kars was detected as 3.63% (CI: 0.76-11.15) in KangalX, 2.12% (CI: 0.23-9.51) in the 1-year group, 3.07% (CI: 0.64-9.50) in females, and 2.5% (CI: 0.70-6.52) in rural dogs.

The prevalence of *H. canis* was highest as 10% (CI: 4.28-19.45) in KangalX, 10% (CI: 2.89-24.34) in the 1-year group, 8.33% (CI: 2.40-20.59) in females, and 7.95% (CI: 3.62-14.98) in rural dogs in Ardahan.

The highest prevalence of *T. ovis* was detected as 9.72% (CI: 4.45-18.14) in KafkasX, 10.52% (CI: 5.10-18.88) in the 3-year and older group, 7.79% (CI: 3.31-15.35) in males, and 9.33% (CI: 4.27-17.45) in shelter dogs in Iğdır.

The highest prevalence of *T. ovis* in Kars was detected as 5.71% (CI: 1.20-17.09) in mixed-breed, 4.76% (CI: 0.51-20.17) in the 2-year group, 3.07% (CI: 0.64-9.50) in females, and 3.33% (CI: 1.13-7.72) in rural dogs.

The prevalence of *T. ovis* was highest as 3.44% (CI: 0.37-15.00) in KafkasX, 4.54% (CI: 0.95-13.79) in the 3-year

Table 2. *Distribution of infectious agents in blood samples according to RLB results*

Study Area		***Theileria ovis***	***Babesia canis***	***Hepatozoon canis***
Iğdır	City Center/Shelter	5	2	12
	Aralik Town Center			
	City Center	2	1	1
	Tuzluca/Ciyrikli	1	2	8
Ardahan	City Center/Shelter			
	Posof/Yurtbekler		1	
	Posof/Turkgozu		2	
	Hanak/Koyunpinar			
	Cildir/Town Center			1
	Cildir/Tasliagil			2
	Cildir/Taskopru			4
	Cildir/Bozyigit	2		
Kars	City Center/Shelter		35	
	City Center/Karacaoren			
	Akyaka/Incedere			2
	Arpacay/Shelter			
	City Center/Pasacayiri	2		
	Selim/Koyunyurdu			
	Selim/Darbogaz		1	
	City Center/Bogatepe	2		1
	City Center/University lodging		6	
	City Center/University farm		2	
Total		**14**	**52**	**31**

Table 3. *The distrubition of TBPs according to the habitat, breed, age and gender of dogs.*

Study Area		Breed						Age						Gender				Habitat			
		KangalX		KafkasX		Mixed		≤1		2		3≥		Male		Female		Rural		Shelter	
		PCR	RLB	PCR	RLB	PCR	RLB	PCR	RLB	PCR	RLB	PCR	RLB	PCR	RLB	PCR	RLB	PCR	RLB	PCR	RLB
Iğdır	City Center/Shelter																			15	23
	Aralik Town Center																				
	City Center	4	5	11	18				1	6	7	9	15	9	15	6	8				
	Tuzluca/Ciyrikli	5	6	2	3		2	4	4		1	3	6	5	8	2	3	7	11		
Ardahan	City Center/Shelter																				
	Posof/Yurtbekler				1								1				1		1		
	Posof/Turkgozu				2						2				2				2		
	Hanak/Koyunpinar																				
	Cildir/Town Center		1						1								1		1		
	Cildir/Tasliagil	1	2								1	1	1		1	1	1	1	2		
	Cildir/Taskopru	1	3		1				2			1	2	1	3		1	1	4		
	Cildir/Bozyigit	1	1		1							1	2	1	2			1	2		
Kars	City Center/Shelter	1	5	11	25	3	5	4	8		3	11	24	6	11	9	24			15	35
	Akyaka/ Incedere	1	1				1		1			1	1			1	2	1	2		
	Arpacay/Shelter																				
	City Center/ Pasacayiri					1	2					1	2			1	2	1	2		
	Selim/ Koyunyurdu	1	1					1	1					1	1			1	1		
	Selim/Darbogaz	1	1									1	1			1	1	1	1		
	City Center/Bogatepe	1	2	1	1					1	1	1	2	2	3			2	3		
	City Center/ University Lodging			5	6							5	6	5	6			5	6		
	City Center/ University Farm					1	1					1	1	1	1			1	1		
Total		17	28	30	58	5	11	9	18	7	15	36	64	31	53	21	44	22	39	30	58

***Table 4**. The infection rates of TBPs by PCR and RLB according to the habitat, breed, age and gender of the dogs*

Risk Factors		*Babesia canis*				*Theileria ovis*				*Hepatozoon canis*				PCR		
		N	Pos	MLE%/CI	P	N	Pos	MLE%/CI	P	N	Pos	MLE%/CI	P	N	Pos	P
Age	≤1	108	11	10.18/5.78-17.32	P>0.05	108	0	0/0-3.43	P<0.05	108	7	6.48/3.17-12.77	P>0.05	108	9	P>0.05
	2	71	7	9.85/4.85-18.98		71	1	1.40/0.24-7.55		71	7	9.85/4.85-18.98		71	7	
	3≥	221	34	15.38/11.22-20.72		221	13	5.88/3.46-9.80		221	17	7.69/4.85-11.97		221	36	
Breed	KangalX	163	8	4.90/2.50-9.38	P<0.05	163	2	1.22/0.33-4.3	P>0.05	163	18	11.04/7.10-16.77	P>0.05	163	17	P>0.05
	KafkasX	180	38	21.11/15.78-27.64		180	9	5.00/2.65-9.22		180	11	6.11/3.44-10.61		180	30	
	Mix	57	6	10.52/4.91-21.12		57	3	5.26/1.80-14.36		57	2	3.50/0.96-11.92		57	5	
Gender	Male	239	22	9.20/6.15-13.54	P<0.05	239	10	4.18/2.28-7.52	P>0.05	239	21	8.78/5.81-13.05	P>0.05	239	31	P>0.05
	Female	161	30	18.63/13.37-25.35		161	4	2.48/0.97-6.21		161	10	6.21/3.40-11.05		161	21	
Habitat	Rural	270	14	5.18/3.11-8.51	P<0.05	270	7	2.59/1.26-5.25	P>0.05	270	18	6.66/4.25-10.29	P>0.05	270	22	P<0.05
	Shelter	130	38	29.23/22.09-37.55		130	7	5.38/2.63-10.69		130	13	10.00/5.93/16.35		130	30	

***N:** Number of samples, **Pos:** Positive samples, **MLE:** Maximum likelyhood estimate, **CI:** Confidence interval*

and older group, 3.44% (CI: 0.72-10.60) in males, and 2.27% (CI: 0.47-7.09) in rural dogs in Ardahan.

The distribution and infection rates of TBPs according to the habitat, breed, age, and gender of the dogs were summarized in *Table 3* and *Table 4*.

Analysis of the overall infection rates, it is seen that *B. canis* infections are concentrated in the east and north, while *H. canis* and *T. ovis* infections are concentrated in the south and east of the study area *(Fig. 2)*.

Relevant gene regions or genotypes of *B. canis*, *T. ovis*, and *H. canis* species detected by RLB were bidirectionally sequenced using BTS18SF2/BTS18SR2 and HepF/HepR primers by a commercial company (BM labosis, Türkiye), and compared with the sequences available in GenBank. In sequence analysis, the *18S rRNA* gene, which is widely used in the identification of *Babesia* and *Theileria* species, was targeted. The isolates belonging to *Babesia* and *Theileria* species and genotypes were amplified using Nbab1F/Nbab1R and BTS18SF2/BTS18SR2 primers, and approximately 1600 bp and 1400 bp length amplification products were obtained, respectively.

In this study, three different pathogens *(T. ovis* in two samples, *B. canis* in two samples, and *H. canis* in two samples) were detected. The raw versions of the relevant sequences were analyzed using the Geneious Prime program and consensus sequences were formed. When compared with the GenBank database; Kars City Center-Shelter 1 (Accession number: OR652378) and Kars City Center-Shelter 20 (Accession number: OR652379) sequences of the present study were found to be identical, and as a result of BLAST analysis, the sequences were found to be 100% identical to the *B. canis* sequences previously reported from Kars (KF499115), Erzurum (KY247107, KY247106, KY247105, MT703876, KP745630, MN704759), Romania (KX711222, MW939359, HQ662634), China (MK571831,

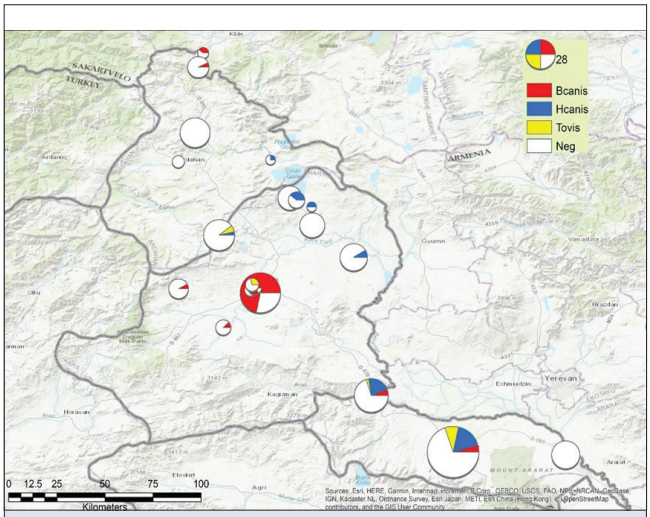

Fig 2. The overall infection rates of TBPs in study area

MK256974, MH143391), Kazakhstan (MK070118), Russia (AY649326, AY962186) and Estonia (KT008057). The *B. canis* sequences reported from Kayseri were found to be 99.86% (MG569903, 2 nucleotide differences) and 98.96% (KJ513199, KJ513200, KJ513201, 4 nucleotide differences) similar.

The two sequences named Bogatepe 18 (Accession number: OR652381) and Iğdır City Center-Shelter 28 (Accession number: OR652382) were found to be identical each other and showed 100% compatible with *T. ovis* sequences reported from China (FJ603460) and Türkiye (AY50845, KT851434, KT851435, KT851433, KT851429). Additionally, these findings were determined to be 99.92% similar to some sequences reported from Türkiye, with only 1 nucleotide difference (MN493111, KT851436).

The two sequences named Iğdır City Center-Shelter 26 (Accession number: OR652383) and Iğdır Tuzluca 17 (Accession number: OR652384) were found to be identical and 100% compatible with the *H. canis* sequences reported from Konya (KX641899), Samsun (KX588232), Ankara (MG077087, MG254611, MG254622, MG254594), Croatia (KT736298), Iran (KX880506, KX880503, KX880502, KT736298), Pakistan (MG209580), and Zambia (LC331054).

Discussion

Several factors including the existing vector population, identification of new vectors, host/reservoir population, animal transfer from endemic areas, climatological changes and the implementation of vector control programs, play a role in the epidemiology of vector-borne diseases [3]. Studies have shown that the northeast Anatolia region of Türkiye has a suitable ecosystem for tick population and a high potential to transmit pathogens [30-32].

Babesiosis in dogs is caused by both large and small *Babesia* species worldwide [2,6,31,40]. Canine babesiosis was first reported in 1935, and *B. vogeli* and *B. gibsoni* were also detected in Türkiye [41]. *Babesia canis* was first reported in the Kars region by Gökçe et al.[31]. In another study conducted in the Kars region [32], *B. canis* was detected in 28 out of 53 (52.8%) dogs by PCR. In studies conducted in Türkiye and different countries around the world, canine babesiosis has been found at rates ranging from 0.1% to 96% [1-3,5-11,41-43]. In addition, *B. canis* has previously been reported in equids in this study area [44]. In this study conducted in the northeast Anatolia region, the prevalence of *B. canis, T. ovis*, and *H. canis* was estimated as 13% (CI: 9.97-16.55), 3.5% (CI: 2.01-5.64) and 7.75% (CI: 5.43-10.67), respectively by RLB. When the study results are evaluated on a provincial scale, the TBP infection rate in Iğdır was 16.05% (22/137) by PCR and 24.81% by RLB (CI: 18.16-32.52). In Kars, the infection rate was 15.97% (27/169) by PCR and 30.17% (CI: 23.63-37.39) by RLB, while it was determined at a rate of 3.19% (3/94) by PCR and 12.76% (CI: 7.17-20.60) by RLB in Ardahan region. While Kars and Ardahan are geographically similar, Iğdır has a different structure compared to these two provinces. In Kars and Iğdır, the severity of the TBP infection has become apparent. The local study on dog tick infestation [45] revealed the presence of potential TBP vectors. Considering the density of tick population, the higher infection rate in the Kars and Iğdır regions compared to the Ardahan region can be attributed to the increased exposure to tick attacks.

TBPs can be seen in all breeds of dogs. Compared to other breeds, German Shepherds and Komondor dogs are more predisposed to TBP infections [2]. In studies [5,7,46] investigating whether dog breeds affect the prevalence of babesiosis, American Pit Bull Terriers were found to be susceptible to *B. gibsoni* infection. Birkkenheuer et al.[47] also stated that the treatment of babesiosis is very challenging, and Tosa Inu and American Staffordshire Terrier breeds are more susceptible than other breeds. As a result of this investigation, the overall infection rates of TBPs were 10.42% (17/163) in KangalX, 16.66% (30/180) in KafkasX and 8.77% (5/57) in mixed-breed dogs by PCR. The prevalence of babesiosis was 4.90% (CI: 2.34-9.04) in KangalX, 21.11% (CI: 15.63-27.50) in KafkasX, and 10.52% (CI: 4.51-20.41) in mixed-breed dogs by RLB. In this region, KafkasX and KangalX dogs are generally bred to guard ruminant herds followed by mixed breeds, and *B. canis* was predominantly detected in KafkasX dogs in all provinces.

According to Solano-Gallego et al.[2], adults are more predisposed to TBP infections than puppies. In a study conducted by Aktaş et al.[8], it was found that age was not a statistically significant factor in canine babesiosis. In our study, the infection rate of TBPs was 8.33% (9/108) in the 1-year group, 9.85% (7/71) in the 2-year group, and 16.28% (36/221) in the 3-year and older group by PCR. Meanwhile the prevalence of *B. canis* was 10.18% (CI: 5.53-16.93) in the 1-year group, 9.85% (CI: 4.51-18.38) in the 2-year group, and 15.38% (CI: 11.09-20.57) in the 3-year and older group by RLB. Although there is unequal distribution among age groups, the prevalence of babesiosis was found to increase with age in RLB results (P>0.05). When infection rates were compared by provinces, *B. canis* infection was most frequently detected in the 3-year and older group in Kars, and in the 2-year group in Ardahan and Iğdır. This situation was once again linked to the density of tick population in the provinces [45].

When the distribution of TBPs was evaluated by gender, there was no significant difference in the prevalence between female and male dogs. However the infection

was more commonly found in male dogs [2,3,28]. In our study, the infection rate of TBPs was 12.97% (31/239) in males and 13.04% (21/161) in females by PCR and the prevalence of babesiosis was 22.17% (CI: 17.26-27.75) in the male group and 27.32% (CI: 20.89-34.57) in the female group by RLB. Although it was statistically insignificant (P>0.05), the distribution of *B. canis* according to gender was found to be concentrated in females in Kars and Iğdır, and males in Ardahan.

Considering the habitat of dogs, in a study, the difference in infection rate of stray and shelter dogs was found insignificant (P>0.05) [8]. However, our study revealed that the infection rate of TBPs was 8.14% in rural and 23.07% in shelter dogs as determined by PCR (P<0.05). Additionally, the prevalence of *B. canis* was found to be 7.50% (CI: 3.77-13.23) in rural and 71.42% (CI: 57.82-82.58) in shelter dogs in Kars, 3.40% (CI: 0.96-8.81) in rural and 50% (CI: 16.68-83.31) in shelter dogs in Ardahan, 3.22% (CI: 0.67-9.94) in rural and 4% (CI: 1.13-10.28) in shelter dogs in Iğdır by RLB (P<0.05). The dogs were not properly cared for and their living conditions were inadequate. They were not received proper protection and control program in terms of TBPs. These findings are overlapped with both the study conducted by Taşçı et al.[45] and the data of this study. In the study conducted by Taşçı et al.[45], it was determined that shelter dogs were more heavily infested with ticks than owned dogs. It has been observed that shelter dogs are infected with *B. canis* at a much higher rate than rural dogs.

Previous studies in Türkiye and various countries around the world have reported the prevalence of hepatozoonosis in dogs to be 1-57.8% [1,3,20,22-29,32,42,48]. In the northeastern Anatolia region of Türkiye, the prevalence of hepatozoonosis in dogs was determined to be 7.75% (CI: 5.43-10.67) by RLB within the reference range. When the results of the study were evaluated at the provincial level, the infection rate of hepatozoonosis was 15.32% (CI: 10.04-22.05) in Iğdır, 1.77% (CI: 0.50-4.66) in Kars, and 7.44% (CI: 3.39-14.06) in Ardahan by RLB. The reason why this determined hepatozoonosis prevalence rate was lower than other regions of Türkiye is believed to be due to both the exposure to tick attacks and the biology of the pathogen. In a study conducted in this region [45], it was determined that *Rhipicephalus sanguineus s.l.*, the vector of *H. canis*, was abundant in Iğdır. Compared to other provinces, the higher hepetozoonosis infection rate in Iğdır could be attributed to the vector tick population density.

Although theileriosis is not a specific infection of dogs, it has a global distribution in dogs. The prevalence of *Theileria* species was found at different rates in previous studies. *T. luwenshuni*, *T. ovis,* and *T. buffeli* were determined in sheepdogs (13%) in Iran [14]. In a study conducted in Myanmar [17], 3 dogs were found to be infected with *T. orientalis*, 3 with *T. buffeli*, 2 with *T.cf. velifera*, 1 with *T. luwenshuni* and 1 with *Theileria sp*. The prevalence of *T. annulata* was found to be 10.78% in Pakistan [4], 1% in Türkiye [8], and 32.5% in Iran [41]. *T. annae* was found to be 3% in Croatia [5], 62.5% in Spain [9], *T. sable* was found to be 13% in Nigeria [15], and *T. equi* was found to be 1.3% in Croatia [5], 19% in France [13], 4% in Nigeria [15], 25.81% in Egypt [18], *T. orientalis* was found to be 0.1% in China [49], while *T. ovis* was found to be 4.7% in South Africa [36], 0.29% in Kyrgyzstan [50]. The mentioned species are typically found in cattle, horses, and sheep as their hosts. However, *T. ovis* was detected at a rate of 3.5% (CI: 2.01-5.64) in dogs in this study. The difference between the infection rates could be attributed to factors such as the treatment for parasitic diseases, geographical locations, number of blood samples and breeding purposes of the dogs.

In conclusion, the health of dogs is not sufficiently taken care of in the northeast Anatolia region. The number of shelter dogs is quite high due to reasons such as lack of infrastructure, unconsciousness, and inadequacy of municipal services. Many rural dogs are bred carelessly and unconsciously. Nowadays, with increasing technological developments, non-specific *Theileria* species have been detected in different hosts. *T. ovis,* an apathogenic agent of sheep, was encountered molecularly for the first time in dogs in this region. Although this situation does not give us information about the actual host of the parasite, it only allowed us to detect the parasite in dogs. The detection of *T. ovis* in dogs indicates a different picture of theileriosis than traditionally thought. Although the findings of our and other studies [5,8,9,15,36,41,50] do not prove that dogs which are used to manage the sheep and cattle herds are hosts of *Theileria* species, they can be interpreted to contribute to the epidemiology of theileriosis. According to our findings, because of close living dogs and ruminant hosts, the lack of host specificity of some species suggests that *T. ovis* may be determined in different hosts and dogs may be natural carriers or reservoirs of *T. ovis.* The results of the study revealed that TBP infection rates were found to be higher in shelter dogs compared to rural dogs. This indicates that the care and rehabilitation conditions of shelter dogs are not healthy enough.

More comprehensive studies should be conducted on the pathogenicity of species or genotypes, distribution of vector ticks, and molecular epidemiology of the detected parasites, and to protect dog health, an effective prevention and control program against parasites in dogs should be implemented as soon as possible.

Declarations

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

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References

1. Düzlü Ö, Abdullah İ, Yildirim A, Önder Z, Çiloğlu A: The investigation of some tick-borne protozoon and rickettsial infections in dogs by Real Time PCR and the molecular characterizations of the detected isolates. *Ankara Üniv Vet Fak Derg,* 61 (4): 275-282, 2014. DOI: 10.1501/Vetfak_0000002642

2. Solano-Gallego L, Sainz A, Roura X, Estrada-Pena A, Miro G: A review of canine babesiosis: The European perspective. *Parasit Vectors,* 9 (1): 336, 2016. DOI: 10.1186/s13071-016-1596-0

3. Guven E, Avcioglu H, Cengiz S, Hayirli A: Vector-borne pathogens in stray dogs in Northeastern Turkey. *Vector Borne Zoonotic Dis,* 17 (8): 610-617, 2017. DOI: 10.1089/vbz.2017.2128

4. Nawab Y, Muhammad I, Ayyub RM, Ahmed A, Muzammil I, Ghumman NZ, Javed MU, Adnan A: Molecular detection of *Theileria annulata* infection: An emerging disease of pet dogs in Pakistan. *Kafkas Univ Vet Fak Derg,* 29 (1): 41-48, 2023. DOI: 10.9775/kvfd.2022.28455

5. Beck R, Vojta L, Mrljak V, Marinculic A, Beck A, Zivicnjak T, Caccio SM: Diversity of *Babesia* and *Theileria* species in symptomatic and asymptomatic dogs in Croatia. *Int J Parasitol,* 39 (7): 843-848, 2009. DOI: 10.1016/j.ijpara.2008.12.005

6. Altay K, Aktaş M, Dumanlı M: Köpek ve Kedilerde Görülen Parazit Hastalıkları. **In,** Özcel MA (Ed): Veteriner Hekimliğinde Parazit Hastalıkları. 2. Baskı, 1061-1065, Türkiye Parazitoloji Derneği, İzmir, 2013.

7. Imre M, Farkas R, Ilie MS, Imre K, Darabus G: Survey of babesiosis in symptomatic dogs from Romania: Occurrence of *Babesia gibsoni* associated with breed. *Ticks Tick Borne Dis,* 4 (6): 500-502, 2013. DOI: 10.1016/j.ttbdis.2013.06.006

8. Aktas M, Ozubek S, Altay K, Ipek ND, Balkaya I, Utuk AE, Kirbas A, Simsek S, Dumanli N: Molecular detection of tick-borne rickettsial and protozoan pathogens in domestic dogs from Turkey. *Parasit Vectors,* 8:157, 2015. DOI: 10.1186/s13071-015-0763-z

9. Miro G, Checa R, Paparini A, Ortega N, Gonzalez-Fraga JL, Gofton A, Bartolome A, Montoya A, Galvez R, Mayo PP, Irwin P: *Theileria annae* (syn. *Babesia microti*-like) infection in dogs in NW Spain detected using direct and indirect diagnostic techniques: Clinical report of 75 cases. *Parasit Vectors,* 8:217, 2015. DOI: 10.1186/s13071-015-0825-2

10. Rene-Martellet M, Moro CV, Chene J, Bourdoiseau G, Chabanne L, Mavingui P: Update on epidemiology of canine babesiosis in Southern France. *BMC Vet Res,* 11:223, 2015. DOI: 10.1186/s12917-015-0525-3

11. Panti-May JA, Rodriguez-Vivas RI: Canine babesiosis: A literature review of prevalence, distribution, and diagnosis in Latin America and the Caribbean. *Vet Parasitol Reg Stud Reports,* 21:100417, 2020. DOI: 10.1016/j.vprsr.2020.100417

12. Muguiro DH, Nekouei O, Lee KY, Hill F, Barrs VR: Prevalence of *Babesia* and *Ehrlichia* in owned dogs with suspected tick-borne infection in Hong Kong, and risk factors associated with *Babesia gibsoni*. *Prev Vet Med,* 214:105908, 2023. DOI: 10.1016/j.prevetmed.2023.105908

13. Fritz D: A PCR study of piroplasms in 166 dogs and 111 horses in France (March 2006 to March 2008). *Parasitol Res,* 106 (6): 1339-1342, 2010. DOI: 10.1007/s00436-010-1804-3

14. Gholami S, Laktarashi B, Shiadeh MM, Spotin A: Genetic variability, phylogenetic evaluation and first global report of *Theileria luwenshuni, T. buffeli*, and *T. ovis* in sheepdogs in Iran. *Parasitol Res,* 115, 2125-2130, 2016. DOI: 10.1007/s00436-016-5005-6

15. Adamu M, Troskie M, Oshadu DO, Malatji DP, Penzhorn BL, Matjila PT: Occurrence of tick-transmitted pathogens in dogs in Jos, Plateau State, Nigeria. *Parasit Vectors,* 7:119, 2014. DOI: 10.1186/1756-3305-7-119

16. Rosa CT, Pazzi P, Nagel S, McClure V, Christie J, Troskie M, Dvir E: Theileriosis in six dogs in South Africa and its potential clinical significance. *J S Afr Vet Assoc,* 85 (1): 1114, 2014.

17. Bawm S, Myaing TT, Thu MJ, Akter S, Htun LL, Win MM, Nonaka N, Nakao R, Katakura K: PCR detection and genetic characterization of piroplasms from dogs in Myanmar, and a possible role of dogs as reservoirs for *Theileria* parasites infecting cattle, water buffaloes, and goats. *Ticks Tick Borne Dis,* 12:101729, 2021. DOI: 10.1016/j.ttbdis.2021.101729

18. Hegab AA, Fahmy MM, Omar HM, Ghattas SG, Mahmoud NE, Abuowarda M: Occurrence and genotyping of *Theileria equi* in dogs and associated ticks in Egypt. *Med Vet Entomol,* 37 (2): 252-262, 2023. DOI: 10.1111/mve.12627

19. Ghauri HN, Ijaz M, Farooqi SH, Ali A, Ghaffar A, Saleem S, Iqbal MK, Aziz MU, Ghani U, Ullah MR, Ahmad HM: A comprehensive review on past, present and future aspects of canine theileriosis. *Microb Pathog,* 126, 116-122, 2019. DOI: 10.1016/j.micpath.2018.10.033

20. Otranto D, Dantas-Torres F, Weigl S, Latrofa MS, Stanneck D, Decaprariis D, Capelli G, Baneth G: Diagnosis of *Hepatozoon canis* in young dogs by cytology and PCR. *Parasit Vectors,* 4:55, 2011. DOI: 10.1186/1756-3305-4-55

21. Altay K, Aktaş M, Dumanlı M: Köpek ve Kedilerde Görülen Parazit Hastalıkları. **In,** Özcel MA (Ed): Veteriner Hekimliğinde Parazit Hastalıkları. 2. Baskı, 1076-1078, Türkiye Parazitoloji Derneği, İzmir, 2013.

22. Rojas A, Rojas D, Montenegro V, Gutierrez R, Yasur-Landau D, Baneth G: Vector-borne pathogens in dogs from Costa Rica: First molecular description of *Babesia vogeli* and *Hepatozoon canis* infections with a high prevalence of monocytic ehrlichiosis and the manifestations of co-infection. *Vet Parasitol,* 199 (3-4): 121-128, 2014. DOI: 10.1016/j.vetpar.2013.10.027

23. Aktas M, Ozubek S, Altay K, Balkaya I, Utuk AE, Kirbas A, Simsek S, Dumanli N: A molecular and parasitological survey of *Hepatozoon canis* in domestic dogs in Turkey. *Vet Parasitol,* 209 (3-4): 264-267, 2015. DOI: 10.1016/j.vetpar.2015.02.015

24. Aydin MF, Sevinc F, Sevinc M: Molecular detection and characterization of *Hepatozoon* spp. in dogs from the central part of Turkey. *Ticks Tick Borne Dis,* 6 (3): 388-392, 2015. DOI: 10.1016/j.ttbdis.2015.03.004

25. Maia C, Almeida B, Coimbra M, Fernandes MC, Cristovao JM, Ramos C, Martins A, Martinho F, Silva P, Neves N, Nunes M, Vieira ML, Cardoso L, Campino L: Bacterial and protozoal agents of canine vector-borne diseases in the blood of domestic and stray dogs from southern Portugal. *Parasit Vectors,* 8:138, 2015. DOI: 10.1186/s13071-015-0759-8

26. Piratae S, Pimpjong K, Vaisusuk K, Chatan W: Molecular detection of *Ehrlichia canis*, *Hepatozoon canis* and *Babesia canis vogeli* in stray dogs in Mahasarakham province, Thailand. *Ann Parasitol,* 61 (3): 183-187, 2015. DOI: 10.17420/ap6103.05

27. Hamel D, Shukullari E, Rapti D, Silaghi C, Pfister K, Rehbein S: Parasites and vector-borne pathogens in client-owned dogs in Albania: Blood pathogens and seroprevalences of parasitic and other infectious agents. *Parasitol Res,* 115 (2): 489-499, 2016. DOI: 10.1007/s00436-015-4765-8

28. Aktas M, Ozubek S: A survey of canine haemoprotozoan parasites from Turkey, including molecular evidence of an unnamed *Babesia*. *Comp Immunol Microbiol Infect Dis,* 52, 36-42, 2017. DOI: 10.1016/j.cimid.2017.05.007

29. Orkun Ö, Nafiye K, Sürsal N, Çakmak A, Nalbantoğlu S, Karaer Z: Molecular characterization of tick-borne blood protozoa in stray dogs

from Central Anatolia Region of Turkey with a high-rate *Hepatozoon* infection. *Kafkas Univ Vet Fak Derg,* 24 (2): 227-232, 2018. DOI: 10.9775/kvfd.2017.18678

30. Aydin N, Vatansever Z, Arslan MO: Molecular epidemiology of *Babesia* and *Theileria* species in sheep in Kars region of Turkey. *Türkiye Parazitol Derg,* 46 (1): 20-27, 2022. DOI: 10.4274/tpd.galenos.2021.09709

31. Gökçe E, Kırmızıgül AH, Taşçı G, Uzlu E, Gündüz N, Vatansever Z: Türkiye'de köpeklerde *Babesia canis canis*' in klinik ve parazitolojik olarak ilk tespiti. *Kafkas Univ Vet Fak Derg,* 19 (4): 717-720, 2013. DOI: 10.9775/kvfd.2013.7598

32. Tasci GT: *Hepatozoon canis* and *Babesia canis canis* infections in dogs in Kars Region: Preliminary study. **In,** *Proceedings of 2nd International Conference on Agriculture, Forest, Food Sciences and Technologies (ICAFOF-2018).* 2-5 April, Çeşme-İzmir, Turkey, 2018.

33. Gubbels JM, de Vos AP, van der Weide M, Viseras J, Schouls LM, de Vries E, Jongejan F: Simultaneous detection of bovine *Theileria* and *Babesia* species by reverse line blot hybridization. *J Clin Microbiol,* 37 (6): 1782-1789, 1999. DOI: 10.1128/JCM.37.6.1782-1789.1999

34. Schouls LM, Van De Pol I, Rijpkema SG, Schot CS: Detection and identification of *Ehrlichia*, *Borrelia burgdorferi* sensu lato, and *Bartonella* species in Dutch *Ixodes ricinus* ticks. *J Clin Microbiol,* 37 (7): 2215-2222, 1999. DOI: 10.1128/JCM.37.7.2215-2222.1999

35. Aydin MF, Aktas M, Dumanli N: Molecular identification of *Theileria* and *Babesia* in sheep and goats in the Black Sea Region in Turkey. *Parasitol Res,* 112 (8): 2817-2824, 2013. DOI: 10.1007/s00436-013-3452-x

36. Matjila PT, Leisewitz AL, Jongejan F, Penzhorn BL: Molecular detection of tick-borne protozoal and ehrlichial infections in domestic dogs in South Africa. *Vet Parasitol,* 155 (1-2): 152-157, 2008. DOI: 10.1016/j.vetpar.2008.04.012

37. Nagore D, Garcia-Sanmartin J, Garcia-Perez AL, Juste RA, Hurtado A: Identification, genetic diversity and prevalence of *Theileria* and *Babesia* species in a sheep population from Northern Spain. *Int J Parasitol,* 34 (9): 1059-1067, 2004. DOI: 10.1016/j.ijpara.2004.05.008

38. Yisaschar-Mekuzas Y, Jaffe CL, Pastor J, Cardoso L, Baneth G: Identification of *Babesia* species infecting dogs using reverse line blot hybridization for six canine piroplasms, and evaluation of co-infection by other vector-borne pathogens. *Vet Parasitol,* 191 (3-4): 367-373, 2013. DOI: 10.1016/j.vetpar.2012.09.002

39. Devleesschauwer B, Torgerson PR, Charlier J, Levecke B, Praet N, Dorny P, Berkvens D, Speybroeck N: Prevalence: Tools for prevalence assessment studies. R package version 0.2.0. 2013.

40. Aysul N, Ural K, Ulutaş B, Eren H, Karagenç T: First detection and molecular identification of *Babesia gibsoni* in two dogs from the Aydın Province of Turkey. *Turk J Vet Anim Sci,* 37 (2): 226-229, 2013. DOI: 10.3906/vet-1201-45

41. Habibi G, Imani A, Afshari A, Bozorgi S: Detection and molecular characterization of *Babesia canis vogeli* and *Theileria annulata* in free-ranging dogs and ticks from Shahriar County, Tehran Province, Iran. *Iran J Parasitol,* 15 (3): 321-331, 2020. DOI: 10.18502/ijpa.v15i3.4196

42. Kaur N, Singh H, Sharma P, Singh NK, Kashyap N, Singh NK: Development and application of multiplex PCR assay for the simultaneous detection of *Babesia vogeli, Ehrlichia canis* and *Hepatozoon canis* in dogs. *Acta Trop,* 212:105713, 2020. DOI: 10.1016/j.actatropica.2020.105713

43. Hirata H, Omobowale T, Adebayo O, Asanuma N, Haraguchi A, Murakami Y, Kusakisako K, Ikeda K, Asakawa M, Suzuki K, Ishihara C, Ikadai H: Identification and phylogenetic analysis of *Babesia* parasites in domestic dogs in Nigeria. *J Vet Med Sci,* 84 (3): 338-341, 2022. DOI: 10.1292/jvms.21-0636

44. Ozubek S, Aktas M: Genetic diversity and prevalence of piroplasm species in equids from Turkey**.** *Comp Immunol Microbiol Infect Dis***,** 59, 47-51, 2018. DOI: 10.1016/j.cimid.2018.08.005

45. Taşçı GT, Aydın N, Ölmez N, Yiğit M, Işık ME, Vatansever Z: Tick infestation in stray dogs: Kars, Ardahan, Iğdır. **In,** *Proceedings of the "1st International Livestock Farming" Conference Dedicated to the 100th Anniversary of the Birth of the National Leader of Azerbaijan Heydar Aliyev and the Republic of Türkiye*. 20-21 October, Lenkeran, Azerbaijan 2023.

46. Teodorowski O, Kalinowski M, Winiarczyk D, Dokuzeylul B, Winiarczyk S, Adaszek L: *Babesia gibsoni* infection in dogs-A European perspective. *Animals,* 12 (6): 730, 2022. DOI: 10.3390/ani12060730

47. Birkenheuer AJ, Levy MG, Breitschwerdt EB: Efficacy of combined atovaquone and azithromycin for therapy of chronic *Babesia gibsoni* (Asian genotype) infections in dogs. *J Vet Intern Med,* 18 (4): 494-498, 2004. DOI: 10.1892/0891-6640(2004)18<494:eocaaa>2.0.co;2

48. Viljoen S, O'Riain MJ, Penzhorn BL, Drouilly M, Vorster I, Bishop JM: Black-backed jackals *(Canis mesomelas)* from semi-arid rangelands in South Africa harbour *Hepatozoon canis* and a *Theileria* species but apparently not *Babesia rossi*. *Vet Parasitol Reg Stud Reports,* 24:100559, 2021. DOI: 10.1016/j.vprsr.2021.100559

49. Xu D, Zhang J, Shi Z, Song C, Zheng X, Zhang Y, Hao Y, Dong H, Wei L, El-Mahallawy HS: Molecular detection of vector-borne agents in dogs from ten provinces of China. *Parasites Vectors,* 8:501, 2015. DOI: 10.1186/s13071-015-1120-y

50. Altay K, Erol U, Sahin OF, Aydin MF, Aytmirzakizi A, Dumanli N: First molecular evidence of *Babesia vogeli*, *Babesia vulpes*, and *Theileria ovis* in dogs from Kyrgyzstan. *Pathogens*, 12 (8):1046, 2023. DOI: 10.3390/pathogens12081046

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Research Article

Assessment of *In-vitro* Cytotoxicity and *In-ovo* Virucidal Antiviral Efficacy of Various Plant Extracts and Bioactive Molecules

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Abstract

The viral diseases that occurred in recent years have increased the interest in non-toxic to healthy cells and naturally isolated agents to struggle with these diseases. The key intention of this research is to examine both antiviral potentials against the Infectious Bronchitis model virus (IBV) and cytotoxic activities on determined cell lines of different active ingredients and medical herbs extracts for developing new antiviral agents or drugs towards SARS-CoV-2. The antiviral potency of the samples against IBV was determined as *in ovo* virucidal antiviral activity in specific pathogen-free (SPF) embryonated chicken eggs (ECEs). To detect antiviral activity, the haemagglutination test was performed after 48 h of incubation for all samples. The cytotoxic activity of the samples was identified on HepG2, Caco-2, HeLa, HEK293, PANC-1, PC-3, A549, MDA-MB-231, and CCD-34Lu cell lines by the MTT protocol. *Hypericum perforatum* extract was found to have a dominant role in cytotoxicity and antiviral activity. In addition, while nobiletin and *Sambucus nigra* do not exhibit cytotoxic activity on cells, they play a significant role in antiviral activity. As a consequence of our investigation, the cytotoxic and antiviral properties of *Laurus nobilis, H. perforatum*, and *S. nigra* extracts were found remarkable and the potential of these extracts was demonstrated.

Keywords: Antiviral, Cytotoxicity, Extract, *In ovo*, Infectious bronchitis virus

Introduction

Population in different regions can be negatively impacted by various viruses, which can induce epidemics and pandemics, deaths, and severe economic losses. Studies conducted by the World Health Organization (WHO) have stated that the population affected by viral pathogens reaches 80%. Therefore, developing efficient treatments against viruses is crucial to prevent these losses. In addition to the current strategies against viruses, the search for alternative approaches has gained momentum. For this purpose, *in vitro* and *in vivo* studies have been used to specify the potential of various natural plant sources as therapeutic agents against different viruses [1]. Antiviral effects for plant-derived components have been broadly evaluated on lots of viruses such as influenza, herpes simplex, and respiratory syncytial virus in the last two decades [2]. One of the critical virus families among these viruses is the *Coronaviridae* which has created major socioeconomic problems and caused serious outbreaks or pandemics worldwide [3]. Severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) which belongs to this family, has swiftly spread into the population and has caused the COVID-19 pandemic [4]. This novel coronavirus, which causes numerous deaths and diseases globally, has once again revealed the necessity of vaccines and therapeutics studies. Despite significantly increasing investment in the pharmaceutical field, prophylactic treatments or existent antiviral approaches for coronaviruses are currently limited. Herbal medicines and naturally-purified molecules may guide new antiviral drugs [3]. Infectious Bronchitis virus (IBV), taking part

in the same virus family as SARS-CoV-2, is known for its high contagion rate, especially among chickens. It is a Gamma coronavirus in the *Coronaviridae* family [5] and causes dangerous respiratory diseases by infecting poultry through the respiratory system [6]. Owing to weak cross-protection of the vaccines among virus serotypes, alternative approaches for effective antiviral agents have become imperative to preventing IBV infection. The research on new antiviral drugs gravitates toward natural products because of resistance against current antiviral agents [7].

In recent years, scientific curiosity has increasingly concentrated on active compounds of plants responsible for biological properties and therapeutic effects [8]. According to research, plant-derived compounds have been used in medicine at a rate of 80% because of active molecules such as alkaloids, glycosides, steroids and tannins. They can exhibit potent bioactivities as antimicrobial, antioxidant, antibacterial, antifungal, and anti-inflammatory in a variety of living systems [9,10]. Furthermore, the cytotoxic and antiviral properties of these plants provide opportunities for studies to discover new therapeutic agents [8,9]. Many studies indicate that phenol content of different herbs plays active role in antiviral activity and affects different RNA viruses generally by inhibition of nucleic acids, gene expression or entry protein [11]. The cytotoxic effect and antiviral activity studies performed by extracts obtained from *Camellia sinensis*, *Hypericum perforatum*, *Sambucus nigra* and molecules such as tangeretin, catechins, nobiletin have also importance in the challenging against viruses.

The focus of this research is to investigate the selected extracts and active ingredients for antiviral activity against IBV. Thus, compounds having anti-IBV activity against model virus system are planned to be pioneers in developing new antiviral agents for SARS-CoV-2.

Material and Methods

Ethical Statement

This study was approved by the Ege University Animal Experiments Local Ethics Committee (Approval no: 2020/051).

Plant Extracts

The plant extracts performing in this study were supplied by NPRO Natural Products Company and prepared by dissolving in Dimethyl sulfoxide (DMSO) to use in all tests.

Cell Lines

Human prostate adenocarcinoma (PC-3), human colorectal adenocarcinoma (Caco-2), human embryonic kidney (HEK293), human cervix adenocarcinoma (HeLa), human alveolar adenocarcinoma (A549), human pancreatic carcinoma (PANC-1), human breast adenocarcinoma (MDA-MB-231), human hepatocarcinoma (HepG2), and a healthy human lung fibroblasts (CCD-34Lu) cells were purchased from ATCC (Manassas, VA, USA) and used for evaluating the cytotoxic activity of samples. The cell lines were cultured at 37°C in a humidified atmosphere containing 5% CO_2 in Dulbecco's Modified Eagle Medium/ Nutrient Mixture F-12 (DMEM/F12) supplemented with 10% fetal bovine serum (FBS), 400 µg/mL penicillin/ streptomycin mixture, and 1% L-glutamine (Gibco, NY, USA).

Virus and Specific Pathogen-Free Embryonated Chicken Eggs (SPF-ECEs)

The IBV D274 strain, present in our laboratory stock was used for antiviral activity assay. Specific pathogen-free embryonated chicken eggs (SPF-ECEs) that were procured from İzmir Bornova Veterinary Control Institute, Türkiye were used for the determination of antiviral potential.

In vitro Cytotoxicity Assay

Cytotoxicity of samples was identified by performing MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] test, a common assay for evaluating cytotoxicity of agents depending on the viability of cells [12]. For the MTT assay, the cell lines having initially $1x10^4$ cells/well concentration were cultivated in 96-well microplates and overnight incubation was performed at 37°C in a humidified atmosphere. Then, the samples at three determined concentrations (0.5, 5, and 50 µg/mL) were treated with the cells and incubated for a further 48 h. While all samples except *S. nigra* were incubated in all selected cell lines, *S. nigra* has been treated with 2 cells (CCD-34Lu and HEK293). Following the incubation for 48 h, MTT solution (diluted from 2.5 mg/mL stock) was added to the cells at 20 µL volume and incubated for 4 h. After the incubation period, formazan crystals were dissolved by using DMSO and the spectrophotometer (Thermo Fisher Scientific) was used to measure the absorbance values at 570 nm. Percent viability of treated cells was assessed by comparing control cells. The half maximal inhibitory concentration (IC_{50}) values were determined by calculation on Prism 5 software (GraphPad5, San Diego, CA, USA).

Preparation of Virus and Inoculation of SPF-ECEs *in ovo*.

Antiviral activity of some plant extracts and molecules were evaluated as virucidal effects against IBV *in ovo* model [13]. Reed and Muench method [14] was used to calculate the Embryo Infective Dose 50% (EID_{50}) by performing the hemagglutination test specified in the WOAH protocol. Based on Reed-Muench calculation, $100EID_{50}$/0.1 mL

virus stock was used in antiviral experiments [15]. The desired final concentration of molecules and extracts diluting by phosphate buffer saline (PBS) was prepared and mixed with the virus 1:1 ratio for 1 h incubation at 25°C. Randomly grouped (n=4) 9-11-day-old SPF-ECEs were inoculated into the chorioallantoic membrane with the 0.1 mL sample-virus mixture. Favipiravir used for clinically antiviral agent was tested in control groups as positive control at 10 mg/g amd 25 mg/g concentrations, respectively. SPF-ECEs were incubated for 48 h and their viability was checked daily. After the incubation period, the SPF-ECEs were stored overnight at +4°C. Following overnight storage, chorioallantoic fluid (CAF) of them were collected to detect HA titer [16].

Hemagglutination (HA) Assay

For the hemagglutination (HA) test, V-bottom 96-well microplates were used according to the WOAH protocol [15]. In the HA assay, 25 mL of PBS was put into all wells. Then, 25 mL of collected CAF was placed in the first column of the microplate for each sample and diluted 2-fold to the last column of the microplate. As a final step, 25 mL chicken red blood cells (1% v/v)(RBC) were added all wells to incubate them for 45 min at 25°C. After incubation, button shape formation for wells was evaluated as HA negative and agglutination was considered as HA positive. The highest HA dilutions and percent viability of the eggs were recorded [15,16].

Statistical Analysis

Statistical analysis of the results was calculated by one-way ANOVA using SPSS (version 23, SPSS Inc., Chicago, IL, USA). Mean and standard deviation (SD) values are expressed and statistical significance (P<0.05) was determined.

Results

In vitro Cytotoxicity Assay

The cytotoxicity assay of samples on different cancerous and noncancerous cell lines was realized. The effect of samples on treated cells was first examined under the microscope and statistically analyzed. According to the MTT assay results the obtained IC_{50} values of samples was given *(Table 1)*. Among the samples, *Abies* spp. essential oil, along with *Laurus nobilis*, *H. perforatum*, and gallic acid had cytotoxic effects on the cells to which they were applied (P<0.05). *H. perforatum* was performed to evaluate the cytotoxic effect for different cancerous and noncancerous cell lines and a remarkable cytotoxic effect has been seen on PANC-1 (IC_{50}=6.69±1.32 mg/mL) (P<0.001) and HeLa (IC_{50}=10.86±0.13 mg/mL) (P<0.001), while both cell lines have approximate values with doxorubicin (IC_{50}=5.52 mg/mL, IC_{50}=9.04 mg/mL, respectively). Also, the results demonstrated that *L. nobilis* extract shows a significant impact on HeLa, PANC-1 and HepG2 cell lines (IC_{50}=11.71±0.12, 7.54±1.20 and 7.33±0.82 mg/mL respectively)(P<0.001). Moreover, the PANC-1 cell line has been observed as more active than doxorubicin (IC_{50}=9.04 mg/mL) which chemotherapeutic agent. *Abies* spp. essential oil against Caco-2 and HeLa cells cytotoxic effect has been not demonstrated (P>0.05). The highest dose of *Abies* spp. essential oil (0.5% v/v) was found to have a percent viability of 50% in PC3, MDA-MB-231 and

Table 1. *The IC_{50} values of C. sinensis, S. nigra, H. perforatum, E. purpurea, M. officinalis, oleuropein, L. nobilis, neohesperidin, nobiletin, gallic acid, tangeretin, catechin hydrate, and doxorubicin µg/mL*

Sample	MDA-MB-231	A549	PANC-1	PC-3	CCD-34Lu	HEK293	HepG2	HeLa	Caco-2
Camellia sinensis	>50	>50	ND	>50	21.65±6.18	>50	>50	>50	ND
Sambucus nigra	-	-	-	-	ND	>50	-	-	-
Echinacea purpurea	>50	ND	ND	ND	ND	>50	>50	>50	ND
Melissa officinalis	>50	ND	ND	>50	ND	>50	ND	>50	ND
Hypericum perforatum	19.53±0.73	18.78±1.75	10.86±0.13	14.27±1.50	13.58±1.17	28.32±1.08	45.38±5.50	6.69±1.32	14.76±1.98
Oleuropein	ND	ND	ND	ND	ND	ND	ND	ND	ND
Laurus nobilis	40.09±0.40	16.14±2.14	7.54±1.20	31.56±6.85	19.82±2.28	27.95±3.23	7.33±0.82	11.71±0.12	46.08±5.21
Neohesperidin	ND	ND	ND	ND	ND	ND	ND	ND	ND
Nobiletin	ND	>50	ND	ND	ND	>50	ND	ND	ND
Gallic acid	42.21±2.6	36.91±5.38	42.06±6.93	38.8±1.65	25.70±1.2	25.70±1.2	40.19±2.6	20.40±0.34	24.14±2.8
Tangeretin	ND	ND	ND	ND	ND	>50	ND	ND	47.36±6.1
Catechin hydrate	ND	ND	ND	ND	ND	ND	ND	ND	>50
Doxorubicin	14.77	2.36	9.04	9.88	4.42	2.76	6.79	5.52	4.45

ND: non detected, -: not analyzed

HEK293 cells among other cells (P<0.01). Besides, it has been found that high cytotoxicity was performed on CCD 34-Lu, PANC-1, A549, HepG2 cells (P<0.001). Although *C. sinensis* and tangeretin demonstrated comparable results, *C. sinensis* showed statistically more significant results than tangeretin in HEK293 and HeLa cell lines (P<0.01). In contrast, tangeretin was more effective than *C. sinensis* in Caco-2 cell line (P<0.01). The other extracts were not active at the highest dose (50 mg/mL) tested.

In ovo Antiviral Activity

The *in ovo* experiment was used to assess the antiviral activity of the plant extracts against IBV [16]. After 48 h incubation of SPF-ECEs, the numbers of embryo deaths, and percentage viability of eggs were recorded and mean HA titers were calculated *(Table 2)*. In addition, embryos after 48 h incubation with sample-virus mixture were evaluated. Deaths in SPF-ECEs were observed between 24-48 h in virus control and vehicle control groups. In

Table 2. *Mortality and HA titers of SPF-ECE's after 48 h incubation*

Sample Groups (n=4)	Concentration	Egg Mortality			Mortality%	Mean HA Titer	Mean HA Titer (log_2-based)
		24 h	48 h	Total			
Untreated control		-	-	0/4	0%	0	0
Virus control		-	1	1/4	25%	2048	11
Vehicle control (5% DMSO)		-	1	1/4	25%	2048	11
Favipiravir (antiviral agent)	10 mg/g	-	-	0/4	0%	512	9
Camellia sinensis	5 mg/g	-	-	0/4	0%	512	9
	10 mg/g	-	-	0/4	0%	512	9
Sambucus nigra	0.1 mg/g			0/4	0%	256	8
	1 mg/g			1/4	25%	32	5
Echinacea purpurea	5 mg/g	-	-	0/4	0%	512	9
	10 mg/g	-	-	0/4	0%	128	7
Melissa officinalis	5 mg/g	-	1	1/4	25%	1024	10
	10 mg/g	-	-	0/4	0%	512	9
Hypericum perforatum	0.1 mg/g	-	-	0/4	0%	64	6
	1 mg/g	-	1	1/4	25%	0	0
	5 mg/g	-	-	0/4	0%	0	0
	10 mg/g	-	-	0/4	0%	0	0
Abies spp. volatile oil	0.5% (v/v)	-	-	0/4	0%	512	9
Oleuropein	5 mg/g	-	-	0/4	0%	512	9
	10 mg/g	-	-	0/4	0%	64	6
Laurus nobilis	5 mg/g	-	1	1/4	25%	256	8
	10 mg/g	-	-	0/4	0%	128	7
Tangeretin	5 mg/g	-	1	1/4	25%	512	9
	10 mg/g	-	1	1/4	25%	128	7
Cateshin hydrade	5 mg/g	-	-	0/4	0%	512	9
	10 mg/g	-	-	0/4	0%	128	7
Neohesperidin	5 mg/g	-	-	0/4	0%	512	9
	10 mg/g	-	-	0/4	0%	256	7
Gallic acid	5 mg/g	-	-	0/4	0%	512	9
	10 mg/g	-	-	0/4	0%	128	8
Nobiletin	5 mg/g	-	-	0/4	0%	512	9
	10 mg/g	-	-	0/4	0%	64	6

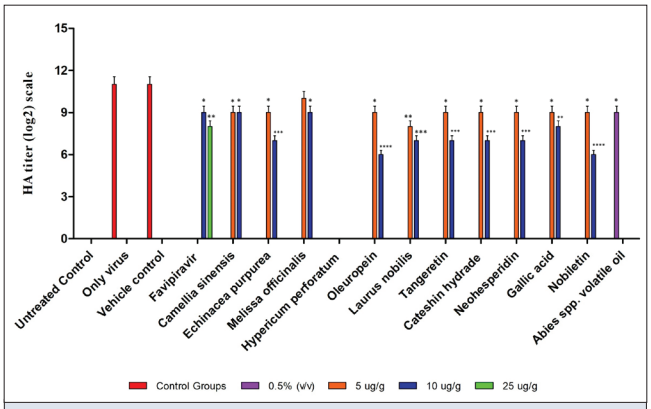

Fig 1. HA titers of IBV-extract/molecule samples on SPF-ECEs (P<0.05, P<0.01, P<0.001, P<0.0001)

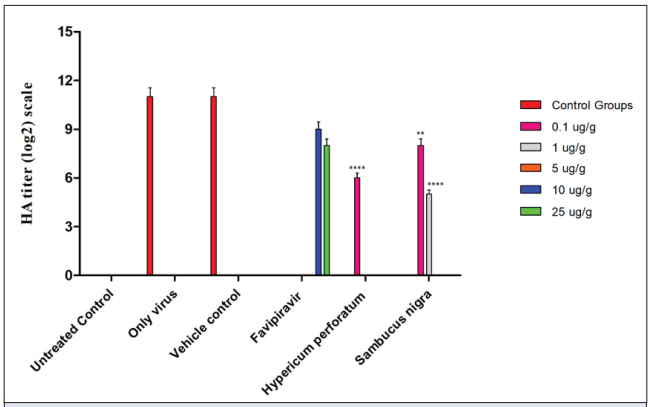

Fig 2. HA titers of IBV-*H. perforatum*, IBV-*S. nigra* samples on SPF-ECEs (P<0.05, P<0.01, P<0.001, P<0.0001)

both groups, the HA titer was calculated as 2048. It was determined that DMSO vehicle control did not affect deaths and HA titer. In the untreated control group, no deaths in SPF-ECEs were detected and HA titers were negative. As a positive antiviral control, favipiravir which can be used commercial antiviral agent, was examined at 10 and 25 mg/g concentrations. It was found that favipiravir could decrease the HA titer compared to virus control. Depending on the results obtained, $\log_2$-based HA titers were determined 3-fold at 10 mg/g and 4-fold at 25 mg/g lower than control. Between 24-48 h observation, 25% embryo deaths were determined for the following groups; *S. nigra* at 1 mg/g, *H. perforatum* at 1 mg/g, *L. nobilis* at 5 mg/g, *Melissa officinalis* at 5 mg/g, tangeretin at 5 mg/g and 10 mg/g. These deaths may be due to some manipulation during inoculation or weak embryo development [17]. All samples except *S. nigra* were tested for antiviral activity at concentrations of 5 mg/g and 10 mg/g. *H. perforatum* was also tested at a concentration of 0.1 and 1 mg/g besides 5 mg/g and 10 mg/g. *In ovo* antiviral activity of *S. nigra* with known antiviral activity was measured at 0.1 and 1 mg/g concentrations. The concentration of *Abies* spp. essential oil was prepared as 0.5% by volume (v/v). The average HA titers of all samples which were analyzed for 5 mg/g, and 10 mg/g were shown *(Fig. 1)*. When all samples were compared, the most potent antiviral activity results were observed in *S. nigra* and *H. perforatum*. Therefore the lower concentrations for the two extracts were attempted *(Fig. 2)*. The efficacy of all groups was evaluated statistically compared to the virus control group. All extracts were statistically significant (P<0.05) except *M. officinalis* (at 5 mg/g). Compared to control groups, it showed that samples at concentrations of 5 mg/g and 10 mg/g had the potential to significantly lower the HA titer by 1 to 5 $\log_2$ HA. Depending on the dose, the extracts showed more effective antiviral activity at a concentration of 10 mg/g. Oleuropein and nobiletin at 10 mg/g had also the highest statistical significance (P<0.0001). Also, *S. nigra* (P<0.01 at 0.1 mg/g) and *H. perforatum* (P<0.0001 at 0.1 mg/g) gave statistically significant results. Antiviral activity was negative in *H. perforatum* at 1, 5 and 10 mg/g concentrations. At 0.1 mg/g, it significantly reduced HA activity with 5 $\log_2$ HA. In the *S. nigra* sample, the antiviral activity value was found between 0.1 mg/g and 1 mg/g. HA activity decreased by 6 $\log_2$ HA at 1 mg/g, while at 0.1 mg/g, HA activity decreased by 3 $\log_2$ HA. This allowed the focus on the potential antiviral activity of both samples.

Discussion

In our study, it has been demonstrated the cytotoxic effects and antiviral activity of medicinal plants and active ingredients. *L. nobilis* was one of these samples and its fruit and leaves have been used in traditional Turkish medicine as antimicrobial, antiseptic, antivenom, diuretic agent and stomach-ached curative [18]. It was reported in studies that aqueous extract obtained from leaves of *L. nobilis* has cytotoxic effects without genotoxicity [19]. According to Oliveira et al.[20], although there is no relationship between cytotoxicity and genotoxic effect, changes in genetic material may occur indirectly. Research findings indicate that costunolide, zaluzanin D, and sesquiterpenes obtained from *L. nobilis*, exhibit potentially inhibitory effects by preventing the growth of human promyelocytic leukemia (HL-60) cells [21]. Compared with other results, the higher cytotoxicity results on PANC-1, HeLa, and HepG2 cells were obtained for *L. nobilis* in our study. Gallic acid which is one of the plant phenols, exhibits selective cytotoxicity to tumor cells compared with a normal cell. In a study performed on HL-60RG cells, it was indicated that gallic acid has the potency to induce apoptosis by way of reactive oxygen forms [22]. This study also evaluated that gallic acid reduced cell viability by 20-50% on different cancerous and non-cancerous cell lines. In parallel with the studies in the literature, obtained results in this experiment emphasize that a certain dose has a high cytotoxic effect on the cell. Spectrometric indications have proven that the activity of *H. perforatum* extracts results from phloroglucinols which

include hyperforin, and zaphthodianthrones which involve hypericin [23]. Hypericin as a potent natural photosensitizer and hyperforin as an inhibitor of the drug-metabolizing enzyme in liver, activate the biological system strongly [24]. *H. perforatum* also involves various active components such as avicularin, rutin, quercetin and derivatives to act as ROS scavengers [25]. It has been shown in another study that hyperoside which is another flavanol of *H. perforatum*, not only prevents of PC12 cells propagation, but also promotes the healthy cell growth [26].

Herbal medicines have been historically preferred for therapy against various viral diseases. These phyto-chemicals are considered to be crucial antiviral compounds for treatment opposed many important viruses. The potential usage of many extracts and bioactive molecules as antiviral agents against the SARS-CoV-2 pandemic is being investigated [27]. As viral infections have become increasingly important, the trend towards antiviral drugs has also accelerated. Assessing the antiviral activity of different plant species or bioactive compounds isolated from them suggests potential therapies [1]. In addition to studies evaluating the *in vitro* and *in vivo* antiviral potential of different plant species, *in ovo* antiviral activity experiments are an effcient method that offers three-dimensional study and provides advantages in ethical issues [13]. In our study, the anti-IBV virucidal activities of the samples were determined as *in ovo*. Compared with previous studies, the antiviral activity of *L. nobilis*, oleuropein and nobiletin compounds exhibit similar results. Serkedjieva et al.[28] evaluated both *S. nigra* and *H. perforatum* extracts *in vitro* and *in vivo*, and reported that the strongest antiviral activity was observed against herpes simplex and influenza A viruses. They stated that the antiviral activity is due to the flavonoid, triterpene, tannin and polysaccharide content, although the mechanism of action is not precise. Chen et al.[29], showed in their study that the *S. nigra* extracts inhibit viral replication and demonstrated the effect of these extracts on avian IBV replication. Treatment of *S. nigra* extracts with the virus before infection to a great extent inhibited the virus at early stage in the infection. In another study, Chen et al.[30] confirmed that ethyl acetate extraction (HPE) of *H. perforatum* exhibited an antiviral effect on IBV. In chicken embryo kidney (CEK) cells, this extract was significantly reduced virus titer by inhibiting mRNA expression.

In our study, *S. nigra* reduced the HA titer by 3-5 $\log_2$ HA between concentrations of 0.1-1 mg/g in parallel with the literature, and for the chloroform phase of ethyl alcohol extraction of *H. perforatum*, the antiviral activity was found to decrease by 5 $\log_2$ HA at 0.1 mg/g. It is thought that the significant antiviral activity of both extracts was due to flavonoid, triterpene, saponin or phenolic acid compounds and exhibits activity by inhibiting viral replication. Phenolic compounds and oleic acids from olives play an effective role in biological activities. Furthermore, oleuropein in olives may serve as an antiviral agent against various viruses through inhibition of replication and entry protein [31,32]. In this study, when the results of cytotoxicity and antiviral activity are compared together, it is suggested that oleuropein can be used as a potential antiviral agent against coronaviruses by promoting cell proliferation in healthy cell lines (CCD-34Lu). It has been stated that nobiletin, the most abundant compound in *Citrus* spp., has potential effects against influenza, hepatitis B and C, human respiratory syncytial, vesicular stomatitis viruses. Antiviral effect against SARS-CoV-2 with protein inhibition is also predicted [27]. This study showed that the nobiletin molecule, which gives promising effects *in ovo*, can be assessed as a potential antiviral agent against IBV. Rosmarinic acid (RA) and its derivatives such as sulfated or hexoside form of rosmarinic acid were identified as the primary bioactive phenolic compound in *M. officinalis* [33]. Lelesius et al.[8] observed *in vitro* virucidal antiviral potential in *M. officinalis* in their experiment. Otherwise, in this study, *M. officinalis* showed low antiviral potency by decreasing HA titer to 2 $\log_2$ HA. This indicated that the samples could exhibit different potential antiviral activity between *in ovo* and *in vitro*. This result is thought to be possible due to the inhibition mechanism targeting the entry and envelope proteins [8]. A computational analysis conducted in a study has revealed the potential of both hesperidin, a flavonoid, and rosmarinic acid, derived from plant extracts, to inhibit SARS-CoV-2 [34]. Moreover, Al-Hatamleh et al. have demonstrated that hesperidin is the only natural compound capable of attaching to the receptor binding domain of the S protein, thereby neutralizing the binding between ACE2 receptor and spike-RBD [35].

In conclusion, studies have proven that traditional medicinal plant extracts and molecules display antiviral activities against numerous viruses. The antiviral activity against IBV and the cytotoxic effect on cancerous cell lines were analyzed for plant extracts and active ingredients in this study. Comparing cytotoxicity and antiviral results, it is shown that *L. nobilis* and *H. perforatum* have a dominant role in both analyses. Also, it is noteworthy that nobiletin and *S. nigra* do not exhibit cytotoxic activity on any cell but play a role in antiviral activity.

Declarations

Availability of Data and Materials: Materials and datasets provided from the study can be obtained from the corresponding author (F. O. Çöven) upon request.

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Author Contributions: All authors participaed to design the methodology, FOÇ, SG, KAHMA: writing the original draft, SG, EU and KAHMA: software and visualization, FK, FÇ and İÇ: data curation, FK, İÇ, AN: investigation and validation, FOÇ, FÇ, AN: reviewing and editing.

References

1. Denaro M, Smeriglio A, Barreca D, De Francesco C, Occhiuto C, Milano G, Trombetta D: Antiviral activity of plants and their isolated bioactive compounds: An update. *Phytother Res,* 34 (4): 742-768, 2020. DOI: 10.1002/ptr.6575

2. Ma L, Yao L: Antiviral effects of plant-derived essential oils and their components: An updated review. *Molecules,* 25 (11): 2627, 2020. DOI: 10.3390/molecules25112627

3. Mani JS, Johnson JB, Steel JC, Broszczak DA, Neilsen PM, Walsh KB, Naiker M: Natural product-derived phytochemicals as potential agents against coronaviruses: A review. *Virus Res,* 284:197989, 2020. DOI: 10.1016/j.virusres.2020.197989

4. Singhal TA: Review of coronavirus disease-2019 (COVID-19). *Indian J Pediatr,* 87 (4): 281-286, 2020. DOI: 10.1007/s12098-020-03263-6

5. Cook JKA, Jackwood M, Jones RC: The long view: 40 years of infectious bronchitis research. *Avian Pathol,* 41 (3): 239-250, 2012. DOI: 10.1080/03079457.2012.680432

6. Westerbeck JW, Machamer CE: The Infectious Bronchitis Coronavirus envelope protein alters golgi pH to protect the spike protein and promote the release of infectious virus. *J Virol,* 93 (11): 15-19, 2019. DOI: 10.1128/jvi.00015-19

7. Civitelli L, Panella S, Marcocci ME, De Petris A, Garzoli S, Pepi F, Vavala E, Ragno R, Nencioni L, Palamara AT, Angiolella L: *In vitro* inhibition of herpes simplex virus type 1 replication by *Mentha suaveolens* essential oil and its main component piperitenone oxide. *Phytomedicine,* 21 (6): 857-865, 2014. DOI: 10.1016/j.phymed.2014.01.013

8. Lelesius R, Karpovaite A, Mickiene R, Drevinskas T, Tiso N, Ragazinskiene O, Kubiliene L, Maruska A, Salomskas A: *In vitro* antiviral activity of fifteen plant extracts against avian Infectious Bronchitis virus. *BMC Vet Res,* 15 (1): 1-10, 2019. DOI: 10.1186/s12917-019-1925-6

9. Ingle KP, Deshmukh AG, Padole DA, Dudhare MS, Moharil MP, Khelurkar VC: Phytochemicals: Extraction methods, identification and detection of bioactive compounds from plant extracts. *J Pharmacogn Phytochem,* 6 (1): 32-36, 2017.

10. Gfeller D, Michielin O, Zoete V: Shaping the interaction landscape of bioactive molecules. *Bioinformatics,* 29 (23): 3073–3079, 2013. DOI: 10.1093/bioinformatics/btt540

11. Singh NA, Kumar P, Jyoti, Kumar N: Spices and herbs: Potential antiviral preventives and immunity boosters during COVID-19. *Phtother Res*, 35 (5): 2745-2757, 2021. DOI: 10.1002/ptr.7019

12. Mosmann T: Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. *J Immunol Methods,* 65 (1-2): 55-63, 1983. DOI: 10.1016/0022-1759(83)90303-4

13. Ghoke SS, Sood R, Kumar N, Pateriya AK, Bhatia A, Mishra A, Dixit R, Singh VK, Desai DN, Kulkarni DD, Dimri U, Singh VP: Evaluation of antiviral activity of *Ocimum sanctum* and *Acacia arabica* leaves extracts against H9N2 virus using embryonated chicken egg model. *BMC Complement Altern Med,* 18 (174): 1-10, 2018. DOI: 10.1186/s12906-018-2238-1

14. Reed LJ, Muench H: A simple method of estimating fifty percent endpoints. *Am J Hyg,* 27 (3): 493-497, 1938. DOI: 10.1093/oxfordjournals.aje.a118408

15. World Organisation for Animal Health (WOAH): Avian Infectious Bronchitis. Manual of Diagnostic Tests and Vaccines for Terrestrial Animals 12th ed. Chapter 3.3.2. Avian Infectious Bronchitis 7-8, 2023.

16. Andleeb R, Ashraf A, Muzammil S, Naz S, Asad F, Ali T, Rafir, Al-Ghanim KA, Al-Misned F, Ahmed Z, Mahboob S: Analysis of bioactive composites and antiviral activity of *Iresine herbstii* extracts against Newcastle disease virus *in ovo*. *Saudi J Biol Sci*, 27 (1): 335-340, 2020. DOI: 10.1016/j.sjbs.2019.10.002

17. Sulaiman LK, Oladele OA, Shittu IA, Emikpe BO, Oladokun AT, Meseko CA: *In-ovo* evaluation of the antiviral activity of methanolic root-bark extract of the African Baobab *(Adansonia digitata* Lin). *AJB,* 10 (20): 4256-4258, 2011.

18. Barla A, Topçu G, Öksüz S, Tümen G, Kingston DGI: Identification of cytotoxic sesquiterpenes from *Laurus nobilis* L. *Food Chem,* 104 (4): 1478-1484, 2007. DOI: 10.1016/j.foodchem.2007.02.019

19. Gomes JV, Teixeira JTDS, De Lima VM, Borba HR: Induction of cytotoxic and genotoxic effects of Guandu River waters in the *Allium cepa* system. *Rev Ambient Agua,* 10 (1): 48-58, 2015. DOI: 10.4136/ambi-agua.1487

20. De Oliveira LM, Voltolini JC, Barbério A: Potencial mutagênico dos poluentes na água do rio Paraíba do Sul em Tremembé, SP, Brasil, utilizando o teste *Allium cepa. Rev Ambient Agua,* 6 (1): 90-103, 2011. DOI: 10.4136/ambi-agua.176

21. Hibasami H, Yamada Y, Moteki H, Katsuzaki H, Imai K, Yoshioka K, Komiya T: Sesquiterpenes (costunolide and zaluzanin D) isolate d from laurel *(Laurus nobilis* L.) induce cell death and morphological change indicative of apoptotic chromatin condensation in leukemia HL-60 cells. *Int J Mol Med,* 12 (2): 147-151, 2003. DOI: 10.3892/ijmm.12.2.147

22. Inoue M, Suzuki R, Koide T, Sakaguchi N, Ogihara Y, Yabu Y: Antioxidant, gallic acid, induces apoptosis in HL-60RG cells. *BBRC,* 204 (2): 898-904, 1994. DOI: 10.1006/bbrc.1994.2544

23. Liu F, Pan C, Drumm P, Ang CYW: Liquid chromatography-mass spectrometry studies of St. John's wort methanol extraction: Active constituents and their transformation. *J Pharm Biomed Anal,* 37 (2): 303-312, 2005. DOI: 10.1016/j.jpba.2004.10.034

24. Vollmer JJ, Rosenson J: Chemistry of St. John's Wort: Hypericin and hyperforin. *J Chem Educ,* 81 (10): 1450-1456, 2004. DOI: 10.1021/ed081p1450

25. Zou Y, Lu Y, Wei D: Antioxidant activity of a flavonoid-rich extract of *Hypericum perforatum* L. *in vitro*. *J Agric Food Chem,* 52 (16): 5032–5039, 2004. DOI: 10.1021/jf049571r

26. Liu Z, Tao X, Zhang C, Lu Y, Wei D: Protective effects of hyperoside (quercetin-3-o-galactoside) to PC12 cells against cytotoxicity induced by hydrogen peroxide and tert-butyl hydroperoxide. *Biomed Pharmacother,* 59 (9): 481-490, 2005. DOI: 10.1016/j.biopha.2005.06.009

27. Gezici S, Sekeroglu N: Novel SARS-CoV-2 and COVID-2019 outbreak: Current perspectives on plant-based antiviral agents and complementary therapy. *IJPER,* 54 (3): 442-456, 2020. DOI: 10.5530/ijper.54.3s.143

28. Serkedjieva J, Manolova N, Zgórniak-Nowosielska I, Zawilinska B, Grzybek J: Antiviral activity of the infusion (SHS-174) from flowers of *Sambucus nigra* L., aerial parts of *Hypericum perforatum* L., and roots of *Saponaria officinalis* L. against influenza and herpes simplex viruses. *Phytother Res*, 4 (3): 97-100, 1990. DOI: 10.1002/ptr.2650040305

29. Chen C, Zuckerman DM, Brantley S, Sharpe M, Childress K, Hoiczyk E, Pendleton AR: *Sambucus nigra* extracts inhibit Infectious Bronchitis virus at an early point during replication. *BMC Vet Res,* 10 (24): 1-12, 2014. DOI: 10.1186/1746-6148-10-24

30. Chen H, Muhammad I, Zhang Y, Ren Y, Zhang R, Huang X, Diao L, Liu H, Li X, Sun X, Abbas G, Li G: Antiviral activity against Infectious Bronchitis virus and bioactive components of *Hypericum perforatum* L. *Front Pharmacol,* 10 (1272): 1-22, 2019. DOI: 10.3389/fphar.2019.01272

31. Omar SH: Oleuropein in olive and its pharmacological effects. *Sci Pharm,* 78 (2): 133-154, 2010. DOI: 10.3797/scipharm.0912-18

32. Imran M, Nadeem M, Gilani SA, Khan S, Sajid MW, Amir RM: Antitumor perspectives of oleuropein and its metabolite hydroxytyrosol: Recent updates. *J Food Sci,* 83 (7): 1781-1791, 2018. DOI: 10.1111/1750-3841.14198

33. Miron TL, Herrero M, Ibáñez E: Enrichment of antioxidant compounds

from lemon balm *(Melissa officinalis)* by pressurized liquid extraction and enzyme-assisted extraction. *J Chromatogr A,* 1288, 1-9, 2013. DOI: 10.1016/j.chroma.2013.02.075

34. Wu C, Liu Y, Yang Y, Zhang P, Zhong W, Wang Y, Wang Q, Xu Y, Li M, Li X, Zheng M, Chen L, Li H: Analysis of therapeutic targets for SARS-CoV-2 and discovery of potential drugs by computational methods. *Acta Pharm Sin B,* 10 (5): 766-788, 2020. DOI: 10.1016/j.apsb.2020.02.008

35. Al-Hatamleh MAI, Hatmal MM, Sattar K, Ahmad S, Mustafa MZ, De Carvalho Bittencourt M, Mohamud R: Antiviral and immunomodulatory effects of phytochemicals from honey against COVID-19: Potential mechanisms of action and future directions. *Molecules,* 25 (21): 1-23, 2020. DOI: 10.3390/molecules25215017

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RESEARCH ARTICLE

Influences of Desflurane on the Central and Hepatic Circadian Clock Persist at 24 Hours Following Anaesthesia in Rats

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Abstract

Circadian rhythm disturbances caused by anaesthesia may be one of the important reasons of many disturbances following surgery. The effects of 6% desflurane on suprachiasmatic nucleus (SCN), liver, and pancreas PER2, CRY1, BMAL, and CLOCK circadian protein levels and serum concentrations of glucose, melatonin, cortisol 24 h following the administration of anaesthesia were determined in this study. 10–12-week-old Wistar albino male rats (n=12) were divided into two groups. Control group (C) was not exposed to desflurane, while the anaesthesia group (D) received desflurane at 6% concentration for 6 h between 07.00 and 13.00 a.m. and was euthanized 24 h after anaesthesia. Tissue samples were collected from both groups at the same time of the day (07.00 a.m.). Serum glucose, melatonin, cortisol and SCN, liver, pancreas CLOCK, BMAL1, PER2, CRY1 levels were determined using commercial kits. CLOCK and PER2 protein levels in SCN and BMAL1 protein concentration in liver decreased, while liver PER2 protein level increased at the 24th h following anaesthesia. Pancreas BMAL1, CLOCK, PER2, CRY1 protein levels were not affected by the anaesthesia protocol applied. No statistically significant alteration was observed in plasma glucose, cortisol and melatonin concentrations. Our findings show that, the circadian clock rhythm in SCN and liver are still affected at the 24th h following exposure to desflurane.

Keywords: Circadian rhythm, Clock proteins, Desflurane, Liver, SCN

Introduction

Determining how anaesthesia affects the circadian rhythm is not only of scientific interest but also of potential clinical importance. Patients often experience sleep disturbances after surgery. Disruption of circadian rhythm and related sleep disorders cause adverse effects on inflammation and immune function, as well as altered mood, behaviour and cognitive dysfunction [1-4]. For these reasons, it is clinically important to elucidate the mechanism of postoperative circadian rhythm disorders.

Twenty-four h physiological processes are controlled by the circadian rhythm through a transcriptional feedback loop of clock genes and proteins [5,6]. The circadian clock is made up of four basic components at the molecular level. In mammals, these are the genes brain and muscle ARNT-like 1 *(Bmal1),* circadian locomotor exit hood *(Clock),* Period 2 *(Per2),* and Cryptochrome 1 *(Cry1)* [7]. While CLOCK and BMAL proteins were demonstrated to promote the transcription of *Per2* and *Cry1*, PER2 and CRY1 proteins inhibit BMAL1:CLOCK transcriptional activity, thus limit their own transcription [8,9]. The protein-mediated disruptions in the circadian rhythm caused by anaesthesia may hinder post-operative recovery [10].

Neurons associated with molecular clocks are located in the ventral region of the suprachiasmatic nucleus (SCN). When photoreceptors of the eye detect light, NMDA receptors in SCN are activated by the retinohypothalamic pathway, resulting in transcription of *Per2* [8,11]. While the "master" of the circadian rhythm is the SCN, peripheral clocks in organs govern processes specific to that tissue in a particular tissue or cell type [12]. For example, the liver circadian clock regulates fasting glycaemic control and glucose clearance, while the pancreatic circadian clock regulates insulin secretion and glucose homeostasis [13].

Sevoflurane, isoflurane and desflurane are the most commonly used inhalation anaesthetics in clinical practice.

Circadian rhythm is affected after general anaesthesia with sevoflurane and isoflurane [14]. However, there is still a lack of information in the literature regarding the effects of desflurane anaesthesia on the circadian clock. Desflurane, one of the third generation inhaled anaesthetic drugs, is frequently preferred in clinical use for providing safe and effective anaesthesia [15] . Desflurane has a significantly lower blood solubility compared to other inhaler agents [8]. With low tissue solubility, there is an acceleration of induction and elimination and more precise control of alveolar desflurane concentration in anaesthesia maintenance. These properties contribute to the rapid onset of desflurane anaesthesia. Anaesthesiologists administer desflurane as a general anaesthetic to wake patients quickly after surgery. Based on these properties, desflurane may have a different effect on the circadian rhythm compared to other inhaled anaesthetics [16]. Human studies have suggested that there may be two separate toxic problems during the metabolism of desflurane. The first one is high current renal failure as a result of biotransformation to free fluoride ion; the second is that it can bind to hepatic tissue macromolecules and cause hepatotoxicity. Desflurane may also induce hepatic microsomal enzymes [16].

Melatonin has important physiological functions such as regulation of the circadian rhythm and the reproductive axis [17]. Melatonin secretion is controlled by the SCN, which regulates the day/night cycle. The administration of anaesthesia regardless of surgery may affect the circadian rhythm of melatonin [18]. Another hormone affected by anaesthesia is the cortisol. When melatonin receptors in the adrenal gland are stimulated with physiological doses, ACTH-mediated cortisol formation is suppressed [19]. The balance between these two hormones is important for health.

In the light of above information, we aimed to investigate SCN, liver and pancreas PER2, CRY1, BMAL and CLOCK protein levels and serum concentrations of glucose, melatonin, cortisol 24 h following the administration of 6% desflurane in 6 L min-1 100% oxygen to rats for 6 h. It is anticipated that the results of the current study may provide important information about how long the effects of desflurane, a widely used anaesthetic in the clinic, on the circadian rhythm and hormonal balance may last.

Material and Methods

Ethical Approval

All experimental procedures were conducted in accordance with institutional animal care guidelines of Pamukkale University and approved by the local Animal Experiments Ethics Committee (PAUHADYEK-2021/33, 24.08.2021-07).

Animals

200-250 g, 10-12-week-old Wistar albino male rats (n=12, Pamukkale University Experimental Animal Unit) were used. The animals were housed in a temperature and humidity controlled (22-23°C, 50±5%) room under a 12 h light-dark cycle. Standard diet food and water were available ad libitum.

Study Design and Anaesthesia Protocol

Rats were divided into two groups (n=6 per group). For adaptation, the animals were placed in the anaesthetic chamber approximately 1 h, for 4 days before beginning the experiments. Control group (C) was not exposed to desflurane but was placed in the anaesthetic chamber, then transferred back to the cages and was euthanized at 07.00 a.m. The anaesthesia exposure group (D) received desflurane at 6% concentration in 6 L min-1% 100% oxygen for 6 h in a transparent anaesthesia box between 07.00-13.00 a.m. and was euthanized 24 h after anaesthesia at 07.00 a.m. Tissue samples were collected from both groups at the same time of the day (07.00 a.m.) in order to avoid the original circadian variation in clock gene expressions. After anaesthesia, the fresh gas flow was decreased to 1 L/min, then rats were transferred to their cages and kept at normal conditions (bright in daytime, dark at night) of the experimental animals unit of the university. In order to maintain their normal body temperature, the chambers housing the mice were placed on a heat sheet. The anaesthesia procedures were performed under dark conditions, and under a dim red light that did not affect the circadian rhythm.

Blood Sample and SCN, Liver and Pancreas Collection

After the experiment, rats were euthanized with an overdose of sodium pentobarbital. SCN, liver and pancreas samples were collected without anaesthesia (C group) and 24 h after exposure to desflurane (D group). The blood samples obtained from the tail vein of all rats at 07.00 a.m. were centrifuged on the same day (1100×g, 20 min). SCN, liver and pancreas tissues were minced and homogenized in PBS (tissue weight (g): PBS (mL) volume=1:9) using a glass homogenizer on ice. The homogenates were then centrifuged for 5 min at 5000×g to get the supernatant. Serum, SCN, liver and pancreas samples were stored at -80°C until experimental analysis.

Determining Glucose, Melatonin, Cortisol, CLOCK, BMAL1, PER2 and CRY1 Levels

Serum glucose (Cat.No E1623Ra, BT Lab), melatonin (Cat.No E0601Ra, BT Lab), cortisol (Cat.No E0828Ra, BT Lab) and SCN, liver, pancreas CLOCK (Cat.No E2920Ra, BT Lab), BMAL1 (Cat.No E2919Ra, BT Lab), PER2 (Cat. No E2917Ra, BT Lab), CRY1 (Cat.No E2918Ra, BT Lab), levels were determined using commercial ELISA kits.

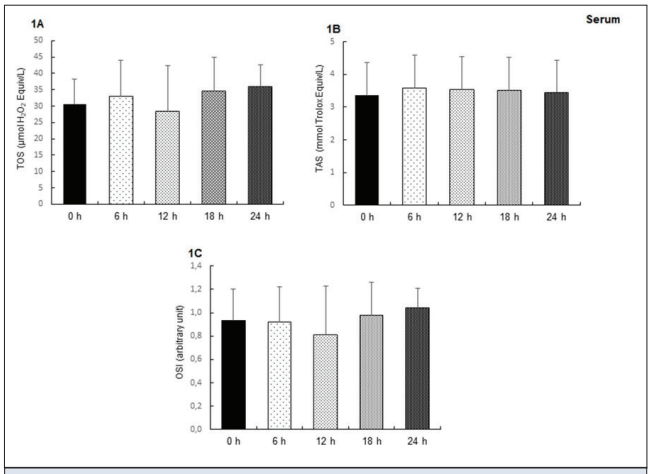

Fig. 1. Serum glucose (A), cortisol (B) and melatonin (C) levels of the groups. C: Control, D: Desfluran group. Values are expressed as mean ± SD. :P<0.05, difference from C

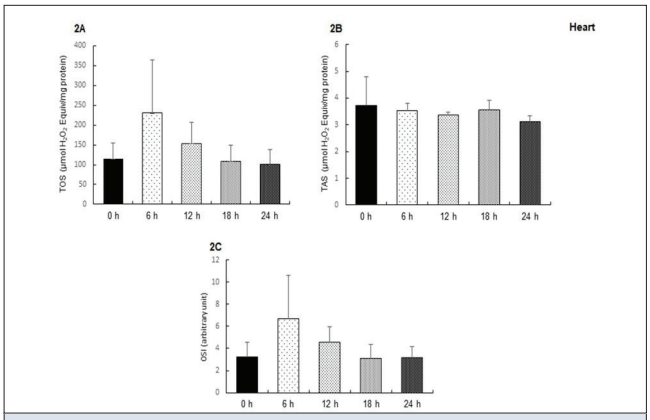

Fig. 2. SCN BMAL1 (A), CLOCK (B), PER2 (C), CRY1 (D) protein levels of groups. C: Control, D: Desfluran group, SCN: Suprachiasmatic nucleus, BMAL1: brain and muscle ARNT-like 1, CLOCK: Circadian Locomoter Output Cycles Protein Kaput, PER2: PERIOD-2, CRY1: Cryptochrome-1. Values are expressed as mean ± SD. :P<0.05, difference from C

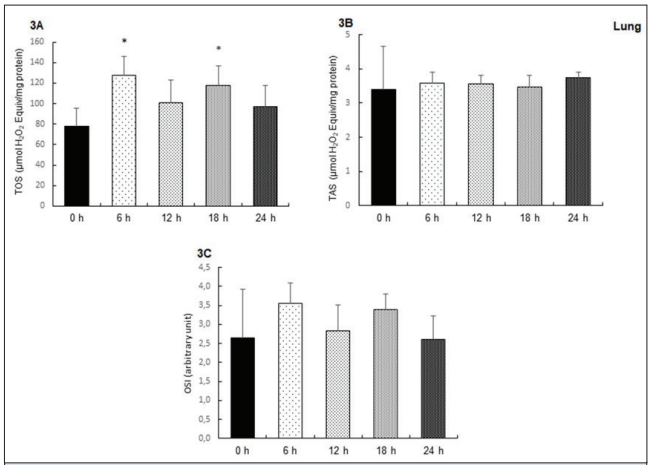

Fig. 3. Liver BMAL1 (A), CLOCK (B), PER2 (C), CRY1 (D) protein levels of groups. C: Control, D: Desfluran group, BMAL1: brain and muscle ARNT-like 1, CLOCK: Circadian Locomoter Output Cycles Protein Kaput, PER2: PERIOD-2, CRY1: Cryptochrome-1. Values are expressed as mean ± SD. :P<0.05, difference from C

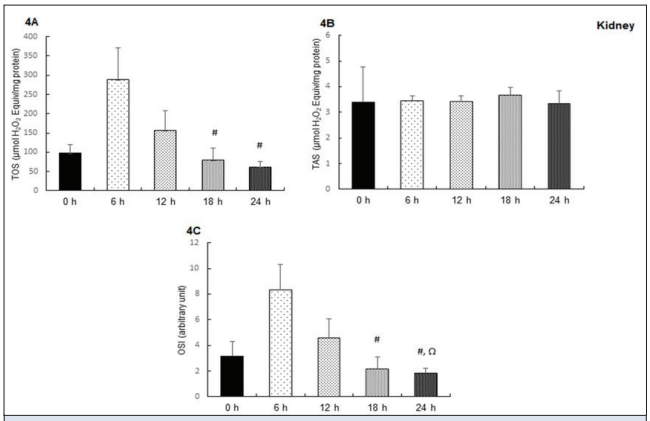

Fig. 4. Pancreas BMAL1 (A), CLOCK (B), PER2 (C), CRY1 (D) protein levels of groups. C: Control, D: Desfluran group, BMAL1: brain and muscle ARNT-like 1, CLOCK: Circadian Locomoter Output Cycles Protein Kaput, PER2: PERIOD-2, CRY1: Cryptochrome-1. Values are expressed as mean ± SD

Statistical Analysis

As a result of the power analysis we performed, assuming that we could obtain a lower power, it was calculated that a power of 80% at a confidence level of 95% could be obtained if at least 12 rats (at least 6 rats for each group) were included in the study and the effect size was d=0.69. All calculations and power analysis were performed by the G-power program (version 3.1.9.2. Heinrich-Heine-Universitat. Duesseldorf. Germany). All statistical analyses were performed using SPSS 25.0 (IBM SPSS Statistics 25 software (Armonk, NY: IBM Corp.). Continuous variables were defined by the mean ± standard deviation. Shapiro Wilk tests were used for determination of normal distribution. For nonparametric test assumptions were provided, Kruskal Wallis Variance Analysis (post hoc: Mann Whitney U test with Bonferroni Correction) were used when parametric test assumptions were not provided. The level of statistical significance was set at P≤0.05.

Results

Fig. 1 demonstrates that serum glucose, cortisol and melatonin levels were not altered significantly by the desflurane applied herein. Anaesthesia induced statistically significant decrements in SCN CLOCK, PER *(Fig. 2)* and liver BMAL1 protein levels, whereas it caused increment in liver PER protein concentration *(Fig. 3)* 24 h after the application compared to control (P<0.05). On the other hand, no statistically significant alteration was determined in pancreas BMAL1, CLOCK, PER2, CRY1 protein levels following desflurane anaesthesia *(Fig. 4)*.

Discussion

Desflurane is one of the most commonly used modern inhaled anaesthetic agents [20]. It provides safer and effective

anaesthesia in clinical use. Since uptake, distribution and elimination of desflurane are faster than similar drugs, it allows rapid changes in the depth of anaesthesia. Its solubility in blood and tissue is lower than the other halogenated anaesthetics [21]. We have administered desflurane at a concentration of 6% in accordance with the dose commonly used in surgeries at our hospital. Desflurane has the least *in vivo* metabolism among inhaled halogenated anaesthetics. However, all halogenated inhaled anaesthetics such as sevoflurane, desflurane and isoflurane may cause metabolic hepatocellular damage. Very serious but few cases of post-anaesthesia hepatic and renal injury have been reported [21]. In the present study, the protein levels which play role in the circadian rhythm (BMAL1, CLOCK, PER2, CRY1) at 07.00 a.m. in the non-anaesthetized group were compared with the same protein levels at 07.00 a.m., 24 h after 6% desflurane anaesthesia. Our results demonstrated that CLOCK and PER2 protein levels in SCN as well as BMAL1 protein concentration in liver decreased, while liver PER2 protein level increased at the 24th h following anaesthesia compared to control group. Pancreas BMAL1, CLOCK, PER2, CRY1 protein levels were not affected by the anaesthesia protocol applied. Similarly, no statistically significant alteration was observed in plasma glucose, cortisol and melatonin concentrations 24 h after desflurane anaesthesia.

The relationship between melatonin and cortisol is especially important in terms of the effects of both hormones on the immune system. General anaesthesia is a sleep-wake state thought to alter circadian rhythms. Different studies have shown conflicting effects of surgery and anaesthesia on melatonin levels which is controlled by the SCN [10,22]. Ozer et al.[23] demonstrated that, 5.7% desflurane administration for six hours during the day and at night does not alter plasma melatonin levels in blood samples collected immediately after anaesthesia in 15 day old rats. Here, we have demonstrated for the first time that, plasma melatonin levels were not changed 24 h after 6% desflurane inhalation. The fact that samples were collected from both groups at the same time of the day (07.00 a.m.) in order to avoid the original circadian variation may have played role in these results. Unfortunately, we could not determine possible time-dependent alterations in melatonin, cortisol and glucose levels in response to desflurane in the current study. Changes in these parameters might have occurred during the 24-hour period, but these alterations might have disappeared when we sampled blood at the 24th hour. Supporting this suggestion, in humans, following 1.5 h of anaesthesia, the elevated cortisol level was reduced to baseline after 24 h [24]. Unchanged plasma cortisol and glucose levels 24 h after exposure to desflurane in healthy rats without any surgical procedure may indicate that, the stress level of our rats was low. Similar to our results, Dikmen et al.[25] stated that, both desflurane and sevoflurane can be used safely during acute hyperglycemic state, because of their non-increasing blood glucose influences.

The presence of common clock and entrainment mechanisms for nocturnal and diurnal species was asserted [26]. The circadian rhythm involves the central clock in the SCN and peripheral clocks in other tissues which regulate local tissue-specific physiological functions [27]. Light entrains the SCN through retinohypothalamic pathway; whereas other stimuli as nutrition, temperature, stress reset the peripheral clocks, and entrainment depends on the timing of stimulation [28]. The core clock system, which co-ordinates the activity of peripheral clocks is made up of a feedback loop whereby BMAL1 and CLOCK induce the expression of *Per2* and *Cry1* genes. Later, the PER-CRY heterodimer is known to repress the transcription of *Clock* and *Bmal1* with negative feedback mechanism [29]. An additional loop consists the ROR and Rev-Erb factors, also regulated by BMAL1-CLOCK complex [30].

Volatile anaesthetics exert their specific actions at the molecular level, with proteins rather than lipids [31]. Imai et al.[5] demonstrated that, 4% desflurane applied for 6 h induces a phase shift in the circadian rhythm being largest in ZT6-12. *Bmal* and *Cry1* expressions were elevated, whereas *Clock* expression was decreased in ZT12 in SCN. *Per2* expression was also increased from ZT2 to ZT8 and decreased later on. These authors collected their last SCN sample at ZT20 following anaesthesia and examined expressions of circadian rhythm genes. The reflection of gene expression alterations on protein levels may take several hours [26]. The innovation our data brings to the literature is that, CLOCK protein level still remains lower in SCN at the 24th h following 6% desflurane anaesthesia in rats. Additionally in line with the results of the above mentioned study, PER2 protein is also decreased in SCN at that time thereby causing a phase shift in the circadian rhythm of mice. This issue should be taken into consideration while using desflurane anaesthesia.

Most tissues of the body exhibit circadian oscillations through mechanisms involving both core clock genes common to all and numerous other tissue-specific genes that cooperate with each other and many circadian epigenetic modifiers are known to function in a tissue-specific manner [32]. We also investigated the effects of 6% desflurane on liver and pancreas circadian clock proteins and have shown for the first time that, while desflurane did not affect pancreas levels of these proteins, liver BMAL1 protein is supressed and PER2 is elevated 24 h after anaesthesia. This finding should be kept in mind when dealing with patients with insulin resistance since it was demonstrated that liver-specific deletion of BMAL1 is known to result in a blunted sensitivity

to insulin [13]. DNA microarray study revealed that, approximately 10% of the sampled liver genome is under circadian control [33]. The regulated genes include the ones encoding cytoskeletal elements and enzymes involved in carbohydrate metabolism emphasizing the importance of liver circadian rhythm on feeding and digestion. Moreover it was demonstrated that, in the case of *mPer2* and *mBmal1*, the anti-phasic relationship observed in the SCN is retained in the liver, with a slightly delayed timing for both clusters [26]. Our resuts lead to the interpretation that following desflurane exposure, elevated liver PER2 protein levels may have caused inhibition of BMAL1 by negative feedback mechanism.

Halogenated ethers may have hepatic and renal side effects via transformation to toxic metabolites [34]. Rare instances of acute liver injury have been reported with all halogenated agents. Cytochrome P450 CYP2El seems to be the specific P450 isoform largely responsible for the defluorination of isoflurane. Although desflurane metabolism is approximately 10% of isoflurane, it is suggested that desflurane may also undergo metabolism by a route similar to that of isoflurane [35]. However, we have observed that liver CRY1 protein levels were not altered at 24th h following desflurane exposure. When all circadian protein findings of the current study are evaluated together, it appears that 6% desflurane administration affects the levels of these proteins not only in the SCN but also in the liver for at least 24 h.

The present study may be considered as a preliminary one giving clues about the prevention and treatment of postoperative complications related to circadian rhythms following desflurane inhalation. Our results demonstrate that, the circadian clock rhythm is affected in SCN and liver for at least 24 h following desflurane anaesthesia. Examination of the effects of desflurane only at the 24th h, the fact that the intraday influences and later effects were not studied constitute the most important limitations of this study. Another limitation is that although the chambers housing the mice were placed on a heat sheet in order to maintain their normal body temperature, the body temperatures of the rats were not notified during the experimental procedure. When the influences of desflurane on circadian protein levels disappears and the time-dependent effects of this anaesthetic on other peripheral clocks as well as the mechanisms of these influences have not been clarified yet. Investigation of the circadian clock genes/proteins in response to desflurane anaesthesia in a variety of clinical situations is also necessary in order to clarify whether desflurane offers significant advantages over the other anaesthetic drugs.

Declarations

Availability of Data and Materials: The authors declare that data supporting the study findings are also available from the corresponding author (I. H. A.) on reasonable request.

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Conflict of Interest: The authors declare that there is no conflict of interest in publishing this article.

Author Contributions: I.H.A., O.K.E.: Conceptualization, methodology, data curation, investigation, resources, project administration; M.B.K.: Methodology, writing, review, and editing. All authors read and approved the final manuscript.

References

1. Logan RW, McClung CA: Rhythms of life: Circadian disruption and brain disorders across the lifespan. *Nat Rev Neurosci*, 20 (1): 49-65, 2019. DOI: 10.1038/s41583-018-0088-y

2. Teichman EM, O'riordan KJ, Gahan CG, Dinan TG, Cryan JF: When rhythms meet the blues: Circadian ınteractions with the microbiota-gut-brain axis. *Cell Metab*, 31 (3): 448-471, 2020. DOI: 10.1016/j.cmet.2020.02.008

3. Walker WH, Walton JC, DeVries AC, Nelson RJ: Circadian rhythm disruption and mental health. *Transl Psychiatry,* 10 (1):28, 2020. DOI: 10.1038/s41398-020-0694-0

4. Zou H, Zhou H, Yan R, Yao Z, Lu Q: Chronotype, circadian rhythm, and psychiatric disorders: Recent evidence and potential mechanisms. *Front Neurosci*, 16:811771, 2022. DOI: 10.3389/fnins.2022.811771

5. Imai R, Makino H, Katoh T, Kimura T, Kurita T: Desflurane anaesthesia shifts the circadian rhythm phase depending on the time of day of anaesthesia. *Sci Rep,* 10 (1):18273, 2020. DOI: 10.1038/s41598-020-75434-6

6. Wang D, Huang Y, Wang X, Chen X, Li J, Zhang S, Wu J, Liu D, Ma D, Mei W: Circadian differences in emergence from volatile anaesthesia in mice: Involvement of the locus coeruleus noradrenergic system. *Br J Anaesth*, 125 (4): 548-559, 2020. DOI: 10.1016/j.bja.2020.07.012

7. Zheng B, Albrecht U, Kaasik K, Sage M, Lu W: Nonredundant roles of the mPer1 and mPer2 genes in the mammalian circadian clock. *Cell*, 105 (5): 683-694, 2001. DOI: 10.1016/s0092-8674(01)00380-4

8. Poulsen RC, Warman GR, Sleigh J, Ludin NM, Cheeseman JF: How does general anaesthesia affect the circadian clock? *Sleep Med Rev*, 37, 35-44, 2018. DOI: 10.1016/j.smrv.2016.12.002

9. Voss LJ, García PS, Hentschke H, Banks MI: Understanding the effects of general anesthetics on cortical network activity using *ex vivo* preparations. *Anesthesiology*, 130 (6): 1049-1063, 2019. DOI: 10.1097/ALN.0000000000002554

10. Cheeseman JF, Winnebeck EC, Millar CD, Kirkland LS, Sleigh J: General anaesthesia alters time perception by phase shifting the circadian clock. *Proc Natl Acad Sci U S A*, 109 (18): 7061-7066, 2012. DOI: 10.1073/pnas.1201734109

11. Tischkau SA, Mitchell JW, Tyan SH, Buchanan GF, Gillette MU: Ca^{2+}/cAMP response element-binding protein (CREB)-dependent activation of *Per1* is required for light-induced signaling in the suprachiasmatic nucleus circadian clock. *J Biol Chem*, 278 (2): 718-723, 2003. DOI: 10.1074/jbc.M209241200

12. Shostak A, Meyer-Kovac J, Oster H: Circadian regulation of lipid mobilization in white adipose tissues. *Diabetes,* 62 (7): 2195-2203, 2013. DOI: 10.2337/db12-1449

13. Lamia KA, Storch KF, Weitz CJ: Physiological significance of a peripheral tissue circadian clock. *Proc Natl Acad Sci U S A,* 105 (39): 15172-15177, 2008. DOI: 10.1073/pnas.0806717105

14. Ocmen E, Erdost HA, Duru LS, Akan P, Cimrin D: Effect of day/night administration of three different inhalational anesthetics on melatonin levels in rats. *Kaohsiung J Med Sci,* 32 (6): 302-305, 2016. DOI: 10.1016/j.kjms.2016.04.016

15. Akbudak IH, Kilic Erkek O, Bor Kucukatay M: Desflurane 6% ınhalation ınhibits erythrocyte deformability and alters oxidative stress in rat lung and kidney in a time-dependent manner. *Kafkas Univ Vet Fak Derg*, 29 (3): 273-279, 2023. DOI: 10.9775/kvfd.2023.29165

16. Brown BR and Frink EJ: Biodegradation and organ toxicity of new volatile anesthetics. *Curr Opin Anesthesiol,* 6 (4): 644-647, 1993.

17. Dispersyn G, Touitou Y, Coste O, Jouffroy L, Lleu JC: Desynchronization of daily rest-activity rhythm in the days following light propofol anaesthesia for colonoscopy. *Clin Pharmacol Ther,* 85 (1): 51-55, 2009. DOI: 10.1038/clpt.2008.179

18. Mihara T, Kikuchi T, Kamiya Y, Koga M, Uchimoto K: Day or night administration of ketamine and pentobarbital differentially affect circadian rhythms of pineal melatonin secretion and locomotor activity in rats. *Anesth Analg*, 115 (4): 805-813, 2012. DOI: 10.1213/ANE.0b013e3182632bcb

19. Kim MH, Lee KY, Bae SJ, Jo M, Cho JS: Intraoperative dexmedetomidine attenuates stress responses in patients undergoing major spine surgery. *Minerva Anestesiol,* 85 (5): 468-477, 2019. DOI: 10.23736/S0375-9393.18.12992-0

20. Zucco L, Santer P, Levy N, Hammer M, Grabitz SD, Nabel S, Ramachandran SK: A comparison of postoperative respiratory complications associated with the use of desflurane and sevoflurane: A single-centre cohort study. *Anaesthesia*, 76 (1): 36-44, 2021. DOI: 10.1111/anae.15203

21. Peyton PJ, Hendrickx J, Grouls RJE, Zundert AV, Wolf AD: End-tidal to arterial gradients and alveolar deadspace for anesthetic agents. *Anesthesiology,* 133, 534-547, 2020. DOI: 10.1097/ALN.0000000000003445

22. Naguib M, Gottumukkala V, Goldstein PA: Melatonin and anaesthesia: A clinical perspective. *J Pineal Res,* 42 (1): 12-21, 2007. DOI: 10.1111/j.1600-079X.2006.00384.x

23. Özer FD, Öçmen E, Akan P, Erdost HA, Korkut S: Effect of day and night desflurane anaesthesia on melatonin levels in rats. *Turk J Anaesthesiol Reanim*, 44 (4): 190-194, 2016. DOI: 10.5152/TJAR.2016.88609

24. Arruda NM, Braz LG, Nogueira FR, Souza KM, Aun AG: Gene and systemic inflammatory effects and neuroendocrine response in surgical patients anesthetized with desflurane-nitrous oxide or desflurane-nitrous oxide-free: A randomized trial. *Int Immunopharmacol*, 90:107163, 2021. DOI: 10.1016/j.intimp.2020.107163

25. Dikmen B, Arpaci AH, Kalayci D, Gunes I, Beskardes E: Are there any effects of Sevoflurane and Desflurane anaesthesia on blood glucose levels in acute hyperglycemic diabetic rats? *Bratisl Lek Listy*, 117 (6): 351-354, 2016. DOI: 10.4149/bll_2016_069

26. Hastings MH, Reddy AB, Garabette M, King VM, Chahad-Ehlers S: Expression of clock gene products in the suprachiasmatic nucleus in relation to circadian behaviour. *Novartis Found Symp*, 253, 203-222, 2003. DOI: 10.1002/0470090839.ch15

27. Bass J, Takahashi JS: Circadian integration of metabolism and energetics. *Science*, 330 (6009): 1349-1354, 2010. DOI: 10.1126/science.1195027

28. Tahara Y, Aoyama S, Shibata S: The mammalian circadian clock and its entrainment by stress and exercise. *J Physiol Sci*, 67 (1): 1-10, 2017. DOI: 10.1007/s12576-016-0450-7

29. Dibner C, Schibler U, Albrecht U: The mammalian circadian timing system: Organization and coordination of central and peripheral clocks. *Annu Rev Physiol*, 72, 517-549, 2009. DOI: 10.1146/annurev-physiol-021909-135821

30. Schiaffino S, Blaauw B, Dyar KA: The functional significance of the skeletal muscle clock: Lessons from *Bmal1* knockout models. *Skelet Muscle*, 6:33, 2016. DOI: 10.1186/s13395-016-0107-5

31. Franks NP, Lieb WR: Mapping of general anaesthetic target sites provides a molecular basis for cut off effects. *Nature,* 316 (6026): 349-351, 1985. DOI: 10.1038/316349a0

32. Merrow M, Spoelstra K, Roenneberg T: The circadian cycle: Daily rhythms from behaviour to genes. *EMBO Rep*, 6 (10): 930-935, 2005. DOI: 10.1038/sj.embor.7400541

33. Panda S, Hogenesch JB, Kay SA: Circadian rhythms from flies to human. *Nature*, 417, 329-335, 2002. DOI: 10.1038/417329a

34. Patel SS, Goa KL: Sevoflurane: A Review of its Pharmacodynamic and Pharmacokinetic Properties and its Clinical Use in General Anaesthesia. *Drugs*, 51 (4): 658-700, 1996. DOI: 10.2165/00003495-199651040-00009

35. Koblin DD, Weiskopf RB, Holmes MA, Konopka K, Rampil IJ: Metabolism of I-653 and isoflurane in swine. *Anesth Analg*, 68 (2): 147-149, 1989. DOI: 10.1213/00000539-198902000-00014

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Research Article

Observations on the Life Stages of the Fruit Fly *Drosophila melanogaster* When Fed Vitamin D3 in Artificial Diet [1]

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Abstract

This study focused on the use of an important micronutrient, vitamin D3 for control of pest insects as environmentally sound alternative management. Vitamin D3 which is a lipophilic vitamin at nutritionally reguired doses not only have important physiological roles but also has a toxic effect at high dietary concentrations. The first instar larvae of *Drosophila melanogaster* (Meigen) (Diptera: Drosophilidae), which is an important pest model organism were reared on artificial diet containing vitamin D3 concentrations of 5.0, 20, 80, 320, 1280 and 2560 mg/L to adult stage in laboratory condition. The effects of this vitamin on developmental stages of the insects were microscopically observed. Morphometric changes depending on increased vitamin D3 concentrations in the developmental stages from larvae to adults were recorded by microscobic and personal observations. Vitamin D3 at increasing concentrations (especially 80-2560 mg/L) in larval diet increased melanization in abdominal region of the insect. This study refers vitamin D3 can play a fortifying role in development as a nutritional additive at low concentrations, or can be used as an insecticide at high concentrations due to its possible toxic effect.

Keywords: Developmental stages, *Drosophila melanogaster*, Microscopic observation, Vitamin D3

Introduction

Vitamin D is the general termination for a group of nutritional substance ensuring in balanced bone and blood calcium levels and immunmodulation function in human and animals. Vitamin D3 is the main dietary resource for vitamin D. A large amount of vitamin D (estimated around 80% but varies with sun exposure) is biosynthesized in the skin from 7-dehydrocholecalciferol as the function of ultraviolet B (UVB) rays. While 25-hydroxy cholecalciferol (25(OH)D) and 1, 25-dihydroxycholecalciferol (1,25(OH)2D) are source of vitamin D from diet, 1,25-dihydroxycholecalciferol (1,25(OH)2D), the most hormonally active form of vitamin D [1]. However, besides the innate immune system, it has autocrine and paracrine roles in cell propagation, differentiation, and programmed cell death [2-4].

There is no direct relationship between the immune system of insects and vitamin D, because it is still unknown whether insects can synthesize vitamin D3 de novo or activate it from precursor molecules with UV-B. Oonincx et al.[5], showed that when four insects which has different in ecology, migratory grasshopper, *Locusta migratoria* (L.), house cricket *Acheta domesticus* (L.), yellow mealworm, *Tenebrio molitor* and the black soldier fly (BSFL) *Hermetia illucens* (L.) were exposed to a low-irradiation UV-B source, vitamin D3 levels in house crickets, vitamin D2 levels in BSFL, and vitamin D2 and vitamin D3 in *T. molitor* were increased. Higher UV-B irradiation increased vitamin D3 concentrations in all insects except *H. illucens*. An increase in vitamin D2 was observed in both *H. illucens* and *L. migratoria*. Bah-Nelson et al.[6], found that the unirradiated Black field

locust *Gryllus bimaculatus* did not have detectable vitamin D3 content (below 0.50 IU/g) and irradiation with UV-B light did not enhance its synthesis, or at least the levels were still undetectable. These studies showed that some insects can synthesize vitamin D de novo and the amounts are dependent on UV-B irradiation and exposure time.

Although its exact physiological role is not known in various insect species where vitamin D has been detected, concentrations varied greatly between species. For example, vitamin D1 was low during larval to pupal transformation (Sláma's sterol reuse theory, 1998) and highest in the pupa ensuring advanced metamorphosis which is responsible for the development of digestive and muscular systems in *Galleria mellonella* adult. It has been revealed that vitamin D1 in *Manduca sexta* has a role in tissue regeneration after epidermis injury [7].

Drosophila melanogaster (Meigen) (Diptera: Drosophilidae) is a major pest for different fruit crops in agricultural area and has developed resistance to insecticide [8]. Therefore, the use of any nutritional micronutrient such as vitamin D or its metabolites can serve as an alternate strategy for *D. melanogaster* management. It has been shown that nutritional vitamin perception is an important determinant of nutritional behavior and plays an important role in development in *D. melanogaster* [9]. There are most important advantages for this insect as model in investigations such as ahving short life cycle, high reproduction capacity and low laboratory culturing costs. Since the fruit fly has similar biological, biochemical, physiological and neurological traits with mammals, this insect is used as an alternative model animal for vertebrates [10,11]. Using this insect as a model, the preventive effect of vitamin D3 on the mutagenicity and carcinogenicity of some chemicals in *D. melanogaster* was studied [12]. Vitamin D3 alone did not increase the overall tumor incidence, but significantly reduced the overall tumor incidence when co-administered with a tumor stimulator, doxorubicin (DXR), in its somatic cells in this insect. There are no studies on the other hand, most of the studies investigated the effects of vitamin A (retinol) and E (α-tocopherol), which are fat-soluble vitamins added to food in low amounts, and water-soluble vitamin C (ascorbic acid) on the longevity of *D. melanosgaster* in relation to its antioxidant properties under oxidative stress conditions [13]. In this study, it has been shown that the intensity of red color in the abdomen increased due to the increase in food intake as a result of feeding the adults with a special dye (0.2% sulforhodamine) added to the foods to which the vitamins were added [13]. For nutrional requirements, 0.00067 g vitamin D3 (500.000 IU/g) was added to the liter of the food to meet the vitamin D requirement for certain foods [14]. In some other pest insects, Amiri et al.[15], showed a decrease in the consumption rate of *Plutella xylostella* to destruxin, fungal toxin showing antifeedant effects in a dose related manner. Increased mortality of *P. xylostella* larvae with increasing concentrations of destruxin A was also reported [16]. In the light of these studies, some of vitamin D metabolites or other micronutrients especially at high concentrations have been demonstrated to have had some deteriorating effects on the insects including *D. melanogaster*. Nevertheless, appearently no researcher has measured or even observed effects of vitamin D3 or other vitamin D metabolites on size or morphological changes in any parts of insects. However, vitamin D3 was registered by the American Environmental Protection Agency in 1984 as a 0.075% nutrient mixture (rodenticide) in and around closed areas, in transport vehicles, against rodents such as rats and mice, by surface or hand application [17].

Melanization is part of the immune defense response after infection or injury in insects and some vertebrates. In this process, melanin pigment is produced and helps neutralize foreign matter or pathogens in the infected or injured area [18]. The prophenoloxidase enzyme in the hemolymph and other tissues is activated in response to the presence of foreign matter or pathogens, and melanin pigment is formed [19]. Melanization in mammals is not as in insects, but similar immune response mechanisms are used.

This study aimed to investigate the effect of dietary vitamin D3 on developmental morphology of *D. melanogaster.* This study is important because it can play a role as a nutritional additive at low concentrations, and it will give an idea whether it can be used as an insecticide at high concentrations due to its toxic effect.

Material and Methods

Ethical Statement

Insect studies do not require ethics committee approval.

Insect Culture

D. melanogaster (Diptera: Drosophilidae) (Oregon R strain) were aseptically reared in 250-mL glass container containing artificial diet in an ES 500 incubator (Nüve, Ankara, Türkiye) at 25±2°C and 60-70% relative humidity (RH) in a 12:12 hour light/dark photoperiod. First instar larvae were selected by removing from the main culture for use in the experiments described here.

The artificial diet used for rearing of the insects and for preparation of treatments was described by Roberts [20]. Briefly, per 1000 mL total volume; diet contained 8 g agar (Merck & Co., New York, USA), 20 g sucrose (Carlo Erba Reagents S.A.S, Sabadell, Barcelona, Spain), 11.78 g dry yeast (Dr. Oetker Food Industry and Trade, Inc., Torbalı-

İzmir, Türkiye), 36 g of potato puree (Knorr, Unilever Co., Ümraniye, İstanbul, Türkiye), 0.8 g L-ascorbic acid (Carlo Erba Reagents S.A.S), 7.72 mL of nipagin (p-hydroxybenzoic acid methyl ester, crystal (Sigma-Aldrich Co., St. Louis, MO, USA) prepared in 3.5% ethanol and 1000 mL of water. The preparation methods of the diet and pouring diets into bottles, and to obtain eggs and larvae and their placement onto diets were described previously by Aslan et al.[21].

Vitamin D3 (C9756; ≥98%; $C_{27}H_{44}O$; Cholecalciferol, Activated 7-dehydrocholesterol, Calciol, Sigma-Aldrich (St. Louis, MO, USA) was added to diets by dissolving in Tween 80 (1% tween 80 in 3% etanol) establish concentrations of 5, 20, 80, 320, 1280 and 2560 mg/L. The control was the diet without vitamin D3 added. Tween 80 control (1% tween 80 in 3% etanol) was also used as solvent control. These vitamin D3 concentrations were calculated depending on to the research with preliminary experiments on *D. melanogaster* or the effect of other nutritional additives on *D. melanogaster* [21,22].

Feeding Experiments

D. melanogaster larvae to be used in the study were obtained from eggs laid by adults placed in glass bottles under condition for maintaining stock culture. The 1st instar larvae hatched in the food from the stock colonies were selected under a stereo microscope. 20 first instar larvae were transferred into 5 mL of diet poured into small 15-mL glass bottles and bottles were covered with hydrophilic cotton. Twenty larvae were transfered on control diet and diets containin vitamin D3 by using a soft-tipped brush (No: 0, Goya Toray). Bottles were kept in incubators maintained at condition used for stock insect cultivation (25±2°C and 60-70% RH on a 12:12 h light/dark photoperiod). We noted that 3rd-instar larvae migrated from the diet to the inside surface of the glass container where they pupated. Newly-formed pupae were marked on the inside surface of the glass and recorded. Survivors to the third instar, pupal stage, and adult stage were photographed (Olympus SZ61, SZ2-LGB) in each rearing bottle to determine the effects of vitamin D3 added into diet at tested concentrations on each developmental stage of the insects. Photographs scale bars were added with the LCmicro (version 5.1) software which is developed by Olympus Soft Imaging Solutions GmbH. The experiments were done as 4 replicates, and 20 larvae were used in each replication.

Statistical Analysis

Average intensity value in pupal stages in each concentrations of vitamin D3 were calculated by applying the photos to Adobe Photoshop [23]. Intensity data were analyzed by one-way "analysis of variance" (ANOVA) [24]. To determine significant differences between means least significant difference (LSD) test was used [24]. Significance was considered at 0.05 level.

Results

Our study showed that increased intensity of melanization occurs towards the last abdominal segments especially in the pupal stage of *D. melanogaster*. The results of the study also show the normal size and appereance of each developmental stage of *D. melanogaster* on the control artificial diet and the small size and abnormal melanization through the last abdominal segments of larval, pupal and adult stages of the insects reared on given dietary concentrations of vitamin D3 as dietary additives *(Fig. 1)*.

Histogram analysis shows average intensity values in pupal stages in different concentrations in comparison to control group *(Table 1)*. High intensive value (92.61±2.39), especially in pupae reared on 20 mg/L was recorded. However low intensity values in pupal stages at 320 and 2560 mg/L of vitamin D3 was recorded in relative to other tested concentrations of vitamin D3.

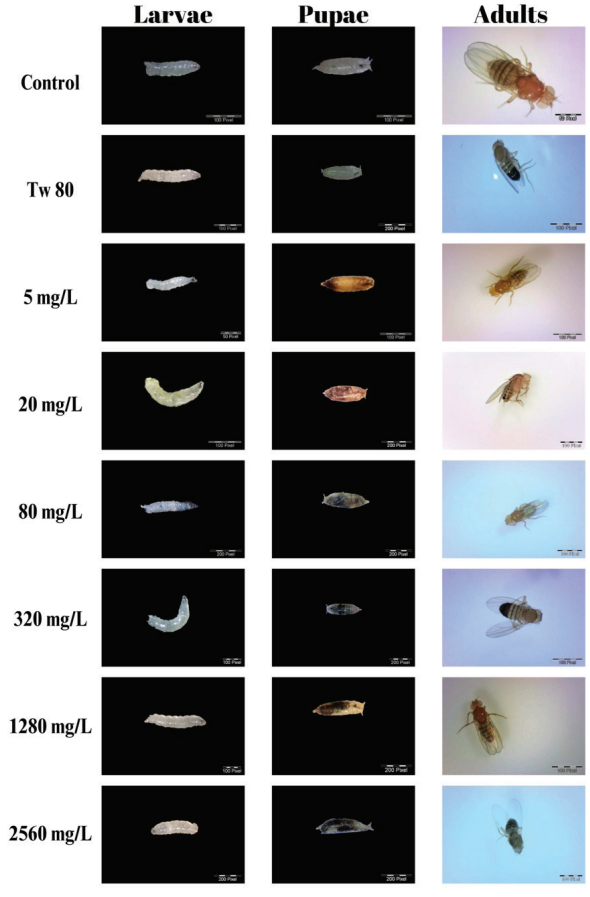

Fig 1. The effects of vitamin D3 on morphology of *D. melanogaster* reared on artificial diet. The magnification was ×20 and the scale bars represent pixels (1 Pixel = 0.14000 µm)

Table 1. *Intensity value of melanization in pupae reraed on artificial diet with vitamin D3*

Vitamin D3 Concentrations (mg/L)	Intensity Value in Pupal Stages (Mean*±S.E)†
0.000§	72.12±1.43[a]
Tw 80	68.74±1.65[a]
5	71.96±2.16[a]
20	92.61±2.39[bc]
80	69.47±3.78[a]
320	59.08±2.87[b]
1280	73.63±3.93[a]
2560	51.19±1.16[b]

**Four replicates with 5 pupae per replicate were used. †Means with the different letter are significantly different (P<0.05). §Control (without Vitamin D3). Tween 80 (Tw 80 solvent control)*

Discussion

The metabolic system of the insect may be affected biochemically by toxic proporties of these fat soluble vitamins at high concentrations as in vertebrates including human and animals. The effects of vitamin D alone have already been known in the some metabolic process of the insects [6]. The feeding experiments might be supported by the imaging techniques to determine their effects on wellfare of insects. Because, determination of only survivorship and developmental period sometimes may be insufficient to ascertain the effects of nutritional impairment of any dietary nutrient [25,26]. This study focused on the use of an important micronutrient, vitamin D3 for control of pest insects as environmentally sound alternative management.

In coincidence with our results with abdominal melanization appearence, abnormalty in vertebrates in relation to melanization is Peutz-Jegher syndrome showing formation of multiple polyps in different regions of the gastrointestinal canals, especially the large intestine [27]. Animals produce melanin pigments for the coloration of their skin and protection from harmful solar radiation. Insects also synthesize melanins even more ingeniously than mammals and use them for exoskeletal pigmentation, cuticular hardening, wound healing and innate immune responses [19]. Firstly, melanin was massively deposited at the wound region in insects for two major reasons with other biochemical processes to prevent further blood loss. Secondly, formed quinonoid products during melanogenic process are well known cytotoxic metabolites to kill any microbial agents entered the insect body through the wound site.

Melanization is an crucial and indispensable phase of the innate immune defence to biotic and abiotic invader [19,28]. The melanization in abdomen region of insects reared vitamin D3 might be attributed to deterioration in guts of larvae which are active feeding stage in response to toxic effects of high concentrations of vitamin D3. For example most insects consumpt the green leaves of plants but excrete black feces in an as yet unknown mechanism similar to our observation after vitamin D3 consumption by larvae. Here we show that the melanization of midgut and most probably hindgut content induced by high concentration of vitamin D3, with its possible toxicity that triggers the melanin synthesis around exces accumulation of vitamin D3 or toxic metabolites or at wound sites formed by toxic effects of vitamin D. Shao et al.[29], found that prophenoloxidase production in hindgut cells and secretion into the hindgut contents causing blackening in a model insect, silkworm *Bombyx mori*. Some researches showed the blackening of the insect feces was due to activated phenoloxidase enzim cascade, which decrease and finally death of bacteria in the hindgut. Our observation of melanization in abdomen region discloses why the fruit fly abdominal region is black showing melanization and provides understanding into innate immunity of hindgut, which is still not known in detailed in insects as stated by Shao et al.[29], for mammals.

High intensive value (92.61±2.39), especially in pupae reared on 20 mg/L may be attributed to melanization reaction as humoral immune adaptation in response to toxicity of this concentration vitamin D3. As in registered rodenticide effects of vitamin D3 [17], compared to other concentrations of vitamin D3, low intensity value of dietary 2560 mg/L vitamin D3 for *D. melanogaster* may be a result of vitamin D3 chronic toxicity because of rapid degradation of melanins. Similar results were obtained by a study with higher animals which reported a correlation between melanotic melanoma and lower survivorship than pigmented melanomas [30]. In the other hand, in consistent with our results showing low intensity value at the highest dose of vitamin D3, melanin might be degraded by a redox mechanism or lysosomally to polycyclic hydrocarbon and may form some reactive oxidant molecules a short after the melanization as suggested by Clancy and Simon [31].

Melanization can also be further induced around wounds to prevent additional mechanical injury in midgut or hindgut of *D. melanogaster* larvae caused by exces consumption of higher dietary vitamin D3 as a possible insecticide. The results of a study with a burrowing bug Cydnidae insects [32], in consistent with our results that this insect synthesize a chemicals such as hydrocarbonate, odorous substance. This causes red-brown volar melanotic pigmented discolored area in diffrent size and appearance at the site of contact with this chemicals on these insects. There is an evidence for our suggestion for vitamin D3 cause pigmentation that when silkworm *Helicoverpa armigera* larvae were orally fed with *Bacillus bombysepticus*, black chest septicaemia

and finally larval death were reported. A mucocutaneous pigmentation (peutz) on the thoracoabdominal region or the first three abdominal segments and then covering the whole body [33].

Consequently, a fat-soluble vitamin D3, as dietary ingredient in quantities above the normal requirement might be crucial nutritional alternatives for pest control. The midgut of insect is the first defensive barrier developing resistance and induces the immune response to fight microbial agents or chemicals exposed such as exsessive accumulation of vitamin D3 in midgut or hindgut of *D. melanogaster* in our study. Absorption rate of other nutrients may be changed as a result of overaccumulation of the vitamin D3 in the gut of the insect as in mammals. It has been already known that bioavailability of vitamin D is important factors including absorption, transportation and metabolism [34].

As Singh and House [35], stated for morphological changes in size of another dipteran pest insect *Agria affinis* with some dietary nonnutritional additive antibiotics; similar detrimental effect on larval, pupal and adult appearance in *D. melanogaster* varied with the level of vitamin D3 in a synthetic diet; for example, in proportion to the level of vitamin D3 was observed. This suggests some caution in use of dietary concentrations of such nutritional additives where normal insect species or damaged insects for pest management are desired. We infer from or results that a model for the process by which vitamin D3 damages the fruit fly midgut and hindgut decreasing survivorship. Although these results may shed a light for the registration of vitamin D3 as an insect control agent like rodenticide [17], further work on biological parameters, mass rearing, formulation, and field application should be undertaken.

Declarations

Availability of Data and Materials: The data sets during and/or analyzed during the current study are available from the corresponding author (E. Ö. Özdoğan) on reasonable request.

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References

1. Jones G: Metabolism and catabolism of vitamin D, its metabolites and clinically relevant analogs. **In,** Holick MF (Ed): Vitamin D. 2nd ed., 99-134, Springer, 2010.

2. Wang TT, Nestel FP, Bourdeau V, Nagai Y, Wang Q, Liao J, Tavera-Mendoza L, Lin R, Hanrahan JW, Mader S, White JH: Cutting edge: 1, 25-dihydroxyvitamin D3 is a direct inducer of antimicrobial peptide gene expression. *J Immunol,* 173, 2909-2912, 2004. DOI: 10.4049/jimmunol.173.5.2909

3. Reichrath J, Lehmann B, Carlberg C, Varani J, Zouboulis C: Vitamins as hormones. *Horm Metab Res,* 39, 71-84, 2007. DOI: 10.1055/s-2007-958715

4. Kamen DL, Tangpricha V: Vitamin D and molecular actions on the immune system: Modulation of innate and autoimmunity. *J Mol Med,* 88, 441-450, 2010. DOI: 10.1007/s00109-010-0590-9

5. Oonincx DGAB, van Keulen P, Finke MD, Baines FM, Vermeulen M, Bosch G: Evidence of vitamin D synthesis in insects exposed to UVb light. *Sci Rep,* 8, 10807, 2018. DOI: 10.1038/s41598-018-29232-w

6. Bah-Nelson I, Newton-Youens J, Ferguson A, Michaels CJ: Calcium accumulation and loss and vitamin D_3 content of feeder black field crickets *(Gryllus bimaculatus)* fed on a high calcium diet with and without UVB irradiation. *J Zool Bot Gard,* 2, 382-387, 2021. DOI: 10.3390/jzbg2030027

7. Slama K: Vitamin D1 versus ecdysteroids: Growth effects on cell regeneration and malignant growth in insects are similar to those in humans. *Eur J Entomol,* 116, 16-32, 2019. DOI: 10.14411/eje.2019.003

8. Morton RA: Evolution of *Drosophila* insecticide resistance. *Genome*, 36, 1-7, 1993. DOI: 10.1139/g93-001

9. Wu Q, Park SJ, Yang M, Ja WW: Vitamin preference in *Drosophila*. *Curr Biol,* 31, 946-947, 2021. DOI: 10.1016/j.cub.2021.06.046

10. Abolaji AO, Kamdem JP, Farombi EO, Rocha JBT: *Drosophila melanogaster* as a promising model organism in toxicological studies. *Arch Bas App Med,* 1, 33-38, 2013.

11. Koon AC, Chan HYE: *Drosophila melanogaster* as a model organism to study RNA toxicity of repeat expansion-associated neurodegenerative and neuromuscular diseases. *Front Cell Neurosci,* 11: 70, 2017. DOI: 10.3389/fncel.2017.00070

12. Vasconcelos MA, Orsolin PC, Oliveiria VC, Lima PMAP, Naves MPC, de Morais CR, Nicolau-Júnior N, Bonetti AM, Spano MA: Modulating effect of vitamin D3 on the mutagenicity and carcinogenicity of doxorubicin in *Drosophila melanogaster* and *in silico* studies. *Food Chem Toxicol,* 143:111549, 2020. DOI: 10.1016/j.fct.2020.111549

13. Bahadorani S, Bahadorani P, Phillips JP, Hilliker AJ: The effects of vitamin supplementation on *Drosophila* life span under normoxia and under oxidative stress. *J Gerontol A Biol Sci Med Sci*, 63, 35-42, 2008. DOI: 10.1093/gerona/63.1.35

14. Lee WC, Micchelli CA: Development and characterization of a chemically defined food for *Drosophila*. *PLoS ONE,* 8:e67308, 2013. DOI: 10.1371/journal.pone.0067308

15. Amiri B, Ibrahim L, Butt TM: Antiffedant properties of destruxins and their potential use with the entomogenous fungus *Metarhizium anisopliae* for improved control of crucifer pests. *Biocont Sci Technol,* 9, 487-498, 1999. DOI: 10.1080/09583159929451

16. Huang S, Ali S, Ren SX: The combination of destruxin A with insecticide chlorantraniliprole increases virulence against *Plutella xylostella* L.. *Pak J Zool,* 48, 1849-1855, 2016.

17. EPA: Vitamin D3, united states environmental protection agency, pesticide fact sheet. No: 42 CAS Number: 434-16-2, 1984.

18. Brenner M, Hearing VJ: The protective role of melanin against UV damage in human skin. *Photochem Photobiol,* 84, 539-549, 2008. DOI: 10.1111/j.1751-1097.2007.00226.x

19. Sugumaran M, Barek H: Critical analysis of the melanogenic pathway in insects and higher animals. *Int J Mol Sci,* 17:1753, 2016. DOI: 10.3390/ijms17101753

20. Roberts DB: Drosophila: A Practical Approach. 2nd ed., 19, Oxford: IRL, 1986.

21. Aslan N, Büyükgüzel E, Büyükgüzel K: Oxidative effects of gemifloxacin on some biological traits of *Drosophila melanogaster* (Diptera: Drosophilidae). *Environ Entomol,* 48, 667-673, 2019. DOI: 10.1093/ee/nvz039

22. Keleş V, Büyükgüzel K, Büyükgüzel E: The effect of streptomycin on survival, development, and some biochemical aspects of *Drosophila*

melanogaster. *Turk J Zool,* 45, 432-441, 2021. DOI: 10.3906/zoo-2101-14

23. Adobe Photoshop, User's Manual. Version 24.7.0, California, USA, 2023.

24. IBM SPSS Statistics: User's Manual. Version 28.0. SPSS, Chicago, IL, USA, 2021.

25. House HL, Barlow JS: The effects of a new salt mixture developed for *Agria affinis* (Fallén) (Diptera: Sarcophagidae) on the growth rate, body weight, and protein content of the larvae. *J Insects Physiol,* 11, 915-918, 1965. DOI: 10.1016/0022-1910(65)90195-2

26. Thompson SN: Nutrition and *in vivo* culture of insect parasitoids. *Annu Rev Entomol,* 31, 197-219, 1986. DOI: 10.1146/annurev.en.31.010186.001213

27. Wu BD, Wang YJ, Fan LL, Huang H, Zhou P, Yang M, Shi XL: Clinical and genetic analyses of 38 chinese patients with peutz-jeghers syndrome. *Biomed Res Int*, 2020:9159315, 2020. DOI: 10.1155/2020/9159315

28. Sugumaran M: Role of insect cuticle in immunity. **In,** Soderhall K, Iwanaga S, Vastha G, (Eds): New Directions in Invertebrate Immunology. 355-374, SOS Publications, Fair Haven, NJ, 1996.

29. Shao Q, Yang B, Xu Q, Li X, Lu Z, Wang C, Huang Y, Söderhäll K, Ling E: Hindgut innate immunity and regulation of fecal microbiota through melanization in insects. *J Biol Chem,* 287, 14270-14279, 2012. DOI: 10.1074/jbc.M112.354548

30. Thomas NE, Kricker A, Waxweiler WT, Dillon PM, Busman KJ, From L, Groben PA, Armstrong BK, Anton-Culver H, Gruber SB, Marrett LD, Gallagher RP, Zanetti R, Rosso S, Dwyer T, Venn A, Kanetsky PA, Orlow I, Paine S, Ollila DW, Reiner AS, Luo L, Hao H, Frank JS, Begg CB, Berwick M; Genes, Environment, and Melanoma (GEM) Study Group: Comparison of clinicopathologic features and survival of histopathologically amelanotic and pigmented melanomas: A population-based study. *JAMA Dermatol,* 150, 1306-1314, 2014. DOI: 10.1001/jamadermatol.2014.1348

31. Clancy CM, Simon JD: Ultrastructural organization of eumelanin from *Sepia officinalis* measured by atomic force microscopy. *Biochemistry,* 40, 13353-13360, 2001. DOI: 10.1021/bi010786t

32. Malhotra AK, Lis JA, Ramam M: Cydnidae (burrowing bug) pigmentation: A novel arthropod dermatosis. *JAMA Dermatol,* 151, 232-233, 2015. DOI: 10.1001/jamadermatol.2014.2715

33. Lin P, Cheng T, Jin S, Wu Y, Fu B, Long R, Zhao P, Xia Q: PC, a novel oral insecticidal toxin from *Bacillus bombysepticus* involved in host lethality via APN and BtR-175. *Sci Rep,* 5, 11101, 2015. DOI: 10.1038/srep11101

34. Maurya VK, Aggarwal M: Factors influencing the absorption of vitamin D in GIT: An overview. *J Food Sci Technol,* 54, 3753-3765, 2017. DOI: 10.1007/s13197-017-2840-0

35. Singh P, House HL: Antimicrobial agents: Their detrimental effects on size of an insect, *Agria affinis*. *Can Entomol*, 102, 1340-1344, 1970. DOI: 10.4039/Ent1021340-10

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Research Article

Antimicrobial Resistance of *Escherichia coli* Involved in Algerian Bovine Carriage, ESBL Detection, Integron Characterization and Genetic Lineages

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Abstract

This study aimed to characterize the fecal carriage of antimicrobial-resistant *Escherichia coli* isolates in healthy bovine in Northern Algeria. Fecal samples of 233 cows were collected and cultured on MacConkey agar. *E. coli* isolates were recovered, identified and tested for antibiotic susceptibility by disk diffusion method. Screening of extended-spectrum-betalactamase (ESBL)-production was performed by double-disk synergy test and characterization of ESBL genes by PCR and sequencing. All isolates were typed for phylogenetic groups and multilocus-sequence-typing (MLST) analysis was performed on phylogroup B2 and ESBL-producing isolates. The presence of antimicrobial resistant genes was analyzed in the collection of *E. coli* isolates and integrons in SXT-resistant isolates. Overall, 39.9% of *E. coli* isolates (89/223) were resistant to at least one antimicrobial agent, and 41.5% of them showed multi-drug resistance (MDR). High resistance rates were detected for tetracycline (32.3%), streptomycin (18.4%), sulphamethoxazole/trimethoprim (15.7%) and ampicillin (15.2%). Two ESBL-producing *E. coli* isolates were identified: A/ST617/CTX-M-15 and A/ST48/SHV-12. Sequence types ST95, ST998 and ST145 were detected among the phylogroup B2 isolates. From 35 SXT^R isolates, class-1 and class-2 integrons were detected in 82.9% (29/35) and 12.9% (1/35), respectively. Six gene-cassette-array structures were detected in the variable region of class-1 *(dfrA1-aadA*; *dfrA12-aadA2, aadA1/2; dfrA12-orfF-aadA2-cml-sul3*-linked and *dfrA17-aadA5)* and class-2 integrons *(dfrA1-sat2-aadA1)*. Our study highlights the potential dynamics of animal *E. coli* isolates in farms.

Keywords: Antimicrobial resistance, bovine, *E. coli*, Integrons, MLST, Phylogenetics

Introduction

In veterinary husbandry, antibiotics are used as therapeutic or prophylactic agents, for the treatment and control of infectious diseases, or, in some countries, as growth promoters to improve weight gain [1]. Nowadays, antibiotics are highly used in dairy industry and in animal farming, and in some countries with scarce control; in this sense, a study refer that 56% of farmers in a sub-Saharan country use non-prescribed antibiotics and about 25% of countries at world level use antibiotics as growth promoters of animals [2,3]. These practices associated with insufficient hygiene and biosecurity led to the emergence and spread of antimicrobial resistance globally [4]. Along the food chain, antimicrobial resistance is considered as a major global public health concern, because many food animals are carrying antibiotic-resistant strains, such as extended spectrum-beta-lactamase (ESBL) producing Enterobacteriaceae [5].

The acquisition of new resistance mechanisms leading to antimicrobial resistance, and the declining flow of new antimicrobial agents continue to threaten our ability to treat common infections, particularly infections caused

by multidrug-resistant (MDR) microorganisms [6,7]. Bacterial infections with MDR are of particular concern because it limits treatment options, can be transferred between pathogenic bacteria, and increases superbug morbidity [8]. ESBL-producing *E. coli* is an emerging MDR bacteria resistant to third-generation cephalosporins and monobactams [9]. *E. coli* is a normal inhabitant of the human intestine, which could under some circumstances cause severe sepsis and urinary tract infections, among hospital-level infections [10]. In animals, diarrhea and several infectious diseases caused by *E. coli* are considered the main causes of economic losses associated with poor growth, drug costs and animal death [11]. The intensification of cattle breeding and the intensive use of antibiotics make cattle important reservoirs of resistant bacteria that can be disseminated at the human-animal-environment interface [12]. Although antimicrobial resistant *E. coli* from cattle have been reported in many parts of the world, information on cattle as potential reservoir of *E. coli* resistant to antimicrobials, particularly in Algeria, is more-scarce. Analysis of antimicrobial resistance genes and molecular typing of *E. coli* isolates from cattle will provide useful data for predicting potential risks associated with mammals' *E. coli* in Algeria.

This study aimed at determining the frequency of ESBL-producing *E. coli*, the genetic characteristics and antibiotic-resistant profiles among *E. coli* recovered from cattle feces in northern Algeria

Material and Methods

Ethical Statement

The study protocol was approved by the Veterinary Science Institute Scientific Committee of the university Saad Dahlab of Blida1 (Ref: CSI/N°12/2015).

Sampling and Bacterial Isolation

From January 2017 to September 2019, 30 farms were visited in three department districts of northern Algeria. Most of the farms (21/30, 70%) were located at Tizi-Ouzou while the remaining were distributed between Algiers (4 farms) and Blida (5 farms). After obtaining consent from the farm's owners, accessible animals inside the stable were submitted to fecal sampling. Up to 50 grams of fecal matter were directly taken from the rectum of each animal in a sterile jar and transported immediately to the laboratory under cold storage for processing.

Fecal samples were diluted (1:10) in buffered peptone water (Pasteur Institute of Algiers, Algeria) and incubated at 37°C for 24 h. The enriched culture was inoculated on MacConkey agar plates (Conda, Spain) and incubated at 37°C for 24 h. One presumptive *E. coli* colony per sample was randomly selected and identified by classical biochemical methods (gram-staining, oxidase test, TSI, indol) and API 20E gallery (BioMerieux, France). The identification was confirmed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry method (MALDI-TOF MS, Bruker) in the Laboratory of Biochemistry and Molecular Biology in the University of La Rioja (Logroño, Spain). One *E. coli* isolate per sample was maintained for further studies. *Table 1* shows the isolates recovered from each of the farms tested.

Antimicrobial Susceptibility and ESBL Phenotypic Tests

Antibiotic susceptibility testing for ampicillin (AMP), amoxicillin/clavulanate (AMC), cefotaxime (CTX), ceftazidime (CAZ), cefoxitin (FOX), imipenem (IMP),

Table 1. *Number of E. coli isolates obtained from faecal samples of bovine of 30 different farms located in 3 different departments of Algeria*

Farm Number	Region	No. of the Tested Samples	No. of *E. coli* Isolates
1	Blida	3	3
2	Blida	3	3
3	Algiers	3	3
4	Tizi-Ouzou	6	6
5	Tizi-Ouzou	7	7
6	Tizi-Ouzou	7	7
7	Tizi-Ouzou	15	15
8	Blida	7	7
9	Tizi-Ouzou	7	7
10	Tizi-Ouzou	9	9
11	Tizi-Ouzou	12	12
12	Tizi-Ouzou	11	11
13	Tizi-Ouzou	14	14
14	Tizi-Ouzou	19	19
15	Tizi-Ouzou	6	6
16	Tizi-Ouzou	8	8
17	Tizi-Ouzou	4	4
18	Tizi-Ouzou	3	3
19	Tizi-Ouzou	8	8
20	Tizi-Ouzou	17	17
21	Tizi-Ouzou	4	4
22	Tizi-Ouzou	8	8
23	Algiers	9	9
24	Algiers	4	4
25	Blida	7	7
26	Tizi-Ouzou	7	7
27	Tizi-Ouzou	4	4
28	Tizi-Ouzou	5	5
29	Blida	4	4
30	Algiers	2	2
Total		223	223

ciprofloxacin (CIP), gentamicin (GEN), chloramphenicol (CHL) and sulfamethoxazole/trimethoprim (SXT) was performed by the disk diffusion method as recommended by EUCAST [13]. For streptomycin (STR) and tetracycline (TET), the CLSI recommendation interpretative criteria were followed [14]. The screening for ESBL production was carried out by double-disk test (DDST), using third generation cephalosporins (CTX and CAZ) and a beta-lactamase inhibitor (AMC). Isolates showing resistance to at least three families of antimicrobial agents were considered as multidrug resistant (MDR).

Characterization of Antimicrobial Resistance Genes

Bacterial DNA was extracted by boiling three to five colonies in 1 mL of sterile Milli-Q water for 8 min. The suspension was centrifuged at 12.000 rpm for 2 min; the supernatant was collected and stored at -20°C for later use. *E. coli* isolates resistant to beta-lactams were tested by PCR for beta-lactamase genes: bla_{TEM}, bla_{SHV}, bla_{OXA-1}, bla_{CTX-M}-universal, and $bla_{CTX-M-1}$ group. The PCRs for bla_{CTX-M}-universal and $bla_{CTX-M-1}$ group were performed for the ESBL-producing isolates. Positive amplicons were sequenced to identify the beta-lactamase gene subtype. *E. coli* isolates were screened for the presence of resistance genes such as: *tet*(A)/*tet*(B) for tetracycline, *sul1/sul2/sul3* for sulphonamide, *cmlA*/*floR* for chloramphenicol, *qnrA/qnrB/qnrS/aac(6')*-Ib-cr for ciprofloxacin and *aac*(3)-II for gentamicin resistance [15].

Integron Analysis

SXT resistant (SXT^R) *E. coli* isolates were tested for the integrase of class 1, 2 and 3 integrons *(intI1, intl2,* and *intl3,* respectively). The variable regions of class 1 and class 2 integrons were amplified by PCR in all *intl*1-positive and *intI2*- positive isolates and amplicons were sequenced to obtain the gene cassette arrays [16].

Phylogenetic Groups and Multi Locus Sequence Typing

E. coli isolates were assigned to one the 8 phylogenetic groups (A, B1, B2, C, D, E, F and Clade I) by using the quadruplex PCR strategy as well as the specific PCRs designed for phylogroups C and E [17]. To identify the genetic lineages of selected *E. coli* isolates (ESBL-producing isolates and those affiliated into the phylogenetic group B2), Multilocus sequence typing (MLST) of seven housekeeping genes *(adk, fumC, gyrB, icd, mdh, purA* and *recA)* was performed by PCR and sequencing to determine the sequence type (ST) *(http://mlst.warwick.ac.uk/)* [18].

Data Analysis

Raw data were entered to Microsoft Excel (2016; Microsoft Corp., Redmond, WA, USA) and imported to MedCalc version 2019 (Ostend, Belgium) for statistical analysis. Binary logistic regression was used to determine the association between the phylogenetic groups and the presence of antimicrobial resistance. In this model phylogroup A was as a reference. A *p*-value of 0.05 was used to determine the significance level.

Results

Antimicrobial Resistance Phenotype and Genotype

A total of 223 *E. coli* isolates were obtained of 223 samples of cattle feces (one isolate per sample) *(Table 1)*. Antibiotic susceptibility results showed that 134 (60.1%) of the isolates were susceptible to all antimicrobial drugs tested, while 89 isolates (39.9%) were resistant to at least one antibiotic. Resistance to cefoxitin and imipenem was not found while resistance levels for other antibiotics were as follows (percentage of resistance): tetracycline (32.3%), streptomycin (18.4%), sulfamethoxazole/trimethoprim (15.7%), ampicillin (15.2%), amoxicillin/clavulanic acid (10.8%), gentamicin (6.7%), chloramphenicol (5.4%), ciprofloxacin (3.1%), and cefotaxime and ceftazidime (0.4%). Two of the 223 *E. coli* isolates showed an ESBL phenotype, and the remaining 221 were ESBL-negative.

Resistance Genes Detected Among the ESBL-negative *E. coli* Strains

Table 2 shows the percentage of antibiotic resistance among the 221 non-ESBL-producing *E. coli* isolates of bovine origin analysed in this study. From the 32 ampicillin resistant isolates, 24 (75%) carried the bla_{TEM} gene and 1 (3.1%) carried the bla_{OXA-1} gene. Tetracycline resistance (70 strains) was associated with the presence of *tet*(A) (22.8%), *tet*(B) (22.8%) or *tet*(A)+*tet*(B) genes (4.3%). The *sul*2, *sul*3, *sul1+sul2* and *sul2+sul3* genes were detected in 54.5%, 3%, 24.2% and 15.1 % of SXT resistant isolates, respectively. The *cmlA* gene was found in 58.3% (7/12) of chloramphenicol-resistant isolates. The *qnrS* gene was identified in 22.8% (1/6) of ciprofloxacin resistant isolates. Finally, the *aac*(3)-II gene was revealed in 28.6% (4/14) of gentamicin resistant isolates. *Table 3* shows the phenotypes of resistance shown by all the *E. coli* isolates of the study.

Out of the 89 tested isolates, 50.5% showed resistance to a minimum of two antibiotics. Upon the resistant strains, fourteen patterns of resistance were identified. Two ESBL producing *E. coli* isolates were obtained in two farms from Tizi-Ouzou and Blida *(Table 3)*. Multi-drug resistance (resistance to at least three families of antibiotics) was observed in 41.5% (37/89) of the tested strains *(Table 4)*.

Characteristics of ESBL-producing Strains

Two ESBL-producing *E coli* isolates were identified in this study and the characteristics are shown in *Table 4*. One of them was ascribed to lineage ST617 and phylogroup A, showed a MDR phenotype [AMP-AMC-CTX-CAZ-TET-

***Table 2**. Percentage of antibiotic resistance among the 221 non-ESBL-producing E. coli isolates of bovine origin analysed in this study*

Antibiotic	No. of Isolates Showing Resistance	Rates of Resistance	Resistance Genes (No. of Isolates/%)
Ampicillin	32	14.5	*bla*$_{TEM}$ (24/75%) *bla*$_{OXA1}$ (1/3.1%)
Amoxicillin/clavulanic acid	23	10.4	*bla*$_{TEM}$ (15/65.2%)
Cefotaxime + ceftazidime	0	0.0	-
Ciprofloxacin	6	2.7	*qnrS* (1/22.8%)
Sulphamethoxazole/ Trimethoprim	33	14.9	*sul2* (18 / 54.5%) *sul3* (1/3%) *sul1+sul2* (8/24.2%) *sul2+sul3* (5/15.1%) *dfrA1* (10/30.3%) *dfrA12* (2/6%)
Tetracycline	70	31.7	*tetA* (16/22.8 %) *tetB* (16/22.8%) *tetA+tetB* (3/4.3%)
Gentamicin	14	6.3	*aac3-II* (4/28.6%)
Streptomycin	41	18.5	*aadA1* (11/26.8%) *aadA2* (2/4.9%) *aadA1/2* (5/12.2%)
Chloramphenicol	12	5.4	*cmlA* (7/58.3%)
Imipenem Cefoxitin	0 0	0.0 0.0	-

***Table 3.** Phenotypes of antimicrobial resistance exhibited by the collection of 223 E. coli isolates obtained of bovine fecal samples*

Phenotype of Antibiotic Resistance[a,b]	No. of Isolates	Percentages
Susceptible	134	60.1
TET	28	12.5
AMP-AMC-TET^{17}-SXT^{13}-STR^{14}-GEN^{4}-CHL^{8}-CIP^{3}	19	8.5
AMP-TET^{5}-SXT^{7}-STR^{7}-CHL^{1}-CIP^{1}	8	3.6
AMP-AMC AMP	4 1	1.8 0.4
AMP-AMC-CTX-CAZ-TET-SXT-CIP-GEN-ESBL^{+} AMP-TET-SXT-ESBL^{+}	1 1	0.4 0.4
TET-SXT-STR-GEN^{2}-CHL^{1}	13	5.8
TET-STR TET-GEN-STR^{2}-CIP^{1}	4 3	1.8 1.3
GEN	3	1.3
GEN-CIP	1	0.4
GEN-STR	1	0.4
CHL	2	0.9

[a] *AMP, ampicillin; AMC, amoxicillin/clavulanicacid, CTX, cefotaxime; CAZ, ceftazidime; FOX, cefoxitin; CIP, ciprofloxacin; SXT, sulfamethoxazole/trimethoprim; TET, tetracycline; GEN, gentamicin; STR, streptomycin; CHL, chloramphenicol. ESBL+: ESBL-producer phenotype*

[b] *Those in superscript indicate the number of isolates that showed the specific resistance for the indicated antibiotic, in case that not all of isolates of the group were resistant*

SXT-CIP-GEN] and carried the gene encoding CTX-M15, as well as the beta-lactamase resistance gene *bla*$_{OXA-1}$, aminoglycoside resistance gene *aac*(3)-II, tetracycline resistance gene *tet*(B) and the sulphamethoxazole resistance genes *sul1* and *sul2*. The second isolate was typed as ST48/phylogroup A, contained the gene encoding

Table 4. Characteristics of the seven E. coli isolates showing an ESBL-phenotype or being included into phylogenetic group B2

Isolate Code	Farm/Region	ESBL-test	Phenotype of Antimicrobial Resistance [a]	Antimicrobial Resistance Genes	Integron 1 (Gene Cassette Array)	MLST	Phylogenetic Group
X2535	29/Blida	+	AMP-AMC-CTX-CAZ-CIP-SXT-TE-GEN	*bla*$_{CTX-M-15}$, *bla*$_{OXA-1}$, *tetB*, *sul1,sul2,aac3-II, aac(6')-Ib-cr*	+ (*dfrA17-aadA5*)	ST617	A
X2525	26/Tizi-Ouzou	+	AMP-SXT-TE	*bla*$_{SHV12}$, *tetA*, *sul3*	+	ST48	A
X2325	3/Algiers	-	AMP-AMC-TE	*bla*$_{TEM}$	-	ST998	B2
X2384	11/Tizi-Ouzou	-	AMP	*bla*$_{TEM}$	-	ST14	B2
X2393	11/Tizi-Ouzou	-	Susceptible	-	-	ST95	B2
X2509	24/Algiers	-	CHL	-	-	ST95	B2
X2515	25/Blida	-	Susceptible	-	-	ST95	B2

[a]*AMP, ampicillin; AMC, amoxicillin/clavulanicacid, CTX, cefotaxime; CAZ, ceftazidime; CIP, ciprofloxacin; SXT, sulfamethoxazole/trimethoprim; TET, tetracycline; GEN, gentamicin; CHL, chloramphenicol*

SHV-12, and carried the genes *tet*(A), *intl1,* and *sul3*; this isolate showed phenotypic resistance to AMP-TET-SXT, but presented a positive screening ESBL test *(Table 4)*.

Characterization of Integrons

Out of 35 SXT^R *E. coli* isolates typed for integrons, 29 (82.8%) carried the *intI1* gene and 1 (2.8%) isolate carried the *intI2* gene, No class 3 integrons were detected. Different gene cassette arrays were found in the class 1 integrons: *aadA1/2* (5 isolates), *dfrA1-aadA1* (10 isolates), *dfrA17-aadA5* (1 isolate), *dfrA12-aadA2* (1 isolate), and *dfrA12-orfF-aadA2-cmlA/aadA1* (1 isolate). In addition, the *dfrA1-sat2-aadA1* array was detected in the variable region of a class 2 integron of one additional *E. coli* isolate *(Fig. 1)*.

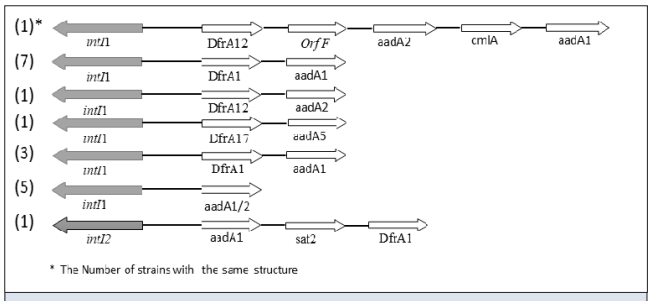

Fig 1. Different structures of class1 and class 2 integrons

Table 5. Distribution of Isolates by phylogenetic groups and correlation with antimicrobial resistance

Phylogroup[a]	No. of Isolates (%)	No. of Isolates (%) Showing Resistance to at Least One Antimicrobial	OR (95% CI)	P Value	No. of isolates Showing Resistance to the Following Number of Antimicrobial Families:						No. and (%) of MDR Isolates	OR (95% CI)	P Value
					1	2	3	4	5	6			
A	70 (31.4)	22 (31.4)	Referent	Referent	9	2	4	3	3	1	11 (15.7)	Referent	Referent
B1	131 (58.7)	59 (45)	1.79 (0.97-1.29)	0.062	27	11	12	4	4	1	21 (16)	1.02 (0.4624-2.27)	0.953
Others	22 (2.2)	8 (22.7)	1.25 (0.46-3.40)	0.667	2	1	1	3	1	-	5 (2.2)	1.19 (0.34-4.20)	0.785
Total	223 (100)	89 (39.9)	NA	NA	38	14	17	10	8	2	37 (16.6)	NA	NA

[a]*Phylogenetic group according to Clermont et al.*[17], [b] *Reference group (Phylogroup A) was chosen arbitrarily, NA = Not applicable for statistical analysis*

Phylogenetic Typing of the *E. coli* Isolates

Seven distinct phylogroups were distinguished among the 223 *E. coli* isolates of this study, with a predominance of the groups B1 and A with 58.7% and 31.4% of isolates, respectively *(Table 5)*. The phylogroups E and B2 represented 4.9% (11/223) and 2.2 % (5/223) respectively, while the phylogroups C, D and F shared three isolates. The 5 isolates of the phylogroup B2 were typed by MLST as ST998, ST14 and ST95. The isolates of phylogroup B2 were recovered from four farms belonging to three different regions of Algeria *(Table 4)*. No statistical correlation was found between phylogenetic groups and the frequency of resistance to at least one antimicrobial agent, or with the rate of resistance to increasing number of antimicrobial families (P>0.05) *(Table 5)*.

Discussion

The unregulated use of antibiotics in bovine farms may enhance the spread of drug-resistant bacteria, particularly ESBL-producing *E. coli*, in the community. These latter

have emerged as a major problem around the world. Primarily, ESBL-producing *E. coli* isolates were only observed in human clinical isolates, but these bacteria have increased drastically in food-producing animals, making them a natural reservoir and contributing to its spread [19]. In the present study, overall, 39.9% of *E. coli* isolates were resistant to at least one antimicrobial agent, whose 41.5% with multi-drug resistant (MDR). The apparent prevalence of resistance to antibiotics recorded in the present survey is lower than those reported in formerly published reports on *E. coli* involved in poultry and pig carriage [20,21]. Only two ESBL-producing *E. coli* isolates were detected in the present study. Unlike to our findings, higher rates of resistance to cefotaxime were observed in *E. coli* isolates recovered from fecal samples of the farms keeping beef cattle (70%) and dairy cattle (85%) in Germany [22]. It is important to remark that no selective media for ESBL-producing *E. coli* recovery was used in our study; so, the prevalence could be higher if antibiotic-supplemented media would be used for ESBL-*E. coli* isolation.

Resistance to tetracycline (31.7%) and streptomycin (18.5%) were the most prevalent phenotypes observed in the tested *E. coli* isolates in our study. Reports from Iran showed higher levels of resistance to streptomycin (98.25%) and tetracycline (98.09%) in *E. coli* isolated from diarrheic calves [23]. The variation between these studies could be due to differences in regulations on antimicrobial use in animals adopted by these countries and therapy traditions followed by veterinarians. Tetracyclines have been used frequently for many decades as efficient and inexpensive antimicrobial agents for animals. The rate of tetracycline resistance detected in our study (31.7%), is in the frame of data obtained in other studies in which higher and lower resistant rates were detected (range4.8-54.5%) [24-27]. Our results concur with the resistance rates to amoxicillin/clavulanic acid (11.62%), sulphamethoxazole/trimethoprim (15.15%) and chloramphenicol (4.04%) reported previously in eastern Algeria [25]. However higher resistance levels were observed in the study of Barour et al.[25] to ampicillin (59.1%) and ciprofloxacin (7.1%).

In the present study, resistance to beta-lactams was mainly associated with the presence of bla_{TEM} gene. This latter was blamed in 24 tested AMP^R isolates while only one AMP^R isolates carried bla_{OXA1}. In a previous report from Tanzania, Madoshi et al.[28] stated that most of beta-lactam resistant *E. coli* isolates recovered from cattle carried bla_{TEM} gene. Sulphonamide resistance genes including *sul2* (51.4%), *sul3* (5.7%), *sul1+sul2* (25.7%), *sul2+sul3* (14.3%) and tetracycline resistance genes *tetA* (23.6%), *tetB* (23.6%) and *tetA+tet B* (4.2%). Accordingly, previous studies reported that sulphonamide resistance genes (*sul1/sul2*) were often found together with tetracycline resistance genes *tet(A) and tet(B)* [29]. The genes *tet(A)* and/or *tet(B),* encoding efflux mechanisms, have been reported to be the most common tetracycline resistance determinant in *E. coli* isolates from humans and animals in many countries [30]. They were associated with 50% (35/70) of the *E. coli* isolates with TET^R phenotypes tested in this study. The number of the isolates harboring exclusively *tet(A)* is similar to those harboring exclusively *tet*(B) genes. Our findings are consistent with the earlier reports showing equal *tet*-gene patterns distribution in *E. coli* isolates recovered from animals, including cattle [30,31]. Other studies reported discordant results with either higher frequencies of *tet(A)* determinant in *E. coli* isolates recovered from cattle [32] or higher frequencies of *tet*(B) genes in *E. coli* isolates [33].

In relation to integron analysis of SXT^R isolates, class 1 integron was detected in 82.8% (29/35) of SXT^R *E. coli* isolates. Five gene-cassette-arrays structures were detected in their variable region: *aadA1/2* (5 isolates), *dfrA1-aadA1*(10 isolates), *dfrA17-aadA5* (1 isolate), *dfrA12-aadA2* (1 isolate), and *dfrA12-OrfF-aadA2-cmlA/aadA1* (1 isolate). One isolate carried the *intI2* with the gene cassette array *dfrA1-sat2-aadA1*. A study conducted in China showed that 66% of *E. coli* strains carried class 1 integron and gene cassette arrays of *aadA1* (most prevalent with 20%), *aadA7, aadA5, aadA17, dfrA1, dfrA5, dfrA1-aadA1, dfrA12-aadA2 and dfrA17-aadA5* [30]. Sequence analysis showed that, the genes *aadA* and *dfrA,* associated to streptomycin and trimethoprim resistance, were dominant in the gene cassette arrays in this study which concurs with previous reports in *E. coli* isolates from cattle [34].

In regards to the phylogenetic groups, B1 (58.7%) and A (31.4%) were the predominant among the *E. coli* isolates. The phylogroups A and B1 are commensals in the intestine and are commonly shed in feces of healthy animals including cattle [35], and blamed in 67.4% of mastitis cases in dairy cattle in China [36]. A study from Brazil showed that most of bovine clinical mastitis associated *E. coli* isolates were assigned to phylogroups A (52%) and B1(38%) [37]. Upon bivariate logistic regression, there was no association between *E. coli* phylogenetic groups and antimicrobial resistance frequencies ($p>0.05$) found during our survey. The major multidrug-resistant *E. coli* isolates belonged to phylogroups A (16%) and B1 (15.7%). In Beijing, 58.6% of antibiotic-resistant *E. coli* strains were affiliated to group B1 and 35.7% were in the group A [38]. Additionally, *E. coli* isolates with MDR were mainly classified in phylogenetic groups A or B1 [39]. The combination of different phylogeny and antimicrobial resistance of *E. coli* may improve the recognition of new subgroups of virulent bacteria.

In cattle, bla_{SHV12} is frequently detected among ESBL producing *E. coli* isolates [19,40]. Molecular analysis of the

two ESBL producing *E. coli* revealed the following patterns [Phylogroup A/ST617/$bla_{CTX-M-15}$] and [Phylogroup A/ST48/bla_{SHV-12}]. Similar findings were reported in Iran [41]. The $bla_{CTX-M-15}$ gene encoding for CTX-M-15 enzyme is often detected in the hospital environment and has been associated with the epidemic lineage ST131/B2 [42]. CTX-M-15 is the most important CTX-M enzyme due to their large diffusion and relation to outbreaks and severe extra-intestinal infections in humans [40]. It has been reported in all continents with reports in all major ecological niches including humans, animals and environment [10,43]. Several studies showed that, the sequence type (ST617) was highly distributed among various livestock species and humans in many African countries [44-47]. The public health threat associated to ESBL-producing CTX-M-15 has to be monitored in different ecological niches and to be considered under the prism of the one health approach. ESBL-producing *E. coli* isolates were multidrug resistant with *blaOXA-1, tetB, sul, sul2, tetA,* and *aac3-II* accessory genes. Similar observation was previously reported by Ibrahim et al.[48] and Lee et al.[49]. These represent a snapshot of resistance genes diversity present in the *E. coli* isolates, including resistance to historically used antibiotics as well as cephalosporins in contemporary use. MLST typing of *E. coli* isolates belonging to the phylogenetic group B2 revealed three ST95 isolates while the remaining belonged to ST14 and ST998. *E. coli* isolates belonging to lineages ST95/B2, ST14/B2, and ST998/B2 are often found in isolates of human origin [50]. Our study highlights an increasing resistance to antibiotics in *E. coli* from cattle carriage. To overcome the problems of multidrug resistant bacteria alternative treatments such as zinc oxide, could be used instead of common antibiotics to treat the *E. coli* and *S. aureus* related diseases [51].

Multi-drug resistance could spread through the food chain if beef meat is contaminated during slaughtering and butchering of cattle as well as through use of livestock feces as manure. Accordingly, hygiene should be adequately enforced at abattoirs to prevent contamination of meat. There is need for formulation and enforcement of policies to regulate use of antimicrobials in the country; antimicrobial surveillance program is also necessary. Public health education about health implications of indiscriminate use of antimicrobials is important.

Declarations

Availabilty of Data and Materials: All data supporting the findings of this study are available from the corresponding author (M. Akkou) upon a reasonable request

Ethical Statement: The study protocol was approved by the Veterinary Science Institute Scientific Committee of the university Saad Dahlab of Blida1 (Ref: CSI/N°12/2015).

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Author Contributions: MS performed the experiments SM-Á, IC, RF-F, and INA contributed significantly to analysis and manuscript preparation. MS and MA performed the data analysis and wrote the manuscript. AH, M-NM and CT helped perform the analysis with constructive discussions.

References

1. Founou LL, Founou RC, Essack SY: Antibiotic resistance in the food chain: A developing cuntry-perspective. *Front Microbiol,* 7:1881, 2016. DOI: 10.3389/FMICB.2016.01881

2. Antók FI, Mayrhofer R, Marbach H, Masengesho JC, Keinprecht H, Nyirimbuga V, Fischer O, Lepuschitz S, Ruppitsch W, Ehling-Schulz M, Feßler AT, Schwarz S, Monecke S, Ehricht R, Grunert T, Spergser J, Loncaric I: Characterization of antibiotic and biocide resistance genes and virulence factors of *Staphylococcus* species associated with bovine mastitis in Rwanda. *Antibiotics,* 9 (1):1, 2020. DOI: 10.3390/antibiotics9010001

3. Góchez D, Raicek M, Ferreira JP, Jeannin M, Moulin G : OIE annual report on antimicrobial agents intended for use in animals : Methods used. *Front Vet Sci,* 6:317, 2019. DOI: 10.3389/fvets.2019.00317

4. Kaczorek E, Małaczewska J, Wójcik R, Rękawek W, Siwicki AK: Phenotypic and genotypic antimicrobial susceptibility pattern of *Streptococcus spp*. isolated from cases of clinical mastitis in dairy cattle in Poland. *J Dairy Sci,* 100, 6442-6453, 2017. DOI: 10.3168/jds.2017-12660

5. Patel SJ, Wellington M, Shah RM, Ferreira MJ: Antibiotic stewardship in food-producing animals: challenges, progress, and opportunities. *Clin Therapeutics,* 42, 1649-1658, 2020. DOI: 10.1016/j.clinthera.2020.07.004

6. Obodoechi LO, Carvalho I, Chenouf NS, Martínez-Álvarez S, Sadi M, Nwanta JA, Chah KF, Torres C: Antimicrobial resistance in *Escherichia coli* isolates from frugivorous *(Eidolon helvum)* and insectivorous *(Nycteris hispida)* bats in Southeast Nigeria, with detection of CTX-M-15 producing isolates. *Comp Immunol Microbiol Infect Dis,* 75:101613, 2021. DOI: 10.1016/j.cimid.2021.101613

7. WHO: WHO 2014 Global report on antimicrobial resistance. https://apps.who.int/iris/bitstream/handle/10665/112642/9789241564748_eng.pdf?sequence=1; *Accessed:* 19.02.2022.

8. Donkor ES, Codjoe FS: Methicillin resistant *Staphylococcus aureus* and extended spectrum Beta-lactamase producing *Enterobacteriaceae:* A therapeutic challenge in the 21st century. *Open Microbiol J,* 13, 94-100, 2019. DOI: 10.2174/1874285801913010094

9. Coque TM, Baquero F, Canton R: Increasing prevalence of ESBL-producing Enterobacteriaceae in Europe. *Euro Surveill,* 13:19044, 2008. DOI: 10.2807/ese.13.47.19044-en

10. Carvalho I, Tejedor-Junco MT, González-Martín M, Corbera JA, Silva V, Igrejas G, Torres C, Poeta P: *Escherichia coli* producing extended-spectrum β-lactamases (ESBL) from domestic camels in the Canary Islands: A one health approach. *Animals,* 10:1295, 2020. DOI: 10.3390/ani10081295

11. Zhou Y, Ji X, Liang B, Jiang B, Li Y, Yuan T, Zhu L, Liu J, Guo X, Sun Y: Antimicrobial resistance and prevalence of extended spectrum β-lactamase-producing *Escherichia coli* from dogs and cats in Northeastern China from 2012 to 2021. *Antibiotics*, 11 (11):1506, 2022. DOI: 10.3390/antibiotics11111506

12. Rousham EK, Unicomb L, Islam MA: Human, animal and environmental contributors to antibiotic resistance in low-resource settings: Integrating behavioural, epidemiological and one health approaches. *Proc Royal Soci B,* 285:20180332, 2018. DOI: 10.1098/rspb.2018.0332

13. EUCAST: European comittee on antimicrobial susceptibility testing: testing breakpoint tables of interpretation of MICs and zone diameters. https://www.eucast.org/Ast_of_Bacteria/, pp.0-77, 2020, [Online]. https://www.eucast.org/fileadmin/src/media/PDFs/EUCASTfiles/Breakpoint tables/v 10.0 Breakpoint Tables.pdf; *Accessed:* 18.02.2022.

14. CLSI: Clinical and Laboratory Standards Institute: Performance Standards for Antimicrobial Susceptibility Testing. Wayne, PA, 2019.

15. Alonso AC, Mora A, Díaz D, Blanco M, González-barrio D, Ruiz-fons F, Simón C, Blanco J, Torres C: Occurrence and characterization of SXT and/or eae-positive *Escherichia coli* isolated from wildlife, including a typical EPEC strain from a wild boar. *Vet Microbiol,* 207, 69-73, 2017. DOI: 10.1016/j.vetmic.2017.05.028

16. Vinué L, Sáenz Y, Rojo-bezares B, Olarte I, Somalo S, Zarazaga M, Torres C, Vinué L, Sáenz Y, Rojo-bezares B, Olarte I, Undabeitia E: Genetic environment of genes and characterisation of integrons in *Escherichia coli* isolates of blood origin in a Spanish hospital. *Int J Antimicrob Agents,* 35 (5): 492-496, 2010. DOI: 10.1016/j.ijantimicag.2010.01.012

17. Clermont O, Christenson JK, Denamur E, Gordon DM: The Clermont *Escherichia coli* phylo-typing method revisited : Improvement of specificity and detection of new phylo-groups. *Envir Microbiol Rep,* 5, 58-65, 2013. DOI: 10.1111/1758-2229.12019

18. Tartof SY, Solberg OD, Manges AR, Riley LW: Analysis of a uropathogenic *Escherichia coli* clonal group by multilocus sequence typing. *J Clin Microbiol,* 43, 5860-5864, 2005. DOI: 10.1128/JCM.43.12.5860

19. Silva N, Carvalho I, Currie C, Sousa M, Igrejas G, Poeta P: Extended-spectrum-β-lactamase and carbapenemase-producing enterobacteriaceae in food-producing animals in Europe. **In,** Capelo-Martínez JL, Igrejas G (Eds): Antibiotic Drug Resistance, 261-273, Wiley, 2019.

20. Tamta S, Vinodh Kumar OR, Pruthvishree BS, Karthikeyan R, Ramkumar N, Rupner, Chethan GE, Dubal ZB, Sinha DK, Singh BR: Faecal carriage of extended spectrum beta-lactamase (ESBL) and New Delhi metallo beta-lactamase (NDM) producing *Escherichia coli* between piglets and pig farmworkers. *Comp Immun Microbiol Infect Dis,* 73:101564, 2020. DOI: 10.1016/j.cimid.2020.101564

21. Vounba P, Arsenault J, Bada-Alambédji R, Fairbrother JM: Prevalence of antimicrobial resistance and potential pathogenicity, and possible spread of third generation cephalosporin resistance, in *Escherichia coli* isolated from healthy chicken farms in the region of Dakar, Senegal. *PLoS One,* 14:e0214304, 2019. DOI: 10.1371/journal.pone.0214304

22. Hille K, Felski M, Ruddat I, Woydt J, Schmid A, Friese A, Fischer J, Sharp H, Valentin L, Michael GB, Hörmansdorfer S, Messelhäußer U, Seibt U, Honscha W, Guerra B, Schwarz S, Rösler U, Käsbohrer A, Kreienbrock L: Association of farm-related factors with characteristics profiles of extended-spectrum β-lactamase-/plasmid-mediated AmpC β-lactamase-producing *Escherichia coli* isolates from German livestock farms. *Vet Microbiol,* 223, 93-99, 2018. DOI: 10.1016/j.vetmic.2018.07.022

23. Shahrani M, Dhkordi FS, Momtaz H: Characterization of *Escherichia coli* virulence genes, pathotyps and antibiotic resistance properties in diarrheic calves in Iran. *Biol Res,* 47:28, 2014. DOI: 10.1186/0717-6287-47-28

24. Amador P, Fernandes R, Prudêncio C, Duarte I: Prevalence of antibiotic resistance genes in portuguese livestock manure. *Antibiotics,* 8, 1-23, 2019. DOI: 10.3390/antibiotics8010023

25. Barour D, Berghiche A, Boulebda N: Antimicrobial resistance of *Escherichia coli* isolates from cattle in Eastern Algeria. *Vet World,* 12, 1195-1203, 2019. DOI: 10.14202/vetworld.2019.1195-1203

26. Shin SW, Byun J, Jung M, Shin M, Yoo HS: Antimicrobial resistance, virulence genes and PFGE-profiling of *Escherichia coli* isolates from South Korean cattle farms. *J Microbiol,* 52, 785-793, 2014. DOI: 10.1007/s12275-014-4166-1

27. Yu ZN, Wang J, Ho H, Wang YT, Huang SN, Han RW: Prevalence and antimicrobial-resistance phenotypes and genotypes of *Escherichia coli* isolated from raw milk samples from mastitis cases in four regions of China. *J Glob Antimicrob Resist,* 22, 94-101, 2020. DOI: 10.1016/j.jgar.2019.12.016

28. Madoshi BP, Kudirkiene E, Mtambo MMA, Muhairwa AP, Lupindu AM, Olsen JE: Characterisation of commensal *Escherichia coli* isolated from apparently healthy cattle and their attendants in Tanzania. *PLoS ONE,* 11:e0168160, 2016. DOI: 10.1371/journal.pone.0168160

29. Zhao J, Dang H: Identification of a globally distributed clinical streptomycin-resistance plasmid and other resistance determinants in a coastal bay of China. *Lett Appl Microbiol,* 52, 1-8, 2010. DOI: 10.1111/j.1472-765X.2010.02958.x

30. Shin SW, Shin K, Jung M, Belaynehe M, Yoo S: Prevalence of antimicrobial resistance and transfer of tetracycline resistance genes in *Escherichia coli* isolates from beef cattle. *Appl Environ Microbiol,* 81, 5560-5566, 2015. DOI: 10.1128/AEM.01511-15

31. Momtaz H, Rahimi E, Moshkelani S: Molecular detection of antimicrobial resistance genes in *E. coli* isolated from slaughtered commercial chickens in Iran. *Vet Med Czech,* 57, 193-197, 2012. DOI: 10.17221/5916-VETMED

32. Sharma R, Munns K, Alexander T, Entz T, Mirzaagha P, Yanke LJ, Mulvey M, Topp E, McAllister T: Diversity and distribution of commensal fecal *Escherichia coli* bacteria in beef cattle administered selected subtherapeutic antimicrobials in a feedlot setting. *Appl Envir Microbiol,* 74, 6178-6186, 2008. DOI: 10.1128/AEM.00704-08

33. Mirzaagha P, Louie M, Sharma R, Yanke LJ, Topp E, McAllister TA: Distribution and characterization of ampicillin- and tetracycline-resistant *Escherichia coli* from feedlot cattle fed subtherapeutic antimicrobials. *BMC Microbiol,* 11:2011. DOI: 10.1186/1471-2180-11-78

34. Bakhshi B, Najibi S, Sepehri-Seresht S: Molecular characterization of enterohemorrhagic *Escherichia coli* isolates from cattle. *J Vet Med Sci,* 76, 1195-1199, 2014. DOI: 10.1292/jvms.13-0237

35. Sheldon IM, Rycroft AN, Dogan B, Craven M, Bromfield JJ, Roberts MH, Price SB, Gilbert RO, Simpson KW: Specific strains of *Escherichia coli* are pathogenic for the endometrium of cattle and cause pelvic inflammatory disease in cattle and mice. *PLoS ONE,* 5:e9192, 2010. DOI: 10.1371/journal.pone.0009192

36. Lan T, Liu H, Meng L, Xing M, Dong L, Gu M, Wang J, Zheng N: Antimicrobial susceptibility, phylotypes, and virulence genes of *Escherichia coli* from clinical bovine mastitis in five provinces of China. *Food Agric Immunol,* 31, 406-423, 2020. DOI: 10.1080/09540105.2020.1736009

37. Tomazi T, Coura FM, Gonçalves JL, Heinemann MB, Santos MV: Antimicrobial susceptibility patterns of *Escherichia coli* phylogenetic groups isolated from bovine clinical mastitis. *J Dairy Sci,* 101, 9406-9418, 2018. DOI: 10.3168/jds.2018-14485

38. Garcia-aljaro C, Moreno E, Antonia A, Prats G, Blanch AR: Phylogroups, virulence determinants and antimicrobial resistance in SXT 2 gene-carrying *Escherichia coli* isolated from aquatic environments. *Res Microbiol,* 160 , 585-591, 2009. DOI: 10.1016/j.resmic.2009.08.004

39. Ramos Sónia Silva N, Canic M, Capelo-Martinez JL, Brito F, Igrejasc G, Poeta P: High prevalence of antimicrobial-resistant *Escherichia coli* from animals at slaughter: A food safety risk. *J Sci Food Agric,* 93 (3): 517-526, 2013. DOI: 10.1002/jsfa.5814

40. Ramos Sónia Silva V, de Lurdes Enes Dapkevicius M, Caniça M, Tejedor-Junco MT, Igrejas G, Poeta P: *Escherichia coli* as commensal and pathogenic bacteria among food-producing animals: Health implications of extended spectrum β-lactamase (ESBL) production. *Animals,* 10:2239, 2020. DOI: 10.3390/ani10122239

41. Taghadosi R, Shakibaie MR, Nave HH: Antibiotic resistance, ESBL genes, integrons , phylogenetic groups and MLVA profiles of *Escherichia coli* pathotypes isolated from patients with diarrhea and farm animals in south-east of Iran. *Comp Immun Microbiol Infect Dis,* 63, 117-126, 2019. DOI: 10.1016/j.cimid.2019.01.004

42. De La Cadena E, Mojica MF, Castillo N, Correa A, Appel TM, García-Betancur JC, J.C. Pallares JC, Villegas MV: Genomic analysis of CTX-M-group-1-producing extraintestinal pathogenic *E. coli* (ExPEc) from patients with urinary tract infections (UTI) from Colombia. *Antibiotics,* 9:899, 2020. DOI: 10.3390/antibiotics9120899

43. Chenouf NS, Carvalho I, Messaï CR, Ruiz-Ripa L, Mama OM, Titouche Y, Zitouni A, Hakem A, Torres C: Extended spectrum β-lactamase-producing *Escherichia coli* and *Klebsiella pneumoniae* from broiler liver, with detection of CTX-M-55 and B2/ST131-CTX-M-15 in *Escherishia coli. Microb Drug Resist,* 27, 268-276, 2020. DOI: 10.1089/mdr.2020.0024

44. Aibinu I, Odugbemi T, Koenig W, Ghebremedhin B: Sequence Type ST131 and ST10 Complex (ST617) predominant among CTX-M-15-producing *Escherichia coli* isolates from Nigeria. *Clin Microbiol Infect,* 18, E49-E51, 2011. DOI: 10.1111/j.1469-0691.2011.03730.x

45. Mshana SE, Falgenhauer L, Mirambo MM, Mushi MF, Moremi N, Julius R, Seni J, Imirzalioglu C, Matee M, Chakraborty T: Predictors of blaCTX-M-15 in varieties of *Escherichia coli* genotypes from humans in community settings in Mwanza, Tanzania. *BMC Infect Dis,* 16:187, 2016. DOI: 10.1186/s12879-016-1527-x

46. Ojo OE, Schwarz S, Michael GB: Detection and characterization of extended-spectrum β-lactamase-producing Escherichia coli from chicken production chains in Nigeria. *Vet Microbiol,* 194, 62-68, 2016. DOI: 10.1016/j.vetmic.2016.04.022

47. Seni J, Falgenhauer L, Simeo N, Mirambo MM, Imirzalioglu C, Matee M, Rweyemamu M, Chakraborty T, Mshana SE: Multiple ESBL-Producing *Escherichia coli* sequence types carrying quinolone and aminoglycoside resistance genes circulating in companion and domestic farm animals in Mwanza, Tanzania, harbor commonly occurring plasmids. *Front Microbiol,* 7:142, 2016. DOI: 10.3389/fmicb.2016.00142

48. Ibrahim DR, Dodd CER, Stekel DJ, Ramsden SJ, Hobman JL: Multidrug resistant, extended spectrum β-lactamase (ESBL) -producing *Escherichia coli* isolated from a dairy farm. *FEMS Microbiol Ecol,* 92:fiw013, 2016. DOI: 10.1093/femsec/fiw013

49. Lee S, Teng L, Dilorenzo N, Weppelmann TA, Jeong KC: Prevalence and molecular characteristics of Extended-Spectrum and AmpC β-Lactamase producing *Escherichia coli* in grazing beef cattle. *Front Microbiol,* 10:3076, 2020. DOI: 10.3389/fmicb.2019.03076

50. Suresh A, Shaik S, Baddam R, Ranjan A, Qumar S, Jadhav S, Semmler T, Ghazi IA, Wieler LH, Ahmed N: Evolutionary dynamics based on comparative genomics of pathogenic *Escherichia coli* lineages harboring polyketide synthase *(pks)* island. *MBio,* 12:e03634-20, 2021. DOI: 10.1128/mBio.03634-20

51. Mwafy A, Youssef DY, Mohamed MM: Antibacterial activity of zinc oxide nanoparticles against some multidrug-resistant strains of *Escherichia coli* and *Staphylococcus aureus. Int J Vet Sci,* 12 (3): 284-289, 2023. DOI: 10.47278/journal.ijvs/2022.181

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Research Article

Chewing Lice (Psocodea: Phthiraptera) Detected in Wild Birds in Hatay, Türkiye, a New Record of the *Colpocephalum ecaudati* Price and Beer from Black Kite *(Milvus migrans)*

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Abstract

This study was carried out to determine chewing lice species of the wild birds, which were brought to the Veterinary Health, Practice and Research Center and Wild Animal Rescue and Rehabilitation Center of Hatay Mustafa Kemal University, between May 2018-August 2022. For this purpose, 75 wild birds injured or sick, which needed medical treatment when they arrived at the hospital, were examined for chewing lice. A total of 356 lice specimens were collected, representing 148 males, 157 females and 51 nymphs. The majority of the bird lice collected (317 out of 356) belonged to Amlyceran suborder, while a small number of them (39 out of 356) were obtained in the Ischnoceran suborder. *Piagetiella titan* (47.8%) detected in *Pelecanus onocrotalus* was the most common louse species. This was followed by *Laemobothrion maximum* (16.0%) detected in *Clanga pomarina, Buteo rufinus*, *Circaetus gallicus. Ciconiphilus quadripustulatus* (16.0%), *Neophilopterus incompletus* (10.4%) and *Colpocephalum zebra* (7.3%) species were detected in *Ciconia ciconia***.** Other species (*Colpocephalum ecaudati, Colpocephalum nanum, Pectinopygus forficulatus*) were in small numbers (2.5%). *Colpocephalum ecaudati* found on black kite *(Milvus migrans)* was reported for the first time in Türkiye. The results of this study contributed to the lice fauna obtained from wild birds in Türkiye.

Keywords: Chewing lice, Phthiraptera, Amblycera, Ischnocera, *Colpocephalum ecaudati*, Hatay, Türkiye

Introduction

There are approximately 5,000 known species of lice, the majority (90%) of which are ectoparasites of birds; the remaining (10%) are parasites of mammals [1]. Chewing lice (Ischnocera, Amblycera) consist of the egg, three nymphal stages and adult in the single host body. All stages are usually host specific and permanently ectoparasites that are common in bird species [2].

Although some species feed on blood, they feed mainly on feathers and dermal debris [3]. When lice are found in large numbers, they cause severe itching, weakness and loss of resistance, breakage and deterioration of feathers, negatively affecting the thermoregulation capacity, body mass, flight performance, metabolic rate, migration, grooming time, survival and sexual selection of birds [3-5]. Additionally, some chewing lice may transmit other parasites to the birds, such as some filarial nematodes, and can also serve as vectors for some bacterial diseases [6]. *Piagetiella titan* (Piaget) can cause stomatitis in white pelicans [7].

Of the 4.000 bird species identified in the world [8], approximately 200 have been reported from 491 bird species [9] in Türkiye [3,9-20]. In some studies conducted worldwide, the incidence of chewing lice in wild birds was found between 10.8%-35% [6]. In some studies conducted in Türkiye, the incidence of chewing lice in wild birds was found to be 40.4% [21] and 41.4% [15]. However, it is believed that not all chewing lice found in birds in Türkiye can be detected, and additional data on the prevalence of chewing lice in wild birds in Türkiye are needed [16].

This study was carried out to determine chewing lice species of the wild birds which were brought to the Veterinary Health, Practice and Research Center and

Wild Animal Rescue and Rehabilitation Center of Hatay Mustafa Kemal University in Türkiye, between May 2018-August 2022.

Materials and Methods

Ethical Approval

This study was approved by Hatay Mustafa Kemal University Animal Experiments Local Ethics Committee (Decision number: 2022/07-07) and Directorate of Nature Conservation and National Parks (12/09/2023-303651).

Study Area

Hatay (37-38°N, 32-35°E) is a 5,403-km^2 area of plains, rivers, streams and high mountains, which is bordered by Syria to the south and the east, and the Mediterranean sea to the west. The summers are hot (20-33°C) with high humidity whereas the winters are wet and mild (5-14°C).

Sampling Data

This study was carried out in 75 wild birds in Hatay Province in Türkiye. The birds were brought from Antakya (10), Kırıkhan (15), Samandağ (17), İskenderun (18), Dörtyol (11) and Reyhanlı (4) districts of Hatay. All of the birds examined in this study were injured and sick, which needed medical attention when they arrived at the hospital.

The identification of birds was conducted according to the guide developed by Heinzel et al. [22]. The naming of the birds was based on Gill et al. [23]. A total of 75 wild birds, consisting of three families (Ciconiidae, Pelecanidae, Accipitridae) in three orders (Ciconiiformes, Pelecaniformes, Accipitriformes) were examined for chewing lice *(Table 1)*.

All the birds were examined immediately following their arrival at the hospital. To sample of chewing lice, the feathers of each bird were examined visually, and then carefully rubbed. The lice detected on the wild birds were collected with a forceps and taken into 70% alcohol, and stored in the laboratory until microscopic examination. The protocols for each bird species and the collected lice from all of the infested birds were recorded.

At the laboratory, the lice specimens were cleared in 10% KOH, rinsed in distilled water, kept for 24 hours in each step in alcohol 70%, 80%, 90%, and 99%, and mounted in Canada balsam on slides. All mounted specimens were examined under trinocular stereo zoom microscope (Nikon SMZ745T) in accordance with the keys or original descriptions utilized by Price and Beer [24], Pilgrim [25], Clay [26], Clayton [27], Martín-Mateo [28], Mey [29] and Adams et al.[30]. Photographs were made using the Leica DM750 trinocular phase contrast microscope with DFC295 camera for all species except larger specimens for which stereomicroscope was used. They were deposited at the Parasitology Department of the Veterinary Faculty of Selçuk University in Konya, Türkiye.

Parasitism Rate Analysis

The prevalence of chewing lice was evaluated for bird families and bird species with a minimum of a single collected individual. The abundance mean and intensity

Table 1. *Chewing lice (Phthiraptera) species detected in the examined wild bird species*

No.	Hosts Order/Family/ Species	Common Name	Suborder	Chewing Lice Species	%	Abundance					
						NI	M	F	N	T	MI
24	CICONIIFORMES Ciconiidae *Ciconia ciconia*	White stork	Amblycera Ischnocera Amblycera	*Ciconiphilus quadripustulatus* *Neophilopterus incompletus* *Colpocephalum zebra*	16.0 10.4 7.3	7 4 7	12 14 10	41 17 15	4 6 1	57 37 26	8.1 9.2 3.7
5	PELECANIFORMES Pelecanidae *Pelecanus onocrotalus*	Great white pelican	Amblycera Ischnocera	*Piagetiella titan* *Pectinopygus forficulatus*	47.8 0.6	3 1	100 1	54 1	16 0	170 2	56.6 2.0
6	ACCIPITRIFORMES Accipitridae *Buteo rufinus*	Long-legged buzzard	Amblycera Amblycera	*Laemobothrion maximum* *Colpocephalum nanum*	14.9 0.8	3 1	10 1	22 2	21 0	53 3	17.6 3.0
11	Accipitridae *Clanga pomarina*	Lesser Spotted eagle	Amblycera	*Laemobothrion maximum*	0.8	1	0	0	3	3	3.0
9	Accipitridae *Circaetus gallicus*	Short-toed snake eagle	Amblycera	*Laemobothrion maximum*	0.3	1	0	1	0	1	1.0
17	Accipitridae *Buteo buteo*	Common buzzard	-	-	-	0	0	0	0	0	0
3	Accipitridae *Milvus migrans*	Black kite	Amblycera	*Colpocephalum ecaudati* [a]	1.1	1	0	4	0	4	4.0
75	Total					29	148	157	51	356	12.2

No: *Number of birds examined;* ***NI:*** *Number of birds infested;* ***M:*** *Male;* ***F:*** *Female;* ***N:*** *Nymph;* ***T:*** *Total,* ***MI:*** *Mean intensity;* [a] *New record for Türkiye*

mean level of each species of chewing lice on the avian hosts were determined.

Results

Twenty one (28%) of 75 birds examined were found to be infested with chewing lice. Eight chewing lice species; six amblyceran species in four genera: *Laemobothrion maximum* (Scopoli), *Ciconiphilus quadripustulatus* (Burmeister), *Piagetiella titan* (Piaget), *Colpocephalum zebra* (Burmeister), *Colpocephalum nanum* (Piaget) and *Colpocephalum ecaudati* (Price and Beer); and two ischnoceran species in two genera: *Neophilopterus incompletus* (Denny) and *Pectinopygus forficulatus* (Nıtzsch) were detected. Fifteen (71.43%) of infested birds with one louse species, four (19.04%) with two lice species and two birds (9.52%) with three lice species were found to be infested.

A total of 356 lice specimens were collected, representing 148 males (41.57%), 157 females (44.10%) and 51 nymphs (14.32%). The majority of the lice (317 out of 356) were in the Amlyceran suborder, while a small number of them (39 out of 356) were in the Ischnoceran suborder.

Piagetiella titan (47.8%) detected in Great white pelican *(Pelecanus onocrotalus)* was the most abundant louse species. This was followed by *L. maximum* (16.0%) detected in *B. rufinus, C. pomarina, C. gallicus. Ciconiphilus quadripustulatus* (16.0%), *N. incompletus* (10.4%), and *C. zebra* (7.3%) detected in *C. ciconia*.

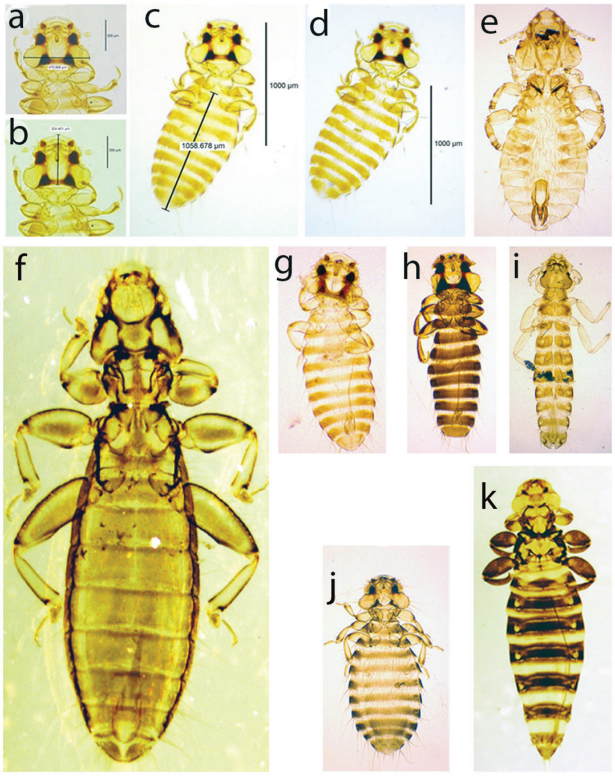

Fig 1. Chewing lice (Phthiraptera) species identified in this study, original. **a-d)** *Colpocephalum ecaudati,* female; **e)** *Neophilopterus incompletus,* male (40x magnification); **f)** *Laemobothrion maximum,* male; **g)** *Colpocephalum nanum,* male (40x magnification); **h)** *Colpocephalum zebra,* male (40x magnification); **i)** *Pectinopygus forficulatus,* male (25x magnification); **j)** *Ciconiphilus quadripustulatus,* female (40x magnification); **k)** *Piagetiella titan,* male

Other species *(C. ecaudati, C. nanum, P. forficulatus)* were identified in small numbers (2.5%). In addition, four female *C. ecaudati* (1.1%) specimens identified according to Price and Beer's description [24] and found on one black kite *(Milvus migrans)* have been reported for the first time in Türkiye with this study *(Table 1)*. The morphological characteristics of female *C. eucaudati* in this study are as follows; the head length is 0.32 mm; head width, 0.47 mm; thorax length, 0.34 mm; thorax width, 0.41 mm; abdomen length, 1.06 mm; abdomen width, 0.60 mm; and total length, 1.55 mm *(Fig. 1)*.

Discussion

Several studies on chewing lice species of wild birds have been conducted in recent years in Türkiye; however, the faunal composition of chewing lice of Turkish wild birds is still far from being understood [3,9,16,17,19,20]. In the present study, it was conducted to determine the chewing lice species found on wild birds which were injured, sick or in need of medical attention in Hatay Province. In this study, the majority of the bird lice (317 out of 356) belonged to the suborder Amblycera *(C. quadripustulatus, C. zebra, C. nanum* and *C. ecaudati, L. maximum, P. titan),* while a small number of them (39 out of 356) were obtained in the Ischnoceran suborder *(N. incompletus, P. forficulatus).* Fifteen (71.43%) of 21 infested birds were infested with one louse species, four birds (19.05%) with two lice species and two birds (9.52%) with three lice species.

Ciconiphilus quadripustulatus (4.40-34.50%), *N. incompletus* (0.46-25.83%), *C. zebra* (0.23-93.63%) and *A. ciconiae* (0.86-20.06%) have been recorded on white stork previously in Türkiye [3,12,15,18,31] and such countries as Algeria [32,33], Romania [34], Poland [35] and Egypt [36]. Twenty four white stork species were examined in this study, and the species of *C. quadripustulatus* (16.0%), *N. incompletus* (10.4%) and *C. zebra* (7.3%) were detected, yet *A. ciconiae* could not be found. According to the findings of the studies; *C. quadripustulatus*, *N. incompletus*, *C. zebra* were the most common lice species on white stork.

Piagetiella titan (1.01-63.00%), *P. forficulatus* (28.90-34.74%), and *C. eucarenum* (0.07-28.09%) in some studies have been recorded on great white pelican. Previous studies performed in Türkiye [3,10,19,20,31,37] and such countries as Irak [38], Iran [39], Central Ciscaucasia [40], and Romania [41]. According to the findings of these studies; *P. titan*, *P. forficulatus* and *C. eucarenum* were the most common lice species on great white pelican. Five great white pelican

species were examined in this study, and *P. titan* (47.8%) was detected as common species. While *P. forficulatus* was found rarely (0.6%) on the infested pelicans, *C. eucarenum* could not be detected in this study.

Many well-known wild carnivorous birds such as the buzzards, eagles, snake-eagles, kites belong to the family of Accipitridae. In various studies conducted in the world and in Türkiye, there are six genera of chewing lice in this family that have been reported: the genera *Laemobothrion*, *Colpocephalum* and *Kurodaia* in the suborder Amblycera; and the genera *Craspedorrhynchus*, *Degeeriella*, *Falcolipeurus* in the subgenera Ischnocera. *Laemobothrion maximum* [3,10,13-15,17,36,41-46], *C. nanum* [3,13,15,17,36,44,45,47], *C. turbinatum* [42,45,48], *C. platystomus* [3,10,13-15,17,36,44,45,48], *D. fulva* [3,10,14,15,17,36,44,45,47,48], *D. leucopleura* [3,45], *D. nisus* [3,45], *K. fulvofasciata* [17,18,36,45], *F. quadripustulatus* [45] and *F. suturalis* [3,45] species have been reported in this family. While *L. maximum* (16.0%) was detected in 6 Long-legged buzzards (14.9%), 11 Lesser Spotted eagles (0.8%), and 9 Short-toed snake eagles (0.3%); *C. nanum* (0.8%) species were detected in 6 Long-legged buzzards examined in this study. In 17 Common buzzards examined, no lice species were found. *Colpocephalum turbinatum*, *C. platystomus*, *D. fulva*, *D. leucopleura*, *D. nisus*, *K. fulvofasciata*, *F. quadripustulatus*, and *F. suturalis* species could not be identified in this study.

In Türkiye, two lice species have been previously reported in *M. migrans*. These are *L. maximum* (one nymph) and *C. milvi* (one female) [15]. In this study, four female *C. ecaudati* (1.1%) specimens obtained from the one black kite *(M. migrans)* were reported for the first time in Türkiye.

In conclusion, a great number of wild birds were infested by numerous lice species in the world. In Türkiye, the number of studies on lice of wild birds has been steadily increasing in recent years. As a result of the studies, it was stated that the number approached approximately 200 with the species identified in later studies [19,20,49,50]. In this study, 356 lice specimens, mostly *P. titan*, *L. maximum*, *C. quadripustulatus*, *N. incompletus* and *C. zebra*, were found in wild bird species of *B. rufinus*, *C. pomarina*, *C. gallicus*, *C. ciconia*. Other species such as *C. ecaudati*, *C. nanum*, *P. forficulatus* were identified in small numbers. In addition, a louse species, *C. ecaudati*, which has not been reported in Türkiye until now, was found in *M. migrans* for the first time in Türkiye.

Declarations

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

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Authors' Contributions: A.Z., I.E., M.Y., M.E.A. contributed to the conception and design of the experiments. A.Z., I.E., M.Y., and M.E.A. performed sample collection. A.Z., I.E., M.Y., and B.D. were involved in data collection and analysis, and helped draft and review the manuscript. All of the authors read and approved the final manuscript.

References

1. Durden LA: Lice (Phthiraptera). **In,** Samuel WM, Pybus MJ, Kocan AA (Eds): Parasitic Diseases of Wild Mammals. 2nd ed., 3-17, Iowa State University Press/Ames, Iowa, USA, 2001.

2. Yilmaz AB, Azizoglu E. Adizel Ö: Chewing louse species (Phthiraptera: Amblycera, Ischnocera) on roadkill wild birds in Van province: Five new species in Turkey. *Indian J Anim Res,* 56 (6): 724-729, 2022. DOI: 10.18805/IJAR.B-1405

3. Girişgin AO, Dik B, Girişgin O: Chewing lice (Phthiraptera) species of wild birds in northwestern Turkey with a new host record. *Int J Parasitol Parasites Wildl,* 2, 217-221, 2013. DOI: 10.1016/j.ijppaw.2013.07.001

4. Tomás A, Palma RL, Rebelo MT, da Fonseca IP: Chewing lice (Phthiraptera) from wild birds in southern Portugal. *Parasitol Int,* 65 (3): 295-301, 2016. DOI: 10.1016/j.parint.2016.02.007

5. Ošlejšková L, Krištofík J, Trnka A, Sychra O: An annotated checklist of chewing lice (Phthiraptera: Amblycera, Ischnocera) from Slovakia. *Zootaxa,* 5069 (1): 1-80, 2021. DOI: 10.11646/ZOOTAXA.5069.1.1

6. Diakou A, Soares, JBPC, Alivizatos H, Panagiotopoulou M, Kazantzidis S, Literák I, Sychra O: Chewing lice from wild birds in northern Greece. *Parasitol Int,* 66 (5): 699-706, 2017. DOI: 10.1016/j.parint.2017.07.003

7. Dik B: Erosive stomatitis in a white pelican *(Pelecanus onocrotalus)* caused by *Piagetiella titan* (Mallophaga: Menoponidae). *J Vet Med B,* 53, 153-154, 2006b. DOI: 10.1111/j.1439-0450.2006.00927.x

8. Price RD, Hellenthal RA, Palma RL, Johnson KP, Clayton DH: The chewing lice: World checklist and biological overview. Illinois Natural History Survey Special Publication, Illinois, 2003.

9. Açıcı M, Erciyas Yavuz K, Gürler T, Eren G, Tuygun T, Koç Ö, Bölükbaş CS, Umur Ş: Kızılırmak deltası kuşlarının bitleri üzerine çalışmalar: Türkiye bit faunası için yeni bildirimler. *Uluslararası 22. Parazitoloji Kongresi*, Didim, Aydın, 11-15 Ekim, 2021.

10. Dik B: Mallophaga species on long-legged buzzards *(Buteo rufinus):* New records from Turkey. *Türkiye Parazitol Derg,* 30 (3): 226-230, 2006.

11. Dik B, Uslu U: Konya'da halkalı sülünlerde *(Phasianus colchicus) Cuclotogaster heterographus* (Mallophaga: Lipeuridae) enfestasyonu. *Türkiye Parazitol Derg,* 30 (2): 125-127, 2006.

12. Dik B, Uslu U: Beyaz leyleklerde *(Ciconia ciconia* Linnaeus, 1758) görülen *Mallophaga* (Insecta) türleri. *Türkiye Parazitol Derg,* 30 (3): 220-225, 2006.

13. Dik B, Aydenizöz Özkayhan M: *Mallophaga* species on longlegged buzzards *(Buteo rufinus)* in Turkey. *Türkiye Parazitol Derg,* 31 (4): 298-301, 2007.

14. Dik B, Uslu U: Konya Hayvanat Bahçesi'ndeki kanatlı hayvanlarda görülen çiğneyici bit (Phthiraptera: Amblycera, Ischnocera) türleri. *Türkiye Parazitol Derg,* 33 (1): 43-49, 2009.

15. İnci A, Dik B, Kibar M, Yıldırım A, Düzlü O: Chewing lice (Phthiraptera) species on wild birds in the Cappadocia region. *Türkiye Parazitol Derg,* 34, 174-178, 2010.

16. Dik B, Kirpik MA, Şekercioğlu ÇH, Şaşmaz Y: Chewing lice (Phthiraptera) found on songbirds (Passeriformes) in Turkey. *Türkiye*

Parazitol Derg, 35, 34-39, 2011.

17. Dik B, Per E, Yavuz KE, Yamaç E: Chewing lice (Phthiraptera: Amblycera, Ischnocera) species found on birds in Turkey, with new records and a new host association. *Turk J Zool,* 39 (5): 790-798, 2015. DOI: 10.3906/zoo-1411-45

18. Göz Y, Dik B, Orunç Kılınç Ö, Yılmaz AB, Aslan L: Chewing lice (Phthiraptera: Amblycera, Ischnocera) on several species of wild birds around the Lake Van Basin, Van, eastern Turkey. *Kafkas Univ Vet Fak Derg,* 21 (3): 333-338, 2015. DOI: 10.9775/kvfd.2014.12484

19. Dik B, Hügül F, Ceylan O: Chewing lice (Phthiraptera: Amblycera, Ischnocera) of some aquatic birds in Konya province, Turkey, new records for Turkish fauna. *Ankara Üniv Vet Fak Derg,* 64, 307-312, 2017. DOI: 10.1501/Vetfak_0000002814

20. Eren G, Özkoç ÖÜ, Açıcı M: Contribution to the knowledge of the diversity of the chewing lice fauna in Turkey. *Turk J Zool,* 46 (6): 444-455, 2022. DOI: 10.55730/1300-0179.3099

21. Girişgin O, Girişgin A, Cimenlikaya N, Saygin B: A survey of the ectoparasites found on wild birds in Northwest Turkey. *Indian J Anim Res,* 57 (8): 1059-1065, 2023. DOI: 10.18805/ijar.bf-1474

22. Heinzel H, Fitter R, Parslow J: Pocket guide to birds of Britain and Europe with North Africa and the Middle East. Harper Collins Publishers Ltd., UK, 1995.

23. Gill F, Donsker D, Rasmussen P (Eds): IOC World Bird List (v13.1). https://www.worldbirdnames.org/new/ioc-lists/crossref/. *Accessed:* 28.08.2023.

24. Price RD, Beer JR: Species of *Colpocephalum* (Mallophaga: Menoponidae) parasitic upon the Falconiformes. *Can Entomol,* 95 (7): 731-763, 1963.

25. Pilgrim RIC: Mallophaga on the Rock Pigeon *(Columba livia)* in New Zealand, with a key to their identification. *N Z Entomol,* 6, 160-164, 1976.

26. Clay T: The *Strigiphilus cursitans* Group (Phthiraptera: Insecta). Rec Queen Victoria Mus, Launceston, 1-4, 1977.

27. Clayton DH: Host specifity of *Strigiphilus* owl lice (Ischnocera: Philopteridae), with the description of new species and host associations. *J Med Entomol,* 27, 257-265, 1990. DOI: 10.1093/jmedent/27.3.257

28. Martín-Mateo MP: Phthiraptera from *Platalea leucorodia* L. (Aves: Ciconiiformes: Threskiornithidae) in Spain. *Res Rev Parasitol,* 54, 109-115, 1994.

29. Mey E: Zur Taxonomie, Lebenweise und parasitophyletischen Evidenz der Federlingsgattung *Struthiolipeurus sensu lato* (Insecta, Phthiraptera, Ischnocera). *Mitt Mus Natkd Berl Zool Reihe,* 74, 65-93, 1998.

30. Adams RJ, Price RD, Clayton DH: Taxonomic revision of old world members of the feather louse genus *Columbicola* (Phthiraptera: Ischnocera), including descriptions of eight new species. *J Nat Hist,* 39, 3545-3618, 2005. DOI: 10.1080/00222930500393368

31. Dik B, Kandir EH: Ectoparasites in some wild birds (Aves) in Turkey. *Prog Nutr,* 23 (2):e2021261, 2021. DOI: 10.23751/pn.v23iS2.11919

32. Bouguessa-Cheriak L, Doumandji S, Messaoud SO, Marniche F: Insect ectoparasites on the white stork, *Ciconia ciconia* (L.) (Ciconiiformes: Ciconiidae), during the breeding period in the extreme east of Algeria. *Oltenia Studii şi comunicări. Ştiinţele Naturii,* 33 (2): 72-78, 2017.

33. Touati L, Athamnia M, Nedjah R, Boucheker A, Samraoui F, El-Serehy HA, Samraoui B: Composition and distribution on a host of avian lice of white storks in North-Eastern Algeria. *Diversity,* 14 (2):77, 2022. DOI: 10.3390/d14020077

34. Adam C: Data on the chewing louse fauna (Phthiraptera: Amblycera, Ischnocera) from some Romanian autochthonous and exotic birds. *Travaux du Muséum National d'Histoire Naturelle "Grigore Antipa",* 50, 145-210, 2007.

35. Fryderyk S, Izdebska JN: Chewing Lice (Insecta, Phthiraptera) of the White Stork *(Ciconia ciconia* L.) in Poland. *Ann Univ Mariae Curie Sk*, 64 (2): 83-85, 2009. DOI: 10.2478/v10067-010-0017-6

36. Riad SA: Ectoparasites associated with migratory birds, Eastern Desert, Red Sea, Egypt. *Egypt Acad J Biol Sci B Zool*, 14 (2): 19-33, 2022. DOI: 10.21608/eajbsz.2022.249312

37. Dik B, Uslu U: The first recording of *Piagetiella titan* (Menoponidae: Mallophaga) on a white pelican *(Pelecanus onocrotalus,* Linneaus) in Turkey. *Türkiye Parazitol Derg,* 30 (2): 128-131, 2006.

38. Mohammad ZA: Some chewing lice (Phthiraptera) species as ectoparasites infested aquatic birds with a new record of three species from Al-Sanaf marsh/southern Iraq. *Iraqi J Vet Sci,* 34 (1): 173-180, 2020. DOI: 10.33899/IJVS.2019.125721.1139

39. Tavassoli M, Salmanzadeh R, Jabbari H: Infestations of *Piagetiella titan* (Menoponidae: Mallophaga) on juvenile white pelicans *(Pelecanus nocrotalus*, L.) in Urmia Lake National Park, northwest Iran. *Int J Vet Res,* 5 (2): 105-108, 2011.

40. Lyakhova OM, Kotti BC: Chewing lice (Mallophaga: Insecta) of birds in the Central Ciscaucasia. *Entomol Rev,* 91, 367-376, 2011.

41. Rékási J, Kiss JB: New data on the lice (Phthiraptera) of some birds in Northern Dobrogea (Romania). *Acrocephalus,* 27 (130/131): 139-145, 2006.

42. Saxena AK: Population characteristics of black kite lice. *J Parasit Dis,* 41 (3): 684-686, 2017. DOI: 10.1007/s12639-016-0866-2

43. Anand KM, Meghna P, Silambarasan R, Navaz S, Latha BR: A report on the occurrence of *Laemobothrion maximum* in black kites *(Milvus migrans). J Vet Parasitol,* 33 (2): 50-52, 2019. DOI: 10.5958/0974-0813.2019.00020.2

44. Yosef R, Strutzer O, Tabibi R, Rózsa L: Infestations of lice of steppe buzzards *(Buteo buteo vulpinus)* differ from those of common buzzards *(Buteo buteo buteo). J Raptor Res,* 53 (1): 102-108, 2019. DOI: 10.3356/JRR-18-21

45. Dik B, Naz S, Sajid MS: Data on the chewing lice (phthiraptera) parasitizing the accipitrid birds (accipitriformes) in Turkey. *J Anim Health Prod,* 10 (4): 443-453, 2022.

46. Adly E, Gustafsson DR, Nasser M, Baeshen R, Kamal M: Host-parasite associations and new records of chewing lice (Phthiraptera: Amblycera, Ischnocera) from raptors (Accipitriformes, Falconiformes, Strigiformes) encountered in Egypt. *J Entomol Sci,* 57 (3): 394-410, 2022. DOI: 10.18474/JES21-75

47. Adam C, Chisamera G, Daróczi SJ, Sándor AD, Gogu-Bogdan M: Data on the chewing louse fauna (Phthiraptera: Amblycera, Ischnocera) from some wild and domestic birds of Romania. *Travaux du Muséum National d'Histoire naturelle "Grigore Antipa",* 52, 177-232, 2009.

48. Gherardi R, D'Agostino C, Perrucci S: Lice, flies, mites, and ticks on raptors (Accipitriformes, Falconiformes and Strigiformes) in rescue centers in Central Italy. *Parasitologia,* 1 (2): 61-68, 2021. DOI: 10.3390/parasitologia1020008

49. Yamaç E, Dik B, Cavus M: The environment and host effects on chewing lice prevalence, richness, and abundance on birds in Turkey. *Ornithol Res,* 31, 193-206, 2023. DOI: 10.1007/s43388-023-00133-5

50. Dik B, Yamaç E, Uslu U: Studies on chewing lice (Phthiraptera: Amblycera, Ischnocera) species from domestic and wild birds in Turkey. *Kafkas Univ Vet Fak Derg,* 19 (4): 553-560, 2013. DOI: 10.9775/kvfd.2012.8207

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Research Article

Effect of Equex on Ram Semen in Different Freezing Extenders [1]

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Abstract

This study was carried out to determine the effects of Equex on ram semen freezability in three different extenders. Semen samples were collected from five rams and divided into six equal aliquots. At first, three non-commercial base extenders **(E1:** Tris-based extender, E2: Tris based extender+ Cystein and E3: Skim Milk Powder (SMP)) were prepared. Each base extender was divided into two parts; a base extender (E1, E2 and E3) or fortified with Equex (E1E, E2E and E3E). The pooled semen was diluted according to the two-step dilution method, and cryopreserved using a programmable freezing machine. Sperm motility parameters were examined by CASA. The morphological properties were evaluated by flow cytometer. It was observed that the addition of Equex had a positive effect on post-thaw motility and live spermatozoa ($P<0.001$), but had no effect on the LIN and Polarized mitochondria. While the Equex addition decreased post-thaw VCL, VSL, VAP and WOB values, it didn't have effect on the post-thaw intact acrosome rates ($P<0.05$). Furthermore, it was observed that Equex had a cryoprotective effect on post-thaw progressive motility in E1E and E3E groups ($P<0.05$), but was not observed in E2E group. In conclusion there was an interaction between Equex and extender ingredient. The cryoprotective effect of Equex was more evident in skim milk-based extender than that in tris-based extender.

Keywords: Cryopreservation, Equex, Kinematic parameters, Ram remen

Introduction

In livestock, artificial insemination (AI) with cryopreserved semen is one of the most important tools to improve the reproductive efficiency. Freeze-thaw process results in biological and functional changes that adversely impairs fertilizing ability by altering sperm mitochondria, acrosome and plasma membrane functional integrity [1]. The composition of semen freezing extenders helps to stabilize the cells during the cooling and storage process. Ram semen has been diluted with various extender ingredients, both defined and undefined, to maintain sperm fertilizing ability during processing and storage [2-6].

Semen collection procedures, extender composition, dilution, cooling, and freezing rates [2,7,8] as well as the type and concentration of cryoprotective agents used [3,5,6], temperature at which cryoprotectant is added to the semen [9], equilibration period [7], and thawing rate [10] can all result in the loss of post-thaw sperm fertilizing ability. Some researchers have attributed this decline in fertility at post-thaw stage to reduced transport ability and survival of ram spermatozoa in the female reproductive tract, along with diminished viability, morphology, and DNA integrity during freezing and thawing procedures [3,6,11]. Another reason for reduced fertility in sheep intra-cervical inseminated with frozen-thawed semen is the anatomical structure of the ewe's cervix and the passage of viable spermatozoa through the cervix. Therefore, there is still a need to optimize cryopreservation and breeding protocols for ram semen [12-14].

Semen extenders have been designed to protect and maintain spermatozoa during the processing and storage

of semen [15]. Various homemade or commercially available extenders have been developed and they are supplemented with different chemicals that reduce cryodamage or oxidative stress with varying levels of success. Skim milk or Tris-based extender are widely used components of semen extenders in most species, showing successful results both *in vitro* and *in vivo* [16,17]. The protective effect of skim milk is provided by the different components that present in the milk [18].

The semen freezing extenders and additives protect spermatozoa metabolism in many ways, including stabilizing the plasma membrane and maintaining intracellular and intramembrane ionic concentrations, thus reducing cold shock damage and osmotic shock. Tris-based extenders with some additives (citrate, glucose, and trehalose, etc) are widely used for liquid/frozen mammalian semen storage [5,6,15,19-24]. The success of Tris-based extender has been attributed to its buffering capacity against changes in pH and tonicity [25]. The other extender components are fructose, citric acid and egg- yolk which are energy sources and protect the cell membrane during cooling process [25,26]. Aisen et al.[20] enriched Salamon and Ritar's Tris based ram semen extender by adding trehalose, and improved the cryo-protective properties of this extender by cystein additives [4].

Equex is a non-permeating cryoprotective agent and a surfactant stabilizes the acrosomal membranes and protects spermatozoa against freeze-thaw toxic effects [27-29]. How Equex stabilizes sperm cell membranes during freeze-thaw process is still being investigated [28]. Equex is a well-known supplement for semen freezing and it has been extensively studied in bull [30], dog [31], stallion [32], boar [27], and gazelle [33]. However, there is a lack of research on the effect of Equex on post-thaw quality of ram semen diluted with various extenders. Also, such a wide range of spermatological features have not been evaluated together in previously published studies [28,34]. Furthermore, in the literature review conducted, no study was found on the effect of Equex in a skim milk-based extender.

The aim of this study, therefore, was to evaluate the effects of Equex on post-thaw motility and motion parameters, plasma membrane and acrosome integrity, and mitochondrial membrane potential (MMP) of ram spermatozoa in extender formulas, that were documented as successful for ram semen freezing.

Material and Methods

Ethical Statement

All procedures were approved by the local ethics committee of Sheep Breeding Research Institute, Ethics Committee on Animal Research (25/05/2018,78255852-050.01.04/0149/1331374).

Animal Management and Semen Collection

The study was carried out during breeding season (September and October) in the Laboratory of Sheep Breeding Research Institute, Bandırma, Türkiye. All chemicals used in this study were purchased from Sigma (St. Louis, MO, USA). Five adult Karacabey Merino rams from Sheep Breeding Research Institute (40.32 latitude, 27.91 longitude), fertility proven aged 2-3 years were used for semen collection. All rams were hosted under uniform management conditions.

Semen Collection and Freezing

Semen samples were collected from five rams by artificial vagina every other day in five replicates. Obtained ejaculates were primarily evaluated for colour, volume, wave motion and initial motility [6]. Normospermic semen (volume ≥0.9 mL, concentrations ≥1800 x 10^6 spz/mL (Photometre Ovine Acucell®- IVM Technologies, France), wave motion ≥4 (1-5), progressive motility ≥70%) were pooled to avoid individual variations.

All the diluent formulas used in the study were prepared according to the formulas stated in the previous studies of Aisen et al.[20], Alçay et al.[4] and Kulaksız et al.[17]. Pooled semen was divided into six equal aliquots. Each aliquot was diluted with one of the extenders below at 400×10^6 spz/mL according to two-step dilution technique [6]. Three base extenders; E1: Tris based extender (Sigma® T6791 Tris 2.71 g, Sigma® F3510 D-Fructose 1.4 g, Sigma® C2404 Citric acid 1.0 g, %20 eggyolk) + Trehalose 50 mM Sigma® T0167) [20], E2: Tris based extender (Sigma® T6791 Tris 2.71 g, Sigma® F3510 D-Fructose 1.4 g, Sigma® C2404 Citric acid 1.0 g, 20% eggyolk, Sigma® T0167 Trehalose 50 mM) +1 mM Cystein (Sigma® C7352) [4] and E3: Skim Milk Powder (SMP) (10 g SMP (Sigma® 70166), 1 g of glucose (Sigma® G7021), 5% egg yolk, 5% glycerol) [17] were used. Each extender was divided into two parts; a base extender (E1, E2 and E3) or fortified with equex (Equex-Paste, Minitube, Tieffenbach Germany, REF.13560/0030) (E1E, E2E and E3E) at a ratio of 0.75% v/v. Diluted semen was cooled to +4°C at a rate of -0.3°C/min in 2 h. Second step dilution was at +4°C at 5 times with 10 min intervals [6].

Then, the samples were allowed to equilibrate for 2 h. Equilibrated semen was filled in 0.25 mL straws (MRS-1 Dual® IMV, France) and frozen (Mini-Digitcool® IMV, France) using IMV advised freezing protocol (4°C to -10°C 5°C/min, -10°C to -55°C 80°C/min, -55°C to -100°C 40°C/min occurred at a rate of 20°C/min from -100°C to -140°C). Frozen straws were stored in liquid nitrogen (-196°C) until thawing for further evaluation. Minimum one week after freezing, at least 3 straws from each group of the independent replicates were thawed at 37°C for 30 sec. for post-thaw semen evaluation.

Post-thaw Semen Evaluation

Sperm Motility: Sperm motility was examined by computer-assisted sperm analyser (CASA) (SCA®, Version 6.3; Microptic, Spain). Tris-based egg yolk free extender at the ratio of $16x10^6$ spz/mL was used to re-dilute semen samples. Progressive motility and total motility values were determined by CASA after 3 µL of re-diluted sample was placed on the slide recommended by the manufacturer (Leja®, Ref. 025107, IMV Technologies, France). While evaluating spermatozoon movement characteristics, field setting was as; min-max 15-70 μm^2, speed settings; set to static <10 µm/s, slow to medium >45 µm/s, fast >75 µm/s, progressive (STR>80). The examined parameters were; total, progressive, rapid and slow motility and the kinematic parameters (linearity (LIN), straightness (STR), curvilinear velocity (VCL), rectilinear velocity (VSL), average path velocity (VAP), wobble (WOB) and amplitude of lateral head displacement (ALH)).

Flow Cytometry Analysis: Plasma and Acrosome Membrane Integrity and Mitochondrial Membrane Potential were analysed by Guava EasyCyte flow cytometry (Guava Technologies Inc., Hayward, CA, USA; distributed by IMV Technologies). At least 5000 sperm cells were counted for each sample. The analyser was routinely checked by the Guava Check kit daily in terms of accuracy (Guava Technologies, Inc., Millipore, Billerica, MA, USA).

Viability and Acrosome Membrane Integrity: The sperm viability and acrosome membrane integrity were evaluated by Easykit 5 (ref. 025293; IMV Technologies) according to the manufacturer recommendations. Briefly, approximately 40000 spermatozoa were added in each well and incubated with 200 µL of EasyBuffer B (Ref. 023862; IMV Technologies) at 37°C for 45 min in the dark. The proportion of intact or damaged sperm membranes was calculated by the software program (EasySoft, ref. 024842; IMV Technologies).

Mitochondrial Membrane Potential: EasyKit 2 (ref. 024864; IMV Technologies) was used to analyse sperm mitochondrial potential according to the manufacturer recommendations. In summary, at the beginning, 10 µL of ethanol was added to the 96 ready-to-use wells to dissolve the fluorochrome. Next, 190 µL of Easybuffer B (Ref. 023862; IMV Technologies) and semen were added, for a total of 50000 sperm. After incubation of the sample in the dark for 30 min at 37°C, analyses were performed until 5000 spermatozoa were counted.

Statistical Analysis

All sperm parameters were considered as Mean ± Standard Error. The data obtained after thawing were analysed by one-way analysis of variance (ANOVA). Tukey comparison method was used to compare the differences between the groups and it was considered significant at the $P<0.05$ level. All statistical analyses were analysed in the SPSS package program.

Results

The effects of Equex in three different freezing extenders were evaluated in five replicates. Pooled semen mean volume, TM, PM and sperm concentration were 4.7 mL, 89.6%, 75.4% and 4.3×10^9 spermatozoa/mL, respectively. There was no statistical difference observed between the mean TM, PM and live sperm with intact acrosome parameters in different extenders E1, E1E, E2, E2E, E3, E3E after dilution, cooling and equilibration.

The effects of different extenders on post-thaw sperm motility-related parameters (TM, PM, RPM, MPM and SPM) are shown in *Table 1*. Freeze-thaw process resulted in reduced TM ($P<0.01$), PM ($P<0.01$) and live sperm with intact acrosome rate ($P<0.001$). The post-thaw TM was not significantly, but numerically higher in the E1 and E2 groups compared to the E3 group. For the post-thaw progressivity (PM, Rapid, Medium and Slow), there was no difference between base extender groups except medium progressivity ($P<0.05$).

Table 1. *The effect of extender and Equex on the post-thaw sperm motility related parameters (Mean±SE)*

Item	n	Total Motil (%)	Progressive Motil (%)	Rapid Motil (%)	Medium Motil (%)	Slow Motil (%)
E1	15	58.0±2.1^{bc}	44.5±1.9^{b}	36.6±1.7^{b}	15.4±1.0^{a}	6.0±0.4
E1E	15	68.2±3.0^{ab}	48.3±2.8^{ab}	40.2±2.3^{b}	19.4±1.6^{a}	8.7±0.9
E2	15	64.3±2.6^{abc}	51.5±2.3^{ab}	42.3±2.1^{b}	14.8±1.3^{a}	7.2±0.8
E2E	15	69.4±2.4^{a}	49.9±2.2^{ab}	44.2±1.9^{ab}	16.7±1.4^{a}	8.5±0.8
E3	15	54.8±2.8^{c}	45.3±2.4^{ab}	42.2±2.2^{b}	5.9±0.7^{b}	6.7±0.8
E3E	15	65.5±2.4^{ab}	54.2±2.0^{a}	50.9±2.0^{a}	7.8±0.9^{b}	6.7±0.5
Total	90	63.4±1.2	49.0±1.0	42.7±0.9	13.3±0.7	7.3±0.3

^{a,b,c} *Different letters in the same column at post-thaw stage indicates significant difference between groups (P<0.05);* ***E1:*** *Tris based extender (Tris 2.71 g, D-Fructose 1.4 g, Citric acid 1.0 g, 20% eggyolk, Trehalose 50 mM);* ***E1E:*** *E1 + Equex at a ratio of 0.75% v/v;* ***E2:*** *Tris based extender + 1 mM Cystein;* ***E2E:*** *E2+ Equex at a ratio of 0.75% v/v;* ***E3:*** *Skim Milk Powder (SMP);* ***E3E:*** *E3 + Equex at a ratio of 0.75% v/v*

***Table 2**. The effect of Equex on the post-thaw kinematics in different extenders (Mean±SE)*

Item	n	LIN	STR	VCL	VSL	VAP	WOB	ALH
E1	15	58.7±1.6^{ab}	67.0±1.8^{a}	98.6±1.5^{b}	55.4±1.8^{ab}	85.7±1.5^{bc}	86.4±0.6^{a}	2.5±0.1^{c}
E1E	15	55.1±1.8^{bc}	63.8±1.8^{ab}	93.2±1.4^{b}	49.4±2.3^{bc}	80.5±1.8^{c}	85.5±0.8^{a}	2.4±0.0^{c}
E2	15	61.6±1.7^{a}	70.6±1.8^{a}	97.0±1.6^{b}	58.8±2.1^{a}	84.5±2.0^{bc}	85.9±0.9^{a}	2.4±0.1^{c}
E2E	15	49.4±1.8^{cd}	58.2±1.9^{b}	94.5±1.5^{b}	44.5±1.8^{c}	80.5±2.0^{c}	84.4±1.0^{a}	2.6±0.1^{c}
E3	15	44.4±1.2^{de}	58.1±1.2^{b}	134.0±2.6^{a}	55.6±1.8^{ab}	101.3±2.2^{a}	74.5±0.6^{b}	4.2±0.1^{b}
E3E	15	41.6±1.2^{e}	59.9±1.9^{b}	132.4±1.8^{a}	51.2±1.9^{abc}	91.1±2.7^{b}	68.8±1.3^{c}	4.6±0.1^{a}
Total	90	51.8±1.0	62.9±0.9	108.3±2.0	52.5±0.9	87.3±1.1	80.9±0.8	3.1±0.1

^{a,b,c,d,e} *Different letters in the same column at post-thaw stage indicates significant difference between groups (P<0.05);* ***E1:*** *Tris based extender (Tris 2.71 g, D-Fructose 1.4 g, Citric acid 1.0 g, 20% eggyolk, Trehalose 50 mM);* ***E1E:*** *E1 + Equex at a ratio of 0.75% v/v;* ***E2:*** *Tris based extender +1 mM Cystein;* ***E2E:*** *E2 + Equex at a ratio of 0.75% v/v;* ***E3:*** *Skim Milk Powder (SMP);* ***E3E:*** *E3 + Equex- at a ratio of 0.75% v/v*

The effects of Equex on the post-thaw sperm motility-related parameters in different extenders are presented in *Table 2*. In general, there was no statistical difference between TM, PM, and slow progressive motility of Equex supplemented groups except for medium and rapid progressive motility (P<0.05). The medium progressive motility of the E3E group was lower and rapid progressive motility was higher than the E1E and E2E groups (P<0.05).

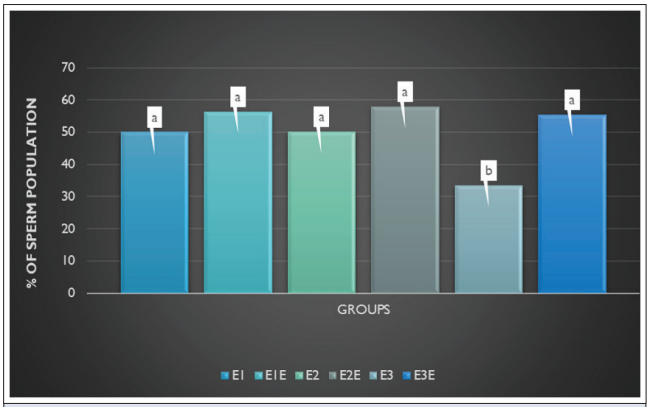

Fig 1. The percentage (%) of sperm with live and intact acrosome parameters at post-thaw stage. ^{a,b}: The difference between groups at post-thaw stage is significant (P<0.05); **E1:** Tris based extender (Tris 2.71 g, D-Fructose 1.4 g, Citric acid 1.0 g, 20% eggyolk, Trehalose 50 mM); **E1E:** E1+ Equex at a ratio of 0.75% v/v; **E2:** Tris based extender + 1 mM Cystein; **E2E:** E2+ Equex at a ratio of 0.75% v/v; **E3:** Skim Milk Powder (SMP); **E3E:** E3 + Equex at a ratio of 0.75% v/v

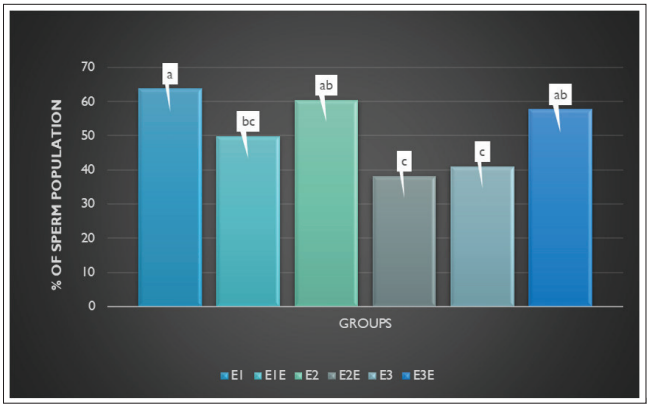

Fig 2. The percentage (%) of sperm with polarized mitochondria at post-thaw stage. Polarized mitochondria is a sperm with high fluoroscense (with Δψm high). ^{a,b,c}: The difference between groups at post-thaw stage is significant (P<0.05); **E1:** Tris based extender (Tris 2.71 g, D-Fructose 1.4 g, Citric acid 1.0 g, 20% eggyolk, Trehalose 50 mM); **E1E:** E1+ Equex at a ratio of 0.75% v/v; **E2:** Tris based extender + 1 mM Cystein; **E2E:** E2+ Equex at a ratio of 0.75% v/v; **E3:** Skim Milk Powder (SMP); **E3E:** E3 + Equex at a ratio of 0.75% v/v

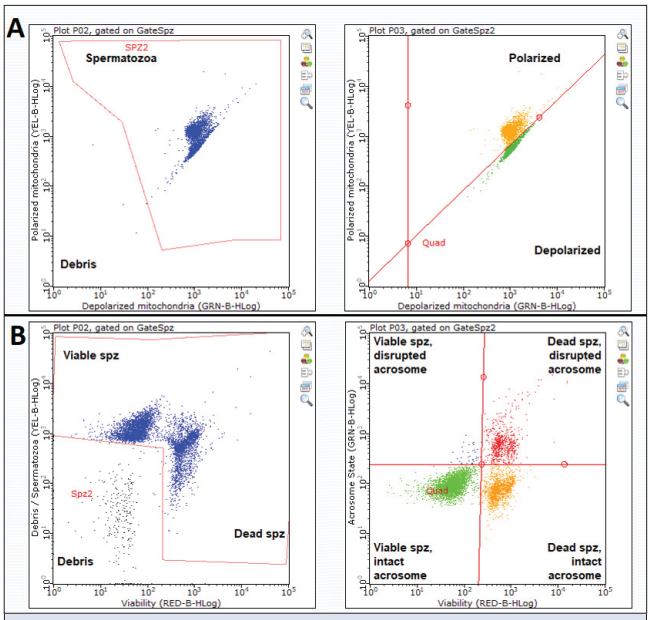

Fig 3. An example for the dotplots of flow cytometer analyses**.** A- Flow cytometry analyses of one experiment showing cells sorted dotplots using Easy kit-2 (mitochondrial activity). Polarized mitochondria is a sperm with high fluoroscense (with Δψm high). At the right dotplot; Orange population; Polarized mitochondria/High Mitochondrial Potential, Green population; Depolarized mitochondria/Low Mitochondrial Potential; B-Flow cytometry analyses of one experiment showing cells sorted dotplots using Easy kit-5 (viability & acrosome integrity). At the right dotplot; Green population: Viable and intact acrosome, Orange population: Dead and intact acrosome, Red population: Dead and damaged acrosome, Blue points: Viable but damage acrosome

The post-thaw mitochondrial activity and live sperm with intact acrosome in function of extender and Equex are represented in *Fig.1*, *Fig. 2*. The extenders ingredients affected post-thaw percentage of sperm with polarized mitochondria and live sperm with intact acrosome. The post-thaw percentage of sperm with polarized

mitochondria and live sperm with intact acrosome of Tris based extender groups (E1 and E2) was higher than that of skim milk based extender group (E3) (P<0.05). It was observed that Equex addition resulted in higher post-thaw live sperm with intact acrosome rates in E1E (proportional), E2E (proportional) and E3E (significant, P<0.05) groups. While the addition of Equex decreased the post-thaw polarized mitochondria rates in the tris based diluent groups (E1E and E2E), it increased in the milk-based extender group (E3E) (P<0.05).

There was an interaction between extenders ingredients and post-thaw sperm LIN and STR *(Table 2)*. The LIN and STR of the E1 and E2 groups were better than that of the E3 group (P<0.05). The Equex supplementation of freezing extender decreased sperm LIN and STR all groups. There was a significant decrease in LIN and STR of the E2E group compared to the E2 group (P<0.05). The post-thaw VCL, WAP and ALH of skim milk based extender group (E3) were higher than that of tris based extender groups (E1 and E2) (P<0.05). The Equex addition was decreased post-thaw VCL, VSL, VAP and WOB values. The ALH values of skim milk based extender groups (E3 and E3E) were higher than that of all the tris based extender groups (P<0.05).

Discussion

In the present study, we investigated the effect of Equex in different laboratory prepared extenders to preserve sperm quality during freeze-thawing. We evaluated sperm motility and motion parameters, live sperm with intact acrosome integrity, and mitochondrial activity. The mean volume of the pooled semen (4.7 mL), the percentage of motile sperm (89.6%), the percentage of progressively motile sperm (75.4%) and the sperm concentration (4.3 × 10^9 spermatozoa/mL) were similar to the results of previous studies [5,19,35,36].

The ingredients of extenders interact with different parts of spermatozoa leading to osmotic and toxic stresses and inducing biochemical changes in cell metabolism immediately after dilution [9,37,38]. Semen extenders provide energy for maintaining sperm motility [39]. As expected, the diluted sperm had slightly higher TM and PM than the pooled semen. There were no differences between the mean TM, PM, and live sperm with intact acrosome parameters in the different extenders (E1, E1E, E2, E2E, E3, E3E) after dilution, cooling, and equilibration.

The freeze-thaw process can damage spermatozoa, causing unfavorable changes in their membrane lipid composition, acrosome status, motility, motion parameters, morphology, and viability [39,40]. In our study, freeze-thaw process resulted in reduced TM (P<0.01), PM (P<0.01) and live sperm with intact acrosome rate (P<0.001) in all groups. So many studies have been conducted using different semen extenders and additives for protecting ram sperm fertilizing ability during freeze-thawing process [5,6]. Tris and Test buffers have been commonly incorporated into extender and help in sustaining sperm motility and fertilizing ability [21-24]. In addition, skim milk and egg yolk based extenders are widely used for preserving ram semen [17,36]. In liquid storage of ram semen, usage of the skim milk-based extender is superior to Tris-based extender [41]. The Tris based extender groups (E1 and E2) resulted in higher post-thaw TM (P>0.05), and live sperm with intact acrosome compared to the skim milk based extender (E3) group (P<0.05). For the post-thaw progressivity related parameters (PM, Rapid, Medium and Slow), there was no difference between groups except E3 medium progressivity (P<0.05).

Equex addition to the extender (0.7 percent Equex in Fiser's extender) improves post-thaw ram sperm motility [34]. It acts as an emulsifier to the egg yolk particles of the extender, and promotes the cryoprotective effect by facilitating the interaction of egg yolk proteins with the sperm plasma membrane [11,42]. Spermatozoa change their behaviour in response to environmental changes and storage. Therefore, it is likely that obtained changes in the sperm motions pattern, reflect the distinctions of the process [33]. The addition of Equex in semen freezing extenders increased post-thaw TM, rapid medium and slow progressive motility numerical in E1E and E2E groups, and significantly in E3E (P<0.05) group.

Sperm motility and membrane integrity are positively correlated with sperm fertilization ability [43]. The extender ingredients and freeze-thaw process modify the structure of the sperm cell membrane that results in mitochondrial damage, and alters the metabolic functions of spermatozoa by reducing the ATP-producing capacity of mitochondria [44]. In our study, post-thaw sperm polarized mitochondria was higher in Tris based extender (E1 and E2) than skim milk based extender (E3) (P<0.05). Equex acts as a non-permeating cryoprotectant agent [11,42]. For the Equex supplemented groups, post-thaw polarized mitochondria rates in the milk-based extender group (E3E) was higher than Tris based extender groups (E2E) (P<0.05). This could be explained by the fact that Equex facilitates the interaction of milk powder proteins such as egg yolk proteins with the sperm plasma membrane [11,42].

The sperm plasma and acrosome membrane integrity is necessary to maintain sperm functionality during storage and also in the female reproductive tract [13]. It was observed that live sperm with intact acrosome in Tris-based extender groups (E1 and E2) was higher than in the skim milk based extender group (E3) (P<0.05). The mechanism whereby Equex exerts its protective effect on the sperm remains unclear [45]. The active ingredient of Equex is sodium dodecyl sulfate (SDS), which has a

detergent property. SDS is known to have a toxic effect on sperm membranes and viability in bull [29]. Extender supplementation with Equex has been reported to promote the post-thaw recovery of motile sperm and intact acrosome in ram [34] and cat epidymal spermatozoa [45]. Equex addition resulted in higher post-thaw live sperm with intact acrosome rates in E1E (proportional), E2E (proportional) and in E3E (significant, P<0.05) groups.

When assessing the potential fertilizing capacity of sperm, it is imperative to keep in mind that each spermatozoon is a multi-compartmental cell required to have various features in order to fertilize an oocyte [46]. For many years, the CASA analysis has been one standard in the laboratory for motility and kinematic parameter assessment [47]. There was an interaction between extender ingredients and post-thaw sperm LIN and STR parameters in our study. The LIN and STR of the tris based extenders groups (E1 and E2) were higher than the skim milk based extender group (E3) (P<0.05). The Equex supplementation of the freezing extender decreased sperm LIN and STR in all groups. In addition, Equex decreased post-thaw VCL, VSL, VAP and WOB values. These results might be due to the increased osmotic environment and the altered sperm membrane functional integrity by the Equex.

Sperm kinematic parameters such as VCL, VSL, ALH, VAP, LIN, STR and WOB are associated with fertility [48,49]. The post-thaw VCL, WAP and ALH of skim milk based extender (E3) were higher than tris based extender groups (E1 and E2) (P<0.05). Sperm velocity-related parameters are an indirect indicator of mitochondrial function [50] and fertility [48]. It was observed that there was a decreasing tendency in sperm velocity parameters of all Equex-supplemented groups.

As a result of our study, it was concluded that there is an interaction between Equex and extender ingredient. The addition of Equex to sperm extenders positively affected post-thaw sperm quality in all groups, but this positive effect was more evident in skim milk-based extender than in tris-based extender. For the tris-based extender groups, the positive effect of Equex addition was less evident in the cystein-containing group (E2E).

Declarations

Availability of Data and Materials: The data underlying this study are available from the corresponding author upon reasonable request (E. Şengül).

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Conflict of Interest: The authors have no conflict of interest to report.

Author Contributions: EŞ, CD: Experimental design, spermatological analyses, writing manuscript; BAK: Spermatological analyses; İÇ: Semen collection; BB, BÜ: Writing and editing manuscript; ZN: Experimental design, writing & editing manuscript, visualization, supervision. All authors read, revised, and approved the final manuscript. All authors have read and agreed to the published version of the manuscript.

References

1. Sharafi M, Borghei-Rad SM, Hezavehei M, Shahverdi A, Benson JD: Cryopreservation of semen in domestic animals: A review of current challenges, applications, and prospective strategies. *Animals,* 12 (23):3271, 2022. DOI: 10.3390/ani12233271

2. Demir K, Ozturk G, Cirit U, Bozkurt HH, Aktas A, Birler S, Ak K, Pabuccuoğlu S: Effects of cooling rate on membrane integrity and motility parameters of cryopreserved ram spermatozoa. *Kafkas Univ Vet Fak Derg,* 21 (1): 61-67, 2015. DOI: 10.9775/kvfd.2014.11726

3. Alcay S, Ustuner B, Cakmak I, Cakmak SS, Nur Z: Effects of various cryoprotctive agents on post-thaw drone semen quality. *Kafkas Univ Vet Fak Derg,* 21 (1): 31-35, 2015. DOI: 10.9775/kvfd.2014.11515

4. Alcay S, Toker MB, Gokce E, Gul Z, Onder NT, Ustuner B, Nur Z, Sagirkaya H, Soylu MK: Improvement of incubation resilience with various antioxidants in cryopreserved. *J Vet Med Erciyes Univ,* 13 (3): 183-190, 2017.

5. Soylu MK, Nur Z, Ustuner B, Dogan I, Sagirkaya H, Gunay U, Ak K: Effects of various cryoprotective agents and extender osmolality on post-thawed ram semen. *Bull Vet Inst Pulawy,* 51 (2): 241-246, 2007.

6. Nur Z, Zık B, Ustuner B, Sagirkaya H, Ozguden CG: Effects of different cryoprotective agents on ram sperm morphology and DNA integrity. *Theriogenology,* 73 (9): 1267-1275, 2010. DOI: 10.1016/j.theriogenology.2009.12.007

7. Santiago-Moreno J, Castaño C, Toledano-Díaz A, Coloma MA, López-Sebastián A, Prieto MT, Campo JL: Semen cryopreservation for the creation of a Spanish poultry breeds cryobank: Optimization of freezing rate and equilibration time. *Poult Sci,* 90 (9): 2047-2053, 2011. DOI: 10.3382/ps.2011-01355

8. Jiménez-Rabadán P, Soler AJ, Ramón M, García-Álvarez O, Maroto-Morales A, Iniesta-Cuerda M, Fernández-Santos MD, Montoro V, Pérez-guzmán MD, Garde JJ: Influence of semen collection method on sperm cryoresistance in small ruminants. *Anim Reprod Sci,* 167, 103-108, 2016. DOI: 10.1016/j.anireprosci.2016.02.013

9. Ak K, Cirit U, Nur Z, Bacinoglu S, Pabuccuoglu S, Ozdas, OB, Birler S: Effects of extender osmolarity, cooling rate, dilution rate and glycerol addition time on post-thaw ram semen characteristics and fertilization. *J Fac Vet Med Istanbul Univ,* 36 (2): 33-46, 2010.

10. Peña AI, Linde-Forsberg C: Effects of Equex, one- or two-step dilution, and two freezing and thawing rates on post-thaw survival of dog spermatozoa. *Theriogenology,* 54 (6): 859-875, 2000. DOI: 10.1016/S0093-691X(00)00397-6

11. Holt WV: Basic aspects of frozen storage of semen. *Anim Reprod Sci,* 62 (1-3): 3-22, 2000. DOI: 10.1016/S0378-4320(00)00152-4

12. Blackburn HD, Gollin D: Animal genetic resource trade flows: The utilization of newly imported breeds and the gene flow of imported animals in the United States of America. *Livest Sci,* 120 (3): 240-247, 2009. DOI: 10.1016/j.livsci.2008.07.006

13. Emamverdi M, Zhandi M, Zare Shahneh A, Sharafi M, Akbari-Sharif A: Optimization of ram semen cryopreservation using a chemically defined

soybean lecithin-based extender. *Reprod Domest Anim,* 48 (6): 899-904, 2013. DOI: 10.1111/rda.12183

14. Vozaf J, Makarevich AV, Baláži A, Vašíček J, Svoradová A, Olexiková L, Chrenek P: Cryopreservation of ram semen: Manual versus programmable freezing and different lengths of equilibration. *Anim Sci J,* 92 (1):e13670, 2021. DOI: 10.1111/asj.13670

15. Paulenz H, Söderquist L, Pérez-Pé R, Berg KA: Effect of different extenders and storage temperatures on sperm viability of liquid ram semen. *Theriogenology,* 57 (2): 823-836, 2002. DOI: 10.1016/S0093-691X(01)00683-5

16. Filho IC, Pederzolli CD, Sgaravatti ÂM, Gregory RM, Filho CS, Jobim MI, Mattos RC: Skim milk-egg yolk based semen extender compensates for non-enzymatic antioxidant activity loss during equine semen cryopreservation. *Anim Reprod,* 6 (2): 392-399, 2009.

17. Kulaksız R, Cebi C, Akcay E: The effect of different extenders on the motility and morphology of ram sperm frozen or stored at 4°C. *Turk J Vet Anim Sci,* 36 (2): 177-182, 2012. DOI: 10.3906/vet-1103-11

18. Manjunath P: New insights into the understanding of the mechanism of sperm protection by extender components. *Anim Reprod*, 9 (514): 809-815, 2012. DOI: 10.1002/mrd.20565

19. Ustuner B, Alçay S, Nur Z, Sagirkaya H, Soylu MK: Effect of egg yolk and soybean lecithin on tris-based extender in post-thaw ram semen quality and *in vitro* fertility. *Kafkas Univ Vet Fak Derg,* 20 (3): 393-398, 2014. DOI: 10.9775/kvfd.2013.10248

20. Aisen EG, Álvarez HL, Venturino A, Garde JJ: Effect of trehalose and EDTA on cryoprotective action of ram semen diluents. *Theriogenology,* 53 (5): 1053-1061, 2000. DOI: 10.1016/S0093-691X(00)00251-X

21. Zhang L, Sohail T, Wang Y, Kang Y, Wang X, Sun X, Li Y: The effect of different storage temperature on Hu ram sperm parameters. *Kafkas Univ Vet Fak Derg,* 28 (2): 201-209, 2022. DOI: 10.9775/kvfd.2021.26676

22. Zhang L, Sohail T, Li Y, Wang Y, Chu C, Feng Y, Ma J: Effect of different extenders on the sperm quality parameters of Hu Ram semen preserved at 16ºC. *Kafkas Univ Vet Fak Derg,* 27 (1): 83-91, 2021. DOI: 10.9775/kvfd.2020.24783

23. Güngör Ş, İnanç ME, Yeni D, Avdatek F, Türkmen R, Taşdemir U: The effect of gallic acid addition to tris-based extender on frozen bull semen. *Kafkas Univ Vet Fak Derg,* 27 (5): 633-639, 2021. DOI: 10.9775/kvfd.2021.26023

24. Gilmore A, Hitit M, Ugur MR, Dinh TTN, Tan W, Jousan FD, Nicodemus M, Kaya A, Memili E: Functional variables of bull sperm associated with cryotolerance. *Kafkas Univ Vet Fak Derg,* 27 (3): 371-379, 2021. DOI: 10.9775/kvfd.2021.25653

25. Salamon S, Ritar AJ: Deep freezing of Angora goat semen: effects of diluent composition and method and rate of dilution on survival of spermatozoa. *Aust J Biol Sci,* 35 (3): 295-303, 1982.

26. Soltanpour F, Moghaddam G: Effects of frozen diluents on storage of ram sperm. *Int J Adv Biol Biomed Res,* 1 (12): 1698-1704, 2013. DOI: 10.26655/IJABBR.2016.6.13

27. Suwimonteerabutr J, Nuntapaitoon M, Tummaruk P: Determination of the optimal concentration of Minitube Equex paste for boar semen cryopreservation based on sperm motility characteristics. *Vet Stanica,* 52 (3): 285-295, 2020. DOI: 10.46419/vs.52.3.8

28. Šterbenc N, Kosec M, Bollwein H, Klinc P: The effect of Equex STM® in freezing media on post thaw motility, viability and DNA integrity of frozen-thawed ram spermatozoa. *Slov Vet Res,* 51 (1): 35-42, 2014. DOI: 10.5167/uzh-107220

29. Arriola J, Foote RH: Glycerolation and thawing effects on bull spermatozoa frozen in detergent-treated egg yolk and whole egg extenders. *J Dairy Sci,* 70 (8): 1664-1670, 1987. DOI: 10.3168/jds.S0022-0302(87)80194-7

30. Chaveiro A, Machado LC, Frijters AC, Engel B, Woelders H: Improvement of parameters of freezing medium and freezing protocol for bull sperm using two osmotic supports. *Theriogenology,* 65 (9): 1875-1890, 2006. DOI: 10.1016/j.theriogenology.2005.10.017

31. Schäfer-Somi S, Binder C, Burak JA, Papadopoulos N, Ilas J, Boersma A, Aurich C: Using egg yolk in a TRIS-Equex STM Paste extender for freezing of dog semen is superior to egg yolk plasma, also after addition of lecithin and catalase. *Cryobiology,* 100, 63-71, 2021. DOI: 10.1016/j.cryobiol.2021.03.009

32. Dorado J, Hidalgo M, Acha D, Ortiz, I, Bottrel M, Azcona F, Carrasco JJ, Gomez-Arrones V, Demyda-Peyrás S: Cryopreservation of Andalusian donkey *(Equus asinus)* spermatozoa: Use of alternative energy sources in the freezing extender affects post-thaw sperm motility patterns but not DNA stability. *Anim Reprod Sci,* 208, 106-126, 2019. DOI: 10.1016/j.anireprosci.2019.106126

33. Abáigar T, Holt WV, Harrison RA, del Barrio G: Sperm subpopulations in boar (*Sus scrofa*) and gazelle *(Gazella dama mhorr)* semen as revealed by pattern analysis of computer-assisted motility assessments. *Biol Reprod,* 60 (1), 32-41, 1999. DOI: 10.1095/biolreprod60.1.32

34. Akourki A, Gil L, Echegaray A, Espinosa E, Josa A, de Blas I, González N, Gallegos de la Hoya M, Meque LC: Effect of the extender supplement Equex-STM on cryopreserved semen in the Assaf sheep. *Cryo Letters,* 25 (2): 147-154, 2004.

35. Ustuner B, Alcay S, Gokce E, Yilmaz MM, Aktar A, Huraydin O, Duman M, Onder NT, Akal E, Nur Z: Lyophilized extender supplemented with Rainbow trout *(Oncorhynchus mykiss)* seminal plasma improves cryopreservation of ram sperm. *Kafkas Univ Vet Fak Derg,* 28 (2): 255-260, 2022. DOI: 10.9775/kvfd.2021.26855

36. Dayanikli C, Sengul E, Bulbul B, Ustuner B, Nur Z: The effect of concentration and storage time on short-term storage of ram sperm sperm. *Kafkas Univ Vet Fak Derg,* 28 (6): 681-690 2022. DOI: 10.9775/kvfd.2022.27636

37. Domingo P, Olaciregui M, González N, Blas ID, Gil L: Comparison of different semen extenders and cryoprotectant agents to enhance cryopreservation of rabbit spermatozoa. *Czech J Anim Sci,* 64 (2): 59-66, 2019. DOI: 10.17221/53/2018-CJAS

38. Yániz JL, Palacín I, Santolaria P: Effect of chamber characteristics, incubation, and diluent on motility of honey bee *(Apis mellifera)* drone sperm. *Apidologie,* 50 (4): 472-481, 2019. DOI: 10.1007/s13592-019-00659-y

39. Hafez ESE: Preservation and Cryopreservation of Gametes and Embryos. **In,** Hafez ESE (Ed): Reproduction in Farm Animals. 7th ed., 431-442, Lea&Febiger, Philadelphia, 1993.

40. Gangwar C, Saxena A, Patel A, Singh SP, Yadav S, Kumar R, Singh VK: Effect of reduced glutathione supplementation on cryopreservation induced sperm cryoinjuries in Murrah bull semen. *Anim Reprod Sci,* 192, 171-178, 2018. DOI: 10.1016/j.anireprosci.2018.03.005

41. Rahman MM, Gofur MR, Bari FY, Juyena N: Effect of skim milk and tris-citrate extenders to preserve the semen of indigenous ram of Bangladesh. *Asian J Biol,* 5 (2): 1-11, 2018. DOI: 10.9734/AJOB/2018/39448

42. Tomás C, Gómez-Fernández J, Gómez-Izquierdo E, Mocé E, de Mercado E: Addition of cholesterol-loaded cyclodextrins to the thawing extender: Effects on boar sperm quality. *Reprod Domest Anim,* 49 (3): 427-432, 2014. DOI: 10.1111/rda.12291

43. Vyt P, Maes D, Dejonckheere E, Castryck F, Van Soom A: Comparative study on five different commercial extenders for boar semen. *Reprod Domest Anim,* 39 (1): 8-12, 2004. DOI: 10.1046/j.1439-0531.2003.00468.x

44. Kritaniya D, Yadav S, Swain DK, Reddy AV, Dhariya R, Yadav B, Anand M, Nigam R: Freezing-thawing induces deprotamination, cryocapacitation-associated changes; DNA fragmentation; and reduced progesterone sensitivity in buck spermatozoa. *Anim Reprod Sci,* 223:106628, 2020. DOI: 10.1016/j.anireprosci.2020.106628

45. Axnér E, Hermansson U, Linde-Forsberg C: The effect of Equex STM paste and sperm morphology on post-thaw survival of cat epididymal spermatozoa. *Anim Reprod Sci,* 84 (1-2): 179-191, 2004. DOI: 10.1016/j.anireprosci.2003.11.003

46. Graham JK, Mocé E: Fertility evaluation of frozen/thawed semen. *Theriogenology,* 64 (3): 492-504, 2005. DOI: 10.1016/j.theriogenology.2005.05.006

47. Mortimer ST, van der Horst G, Mortimer D: The future of computer-aided sperm analysis. *Asian J Androl,* 17 (4): 545-553, 2015. DOI: 10.4103/1008-682X.154312

48. Aitken RJ, West KM: Analysis of the relationship between reactive

oxygen species production and leucocyte infiltration in fractions of human semen separated on Percoll gradients. *Int J Androl,* 13 (6): 433-451, 1990. DOI: 10.1111/j.1365-2605.1990.tb01051.x

49. Fernández-López P, Garriga J, Casas, I, Yeste M, Bartumeus F: Predicting fertility from sperm motility landscapes. *Commun Biol,* 5 (1): 1-11, 2022. DOI: 10.1111/j.1365-2605.1990.tb01051.x

50. Nesci S, Spinaci M, Galeati G, Nerozzi C, Pagliarani A, Algieri C, Tamanini C, Bucci D: Sperm function and mitochondrial activity: An insight on boar sperm metabolism. *Theriogenology,* 144, 82-88, 2020. DOI: 10.1016/j.theriogenology.2020.01.004

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Research Article

Evaluation of Antimicrobial Efficacy and Phenolic Compound Profiles in Geopropolis Samples from Bolivia and Venezuela

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Abstract

In this study, we investigated the balsamic contents, total phenolic contents, flavone-flavonol contents, and flavanones-dihydroflavonols contents, and antimicrobial effects of seven geopropolis samples produced by four neotropical stingless bee species: Venezuelan *Melipona favosa*, and Bolivian *M. grandis*, *Scaptotrigona depilis*, *S. polysticta*. The balsamic content value was found highest in the sample of *S. polysticta* (sample 7) collected from Bolivia, highest total phenolic content in *M. favosa* geopropolis (sample 1) from Venezuela, highest flavone-flavonol content in *M. favosa* geopropolis (sample 2) collected from Venezuela and highest flavones-dihydroflavonols content in S. *polystica* (sample 7) from Bolivia. As a result, except for balsamic content values, other investigated values were lower compare to the previous researches about different stingless bee species geopropolis. This is proved that climatic conditions, bee species and collecting area affected the chemical content of geopropolis significantly. The antimicrobial findings indicated that the examined geopropolis extracts displayed different degrees of inhibition against the growth of Gram-positive bacteria and fungi, which correlated with their phenolic contents. Nevertheless, these extracts did not demonstrate a comprehensive inhibitory effect on Gram-negative bacteria. Standardized geopropolis samples, rich in phenolic content, can complement antibiotics naturally for preventing and treating infections from Gram-positive bacteria and *Candida albicans*. However, further studies are still needed regarding the clinical applications of geopropolis in various infections.

Keywords: Geopropolis, *Melipona favosa, Melipona grandis, Scaptotrigona depilis, Scaptotrigona polysticta,* Antimicrobial activity, Phytochemicals

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Introduction

Plants produce resinous secretions from various parts such as buds, bark, flowers, and fruits, to protect against microbial clay and provide thermal isolation. This resinous substance collected by honey bees and processed with digestive enzymes and beeswaxes is called propolis. It is used for various functions in the stingless bee nest [1,2].

Geopropolis, generated by some species of stingless bees belonging to the *Meliponini tribe,* represents a distinct variety of propolis. Stingless bees produce geopropolis by combining the plant resinous substances with wax, digestive enzymes, and soil. On the other hand, there is no soil in the propolis produced by *Apis mellifera* [3,4].

In tropical and subtropical regions worldwide, there are 56 genera and 605 species of stingless bees [5,6]. It was found that more than some 500 species are in the Neotropical region and 259 species in Brazil [7].

The stingless bee species *Melipona favosa* is an important species for Venezuela, living in the plains and along the coastlines of the country. This gentle stingless bee species is locally known as "erica" and "maba." These bees typically construct their nests in trees, on walls, and on fence posts, and are managed in hives by traditional stingless beekeepers [8].

Stingless bees are social insects like honey bees, they also produce honey, pollen, beeswax, and cerumen besides

geopropolis. Furthermore, they are very important pollinators of many crops like, lychee, avocado, macadamia, mango, coffee [9-12]. For these reasons stingless bees are used locally and beekeping with stingless bees is called meliponiculture [5].

Meliponiculture is an industry that develops in tropical countries and holds high economic value due to the increased productivity of agricultural products through pollination services. It also allows for the extraction of commercially valuable products like beeswax, pollen, propolis, and royal jelly. Therefore, meliponiculture is a valid activity that can generate income for local communities in the Amazon region [6,13].

Propolis has many functions in the beehive, such as thermal isolation, closing the holes crevices, and disinfection of the hive with its antimicrobial effects [1]. Also, the geopropolis is using for has similar functions in the hive by stingless bee nest.

Geopropolis, similar to propolis, possesses a highly complex and variable chemical composition, which is influenced by factors like flora, climate, and the species of bees [14]. Studies have shown that geopropolis is rich in many components such as phenolic acids, flavanoids, terpenes, fatty acids and steroids, organic acids and alcohols [7,14-16]. Thanks to these compounds, antibacterial, antifungal, cytotoxic, antioxidant effects have been reported to have a very therapeutic effect [7,16].

By this study, we aimed to investigate the balsamic, total phenolic, flavone-flavonol, flavanone-dihydroflavonol contents and antimicrobial effects of geopropolis collected from Venezuela and Bolivia that are collected by *M. favosa, M. grandis, Scaptotrigona depilis, S. polysticta*.

Material and Methods

Geopropolis Samples

Several geopropolis samples from various stingless bee species were gathered in different regions from Bolivia and Venezuela *(Table 1)*.

Table 1. *Countries where samples collected and Stingless bee species and countries of origin of propolis samples*

Sample No	Stingless Bee Species	Country
1	*Melipona favosa*	Venezuela
2	*Melipona favosa*	Venezuela
3	*Melipona favosa*	Venezuela
4	*Melipona grandis*	Bolivia
5	*Scaptotrigona depilis*	Bolivia
6	*Scaptotrigona depilis*	Bolivia
7	*Scaptotrigona polysticta*	Bolivia

Stingless bee samples were collected using isopropyl alcohol, dried at ambient temperature, prepared using tissues in rigid plastic boxes, and entomological identifications were conducted.

The propolis samples were obtained through collaboration with Apitherapy and Bioactivity (APIBA), University of the Andes, Mérida, Venezuela. *M. favosa* propolis was collected from the Paraguaná Peninsula. Paraguaná Peninsula is located in the north of Falcón State, alongside the Caribbean Sea. It is one of the driest areas in Venezuela, situated within an arid and semi-arid bioclimatic environment, characterized by strong winds and an average annual precipitation of about 340 mm. It is a xerophytic area of about 3.405 km² with almost entirely arid soil. Cerro de Santa Ana 830 masl is surrounded by predominant plains with savannah xerophytic vegetation. The geopropolis samples of *M. favosa* were collected at Cerro Santa Ana piedmont from the meliponary of the Agenda Petroleo Project.

M. grandis, *S. depilis*, and *S. polysticta* propolis were collected from the Amboró National Park in Bolivia at the beginning of the winter season. Amboró National Park covers 636.000 hectares from 320 to 3.300 masl, located in the eastren lowlands of Bolivia, near to San Carlos, with high biodiversity of the Bolivian Amazonian Forest. Environmental problems are timber trafficking and illegal coca cultivation. Geopropolis samples were collected near to Pirai river, from the stingless bee keepers of the sustainable meliponiculture project promoted by the Ecological Association of the East (ASEO), and the Association of Native Honey Producers (APROMIN). The geographical localization of the stingless bee nests producing the collected geopropolis samples is shown in *Fig. 1*.

Propolis Extraction

The extraction of propolis was conducted following the method outlined by Popova et al.[17]. Initially, propolis was shredded, and a 1-gram sample of propolis powder was combined with a 70% ethanol solution (Merck, Germany). After the dilution process, an ultrasound bath treatment

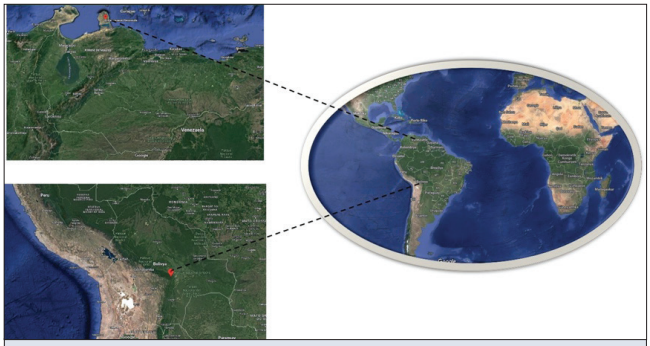

Fig 1. The geographical localization of the collected geopropolis samples

was conducted with a 300 W ultrasonic bath (ISOLAB, Germany, model: 621.05.022s). Subsequently, The blend was filtrated using Whatman No. 4 filter paper (Millipore, USA), and the remaining solid residue underwent a second extraction. After this step, the ethanol extracts were combined and diluted to a final volume of 100 mL using 70% ethanol.

Balsamic Content

Three parallel extracts were arranged for each crude sample using 70% ethanol. Two milliliters from each extracts were evaporated until they reached a consistent weight after drying. The ethanol-soluble fraction was used to calculate the proportions of balsam in the extracts. The mean of these values was determined [17] and expressed as a percentage (%).

Estimation of Total Polyphenol Content by Folin-Ciocalteu Colorimetric Method

The Folin-Ciocalteu colorimetric method, as outlined by Slinkard and Singleton [18], was employed to determine the total polyphenol content in the ethanolic extract of propolis (EEP). Absorbance measurements were recorded at 760 nm utilizing a UV-VIS spectrophotometer (Genesys 10S UV-VIS Spectrophotometer). For the calibration curve, gallic acid served as the standard. The total content of the extracts was quantified by comparing the results with a calibration curve established with gallic acid as the standard (r^2=0.997) and stated as milligrams of gallic acid equivalents (GAE) per gram of propolis extract (mgGAE/g). Each extract was measured three times for accuracy.

Flavone and Flavonol Content

The analysis of flavone and flavonol content followed the procedure outlined by Popova et al.[17]. Absorbance measurements were recorded at 425 nm using a UV-VIS spectrophotometer (Genesys 10S UV-VIS Spectrophotometer). Calibration was carried out with quercetin as the reference compound (r^2=0.999). Three measurements were conducted for each extract, and the results were expressed as a percentage (%).

Flavanone and Dihydroflavonol Content

For the analysis, 1 mL of the extract was combined with 2 mL of a DNP (2,4-dinitrophenylhydrazine) solution. The DNP solution was rearranged by dissolving 1 g of DNP in 2 mL of 96% sulfuric acid, consequently, the mixture was diluted to a final volume of 100 mL with methanol using a volumetric flask. The blend was then subjected to heating at 50°C during 50 min. Upon reaching room temperature, the solution was further diluted to 10% KOH in methanol. Afterward, 0.5 mL of the resultant solution was introduced into 10 mL of methanol, followed by additional dilution to achieve a final volume of 25 mL with methanol. The absorbance was measured at 486 nm employing a UV-VIS spectrophotometer (Genesys 10S UV-VIS Spectrophotometer). Calibration was conducted with naringenin serving as the reference compound. Three measurements were taken for each extract [19] and expressed as a percentage (%).

***Table 2**. Bacterial and fungal strains tested for propolis bioactivity*

Agent	Microorganisms
Gram positive bacteria	*Staphylococcus aureus* ATCC 29213
	Methicillin-Resistant *Staphylococcus aureus* (MRSA) NCTC 10442
	Enterococcus faecalis ATCC 29212
Gram negative bacteria	*Escherichia coli* ATCC 25922
	Klebsiella pneumoniae ATCC 13883
	Pseudomonas aeruginosa ATCC 27853
	Acinetobacter baumannii ATCC 19606
Fungi	*Candida albicans* ATCC 10231

Antimicrobial Activity Assay

The test organisms employed in the study are detailed in *Table 2*. Before the research commenced, all strains were stored and preserved through cryopreservation at -86°C in the Microbiology Laboratories of Gülhane Faculty of Medicine. Bacterial strains were cultivated on 5% Sheep Blood Agar (RTA Labs, Gebze, Türkiye) at 37°C for 24 h, whereas yeasts were cultured in RPMI-1640 medium (Thermo Fisher Scientific, USA) at 25°C for 48 h. Propolis extracts, diluted in 70% ethanol, were subjected to broth microdilution assays to ascertain their Minimal Inhibitory Concentration (MIC) values, in accordance with the guidelines outlined by the Clinical and Laboratory Standards Institute (CLSI) standart M7-A9 and M27-A3.

Briefly, the ethanolic extracts of propolis (EEP) were prepared with two-fold serial dilutions in Mueller Hinton Broth (RTA Labs, Gebze, Türkiye), and then transferred to the wells of U-bottom microdilution plates. Bacterial and fungal inoculum suspensions were adjusted to final concentrations of 10^5 CFU/mL and 10^4 CFU/mL, respectively, and dispensed into microdilution wells containing various concentrations of EEP in 20 µL. Afterward, microdilution plates inoculated with bacteria were incubated in a 37°C incubator for 24 h whereas those inoculated with *Candida albicans* were kept at 25°C for 48 h. Positive controls were established using Meropenem (Sigma-Aldrich, USA) for bacteria and Fluconazole (Sigma-Aldrich, USA) for fungal strains. In each experiment, a positive control and a negative control and ethanol control (2% ethanol) were used. The Minimal Inhibitory Concentration (MIC) was determined as the lowest concentration of each extract that inhibited visible

growth of microorganisms. The Tetrazolium/formazan test (TTC) was used to evaluate the viability of the tested microorganisms [20]. The experiments were conducted in triplicate, and the outcomes were reported in mg/mL.

Statistical Analysis

The statistical analysis was conducted with Statistical Package for Social Sciences (SPSS) Version 25 (SPSS, Inc.). The normal distribution suitability of variables was assessed through the Shapiro-Wilk and Kolmogorov-Smirnov tests, skewness and kurtosis values, histogram graphs, and decisions were made based on mean ± standard deviation and median values. If the p-value was below 0.05, it was interpreted as "significant". Spearman correlation analysis was performed to assess the correlation of numerical data that does not conform to normal distribution. Likewise, the Mann-Whitney U test was employed to compare numerical data between groups that do not conform to normal distribution.

Results

In this study, balsamic contents, total phenolic contents, flavone-flavonol contents, flavanone-dihydroflavonol contents, and antimicrobial effects of seven geopropolis samples belonging to four stingless bee species *(M. favosa*, *M. grandis*, *S. depilis*, *S. polysticta)* collected from Bolivia and Venezuela were analyzed.

According to our results, the balsamic content of geopropolis samples ranged from 3.33% to 30.15% *(Table 3)*. The maximum value was found in geopropolis of *S. polysticta* stingless bee species collected from Bolivia.

While the maximum flavone and flavonol content (0.89±0.01) was detected in the geopropolis (sample 2) belonging to the *M. favosa* stingless bee species collected from Venezuela, no flavone and flavonol content was found in samples 4 *M. grandis* and 7 *S. polysticta (Table 3)*.

Although the flavone-dihydroflavonol contents of geopropolis collected from Bolivia varied between 2.02±0.01 and 7.43±0.01, no flavanone and dihydroflavonol contents were detected in geopropolis collected from Venezuela *(Table 3)*.

In our study, we determined total polyphenol content according to Folin-Ciocalteu colourimetric method Slinkard and Singleton [18] and we found values between 1.66±0.00 and 16.73±0.00 mgGAE/g. The highest total phenolic value was determined to be 16.73 mgGAE/g, and it is associated with sample number 1 derived from *M. favosa*. The lowest total total phenolic content value was found to be in 1.66 mgGAE/g belong to the sample 3 collected by *M. favosa*.

The antimicrobial effect of seven geopropolis extracts obtained from four stingless bee species was tested against Gram-negative bacteria *(Escherichia coli, Klebsiella*

Table 3. *Bee species, and analytical results*

Sample No	Bee Species	Balsamic Content%	Total Phenolic Content (mgGAE/g)	Flavone and Flavonol Content (%)	Flavanones and Dihydroflavonols Content (%)
1	*Melipona favosa*	4.33	16.73±1.36	0.62±0.01	-
2	*Melipona favosa*	3.50	6.93±0.01	0.89±0.01	-
3	*Melipona favosa*	6.16	1.66±0.01	0.49±0.01	-
4	*Melipona grandis*	15.66	12.44±0.01	-	2.02±0.01
5	*Scaptotrigona depilis*	3.33	2.13±0.009	0.12±0.06	3.07±0.01
6	*Scaptotrigona depilis*	22.99	8.59±0.01	0.25±0.02	4.25±0.01
7	*Scaptotrigona polysticta*	30.15	5.91±0.01	-	7.43±0.01

Table 4. *Minimum inhibitory concentration (mg/mL) for the studied microorganisms*

Sample	*E. coli* ATCC 25922	*K. pneumonia* ATCC 13883	*P. aeruginosa* ATCC 27853	*A. baumannii* ATCC 19606	*S. aureus* ATCC 29213	*MRSA* NCTC 10442	*E. faecalis* ATCC 29212	*C. albicans* ATCC 10231
PROPOLIS 1	>41	>41	>41	>41	5.125	5.125	5.125	5.125
PROPOLIS 2	>41	>41	>41	>41	5.125	5.125	10.25	5.125
PROPOLIS 3	>41	>41	>41	>41	10.25	10.25	20.5	20.5
PROPOLIS 4	>41	>41	>41	>41	5.125	5.125	10.25	10.25
PROPOLIS 5	>41	>41	>41	>41	20.5	20.5	20.5	20.5
PROPOLIS 6	>41	>41	>41	>41	5.125	5.125	5.125	10.25
PROPOLIS 7	>41	>41	>41	>41	10.25	10.25	10.25	5.125

Table 5. *Statistical analysis between geographical region and balsamic content, total phenolic content and antibacterial effect*

Variables	Venezuela n=3 (42.9%)	Bolivia n=4 (57.1%)	P-Value*
Balsamic content (%)	4.33 (3.50-6.16)	19.33 (3.33-30.15)	0.400
Total phenolic content, (mgGAE/g)	6.93 (1.66-16.73)	7.25 (2.13-12.44)	1.000
S. aureus (ATCC 29213) MIC (mg/mL)	5.13 (5.13-10.25)	7.69 (5.13-20.50)	0.629
MRSA (NCTC 10442) MIC (mg/mL)	5.13 (5.13-10.25)	7.69 (5.13-20.50)	0.629
E. faecalis (ATCC 29212) MIC (mg/mL)	10.25 (5.13-20.50)	10.25 (5.13-20.50)	1.000
C. albicans (ATCC 10231) MIC (mg/mL)	5.13 (5.13-20.50)	10.25 (5.13-20.50)	0.629

**Mann-Whitney U test, Data are expressed as median (min-max)*
Since no antibacterial effect was detected on Gram-negative bacteria, it was not included in the analysis

pneumoniae, Pseudomonas aeruginosa, and *Acinetobacter baumannii),* Gram-positive bacteria *(Staphylococcus aureus*, Methicillin-resistant *Staphylococcus aureus* (MRSA), and *Enterococcus faecalis),* and the fungus *C. albicans*. The MIC values for the tested microorganisms ranged from 5.125 to >41.0 mg/mL. Detailed findings are provided in *Table 4*. The propolis sample obtained from the stingless bee *M. favosa* (sample 1) exhibited significant and broad-spectrum antimicrobial activity, primarily attributed to its high phenolic content. Remarkably, geopropolis extracts demonstrated increased sensitivity against Gram-positive bacteria and fungi when compared to Gram-negative bacteria. The strains *S. aureus* ATCC 29213 and MRSA NCTC 10442 proved to be the most susceptible microorganisms, with minimum inhibitory concentrations ranging from 5.125 to 20.5 mg/mL.

The Spearman correlation analysis revealed a statistically significant high degree of negative correlation between the total phenolic content and MIC values of *S. aureus*, MRSA, and *E. faecalis* strains (r=-0.837, P=0.019; r=-0.837, P=0.019; r=-0.850, P=0.015, respectively). Simultaneously, a statistically significant high degree of positive correlation was found between the MIC value of *E. faecalis* and the MIC values of *S. aureus* and MRSA strains (r=0.791, P=0.034; r=0.791, P=0.034, respectively). No statistically significant correlation was detected between balsamic content, total phenolic content, and MIC values of the strains. In the regional comparison conducted, no statistically significant difference was observed concerning balsamic content, total phenolic content, and MIC values *(Table 5)*.

Discussion

Propolis, also known as bee glue, is a sticky substance used by bees as a hygienic building material in their hives, derived from a mixture of insect secretions and plant resins. Numerous studies have demonstrated various pharmacological activities of propolis, including antimicrobial, anti-inflammatory, anticancer, antioxidant, hepatoprotective, cytotoxic, and immunomodulatory properties. The majority of these pharmacological activities are generally attributed to propolis produced by *A. mellifera*, the most widespread bee species worldwide [21,22].

Nevertheless, the chemical composition and pharmacological activities of geopropolis, produced by stingless bees, commonly referred to as meliponines, have been inadequately explored in existing research. Unlike honeybees, some stingless bees mix their propolis with clay or soil, resulting in a softer resinous material known as geopropolis. Despite the compositional differences, geopropolis exhibits similar effects to *A. mellifera* propolis [23].

The geographical features and bee species significantly influence the chemical and biological characteristics of geopropolis. Geopropolis contains fatty acids, organic acids, sugars, alcohols, steroids, as well as polyphenolic compounds, triterpenes, and saponins [3].

In this study, seven geopropolis samples from four bee species collected from Venezuela and Bolivia were examined for their balsamic, total phenolic, flavone-flavonol, flavone-dihydroflavonol contents, and antimicrobial effects.

In our study, the balsamic content of geopropolis collected from Venezuela was generally found to be low. The balsamic content of all geopropolis samples ranged from 3.33% to 30.15%. In the literature, there is no available data regarding the balsamic content of geopropolis. When comparing our results with *A. mellifera* propolis, it is observed that the geopropolis fall within the lower range of balsamic content seen in *A. mellifera* propolis but do not reach the higher levels. In a study on poplar-type propolis from different geographic regions, 114 samples were investigated, and the minimum value was reported as 18%, the maximum value as 82%, and the mean value as 57% [17]. In contrast, our mean value is only 11.6%. It is evident that the balsamic content of geopropolis is significantly lower than that of propolis caused by the admixture with clay, a material without balsamics.

The reason of this diferrences is caused probably from

the source of geopropolis. While the source of propolis is resins of plants, the source of geopropolis is both resin and soil. Owing to this feature, geopropolis can include resin in lower ratios compare to the propolis of *A. mellifera*. As a result of lower resin, it has lower balsamic content than propolis of *A. mellifera*.

The total phenolic content of geopropolis samples ranged from 1.66±0.00 to 16.73±0.00 mgGAE/g. At in previous studies, total phenolic content of geopropolis samples belong to the *M. fasciculata* geopropolis was found between126.60±0.84 and 631.26±4.22 mgGAE/g, geopropolis of *M. orbigny* between 211.0±7.5 and 23±1.0 QE/100 g, and geopropolis of *M. scutellaris* as 620.01±6.45 mgGAE/g [15,16,24].

Compared to the previous researches, our results for total phenolic contents are so lower. This can be sourced from bee species, climatic conditions, flora of the location geopropolis where plant resins were collected.

As a result of literature scanning search, we can say that researchers invetigated the total phenolic contents of geopropopolis collected by *M. fasciculata*, *M. orbigny*, *M. fasciolata*, *M. scutellaris* but there is no any data about the total phenolic content of *M. favosa*, *M. grandis*, *S. depilis*, *S. polysticta* geopropolis. In this case our results will be the first data for these stingless bees species.

Similar to the balsamic content, there is no any information available about flavone-flavonol and flavanones-dihydro-flavonols contents of geopropolis. The previous researches about geopropolis were mostly based on its total phenolic content. Geopropolis has a very rich chemical content, especially phenolic content affected the antioxidant capacity in a positive way.

Researches about biological activities of geopropolis evaluated that geopropolis has also so many bioactivities as propolis, due to its phytochemical content (benzoic acids, dihydrocinnamic acids, cinnamic acids prenylated coumaric acids, diterpenic acids, aliphatic acid and esters, alcohols, aromatic acids, hydrocarbons, terpenes triterpenic alcohols, and sugars). The presence of these chemical compounds shows differences between samples, and this variability is probably caused by different from bee species [7,25]. It was shown that *M. fasciculata* geopropolis, contains fatty acids, organic acids, sugars, alcohols, steroids gallic acid, elagic acid and hydrolyzable tannins and due to these compound it has antiviral, anticarcinogenic, anti-inflammatory and antioxidant properties [14,24,26]. The absence of detailed phytochemical analyses in our study, which would have provided a comprehensive demonstration of the components responsible for biological activity in geopropolis samples collected from two different regions, can be considered a limitation of the study.

Geopropolis, which differs structurally from propolis with no soil, shows physicochemical differences too. Compared to chemical content, it is known that geopropolis contains much lesser phenolic acid than propolis [27]. This explains that geopropolis has lower antioxidant and antimicrobial activity than propolis. There is a need for comprehensive research comparing the antioxidant and antimicrobial activities of propolis and geopropolis, as well as investigating their anticarcinogenic, anti-inflammatory, analgesic, antidepressant, anxiolytic, and immunomodulatory effects. Such studies would help identify the specific components responsible for these effects.

In our research, we evaluated the antimicrobial efficacy of ethanol extracts derived from seven geopropolis samples collected from four stingless bee species. The testing included Gram-negative and Gram-positive bacteria, along with reference strains of *C. albicans*. Specifically, it was noted that geopropolis extracts displayed increased sensitivity against Gram-positive bacteria and fungi compared to Gram-negative bacteria. The antibacterial effect of geopropolis samples on Gram-positive bacteria was found to be dependent on phenolic contents. However, it was determined that there was no positive correlation between antibacterial effect, balsamic content, and the geographical region and bee species from which they were collected.

Previous studies have consistently noted the resistance of Gram-negative bacteria to ethanol extracts of propolis compared to Gram-positive bacteria, attributed to differences in their cell wall structures [21,22,28-32]. The antimicrobial activity studies have demonstrated that the antibacterial effect of propolis types on Gram-positive bacteria is strongly linked to the content of propolis, and it can vary widely with a broad range of MIC values (between 6 and 20000 µg/mL) [29,33]. However, the MIC values we determined for the geopropolis extracts tested in our study may be found to be relatively high when compared with other research on the antimicrobial activity of geopropolis. This inconsistency could be attributed to the relatively low phenolic content present in our geopropolis samples, which plays a significant role in antimicrobial activity.

In our prior study investigating the antimicrobial activity of pollen samples, we observed that pollen extracts exhibited no activity against the *C. albicans* ATCC 10231 strain [34]. Interestingly, conversely, geopropolis extracts exhibited the capacity to hinder the growth of *C. albicans*, with MIC values ranging from 5.125 to 20.5 mg/mL. Previous research has indicated that geopropolis extracts possess antifungal activity at MIC values ranging from 1 to 3.4 mg/mL [22,29,32,35-38]. However, a more comprehensive phytochemical analysis is warranted to identify the active

metabolites responsible for the antifungal effects of geopropolis.

Completely natural and non-toxic propolis is not only used in traditional and complementary medicine but is also widely utilized in various applications such as food, functional foods, pharmaceuticals, livestock, cosmetics, and particularly in oral health practices in dentistry, thanks to its effective antibacterial properties [22,39,40]. However, the low water solubility of propolis limits its use in several other areas. It has been shown that propolis has the capacity to enhance the effectiveness of antibacterial agents, exhibiting a synergistic effect with antibiotics, allowing for high antimicrobial efficacy at low doses [41].

Based on these findings, we believe that standardized geopropolis samples with high phenolic content could serve as a natural complement to antibiotics in the prevention and/or treatment of infections caused by Gram-positive bacteria and *C. albicans*. Additionally, the statistically significant positive correlation found among the MIC values of Gram-positive bacterial standard strains in our study suggests that geopropolis samples could be used in the treatment of mixed infections involving Gram-positive bacteria. Nevertheless, additional research is required to investigate the clinical applications of geopropolis in various infections. In particular, evaluating clinical strains with different sensitivity patterns in vitro alongside standard strains in studies would provide more accurate results. Additionally, there is a need for comprehensive geopropolis and antibiotic synergy studies to ensure the control of resistant pathogenic bacterial species, such as MRSA.

Unfortunately, despite the natural, non-toxic, and highly effective nature of this product that God has bestowed upon humans, there are some challenges in its clinical use. Similar to propolis produced by *A. mellifera*, the inability to establish a standard for the chemical characterization of geopropolis due to its different chemical compositions based on geographical regions and botanical sources, and the lack of standardization in the extraction procedure, can be emphasized as significant challenges in clinical applications. Overcoming these challenges in the clinical use of this product requires advanced laboratory analyses, comprehensive pharmacodynamic and pharmacokinetic studies, laboratory animal research, and clinical trials.

Declarations

Availability of Data and Materials: Datasets analyzed during the current study are available with the corresponding author (Ö. Kuru) on reasonable request.

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

Author Contributions: Methodology and collection of sample, PV, ÖGÇ; propolis extraction and phenolic analysis, ÖGÇ and NM; microbiological analysis, ÖK and OB; writing - review and editing ÖGÇ, ÖK, and PV. All authors have read and agreed to the published version of the manuscript.

References

1. Franchin M, Saliba ASMC, Giovanini de Oliveira Sartori A, Orestes Pereira Neto S, Benso B, Ikegaki M, Wang K, Matias de Alencar S, Granato D: Food-grade delivery systems of Brazilian propolis from *Apis mellifera:* From chemical composition to bioactivities *in vivo. Food Chem,* 432:137175, 2024. DOI: 10.1016/j.foodchem.2023.137175

2. Salatino A, Salatino MLF, Negri G: How diverse is the chemistry and plant origin of Brazilian propolis? *Apidologie,* 52 (6): 1075-1097, 2021. DOI: 10.1007/s13592-021-00889-z

3. Ferreira BL, Gonzaga LV, Vitali L, Micke GA, Maltez HF, Ressureição C, Costa ACO, Fett R: Southern-Brazilian geopropolis: A potential source of polyphenolic compounds and assessment of mineral composition. *Food Res Int,* 126:108683, 2019. DOI: 10.1016/j.foodres.2019.108683

4. Zullkiflee N, Taha H, Usman A: Propolis: Its role and efficacy in human health and diseases. *Molecules,* 27 (18):6120, 2022. DOI: 10.3390/molecules27186120

5. Adler M, Escóbar-Márquez L, Solis-Soto MT, Pinto CF: Stingless bees: Uses and management by meliponiculturist women in the Chaco region of Bolivia. *J Ethnobiol Ethnomed,* 19 (1):5, 2023. DOI: 10.1186/s13002-022-00574-0

6. Engel MS, Rasmussen C, Ayala R, de Oliveira FF: Stingless bee classification and biology (Hymenoptera, Apidae): A review, with an updated key to genera and subgenera. *ZooKeys,* 1172, 239-312, 2023. DOI: 10.3897/zookeys.1172.104944

7. Nogueira DS: Overview of stingless bees in Brazil (Hymenoptera: Apidae: Meliponini). *Entomo Brasilis,* 16, e1041:1-13, 2023. DOI: 10.12741/ebrasilis.v16.e1041

8. Vit P: *Melipona favosa* Pot-Honey from Venezuela. **In,** Vit P, Pedro S, Roubik D (Eds): Pot-Honey. 2nd ed., 360-373, Springer, New York, NY. 2013.

9. Can-Alonzo C, Quezada-Euán JJG, Xiu-Ancona P, Moo-Valle H, Valdovinos-Nunez GR, Medina-Peralta S: Pollination of 'criollo'avocados *(Persea americana)* and the behaviour of associated bees in subtropical Mexico. *J Apic Res,* 44 (1): 3-8, 2005. DOI: 10.1080/00218839.2005.11101138

10. Heard TA: The role of stingless bees in crop pollination. *Annual Rev Entomol,* 44 (1): 183-206, 1999. DOI: 10.1146/annurev.ento.44.1.183

11. Felix JA, Freitas BM: Richness and distribution of the meliponine fauna (Hymenoptera: Apidae: Meliponini) in the State of Ceará, Brazil. *An Acad Bras Ciênc,* 93 (3):2021. DOI: 10.1590/0001-3765202120190767

12. Klein AM, Steffan-Dewenter I, Tscharntke T: Fruit set of highland coffee increases with the diversity of pollinating bees. *Proc Biol Sci,* 270 (1518): 955-961, 2003. DOI: 10.1098/rspb.2002.2306

13. Carvalho RM, Martins CF, Mourão Jda S: Meliponiculture in Quilombola communities of Ipiranga and Gurugi, Paraíba state, Brazil: An ethnoecological approach. *J Ethnobiol Ethnomed,* 10:32014, 2014. DOI: 10.1186/1746-4269-10-3

14. Dutra RP, Abreu BV, Cunha MS, Batista MC, Torres LM, Nascimento FR, Ribeiro MN, Guerra RN: Phenolic acids, hydrolyzable tannins, and antioxidant activity of geopropolis from the stingless bee *Melipona*

fasciculata Smith. *J Agric Food Chem,* 62 (12): 2549-2557, 2014. DOI: 10.1021/jf404875v

15. Araujo KSS, Santos JJF, Sato MO, Finco FDBA, Soares IM, Barbosa RdS, Alvim TC, Ascencio SD, Mariano SMB: Physicochemical properties and antioxidant capacity of propolis of stingless bees (Meliponinae) and Apisfrom two regions of Tocantins, Brazil. *Acta Amazonica,* 46 (1): 61-68, 2016. DOI: 10.1590/1809-4392201501045

16. Campos JF, dos Santos UP, Macorini LF, de Melo AM, Balestieri JB, Paredes-Gamero EJ, Cardoso CA, de Picoli Souza K, dos Santos EL: Antimicrobial, antioxidant and cytotoxic activities of propolis from *Melipona orbignyi* (Hymenoptera, Apidae). *Food Chem Toxicol,* 65, 374-380, 2014. DOI: 10.1016/j.fct.2014.01.008

17. Popova MP, Bankova VS, Bogdanov S, Tsvetkova I, Naydenski C, Marcazzan GL, Sabatini AG: Chemical characteristics of poplar type propolis of different geographic origin. *Apidologie,* 38 (3): 306-311, 2007. DOI: 10.1051/apido:2007013

18. Slinkard, K, Singleton VL: Total phenol analysis: Automation and comparison with manual methods. *Am J Enol Vitic,* 28 (1): 49-55, 1977. DOI: 10.5344/ajev.1977.28.1.49

19. Trusheva B, Trunkova D, Bankova V: Different extraction methods of biologically active components from propolis: A preliminary study. *Chem Cent J,* 1 (1):13, 2007. DOI: 10.1186/1752-153X-1-13

20. Moussa SH, Tayel AA, Al-Hassan AA, Farouk A: Tetrazolium/formazan test as an efficient method to determine fungal chitosan antimicrobial activity. *J Mycol,* 2013:753692, 2013. DOI: 10.1155/2013/753692

21. Zulhendri F, Lesmana R, Tandean S, Christoper A, Chandrasekaran K, Irsyam I, Suwantika AA, Abdulah R, Wathoni N: Recent update on the anti-inflammatory activities of propolis. *Molecules,* 27 (23):8473, 2022. DOI: 10.3390/molecules27238473

22. Zulhendri F, Chandrasekaran K, Kowacz M, Ravalia M, Kripal K, Fearnley J, Perera CO: Antiviral, antibacterial, antifungal, and antiparasitic properties of propolis: A review. *Foods,* 10 (6):1360, 2021. DOI: 10.3390/foods10061360

23. Lavinas FC, Macedo EHBC, Gabriel BLSá, Amaral ACF, Silva JRA, Azevedo MMB, Vieira BA, Domingos TFS, Vermelho AB, Carneiro CS, Rodrigues IA: Brazilian stingless bee propolis and geopropolis: Promising sources ofbiologically active compounds. *Rev Bras Farmacog,* 29 (3): 389-399, 2018. DOI: 10.1016/j.bjp.2018.11.007

24. Batista MCA, Abreu BVB, Dutra RP, Cunha MS, Amaral FMM, Torres LMB, Ribeiro MNS: Chemical composition and antioxidant activity of geopropolis produced by *Melipona fasciculata* (Meliponinae) in flooded fields and cerrado areas of Maranhão State, northeastern Brazil. *Acta Amazonica,* 46 (3): 315-322, 2016. DOI: 10.1590/1809-4392201600034

25. Celemli OG: Chemical properties of propolis collected by stingless bees. **In,** Vit P, Pedro S, Roubik D (Eds): Pot-Honey. 525-537, Springer, NY, 2013.

26. Melekoğlu A, Ceviz AB, Eronat AP, Öztürk T, Pehlevan F, Yilmaz-Aydoğan H, Öztürk O: Synergistic and dose-dependent effects of pinostrobin, pinocembrin and pinobanksin on different breast cancer cell lines. *Kafkas Univ Vet Fak Derg,* 30 (1): 23-30, 2024. DOI: 10.9775/kvfd.2023.30187

27. Barth OM, de Freitas AS: Palynology as a tool to distinguish between propolis and geopropolis: Southern Brazilian samples. *Open Access Libr J*, 2 (12):1, 2015. DOI: 10.4236/oalib.1102217

28. Torres AR, Sandjo LP, Friedemann MT, Tomazzoli MM, Maraschin M, Mello CF, Santos ARS: Chemical characterization, antioxidant andantimicrobial activity of propolis obtained from *Melipona quadrifasciata* quadri-fasciata and *Tetragonisca angustula* stingless bees. *Braz J Med Biol Res,* 51, 1-10, 2018. DOI: 10.1590/1414-431x20187118

29. Przybyłek I, Karpiński TM: Antibacterial properties of propolis. *Molecules,* 24 (11):2047, 2019. DOI: 10.3390/molecules24112047

30. Ozen T, Kilic A, Bedir O, Koru O, Sorkun K, Tanyuksel M, Kilic S, Gencay O, Yildiz O, Baysallar M: *In vitro* activity of Turkish propolis samples against anaerobic bacteria causing oral cavity infections. *Kafkas Univ Vet Fak Derg,* 16 (2): 293-298, 2010. DOI: 10.9775/kvfd.2009.707

31. Burgut A: Effects of propolis extracts on biogenic amine production by food-borne pathogens. *Kafkas Univ Vet Fak Derg,* 26 (2): 193-200, 2020. DOI: 10.9775/kvfd.2019.22718

32. Campos JF, dos Santos UP, da Rocha PS, Damião MJ, Balestieri JBP, Car-doso CAL, Paredes-Gamero EJ, Estevinho LM, Souza KP, Santos EL: Antimicrobial, antioxidant, anti-inflammatory, and cytotoxic activities of propolis from the stingless bee *Tetragonisca fiebrigi* (Jataí). *Evid Based Complement Alternat Med,* 2015:296186, 2015. DOI: 10.1155/2015/296186

33. Salatino A: Perspectives for uses of propolis in therapy against ınfectious diseases: A review. *Molecules,* 27 (14):4594, 2022. DOI: 10.3390/molecules27144594

34. Ozkok A, Koru O, Bedir O, Cetinkaya S, Gencay Celemli O, Ozenirler C, Mayda N, Sorkun K: Total bioactive compounds and antimicrobial capacities of bee pollen with different botanical origins. *Bull Univ Agric Sci Vet Med Cluj-Napoca, Food Sci Technol,* 78 (1):57, 2021. DOI: 10.15835/buasvmcn-fst:2020.0061

35. Araújo MJ, Bosco SM, Sforcin JM: *Pythium insidiosum:* Inhibitory effectsof propolis and geopropolis on hyphal growth. *Braz J Microbiol,* 47, 863-869, 2016. DOI: 10.1016/j.bjm.2016.06.008

36. Souza ECA, da Silva EJG, Cordeiro HKC, Filho NML, da Silva FMA, dos Reis DLS, Porto C, Pilau EJ, da Costa LAMA, de Souza ADL, Menezes C, Flachauim A: Chemical compositions and antioxidant and antimicrobial activities of propolis produced by *Frieseomelitta longipes* and *Apis mellifera* bees. *Quim Nova,* 41, 485-491, 2018. DOI: 10.21577/0100-4042.20170208

37. da Cunha MG, de Cássia Orlandi Sardi J, Freires IA, Franchin M, Rosalen PL: Antimicrobial, anti-adherence and antibiofilm activity against *Staphylococcus aureus* of a 4-phenyl coumarin derivative isolated from Brazilian geopropolis. *Microb Pathog,* 139:103855, 2020. DOI: 10.1016/j.micpath.2019.103855

38. Sa-Eed A, Donkor ES, Arhin RE, Tetteh-Quarcoo PB, Attah SK, Kabotso DEK, Kotey FCN, Dayie NTKD: *In vitro* antimicrobial activity of crude propolis extracts and fractions. *FEMS Microbes,* 20:4, 2023. DOI: 10.1093/femsmc/xtad010

39. El-Guendouz S, Lyoussi B, Miguel MG: Insight on propolis from Mediterranean countries: Chemical composition, biological activities and application fields. *Chem Biodivers,* 16:e1900094, 2019. DOI: 10.1002/cbdv.201900094

40. da Silva Barboza A, Aitken-Saavedra JP, Ferreira ML, Fábio Aranha AM, Lund RG: Are propolis extracts potential pharmacological agents in human oral health? - A scoping review and technology prospecting. *J Ethnopharmacol,* 271:113846, 2021. DOI: 10.1016/j.jep.2021.113846

41. Almuhayawi MS: Propolis as a novel antibacterial agent: A review. *Saudi J Biol Sci,* 27 (11): 3079-3086, 2020. DOI: 10.1016/j.sjbs.2020.09.016

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Research Article

Complete Mitochondrial Genome Analyses of *Forcipomyia pulchrithorax* (Diptera: Ceratopogonidae): Genome Orientation and Phylogenetic Implications

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Abstract

Forcipomyia Meigen, 1818, is the second-largest genus of the family Ceratopogonidae, having medico veterinary and agricultural importance. A few mitogenomes of Ceratopogonidae have previously been available. Here, we aimed to characterize the whole mitogenome of *Forcipomyia pulchrithorax* Edwards, 1924, collected from Central Anatolia Region of the Türkiye using NGS to contribute to the genetic diversity within this family. The mitogenome of *F. pulchrithorax* (OR666457) consisted of a circular DNA molecule spanning 15,930 bp and containing 72.7% AT content. It includes 13 protein-coding genes (PCGs), 22 transfer RNA (tRNA) genes, 2 ribosomal RNA (rRNA) genes, and 1 control region. All PCGs exhibited typical ATN start codons and followed the conventional TAN stop codons, except COX1, COX2, COX3, ND1, and ND5, which ended with incomplete codons. The 22 tRNA genes demonstrated the ability to form a cloverleaf structure, except tRNA-Ser1, which lacked DHU arm, similar to most insect mitogenomes. The ML phylogenetic analyses clearly delineated the species of Culicomorpha into well supported monophyletic clades. *Forcipomyia pulchrithorax* was clustered with the Ceratopogonidae species constituting an outer taxon among the Culicomorpha. The comprehensive mitogenome of *F. pulchrithorax* will be valuable for future studies focused on the phylogenetic characterization and diversity of Ceratopogonidae within the Culicomorpha.

Keywords: Culicomorpha, Diptera, *Forcipomyia pulchrithorax*, Mitochondrial genome, Phylogeny

Introduction

The genus *Forcipomyia* Meigen, 1818 of Forcipomyiinae (Diptera: Ceratopogonidae) comprises medium-sized midges. The members of this subfamily have a considerable economic impact due to their role in pollinating cocoa and rubber trees [1].

Biting midges belonging to this genus have a worldwide distribution, inhabiting diverse environments such as paddy fields, bamboo forests, and marshlands. *Forcipomyia* is one of the most species-rich genera of the family Ceratopogonidae, with 1.186 described species in 36 subgenera [2]. Other insect orders (e.g., Phasmida, Orthoptera, Lepidoptera, Heteroptera, Odonata) often serve as hosts for adult female ectoparasites [3,4]. Along with the genera *Culicoides* and *Leptoconops* of the Ceratopogonidae, the females of the subgenus *Forcipomyia* also feed on vertebrate blood [3,5].

In insects, the mitochondrial genome is small, typically ranging from 15 to 20 kb, and encompasses 37 genes. These genes comprise 13 protein-coding genes (PCGs), 22 transfer RNA genes (tRNAs), and the large and small ribosomal RNA unit genes (rrnL and rrnS, respectively). Besides, the mitochondrial genome has a single, large non-coding region that contains controlling elements for replication and transcription, along with various spacer and overlapping regions [6-9]. Unlike the nuclear genome, mitogenomes are maternally inherited, occur in hundreds to thousands of copies per cell, and exhibit

minimal recombination in animals [10-12]. By comparing the mitochondrial gene arrangements of animals, scientists can infer long-term evolutionary relationships since rearrangements are unique and generally rare events that are unlikely to occur independently in separate evolutionary lineages [6].

Modern molecular tools have been successfully used to evaluate Ceratopogonidae's including the genus *Forcipomyia* through genetic markers such as partial or complete mitochondrial sequences [13-15]. Although Ceratopogonidae is one of the largest groups of Diptera insects, only a few complete mitogenomes have been characterized or published so far [14-17]. In the *Forcipomyia* genus, *F. makanensis*, which has a wide distribution in certain regions of China, is the only species with a published mitogenome characterization [10].

Considering the lack of mitogenome-based genomic information on the genus *Forcipomyia* of Ceratopogonidae, which has potential epidemiological interest and economic impact in many regions of the world, in this study, we performed for the first time the characterization of the mitochondrial genome of the species *F. pulchrithorax* Edwards, 1924, collected from the Central Anatolia Region of Türkiye. We reconstructed the phylogenetic relationship of the species within the families of Culicomorpha based on the concatenation of all 13 PCGs and two rRNA genes.

Material and Methods

Ethical Statement

This study does not require the approval of the Local Ethics Committee for Animal Experiments.

Collection of Specimens and DNA Extraction

Adult midges were sampled in August 2022 from a location in Sivas province of the Central Anatolia Region of Türkiye (Coordinates: 39.327151, 37.391583). The midges were captured using Onderstepoort-type 220 V blacklight traps with 8 W UV light tubes and stored in absolute ethanol for identification. Initial identification of the midges relied on their morphological characteristics using appropriate keys [18,19]. After morphological identification, the midges were preserved in absolute ethanol at -20°C. Genomic DNA (gDNA) was extracted from the whole body of the midges using a PureLink™ Genomic DNA Mini Kit (Thermo Scientific, Waltham, MA, USA) following the manufacturer's protocol. The obtained gDNA was quantified using a Qubit 3.0 Fluorometer (Thermo Scientific, Waltham, MA, USA) and stored at −20°C.

Long-range PCR Amplification of Mitogenome and Sequencing

To amplify the complete mitogenome of the specimens in two overlapping fragments, we first downloaded all full or partial mitochondrial sequences of Culicomorpha species available in GenBank and conserved regions were determined by multiple alignment of all sequences. Two sets of long-range PCR primers were designed from the corresponding gene regions using Primer3 v.2.3.7. [20], which was integrated with Geneious Prime software *(https://www.geneious.com)* (*Table 1*).

Long-range PCR was performed using LongAmp® Taq 2x Master Mix (New England Biolabs, Ipswich, MA, USA) under the following cycling parameters: 1 min at 94°C; 35 cycles of 30 s at 94°C, 30 s at 50°C (First fragment)/53°C (Second fragment), and 17 min (First fragment)/7 min (Second fragment) at 60.5°C; and 60.5°C for 10 min. Each amplification products (5 µL) electrophoretically resolved, purified, and quantified as described by Ciloglu et al.[21].

Preparation of Libraries, NGS, Annotation, and Analysis of Mitochondrial DNA

We diluted the PCR products with ddH_2O to 0.2 ng/µL of DNA concentration. Two diluted PCR products were pooled together in equimolar ratios. We used the Nextera XT DNA Library Prep Kit (Illumina, San Diego, USA) and the Nextera XT DNA Library Preparation Index Kit v2 Set A (Illumina, San Diego, USA) for the construction of the libraries following the protocols of the manufacturer. The amplicons of each library were quantified and diluted to approximately equal concentrations and were pooled. The pooled multiplexed libraries were sequenced on an Illumina MiSeq platform (Illumina, San Diego, USA) with the MiSeq Reagent Kit v2 (500 cycles).

FastQC [22] was used to assess the quality of the raw sequence data. We filtered out sequences shorter than 50

Table 1. *Primers used for the amplification of the mitogenome of F. pulchrithorax*

Organism	Primer set	Primer Name	Primer Sequence	Target Gene	TM Degree (°C)	Extension Time (min)	Size (bp)
Forcipomyia pulchrithorax	1	MidgeF1	TTATAATTGGRGGATTYGGWAATTG	COI	50	17	13.000
		MidgeF2	TTAAGTTACTTTAGGGATAACAGC	16SrRNA			
	2	MidgeF3	ATTACGCTGTTATCCCTAAAGTAAC	COI	53	7	5.165
		MidgeF4	GTTCAKCCRGTTCCDGCYCCAT	16SrRNA			

TM: melting temperature

bp, trimmed low-quality bases (Q-score <25) at the end of reads, and removed adaptors using the BBDuk plug-in of Geneious Prime to The paired-end clean reads (~400.000 reads) of *F. pulcrithorax* were assembled in Geneious Prime using the mitogenome of *F. makanensis* from GenBank (Accession number: MK000395) as a reference sequence [15]. The tool embedded in Geneious Prime, Map to Reference, was utilized with the Highest Sensitivity/Medium Geneious Mapper Algorithm and up to 25 iterations. To ensure coverage and completeness, we performed *de novo* assembly using GetOrganelle v1.7.7.0 [23]. Finally, the aliging of *de novo* contigs with those generated by mapping to the reference sequence of *F. makanensis* confirmed that the assembly method did not influence the final mitogenome sequences.

The mitochondrial genome of *F. pulcrithorax* was annotated using MITOS2 [24] and the invertebrate mitochondrial genetic code. tRNA genes were identified using tRNAscan-SE [25] and MITOS2. Gene borders were manually curated and checked using MITOS2. The tRNA visualization was performed with VARNA [26]. PCGs and rRNA genes were determined based on alignments with available Culicomorpha mitogenomes in GenBank. The graphical mitogenome circular map was drawn using Proksee Server [27]. RSCU, nucleotide, and translation statistics were calculated using Geneious Prime and MEGA 11 programs [28].

Phylogenetic Analyses

A total of 18 Culicomorpha species representing seven families and 15 genera were retrieved from GenBank to reconstruct the phylogenetic relationships of the *F. pulcrithorax* (*Table 2*). *Phlebotomus chinensis* was used as an outgroup taxon. The phylogeny data was based on the concatenated sequence alignments of nucleotide sequences of 13 protein-coding genes and two rRNA genes. Initially, the mitogenome sequences in the data set were aligned using MAFFT [34] in Geneious Prime. Poorly aligned sites were subsequently removed using the Gblocks server [35] with stringent criteria. The phylogenetic tree was constructed using the maximum likelihood (ML) method in MEGA 11 with the optimal nucleotide substitution model (GTR +I +G with 1000 replicates), determined by the jModel test v.0.1.1 [36].

Results

Mitogenome Organization

The complete mitogenome of *F. pulcrithorax* is 15.930 bp in length, including 13 PCGs, 22 tRNAs, and two rRNAs (rrnL and rrnS) (*Fig. 1*). The heavy strand (H-strand) encodes most of the genes (nine PCGs and 14 tRNAs) while, the light strand (L-strand) contains the remaining reverse complementary genes (four PCGs, eight tRNAs, and two rRNAs) as shown in *Table 3*. The AT content of the *F. pulcrithorax* mitogenome is 72.7% (*Table 3*).

Table 2. *Summary of taxonomic groups used in this study*

Family	Genus	Species	Whole Genome	Genbank Accession	References
Ceratopogonidae	*Forcipomyia*	*Forcipomyia pulchrithorax*	15.590	OR666457	This study
		Forcipomyia sp.	15.584	MK000395	Jiang [15]
	Culicoides	*Culicoides arakawae*	15.412	AB361004	Matsumoto et al.[14]
Dixidae	*Dixella*	*Dixella aestivalis*	16.465	KT878382	Briscoe et al.[29]
		Dixella sp.	15.574	KM245574	Kang et al.[30]
Culicidae	*Culex*	*Culex pipiens molestus*	16.323	MN389460	Unpublished
	Aedes	*Aedes koreicus*	15.843	MZ460582	Unpublished
	Anopheles	*Anopheles cruzii*	15.877	KJ701506	Unpublished
	Ochlerotatus	*Ochlerotatus vigilax*	16.445	KP721463	Hardy et al.[31]
Corethrellidae	*Corethrella*	*Corethrella condita*	14.520	MK281357	Zhang et al.[17]
Chaoboridae	*Chaoborus*	*Chaoborus sp.*	14.746	MK281356	Zhang et al.[17]
Simuliidae	*Simulium*	*Simulium variegatum*	15.575	KU252587	Unpublished
		Simulium noelleri	16.190	MT410847	Unpublished
Chironomidae	*Polypedilum*	*Polypedilum nubifer*	15896	MZ747090	Xiao et al.[32]
	Chironomus	*Chironomus flaviplumus*	15.739	MW770891	Unpublished
	Limnophyes	*Limnophyes minimus*	15.607	MZ041033	Unpublished
	Microchironomus	*Microchironomus tabarui*	15.667	MZ261913	Kong et al.[33]
Thaumaleidae	*Thaumalea*	*Thaumalea sp.*	14.610	MK281359	Zhang et al.[17]

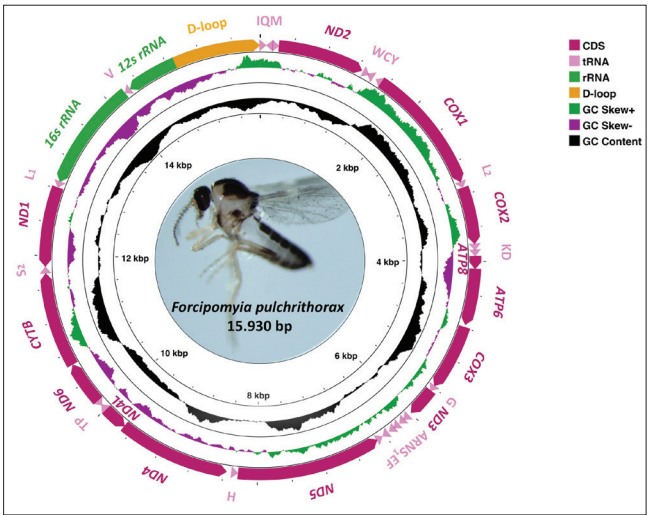

Fig 1. Circular mitogenome map of *F. pulcrithorax*. The internal values indicate the overall content of the nucleotide bases. The magenta, pink, and green blocks indicate PCGs, tRNAs, and rRNAs, respectively. Each tRNA is identified by a unique letter abbreviation. The genes located in the H (Forward) and L (Reverse) strands are shown in opposite directions

Protein-coding Genes (PCGs) and Codon Usage

The total length of 13 PCGs is 11.175 bp, accounting for 70.15% of the whole mitogenome length of *F. pulcrithorax*. All 13 PCGs of *F. pulcrithorax* initiate translation using ATN codons (ATG for *ND2*, *ND3*, *ND4*, *COX2*, *COX3*, *ATP6*, *ATP8*, and *CYTB*; ATA for *ND4L*; ATT for *COX1*, *ND1*, *ND5*, and *ND6*). For the stop codon, seven PCGs of *F. pulcrithorax* had the common mitochondrial stop codon TAA, while *ND4* was terminated with the stop codon TAG, *COX3* ended with "TA-", and *COX1*, *COX2*, *ND1*, and *ND5* ended with a single "T" *(Table 3)*.

The *F. pulcrithorax* mitogenome encodes 3.732 amino acids, excluding incomplete stop codons. This reflects the high A+T content observed throughout the genome, particularly in codon usage. In the *F. pulcrithorax* mitogenome *(Fig. 2)*, NNA and NNU codons (78.05%) are significantly more prevalent than NNC and NNG codons (21.95%). Among the 13 PCGs in *F. pulcrithorax*, Leu1 (403) is the most frequently used amino acid codon, followed by Thr (313). Cys (25) is the least frequently used amino acid codon *(Fig. 2)*.

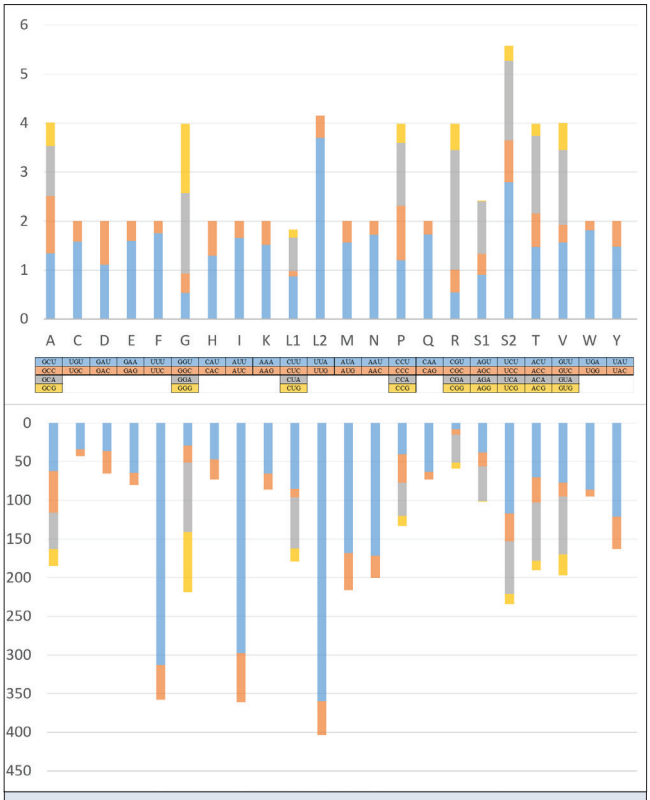

Fig 2. RSCU and codon usage of the *F. pulcrithorax* mitogenome. The graphics indicate (A): RSCU, (B): Codon usage counts. Amino acids were shown based on the IUPACIUB one-letter abbreviation. Codon families were given on the X-axis. The stop codons were not included

Transfer RNAs

The mitogenome of *F. pulcrithorax* contains all 22 tRNAs *(Fig. 3)*. The tRNAs exhibit a size range of 61 to 72 bp, with 13 tRNA genes located on the H-strand and the remaining nine tRNA genes on the L-strand *(Table 4)*. Most tRNA genes display classical clover-leaf secondary structures, except for trnS1(AGN), which possesses a simplified DHU arm *(Fig.3)*.

Phylogenetic Analyses

The phylogenetic analyses of the mitogenome concatenated PCGs and ribosomal genes separated the species in each family with an overall 36% mean genetic distance (10.6% to 53.4%) *(Table 5)*. The ML tree indicated the monophyly of all families in Culicomorpha with high bootstrap supports

Table 3. *Overall nucleotide composition in the mitogenome of F. pulcrithorax*

Forcipomyia pulchrithorax	**Size**	**%**	**%A**	**%T**	**%G**	**%C**	**%A+T**	**%G+C**
Total Length	15.930	100%	37.50%	35.20%	10.40%	16.90%	72.70%	27.30%
PCGs	11.175	70.32%	36.40%	33.70%	11.60%	18.30%	71.10%	29.90%
12s rRNA	614	3.85%	37.00%	38.90%	7.80%	16.30%	75.90%	24.10%
16s rRNA	1.320	8,28%	41.70%	41.00%	5.30%	12.00%	82.70%	17.30%
tRNAs	1.450	9.07%	37.70%	37.10%	10.90%	14.30%	74.80%	25.20%
Control Region (D-loop)	1.037	6.50%	44.10%	37.90%	6.30%	11.80%	82.00%	18.00%

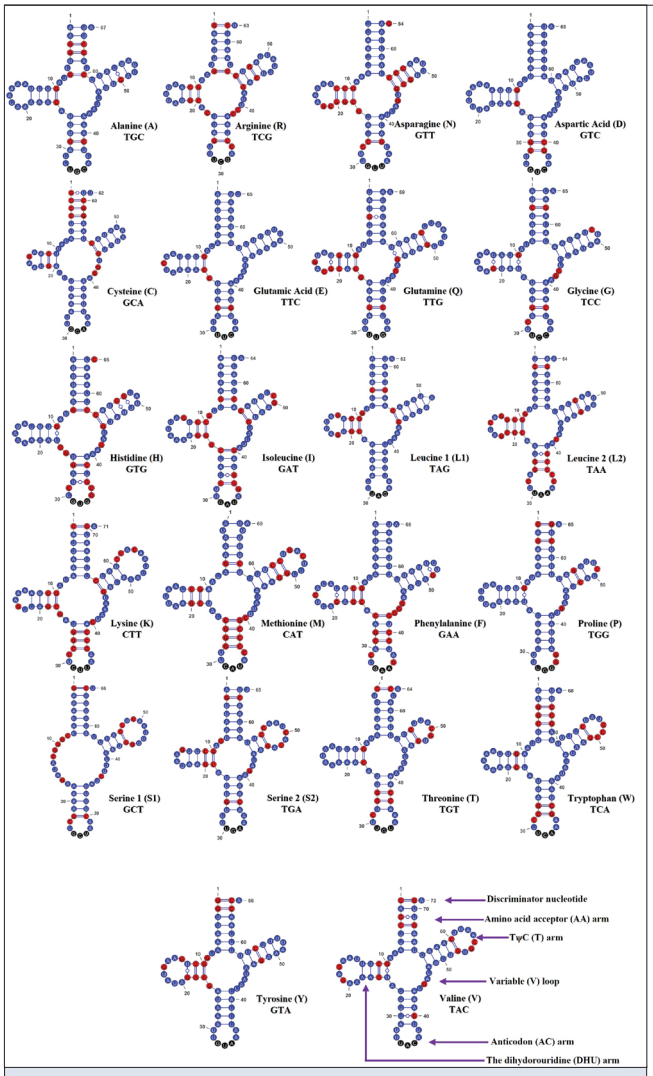

Fig 3. Predicted secondary cloverleaf structure for the tRNAs of *F. pulcrithorax*

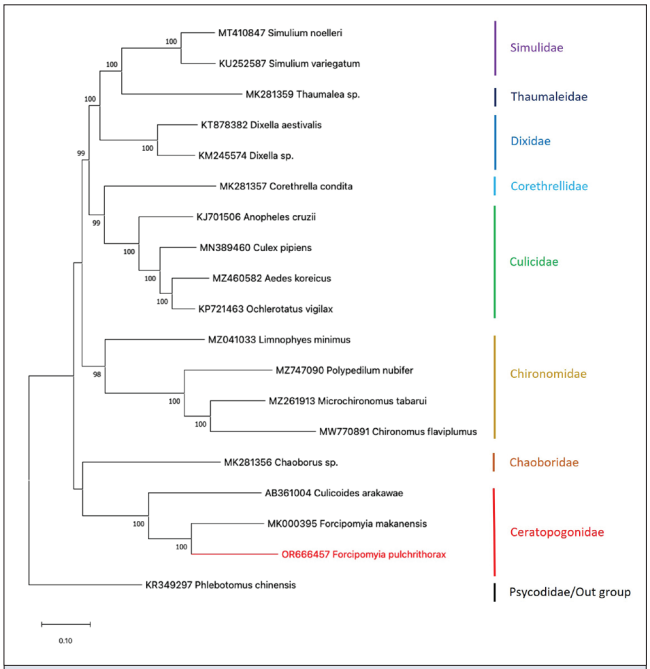

Fig 4. The reconstructed ML tree of mitogenome sequences of *F. pulcrithorax* (in red character), along with the representatives of other genera in the families of Culicomorpha is available in GenBank. The ML tree is based on the concatenated sequence alignments of nucleotide sequences of 13 protein-coding genes and two rRNA genes using GTR+I+G nucleotide substitution model. The bootstrap values over 70.0% are indicated at each node. *Phlebotomus chinensis* was employed as an outgroup taxa

(98.0% to 100.0%) *(Fig. 4). Forcipomyia pulcrithorax* was clustered with *F. makanensis*, and this clade was placed as a sister to *C. arakawae* with high bootstrap values (100.0%). In our analyses, Ceratopogonidae, including the newly characterized *F. pulcrithorax* constituted an outer taxon among the Culicomorpha. Chironomidae was recognized as a sister taxon to Culicidae + Dixidae + (Thaumaleidae + Simuliidae).

Discussion

The nucleotide composition of *F. pulcrithorax* is AT-biased, similar to the mitogenomes of several Culicomorpha species [15-17,29,30]. The AT content of *F. pulcrithorax* mitogenome was found to be 72.7% *(Table 3)*. The high AT content is widely attributed to the evolution of mitochondrial origin [37].

The whole mitogenome length of *F. pulcrithorax* is similar to *F. makanensis* [15] and relatively lower compared to *C. arakawae* [14] within the same family, Ceratopogonidae. Jiang et al.[15] identified all PCGs with the standard stop codon pattern "TAA," except *COX3*, which ended with an incomplete termination codon "TA-" in the mitogenome of *F. makanensis*. The COX3 gene of the *F. pulcrithorax* was also ended with incomplete stop codon "TA-" similar to *F. makanensis*. However further incomplete termination codon "T--" was also identified in the *COX1*, *COX2*, *ND1*, and *ND5* genes of *F. pulcrithorax*. Incomplete stop codons are also present in the PCGs of Culicomorpha species listed in *Table 3* and other common insect mitogenomes [14,15,17,29,33]. They are considered to produce functional stop codons in polyadenylation mechanisms and polycistronic transcription cleavage [38,39].

High A+T content was identified throughout the *F. pulcrithorax* mitogenome, particularly in PCGs. The relative synonymous codon usage (RSCU) matrix indicated the dominancy of NNA and NNU compared with the codons NNC and NNG. This type of RSCU is also frequently observed in other metazoan mitochondrial genomes [40-42].

The mitogenome of *F. pulcrithorax* contains all 22 tRNAs characteristic of dipteran mitogenomes. Most tRNA genes display classical clover-leaf secondary structures except for trnS1(AGN). This peculiarity of tRNASer is prevalent among insect and metazoan mitogenomes [8,43].

Table 4. *The mitogenome organization of F. pulcrithorax*

Gene	Direction	Minimum	Maximum	Size	Anticodon	Start Codon	Stop Codon	Intergenic Nucleotides
tRNA-Ile	H	1	64	64	GAT	-	-	13
tRNA-Gln	L	78	146	69	TTG	-	-	5
tRNA-Met	H	152	220	69	CAT	-	-	0
ND2 CDS	H	221	1252	1032	-	ATG	TAA	20
tRNA-Trp	H	1273	1340	68	TCA	-	-	-8
tRNA-Cys	L	1333	1394	62	GCA	-	-	53
tRNA-Tyr	L	1448	1513	66	GTA	-	-	34
COX1 CDS	H	1548	3045	1498	-	ATT	T--	0
tRNA-Leu	H	3046	3109	64	TAA	-	-	4
COX2 CDS	H	3114	3798	685	-	ATG	T--	0
tRNA-Lys	H	3799	3867	69	CTT	-	-	-1
tRNA-Asp	H	3869	3936	68	GTC	-	-	0
ATP8 CDS	H	3937	4095	159	-	ATG	TAA	-7
ATP6 CDS	H	4089	4766	678	-	ATG	TAA	24
COX3 CDS	H	4791	5578	788	-	ATG	TA-	0
tRNA-Gly	H	5579	5643	65	TCC	-	-	0
ND3 CDS	H	5644	5997	354	-	ATG	TAA	45
tRNA-Ala	H	6043	6109	67	TGC	-	-	3
tRNA-Arg	H	6113	6175	63	TCG	-	-	24
tRNA-Asn	H	6200	6263	64	GTT	--	-	0
tRNA-Ser	H	6264	6329	66	GCT	-	--	31
tRNA-Glu	H	6361	6425	65	TTC	-	-	15
tRNA-Phe	L	6441	6506	66	GAA	-	-	0
ND5 CDS	L	6507	8229	1723	-	ATT	T--	6
tRNA-His	L	8236	8300	65	GTG	-	-	60
ND4 CDS	L	8361	9701	1341	-	ATG	TAG	-7
ND4L CDS	L	9695	9997	297	-	ATA	TAA	4
tRNA-Thr	H	10002	10062	61	TGT	-	-	2
tRNA-Pro	L	10068	10132	65	TGG	-	-	1
ND6 CDS	H	10134	10655	522	-	ATT	TAA	-1
Cytb CDS	H	10655	11791	1137	-	ATG	TAA	4
tRNA-Ser	H	11796	11860	65	TGA	-	-	6
ND1 CDS	L	11867	12821	956	-	ATT	T--	4
tRNA-Leu	L	12826	12887	62	TAG	-	-	0
16S rRNA	L	12888	14207	1320	-	--	-	0
tRNA-Val	L	14208	14279	72	TAC	-	-	0
12S rRNA	L	14280	14893	614	-	-	-	0
Control Region	H	14894	15930	1037	-	-	-	0

Table 5. *Mean pairwise genetic distance matrix of the mitogenome, for the clades Culicomorpha species. Evolutionary analyses were conducted using the Kimura-2-parameter (K2P) distance model with MEGA version 11*

No	Species with Accession Number	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
1	OR666457 *Forcipomyia pulchrithorax*																	
2	MK000395 *Forcipomyia makanensis*	0.264																
3	AB361004 *Culicoides arakawae*	0.347	0.332															
4	MK281356 *Chaoborus* sp.	0.442	0.418	0.410														
5	MW770891 *Chironomus flaviplumus*	0.534	0.510	0.496	0.468													
6	MZ261913 *Microchironomus tabarui*	0.469	0.450	0.447	0.406	0.250												
7	MZ747090 *Polypedilum nubifer*	0.479	0.442	0.448	0.413	0.316	0.268											
8	MZ041033 *Limnophyes minimus*	0.436	0.389	0.391	0.362	0.401	0.357	0.363										
9	KP721463 *Ochlerotatus vigilax*	0.410	0.365	0.378	0.316	0.393	0.367	0.363	0.291									
10	MZ460582 *Aedes koreicus*	0.416	0.389	0.388	0.340	0.417	0.381	0.375	0.312	0.106								
11	MN389460 *Culex pipiens*	0.406	0.381	0.372	0.337	0.396	0.369	0.370	0.305	0.115	0.141							
12	KJ701506 *Anopheles cruzii*	0.404	0.373	0.371	0.330	0.411	0.374	0.379	0.305	0.166	0.190	0.178						
13	MK281357 *Corethrella condita*	0.428	0.392	0.411	0.359	0.437	0.388	0.407	0.337	0.269	0.295	0.294	0.278					
14	KM245574 *Dixell*a sp.	0.423	0.382	0.394	0.343	0.436	0.387	0.388	0.310	0.266	0.279	0.271	0.275	0.308				
15	KT878382 *Dixella aestivalis*	0.430	0.391	0.398	0.347	0.436	0.400	0.398	0.316	0.273	0.299	0.284	0.286	0.323	0.139			
16	MK281359 *Thaumalea* sp.	0.461	0.467	0.451	0.428	0.526	0.449	0.445	0.379	0.354	0.362	0.362	0.360	0.381	0.341	0.345		
17	KU252587 *Simulium variegatum*	0.431	0.417	0.416	0.377	0.454	0.402	0.407	0.330	0.292	0.305	0.302	0.306	0.341	0.299	0.303	0.343	
18	MT410847 *Simulium noelleri*	0.432	0.404	0.420	0.378	0.445	0.396	0.401	0.326	0.294	0.298	0.304	0.308	0.339	0.293	0.300	0.331	0.127

According to Henning's (1973) classification, Ceratopogonidae was placed within the superfamily Chironomoidea and identified as the sister taxon to Chironomidae [44]. Some subsequent studies [45,46] also supported this relationship. However, Ceratopogonidae was placed in the superfamily Simulioidea with Thaumaleidae and Simuliidae in Borkent's analysis based on six pupal and one adult synapomorphies [47]. Zhang et al.[17] also reported compatible findings with Borkent's analyses without strong Bayesian support for the sister-group relationship between Ceratopogonidae and Thaumaleidae + Simuliidae. In our analyses, Ceratopogonidae, including the newly characterized *F. pulcrithorax* constituted an outer taxon among the Culicomorpha. Chironomidae was recognized as a sister taxon to Culicidae + Dixidae + (Thaumaleidae + Simuliidae), which was congruent with the findings of the phylogenetic reconstruction of Miller et al.[48] and Zhang et al.[17]. On the other hand, the family Chironomidae, was also reported as a paraphyletic taxon by Zhang et al.[17] when using PCGs, rRNAs, and tRNAs in the phylogenetic analyses with different evolutionary models.

In conclusion, our study provides the first data on the mitogenome characterization and phylogenetic analyses of *F. pulcrithorax*, and the findings contribute to the taxonomy and phylogeny of Culicomorpha, which is considered a complex taxon. We also conclude that the mitogenome data of *F. pulchrithorax* can be used to identify genetic markers for species identification. Although Ceratopogonidae contains many described species, there are only three mitogenome data from the species of this family, including *F. pulchritorax* characterized in our study. Therefore, broader taxonomic sampling and mitogenome characterization are needed to better understand of the

phylogenetic and taxonomic evaluation of the members of Ceratopogonidae.

Availability of Data and Materials

The sequence of mitogenome was deposited in the GenBank under accession number of OR666457.

Ethical Statement

This study does not require the approval of the Local Ethics Committee for Animal Experiments.

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Conflict of Interest

The authors declared that there is no conflict of interest.

Author Contributions

Conceptualization, G.K.K, A.Y., and O.I; methodology, G.K.K., C.C.K., and A.M.T.; investigation, G.K.K. and S.T.; data curation, G.K.K. and A.M.T; writing-original draft preparation, G.K.K. and S.T.; writing-review and editing, G.K.K., A.Y., and O.I.; supervision, G.K.K. and A.Y. All authors have read and agreed to the published version of the manuscript.

References

1. Bystrak PG, Wirth WW: The North American species of *Forcipomyia*, Subgenus *Euprojoannisia* (Diptera: Ceratopogonidae). *Tech Bull,* 56, 1978. DOI: 10.22004/ag.econ.157737

2. Borkent A, Dominiak P: Catalog of the biting midges of the world (Diptera: Ceratopogonidae). *Zootaxa*, 4787:2020. DOI: 10.11646/zootaxa.4787.1.1

3. Borkent A, Spinelli GR: Neotropical Ceratopogonidae (Diptera: Insecta). **In,** Adis J, Arias JR, Rueda-Delgado G, Wnatzen KM (Eds): Neotropical Ceratopogonidae (Diptera: Insecta). 4th ed., Pensoft Publishers, Sofia-Moscow, 2007.

4. Clastrier J, Wirth WW: Révision des Forcipomyia du sous-genre microhelea de la région néotropicale, parasites de phasmes (Diptera: Ceratopogonidae). **In,** Proceedings of the Annales de la Société Entomologique de France (NS). *J Entomol,* 31 (2): 97-150, 1995. DOI: 10.1080/21686351.1995.12277880

5. Deniz A, Korkmaz C, Bedir H, Vatansever Z, Ekinci AI, Sezer O, Erol U, Tuncer, S: Host preferences of vector *Culicoides* (Diptera, Ceratopogonidae, *Culicoides* Latreille) species in Türkiye. *Kafkas Univ Vet Fak Derg*, 29 (1): 63-69, 2023. DOI: 10.9775/kvfd.2022.28539

6. Boore JL: Animal mitochondrial genomes. *Nucleic Acids Res*, 27, 1767-1780, 1999. DOI: 10.1093/nar/27.8.1767

7. Shadel GS, Clayton DA: Mitochondrial DNA maintenance in vertebrates. *Annu Rev Biochem,* 66, 409-435, 1997. DOI: 10.1146/annurev.biochem.66.1.409

8. Wolstenholme DR: Animal mitochondrial DNA: Structure and evolution. *Int Rev Cytol,* 141, 173-216,1992. DOI: 10.1016/S0074-7696(08)62066-5

9. Zhang DX, Hewitt GM: Insect mitochondrial control region: A review of Its structure, evolution and usefulness in evolutionary studies. *Biochem Syst Ecol,* 25, 99-120, 1997. DOI: 10.1016/S0305-1978(96)00042-7

10. Sato M, Sato K: Maternal inheritance of mitochondrial DNA by diverse mechanisms to eliminate paternal mitochondrial DNA. *Biochim Biophys Acta,* 1833, 1979-1984, 2013. DOI: 10.1016/j.bbamcr.2013.03.010

11. Berlin S, Smith NG, Ellegren H: Do avian mitochondria recombine? *J Mol Evol*, 58, 163-167, 2004. DOI: 10.1007/s00239-003-2537-z

12. Hagström E, Freyer C, Battersby BJ, Stewart JB, Larsson NG: No recombination of mtDNA after heteroplasmy for 50 generations in the mouse maternal germline. *Nucleic Acids Res*, 42, 1111-1116, 2013. DOI: 10.1093/nar/gkt969

13. Allegrucci G, Carchini G, Todisco V, Convey P, Sbordoni V: A molecular phylogeny of antarctic chironomidae and its implications for biogeographical history. *Polar Biol*, 29, 320-326, 2006. DOI: 10.1007/s00300-005-0056-7

14. Matsumoto Y, Yanase, T, Tsuda T, Noda H: Species-specific mitochondrial gene rearrangements in biting midges and vector species identification. *Med Vet Entomol*, 23, 47-55, 2009. DOI: 10.1111/j.1365-2915.2008.00789.x

15. Jiang X, Han X, Liu Q, Hou X: The mitochondrial genome of *Forcipomyia Makanensis* (Insecta: Diptera: Ceratopogonidae). *Mitochondrial DNA B Resour*, 4, 344-345, 2019. DOI: 10.1080/23802359.2018.1544048

16. Deviatiiarov R, Kikawada T, Gusev O: The complete mitochondrial genome of an anhydrobiotic midge *Polypedilum Vanderplanki* (Chironomidae, Diptera). *Mitochondrial DNA A Resour*, 28, 218-220, 2017. DOI: 10.3109/19401736.2015.1115849

17. Zhang X, Kang Z, Ding S, Wang Y, Borkent C, Saigusa T, Yang D: Mitochondrial genomes provide insights into the phylogeny of Culicomorpha (Insecta: Diptera). *Int J Mol Sci,* 20:747, 2019. DOI: 10.3390/ijms20030747

18. Duan C, Jiang XH, Han XJ, Hou XH: Description of a new species of *Forcipomyia (Forcipomyia)* (Diptera: Ceratopogonidae) and a key to species of the subgenus from the Chinese Mainland. *J Med Entomol*, 56, 1614-1622, 2019. DOI: 10.1093/jme/tjz108

19. Mathieu B, Cêtre-Sossah C, Garros C, Chavernac D, Balenghien T, Carpenter S, Setier-Rio ML, Vignes-Lebbe R, Ung V, Candolfi E, Delécolle JC: Development and validation of IIKC: An interactive identification key for *Culicoides* (Diptera: Ceratopogonidae) females from the Western Palaearctic Region. *Parasit Vectors*, 5:137, 2012. DOI: 10.1186/1756-3305-5-137

20. Rozen S, Skaletsky H: Primer3 on the WWW for general users and for biologist programmers. *Methods Mol Biol*, 132, 365-386, 2000. DOI: 10.1385/1-59259-192-2:365

21. Ciloglu A, Ibis O, Yildirim A, Aktas M, Duzlu O, Onder Z, Simsek E, Yetismis G, Ellis VA, Inci A: Complete mitochondrial genome characterization and phylogenetic analyses of the main vector of *Crimean-Congo Haemorrhagic Fever Virus*: *Hyalomma Marginatum* Koch, 1844. *Ticks Tick Borne Dis,* 12:101736, 2021. DOI: 10.1016/j.ttbdis.2021.101736

22. Andrews S: A Quality Control Tool for High Throughput Sequence Data [Online]. http://www.bioinformatics.babraham; *Accessed:* 29 August 2023.

23.Jin JJ, Yu WB, Yang JB, Song Y, DePamphilis CW, Yi TS, Li DZ: GetOrganelle: A fast and versatile toolkit for accurate de novo assembly of organelle genomes. *Genome Biol*, 21, 1-31, 2020. DOI: 10.1186/s13059-020-02154-5

24. Donath A, Jühling F, Al-Arab M, Bernhart SH, Reinhardt F, Stadler PF, Middendorf M, Bernt M: Improved annotation of protein-coding genes boundaries in metazoan mitochondrial genomes. *Nucleic Acids Res*, 47, 10543-10552, 2019. DOI: 10.1093/nar/gkz833

25. Chan PP, Lowe TM: tRNAscan-SE: Searching for tRNA genes in genomic sequences. *Methods Mol Biol*, 1962, 1-14, 2019. DOI: 10.1007/978-1-4939-9173-0_1

26. Darty K, Denise A, Ponty Y: VARNA: Interactive drawing and editing of the RNA secondary structure. *Bioinform*, 25:1974, 2009. DOI: 10.1093/bioinformatics/btp250

27. Grant JR, Enns E, Marinier E, Mandal A, Herman, EK, Chen C, Graham M, Van Domselaar G, Stothard P: Proksee: In-depth characterization and visualization of bacterial genomes. *Nucleic Acids Res*, 51 (W1): W484-W492, 2023. DOI: 10.1093/nar/gkad326

28. Tamura K, Stecher G, Kumar S: MEGA11: Molecular evolutionary genetics analysis version 11. *Mol Biol Evol*, 38 (7): 3022-3027, 2021. DOI: 10.1093/molbev/msab120

29. Briscoe AG, Sivell D, Harbach RE: The complete mitochondrial genome of *Dixella aestivalis* (Diptera: Nematocera: Dixidae). *Mitochondrial*

DNA A Reseour, 28 (1): 83-84, 2017. DOI: 10.3109/19401736.2015.1110809

30. Kang Z, Li X, Yang D: The complete mitochondrial genome of *Dixella* sp. (Diptera: Nematocera, Dixidae). *Mitochondrial DNA A Reseour*, 27 (2): 1528-1529, 2016. DOI: 10.3109/19401736.2014.953123

31. Hardy CM, Court LN, Morgan MJ: The complete mitochondrial DNA genome of *Aedes vigilax* (Diptera: Culicidae). *Mitochondrial DNA A Reseour*, 27 (4): 2552-2553, 2016. DOI: 10.3109/19401736.2015.1038800

32. Xiao YL, Xu ZG, Wang JX, Fang XL, Fu Y: Complete mitochondrial genome of a eurytopic midge, *Polypedilum nubifer* (Diptera: Chironomidae). *Mitochondrial DNA B Reseour*, 7 (11): 1936-1938, 2022. DOI: 10.1080/23802359.2022.2122746

33. Kong FQ, Zhao YC, Chen JL, Lin XL: First report of the complete mitogenome of *Microchironomus Tabarui* Sasa, 1987 (Diptera, Chironomidae) from Hebei province, China. *Mitochondrial DNA B Resour*, 6 (10): 2845-2846, 2021. DOI: 10.1080/23802359.2021.1970638

34. Katoh K, Standley DM: A simple method to control over-alignment in the MAFFT multiple sequence alignment program. *Bioinform*, 32 (13): 1933-1942, 2016. DOI: 10.1093/bioinformatics/btw108

35. Castresana J: Selection of conserved blocks from multiple alignments for their use in phylogenetic analysis. *Mol Biol Evol*, 17 (4): 540-552, 2000. DOI: 10.1093/oxfordjournals.molbev.a026334

36. Posada D: jModelTest: phylogenetic model averaging. *Mol Biol Evol*, 25 (7): 1253-1256, 2008. DOI: 10.1093/molbev/msn083

37. Rodríguez-Trelles F, Tarrío R, Ayala FJ: Fluctuating mutation bias and the evolution of base composition in *Drosophila*. *J Mol Evol*, 50, 1-10, 2000. DOI: 10.1007/s002399910001

38. Ojala D, Montoya J, Attardi G: tRNA Punctuation model of rna processing in human mitochondria. *Nature*, 290 (5806): 470-474, 1981. DOI: 10.1038/290470a0

39. Boore JL, Brown WM: Mitochondrial genomes of *Galathealinum*, *Helobdella*, and *Platynereis*: sequence and gene arrangement comparisons indicate that pogonophora is not a phylum and annelida and arthropoda are not sister taxa. *Mol Biol Evol*, 17 (1): 87-106, 2000. DOI: 10.1093/oxfordjournals.molbev.a026241

40. Chen Y, Huang D, Wang Y, Zhu C, Hao J: The complete mitochondrial genome of the endangered apollo butterfly, *Parnassius Apollo* (Lepidoptera: Papilionidae) and its comparison to other Papilionidae Species. *J Asia Pac Entomol*, 17 (4): 663-671, 2014. DOI: 10.1016/j.aspen.2014.06.002

41. Sun WY, Xu DL, Chen HX, Shi W, Sundberg P, Strand M, Sun SC: Complete mitochondrial genome sequences of two parasitic/commensal nemerteans, *Gononemertes Parasita* and *Nemertopsis Tetraclitophila* (Nemertea: Hoplonemertea). *Parasit Vectors*, 7:273, 2014. DOI: 10.1186/1756-3305-7-273

42. Salvato P, Simonato M, Battisti A, Negrisolo E: The complete mitochondrial genome of the bag-shelter moth *Ochrogaster Lunifer* (Lepidoptera, Notodontidae). *BMC Genom*, 9 (1): 1-15, 2008. DOI: 10.1186/1471-2164-9-331

43. Cameron S: How to sequence and annotate insect mitochondrial genomes for systematic and comparative genomics research. *Syst Entomol*, 39 (3): 400–411, 2014. DOI: 10.1111/syen.12071

44. Hennig W: Diptera (Zweiflugler). *Handbuch der Zoologie*, 4, 1–337, Berlin, 1973.

45. Oosterbroek P, Courtney G: Phylogeny of the nematocerous families of Diptera (Insecta). *Zool. J Linn Soc*, 115 (3): 267-311, 1995. DOI: 10.1111/j.1096-3642.1995.tb02462.x

46. Wiegmann BM, Trautwein MD, Winkler IS, Barr NB, Kim JW, Lambkin C, Bertone MA, Cassel BK, Bayless KM, Heimberg AM: Episodic radiations in the fly tree of life. *PNAS*, 108 (14): 5690-5695, 2011. DOI: 10.1073/pnas.1012675108

47. Borkent A: The pupae of Culicomorpha morphology and a new phylogenetic tree. *Zootaxa*, 3396 (1): 1-98, 2012.

48. Miller BR, Crabtree MB, Savage HM: Phylogenetic relationships of the Culicomorpha inferred from 18S and 5.8S Ribosomal DNA sequences (Diptera: Nematocera). *Insect Mol Biol*, 6 (2): 105-114, 1997. DOI: 10.1111/j.1365-2583.1997.tb00078.x

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RESEARCH ARTICLE

Horse Surgery and Survival Prediction with Artificial Intelligence Models: Performance Comparison of Original, Imputed, Balanced, and Feature-Selected Datasets

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Abstract

Artificial intelligence (AI) technology, while less advanced than in human medicine, holds significant potential in the field of veterinary medicine. This technology offers a range of essential benefits, such as disease diagnosis, treatment planning, disease control, and overall animal health improvement. Based on clinical data, this study uses 15 AI models to predict the necessity of surgery and the likelihood of survival in horses displaying symptoms of acute abdominal pain (colic). By comparing surgical and survival predictions across the original, imputed missing values, and balanced datasets, we determine the most effective dataset based on the average accuracy of the 15 AI models. Furthermore, we explore the potential for improved accuracy with a reduced feature set by calculating feature importance scores for surgery and survival predictions. Our results indicate that the balanced dataset achieved the highest average accuracy for predicting surgery and survival, with 80.76% and 77.96%, respectively. The Random Forest (RF) model outperformed others as the most accurate model for both surgery (accuracy = 85.83, Area Under the Curve [AUC] = 0.906) and survival prediction (accuracy = 80.75, AUC = 0.888). It was observed that reducing the number of features in the dataset by 56% led to an increase in surgery prediction accuracy to 86.38%. Similarly, when the number of features was reduced by 24% for survival prediction, the prediction performance increased to 83.75%. This study emphasizes the importance of the precise implementation of artificial intelligence techniques in veterinary medicine, which can significantly enhance model performance.

Keywords: Artificial intelligence, Data balancing, Feature selection, Horse colic, Prediction, SMOTE

INTRODUCTION

In recent years, research in artificial intelligence has gained significant attention and made notable advancements. The most popular tasks performed using AI models are prediction/classification [1,2] and regression problems [3,4]. However, in veterinary medicine, the use of this technology has not yet been as developed as in other areas. Nonetheless, studies conducted in veterinary medicine have demonstrated the potential for achieving successful results with this technology [5,6].

Horse health is one of the essential fields where artificial intelligence technology should be applied. This is because horses are valuable animals, often used in racing. Acute abdominal (colic) is one of the horses' most commonly encountered health issues. Colic presents a severe, health problem for horses and can result in death if not treated promptly. This issue is closely tied to a substantial economic impact and is a significant concern for horse owners [6]. Approximately 90% of colic cases in horses resolve spontaneously or with medical treatment, but the remaining 10% of colic cases can be fatal if not treated surgically [7]. Therefore, veterinarians strive to protect horses' health by recognizing colic symptoms and providing rapid intervention. At this point, artificial intelligence models come into play, offering fast and accurate predictions, and they can be used as a supportive tool in veterinarians' decision-making processes.

Artificial intelligence systems are based on mathematical models and require training using sample data. This training data includes the information that helps the model learn the desired outputs. Artificial intelligence is

used to enhance computers' learning, decision-making, and problem-solving capabilities, and new models and applications are continuously being developed.

Artificial intelligence has been used in the field of veterinary medicine for body weight prediction [8], diagnostic radiology [9], blood sample value estimation [10], diagnosis of infectious and inflammatory disorders [11], classification of radiographs [12], animal diagnosis [13], milk yield prediction [14], and determination of bone fracture locations [15]. In a search conducted in the WOS database using the keywords "Horse colic + artificial intelligence," "Horse colic + machine learning," and "Horse colic + data mining," a relevant study was found. In this study, Fraiwan used artificial intelligence methods to predict with an accuracy rate of 76% and 85% the need for surgery and survival of horses with colic [6].

This study aims to make accurate surgery and survival predictions for -horse colic. For this purpose, the dataset used in the study contains missing values and is imbalanced. To address the difficulties posed by the dataset and achieve accurate predictions, the artificial intelligence methodology was meticulously implemented, and the prediction outcomes were evaluated at each stage.

The key contributions of this study are summarized as follows:

· This study demonstrates that accurately completing missing values in the dataset using the missForest method enhances prediction performance.

· This study addresses the issue of data imbalance by employing the SMOTE method and discovers that a balanced dataset enhances prediction performance.

· The study evaluates the performance of 15 AI models using accuracy and AUC statistical measures, and as a result, it indicates that the RF model outperforms other models.

· The study shows that a higher degree of surgical success and accuracy in predicting survival may be achieved by using fewer features.

Material and Methods

System Architecture

This study applied a series of steps to predict the need for surgical intervention and the probability of survival for horses with colic. The results obtained from the models may vary according to the dataset and the applied process steps [16]. Therefore, researchers can reduce or augment these steps according to the dataset and needs. The flowchart showing the classification process is shown in *Fig. 1*, and the subsequent sections provide a thorough explanation of each stage.

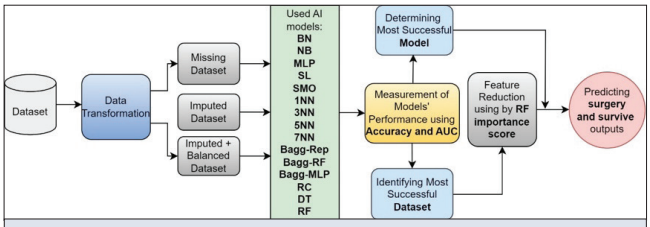

Fig 1. Flowchart for predicting of horse surgery and survival

Used Dataset

The dataset used in this study is publicly accessible online [17]. The dataset, containing medical data for 299 horses, consists of 27 features, with 25 used as inputs and two as outputs. The 'surgery' variable used as an output indicates whether the horses underwent surgery. The other output variable contains information about whether the horse survived. *Table 1* provides the characteristics of the features in the dataset, their types, feature information, missing value statistics, and information on using them as input/output in artificial intelligence models.

Data Transformation

Data transformation is the process of modifying or reshaping data to make it more suitable for dataset analysis or modeling [18]. This process improves data quality, ensures better model performance, and enhances data comprehensibility. Common data transformation techniques include feature scaling, normalization, transformation of categorical data, and handling outliers.

In this study, the dataset was transformed using categorical data transformation techniques. For example, the temperature of the extremities feature, with values normal, warm, cool, and cold, was transformed into 0, 1, 2, and 3, respectively. All categorical features underwent this transformation. Additionally, since the study aims to predict the survival status, the survival variable was assigned 0 for lived and 1 for dead and euthanized horse samples. The dataset used for predicting surgery and survival after data transformation was referred to as the *original dataset*.

Missing Value Imputation

In scientific research, researchers may encounter incomplete data collection, which may deviate from their anticipated parameters. Imputation is one of the popular data analysis approaches aimed at filling in missing data to make them usable [19]. The presence of missing values in the dataset presents a challenge for the planned analysis. The reason for this is that almost all classical and contemporary statistical techniques have been developed based on the assumption that the dataset is comprehensive [20]. In this study, Cihan [20] compared the accuracy performances of mean, kNN, SVD, bPca, and

Table 1. Characteristics and Information about the Horse Colic dataset

Feature	Type	Feature Information	#NA	Missing Rate	Direction
Age	Categoric	Adult, Young (<6 months)	0	0%	input
Temperature of extremities		Normal, warm, cool, cold	56	19%	
Peripheral pulse		Normal, increased, reduced, absent	69	23%	
Mucous membranes		normal pink, bright pink, pale pink, pale cyanotic, bright red, dark cyanotic	47	16%	
Capillary refill time		< 3 seconds, >= 3 seconds	32	11%	
Pain		No pain, depressed, mild pain, severe pain, extreme pain	55	18%	
Peristalsis		Hypermotile, normal, hypomotile, absent	44	15%	
Abdominal distension		None, slight, moderate, severe	56	19%	
Nasogastric tube		None, slight, significant	104	35%	
Nasogastric reflux		None, > 1 liter, < 1 liter	106	35%	
Rectal examination - feces		Normal, increased, decreased, absent	102	34%	
Abdomen		Normal, other, firm, small intestine, large intestine	118	39%	
Abdominocentesis appearance		Clear, cloudy, serosanguinous	165	55%	
Surgical lesion		No: Non-surgical lesion/Yes: Surgical lesion	0	0%	
Cp_data		No: Pathology data not present/Yes: data present	0	0%	
Rectal temperature	Numeric	Min: 35.4 - Max: 40.8	60	20%	
Nasogastric reflux ph		Min: 1 - Max: 7.5	246	82%	
Pulse		Min: 30 - Max: 184	24	8%	
Respiratory rate		Min: 8 - Max: 96	58	19%	
Packed cell volume		Min: 23 - Max: 75	29	10%	
Total protein		Min: 3.3 - Max: 89	33	11%	
Abdomcentesis total protein		Min: 0.1 - Max: 10.1	198	66%	
Lesion_1		Min: 0 - Max: 41110	0	0%	
Lesion_2		Min: 0 - Max: 7111	0	0%	
Lesion_3		Min: 0 - Max: 2209	0	0%	
Surgery	Categoric	No: horse had surgery/Yes: without surgery	0	0%	output
Outcome (Survive)	Categoric	Lived, Died, Euthanized	0	0%	output

missForest imputation methods. The analysis revealed that the missForest method successfully handled missing data in all different datasets. As a result, in this study, the missing values in the horseColic dataset were imputed using the missForest method.

The MissForest method utilizes Random Forest to predict missing values. It creates a separate Random Forest model for each missing variable. It uses these models to predict the missing values by considering the relationships between the missing variables, other complete variables, and the output variable [21]. This helps in accurately and reliably imputing missing data. The 'missForest' function in R programming was used to implement this method. This function completed the missing values in the dataset containing the 'surgery' output variable and then in the dataset containing the 'survive' output variable. In this study, the dataset version with missing values filled using the missForest method was referred to as the *imputed dataset*.

When applying artificial intelligence methods in WEKA, you can work with datasets that have missing values. WEKA ignores the missing data and does not use these instances in classification or artificial intelligence processes. Ignoring missing data results in a loss of information in the dataset. Therefore, it is essential to handle missing data properly.

Data Balancing

The situations where the output features (decision variables) in the dataset are not evenly distributed indicate that the dataset is imbalanced. Real-world datasets often

imbalanced [22]. In imbalanced datasets, the class with a small number of samples is referred to as the minority class, while the class with a large number of samples is called the majority class [23]. Imbalanced datasets can mislead influence classification results, so it is desirable in artificial intelligence studies that the decision variable is evenly distributed in the datasets used. There are various methods that can be applied to eliminate imbalance in the preprocessing step of the data. The Synthetic Minority Over Sampling Technique (SMOTE) is one of the methods that can be used to address this issue, and it has been used in this study to address the data imbalance.

SMOTE is one of the most well-known and commonly used resampling methods. SMOTE creates new artificial minority class samples by interpolating among the existing minority class examples. This approach to generating synthetic samples was inspired by a technique used in handwritten character recognition. The method first finds the k nearest neighbors for each minority class example; then, it selects a random nearest neighbor. Subsequently, a new minority class example is created using the straight segment between a minority class example and its nearest neighbor. This process is repeated until both classes have an equal number of examples [24].

In this study, data balancing was performed using the SMOTE method after completing the missing values in the dataset. This dataset was referred to as a balanced dataset. For the survival output variable, the number of minority samples was increased by 47%, from 121 to 177, to balance the output sample size. Similarly, for the surgery output variable, the number of samples belonging to the minority class was increased by 48%, resulting in 180 'yes' and 180 'no' instances to balance the dataset.

Artificial Intelligence Models

In this study, colic horses' surgery and survival statuses were predicted using fifteen artificial intelligence classification methods. A list of the artificial intelligence classification algorithms used in this study, along with their brief descriptions, is presented in *Table 2*.

Table 2. *AI models used in this study*

Categories	Model	Abbr.	Description
Bayes	Bayes Network	BN	It is a probability theory-based graphical modeling approach. It uses a network structure that illustrates the relationships between variables and utilizes Bayes' theorem to make predictions [25].
	Naive Bayes	NB	Naive Bayes makes classifications based on the Bayes theorem and assumes independence between variables [26].
Functions	Multilayer Perceptron	MLP	It is one of the types of artificial neural networks. It makes complex decisions by mimicking the way the human brain works. It typically consists of input, hidden, and output layers.
	Simple Logistic	SL	It is a classification algorithm that fits the data with a suitable curve and has the ability to express class predictions of input features using probability distributions.
	Sequential Minimal Optimization	SMO	SMO is a classification algorithm based on Support Vector Machines (SVM). It maps the data into a high-dimensional space and provides a fit. It is developed to accelerate and optimize SVM training [27].
Lazy-learning algorithms	K-NN (K=1)	1NN	K-NN, to classify an instance, compares it with the classes of the K nearest neighbors. When a new data point arrives, K-NN calculates the K closest neighbors to this point, and by examining the classes of these neighbors, it determines the class of the new point. Since the choice of the K value can affect the classification results [28], different K values (1, 3, 5, 7) were tested in the study.
	K-NN (K=3)	3NN	
	K-NN (K=5)	5NN	
	K-NN (K=7)	7NN	
Meta-learning algorithms	Bagging REP Tree	Bagg-Rep	Bagging, each base classifier is trained independently and combines their results to create a stronger and more stable classifier. This can enhance prediction performance. Since each base classifier is trained on a different training subset, Bagging can reduce variance, thereby improving the model's prediction performance. In this study, prediction performances were tested using REP Tree, Random Forest, and Multilayer Perceptron as base classifiers.
	Bagging Random Forest	Bagg-RF	
	Bagging Multilayer Perceptron	Bagg-MLP	
	Random Committe	RC	It is a method that combines different classifiers to create a stronger classifier. First, it trains the dataset with random subsampling. Then, each classifier makes its own predictions. In the final step, the majority of these predictions are used to obtain the result [29]. In this study, Random Tree was used as the classifier.
Tree-based algorithms	Decision Tree	DT	A decision tree is represented as a tree structure, and each internal node is associated with a feature. As the dataset progresses through this tree structure, a decision is made at each node, and the data point is directed along a branch based on the relevant feature's value [13]. The C4.5 decision tree algorithm, known as J48 in WEKA, was used in this study.
	Random Forest	RF	It is an ensemble learning model that combines multiple decision trees. Each tree is trained on a random subset of the dataset, and this process is repeated randomly. Then, each tree classifies the data points, and the result of these classifications is subjected to majority voting [30].

This study used the R programming language [31] and the WEKA 3.8.5 (Waikato Knowledge Analysis Environment) tool [32]. The R programming language was employed for imputing missing data and determining feature importance scores. The WEKA tool was used for data balancing and classification tasks. The K-fold cross-validation (CV) technique was employed for both the training and testing phases of the classification methods. This technique divides the dataset into K equally sized subsets. While K-1 subsets are used for model training, the remaining subset is reserved for testing the model's performance. This process is repeated K times, with each subset used once for testing. The average of the K results is used to combine classification outcomes and assess overall performance. The CV technique is crucial for impartially evaluating model performance because it utilizes the entire dataset for model training and performance testing. In this study, a 10-fold cross-validation was implemented.

Measurement of Models Performance

The study used the Accuracy (ACC) and the Area Under the ROC Curve (AUC) metric to evaluate the prediction performance of artificial intelligence models for surgery and survival. Accuracy represents the ratio of correctly identified cases to the total number of cases (Equation 1). In this study, accuracy reflects the ability to correctly identify the need for surgical intervention and to accurately describe the horse's survival status.

$$Accuracy = \frac{Correct\ Predictions}{All\ Predictions} \quad (1)$$

The ROC curve is a graph that illustrates the variation in sensitivity and specificity at different threshold values, and the AUC measures the area under this curve. A high AUC value indicates that the model performs well and can classify accurately [33]. AUC was used to evaluate the ability to accurately predict the need for surgical intervention and the likelihood of a horse's survival.

Feature Selection

Feature selection is a process that involves choosing the most relevant and informative features from a dataset while excluding less important ones. By doing so, the dimensionality of the dataset is reduced, leading to faster AI model execution, lower memory consumption, and a streamlined analysis process. However, model performance may not always improve after the feature reduction process. While removing some features may enhance predictive performance, in some cases, it risks losing important information.

In fields such as veterinary medicine, data collection can be a challenging and costly process. The more accurate predictions can be made with less data, the more time and cost savings can be achieved. In this study, we calculated feature importance scores for the surgery and survival variables. We used the "importance" function in R programming for this purpose [34]. This method measures the importance of each feature and provides an importance ranking. Feature importance was calculated based on the Gini importance criterion, which indicates each feature's contribution to the model's predictive performance. After calculating the feature importance scores, we measured predictive performance by iteratively removing features, starting with the least important ones. This process identified the feature group that yielded the highest predictive performance.

Results

In this study, the prediction performances of AI models were analyzed after applying pre-processing and post-processing steps to achieve highly accurate predictions. In the pre-processing step, features in the dataset were transformed, missing values were handled, and data balancing procedures were carried out. In the post-processing step, a feature selection process was performed.

For the prediction of surgery and survival, a comparative performance analysis was conducted using 15 different artificial intelligence models on three different versions of the dataset. The original dataset containing missing data (referred to as original), the dataset in which missing values were imputed using the missForest method (referred to as imputed), and the dataset that was balanced after imputation (referred to as balanced) were compared in terms of the prediction performance of 15 AI models in *Table 3*.

According to the findings obtained, it was concluded that the 'balanced' dataset had the highest average accuracy for both surgery and survival predictions (80.76% and 77.96%, respectively). The dataset with the lowest average accuracy for both output variables was the original dataset (78.73% for surgery and 72.44% for survival).

According to the results presented in *Table 3*, the high accuracy value of the balanced dataset indicates that the processed and balanced version improved the accuracy of predictions. Therefore, the balanced dataset will be used for further procedures in the subsequent steps of the study. In evaluating the impact of datasets on prediction performance, using the average of 15 different AI models, rather than a single model, demonstrates that success is model-independent.

Furthermore, to determine the most successful model in making surgery and survival predictions, the prediction performance of 15 AI models was compared internally. The accuracy of surgery and survival predictions for the balanced dataset can be seen in *Table 3*, and the AUC values are presented in *Fig. 2*.

Table 3. *Accuracy (%) results of models on different datasets for surgery and survival prediction*

Model	Prediction of Surgery			Prediction of Survival		
	Original Dataset	Imputed Dataset	Balanced Dataset	Original Dataset	Imputed Dataset	Balanced Dataset
BN	77.59	76.92	75.27	72.57	75.58	79.15
NB	71.57	73.24	73.33	73.57	75.58	78.02
MLP	77.25	76.92	78.61	68.89	70.90	73.23
SL	80.60	80.26	81.66	70.23	70.24	77.46
SMO	79.93	80.60	81.66	69.89	73.24	77.18
1NN	73.24	74.91	79.72	68.56	72.57	78.87
3NN	74.58	75.91	81.11	68.56	75.58	80.00
5NN	77.59	79.59	83.05	68.56	74.25	79.72
7NN	77.59	79.26	82.22	69.23	73.24	80.28
Bagg-Rep	80.94	80.6	81.94	73.57	74.58	76.34
Bagg-RF	82.60	82.94	84.16	78.26	79.26	80.56
Bagg-MLP	80.26	81.93	80.83	73.91	75.25	76.34
RC	83.27	78.59	82.50	76.92	73.91	80.00
DT	81.27	79.59	79.44	77.25	74.58	71.55
RF	82.6	84.28	**85.83**	76.58	73.25	**80.75**
Average	78.73	79.04	**80.76**	72.44	74.13	**77.96**

Feature selection was carried out to examine the impact of features in the dataset on prediction performance and, if possible, to make predictions with fewer features while achieving higher accuracy. The importance scores of features in the dataset were calculated using the *importance* function. In *Fig. 3* and *Fig. 4*, the importance scores of features for surgery and survival datasets and the prediction accuracy obtained through RF classification are presented.

When examining *Fig. 3*, it can be observed that the feature with the lowest importance for surgery prediction is lesion_3 (gini index = 0). Starting with the feature of lowest importance, features were iteratively removed from the dataset, and surgery prediction was made using the RF method. When all features were used, surgery was predicted with an accuracy of 85.83%, but when the 14 features with the lowest importance were removed from the dataset, the prediction accuracy increased to 86.38%.

As seen in *Fig. 4*, the accuracy rate of the survival prediction made using all features with the RF method is 80.75%. However, removing the least important six features from the dataset, increased the prediction accuracy to 83.85%. These results demonstrate that removing the

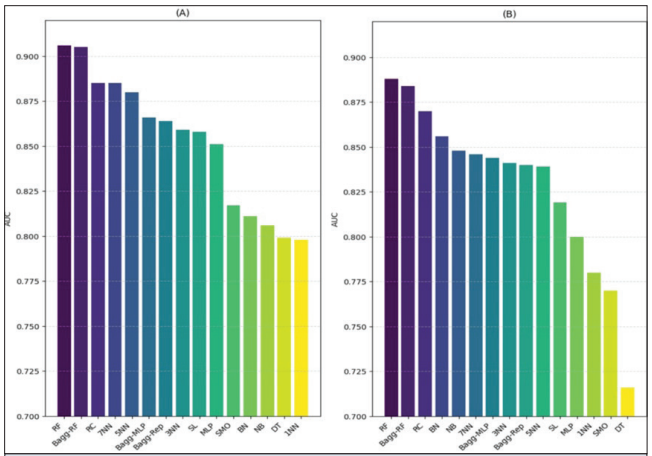

Fig 2. AUC results of 15 AI models for A- surgery and B- survival prediction

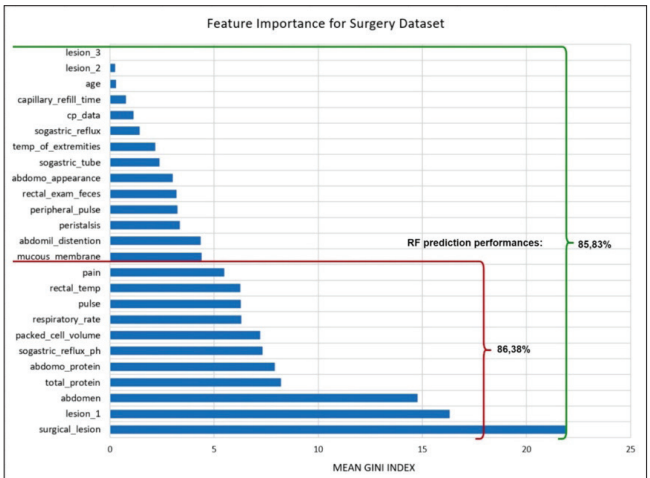

Fig 3. Impact of variables and RF prediction results on the Surgery Dataset

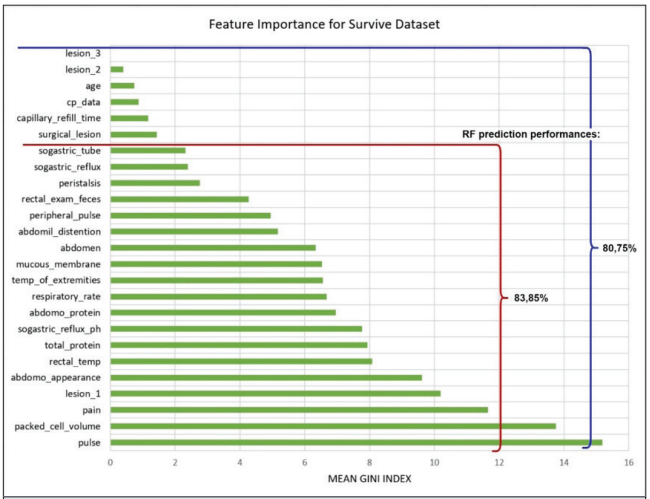

Fig 4. Impact of variables and RF prediction results on the Survive Dataset

least important features from the dataset enhances the performance of survival prediction.

These findings also indicate that feature selection improves the accuracy of surgery and survival predictions and that removing unimportant features can enhance model performance. The results also emphasize the importance of reducing model complexity to make more effective predictions in AI classification problems.

Discussion

In recent years, artificial intelligence methods have become widely used in various disciplines. However, in the field of veterinary medicine, the use of this technology has lagged behind and has yet to gain the expected popularity [6,35]. One of the primary reasons for the limited use of artificial intelligence in this field is that datasets are typically private and have limited accessibility. In human medicine, artificial intelligence has been extensively researched for many years, and there are many publicly available datasets. However, researchers often use data collected on animals in their own studies. The privacy of datasets hinders the development of interdisciplinary studies and restricts the transparency and accuracy of the obtained results [36]. We believe that sharing data publicly is a necessity for more interdisciplinary work in the field of veterinary medicine.

This study systematically applies artificial intelligence steps to the publicly accessible Horse Colic dataset on Kaggle, providing comparative results. Despite its publication many years ago, the Horse Colic dataset has yet to receive extensive study due to its complexity. The dataset includes data for 27 features from 299 horses, encompassing three types of data (continuous, discrete, and nominal). It also exhibits a relatively high rate of missing values *(Table 1)* and is imbalanced.

Missing values are a common issue in real-world dataset [37]. In some artificial intelligence applications, specific tools do not allow for model development on datasets containing missing data. In WEKA, artificial intelligence models can be used with datasets that include missing instances, but the missing samples are ignored. Both scenarios present challenges for researchers. Refrain from missing values further reducing the already limited number of examples in this field. Additionally, especially in medical datasets, accurately completing missing values is crucial to avoid making incorrect predictions. One of the primary objectives of this study is to fill in missing values in the dataset accurately. In many artificial intelligence applications, missing values in the dataset are either ignored or replaced with the mean value. However, several successful methods have been developed to complete missing values accurately. Mishandling missing values in a wrong or incomplete manner can lead to misleading or unreliable results. In this study, the missForest method, which has previously demonstrated its effectiveness, was used to impute missing values. The findings from the study indicate that the imputed dataset leads to more successful accuracy in surgery and survival predictions compared to predictions made with the original dataset.

The study's secondary aim is to deal with the challenge of imbalanced data. This is because dealing with dataset imbalances is important in improving prediction performance. Data imbalance issues arise in fields like veterinary medicine, where rare events like death are represented by a limited number of examples in the datasets. These imbalances negatively affect the model's learning process and prediction performance [38]. The SMOTE method was used in the study to address the dataset imbalance. When the prediction success of the balanced dataset is compared with the other datasets (original and imputed), the highest accuracy rates are obtained from the balanced dataset. These results demonstrate that carefully addressing data imbalance issues and using data balancing methods have a positive effect on the performance of model predictions.

The third main objective of the study is to identify the most effective artificial intelligence model for surgery and survival predictions. For this purpose, predictions were made using 15 different models. When comparing the performance of the models for surgery and survival predictions, it was concluded that the RF method is more successful than the other methods. The RF method has demonstrated successful results in solving various problems. Therefore, the RF method can be an effective option in various application areas in veterinary medicine.

The ultimate goal of the study is to make more successful predictions with fewer features in the dataset. This is because numerous dataset features can increase model complexity and raise the risk of overfitting. Therefore,

feature selection makes the model more straightforward and effective. Additionally, since collecting medical data is a challenging process, making more accurate predictions with fewer features is important in data collection and resource utilization. In this study, feature importance scores were calculated using RF importance. It was concluded that when features with low importance were removed from the dataset, more accurate predictions could be made with fewer features *(Fig. 2)*. This is a crucial aspect to be considered in data analysis and artificial intelligence studies in veterinary medicine.

In conclusion, this study was conducted to explore the potential of artificial intelligence methods in veterinary medicine and address significant challenges in this area. Artificial intelligence holds great potential in veterinary medicine as well. However, its use in this field is still limited and faces essential barriers, such as the private and limited accessibility of datasets. The objective of this study was to enhance artificial intelligence research in veterinary medicine by showcasing methods to handle missing data, alleviate data imbalances, and simplify model complexity. In the future, creating and sharing more publicly accessible datasets could encourage the wider adoption of artificial intelligence methods in veterinary medicine. Additionally, interdisciplinary studies in this field could make valuable contributions to animal health and treatment.

Declarations

Availability of Data and Materials: The dataset used in the study is publicly available at: https://www.kaggle.com/datasets/uciml/horse-colic

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Competing Interests: The author declared that there is no conflict of interest.

References

1. Cihan P, Ozger ZB: A new approach for determining SARS-CoV-2 epitopes using machine learning-based *in silico* methods. *Comput Biol Chem,* 98:107688, 2022. DOI: 10.1016/j.compbiolchem.2022.107688

2. Nasir N, Kansal A, Alshaltone O, Barneih F, Sameer M, Shanableh A, Al-Shamma'a A: Water quality classification using machine learning algorithms. *J Water Process Eng,* 48:102920, 2022. DOI: 10.1016/j.jwpe.2022.102920

3. Cihan P, Ozel H, Ozcan HK: Modeling of atmospheric particulate matters via artificial intelligence methods. *Environ Monit Assess,* 193, 1-15, 2021. DOI: 10.1007/s10661-021-09091-1

4. Tüfekci P: Prediction of full load electrical power output of a base load operated combined cycle power plant using machine learning methods. *Int J Electr Power Energy Syst,* 60, 126-140, 2014. DOI: 10.1016/j.ijepes.2014.02.027

5. Vinicki K, Ferrari P, Belic M, Turk R: Using convolutional neural networks for determining reticulocyte percentage in cats. *arXiv:*180304873, 2018. DOI: 10.48550/arXiv.1803.04873

6. Fraiwan MA, Abutarbush SM: Using artificial intelligence to predict survivability likelihood and need for surgery in horses presented with acute abdomen (colic). *J Equine Vet Sci,* 90:102973, 2020. DOI: 10.1016/j.jevs.2020.102973

7. Hillyer M, Taylor F, French N: A cross-sectional study of colic in horses on Thoroughbred training premises in the British Isles in 1997. *Equine Vet J,* 33 (4): 380-385, 2001. DOI: 10.2746/042516401776249499

8. Topai M, Macit M: Prediction of body weight from body measurements in Morkaraman sheep. *J Appl Anim Res,* 25 (2): 97-100, 2004. DOI: 10.1080/09712119.2004.9706484

9. Müller TR, Solano M, Tsunemi MH: Accuracy of artificial intelligence software for the detection of confirmed pleural effusion in thoracic radiographs in dogs. *Vet Radiol Ultrasound,* 63 (5): 573-579, 2022. DOI: 10.1111/vru.13089

10. Cihan P, Gökçe E, Atakişi O, Kırmzıgül AH, Erdoğan HM: Prediction of immunoglobulin G in lambs with artificial intelligence methods. *Kafkas Univ Vet Fak Derg,* 27 (1): 21-27, 2021. DOI: 10.9775/kvfd.2020.24642

11. Ferrini S, Rollo C, Bellino C, Borriello G, Cagnotti G, Corona C, Di Muro G, Giacobini M, Iulini B, D'Angelo A: A novel machine learning-based web application for field identification of infectious and inflammatory disorders of the central nervous system in cattle. *J Vet Intern Med,* 37 (2): 766-773, 2023. DOI: 10.1111/jvim.16664

12. Costa da Silva RG, Mishra AP, Riggs CM, Doube M: Classification of racehorse limb radiographs using deep convolutional neural networks. *Vet Rec Open,* 10 (1):e55, 2023. DOI: 10.1002/vro2.55

13. Cihan P, Kalipsiz O, Gökçe E: Yenidoğan kuzularda bilgisayar destekli tanı. *Pamukkale Üniv Müh Bilim Derg,* 26 (2): 385-391, 2020. DOI: 10.5505/pajes.2019.51447

14. Topuz D: Lactation milk yield prediction with possibilistic logistic regression analysis. *Kafkas Univ Vet Fak Derg,* 27 (5): 547-557, 2021. DOI: 10.9775/kvfd.2020.25171

15. Baydan B, Barıscı N, Unver HM: Determining the location of tibial fracture of dog and cat using hybridized mask R-CNN architecture. *Kafkas Univ Vet Fak Derg,* 27 (3): 347-353, 2021. DOI: 10.9775/kvfd.2021.25486

16. Kumar Y, Koul A, Singla R, Ijaz MF: Artificial intelligence in disease diagnosis: A systematic literature review, synthesizing framework and future research agenda. *J Ambient Intell Humaniz Comput,* 14, 8459-8486, 2022. DOI: 10.1007/s12652-021-03612-z

17. Kaggle: Horse colic dataset: Can you predict the life or death of a horse?. https://www.kaggle.com/datasets/uciml/horse-colic; *Accessed:* 07.07.2023.

18. Kandel S, Heer J, Plaisant C, Kennedy J, Van Ham F, Riche NH, Weaver C, Lee B, Brodbeck D, Buono P: Research directions in data wrangling: Visualizations and transformations for usable and credible data. *Inf Vis,* 10 (4): 271-288, 2011. DOI: 10.1177/14738716114159

19. Little RJ, Rubin DB: Statistical Analysis with Missing Data. John Wiley & Sons, 2019.

20. Cihan P: A comparison of five methods for missing value imputation in data sets. *Int Sci Voc Stud J,* 2 (2): 80-85, 2018.

21. Cihan P, Kalıpsız O, Gökce E: Veri madenciliği yöntemleriyle hayvan hastalıklarında teşhis, prognoz ve risk faktörlerinin belirlenmesi. *PhD Thesis*, Yıldız Teknik Univ., Fen Bil. Enst., 2018.

22. Cateni S, Colla V, Vannucci M: A method for resampling imbalanced datasets in binary classification tasks for real-world problems. *Neurocomputing,* 135, 32-41, 2014. DOI: 10.1016/j.neucom.2013.05.059

23. Saptono R, Winarno W, Drajati DP: Comparison of under-sampling and over-sampling techniques in diabetic mellitus (DM) patient data classification by using naive bayes classifier (NBC). *J Telecommun Electron Comput Eng,* 10, 145-148, 2018.

24. Chawla NV, Bowyer KW, Hall LO, Kegelmeyer WP: SMOTE: Synthetic minority over-sampling technique. *J Artif Intell Res,* 16, 321-357, 2002. DOI: 10.1613/jair.953

25. Friedman N, Geiger D, Goldszmidt M: Bayesian network classifiers. *Mach Learn,* 29, 131-163, 1997. DOI: 10.1023/A:1007465528199

26. Sallam NM, Saleh AI, Arafat Ali H, Abdelsalam MM: An efficient strategy for blood diseases detection based on grey wolf optimization as feature selection and machine learning techniques. *Appl Sci,* 12 (21):10760, 2022. DOI: 10.3390/app122110760

27. Awad M, Khanna R, Awad M, Khanna R: Support vector machines for

classification. In, Efficient Learning Machines: Theories, Concepts, and Applications for Engineers and System Designers. 39-66, Apress Open, 2015.

28. Sanlı T, Sıcakyüz Ç, Yüregir O: Comparison of the accuracy of classification algorithms on three data-sets in data mining: Example of 20 classes. *Int J Eng Sci Technol,* 12 (3): 81-89, 2020. DOI: 10.4314/ijest.v12i3.8

29. Niranjan A, Nutan D, Nitish A, Shenoy PD, Venugopal K: ERCR TV: Ensemble of random committee and random tree for efficient anomaly classification using voting. *2018 3rd International Conference for Convergence in Technology (I2CT). 06-08 April,* IEEE, 2018.

30. Breiman L: Random forests. *Mach Learn,* 45, 5-32, 2001. DOI: 10.1023/A:1010933404324

31. Venables W SD, R Core Team An Introduction to R-Version 3.0.2. 2013. http://cran.r-project.org/doc/manuals/R-intro.pdf; *Accessed:* 03.09.2023.

32. WEKA: Machine Learning Software in Java. https://www.cs.waikato.ac.nz/ml/weka/; *Accessed:* 05.09.2023.

33. Hajian-Tilaki K: Receiver operating characteristic (ROC) curve analysis for medical diagnostic test evaluation. *Caspian J Intern Med,* 4 (2): 627-635, 2013.

34. LIAW A: Importance: Extract variable importance measure. 2020. https://www.rdocumentation.org/packages/randomForest/versions/4.6-14/topics/importance; *Accessed:* 03.09.2023.

35. Cihan P, Gokce E, Kalipsiz O: A review of machine learning applications in veterinary field. *Kafkas Univ Vet Fak Derg,* 23 (4): 673-680, 2017. DOI: 10.9775/kvfd.2016.17281

36. Appleby RB, Basran PS: Artificial intelligence in veterinary medicine. *J Am Vet Med Assoc,* 260 (8): 819-824, 2022. DOI: 10.2460/javma.22.03.0093

37. Tran CT, Zhang M, Andreae P, Xue B, Bui LT: Improving performance of classification on incomplete data using feature selection and clustering. *Appl Soft Comput,* 73, 848-861, 2018. DOI: 10.1016/j.asoc.2018.09.026

38. Ali H, Salleh MNM, Saedudin R, Hussain K, Mushtaq MF: Imbalance class problems in data mining: A review. *Indones J Electr Eng Comput Sci,* 14 (3): 1560-1571, 2019. DOI: 10.11591/ijeecs.v14.i3.pp1552-1563

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Research Article

Morphological and Molecular Characterization of Mucormycosis and Other Fungal Agents in Cattle

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Abstract

Mucormycosis is a type of opportunistic fungal infection caused by the Mucorales order of Zygomycetes. The study's goal was to characterize *Lichtheimia* and other fungal agents in Tekirdağ province of Türkiye by morphological and molecular methods. Head hair and skin scrapings of 13 cattle with mucormycosis lesions inoculated onto Rose Bengal Agar, Potato Dextrose, and Malt Extract Agar. After the incubation at 25°C and 27°C, pure colonies were evaluated morphologically and microscopically. For molecular identification, DNA isolation and PCR studies were followed by sequence analysis and the results were compared with the data in GeneBank using the nBLAST tool. ITS1/ITS4 primers used in PCR study. Fungal species were identified with data verified after morphological and molecular identification. The sequence analyses revealed that 12 samples had *L. ramosa* HBF570, 7 samples contained *A. niger* HBF572 and *P. crustosum* HBF571, 2 samples contained *A. chevalieri* HBF573 and *A. flavus* HBF576, and one sample contained *A. pseudoglaucus* HBF577 and *Aspergillus* sp. HBF570. The study's causative agents emerged were environmental fungus species. In conclusion, because of the fungal diversity in the environment, hygiene investigations must be conducted and implemented for the protection of mucormycosis.

Keywords: Cattle, *Lichthemia ramose*, Mucormycosis, Sequencing

Introduction

Mucormycosis is a major cause of death in humans and animals caused by opportunistic infections present in the environment. Mucormycosis is a dangerous fungal infection that primarily affects the immunocompromised. Infections can be distinguished by widespread angioinvasion and necrosis. Mucor pathogens are zoonotic pathogens found in soil, decaying debris and cow feed, such as straw [1]. Mucormycosis is caused mostly by Mucorales (Mucoromycotina), which includes *Mucor, Rhizomucor, Rhizopus*, and *Lichtheimia* (previously *Absidia),* and Mortierellales *(Mortirellomycotina),* which includes *Mortierella* [2].

Fungi from the order Mucorales cause zygomycosis more frequently in people than fungi from the order Entomophthorales. Zygomycosis is classified into several infection kinds based on the location of the lesions. Zygomycosis, regardless of the kind of fungal infection, is a deadly threat which demands prompt, correct diagnosis and treatment. Mucormycosis is the most common cause of zygomycosis in humans and cattle. Bovine zygomycosis typically causes isolated lesions (most commonly lymphadenitis or stomach zygomycosis) and is usually discovered inadvertently during a necropsy or slaughterhouse check. Cattle deaths from zygomycosis are uncommon [3]. Mucormycoses are divided into rhinocerebral, pulmonary, cutaneous, gastrointestinal, and

disseminated forms based on the location of the lesions. In cattle, these infections have been linked to gastroenteritis, meningoencephalitis, and endometritis [4]. Mucormycosis spreads quickly. It causes fungal emboli and necrotic suppurative lesions. *Mucoromycotina* is extremely invasive in the circulatory system. The agent is rarely discovered in suspect material for fungal isolation during diagnosis, and the fungus required for identification cannot be produced in the majority of cases. Due to the lack of clinical signs in cows and the complexity of the diagnostic process, diagnostic examination for fungal agents is frequently overlooked [5].

Although *Lichtheimia* species (particularly *L. ramosa* and *L. corymbifera*) are prominent mucormycosis causes, it is uncertain whether the same risk factors underpin fungal infection caused by different mold genera and species [6]. *L. ramosa* is a thermotolerant soil fungus commonly found near cattle in hay, grain, bedding, and silage. *L. ramosa* is also a common part of the stomach fungal flora [7]. The genus *Lichtheimia* (*Mucorales, Lichtheimiaceae*) is made up of saprotrophic fungi that live in soil, plants, indoor air, food products, and excrement, and it contains major mucormycosis causal agents in humans and animals. *Lichtheimia* species are found on all continents, with isolates from both environmental and clinical sources [8]. Fungal spores and hyphae are abundant in cattle feed, and oral intake appears to be the most likely route of infection. Endogenous infections may also occur since mucoralean species, including *L. corymbifera* and *L. ramosa*, are among the filamentous fungi found in healthy bovine rumen fluid [6]. Rhinocerebral mucormycosis has been observed as a result of a possible postnatal infection with *L. ramosa* [9]. Striatal necrosis caused by *L. ramosa* was reported in a newborn calf [7]. In humans, nasal *L. ramosa* infection arises as a result of inhaling asexual spores and leads to cerebral lesions [6].

Morphological, microscopic and molecular methods need to be evaluated together in the identification of fungi. Nowadays, molecular methods with the support of universal databases are quite successful in obtaining accurate results. Specialized regions of Fungal nuclear ribosomal DNA are used in the molecular identification of fungi. Fungal nuclear ribosomal DNA (rDNA) consists of three parts: the large subunit gene (25S), the small subunit gene (18S) and the 5.8S gene, separated by internal transcribed spacer (ITS) regions. ITS regions play an important role in the molecular identification of fungi. Primers targeting the amplification of these ITS regions are generally used to identify fungi, and these primers are universally used to identify many fungal species [10,11].

This study aimed to determine the morphological and molecular characteristics of fungal agents that induce keratinization lesions of varying sizes and numbers on the skin of cattle bred in Tekirdağ province, Türkiye.

Material and Methods

Ethical Statement

"Informed Consent Form" was obtained from animal owners for sampling the material of the study, and "Animal Experiments Local Ethics Committee Approval" is not required for the study.

Hair and Skin Rash (Scraping) Samples

The material of the study was selected from a private enterprise engaged in mixed cattle and dairy breeding in Kırkkepenek village of Tekirdağ province (Türkiye), which had a total of 35 cattle, including 22 cattle with keratinization lesions of various sizes and numbers on skin. Hair and skin scraping samples were taken from 13 cattle with at least five lesions of similar size *(Fig. 1)*. Before sampling, the areas around the lesions were cleaned with 70% alcohol, the skin debris was scraped with a sterile scalpel, and the hairs were pulled with a sterile forceps and placed in sterile containers. The samples taken were stored at +4°C until the laboratory studies [12].

Fungus Isolation

From the samples brought to the laboratory, a certain amount of hair and skin scrapings were taken with sterile forceps under aseptic conditions and inoculated onto Rose Bengal Agar and incubated at 25°C and 27°C for 30 days. During the incubation, the samples were checked at certain periods (every 7 days) and their growth was observed. Different colonies or mycelia were planted on Potato Dextrose Agar (PDA) and Malt Extract Agar (MEA) and pure colonies were obtained by incubating the petri dishes at 25°C and 27°C.

Morphological and Microscopic Identification

Morphological characteristics of pure colonies obtained in PDA and MEA were evaluated. For the morphological evaluation, visual characteristics of their colonies, such

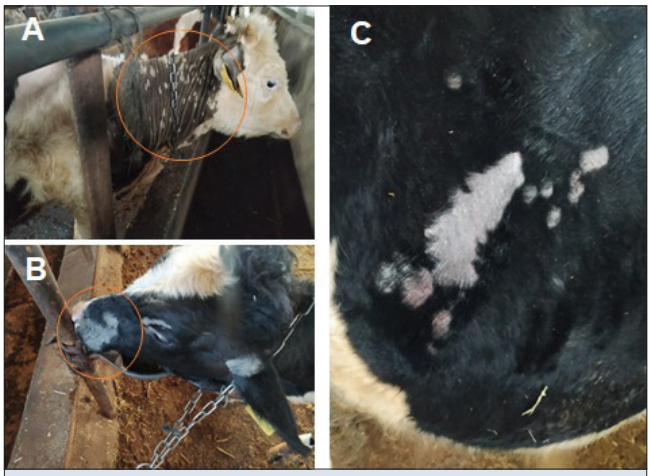

Fig 1. Macroscopic view of the sampled lesions **(A-C)**

as color, shape, size, and surface texture, were taken into account [14]. Fungal samples were stained with lactophenol cotton blue. The cellular structures, hyphae and spores were measured under the microscope.

Molecular Identification

Each of the pure fungal colonies was incubated at 25°C and 27°C for 5-7 days, and after mycelial development was achieved, the micelles were used in DNA isolation. For DNA isolation, mycelial samples were frozen at -20°C and then the cell walls were disrupted by applying liquid nitrogen. Following this physical fragmentation, the Phenol-Chloroform Isoamyl Alcohol method was used for DNA isolation [12]. After DNA isolation, the total DNA amount of the isolates was measured at 260-280 nm absorbance with a nanodrop spectrophotometer. DNAs were stored at -20°C until used in the PCR.

For the fungal isolates, the PCR method was performed as described by Bıyık et al.[15] and White [14]. For this purpose, 25 µL PCR buffer was prepared for each 35-cycle PCR reaction. Mastermix (AMPLIQON) was used for the PCR reaction. ITS1 (5'-TCCGTAGGTGAACCTGCGG-3')/ ITS4 (5'-TCCTCCGCTTATTGATATGC-3') primers were used in the PCR mix was adjusted to 25 µL with containing 12.5 µL of master mix, 9.5 µL of dH2O, 0.5 µL of each primers and 2 µL of DNA. PCR conditions were denaturation at 95°C for 2 min, annealing at 56.9°C for 30 sec, and extension at 72°C for 1 min. Sequence analysis of the amplicons whose PCR studies were completed was outsourced to Innopenta Biotechnology company. The analyzed DNA sequence results were compared with the data in GeneBank using the nBLAST program *(https://blast.ncbi.nlm.nih.gov/)* and molecular identifications were made.

The analyzed DNA sequence results were compared with the data in GenBank using the nBLAST program *(https://blast.ncbi.nlm.nih.gov/)* and molecular identification was performed. The identified isolates were registered in GenBank. A phylogenetic tree was created for the evolutionary relationships of the isolates identified using the MEGA X program. Phylogenetic tree created with the maximum likelihood method. The percentage of trees in which related taxa were brought together was determined using the bootstrap (1000 repetitions) test. Evolutionary history was evaluated using the Tamura-3 parameter model and the Maximum likelihood method. Gamma distribution (G+ parameter 1) was used to model the difference in evolutionary rates between regions.

Results

Isolation Results

Images of hair and skin rash (scraping) samples taken from 13 animals with at least five lesions after Rose Bengal

Fig 2. Images of the samples after 7 and 30 days of incubation on Rose Bengal Agar Chloramphenicol Agar, respectively

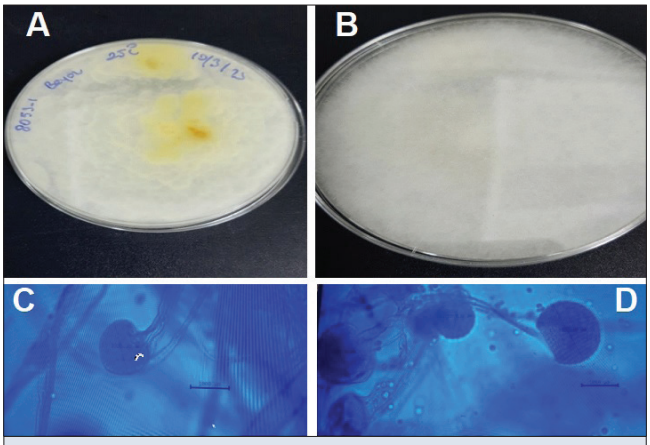

Fig 3. Morphological and microscopic images of *L. ramosa*. **A, B**- lower and upper surface of the colony; **C, D**- columella structures

Agar Chloramphenicol Agar incubation are presented in *Fig. 2*. Samples with different morphologies developed in petri dishes were purified. Purified samples were identified as *Lichthemia ramosa, Penicillium crustosum, Aspergillus niger, Aspergillus chevalieri, Aspergillus cristatus, Aspergillus flavus, Aspergillus pseudoglaucus* and *Aspergillus* sp. according to their morphological and microscopic examinations.

The fungi identified as *L. ramosa* (Zopf) Vuill. (1903) grow slowly on PDA medium in the first 5 days and in the 7^{th}-10^{th} days. It grew to cover the entire petri dish within days. The hyphae covered the entire petri dish and reached the petri lid and were white in color. The bottom surface of the colony contained yellow color in certain areas *(Fig. 3-A,B)*. The columella was spherical and measured 1000 µm *(Fig. 3-C,D,* 40X). Sporangiospores were observed as flat spheroids and mostly hemispherical.

Colonies of the fungi identified as *A. cristatus* (Raper & Fennell) formed after 7 days of incubation in MEA at 25°C were examined. The lower part of the colony was yellow, fragmented, and the edges on the colony surface were surrounded by bright yellow color, and the middle part was brown *(Fig. 4-A,B)*. Colony diameter was measured as 15-18 mm. The cleistothecium structure and the ascus structures within it were seen *(Fig. 4-C)*. Ascus and ascospores are seen in *Fig. 4-D*, and conidiaspore and conidiophore structures are seen in *Fig. 4-E*.

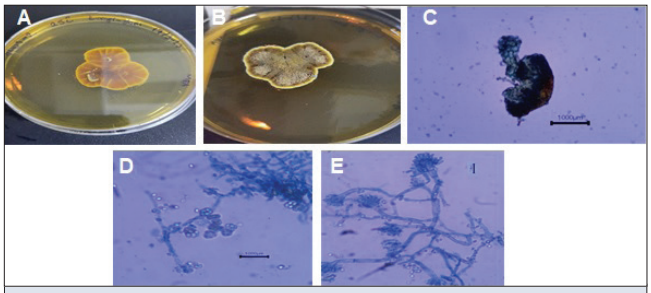

Fig 4. Morphological and microscopic images of *A. cristatus*. **A,B** - bottom of colony surface; **C**- cleistothecium and ascus structures within it; **D**- ascospores with ascus; **E**- conidiaspore and conidiophore structures

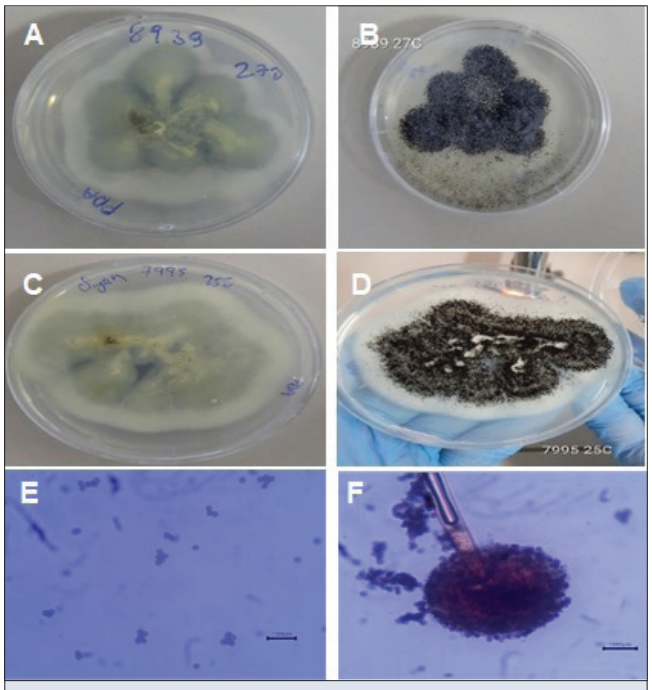

Fig 5. Morphological (A-D) and microscopic **(E, F)** images of *A. niger*

The fungi identified as *A. niger* (sensu auct. pro parte, pre 2007) spread and formed colonies on PDA medium during an incubation period of 7 days at 27°C and 25°C. Colony sizes varied between 15-25 mm in MEA and 50-60 mm in PDA medium. The lower surface of the colony was flat, surrounded by a yellow-white zone *(Fig. 5-A,C)*, and the colony surface had a black wooly structure *(Fig. 5-B,D)*. Conidia and conidiaspores were brown-black in color *(Fig. 5-E,F)*. Conidiaspores were 3-4 µm in diameter and had a rough-spiny surface.

The fungi identified as *A. chevalieri* (L. Mangin, Thom & Church 1926) formed a colony on PDA medium during 7 days of incubation at 25°C, with a yellow, shiny appearance on the lower surface and a light yellow middle part on the upper surface, surrounded by a light brown line *(Fig. 6-A,B)*. Colony sizes in MEA was measured between 15-20 mm. The cleistothecium structure was measured as 520 µm *(Fig. 6-C)*.

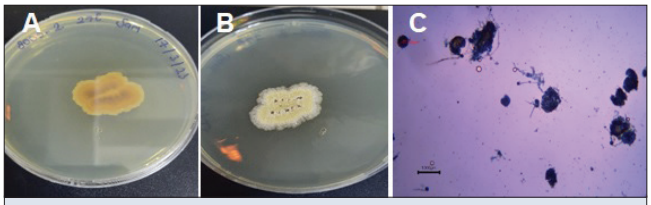

Fig 6. Morphological **(A, B)** and microscopic **(C)** images of *A. chevalieri*

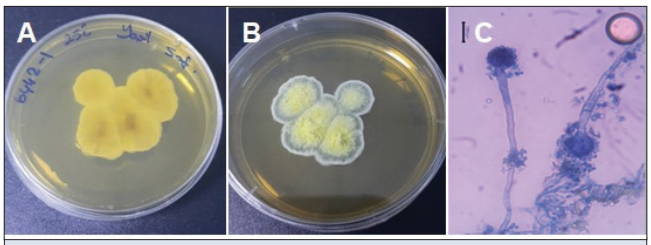

Fig 7. Morphological **(A, B)** and microscopic **(C)** images of *Aspergillus* sp.

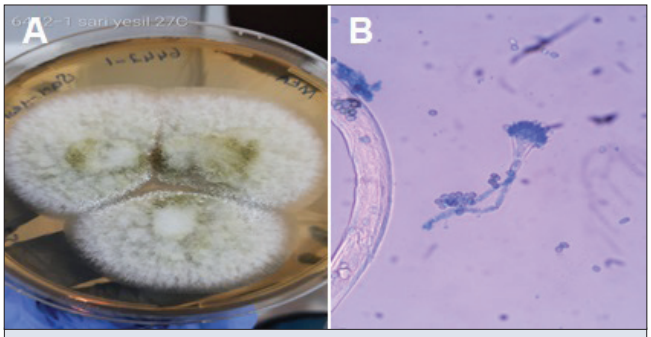

Fig 8. Morphological **(A)** and microscopic images **(B)** of *A. flavus*

The fungi identified as *Aspergillus* sp. formed a colony of 15 mm with yellow, slightly fragmented lower surface in the MEA. The upper surface of the colony was surrounded by green lines and the middle was yellow *(Fig.7-A,B)*. As a result of microscopic examination, hyphae, conidia and vesicle structures were seen, with septa and spores in a circular structure *(Fig.7-C)*.

Fungi identified as *A. flavus* (Link, 1809) formed green-white colonies with a diameter of 30-35 mm on a 60 mm petri dish in MEA *(Fig. 8-A)*. The stem of the vesicle, which had a spherical structure and a diameter of 20-30 µm, was seen to be long and the conidia had rough surfaces with a diameter of 3-4 µm *(Fig. 8-B)*.

The fungi identified as *A. pseudoglaucus* (Blochwitz) were observed to form yellow-light brown colored colonies on

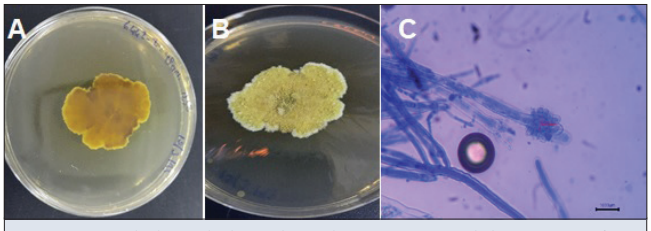

Fig 9. Morphological **(A, B)** and microscopic **(C)** images of *A. pseudoglaucus*

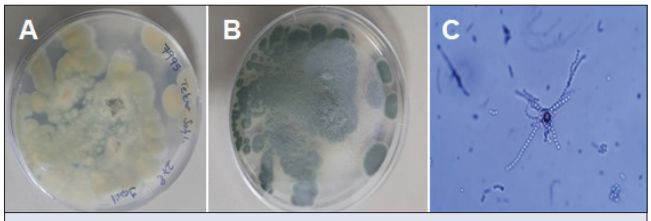

Fig 10. Morphological **(A, B)** and microscopic **(C)** images of *P. crustosum*

the PDA medium with dull yellow and short mycelium on the lower surface *(Fig. 9-A,B)*. The colony diameter was measured as 30 mm and the vesicle section at the end of the long stalk was measured as 700 µm with a 40X objective *(Fig. 9-C)*. The conidia were 4-7 µm long, had an ellipsoid structure and a rough surface.

The fungi identified as *P. crustosum* (Thom, 1930) formed colonies with a yellow-cream colored lower surface and a green powdery structure on the upper surface of the colonies growing on PDA medium *(Fig.10-A,B)*. A colony surrounded by a white thin structure and forming a light yellow pigmentation was observed. Conidia were spherical and in long chains *(Fig.10-C)*.

Molecular Identification Findings

Thirteen fungus samples, which were predicted to be morphologically different from the fungus samples growing in petri dishes, were sent to the sequencing service. As a result of the sequencing, 8 different species, some of which were the same species, were identified from these 13 fungal samples. As a result of the identification, of the 13 samples examined in the study (hair and skin rash/scratching), 12 samples contained *L. ramosa* HBF570, 7 samples contained *A. niger* HBF572 and *P. crustosum* HBF571, two samples each contained *A. chevalieri* HBF573 and *A. flavus* HBF576, and one sample each contained *A. pseudoglaucus* HBF577 and *Aspergillus* sp. HBF570 was determined.

The species obtained as a result of the sequence were recorded in GeneBank. GeneBank registration codes and accession numbers of the species are given in *Table 1*. The

Table 1. *Data obtained as a result of the sequence*

Species Name	Accession Number	Blast Similarity (%)
Lichthemia ramosa HBF570	OR588056	98
Penicillium crustosum HBF571	OR588057	99
Aspergillus niger HBF572	OR588058	99
Aspergillus chevalieri HBF573	OR588059	97
Aspergillus cristatus HBF574	OR588060	99
Aspergillus sp. HBF575	OR588061	96
Aspergillus flavus HBF576	OR588062	98
Aspergillus pseudoglaucus HBF577	OR588063	100

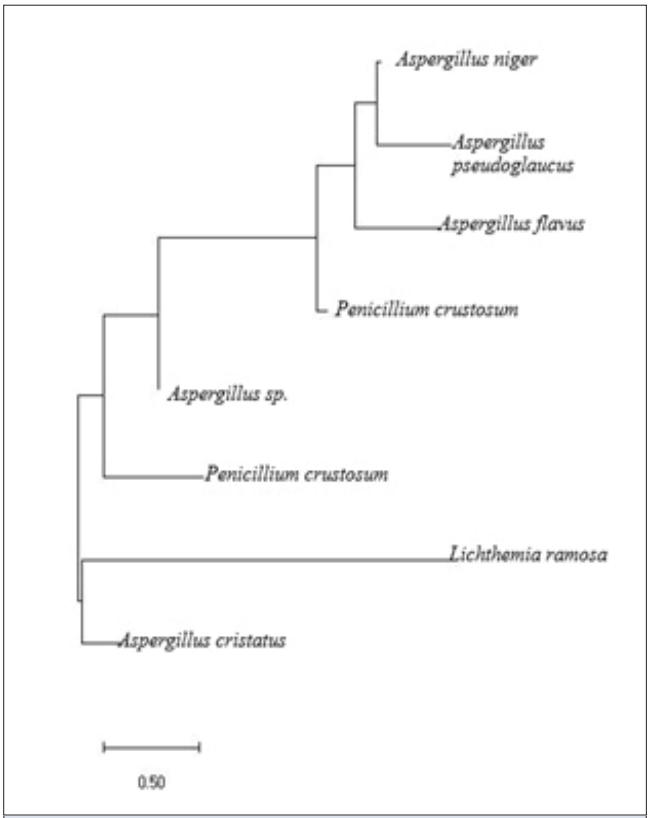

Fig 11. Phylogenetic tree model of isolated species

data obtained after molecular identification was analyzed in the MEGA X program and a phylogenetic tree was created *(Fig. 11)*.

After inoculation and incubation of the study samples by serial dilution, colonies with different morphological characteristics developed in the petri dishes. Considering the development density of these colonies in the petri dish, the development density of the species identified in each sample is expressed in *Fig. 12*. In the figure, the different species isolated and identified from each sample are given in their own column. The isolated species were expressed by dividing them according to a numerical ratio within the column, depending on the number of colonies in the petri dishes.

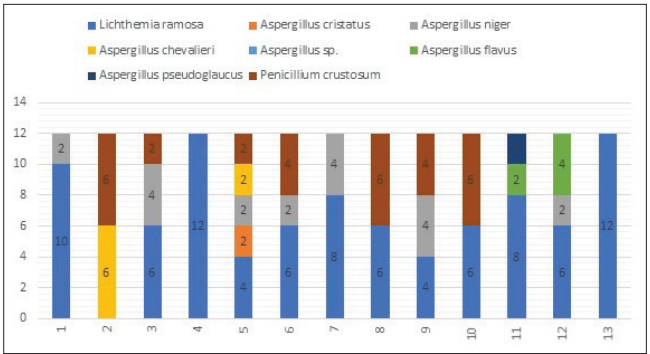

Fig 12. Number of colonies (density/distribution) of species isolated from hair and skin samples

Discussion

Mucormycosis (zygomycosis) is a dangerous but uncommon fungal infection caused by the Mucormycetes mold class. These fungi can be found all over the place. They are found in soil and decaying organic materials such as leaves, compost piles, and rotten wood [15]. Mucormycosis is caused by *Rhizopus, Mucor, Rhizomucor, Syncephalastrum, Cunninghamella Bertholletiae, Apophysomyces*, and *Lichtheimia* species [18]. The main source of infection is direct contact with fungus spores in the environment. *L. ramosa* is a fungus species that has been discovered as the dominating (dense) species in hair and skin scraping samples in this investigation. It is an opportunistic pathogen in both animals and humans. This type of fungus produces mucormycosis, which damages several organs. The only report on *L. ramosa* in our nation was of pulmonary mucormycosis in a human with HIV infection [19], but no reports on animals were discovered. Many diseases caused by *L. ramosa* are being studied around the world. *L. ramosa* was found in the internal organ tissues of a pregnant cow in a study conducted in Korea when different tissue samples were evaluated after death. This study documented the case of angioinvasive mucormycosis in cattle [3]. Tanaka et al.[9] investigated a case of rhinocerebral zygomycosis in calves caused by *L. ramosa* infection. The fungus infiltrated the brain via the olfactory nerves, according to histopathological analysis. This study was reported as the case of rhinocerebral zygomycosis affecting cattle. A recent study on the identification of *L. ramosa* in tissues was conducted and presented by Iwanaga et al.[7]. The researchers claimed that the histological study performed after the necropsy of a 12-day-old male calf that died as a result of different neurological abnormalities was the report of striatal necrosis induced by *L. ramosa* in a newborn calf. Furthermore, mucoral species such as *L. corymbifera* and *L. ramosa* have been detected in the rumen fluid of healthy calves and may cause endogenous infections. *L. ramosa* was found in samples taken from the calf neck area of a study in which dermophyte and non-dermophyte fungi isolated from lesional areas of different animals were identified. ITS regions were used in fungus molecular identification investigations [20]. This study's technique and findings sections overlap with ours. According to the research, *L. ramosa* has been detected in several cases in animals. *L. ramosa* was found in all but one of the hair follicles and skin scrapings/rash collected from lesional regions in cattle in our investigation.

Aspergillus species produce diseases known as "aspergillosis" in mammals. In pregnant animals, aspergillosis causes gastrointestinal disorders, lung tissue damage, and waste [21]. When *Aspergillus* instances in cattle were evaluated, mycotic abortion was frequently seen. *A. flavus, A. fumigatus, A. nidulans, A. terreus, A. niger*, and *A. versicolar* are the species that induce waste in cattle [22]. *A. cristatus* is a species that has been isolated from many different parts of the world. This fungus, known as golden blossom in China, is utilized as a flavour during the fermentation stage of a local tea [23]. It's been isolated from strawberry puree and Philippine peanuts as well [11]. These fungi have been found in the Kocaeli-Çatalca region of Türkiye [24]. *A. niger* is a fungus that has been thoroughly studied and has numerous features defined among all fungal species. It produces mycotoxin and has a classic mold appearance with its black conidia. Although this species is common in fruits, vegetables, cereals, and nuts around the world, it has also been detected in a variety of herbal items in our country. *A. chevalieri* is a common fungus species that causes spoiling in various plant products and foods (cereals, chocolate, fatty nuts, meat, and fruits) due to its enzymatic activities. *A. pseudoglaucus* is another species that causes food deterioration. This strain was identified from cheese, processed meat, and grains. It has been observed in our country from the Aegean, the Çatalca-Kocaeli, and Sakarya. Previously, *Penicillium crustosum* was isolated from animal feed and has been linked to crop losses in cereals [11]. In Türkiye, *P. crustosum* has also been isolated from rice and wheat [25].

It is thought that the *Aspergillus* species identified in the study contaminated calf hair and skin from their spores found in the barn environment. Likewise, it is predicted that *L. ramosa* HBF570 may be present in the barn environment and cause lesions by contaminating calf hair and skin. The pathogenicity of *Aspergillus* and *L. ramosa* species in animals and humans may have been learned earlier. These species include lung, nasal sinus, brain, eye, skin, gastrointestinal tract and multiple causes systemic ethnicity [26]. Fungi belonging to the order *Mucorales* cause saprophytic opportunistic infections [27]. In the study, the sequences were documented in GeneBank as a report on the isolation and identification of *L. ramosa* HBF570 from cow hair and skin scrapings. Other species isolated in the study were molecularly identified from bovine lesional skin by comparison with the updated data of Asan [28] and entered into the gene bank records. Control of these pathogenic agents is important for animal health. These agents can enter the body system through the air and skin and cause a systematic disease. For this reason, hygiene studies should be carried out by taking into account the richness of fungi in the barn environment, and diseases should be prevented from infecting the internal organs of the cow through the hair-skin surface and air.

Declarations

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

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Ethical Statement: Animal Experiments Local Ethics Committee Approval is not required for the study.

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Author Contributions: Experimental design was performed by BB, TG, and AÇ; material preparation and analysis were performed by HHB, SA and SÖK; results were interpreted by AÇ, TG, BB and HHB; the first draft of the manuscript was written by AÇ, BB and HHB; all authors contributed to the final version of the manuscript.

References

1. Ali R, Khan IH: Mycotic abortion in cattle. *Pak Vet J,* 26 (1): 44-46, 2006.

2. de Hoog G, Guarro J, Gené J, Figueras MJ: Atlas of Clinical Fungi. 2nd ed., American Society for Microbiology, Washington, DC, 2000.

3. Lee K, Kim H, Sohn J, So B, Choi EJ: Systemic mucormycosis caused by *Lichtheimia ramosa* in a pregnant cow. *Vet Med,* 65 (11): 506-510, 2020. DOI: 10.17221/8/2020-VETMED

4. Jensen H, Olse SN, Aalbaek B: Gastrointestinal aspergillosis and zygomycosis of cattle. *Vet Pathol,* 31, 28-36, 1994. DOI: 10.1177/030098589403100104

5. Pesca C, Cruciani D, Agostini L, Rossi E, Pavone S, Stazi M, Mangili P, Crotti S: Simultaneous detection of *Aspergillus nidulans*, *Aspergillus luchuensis and Lichtheimia* sp. in a bovine abortion. *J Mycol Med,* 30:100923, 2020. DOI: 10.1016/j.mycmed.2019.100923

6. Schwartze VU, Jacobsen ID: Mucormycoses caused by *Lichtheimia* species. *Mycoses,* 57, 73-78, 2014. DOI: 10.1111/myc.12239

7. Iwanaga M, Kamikawa A, Imai N, Shimada K, Degawa Y, Hanafusa Y, Shibahara T: Striatal necrosis caused by *Lichtheimia ramosa* in a neonatal calf. *J Vet Med Sci,* 83 (12): 1943-1947, 2021. DOI: 10.1292/jvms.21-0499

8. Nguyen TTT, Santiago ALCM, Kirk PM, Lee HB: Discovery of a new *Lichtheimia (Lichtheimiaceae, Mucorales)* from invertebrate niche and its phylogenetic status and physiological characteristics. *J Fungi,* 9:317, 2023. DOI: 10.3390/jof9030317

9. Tanaka Y, Toyotome T, Inokuma H, Watanabe KI, Kobayashi Y, Horiuchi N: *Rhinocerebral zygomycosis* due to a *Lichtheimia ramosa* infection in a calf: Neural spread through the olfactory nerves. *Mycopathologia,* 184, 141-146, 2019. DOI: 10.1007/s11046-018-0296-z

10. Tsui CKT, Woodhall J, Chen W, Lévesque CA, Lau A, Schoen CD, Baschien C, Najafzadeh MJ, de Hoog GS: Molecular techniques for pathogen identification and fungus detection in the environment. *IMA Fungus,* 2, 177-189, 2011. DOI: 10.5598/imafungus.2011.02.02.09

11. Njambere EN, Attanayake RN, Chen W: Applications of Molecular Markers and DNA Sequences in Identifying Fungal Pathogens of Cool Season Grain Legumes. **In,** Gherbawy Y, Kerstin Voigt K (Eds): Molecular Identification of Fungi. 79-92, Springer, 2010.

12. Balıbay Ö, Tel OY: Sığırlardan *Trichophyton verrucosum*'un kültür ve MALDI-TOF MS yöntemleri ile identifikasyonu. *Dicle Üniv Vet Fak Derg,* 14 (1): 43-47, 2021. DOI: 10.47027/duvetfd.836008

13. Pitt JI, Hocking AD: Fungi and Food Spoilage. 3rd ed., Springer, 2009.

14. Kaufman PB, Wu W, Kim D, Cseke LJ: Handbook of molecular and cellular methods in biology and medicine. CRC Press, Boca Raton, Florida, 1995.

15. Bıyık H, Törün B, Geroğlu Y, Poyrazoğlu ÇE, Başbülbül G: Preservation and molecular identification of *Aspergillus* and *Penicillium* species with ITS-PCR. *Eur J Biotechnol Biosci*, 4 (11): 4-7, 2016.

16. White TJ: Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. **In,** PCR Protocols: A Guide to Methods and Applications. 315-322, Scientific Research Publisher, 1990.

17. Richardson M: The ecology of the *Zygomycetes* and its impact on environmental exposure. *Clin Microbiol Infect*, 15 (5): 2-9, 2009. DOI: 10.1111/j.1469-0691.2009.02972.x

18. Roden MM, Zaoutis TE, Buchanan WL, Knudsen TA, Sarkisova TA, Schaufele RL, Sein M, Sein T, Chiou CC, Chu JH, Kontoyiannis DP, Walsh TJ: Epidemiology and outcome of zygomycosis: A review of 929 reported cases. *Clin Infect Dis*, 41 (5): 634-653, 2005. DOI: 10.1086/432579

19. Kutlu M, Ergin Ç, Bir F, Hilmioğlu-Polat S, Gümral R, Necan C, Sayın-Kutlu S: Pulmonary mucormycosis due to *Lichtheimia ramosa* in a patient with HIV infection. *Mycopathol*, 178, 111-115, 2014. DOI: 10.1007/s11046-014-9761-5

20. Rahimi T, Mohammadi R: Identification of dermatophyte and nondermatophyte molds isolated from animal lesions suspected to dermatomycoses. *Adv Biomed Res*, 9:4, 2020. DOI: 10.4103/abr.abr_230_19

21. Headley SA, Müller MC, de Oliveira TES, Duarte CABG, Pereira PFV, Vieira MV, Pretto-Giordano LG: Diphtheric aspergillosis tracheitis with gastrointestinal dissemination secondary to viral infections in a dairy calf. *Microb Pathog*, 149:104497, 2020. DOI: 10.1016/j.micpath.2020.104497

22. Prameela DR: Mycotic abortions in bovine: A review. *J Anim Feed Sci Technol*, 7 (2): 87-91, 2019. DOI: 10.21088/jafst.2321.1628.7219.5

23. Tan Y, Wang Y, Ge Y, Ren X, Wang Y, Liu Z: Isolation and molecular identification of *Aspergillus cristatus* in fermented fuzhuan brick tea from Guizhou Province. *Mycosyst*, 36, 154-163, 2017.

24. Abacı GÖ, Akata I, Akgül H, Aktaş S, Alkan S, Allı H, Aydoğdu H, Berikten D, Demirel K, Demirel R, Doğan HH, Erdoğdu M, Ergül CC, Eroğlu G, Giray G, Uztan HA, Kabaktepe Ş, Kadaifçiler D, Kalyoncu F, Karaltı İ, Kaşık G, Kaya A, Keleş A, Kırbağ S, Kıvanç M, Ocak İ, Ökten S, Özkale E Öztürk C, Sevindik M, Şen B, Şen İ, Türkekul İ, Ulukapı M, Uzun Y, Yoltaş A: Türkiye mantarları listesi (The checklist of fungi of Turkey). **In,** Sesli E, Asan A, Selçuk F (Eds): Türkiye Mantarları Listesi. İstanbul, Ali Nihat Gökyiğit Vakfı Yayını, 1177 p. İstanbul, Türkiye, 2020.

25. Kocer F, Imali A: Microfungus flora of rice *(Oryza sativa* L.) and wheat *(Triticum durum* L.) grains from Çorum region (Turkey). *Int J Sci Technol Res*, 5 (9), 93-98, 2019. DOI: 10.7176/JSTR/5-9-12

26. Walsh TJ, Hayden RT, Larone DH: Mucormycosis (Zygomycosis). **In,** Westblade LF, Burd EM, Lockhart SR, Procop GW (Ed): Larone's medically important fungi: A Guide to Identification. 7th ed., 44, John Wiley & Sons, 2018.

27. Slaviero M, Vargas TP, Bianchi MV, Ehlers LP, Spanamberg A, Ferreiro L, Pavarini SP: Rhizopus microsporus segmental enteritis in a cow. *Med Mycol Case Rep*, 28, 20-22, 2020. DOI: 10.1016/j.mmcr.2020.04.002

28. Asan A: *Aspergillus*, *Penicillium* and related species reported from Turkey. *Mycotaxon*, 89 (1), 155-157, 2004.

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Research Article

Analysis of Effect of Hyperthermia on White Blood Cells of Experimental Rats Using Geometric Morphometrics Method

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Abstract

An elevated temperature in the environment increases the core body temperature which stimulates thermoregulatory mechanisms. The aim of this study was to examine the effect of elevated temperature on number and shape of leukocytes using descriptive statistics and geometric morphometrics. The study included 32 Wistar rats divided into two groups according to exposed temperature (41°C and 44°C) and more two subgroups in groups associated with death (antemortem and postmortem). Peripheral blood smears were performed before exposure to temperature and after exposure to temperature. The number of leukocytes was determined. Images of peripheral blood smears were taken, on which eight landmarks on the outside of leukocytes were marked in tpsDig program. In the MorphoJ program, we analyzed shape of leukocytes. The results showed significant differences in the number of neutrophils and lymphocytes in subgroup antemortem and in subgroup postmortem exposed to a temperature of 41°C. In group exposed to a temperature of 44°C, a significant difference was found in the number of all leukocytes, except monocytes in the antemortem subgroup, and except basophils and eosinophils in the postmortem subgroup. The results of geometric morphometrics shape analysis showed significant differences in the shape of lymphocytes between subgroups antemortem and postmortem.Exposure to elevated temperature resulted with number and shape changes of leukocytes.

Keywords: hyperthermia, white blood cells, geometric morphometrics, experimental rats

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Introduction

The negative effects of elevated air temperature on the body are numerous and are noticeable in both healthy and sick individuals. The body's internal temperature can reach 40.5°C, which can result in significant damage to all organ systems, and the most severe consequences will occur if the collapse of the central nervous system develops as a result of hyperthermia, which will eventually lead to an imbalance within the entire organism [1].

Precisely for this reason, there was a need to research the effects of hyperthermia on certain cells, organs, and organ systems. These studies can be performed on different cells, but due to the fact that changes in blood cells can be found very quickly after exposure to high temperatures, the hematological parameters play a very important role in the diagnosis and prognosis of the effects of heat stress on the body [2]. The established daily rhythm of body temperature is regulated by a complex thermoregulatory center, localized in the preoptic nucleus of the front part of the hypothalamus [3]. With constant exposure to high temperatures, the organism must invest a certain amount

of energy to activate various adaptation mechanisms in order to prevent its collapse or damage. The activity of these mechanisms depends on the preservation and functionality of the respiratory, circulatory, nervous, and endocrine systems, and on the function of certain body enzymes, which represent very important links that contribute to the complex process of adaptation of the organism to elevated temperatures, with the least possible harmful consequences on the organism itself [4]. However, thermoregulation can only be carried out within certain limits of ecological valence, below that limit hypothermia occurs, and above that limit hyperthermia occurs, i.e. overheating, which acts as a stressogenic factor on the animal's organism [5].

The increased temperature of the environment affects the general condition of the organism, which results in reduced physical activity, reflects on the motor and cognitive functions of the organism, and increases the risk of health problems, which has been proven by numerous scientific studies [6-8]. Taking into account global warming, it is necessary to examine how the increased temperature of the environment is reflected on the organs and organ systems, as well as on the cells of the organism exposed to the increased temperature. It is also necessary to investigate how the organism adapts to conditions of increased environmental temperature.

Disorders of the leukocyte lineage after exposure to heat stress are noticeable in the form of an increase in the number of granulocytes and the ratio of granulocytes to lymphocytes, which is a feature of inflammation [9]. Numerous cellular and physiological effects occur after long-term exposure to high temperature, which are used to improve temperature tolerance, but also to change the innate immunological response. Increased temperature causes activation of phagocytes, granulocytes and infiltration of lymphocytes, and expression of NK cells [10,11].

It has also been proven that heat can lead to disturbances at the molecular level, causing numerous changes in the leukocyte genes of the peripheral blood, which consequently leads to a series of other changes within the organism [12].

In the literature, there are studies where the authors examined the impact of increased environmental temperature on animals. In a study conducted by Rana et al.[13] in 2014, the authors examined the effect of exposure to increased sunlight on indigenous sheep from Bangladesh. The authors monitored how exposure to elevated temperature over a long period of time affects red blood cells (RBC), hemoglobin (Hb%), compacted cell volume (PCV%) and white blood cells (WBC). This research is of great importance considering that sheep are exposed to high sunlight when grazing grass, which has a great impact on the economy in the sheep industry. The results of the study showed that exposure to elevated temperature for a long time resulted in increased values of the number of RBC, Hb% and PCV% which wa statistically significant, while changes in WBC were not statistically significant.

In a study conducted in 2019, Mofizur and the authors examine the impact of increased water temperature on juvenile Red Spotted Grouper *Epinephelus akaara* by analyzing blood elements. The study included 180 tested fish that were exposed to water temperatures of 25°C, 28°C, 31°C and 34°C for 6 weeks. Blood analysis was performed after the second, seventh and forty-second days. An increase in the number of red blood cells and leukocytes was observed in the groups that were exposed to temperature 31°C and 34°C after six weeks, and there were abnormalities in erythrocyte cells. In the group exposed to temperature 34°C, the analysis of number of leukocytes showed an increase in the number of neutrophils and a decrease in the number of lymphocytes. The authors concluded that exposure to elevated water temperature 31°C and 34°C changes the morphology and number of blood cells in red spotted grouper, which may affect their immune system [14].

Exposure to elevated temperature that conditions hyperthermia was used for therapeutic purposes in cancer patients, treated with whole body hyperthermia and chemotherapy, before, during and one day after the treatment. Apoptosis rates of the entire lymphocytes and natural killer cells were determined. During treatment, there was a significant increase in apoptosis in the entire lymphocytes. In contrast, an increased rate of apoptosis in natural killer cells was observed 20 hours after the finished therapy [15]. Two treatment whole body hyperthermia protocols are most commonly used: long-term exposure to slightly elevated temperature and short-term exposure to very elevated temperature [16].

It was established that the number of blood cells cannot be a realistic indicator of the true effect of heat, and that morphological changes in blood cells should also be taken into account in order to reach a conclusion about the real effects of heat. The number of blood elements may be within the reference values, but their function and form are completely incorrect and ineffective [17].

The aim of this study was to examine the effect of elevated temperature on number and shape of leukocytes on experimental rats using descriptive statistics and geometric morphometrics method.

Material and Methods

Ethical Statement

The study was conducted at the Faculty of Veterinary Medicine of the University of Sarajevo, by valid ethical principles on biomedical research on animals and after obtaining approval from the Ethical Committee at the Faculty of Veterinary Medicine of the University of Sarajevo (number: 07-03-850-4/22).

In the study was included 32 albino Wistar rats of both sexes, six months old and weighing from 250 to 300 g. All animals were kept under the same laboratory conditions and for seven days before the experiment they were kept in a vivarium with a 12-h light regime day-night and at room temperature (20°C±2°C) for the purpose of acclimatization and adaptation. During the duration of the experiment, the animals received commercial feed for laboratory animals and running water ad libitum. Keeping and caring for animals, as well as conducting all experimental procedures, were carried out in compliance with international guidelines for biomedical research on animals - CIOMS (The Council for International Organizations of Medical Sciences) and ICLAS (The International Council for Laboratory Animal Science).

The state of hyperthermia was achieved by immersing the rats in water of temperature 41°C and 44°C. Before the experiment, the rats were anesthetized with 10 mg/kg xylazine HCl (Rompun® 2%, Bayer) and 50 mg/kg ketamine HCl (Ketaminol® 100 mg/mL, MSD Animal Health), by intramuscular injection into the thigh muscle (m. quadriceps). We filled the water bath with water and heated it to the target water temperature. The temperature of the water was continuously monitored on the display with an additional measurement with a probe immersed in the water and a reading on the thermometer. We immersed a previously anesthetized rat with its head above the water level in pre-heated water of the target temperature. The survival time was recorded, which included the time from the immersion of the rats in the water of the given temperature (41°C and 44°C) until the moment when death was established. We defined hyperthermia as an increase of 0.5°C in internal temperature, and heat stroke as an increase in internal temperature above 40.5°C.

Experimental rats was divided into two groups according to exposed temperature (41°C and 44°C) which were further divided into two subgroups (antemortem and postmortem). Before exposure to temperature, peripheral blood smears were performed on each experimental rat so that the animals themselves were the control.

Immediately before immersion in water, an esophageal probe for measuring the internal temperature (RET-4 Probe for mice and rats) was placed in the esophagus (5 cm) of an anesthetized rat, and the core temperature was continuously read on a thermometer (Physitemp Thermalert Model TH-8) and recorded before immersion, immediately after immersion, at the 20th min, and at the moment of death. The thermometer and probe for temperature measurement is manufactured by Physitemp, Instruments Clifton, USA.

Blood for obtaining serum was sampled on two occasions: 7 days before the planned experiment from the tail vein of anesthetized rats (control group) and another blood sampling from the abdominal aorta during the duration of the experiment. Peripheral blood smear with standard staining (Giemsa) was performed. On peripheral blood smears for each experimental rat in subgroups the number of leukocytes (neutrophils, lymphocytes, basophils, monocytes, eosinophils) was determined for analysis of changes of the numbers of leukocytes. For analysis of changes of morphology of leukocytes, images of leukocytes on peripheral blood smears were taken. On twodimensional models of leukocytes eight landmarks on the outside were marked in tpsDig program. Position of the landmarks is defined by values of x and y axis in coordinate system. This values for all landmarks and for all leukocytes we entered in the MorphoJ program where we analyzed shape of leukocytes and their difference between groups.

Statistical Analysis

All statistical analyses were performed using SPSS version 13.0 for Windows (Chicago, IL, USA).

The distribution of quantitative variables was tested using the Kolmogorov-Smirnov test. Results of descriptive statistics for continuous variables were expressed as mean and standard deviation (SD) for normally distributed variables or as median and interquartile range for variables with skewed distributions.

The categorical variables were reported as frequencies and percentages. An independent two-sample Student t-test assessed the significance of the mean differences between the two groups. The Mann-Whitney U-test assessed the difference in the values of parameters that showed a non-normal distribution. The differences between categorical variables were assessed using the Chi-square test. P values less than 0.05 were considered statistically significant.

For shape analysis of leukocytes we used geometric morfpmetrics method. On two-dimensional models of leukocytes we marked 8 landmarks on each leukocytes cells using tpsDig program. In MorphoJ program we analysed differences on the shape of leukocytes between experimental groups using Principal Component Analysis (PCA) and Discriminant Functional Analysis (DFA).

Table 1. *Mean values of the number of leukocytes in the control group and in the antemortem group of rats exposed to temperature 41°C*

Type of Leucocytes	Basal Values in Control Group Antemortem-41 (n=6)	Antemortem (n=6)	P
Neutrophils x 10^9/L	19.7±8.2	6.3±4.6	0.004
Lymphocytes x 10^9/L	73.3±8.4	86.0±6.6	0.009
Basophils x 10^9/L	1.17±2.4	1.3±3.3	0.931
Monocytes x 10^9/L	1.17±1.8	1.67±1.5	0.681
Eosinophils x 10^9/L	4.67±2.9	4.67±2.3	1.0

Table 2. *Mean values of the number of leukocytes in the control group and in the postmortem group of rats exposed to temperature 41°C*

Type of Leucocytes	Basal Values in Control Group Postmortem-41 (n=7)	Postmortem (n=7)	P
Neutrophils x 10^9/L	18.0±5.4	7.0±6.9	0.022
Lymphocytes x 10^9/L	76.0±5.9	81.3±6.4	0.183
Basophils x 10^9/L	0.29±0.49	1.0±1.53	0.220
Monocytes x 10^9/L	1.7±1.5	7.1±4.0	0.011
Eosinophils x 10^9/L	4.0±1.5	3.6±1.6	0.667

Table 3. *Mean values of the number of leukocytes in the control group and in the antemortem group of rats exposed to temperature 44°C*

Type of Leucocytes	Basal Values in Control Group Antemortem-44 (n=6)	Antemortem (n=6)	P
Neutrophils x 10^9/L	18.3±2.7	3.8±4.2	< 0.001
Lymphocytes x 10^9/L	73.4±4.4	93.7±5.7	< 0.001
Basophils x 10^9/L	0.67±0.52	0.0±0.0	0.025
Monocytes x 10^9/L	3.5±2.6	1.0±1.7	0.161
Eosinophils x 10^9/L	4.17±1.3	1.5±1.5	0.01

Results

The number of neutrophils decreased statistically significantly, and the number of lymphocytes increased statistically significantly in the group of rats exposed to temperature 41°C- antemortem *(Table 1)*.

The number of neutrophils decreased statistically significantly and the number of monocytes increased statistically significantly in the postmortem group of rats exposed to a temperature of 41°C *(Table 2)*.

The number of neutrophils, basophils, and eosinophils decreased statistically significantly and the number of lymphocytes increased statistically significantly in the antemortem group of rats exposed to a temperature of 44°C *(Table 3)*.

The number of neutrophils and eosinophils decreased statistically significantly and number of lymphocytes increased statistically significantly in the postmortem group of rats exposed to temperature of 44°C *(Table 4)*.

Table 4. *Mean values of the number of leukocytes in the control group and in the postmortem group of rats exposed to temperature 44°C*

Type of Leucocytes	Basal Values in Control Group Postmortem- 44 (n=7)	Postmortem (n=7)	P
Neutrophils x 10^9/L	15.6±1.99	3.6±2.8	< 0.001
Lymphocytes x 10^9/L	78.1±1.6	94.3±3.99	< 0.001
Basophils x 10^9/L	0.79±0.91	0.0±0.0	0.062
Monocytes x 10^9/L	2.1±1.6	1.4±1.6	0.526
Eosinophils x 10^9/L	3.4±1.3	1.0±1.4	0.001

Table 5. *Results of correct classification test of lymphocytes of rats using geometric morphometrics for external surface shape analysis*

True	Allocated to Lymphocytes-Antemortem	Allocated to Lymphocytes-Postmortem	Total Number
Number of lymphocytes-antemortem	**33**	16	49
Number of lymphocytes-postmortem	18	**39**	57

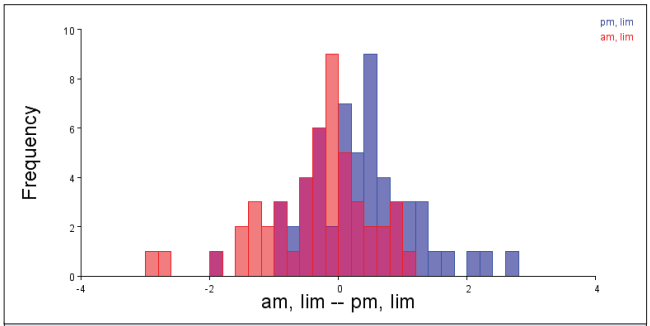

Fig 1. Results of discriminant functional analysis of the shape of lymphocytes between antemortem and postmortem groups of rats

Geometric morphometrics method is used for analysis of shape of leukocytes where provided general Procrustes analysis, test of correct classification and discriminant functional analysis.

Test of correct classification based on the shape of external surface of lymphocytes using geometric morphometrics correct classified 33 of 49 lymphocytes in the group antemortem (67.35% accuracy), and 39 of 57 lymphocytes in group of postmortem (68.42% accuracy). Results of test of correct classification showed in *Table 5*. These differences are statistically significant, P value was 0.0104.

Fig. 1 showed the results of discriminant functional analysis of external surface shape analysis of lymphocytes in rats exposed to temperature.

Discussion

After the target organism is exposed to long-term hyperthermia, a whole series of negative effects arise in its immune system. Long-term exposure to hyperthermia changes the immune response of organs and tissues, leading to the activation of neutrophils at the site of inflammation, infection or injury caused by elevated temperature [18].

The present study showed a statistically significant decrease in the number of neutrophils in the antemortem and postmortem groups after exposure to temperatures of 41 and 44 degrees, while in both groups there was an increase in the number of lymphocytes. Since neutrophils have the shortest lifespan of all leukocytes, it is believed that this could be the reason for the statistically significant decrease in their number, which was confirmed by the results of our study. The lifespan of neutrophils in the blood lasts an average of 8 hours, while in the tissues they can last from 1 to 4 days [19]. Exposure of neutrophils to high temperatures leads to the creation of mediators that accelerate their apoptosis, which results in a decrease in their number. There are many such mediators; among them, cytokines, chemokines, and some conditions such as acidosis and hyperthermia stand out [20]. Raheim et al.[21] stated that such changes in neutrophils may be the result of changes in the action of the Na/K pump, which results in a change in intracellular osmolarity, but it is not excluded that hyperthermia may lead to direct mechanical damage to the cell membrane.

Although a statistically significant change in the number of neutrophils was observed, no statistically significant difference in the shape of neutrophils was found using geometric morphometrics. The reason for this may be because hyperthermia led to the destruction of a larger number of neutrophils, which ultimately led to a low absolute number of relatively preserved neutrophils on which shape could be assessed by geometric morphometrics. In a pilot study on rats, Iba et al.[22] neutrophils were presented with a hypersegmented, clustered nucleus, but no changes in the shape of the outer surface of neutrophils were observed. In our study, in the geometric morphometrics program, we marked the landmark points on the outer surface of the cell membrane and not on the nuclei. Unlike neutrophils, lymphocytes showed statistically significant shape changes using geometric morphometrics. Exposure of rats to hyperthermia consequently led to an increase in the number of lymphocytes, which allowed us to initially have a higher absolute number of lymphocytes that we could subject to geometric morphometrics analysis.

In our study, eosinophils were more sensitive and showed a significant decrease in number compared to basophils, after exposure to a temperature of 44°C. A study by Edwards et al.[23] on pigs showed a special sensitivity of eosinophils to an increase in temperature, in which necrobiotic changes and extreme lobulation of the nucleus occurred after exposure to hyperthermia for one h. Such changes undoubtedly reduce their number in the peripheral blood smear, which agrees with our results. Salanova et al.[24] state that exposure to heat also affects the internal processes of neutrophils, interfering with signal transmission within the cell itself. This type of destabilization of cellular processes can occur even during short-term exposure to heat stress.

In the blood of rats under physiological conditions, the number of eosinophils, basophils and monocytes is low. Considering this, it is very difficult to determine the effect of elevated temperature on these cells. In our study, too, we only found a decrease in the number of eosinophils in two groups of rats after exposure to a temperature of 44°C, while an increase in the number of monocytes was recorded only in one group [25]. Our results correlate with the results of the study by Mahmutovic and associates, where it was found that eosinophils did not show statistically significant variations during the test, which is expected, according to their role in the body [26].

In vitro studies showed that an increase in temperature leads to increased production of lymphocytes and antibodies [27]. In our study, there was also a significant increase in the number of lymphocytes in all tested groups, regardless of the exposed temperature. An exception is the postmortem group after exposure to a temperature of 41°C. The results of the previous research showed a significant decrease in the number of T lymphocytes in organisms exposed to hyperthermia in a standardized hyperthermic spa with a mean increase in rectal temperature of 1.35°C, but without a significant change in the total number of lymphocytes [28].

In our study, we did not classify lymphocytes into B or T lymphocytes. Therefore, the unchanged number of lymphocytes in one group may be the reason for the missing additional classification. Also, Ashman et al.[29] state that blood T-lymphocytes incubated at 39°C show an increased response to the stimulatory effect of IL1 and IL2 on their proliferation, compared to those incubated at a temperature of 37°C. The same response to stimulation by the mentioned interleukins in B-lymphocytes was not recorded. In this study, it is stated that hyperthermia predominantly affects the proliferation of T-lymphocytes. Using the ELISPOT technique, Huang et al.[30] found that hyperthermia has a stimulating effect on B-lymphocytes, leading to increased production of IgG, IgA and IgM antibodies. This effect on B-lymphocytes refers to the effect of temperatures up to 42°C, because temperatures above 42°C oposite effect. Exposure to heat stress leads to an increase in the number of granulocytes and to an

increased ratio of granulocytes to lymphocytes, which is characteristic of inflammation [6].

An increased number of monocytes was found only in the postmortem group of rats exposed to a temperature of 41°C, without statistically significant changes in the shape of monocytes. Previous studies have shown that monocytes are most often attached to platelets, which indicates their interaction during heat stress [30]. This can ultimately explain such a small number of observed monocytes in our study has an inhibitory effect. Bouchama et al.[31] stated that hyperthermia is related to an increase in the number of total leukocytes as well as an increase of the percentage of its subpopulation. This increase in the number of leukocytes is mostly due to the increase in the number of suppressor T cells and natural killer cells. The study confirmed a significant correlation of the total number of circulating lymphocytes with the severity of hyperthermia.

When interpreting the results, it should be kept in mind that rats are much more sensitive to the effects of high temperature and develop a shock state much faster with a fatal outcome. This is very important to keep in mind when presenting and comparing the results of the study on the human heat stroke model. It is also important to note that lymphocytes are the most numerous type of leukocytes in this type of animal, and regardless of the fact that they are the smallest type of leukocytes, their importance in the body's defense against various pathogens is immeasurable.

In our study, rats were briefly exposed to hyperthermia. As a consequence of the organism's stress reaction, there was an increase in the number of lymphocytes.

Also, the results of our study showed a changed shape of lymphocytes between subgroups antemortem and postmortem *(Fig. 1)*, which indicates that altered lymphocytes may have altered function. The result is a changed reaction of the immune system in organisms exposed to elevated temperature.

Declarations

Availability of Data and Materials: The datasets generated during the current study are available from the corresponding author (Z. Ajanović) on reasonable request.

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

Author Contributions: ZA, ED and LD conceived and designed the study. ED, MK and ZA executed the experiment and analyzed the serum and tissue samples. AD and ZA analyzed the data. All authors interpreted the data, critically revised the manuscript for important intellectual contents and approved the final version.

Ethical Approval: The study was conducted at the Faculty of Veterinary Medicine of the University of Sarajevo, by valid ethical principles on biomedical research on animals and after obtaining approval from the Ethical Committee at the Faculty of Veterinary Medicine of the University of Sarajevo (Number: 07-03-850-4/22).

References

1. Hussen J: Changes in cell vitality, phenotype, and function of dromedary camel leukocytes after whole blood exposure to heat stress *in vitro*. *Front Vet Sci,* 8:647609, 2021. DOI: 10.3389/fvets.2021.647609

2. Dervišević E, Dervišević L, Ajanović Z, Bešić A, Salihbegović A, Mujarić E, Dervišević M: Forensic meaning of core temperature- an indicator for assessing the severity of heatstroke in an animal model. *Veterinaria,* 72 (2): 183-191, 2023. DOI: 10.51607/22331360.2023.72.2.183

3. Dervisevic E, Doric M, Radovic S, Dervisevic L, Katica M, Ajanovic Z, Salihbegovic A, Sarajlic N: Morphological changes in myocardium of Wistar rat caused by hyperthermia. *J King Saud Univ Sci,* 35 (2):102473, 2023. DOI: 10.1016/j.jksus.2022.102473

4.Dervisevic E, Hasic S, Kiseljakovic E, Jadric R, Dervisevic L, Ajanovic Z, Salihbegovic A: Cellular network of damage caused by exposure to high ambient temperature in Wistar rats: The role of Hsp70. *J King Saud Univ Sci,* 35 (5):102654, 2023. DOI: 10.1016/j.jksus.2023.102654

5. Dervisevic E, Hasic S, Katica M, Dervisevic L, Ajanovic Z, Salihbegovic A: Heat-related biomarkers: Focus on the correlation of troponin I and 70 kDa heat shock protein. *Heliyon,* 9 (4):e14565, 2023. DOI: 10.1016/j.heliyon.2023.e14565

6. Ebi KL, Capon A, Berry P, Broderick C, de Dear R, Havenith G, Honda Y, Kovats RS, Ma W, Malik A, Morris NB, Nybo L, Seneviratne SI, Vanos J, Jay O: Hot weather and heat extremes: health risks. *Lancet,* 398 (10301): 698-708, 2021. DOI: 10.1016/S0140-6736(21)01208-3

7. Wells JCK: Environmental temperature and human growth in early life. *J Theor Biol*, 204 (2): 299-305, 2000. DOI: 10.1006/jtbi.2000.2015

8. Krau SD: The impact of heat on morbidity and mortality. *Crit Care Nurs Clin North Am,* 25 (2): 243-250, 2013. DOI: 10.1016/j.ccell.2013.02.009

9. Gürağaç A, Demirer Z: The neutrophil-to-lymphocyte ratio in clinical practice. *Can Urol Assoc J*, 10 (3-4): 141-142, 2016.

10. Dervisevic E, Katica M, Ajanovic Z, Joguncic A, Dervisevic L, Dervisevic M, Salihbegovic A: Influence of hyperthermia on dimensions of erythrocytes: Experimental preliminary study on rats. *Veterinaria,* 71 (1): 41-52, 2022. DOI: 10.51607/22331360.2022.71.1.41

11. Bridges TM, Tulapurkar ME, Shah NG, Singh IS, Hasday JD: Tolerance for chronic heat exposure is greater in female than male mice. *Int J Hyperthermia*, 28 (8): 747-755, 2012. DOI: 10.3109/02656736.2012.734425

12. Dervišević E, Hasić S, Katica M, Salihbegović A, Ajanović Z, Sarajlić N: Forensic significance of cTnI serum for the detection of terminal myocardial damage in rats *(Rattus norvegicus)* caused by hyperthermia. *J King Saud Univ Sci,* 34 (2):101753, 2021. DOI: 10.1016/j.jksus.2021.101753

13. Rana MS, Hashem MA, Sakib MN, Kumar A: Effect of heat stress on blood parameters in indigenous sheep. *J Bangladesh Agril Univ,* 12 (1): 91-94, 2014. DOI: 10.22004/ag.econ.209902

14. Mofizur RM, Bae KH, Ja BH: Changes in blood cell morphology and number of red spotted grouper, *Epinephelus akaara* in response to thermal stress. *Dev Reprod*, 23 (2): 139-148, 2019. DOI: 10.12717/DR.2019.23.2.139

15. Dieing A, Ahlers O, Kerner T, Wust P, Felix R, Löffel J, Riess H, Hildebrandt B: Whole body hyperthermia induces apoptosis in subpopulations of blood lymphocytes. *Immunobiology*, 207 (4): 265-273, 2003. DOI: 10.1078/0171-2985-00236

16. Ostberg JR, Repasky EA: Comparison of the effects of two different whole body hyperthermia protocols on the distribution of murine leukocyte populations. *Int J Hyperthermia*, 16 (1): 29-43, 2000. DOI: 10.1080/026567300285402

17. Dervisevic E, Hasic S, Dervisevic L, Ajanovic Z, Latica M, Salihbegovic A: Animal models and experimental research in medicine: Impact of temperature on morphological characteristics of erythrocytes and heart weight: Experimental study on Wistar rats. *IntechOpen*, 2023. DOI: 10.5772/intechopen.105101

18. Adewoyin AS, Nwogoh B: Peripheral blood film - A review. *Ann Ib Postgrad Med,* 12 (2): 71-79, 2014.

19. Anđelković Z, Somer L, Avramović V, Milosavljević Z, Tanasković I, Matavulj M: Histologija, Niš: Impressum; 2009.

20. Rosenspire AJ, Kindzelskii AL, Petty HR: Cutting edge: Fever-associated temperatures enhance neutrophil responses to lipopolysaccharide: A potential mechanism involving cell metabolism. *J Immunol*, 169 (10): 5396-5400, 2002. DOI: 10.4049/jimmunol.169.10.5396

21. Ranheim EA: Leukocyte abnormalities associated with hyperthermia. *Int J Hematolog,* 97, 545-546, 2013. DOI: 10.1007/s12185-013-1310-3

22. Iba T, Tomohiro S, Kondo Y, Kondo K, Levy HJ: Morphological changes in blood cells in a rat model of heatstroke: A pilot study. *J Clin Med,* 11 (16):4821, 2022. DOI: 10.3390%2Fjcm11164821

23. Edwards MJ, Penny RH: Effects of hyperthermia on the myelograms of adult and fetal guinea-pigs. *Br J Haematol,* 59 (1): 93-101, 1985. DOI: 10.1111/j.1365-2141.1985.tb02968.x

24. Salanova B, Choi M, Rolle S, Wellner M, Scheidereit C, Luft FC, Kettritz R: The effect of fever-like temperatures on neutrophil signaling. *FASEB J,* 19 (7): 816-818, 2005. DOI: 10.1096/fj.04-2983fje

25. Suljević D, Eminović I, Hasković E, Mitrašinović-Brulić M: Hematological status of the rat *Rattus norvegicus* in hypothermia. *Veterinaria*, 60 (3-4): 179-186, 2011.

26. Mahmutović L, Hasković E, Suljević D, Mitrašinović-Brulić M: Efekt hipertermije na diferencijalnu krvnu sliku štakora *(Rattus norvegicus). Veterinaria,* 62 (1-2): 47-54, 2013.

27. Litman RS, Rosenberg H: Malignant hyperthermia: Update on susceptibility testing. *JAMA*, 293 (23): 2918-2924, 2005. DOI: 10.1001/jama.293.23.2918

28. Yang L, Dedkova EN, Allen PD, Jafri MS, Fomina AF: T lymphocytes from malignant hyperthermia-susceptible mice display aberrations in intracellular calcium signaling and mitochondrial function. *Cell Calcium,* 93:102325, 2021. DOI: 10.1016/j.ceca.2020.102325

29. Ashman RB, Müllbacher A: Infectious disease, fever, and the immune response. *Immunol Today*, 5 (9): 268-271, 1984. DOI: 10.1016/0167-5699(84)90138-5

30. Huang YH, Haegerstrand A, Frostegård J: Effects of *in vitro* hyperthermia on proliferative responses and lymphocyte activity. *Clin Exp Immunol,* 103 (1): 61-66, 1996. DOI: 10.1046/j.1365-2249.1996.00932.x

31. Bouchama A, al Hussein K, Adra C, Rezeig M, al Shail E, al Sedairy S: Distribution of peripheral blood leukocytes in acute heatstroke. *J Appl Physiol,* 73 (2): 405-409, 1992. DOI: 10.1152/jappl.1992.73.2.405

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Research Article

Antennal Transcriptome and Proteome Analysis of Olfactory Genes and Tissue Expression Profiling of Odorant Binding Proteins in *Wohlfahrtia magnifica*

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Abstract

Wohlfahrtia magnifica is a species of fly that parasitizes Bactrian camels. The adult flies lay their larvae near the vulva of the camels, and these larvae develop and cause damage to the vaginal tissues, resulting in vaginal myiasis. Olfactory organs play an important role in the identification and location of host, foraging, mating and oviposition behavior of *W. magnifica*. Olfactory genes were identified by antennal transcriptome analysis. Twenty-four odor-binding proteins (OBPs) and two chemosensory proteins (CSPs) were identified in the antenna transcriptome of *W. magnifica*, and then the phylogenetic analysis of the olfactory genes of *W. magnifica* and other species was carried out. RT-qPCR was used for the first time to analyze the expression profile of OBPs in the antenna tissues of the *W. magnifica*. In the tissue expression analysis of OBP genes, it was found that many of them showed obvious gender bias in antennae, indicating their different roles in identifying pheromones. These results will help to lay a foundation for the future research on the sense of smell of *W. magnifica* and help to better reveal the change of odor reception of *W. magnifica* and provide new ideas for the research on biological prevention and control of vaginal myiasis.

Keywords: Antenna, Olfactory genes, Transcriptome, *W. magnifica*

Introduction

Wohlfahrtia magnifica belongs to the order Diptera, family Sarcophagidae, genus Wohlfahrtia Brauer. *W. magnifica* is the main pathogen of traumatic myiasis of flies, which is widely distributed in many countries in Asia, Europe, Africa and Northern China [1-3]. In China, *W. magnifica* is the sole pathogen responsible for causing vaginal myiasis in Bactrian camels. Female flies produce larvae near the vulva of Bactrian camels, and the larvae of *W. magnifica* parasitize and develop gradually in the vagina of Bactrian camels. The labia of the sick camel is swollen and closed, often accompanied by blood outflow. Upon opening the labia, it becomes evident that the lesion contains larvae at different stages of development. If it is not treated in time, the presence of *W. magnifica* in the environment will produce maggots at the focus, and the physical trauma will increase, which can lead to paralysis or even death of

the host. At present, there are no effective prevention and control measures, and it is urgent to seek new prevention and control methods [4].

Highly developed sensory organs help the *W. magnifica* adapt to the complex and changeable environment, and play a key role in its life, such as identifying and locating the host, foraging, mating and spawning [5,6]. As the most important olfactory organ of the *W. magnifica*, the main task of antenna is to recognize and perceive pheromones. The recognition of odor molecules in insects is a complex process, involving a variety of proteins related to smell, such as odorant binding proteins (OBPs), chemosensory proteins (CSPs), olfactory receptors (ORs), gustatory receptors (GRs), ionotropic receptors (IRs) and sensory neuron membrane proteins (SNMPs) [7-10]. The task of identifying pheromones and odors is mainly accomplished by odorant binding proteins (OBPs) and chemosensory proteins (CSPs) [11]. OBPs are mainly expressed in antenna and are small water-soluble acidic proteins. They exhibit a conserved structure comprising six cysteine residues that form three intertwined disulfide bridges. Typically, OBPs consist of around 120 to 150 amino acids and exhibit a mass of approximately 14 kDa [12-15]. CSPs are present at high concentrations in chemosensory sen-silla lymphs and are broadly expressed in non-sensory tissues. CSPs are soluble small molecule proteins with four cysteines that form two disulfide bridges [16,17]. It is considered that insect OBPs can bind and transport hydrophobic odorant molecules across the hydrophilic sensillum lymph to corresponding receptors on olfactory sensory neurons. Further, OBP-odorant complexes (or odorant itself) activate receptors (ORs or IRs) to stimulate a cascade of reaction, which converts chemical signals into electric signals and eventually lead to specific behaviors [18,19].

The OBPs are subdivided into three subfamilies: pheromone-binding proteins (PBPs), general odorant-binding proteins (GOBPs) and antennal-binding proteins (ABPs). PBPs are expressed in long sensillum trichodea and show a male antennae-biased expression pattern. PBPs are involved in the detection of female sex pheromones, playing a crucial role in mate location and mating behavior [20]. GOBPs, including GOBP1 and GOBP2, are usually distributed in sensillum basiconica [21]. GOBPs are thought to bind general odorants such as host plant volatiles and other environmental chemical cues. However, a few studies reported that GOBPs may also be involved in sex pheromone detection [22]. Research on olfactory-related proteins, including OBPs, has been conducted in various insect species. Over 150 species of OBPs have been identified in 35 species of lepidoptera insects alone [23]. With the deepening of their research, many insects have carried out research on the types, functions and olfactory mechanisms of olfactory related proteins. Olfactory-related proteins of insects are potential targets for the control of *W. magnifica* in the future [24-27].

In this study, we sequenced the antenna transcriptome of *W. magnifica*, analyzed and identified the genes encoding olfactory protein, and measured the transcription expression of important OBP genes in the antenna tissues of male and female adults of *W. magnifica* by fluorescence quantitative real-time PCR. The research aimed to identify and compare the olfactory genes of *W. magnifica* by analyzing the antenna transcriptome. This analysis provides a foundation for further understanding the underlying molecular mechanisms involved in olfactory perception in *W. magnifica*. Knowing these information can provide target genes for the biological control of myiasis, and then provide a good start for the integrated management of *W. magnifica* [28,29].

Material and Methods

Ethical Statement

All experimental procedures were approved by the Animal Protection and Use Committee of Inner Mongolia Agricultural University and strictly followed animal welfare and ethical guidelines.

Source of *W. magnifica* and Its Antenna Collections

In this study,we used the *W. magnifica* raised in the laboratory. The populations were kept in a laboratory insect rearing box (aluminium alloy frame, 120 mesh nylon sand mesh cover, space volume: 35 cm x 35 cm x 35 cm). The laboratory rearing conditions were kept at 25°C, relative humidity was 50%~60%, and the illumination period was L: D=15:9 h. For the transcriptome sequencing analysis, the hatched adults were immediately separated by sex. Both of 50 pairs of antennae of 3-day-old female and male *W. magnifica* were collected by dissecting microscope, and immediately frozen in liquid nitrogen and stored at −80°C until RNA was isolated, and further use.

RNA Extraction, CDNA Library Construction and Illumina Sequencing

In this study, total RNA was extracted from the male and female antenna tissue samples using a Total RNA Extractor kit from TaKaRa (Dalian, China). The concentration of RNA was measured using a Qubit 2.0 fluorometer. The integrity of the RNA and the presence of genomic DNA contamination were assessed using agarose gel electrophoresis. After the RNA extraction, library construction was performed. The constructed library was quantified using a Qubit 2.0 fluorometer and diluted to a concentration of 1 ng/L. The sequencing of the library was carried out by Sheng gong Bioengineering (Shanghai) Company.

Transcriptome Data Analysis and Gene Functional Annotation

The quality of the original sequencing data was evaluated by FastQC, and then the quality was cut by Trimmomatic, and relatively accurate and effective data were obtained. Trinity was used to assemble the clean data of all samples from scratch, and the assembly results were optimized and evaluated. The transcript was compared with databases such as CDD, KOG, COG, NR, NT, PFAM with NCBI Blast+, and its functional annotation information was obtained. Using KAAS to get KEGG annotation information of transcript. The CDS prediction was made according to the transcript and database Blast comparison results and Trans Decoder. Use ggplot2 software package v.3.5 for data visualization and drawing.

Annotation, Sequence Alignment and Phylogenetic Analysis of Olfactory Genes

All candidate genes of OBP and CSP were manually checked by BLASTx and BLASTn software of NCBI Online. ORF Finder online software is used to predict the open reading frame of genes. The candidate OBPs and CSPs were searched for the presence of N-terminal signal peptides using SignalP5.0. Using the nucleotide sequences of OBP and CSP as queries (BLASTx) in GenBank database, the sequences of different insect species were retrieved from GenBank database and used to construct phylogenetic trees. DNAMAN software was used for multiple alignment of amino acid sequences. Edit phylogenetic tree with MEGA software. The expression level is displayed as TPM value (the expression amount obtained after homogenization of gene length and sequencing depth), which is calculated by Saimon 0.8.2.

Analysis of Antenna Expression of OBP Genes

The relative expression level of OBP genes in male and female antennae was analyzed by fluorescence quantitative real-time PCR. Total RNA extraction is the same as above. Primer 3 plus designed specific primers. RT-qPCR data were analyzed by $2^{-(\Delta\Delta Ct)}$ method to calculate the relative expression level, and graph-pad was used to draw the result graph. SPSS 23.0 was used for one-way ANOVA, and Tukey method was used to test the significance of the difference ($P<0.05$).

Results

Transcriptome Assembly and Annotation

In order to identify the olfactory genes, the antennae of male and female adults of *W. magnifica* were sequenced by Illumina HiSeq, and 61,868,724 and 68,888,578 original readings were obtained from the antennae of the male and female *(Table 1)*. The raw data obtained by sequencing contains low-quality sequences with connectors.In order to ensure the quality of information analysis, the original data must be filtered.By removing the low-quality and trimmed readings with the length less than 20 nt, 59,685,374 and 65,806,910 male and female clean readings were obtained. The percentage of Q30 bases exceeded 94.81% and the GC content was 40.64-40.98% *(Table 2)*. After filtering out adapters and low-quality original sequences and assembling the readings from male and female antennae into a single transcription group, assembled into 153,779 unigenes with a total length of 76,933334bp, and 568 N50 500.29bp unigenes with a length between 201bp and 29622bp were obtained, of which 12605 unigenes with a length exceeding 1000bp accounted for 8.19% of all unigenes *(Table 3)*.

In total, 99085 unigenes from *W. magnifica* (64.43% of 153779 unigenes) were annotated in at least one of the data-

Table 1. *Sample statistics of Original Data Information*

Parameter		Male Antenna	Female Antenna
Total Reads Count (#)		61868724	68888578
Total Bases Count (bp)		9280308600	10333286700
Average Read Length (bp)		150	150
Q10 Bases	Count (bp)	9277656490	10329662377
	Ratio (%)	99.97%	99.96%
Q20 Bases	Count (bp)	9120877203	10129798266
	Ratio (%)	98.28%	98.03%
Q30 Bases	Count (bp)	8850535758	9796998975
	Ratio (%)	95.37%	94.81%
N Bases	Count (bp)	2652110	3624323
	Ratio (%)	0.03%	0.04%
GC Bases	Count (bp)	3803323380	4199868275
	Ratio (%)	40.98%	40.64%

Table 2. *Sample statistics of QC data information*

Parameter		Male Antenna	Female Antenna
Total Reads Count (#)		59685374	65806910
Total Bases Count (bp)		8771287389	9708026321
Average Read Length (bp)		146.96	147.52
Q10 Bases	Count (bp)	8770976692	9707581265
	Ratio (%)	100.00%	100.00%
Q20 Bases	Count (bp)	8698509459	9622028526
	Ratio (%)	99.17%	99.11%
Q30 Bases	Count (bp)	8507137252	9396553581
	Ratio (%)	96.99%	96.79%
N Bases	Count (bp)	310697	445056
	Ratio (%)	0.00%	0.00%
GC Bases	Count (bp)	3589396721	3946965602
	Ratio (%)	40.92%	40.66%

Table 3. *Summary of assembled transcript and unigenes*

Parameter	No.	Length Range/bp							
		>=500bp	>=1000bp	N50	N90	Max. Length	Min. Length	Total Length	Average Length
Transcript	262628	77017	32060	800	255	29622	201	155409503	591.75
Unigene	153779	36021	12605	568	240	29622	201	76933334	500.29

Table 4. *Functional annotation of the unigenes in different Databases*

Database	Number of Genes	Percentage (%)
Annotated in CDD	6793	4.42
Annotated in PFAM	8126	5.28
Annotated in KEGG	4320	2.81
Annotated in KOG	8246	5.36
Annotated in Swissprot	13165	8.56
Annotated in GO	11516	7.49
Annotated in NR	37603	24.45
Annotated in NT	93604	60.87
Annotated in at least one database	99085	64.43
Annotated in all database	1536	one
Total genes	153779	100

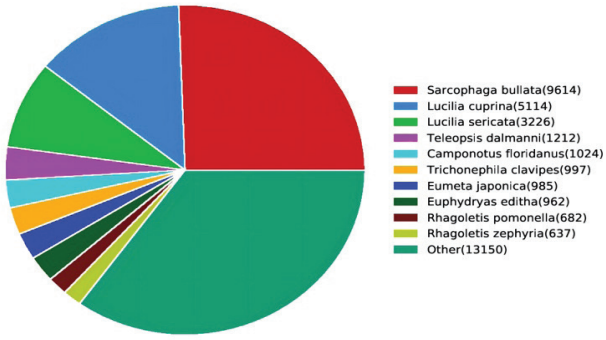

Fig 1. Pie chart of homologous species distribution

bases searched (NT, NR, PFAM, KOG, Swiss-Prot, KEGG, CDD and GO databases) *(Table 4)*. Homology searches againstthe Nr database showed that the *W. magnifica* antennal tran-scriptome shared the greatest homology with sequences from *Sarcophage bullate* (25%), followed by *Lucilia cuprina* (13.6%) and *Lucilia sericate* (8.6%) *(Fig. 1)*. Of the 153,779 unigenes, 11,516 (7.49%) correspond to at least one specific term in "biological process", "cellular component" and "molecular function". In the molecular function category, 6687 (58.1%) and3985 (34.6%) unigenes were linked to binding and catalytic activity. In terms of the biological process, 9696 (84.2%), 7453 (64.7%) unigenes were related to cellular processesand metabolic processes. In the cellular component category, 9683 (84.1%) unigenes were assigned to the terms cell and cell part *(Fig. 2)*.

Identification of Candidate Odor Binding Proteins

In the study, a total of 24 candidate OBP transcripts were identified in the antennal transcriptome of both male and female *W. magnifica* flies. These OBP gene sequences were found to be over 240 bp in length and possessed complete open reading frames (ORFs). Among them, 17 kinds of protein were identified as PBPs. These PBPs were found to be male-specific and associated with pheromone-sensitive neurons, suggesting their potential role in olfactory perception and mate recognition. To determine the homology of these OBP sequences, the BLASTx analysis was performed. The results revealed that Wmag36544 exhibited 96.34% orthology with the OBP sequence from *Sarcophaga caerulescens*, indicating a high level of

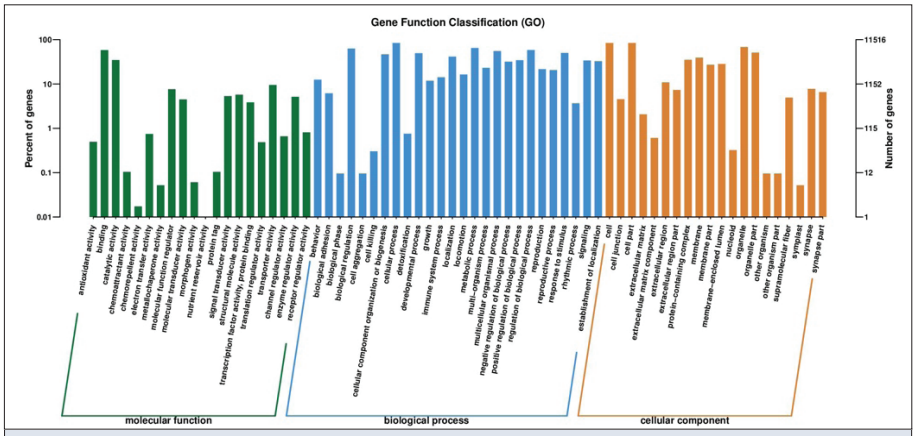

Fig 2. Gene function classification (GO)

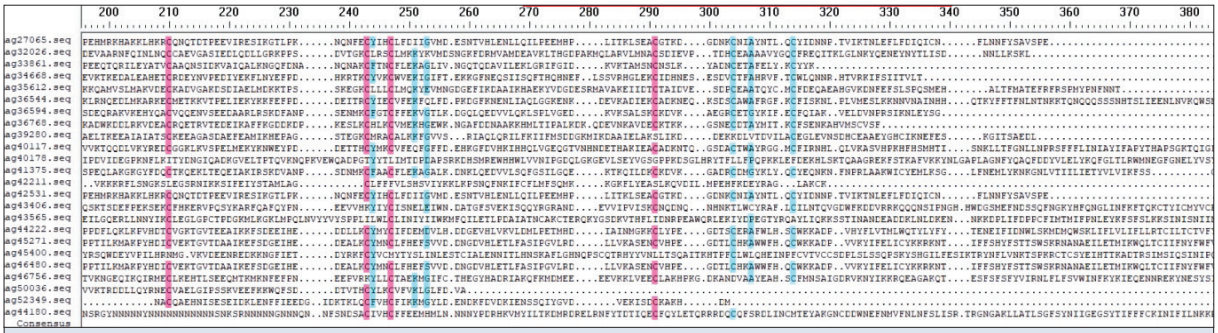

Fig 3. Amino acid sequence comparison of 24 odor-binding proteins of *W. magnifica*

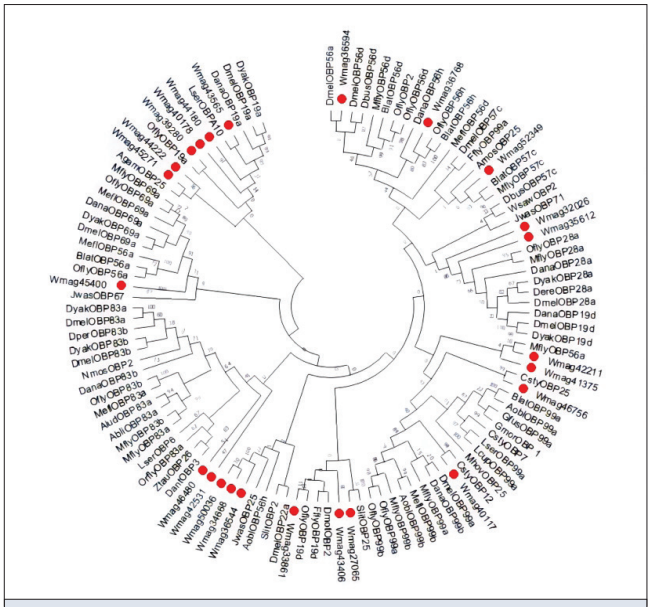

Fig 4. Neighbor-Joining method phylogenetic tree of odorant binding proteins of *W. magnifica* and other insects. Include those from the Zeugodacus cucurbitae (melon fly), Bactrocera dorsalis (oriental fruit fly), Drosophila busckii, Bactrocera latifrons, Anastrepha ludens (Mexican fruit fly), Drosophila ananassae, Cephus cinctus (wheat stem sawfly) OBPs. Marked a specific gene. Evaluate node support through 1000 boot replications

similarity between the two sequences. Additionally, the orthologs of other OBP sequences showed more than 80% similarity with their respective counterparts in other species.

According to the TPM value (measured as the proportion of a transcript in the RNA pool), 13 transcripts encoding OBPs were highly expressed in the male antenna and 11 transcripts encoding OBPs were highly expressed in the female antenna (TPM>100). Among these transcripts, Wmag45271 exhibited the highest expression level in the male antenna, indicating it was highly expressed in males. On the other hand, Wmag43565 showed the highest expression level in the female antenna, indicating it was highly expressed in females. Interestingly, Wmag52349 demonstrated the lowest expression level in both the female and male antennae, suggesting it had relatively low expression in both sexes.

The results of the amino acid sequence alignment revealed that all 19 OBPs identified in the study possessed six conserved cysteine sites. Additionally, there were three amino acid residues located between the second and third cysteine, which is a characteristic feature of typical OBPs. The other four OBPs (Wmag42211, Wmag40178, Wmag44180, Wmag45400) were found to lack the first cysteine site *(Fig. 3)*. Among the OBPs, only seven of them (Wmag36768, Wmag44222, Wmag34668, Wmag3659, Wmag27065, Wmag43565, and Wmag50036) exhibited the presence of signal peptides, indicating their potential role in secretion or membrane targeting. Phylogenetic tree shows the evolutionary relationship between insect OBP genes *(Fig. 4)*. Based on the phylogenetic tree analysis, the OBPs clustered together based on their respective subfamilies, forming eight distinct branches corresponding to different functional groups. In the phylogenetic tree, Wmag40117 is 99% similar to *Calliphora stygia* OBP25, and Wmag40117 is 98% similar to *Calliphora stygia* OBP12. These findings suggest a close evolutionary relationship between these OBP sequences.

Identification of Candidate Chemosensory Proteins

Two candidate CSPs were identified in male and female antenna transcriptome, which were named Wmag44066 and Wmag72266 respectively. The two putative CSP genes are both over 470 bp in length and have complete ORFs, among which Wmag72266 contains signal peptide. By performing a BLASTx analysis to assess gene homology, it was determined that the two CSP sequences exhibited approximately 80% similarity to CSP sequences from other species, indicating their conservation across different organisms. Significant differences were observed in the abundance of these CSP transcripts between the sexes. According to the TPM (Transcripts Per Million) values, the expression of Wmag44066 in the male antenna was higher (TPM>200) compared to its expression in the female antenna (TPM<50). This suggests a potential sex-specific role for Wmag44066 in chemosensory processes. The two CSPs have four highly conserved cysteine residues, which are consistent with the “CSP sequence motif” C1-X6-8-C2-X16-21-C3-X2-C4, where X represents any amino

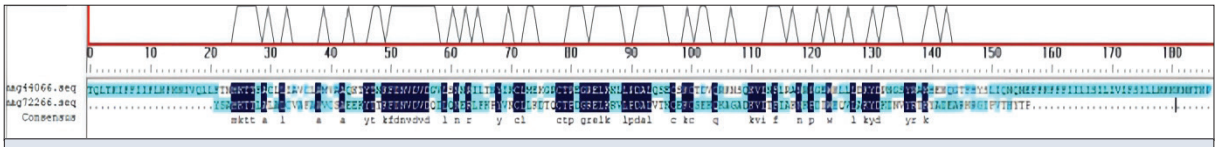

Fig 5. Amino acid sequence comparison of two typical chemosensory proteins of *W. magnifica*

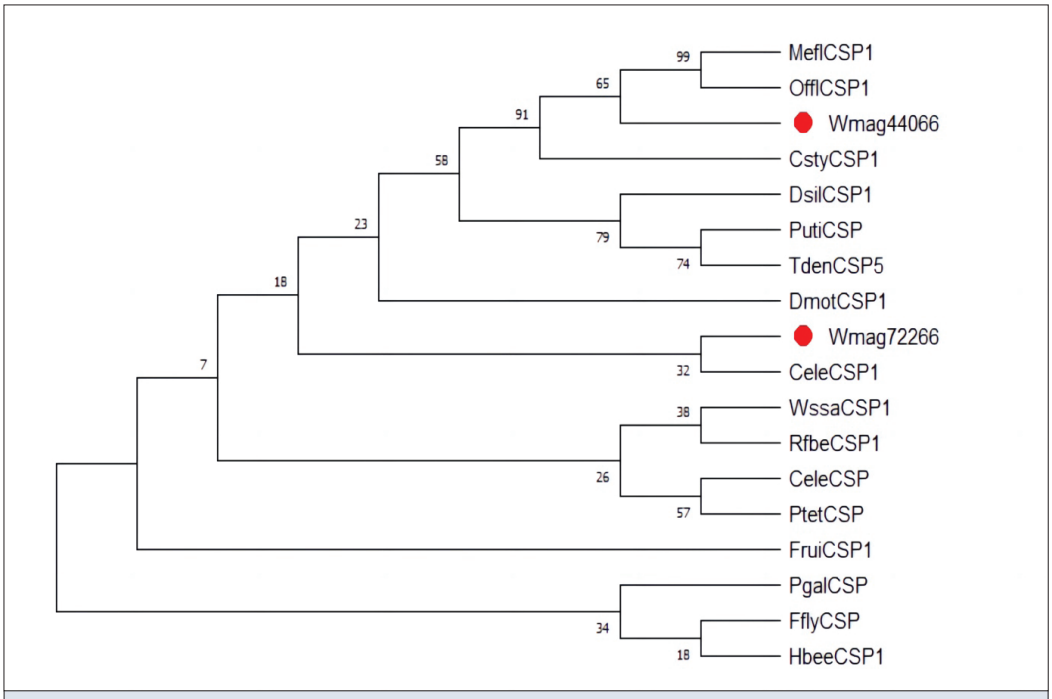

Fig 6. Neighbor-Joining method phylogenetic tree of chemosensitive proteins of *W. magnifica* and other insects. Include those from *Zeugodacus cucurbitae* (melon fly), Bactrocera dorsalis (oriental fruit fly), Drosophila melanogaster (fruit fly), *Plutella xylostella* (diamondback moth), *Cephus cinctus* (wheat stem sawfly) CSPs. Marked for specific genes. Node support was assessed by 1.000 guided copies

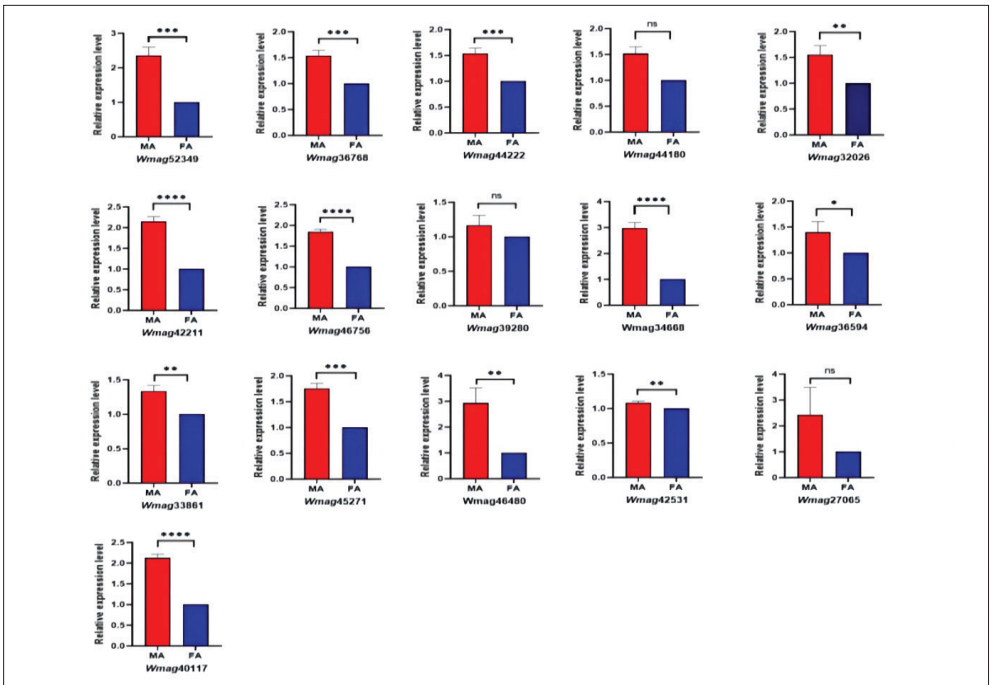

Fig 7. Expression profile of odor-binding protein in antenna tissue of *W. magnifica.* MA: male antennae; FA: female antennae. Asterisks above the bars indicate a significant difference (P<0.05, t-test); NS, no significant difference

acid, which is consistent with the structural characteristics of insect CSP gene *(Fig. 5)*. The phylogenetic tree analysis, which depicts the evolutionary relationship between chemosensory proteins in various insects, revealed that Wmag44066 and Wmag72266 occupied distinct positions in the tree *(Fig. 6)*. This indicates that these two CSPs have undergone divergent evolutionary paths and have unique evolutionary contexts within the chemosensory protein family.

Analysis of Antenna Tissue Expression of OBPs

In order to verify the expression profile of OBPs in the female and male antennae of *W. magnifica*, we carried out RT-qPCR on 16 kinds of OBPs. We compared the results of TPM and RT-qPCR, and found that the expression trend of most OBPs in male and female antennae was the same, which further proved the accuracy of transcriptome data. QPCR results showed that Wmag42531 and Wmag39280 were highly expressed in the antenna, indicating that OBPs may play a role in the combination and transmission of odor signals in the antenna. Moreover, significant gender-biased expression of several OBP genes was observed. Except for 3 OBP (Wmag44180, Wmag39280, Wmag27065), the other 13 OBPs were significantly different, and they were gender-biased in the antenna *(Fig.7)*. This gender bias indicates that these OBPs may have different functions in males and females, such as perception of the opposite sex or involvement in oviposition behavior.

Discussion

It is very important to understand how OBPs and CSPs participate in the process of insect olfactory perception and recognition of external environmental chemical stimuli for formulating effective pest management strategies [30,31]. Before this study, most of the research on *W. magnifica* focused on the field of morphology [32,33]. In this study, 24 OBPs and 2 CSPs were identified from the database of antenna transcriptome of adult *W. magnifica* by bioinformatics. These genes all play a key role in the olfactory perception of insects.Compared to the total number of insects in the world, OBPs have been identified in only a few species of insects to different degrees, such as *Drosophila melanogaster* (51 OBPs), *Anopheles gambiae* (57 OBPs) and *Parasitoid Wasp Nasonia vitripennis* (82 OBPs) [34-36]. In this study, 24 candidate OBPs were identified in *W. magnifica*, with most of them classified as PBP (pheromone binding proteins). The variation in the number of OBPs among different species suggests differences in olfactory discrimination and perception mechanisms among different insect species.

According to TPM value, there are also differences in the expression of antenna homologous genes between female and male *W. magnifica*. For example, Wmag43565 is abundantly expressed in both male and female antennae, but the expression level is higher in female antennae. This prompted further exploration of the expression profile of OBPs in the antenna. Through RT-qPCR analysis, it was discovered that Wmag45271 and Wmag46480 were highly expressed in the antenna of female flies. This suggests that these OBPs may play a crucial role in identifying the volatiles emitted by Bactrian camels or in locating suitable spawning sites. However, further functional verification is necessary to confirm these hypotheses. Notably, the expression level of Wmag45271 in male antennae was higher than that in female antennae, indicating its potential involvement in female sex pheromone recognition. Lepidoptera PBPs and GOBPs form a monophyletic lineage with a single ancestral origin. They have undergone divergence by gene duplication under different selection pressures [37]. Phylogenetic tree showed that Wmag40117 was 99% similar to *Calliphora stygia* OBP25, and Wmag40117 was 98% similar to *Calliphora stygia* OBP12. The evolutionary relationship of OBPs in insect specialization is further explained. Overall, the study's findings shed light on the expression patterns and potential functions of OBPs in *W. magnifica*, highlighting their role in olfactory perception and chemical communication in the species.

CSPs are widely expressed in insect antennae and other parts of the body, and participate in insect recognition pheromone perception and related behaviors [38,39]. In addition, CSPs has many functions. For example, CSPs may be involved in dissolving hydrocarbons in the stratum corneum to identify their symbiotic relationship [40]. This suggests that CSPs are important for maintaining specific ecological interactions. The competitive binding analysis of tryptophan fluorescence spectrum and molecular docking showed that some CSPs in *Bemisia tabaci* was very important to promote the transport of fatty acids, thus regulating some metabolic pathways of insect immune response [41]. These findings highlight the diverse roles of CSPs in insect physiology and behavior, showing their involvement in chemical communication, symbiosis, and immune response regulation.

In this study, two candidate CSP genes were identified. The number of CSPs is less than that of *Sclerodermus sp.* (10), *C. cunea* (11), *M. pulchricornis* (8) and *B. dorsalis* (4) [42-45]. According to TPM value, Wmag44066 is highly expressed in antenna, which may be related to chemical acceptance. Based on the TPM values, Wmag44066 was found to be highly expressed in the antennae, suggesting its potential involvement in chemical acceptance. Interestingly, the TPM value of Wmag44066 in male antennae was approximately four times higher than in female antennae, indicating its possible role in male *W. magnifica*'s recognition of female pheromones. On the other hand,

Wmag72266 was almost exclusively expressed in female antennae, suggesting its potential role in host location and oviposition behavior of female *W. magnifica*. Similar biased expression patterns have been observed in other species as well. The phylogenetic tree analysis revealed that Wmag44066 and Wmag72266 belong to different branches, indicating that they likely play different roles in recognition of pheromones and related behaviors. Overall, these findings suggest that the identified CSP genes in *W. magnifica* may have distinct functions in the perception and response to pheromones, potentially contributing to the species' mating behavior and host-seeking behavior.

In this study, we used the Illumina Hiseq™ 4000 high-throughput sequencing platform to sequence the transcriptome of the male and female antennae of *W. magnifica*. We identified 26 olfactory related genes and obtained the expression of 16 OBP genes in male and female antennae by RT-qPCR experiment, which confirmed the accuracy of our transcriptome data. It enables us to predict their functions and provides insights into the mechanism of *W. magnifica* looking for hosts. These studies also provide a molecular basis for the development of attractants and repellents for *W. magnifica* and provide a new idea for biological control of *W. magnifica*.

Declarations

Availability of Data and Materials: The datasets generated and/or analysed during the study are available from the corresponding authors and can be provided upon request.

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Competing Interests: The authors declared that there is no conflict of interest.

Author Contributions: LFL performed the experiments, analysed the results, and drafted the manuscript. RW and LXZ assisted in the experimental design and summarized the experimental results. SM data interpretation and manuscript preparation. JYT and CG put forward valuable suggestions for the revision and improvement of the paper. DE and WMQ conceived and designed the study, revised the manuscript and funded the study. All authors have read and agreed to the published version of the manuscript.

References

1. Xiwen A, Bin Y, Haur B, Gowa O, Xiuzhen W, Demtu E: Morphological observation of the larva of the alax bactrian camel vaginal myiasis. *J Camel Pract Res,* 26 (1): 57-62, 2019. DOI: 10.5958/2277-8934.2019.00008.0

2. Haobo L, Jiaqi X, Baoxiang H, Demtu E: Studies on overwintering behavior and cold stress related unigenes of *Wohlfahrtia magnifica*. *Kafkas Univ Vet Fak Derg,* 28 (1): 75-85, 2022. DOI: 10.9775/kvfd.2021.26481

3. Likang Z, Dongdong A, Ming Y, Huar B, Baoxiang H, Bo S, Ya T, Demtu E: The efects of genital myiasis on the diversity of the vaginal microbiota in female Bactrian camels. *BMC Vet Res,* 18 (1):87, 2022. DOI: 10.1186/s12917-022-03189-5

4. Hadani A, Ben Yaakov B, Rosen S: Myiasis caused by *Wohlfahrtia magnifica* (Schiner, 1862) in the Arabian camel *(Camelus dromedarius)* in the Peninsula of Sinai. *Rev Elev Med VetPays Trop,* 42 (1): 33-38, 1989. DOI: 10.19182/REMVT.8876

5. Tanaka K, Shimomura K, Hosoi A, Sato Y, Oikawa Y, Seino Y, Kuribara T, Yajima S, Tomizawa M: Antennal transcriptome analysis of chemosensory genes in the cowpea beetle, *Callosobruchus maculatus* (F.). *PLoS One,* 17 (1):e0262817, 2022. DOI: 10.1371/journal.pone.0262817

6. Dong JF, Wang K, Sun YL, Tian CH, Wang SL: Antennal transcriptome analysis of odorant-binding proteins and characterization of GOBP2 in the variegated cutworm *Peridroma saucia*. *Front Physiol,* 14:1241324, 2023. DOI: 10.3389/fphys.2023.1241324

7. Ma Y, Li Y, Wei Z, Hou J, Si Y, Zhang J, Dong S, Yan Q: Identification and functional characterization of general odorant binding proteins in *Orthaga achatina*. *Insects,* 14 (3): 216, 2023. DOI: 10.3390/insects14030216

8. Li GW, Chen XL, Chen LH, Wang WQ, Wu JX: Functional analysis of the chemosensory protein GmolCSP8 from the oriental fruit moth, *Grapholita molesta* (Busck) (Lepidoptera: Tortricidae). *Front Physiol,* 10:552, 2019. DOI: 10.3389/fphys.2019.00552

9. Cassau S, Krieger J: The role of SNMPs in insect olfaction. *Cell Tissue Res,* 383 (1): 21-33, 2021. DOI: 10.1007/s00441-020-03336-0

10. Leal WS: Odorant reception in insects: roles of receptors, binding proteins, and degrading enzymes. *Annu Rev Entomol,* 58, 373-391, 2013. DOI: 10.1146/annurev-ento-120811-153635

11. Fan J, Francis F, Liu Y, Chen JL, Cheng DF: Review: An overview of odorant-binding protein functions in insect peripheral olfactory reception. *Genet Mol Res,* 10 (4): 3056-3069, 2011. DOI: 10.4238/2011.December.8.2

12. Brito NF, Moreira MF, Melo AC: A look inside odorant-binding proteins in insect chemoreception. *J Insect Physiol,* 95, 51-65, 2016. DOI: 10.1016/j.jinsphys.2016.09.008

13. Leal WS, Nikonova L, Peng G: Disulfide structure of the pheromone binding protein from the silkworm moth, *Bombyx mori*. *FEBS Lett,* 464 (1-2): 85-90, 1999. DOI: 10.1016/s0014-5793(99)01683-x

14. Scaloni A, Monti M, Angeli S, Pelosi P: Structural analysis and disulfide-bridge pairing of two odorant-binding proteins from *Bombyx mori*. *Biochem Biophys Res Commun*, 266 (2): 386-391, 1999. DOI: 10.1006/bbrc.1999.1791

15. Laughlin JD, Ha TS, Jones DNM, Smith DP: Activation of pheromone-sensitive neurons is mediated by conformational activation of pheromone-binding protein. *Cell,* 133 (7): 1255-1265, 2008. DOI: 10.1016/j.cell.2008.04.046

16. Angeli S, Ceron F, Scaloni A, Monti M, Monteforti G, Minnocci A, Petacchi R, Pelosi P: Purification, structural characterization, cloning and immunocytochemical localization of chemoreception proteins from *Schistocerca gregaria*. *Eur J Biochem,* 262 (3): 745-754, 1999. DOI: 10.1046/j.1432-1327.1999.00438.x

17. Pelosi P, Zhou JJ, Ban LP, Calvello M: Soluble proteins in insect chemical communication. *Cell Mol Life Sci,* 63 (14): 1658-1676, 2006. DOI: 10.1007/s00018-005-5607-0

18. Jacquin-Joly E, Merlin C: Insect olfactory receptors: Contributions of molecular biology to chemical ecology. *J Chem Ecol,* 30 (12): 2359-97, 2004. DOI: 10.1007/s10886-004-7941-3

19. Xu P, Atkinson R, Jones DN, Smith DP: *Drosophila* OBP LUSH is required for activity of pheromone-sensitive neurons. *Neuron,* 45 (2): 193-200, 2005. DOI: 10.1016/j.neuron.2004.12.031

20. Maida R, Mameli M, Müller B, Krieger J, Steinbrecht RA: The expression pattern of four odorant-binding proteins in male and female silk moths, *Bombyx mori*. *J Neurocytol,* 34 (1-2): 149-163, 2005. DOI: 10.1007/

s11068-005-5054-8

21. Vogt RG, Rybczynski R, Lerner MR: Molecular cloning and sequencing of general odorant-binding proteins GOBP1 and GOBP2 from the tobacco hawk moth Manduca sexta: comparisons with other insect OBPs and their signal peptides. *J Neurosci,* 11 (10): 2972-2984, 1991. DOI: 10.1523/JNEUROSCI.11-10-02972.1991

22. Zhou JJ, Robertson G, He X, Dufour S, Hooper AM, Pickett JA, Keep NH, Field LM: Characterisation of *Bombyx mori* Odorant-binding proteins reveals that a general odorant-binding protein discriminates between sex pheromone components. *J Mol Biol,* 389 (3): 529-545, 2009. DOI: 10.1016/j.jmb.2009.04.015

23. Zhou JJ: Odorant-binding proteins in insects. *Vitam Horm,* 83, 241-272, 2010. DOI: 10.1016/S0083-6729(10)83010-9

24. Guo B, Hao E, Qiao H, Wang J, Wu W, Zhou J, Lu P: Antennal transcriptome analysis of olfactory genes and characterizations of odorant binding proteins in two woodwasps, *Sirex noctilio* and *Sirex nitobei* (Hymenoptera: Siricidae). *BMC Genomics,* 22 (1):172, 2021. DOI: 10.1186/s12864-021-07452-1

25. Lu P, Hao E, Bao M, Liu R, Gao C, Qiao H: Mating behavior and identification of male-produced pheromone components in two woodwasps, *Sirex noctilio* and *Sirex nitobei*, in China. *Insects,* 13 (10):966, 2022. DOI: 10.3390/insects13100966

26. Faal H, Silk PJ, Mayo PD, Teale SA: Courtship behavior and identification of a sex pheromone in *Ibalia leucospoides* (Hymenoptera: Ibaliidae), a larval parasitoid of *Sirex noctilio* (Hymenoptera: Siricidae). *Peer J,* 9:e12266, 2021. DOI: 10.7717/peerj.12266

27. Xue J, Ai D, Xu X, Wang C, Jiang X, Han T, Er D: Isolation and identification of volatile substances with attractive effects on *Wohlfahrtia magnifica* from vagina of Bactrian camel. *Vet Sci,* 9 (11):637, 2022. DOI: 10.3390/vetsci9110637

28. Huang ZY, Wang XY, Lu W, Zheng XL: Sensory gene identification in the transcriptome of the ectoparasitoid *Quadrastichus mendeli*. *Sci Rep,* 11 (1):9726, 2021. DOI: 10.1038/s41598-021-89253-w

29. Song YQ, Gu HZ, Song ZY, Sun HZ: Identification of candidate chemosensory receptors in the antennal transcriptome of *Tropidothorax elegans*. *Mol Biol Rep,* 48 (4): 3127-3143, 2021. DOI: 10.1007/s11033-021-06327-8

30. Rihani K, Ferveur JF, Briand L: The 40-year mystery of insect odorant-binding proteins. *Biomolecules,* 11 (4):509, 2021. DOI: 10.3390/biom11040509

31. Han WK, Yang YL, Si YX, Wei ZQ, Liu SR, Liu XL, Yan Q, Dong SL: Involvement of GOBP2 in the perception of a sex pheromone component in both larval and adult Spodoptera litura revealed using CRISPR/Cas9 mutagenesis. *Insect Biochem Mol Biol1,* 41:103719, 2022. DOI: 10.1016/j.ibmb.2022.103719

32. Wang C, Hailing L, Gowa O, Huar B, Yunzhang L, Bin Y, Tuya L, Tu Y, Demtu E: Morphological and scanning electron microscopic (SEM) studies of the pupae of *Wohlfahrtia magnifica*. *J Camel Pract Res*, 27 (1): 17-22, 2020. DOI: 10.5958/2277-8934.2020.00003.X

33. Hailing L, Xiwen A, Junwen Z, La B, Hur C, Huar B, Bin Y, Yunzhang L, Demtu E: Morphological observation of the *Wohlfahrtia magnifica* in Mongolia plateau. *J Camel Pract Res*, 27 (3): 351-357, 2020. DOI: 10.5958/2277-8934.2020.00051.X

34. Hekmat-Scafe DS, Scafe CR, McKinney AJ, Tanouye MA: Genome-wide analysis of the odorant-binding protein gene family in *Drosophila melanogaster*. *Genome Res,* 12 (9): 1357-1369, 2002. DOI: 10.1101/gr.239402

35. Xu, P X, Zwiebel, L J & Smith DP: Identification of a distinct family of genes encoding atypical odorant-binding proteins in the *malaria vector mosquito, Anopheles gambiae. Insect Mol Biol,* 12 (6): 549-560, 2003. DOI: 10.1046/j.1365-2583.2003.00440.x

36. Vieira FG, Forêt S, He XL, Rozas J, Field LM, Zhou JJ: Unique features of odorant-binding proteins of the *Parasitoid Wasp Nasonia vitripennis* revealed by genome annotation and comparative analyses. *PLoS ONE,* 7 (8):e43034, 2012. DOI: 10.1371/journal.pone.0043034

37. Vogt RG, Große-Wilde E, Zhou JJ: The Lepidoptera odorant binding protein gene family: Gene gain and loss within the GOBP/PBP complex of moths and butterflies. *Insect Biochem Mol Biol,* 62, 142-153, 2015. DOI: 10.1016/j.ibmb.2015.03.003

38. Sánchez-Gracia A, Vieira FG, Rozas J: Molecular evolution of the major chemosensory gene families in insects. *Heredity (Edinb),* 103 (3): 208-216, 2009. DOI: 10.1038/hdy.2009.55

39. Picimbon JF, Dietrich K, Breer H, Krieger J: Chemosensory proteins of *Locusta migratoria* (Orthoptera: Acrididae). *Insect Biochem Mol Biol,* 30 (3): 233-241, 2000. DOI: 10.1016/s0965-1748(99)00121-6

40. Ozaki M, Wada-Katsumata A, Fujikawa K, Iwasaki M, Yokohari F, Satoji Y, Nisimura T, Yamaoka R: Ant nestmate and non-nestmate discrimination by a chemosensory sensillum. *Science,* 309 (5732): 311-314, 2005. DOI: 10.1126/science.1105244

41. Liu G, Ma H, Xie H, Xuan N, Guo X, Fan Z, Rajashekar B, Arnaud P, Offmann B, Picimbon JF: Biotype characterization, developmental profiling, insecticide response and binding property of *Bemisia tabaci* chemosensory proteins: Role of CSP in insect defense. *PLoS One,* 11 (5):e0154706, 2016. DOI: 10.1371/journal.pone.0154706

42. Zhou CX, Min SF, Yan-Long T, Wang MQ: Analysis of antennal transcriptome and odorant binding protein expression profiles of the recently identified *parasitoid wasp*, *Sclerodermus sp*. *Comp Biochem Physiol Part D Genomics Proteomics,* 16, 10-19, 2015. DOI: 10.1016/j.cbd.2015.06.003

43. Zhao Y, Wang F, Zhang X, Zhang S, Guo S, Zhu G, Liu Q, Li M: Transcriptome and expression patterns of chemosensory genes in antennae of the *Parasitoid Wasp Chouioia cunea*. *PLoS One,* 11 (2):e0148159, 2016. DOI: 10.1371/journal.pone.0148159

44. Sheng S, Liao CW, Zheng Y, Zhou Y, Xu Y, Song WM, He P, Zhang J, Wu FA: Candidate chemosensory genes identified in the endoparasitoid *Meteorus pulchricornis* (Hymenoptera: Braconidae) by antennal transcriptome analysis. *Comp Biochem Physiol Part D Genomics Proteomics,* 22, 20-31, 2017. DOI: 10.1016/j.cbd.2017.01.002

45. Wu Z, Zhang H, Wang Z, Bin S, He H, Lin J: Discovery of chemosensory genes in the oriental fruit fly, *Bactrocera dorsalis*. *PLoS One,* 12 (10):e0129794, 2015. DOI: 10.1371/journal.pone.0129794

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Research Article

Evaluation of Effectiveness of Neutral-pH Superoxidized Solution (NSOS) with Peritoneal Lavage in Rat Fecal Peritonitis Model: An Experimental Study

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Abstract

This experimental study aimed to evaluate the effectiveness of NSOS (Neutral-pH superoxidized solution) with peritoneal lavage in rat fecal peritonitis models. Forty Wistar rats weighing between 250-300 g were used for the study. All rats were inducted for fecal peritonitis formation. The rats were divided into five groups as follows; Group 0: control, no intervention. Group 1: Application of 10 mg/kg NSOS into the peritoneal cavity after 6 h of induction of peritonitis. Group 2: 10 mg/kg NSOS application and repeated at 24 and 48 h. Group 3: NSOS + Antibiotic treatment (Seftriakson 30 mg/kg/day IM 2x1 + metronidazol 15 mg/kg/day IM 2x1). Group 4: Only antibiotic treatment (Seftriakson 30 mg/kg/day IM 2x1 + metronidazol 15 mg/kg/day IM 2x1). The animals were examined for peritoneal and thoracal abscess formation, adherences, and any abnormality with inspection after sacrificing on the 7th day. The peritoneal lavage fluid culture for microbiological analysis and blood samples were taken for blood cultures, biochemical and infectious parameters of WBC, CRP, TNF- α, IL-6, IL-1 β, and IL-10. Peritonitis was developed in all rats at the end of follow-up. No death was observed in rats on the seventh day of the experiment. Group 3 (NSOS + Antibiotic treatment) showed the most significant improvement in the infection of peritoneal fluid. NSOS and antibiotic together (Group 3) were found to be more effective against *Klebsiella* than *Enterococcus* sp. The blood cultures showed a significant reduction in all groups. The infectious parameters including IL-6, IL-1 β, and IL-10 showed no significant difference in the first week of treatment between all groups. Only TNF- α was observed significantly lower in group 3 when compared to the other groups (P=0.001). Peritoneal lavage with neutral pH-superoxidized water plus an antibiotic regimen is the most effective treatment in the rat fecal peritonitis model. Further studies including human subjects are needed to investigate its effectiveness and validity.

Keywords: Fecal peritonitis, Neutral pH, NSOS, Rat, Antibiotic treatment

Introduction

Intraabdominal infections and sepsis are one of the leading causes of morbidity and mortality in patients with surgical interventions [1]. Peritonitis is usually caused by the bacterial contamination of the visceral peritoneum due to gastrointestinal organ perforations [2]. The infections in peritonitis usually come from the duodenum, stomach, gallbladder, pancreas, small intestine, colon, bladder, ovary, and renal system. Generalized peritonitis is an ominous sign of health status and requires prompt surgical therapy. If left untreated, the peritonitis can cause sepsis development quickly and sepsis is associated with a high rates of mortality and morbidity resulting from multiple organ failures [3].

Fecal peritonitis occurs due to the contamination of fecal material in the case of the bowel perforation. Enterococcus and Klebsiella are one of the most seen microorganisms in peritoneal infections and abscesses formation [4]. Surgical therapy is the main factor in the treatment of fecal peritonitis. During this surgery, the organ perforations are repaired and peritoneal lavage with normal saline irrigation is the most widely used method. In the literature, several studies showed the effect of different agents used for peritoneal lavage including ropivacaine, hydrogen-

rich saline, glycyrrhiza glabra cold atmospheric plasma, boric acid-linked ampicillin and lidocaine [5-11].

Neutral-pH superoxide water (NSOS) is formed by applying an electric current to salty water, followed by an electrochemical process in aqueous solutions from tap water and NaCI [12]. It has been used as a broad-spectrum disinfectant and shown to be non-toxic to human bodies. There are several studies reported that it has a significant antimicrobial effectiveness against bacteria and viruses [13]. The super-oxidized solutions have been used on ocular surfaces or wound infections and showed the confirmation of feasibility and safety in experimental studies [14].

In our previous experimental rat study on healthy peritoneal surfaces showed that pH-neutral superoxidized water does not result in any remarkable toxicity or complications [15]. Thus, we aimed to investigate its safety and effectiveness for peritoneal irrigation in the fecal peritonitis model.

Material and Methods

Ethical Approval

This study was approved by the Ethics Committee for Animal Experiments of Van Yüzüncü Yıl University, reference number 2020/10-11, dated 28.10.2020, and was performed in the Experimental Animal Research Laboratory of the university, located in Van, Türkiye.

Animals

Forty Wistar-albino rats were used in this experimental study. All rats were treated humanely in accordance with the Declaration of Helsinki. The animals were fed in accordance with the standards determined by the institution. The rats were randomly divided equally into 5 groups.

Group 0: Control group, fecal peritonitis was established and no other treatment was applied.

Group 1: *10 mg/kg NSOS applied into the peritoneal* cavity 6 h after the establishment of fecal peritonitis.

Group 2: 10 mg/kg NSOS applied into peritoneal cavity 6 h after establishment of fecal peritonitis and repeated at 24 and 48 h.

Group 3: 10 mg/kg NSOS and Antibiotic treatment (Seftriakson [Novasef®, Sanofi] 30 mg/kg/day IM 2x1 + metronidazol [Flagyl®, Eczacıbaşı] 15 mg/kg/day IM 2x1) applied into the peritoneal cavity 6 h after the establishment of fecal peritonitis.

Group 4: Only Antibiotic treatment (Seftriakson 30 mg/kg/day IM 2x1 + metronidazol 15 mg/kg/day IM 2x1) was applied 6 h after the establishment of fecal peritonitis.

Establishment of Fecal Peritonitis

Fecal peritonitis was created by the method defined by Buyne OR et al[16]. Fresh faeces from Wistar rats were obtained and suspended in twice the volume sterile water. An autoclave was used for sterilization of suspension. Then centrifugation process was performed and upper two layers were autclaved again and stored at 4°C. At the day of study 1 mL of fecal suspension was mixed with 0.5 mL of Schaedler solution including Escherichia coli bacillus level 10^4-10^8 cfu and B. fragilis level 10^4 cfu isolate which were obtained from the laboratory and then, 10 mL/kg of this fecal suspension was injected into the peritoneal cavity of the subjects. 6 h after injection of fecal material, the peritoneal cavity was irrigated with 10 mL/kg NSOS in groups 1, 2, and 3.

The rats were followed up daily for fever, tacyhpnea, weight, feeding status, and general appearance (flat hair, drowsiness, anxiety, blackness around the eyes). The rats were fed as Ad-Libitum. Single dose of 1 mg/kg of xylazine intraperitoneally administered after the operation for pain management. 24 h after the establishment of fecal peritonitis, 5-10 mL of saline was injected into the peritoneal cavity with massage and so distribution was created. 2-3 min after that procedure, 1-3 mL of peritoneal fluid was withdrawn. This fluid was kept for biochemical and microbiological analysis. Also, blood samples were taken for culture analysis.

There was no death in the subjects until Day 7. On the 7th day, the subjects were anesthetized with 50 mg/kg ketamine HCl (Ketalar®, Pfizer) and 10 mg/kg xylazine HCl (Rompun®, Bayer), and the abdominal and thorax cavities were opened, abscesses were investigated in the abdomen, and whether there was any adhesion was investigated. Fluid and tissue samples were taken for culture in the peritoneal cavity. Any abscess was investigated in the thoracic cavity. Blood samples were taken for biochemical analysis and culture, and then the animals were sacrificed. Among the biochemical parameters, CRP, TNF-α, IL-6, IL-1β, and IL-10 were evaluated by ELISA method. The hemogram was checked on an autoanalyzer.

Statistical Analysis

Descriptive statistics for continuous variables among the features emphasized expressed as Mean, Standard Deviation, Minimum and Maximum values, and also it is expressed as numbers and percentages for categorical variables. The Kruskal-Wallis test was used for comparisons made by groups in terms of continuous variables. The Chi-square test and multiple correspondence analysis were used to determine the relationship between groups and categorical variables. In the calculations, the statistical significance level was taken as 5% and the SPSS (ver:21) statistical package program was used for the calculations.

Results

All rats survived during the first week of the experiment after peritoneal lavages. The peritoneal infections developed in all groups. All groups were observed to have blood culture positivity at 24 h after the induction of peritonitis. Group 0, the control group, showed similar results on the first day and seventh days of the experiment. Although it was not statistically significant, group 1 showed a significant improvement of NSOS on the *Klebsiella* when compared the first day to seventh day. In group 2, the repeated use of NSOS did not show the additional effect on the *Klebsiella,* however, it showed some positive effect on the Enterococcus species *(Table 1)*.

Group 3 showed the most effectiveness and NSOS plus antibiotic regimen was the most effective treatment for *E. coli* and *Klebsiella*. This was statistically significant with p value of 0.016. First day of positive peritoneal culture's number was 17/24 and the seventh day was 4/24 (70% vs 16%, P=0.016). The peritoneal culture results showed that NSOS plus antibiotic regimens were the most effective method for the rat peritonitis model.

When analyzing the blood cultures, all groups had positive cultures for infection on first day of experiment. All groups showed a significant reduction for infections on the seventh day. Group 4 was observed no effectivity against *Enterococcus* species. However, the most significant improvement in the infections with regards to blood culture positivity taken for all three types of microorganisms *(Klebsiella, E. coli,* and *Enterococcus)* was observed in group 3 as 10/24 at first day vs 1/24 at seventh day with P=0.01) *(Table 2)*.

The peritoneal and blood cultures were taken at first day and seventh day of experiment. In total, 48 cultures were taken for each group. *Table 3* shows the comparison of Peritoneal Fluid and blood culture positivity *(Klebsiella, E. coli,* and *Enterococcus)* of first and seventh day of experiment in groups. All groups showed a decrease in culture positivity numbers. However, only group 3 (NSOS + antibiotic regimen) had a statistically significantly reduction in culture positivity (27/48, (56%) vs 5/48, (10%), P=0.022) *(Fig. 1)*.

The blood parameters including WBC, CRP, TNF-α, IL-6, IL-1β, IL-10 were analyzed at seventh day of experiment as shown in *Table 4*. It was observed that IL-6, IL-1β and IL-10 showed no significant difference in first week of treatment between all groups. TNF-α was observed significantly lower in group 3 when compared to the other

Table 1. *Peritoneal fluid cultures of groups at the 7th day*

Causative Bacteria		Group 0 (n:8) n (%)	Group 1 (n:8) n (%)	Group 2 (n:8) n (%)	Group 3 (n:8) n (%)	Group 4 (n:8) n (%)	P Value
Klebsiella	Positive Negative	4 (50%) 4 (50%)	2 (25%) 6 (75%)	5 (62%) 3 (37%)	1 (12%)[a] 7 (87%)	6 (75%) 2 (25%)	0.03
Enterococcus	Positive Negative	4 (50%) 4 (50%)	4 (50%) 4 (50%)	2 (25%) 6 (74%)	3 (37%) 5 (62%)	5 (62%) 3 (37%)	0.12
E. coli	Positive Negative	3 (37%) 5 (62%)	3 (37%) 5 (62%)	4 (50%) 4 (50%)	0[a] 8 (100%)	2 (25%) 6 (75%)	0.02
Total cultures	Positive Negative	11 (45%) 13 (55%)	9 (37%) 15 (62%)	11 (45%) 13 (55%)	4 (16%)[a] 20 (84%)	13 (55%) 11 (45%)	0.016

P<0.05 shows statistical significance

Table 2. *Blood Cultures of groups at the 7th day*

Causative Bacteria		Group 0 (n:8) n (%)	Group 1 (n:8) n (%)	Group 2 (n:8) n (%)	Group 3 (n:8) n (%)	Group 4 (n:8) n (%)	P Value
Klebsiella	Positive Negative	2 (25%) 6 (75%)	1 (12.5%) 7 (87.5%)	1 (12.5%) 7 (87.5%)	0 [a] 8 (100%)	2 (25%) 6 (75%)	0.002
Enterococcus	Positive Negative	3(37.5%) 5(62.5%)	2 (25%) 6 (75%)	2 (25%) 6 (75%)	0 [a] 8 (100%)	5 (62.5%) 3 (37.5%)	0.032
E. coli	Positive Negative	2(25%) 6(75%)	3 (37.5%) 5 (62.5%)	2 (25%) 6 (75%)	1 (12.5%) 7 (87.5%)	2 (25%) 6 (75%)	0.082
Total cultures	Positive Negative	7(29%) 17(70%)	6 (25%) 18 (75%)	5 (20%) 19 (79%)	1 (4%)[a] 23 (96%)	9 (37.5%) 15 (62.5%)	0.01

P<0.05 shows statistical significance, a indicates the statistically significant difference

Table 3. *Comparison of total number of peritoneal Fluid and blood culture positivity (Klebsiella, E. coli, and Enteroccocus) of first and seventh day of experiment in groups*

Groups	First Day n (%)	Seventh Day n (%)	P Value
Group 0	22/48 (45%)	18/48 (37%)	0.794
Group 1	24/48 (50%)	15/48 (31%)	0.114
Group 2	24/48 (50%)	16/48 (33%)	0.446
Group 3	27/48 (56%)	5/48 (10%)	0.022
Group 4	23/48 (47%)	20/48 (41%)	0.999

P<0.05 shows statistical significance

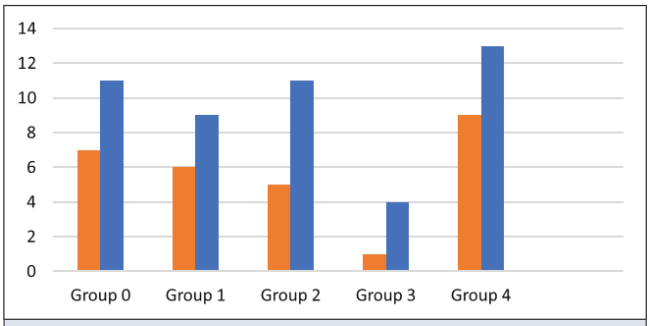

Fig 1. Total number of blood and peritoneal cultures positivity *(Klebsiella, E. coli,* and *Enterococcus)*

Table 4. *Comparison of biochemical-infectious-blood parameters in groups at the 7th day*

Parameters	Group 0 (n:8)	Group 1 (n:8)	Group 2 (n:8)	Group 3 (n:8)	Group 4 (n:8)	P Value
WBC, x10^3	11.09±1.55	8.20±3.81	7.32±1.04	6.68±0.84[a]	6.62±1.15[a]	0.002
CRP, mg/L	12.83 ±0.61	12.72±1.57	13.01±0.88	13.76±0.37	13.26±0.75	0.082
TNF-alpha, ng/L	125.27±23.54	131.04±22.78	127.86±20.61	98.53±17.52	125.89±9.34	0.082
IL-1, ng/mL	4.95±0.57	5.40±0.95	4.86±0.46	4.97±0.88	4.78±0.29	0.625
IL-6, ng/L	2.35±1.76	2.26±0.86	1.89±0.49	2.33±1.38	1.66±0.20	0.516
IL-10, pg/mL	44.11±0.41	43.85±0.13	44.00±0.45	43.72±0.03	43.76±0.10	0.121

P<0.05 shows statistical significance, WBC: White blood cell, CRP: C-reactive protein, TNF: Tumor necrosis factor, IL: Interleukin The measurements are shown as mean ± Standard deviation, a showed statistical difference

groups (98.5 3±17.52 ng/L, P=0.001). WBC was found to be significantly higher in Group 0, control group when compared to the other groups (11.09±1.55 x 10^3, P=0.002).

Discussion

Our previous animal study showed that pH-neutral SOS(NSOS) does not result in any significant toxicity or complications on the liver and peritoneal surfaces [15]. Moreover, in this study, we examined its effect on the infectious parameters in the fecal rat peritonitis model. This study showed that washing the peritoneal cavity with an NSOS plus antibiotic regimen has significant effectiveness in the treatment of rat fecal peritonitis model. Intraabdominal infections and abscess formations are one of the main reasons for morbidity and mortality after perforated appendicitis, tuba ovarian infections, or other peritoneal diseases [17]. Peritoneal irrigation is a challenging issue in the field of surgery and normal saline is traditionally a mostly used agent for peritoneal washings, however, this saline has no microbicidal activity. In the literature, there are many agents like lidocaine, ropivacaine, meropenem and moxifloxacin used to t reat this infection in rat peritonitis and also human subjects [6,7,18,19].

The peritoneum has a unique feature for its ability to deal with infections. The main mechanisms that have bactericidal activity are clearance from the diaphragmatic lymph system, phagocytization by host White blood cells and walled-off infection between the omentum and viscera [4,20]. These three factors can prevent intra-

abdominal infections at a certain level of bacterial load, however, the fecal contamination of the abdominal cavity in which a high load of microorganisms is encountered resulted in fail of defense systems. This eventually may lead to the development of sepsis and organ failures. Therefore studies have addressed the evaluation of effectiveness of different agents used for irrigation of peritoneal cavity during surgery. Aminoglycosides are the most used agent for peritoneal irrigation in cases of perforated appendicitis. A study by Shuaib et al.[21] evaluated the effectiveness of intraperitoneal gentamicin and clindamycin with peritoneal lavage in rat fecal peritonitis model and concluded that antibiotic peritoneal lavage alone reduces mortality rate when compared to a normal saline peritoneal lavage. Although the use of intraperitoneal antibiotics is thought to be safe and effective, studies focused on their bioavailability, long-term side effects, and cost-effectiveness.

Superoxidized water is a broad-spectrum antimicrobial and has been used in burn wounds, mediastinitis and open heart surgeries. Kubato et al.[12] studied the effect of strong acid electrolyzed water which is the older form of superoxide solutions on the surgical site infection of patients with perforated appendicitis and they found that peritoneal lavage and wound washing with superozidized water has no adverse effects and are effective for preventing surgical site infection. However, they used a strong acid agent that may be corrosive in mucoal surfaces. Therefore, NSOS which is used in our experimental study is a pH-neutral superoxidized water and formed by applying an electric current to salty water, followed by electro-chemical processed in aqueous solutions from pure water and sodium chloride. It is widely used as broad spectrum disinfectant and is non-toxic to human tissues unlike to the first supeoxide solutions which have low-pH values and corrosive features [22].

Landa-Solis et al.[23] studied the NSOS and reported that it has good antimicrobial activity, with significant advantages over strong acidic superozidized solutions, including neutral pH, decreased free active chlorine and a long shelf life. Our study confirmed this results that the Group 3 in which combined regimen of NSOS plus antibiotics showed the most significant improvement in the infectious state of rat peritonitis model. To date, this is the first randomized experimetal study that evaluated the effect of NSOS plus antibiotic regimen on rat peritonitis model. The results are promising that by applying and adding this non-toxic anti-bacterial solution to the intraperitoneal antibiotic lavage may prevent infections more effectively than other strategies.

It was worth noting that the standard treatment of peritonitis with antibiotics may be improved by adding the NSOS which is shown to be safe and non-toxic to human bodies and found to be effective when given with seftriakson-metronidazole combination together. Another point should be noted that our previous study is validated from the aspect of effectiveness, however, further clinical studies on human subjects is mandatory to implement its clinical use [15].

Some limitations should be declared about this study. Firtsly, it has a short term follow-up time of one week. Because some toxic effects or healing processes can be seen in a long-term time frame, this short time can be a weakness of our study. Secondly, the response to treatment was not measured by an objective method like bacteria colony number counting. Instead of it, the culture positivity was used. The main strength of this study is that all the procedures were performed by the same surgeon and culture analyses were done in the same hospital's laboratory.

In conclusion, this study's goal was to determine the effectiveness of NSOS in combination with antibiotics in the treatment of rat peritonitis model. Our findings revealed that peritoneal irrigation with NSOS plus an antibiotic regimen could improve the infectious state and survival of rats with peritonitis. Further studies including human subjects are needed to clarify our results and to determine its use in clinical practice.

Declarations

Availability of Data and Materials: The data used in this article will be provided by the corresponding author (A. Aras) upon request.

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

Author Contributions: Forming the hypothesis and planning the study: AA; Carrying out the experimental phase: AA, EK, SÇ, HS; Obtaining data and writing the article: AA, EK.

References

1. Martin GS, Mannino DM, Eaton S, Moss M: The epidemiology of sepsis in the United States from 1979 through 2000. *New Engl J Med,* 348 (16): 1546-1554, 2003. DOI: 10.1056/NEJMoa022139

2. Merrell RC, Latifi R: The abdomen as a source of sepsis in critically ill patient. **In,** Holzheimer RG, Mannick JA (Eds): Surgical Treatment: Evidence-Based and Problem-Orienteded. W. Zuckschwerdt Verlag GmbH, 2001.

3. Luo XJ, Peng J, Li YJ: Recent advances in the study on capsaicinoids and capsinoids. *Eur J Pharmacol,* 650 (1): 1-7, 2011. DOI: 10.1016/j.ejphar.2010.09.074

4. Malangoni MA, Song J, Herrington J, Choudhri S, Pertel P: Randomized controlled trial of moxifloxacin compared with piperacillin-tazobactam and amoxicillin-clavulanate for the treatment of complicated intra-

abdominal infections. *Ann Surg,* 244 (2): 204-211, 2006. DOI: 10.1097/01.sla.0000230024.84190.a8

5. Sada H, Egi H, Ide K, Sawada H, Sumi Y, Hattori M, Sentani K, Oue N, Yasui W, Ohdan H: Peritoneal lavage with hydrogen-rich saline can be an effective and practical procedure for acute peritonitis. *Surg Today,* 51 (11): 1860-1871, 2021. DOI: 10.1007/s00595-021-02271-z

6. Brocco MC, Gomez RS, Paulo DN, Almeida CE, Baptista JF: Histological features of peritoneal lavage with ropivacaine in rats with fecal peritonitis. *Acta Cir Bras,* 27 (2): 193-199, 2012. DOI: 10.1590/s0102-86502012000200016

7. Brocco MC, Paulo DN, Baptista JF, Ferrari TA, Azevedo TC, Silva AL: Effects of peritoneal lavage with lidocaine on survival of rats with fecal peritonitis. *Acta Cir Bras,* 23 (1): 42-47, 2008. DOI: 10.1590/s0102-86502008000100008

8. Zhou Q, Meng W, Ren Y, Li Q, Boermeester MA, Nthumba PM, Rickard J, Zheng B, Liu H, Shi Q, Zhao S, Wang Z, Liu X, Luo Z, Yang K, Chen Y, Sawyer RG: Effectiveness of intraoperative peritoneal lavage with saline in patient with intra-abdominal infections: A systematic review and meta-analysis. *World J Emerg Surg,* 18 (1): 24, 2023. DOI: 10.1186/s13017-023-00496-6

9. Rakhshandeh H, Rahimi VB, Dehghan-Naieri B, Rahmanian-Devin P, Jebalbarezy A, Hasanpour M, Iranshahi M, Askari VR: Peritoneal lavage with is effective in preventing peritoneal adhesion in a rat model. *Inflammopharmacology,* 31 (2): 899-914, 2023. DOI: 10.1007/s10787-023-01139-7

10. Oztan MO, Ercan UK, Aksoy Gokmen A, Simsek F, Ozdemir GD, Koyluoglu G: Irrigation of peritoneal cavity with cold atmospheric plasma treated solution effectively reduces microbial load in rat acute peritonitis model. *Sci Rep,* 12 (1): 3646, 2022. DOI: 10.1038/s41598-022-07598-2

11. Idiz UO, Aysan E, Firat D, Ercan C, Demirci S, Sahin F: Effects of boric acid-linked ampicillin on the rat intra-abdominal sepsis model. *Pak J Pharm Sci,* 32 (2): 477-481, 2019.

12. Kubota A, Goda T, Tsuru T, Yonekura T, Yagi M, Kawahara H, Yoneda A, Tazuke Y, Tani G, Ishii T, Umeda S, Hirano K: Efficacy and safety of strong acid electrolyzed water for peritoneal lavage to prevent surgical site infection in patients with perforated appendicitis. *Surg Today,* 45 (7): 876-879, 2015. DOI: 10.1007/s00595-014-1050-x

13. Martinez-Munive A, Menedez-Skertchly A, Toiber M: Super-oxidized water (Microcyn 60) for mesh hernioplasty in grossly contaminated fields: An experimental study. *American College of Surgeons 91st Annual Clinical Congress,* San Francisco, 2005.

14. Nakae H, Inaba H: Effectiveness of electrolyzed oxidized water irrigation in a burn-wound infection model. *J Trauma,* 49 (3): 511-514, 2000. DOI: 10.1097/00005373-200009000-00020

15. Aras A, Karaman E, Yildirim S, Yilmaz O, Kiziltan R, Karaman K: Intraperitoneal infusion of neutral-pH superoxidized solution in rats: Evaluation of toxicity and complications on peritoneal surface and liver. *Med Sci Monit,* 23, 960-965, 2017. DOI: 10.12659/msm.899453

16. Buyne OR, Bleichrodt RP, Verweij PE, Groenewoud HM, van Goor H, Hendriks T: A peritonitis model with low mortality and persisting intra-abdominal abscesses. *Int J Exp Pathol,* 87 (5): 361-368, 2006. DOI: 10.1111/j.1365-2613.2006.00488.x

17. Owens CD, Stoessel K: Surgical site infections: Epidemiology, microbiology and prevention. *J Hosp Infect,* 70 (Suppl. 2): 3-10, 2008. DOI: 10.1016/S0195-6701(08)60017-1

18. Mascena GV, Figueiredo Filho CA, Lima Junior MAX, Oliveira TKB, Gadelha DNB, Melo M, Brandt CT: Fecal peritonitis in aging rat model: Therapeutic response to different antibiotic strategies. *Acta Cir Bras,* 33 (5): 446-453, 2018. DOI: 10.1590/s0102-865020180050000007

19. Sakcak I, FM A, EU Y, Atas H, Kilic K, Cosgun E, Mumcuoglu I, Sezer S, Bostanoglu S, Isgoren A: The prevention of the development of peritonitis with different disinfectants to compare the efficacy of gastric lavage at the NOTES. *Kafkas Univ Vet Fak Derg,* 17 (Suppl. A): S37-S42, 2011. DOI: 10.9775/kvfd.2010.3080

20. Dunn DL, Barke RA, Ahrenholz DH, Humphrey EW, Simmons RL: The adjuvant effect of peritoneal fluid in experimental peritonitis. Mechanism and clinical implications. *Ann Surg,* 199 (1): 37-43, 1984. DOI: 10.1097/00000658-198401000-00007

21. Shuaib A, Shuaib A, Alown F, Abdulhussain H, Albanoun A, Edward M, Alduhaim T: Evaluation of effectiveness of intraperitoneal gentamicin and clindamycin with peritoneal lavage in rat fecal peritonitis model. *Surg Chron,* 25 (1): 7-11, 2020.

22. Rutala WA, Weber DJ: New disinfection and sterilization methods. *Emerg Infect Dis,* 7 (2): 348-353, 2001. DOI: 10.3201/eid0702.010241

23. Landa-Solis C, González-Espinosa D, Guzmán-Soriano B, Snyder M, Reyes-Terán G, Torres K, Gutierrez A: Microcyn™: A novel super-oxidized water with neutral pH and disinfectant activity. *J Hosp Infect,* 61 (4): 291-299, 2005. DOI: 10.1016/j.jhin.2005.04.021

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Research Article

Effect of Dietary Glycerol Addition on Growth Performance, Serum Biochemical Indexes, Carcass Traits, Fat Deposition, and Meat Quality in Fattening Period Kazakh Sheep

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Abstract

Glycerol has been evaluated as a safe and promising animal feed. It can replace traditional energy foods and reduce competition with major animal husbandry systems. In this study, 40 fattening Kazakh ewes (18 months of age) were selected to investigate the dynamic response of growth performance, serum biochemical indexes, fat deposition, carcass traits and meat quality to different levels of glycerol (0, 1%, 7%, and 12% DM). The results showed that glycerol could improve sheep growth performance, increase fat deposition, increase carcass dressing percentage and improve meat quality. Among them, the Gly7% group had significant positive effects (P<0.05) on FBW, ADG, GLU, TP, ALB, TG, HDL-C, fat deposition, HCW, CCW, HCD and EE; and significant negative effects (P<0.05) on FCR, ADFI, TC, LDL-C, a*, b* and SF. In conclusion, the recommended supplementation of glycerol in the diet of Kazakh ewes is 7% DM for optimal fattening performance without compromising health. This study may provide a theoretical basis for the rational utilization of food-grade glycerol in sheep diets.

Keywords: Kazakh sheep, Glycerol, Fat deposition, Growth performance, Carcass traits

Introduction

Kazakh sheep is a meat and fat dual-purpose rough wool sheep breed, with adaptability, cold resistance, rough feeding, stable genetic traits, disease resistance, and other excellent characteristics. They are quick to catch fat in summer and fall and have high meat and fat production performance [1]. Inadequate forage in winter and spring leads to insufficient nutritional intake and poor body condition of sheep, which seriously affects the production and reproductive performance of ewes in the coming year. Therefore, empty ewes need to supply energy and restore body condition in the spring. Crude glycerol is a by-product of the conversion of vegetable oils or animal fats into biodiesel, and its main component is glycerol [2]. Glycerol has an energy value similar to corn. It is a safe and promising animal feed that can enter the animal feed market as a safe dietary ingredient for animals, thus replacing traditional energy feeds [3,4].

In recent years, a large number of studies have explored the potential of adding glycerol to ruminant diets. Cheng and Huang [5] reported that glycerol can improve the economic efficiency of beef cattle rearing. Barros et al.[6] and Van Cleef et al.[7] found that the use of crude glycerol only reduced the nutrient intake of the animals and had no significant effect on growth performance. Orrico et al.[8] concluded that the addition of 7.5% crude glycerol to the diet of fattening lambs had a more positive effect in terms of reducing dry matter intake, improving feed conversion and reducing feeding costs. Ribeiro et al.[9] suggested that the addition of 70 g/kg of crude glycerol to lamb diets contributed more positively to increases in slaughter weight, carcass weight and improved carcass traits. However, Brant et al.[10] demonstrated that replacing energy diets with glycerol significantly reduced lamb final weight, average daily gain and cold carcass yield. The effect of glycerol addition to ruminant diets on the above

indicators varied from experiment to experiment, thus more in-depth studies are needed.

Current research on glycerol has focused on exploring its effects on feed intake, nutrient digestibility, rumen fermentation and sensory traits. However, the effect of food-grade glycerol as an energy supplement on fat deposition and fattening in Kazakh ewes has not been reported. Therefore, this study aimed to evaluate the effect of glycerol as an energy supplement in feed on growth performance, serum biochemical indexes, carcass traits, fat deposition and meat quality of fattening Kazakh sheep and to determine the appropriate dosage in diets. Ultimately, a theoretical basis was laid for the application of food-grade glycerol in sheep farming.

Material and Methods

Ethical Statement

This study was approved by the Bioethics Committee of Shihezi University (Approval no: A2021-38). All sheeps were kept experimentally and euthanized in strict accordance with the committee's guidelines. During the experiment, every effort was made to minimize suffering by the animals.

Animals, Experimental Design and Feed

Forty healthy Kazakh ewes (18 months of age; initial body weight = 32.55±3.13 kg) were selected in the spring and randomly divided into 4 groups of CON, Gly1%, Gly7% and Gly12% according to the level of glycerol supplementation, with 10 replicates in each group. All sheep were fed a basal diet for a 104-d feeding period (comprising a 14-d adaptation period and a 90-d experimental period). The diet was formulated according to The National Research Council (NRC) (2007) ewes nutritional standard, and its composition and nutritional levels are listed in *Table 1*. And the glycerol used in this experiment was food-grade glycerol (License No. SC20137148200057, purity: 99.88%, combustion energy: 4.32 Mcal/kg), which was supplied by Shandong Xuanyang Biotechnology Co. Ltd (Shandong, China).

Forty sheep were shorn and vaccinated before the experiment and weighed and housed in groups in a semi-open barn with a consistent feeding environment in terms of temperature, ventilation and light. Sheep were fed the basal diet twice a day (at 08:00 and 19:00) with ad libitum feeding and watering. Glycerol was dissolved in water and administered 1 h after morning feeding each day according to the glycerol concentration corresponding to the different groups. The amount of sheep fed to each group was adjusted to exceed the total feed intake of each group by about 3% on the previous day. At the end of the feeding experiment, six sheep from each group with final body weights close to the group average were selected and euthanized after 12 h of fasting and water deprivation.

Determination of Growth Performance

The body weight was recorded as initial body weight (IBW) on the 1st day and final body weight (FBW) on the 90th day of the experiment period and was weighed every 30d during the experiment period. The amount of feed and leftovers from each group were collected and weighed daily to calculate the average daily feed intake of sheep in the group (ADFI=(amount fed on the day-leftovers on day 2)/10). Finally feed conversion ratio (FCR=ADFI/ADG) was calculated.

Serum Collection and Analysis

Serum was prepared by collecting 5 mL of jugular vein blood before morning feeding on days 1, 30, 60, and 90

Table 1. *Ingredients and chemical compositions of basal diet (%, of DM)*

Ingredients	Content, %	Chemical Composition[2]	Content, %
Alfalfa hay	40.00	Net energy (Mcal/kg)	2.62
Alfalfa silage	35.00	Dry Matter (DM)	83.375
Oat grass	5.00	Crude protein (CP)	17.50
Soybean meal	7.90	Ash	8.63
Corn	8.50	Ether extract (EE)	2.90
Baking soda	1.50	Calcium	0.49
Premix[1]	1.00	Phosphorus	0.41
Sodium chloride	0.80	Neutral detergent fiber(NDF)	44.94
Lysine	0.30	Acid detergent fiber(ADF)	35.59
Total	100.00		

[1] *Provided the following per kilogram of diet: 3600 mg of Fe (as ferrous sulfate), 4000 mg of Zn (as zinc sulfate), 400 mg of Cu (as copper sulfate), 20 mg of Na (as sodium chloride), 2000 mg of Mn (as manganese), 40 mg of I (as potassium iodide), 20 mg of Co (as cobalt sulfate), 400,000 IU of vitamin A, 40,000 IU of vitamin D_3, and 8,000 IU of vitamin E.*
[2] *Energy level were predicted and the rest nutrient levels were measured*

of the positive test period, respectively. Total protein (TP), albumin (ALB), globulin (GLB), creatinine (Cre), blood urea nitrogen (BUN), glucose (GLU), triglycerides (TG), high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C), total cholesterol (TC), alanine aminotransferase (ALT), aspartate aminotransferase (AST), and alkaline phosphatase (AKP) were measured using commercially available kits from the Institute for Construction Bioengineering (Nanjing, Jiangsu, China). Experiments were performed on a full-wavelength enzyme labeler-microplate spectrophotometer (Thermo Fisher Scientific, USA) instrument as described by Zheng et al.[11].

Determination of Fat Deposition and Carcass Traits

Live weight before slaughter (LWBS), organ weights, and fat weights of each site (pericardial fat, perirenal fat, and tail fat) were weighed and recorded, and then an index was calculated for each organ (organ index= (organ weight/LWBS) × 100). The carcasses were weighed as hot carcass weights (HCW) after standing for 30 min and cold carcass weights (CCW) after 24 h of acid drainage at 4°C, and the hot carcass dressing percentage (HCD = (HCW/LWBS) × 100) and cold carcass dressing percentage (CCD = (CCW/LWBS) × 100) were calculated. Loin eye muscle area (LEA = Length × width × 0.7) and back-fat thickness (BFT) were measured as described by Coria et al.[12] at the longissimus dorsi between the 12th and 13th ribs on the left side of the carcass.

Determination of Meat Quality

A sample of the longissimus dorsi of each sheep was taken and measured for meat color and pH. PH was determined at 45 min and 24 h after slaughter using a portable acidimeter. Meat color was recorded 45 min after slaughtering with a meat colorimeter (MATTHAUS, OPTP-STAR, Germany), including L* (brightness), a* (red) and b* (yellow). Meat color and pH were measured three times at different parts of the sample and the average of the three points was taken to determine the final values. Samples of the longissimus dorsi were taken for the determination of drip loss, cooking loss and shear force. Each sample was trimmed into a 2 cm square, weighed and recorded as W1, hung in a bag, stored at 4°C for 24 h and then blotted and weighed as W2, which was used to calculate the drip loss (DL = (W1-W2)/W1 × 100). About 50 g of each muscle sample was taken, weighed and recorded as w1, and heated in a sealed water bath at 80°C for 1 h. The meat samples were removed and cooled to room temperature in cold water, drained and weighed and recorded as w2, which was used to calculate the cooking loss (CKL = (w1-w2)/w1 × 100). The meat samples after cooking loss determination were cut into 1cm×1cm×3cm sizes along its muscle fiber direction for shear force (SF) determination. Each sample was randomly selected at three different locations and cut perpendicular to the muscle fiber direction using a digital tenderizer (Model: C-LM3B), and the average value was taken at the end.

The chemical composition of the longissimus dorsi and diets was determined according to the recommendations of AOAC (2008). Conventional nutrients were determined according to the following methods: dry matter (method 934.01); crude protein (CP) (N × 6.25; method 984.13); ether extract (EE) (method 920.39); crude ash (Ash) (method 942.05) were determined [13].

Statistical Analysis

The experimental data were analyzed using SPSS 26.0 software. Data on ADG, ADFI, FCR and serum biochemical indexes were analyzed using a linear mixed-effects model. The method used comprised treatment, period, and treatment × period as fixed factors, and sheep as a random factor to assess the effect of different glycerol levels of repeated measures over time on the same sheep. Means were estimated using least-square means, with statistical significance between groups determined using the Duncan test [14,15]. The remaining data were analyzed by one-way ANOVA and Duncan test. The results were expressed as the mean ± SEM. A statistical significance was considered as $P<0.05$, and a trend was defined as $0.05\leq P<0.10$.

Results

As can be seen in *Table 2*, the difference in IBW between the groups was not significant ($P>0.05$), and FBW was significantly increased in the Gly7% group ($P<0.05$). Period and treatment × period had no significant effect on ADG and FCR ($P>0.05$), while Period had a significant difference on ADFI ($P<0.05$), especially, ADFI was significantly reduced on the 60th and 90th d. The differences in ADG, ADFI and FCR between the groups were significant ($P<0.05$), with the Gly 7% group significantly increasing ADG by about 28.40% and decreasing ADFI and FCR by about 1.63% and 35.57%, respectively, compared with the CON group.

The blood biochemical indexes of Kazakh sheep are shown in *Table 3*. Treatment × period had a significant effect on serum GLU, TG, HDL-C and LDL-C levels ($P<0.05$), but not on other indexes ($P>0.05$). In terms of treatment, serum concentrations of GLU, TP, ALB, TG, HDL-C and TG were significantly higher and serum concentrations of TC and LDL-C were significantly lower in the Gly7% group compared to the CON group. However, other biochemical indexes were not significantly different between the groups ($P>0.05$). The effects of the experiment period on serum GLU, LDL-C, TG, TC and AKP parameters were all significantly different ($P<0.05$). Among them, serum

Table 2. *Effect of glycerol supplementation in diets on growth performance of Kazakh sheep (n=10)*

Item	Experimental Diets				SEM	Period			SEM	P-Value		
	CON	Gly1%	Gly7%	Gly12%		30d	60d	90d		Treatment	Period	Treatment × Period
IBW/kg	32.58	32.52	32.54	32.56	0.450	-	-	-	-	1.000	-	-
FBW/kg	45.30^{b}	46.66^{ab}	48.60^{a}	45.02^{b}	0.501	-	-	-	-	0.039	-	-
ADG/(g/d)	142.78^{b}	155.56^{ab}	183.33^{a}	131.67^{b}	6.640	153.00	157.83	150.67	6.606	0.018	0.891	0.472
ADFI/(g/d)	1.23^{a}	1.22^{ab}	1.21^{b}	1.22^{ab}	0.003	1.29^{a}	1.19^{b}	1.18^{b}	0.001	0.004	<0.001	0.154
FCR	11.16^{a}	8.56^{bc}	7.19^{c}	10.33^{ab}	0.399	9.09	9.18	9.67	0.397	0.001	0.780	0.565

^{a,b} *Values within a row with different superscripts differ significantly at P<0.05; CON, Gly 1%, Gly 7% and Gly 12% group was fed the basal diet supplemented with glycerol at 0%, 1%, 7%, and 12% (DM basis) per sheep*

Table 3. *Effect of glycerol supplementation in diets on blood biochemical indexes of Kazakh sheep (n=10)*

Item	Experimental Diets				SEM	Period				SEM	P-Value		
	CON	Gly1%	Gly7%	Gly12%		0d	30d	60d	90d		Treatment	Period	Treatment × Period
GLU (mmol/L)	2.37^{b}	2.54^{ab}	2.60^{a}	2.48^{ab}	0.034	2.24^{c}	2.48^{b}	2.73^{a}	2.53^{b}	0.031	0.040	<0.001	0.016
Cre (μmol/L)	49.11	55.71	59.25	52.53	1.583	49.65	59.06	55.25	52.65	1.554	0.100	0.128	0.713
TP (g/L)	55.29^{b}	55.64^{b}	56.44^{a}	55.39^{b}	0.137	55.52	55.98	55.81	55.44	0.116	0.011	0.400	0.985
ALB (g/L)	21.61^{b}	22.26^{ab}	22.63^{a}	22.58^{a}	0.132	21.96	22.03	22.44	22.64	0.130	0.015	0.152	0.468
GLB (g/L)	33.68	33.38	33.81	32.81	0.179	33.56	33.96	33.37	32.80	0.175	0.173	0.118	0.896
BUN (mmol/L)	5.15	5.13	5.13	5.19	0.010	5.13	5.13	5.16	5.18	0.010	0.080	0.107	0.249
TG (mmol/L)	0.55^{b}	0.57^{ab}	0.60^{a}	0.54^{b}	0.008	0.55^{ab}	0.53^{b}	0.60^{a}	0.58^{ab}	0.008	0.014	0.013	0.030
HDL-C (mmol/L)	0.97^{b}	0.99^{ab}	1.02^{a}	0.95^{b}	0.010	0.96	0.97	1.02	0.98	0.010	0.023	0.090	0.030
LDL-C (mmol/L)	0.61^{a}	0.56^{ab}	0.54^{b}	0.61^{a}	0.012	0.65^{a}	0.61^{a}	0.52^{b}	0.53^{b}	0.011	0.039	<0.001	0.034
TC (mmol/L)	1.58^{a}	1.51^{ab}	1.34^{b}	1.31^{b}	0.037	1.28^{b}	1.45^{ab}	1.54^{a}	1.48^{ab}	0.036	0.014	0.048	0.954
ALT (U/L)	22.46	21.63	21.49	21.91	0.240	22.60	22.19	21.07	21.64	0.235	0.466	0.105	0.981
AST (U/L)	89.25	89.12	89.88	88.67	0.472	89.92	89.52	89.53	87.95	0.441	0.748	0.185	0.492
AKP (U/L)	86.92	85.63	85.08	87.24	0.419	87.65^{a}	86.82^{ab}	85.07^{b}	85.33^{b}	0.410	0.161	0.012	0.774

^{a,b,c} *Values within a row with different superscripts differ significantly at P<0.05; CON, Gly 1%, Gly 7%, and Gly 12% group was fed the basal diet supplemented with glycerol at 0%, 1%, 7% and 12% (DM basis) per sheep*

Table 4. *Effect of glycerol supplementation in diets on fat deposition of Kazakh sheep (n=6)*

Item	Experimental Diets				SEM	P-Value
	CON	Gly1%	Gly7%	Gly12%		
Pericardial fat	67.42	73.24	75.35	68.88	1.885	0.433
Perirenal fat	96.70^{b}	119.62^{a}	124.55^{a}	98.19^{b}	4.044	0.012
Tail fat	939.17^{b}	976.55^{ab}	1048.10^{a}	923.95^{b}	17.126	0.036
Total fat	1003.29^{b}	1169.40^{ab}	1248.00^{a}	1091.02^{b}	19.427	0.007

^{a,b} *Values within a row with different superscripts differ significantly at P<0.05; CON, Gly 1%, Gly 7%, and Gly12% group was fed the basal diet supplemented with glycerol at 0%, 1%, 7% and 12% (DM basic) per sheep*

GLU, TG, and TC levels were significantly higher, and LDL-C and AKP levels were significantly lower on 60 d. However, HDL-C content tended to increase (P=0.090) on 60 d (1.02 mmol/L vs. 0.96 mmol/L).

As shown in *Table 4*, perirenal fat, tail fat and total fat weights differed significantly (P<0.05) among the groups. Compared to the CON group, perirenal fat was significantly increased by about 23.70% and 28.80% in the Gly 1% and Gly 7% groups, and tail fat and total fat were significantly increased by about 11.60% and 24.39% in the Gly 7% group. Fat weight in the CON group was lower than in each glycerol group, except in the tail fat, where fat weight was slightly higher than in the Gly 12% group. The

Table 5. *Effect of glycerol supplementation in diets on carcass traits of Kazakh sheep (n=6)*

Item	Experimental Diets				SEM	P-Value
	CON	Gly1%	Gly7%	Gly12%		
LWBS (kg)	45.40	46.53	48.50	44.90	0.537	0.073
HCW (kg)	19.89^{b}	20.61^{b}	22.18^{a}	19.83^{b}	0.323	0.022
HCD (%)	43.79^{b}	44.28^{b}	45.71^{a}	44.17^{b}	0.260	0.035
CCW (kg)	18.70^{b}	19.67^{ab}	21.00^{a}	18.77^{b}	0.332	0.037
CCD (%)	41.19	42.22	43.35	41.83	0.477	0.465
BFT (mm)	4.88	5.27	5.65	5.15	0.112	0.103
LEA (cm^2)	14.24	13.78	13.42	13.93	0.150	0.296

^{a,b} *Values within a row with different superscripts differ significantly at P<0.05; CON, Gly 1%, Gly 7%, and Gly 12% group was fed the basal diet supplemented with glycerol at 0%, 1%, 7% and 12% (DM basis) per sheep*

Table 6. *Effect of glycerol supplementation in diets on organ weights and indexes of Kazakh sheep (n=6)*

Organ	Item	Experimental Diets				SEM	P-Value
		CON	Gly1%	Gly7%	Gly12%		
Heart	Weight (g)	260.97	256.53	272.33	258.50	2.492	0.101
	PLWBS (%)	0.58	0.55	0.56	0.58	0.010	0.463
Liver	Weight (g)	503.27	570.15	631.87	563.95	18.826	0.111
	PLWBS (%)	1.12	1.30	1.33	1.09	0.050	0.230
Spleen	Weight (g)	75.27	78.07	77.20	72.70	1.111	0.346
	PLWBS (%)	1.17	1.17	1.16	1.16	0.003	0.890
Lungs	Weight (g)	467.40	470.57	480.60	463.37	4.481	0.596
	PLWBS (%)	1.03	1.01	0.99	1.04	0.013	0.704
Kidneys	Weight (g)	170.50	176.77	180.90	171.53	2.524	0.455
	PLWBS (%)	0.38	0.38	0.37	0.38	0.006	0.946

CON, Gly 1%, Gly 7% and Gly 12% group was fed the basal diet supplemented with glycerol at 0%, 1%, 7%, and 12% (DM basic) per sheep

Table 7. *Effect of glycerol supplementation in diets on meat quality of Kazakh sheep (n=6)*

Item	Experimental Diets				SEM	P-Value
	CON	Gly1%	Gly7%	Gly12%		
pH_{45min}	6.19	6.24	6.33	6.24	0.035	0.592
pH_{24h}	5.73	5.47	5.61	5.69	0.049	0.240
L*	34.13	38.60	36.57	36.00	0.658	0.111
a*	13.43^{a}	12.32^{b}	12.31^{b}	12.08^{b}	0.180	0.023
b*	9.83^{a}	9.59^{ab}	9.29^{b}	9.15^{b}	0.088	0.016
SF (N)	32.97^{a}	30.03^{ab}	28.13^{b}	30.67^{ab}	0.603	0.029
DL (%)	2.51	2.29	2.09	2.35	0.063	0.118
CKL (%)	30.67	29.45	28.35	29.80	0.328	0.082

^{a,b} *Values within a row with different superscripts differ significantly at P<0.05; CON, Gly 1%, Gly 7%, and Gly12% group was fed the basal diet supplemented with glycerol at 0%, 1%, 7% and 12% (DM basic) per sheep*

above results indicate that glycerol supplementation in the diet had a promotional effect on fat deposition, which was more pronounced in the Gly 7% group.

Carcass traits are shown in *Table 5*. Significant differences were observed in HCW, HCD and CCW between the groups (P<0.05). HCD increased in all glycerol groups compared to the CON group and increased with increasing glycerol levels in the Gly1% and Gly7% groups. However, there was a tendency for the Gly1% and Gly7% treatment groups to have higher LWBS (P=0.073) than the CON group (46.53 and 48.50 vs. 45.40 kg). In terms of organ weights and organ indexes *(Table 6)*, there were no

Table 8. *Effect of glycerol supplementation in diets on the chemical composition of the longissimus dorsi of Kazakh sheep (n=6)*

Item	Experimental Diets				SEM	P-Value
	CON	Gly1%	Gly7%	Gly12%		
Moisture (%)	68.61	70.29	71.01	69.73	0.372	0.129
CP (%)	23.27	23.63	24.64	23.17	0.229	0.080
EE (%)	5.05^{b}	5.22^{ab}	5.40^{a}	5.20^{ab}	0.043	0.025
Ash (%)	1.28	1.24	1.24	1.25	0.006	0.176

^{a,b} *Values within a row with different superscripts differ significantly at P<0.05; CON, Gly 1%, Gly 7% and Gly12% group was fed the basal diet supplemented with glycerol at 0%, 1%, 7% and 12% (DM basic) per sheep*

significant differences (P>0.05) between the groups. Liver and kidneys weights were increased in all glycerol groups as compared to the CON group, with the Gly7% group maximizing the increase by about 25.55% and 6.10%. All glycerol groups decreased heart and lungs indexes and increased liver index.

The meat quality is shown in *Table 7*. The pH value of the slaughtered muscle was less than 7.0, which was weakly acidic, and the pH value decreased with the cooling time of the muscle, but the difference between the groups was not significant (P>0.05). The a*, b* and SF decreased significantly with increasing glycerol levels (P<0.05). There was a trend of decreasing CKL in the treatment group compared to the CON group (29.45%, 28.35% and 29.80% vs. 30.67%) (P=0.082). In terms of muscle chemical composition *(Table 8)*, after glycerol supplementation, the EE content of muscle was increased in all groups, including a significant increase of 6.93% in the Gly7% groups (P<0.05). In addition, the content of CP in the Gly1% and Gly7% groups tended to increase compared with the CON group (23.63% and 24.64% versus 23.27%) (P=0.080). However, there was no significant difference in moisture and Ash content between the groups (P>0.05).

Discussion

Piao et al.[16] found that replacing a portion of the molasses in the diet with 3.17% glycerol increased the intake of their concentrates, and hypothesized that glycerol was more effective than molasses in improving appetite. Studies have shown that adding 5% and 10% glycerol to diets can increase ADG, decrease FCR and limit feed intake [16-19]. Our experimental results were similar in that the increase in glycerol supplementation in the diet was accompanied by a significant limitation of ADFI, which gradually decreased over time, reaching a minimum on the 90 d of the experiment. We speculate that this may be because absorbed glycerol is metabolized in the liver to 3-phosphoglycerol, which then participates in the gluconeogenesis or glycolysis pathway, increasing the number of hepatic tricarboxylic acid cycles, which in turn stimulates satiety in sheep, leading to a decrease in feed intake. And Wang et al.[18] reported that ADG increases with increasing dietary energy levels. However, we found that the body weight and ADG of the highest energy Gly12% group did not follow this pattern. We considered that the excessive nutrient levels negatively affected the digestion and metabolism of the sheep, thus affecting their growth performance. Meanwhile, ADG was highest on d 60, suggesting that this is when sheep are physiologically at their best. A similar report is: Ding et al.[20] found that ADG and FCR were highest on d 45 in a 60-d experiment.

Serum biochemical indexes can reflect the body's absorption and metabolism of nutrients [21]. GLU levels are positively correlated with dietary energy levels, with low GLU levels indicating a low level of dietary nutrition or a reduced ability of the body to digest, absorb and utilize sugars. GLU content is influenced by dietary energy levels, with low GLU indicating a low level of dietary nutrition or a reduced ability of the body to digest, absorb and utilize sugars [22]. In the present study, the CON and Gly12% groups had lower GLU levels, which was consistent with their lower body weights and ADGs, suggesting that these groups had inadequate energy intake or lower utilization of sugars. Melanson et al.[23] demonstrated for the first time that starvation is concomitant with a decrease in blood glucose, which in turn regulates animal feeding, and the relationship between the level of ADFI and GLU concentration in the present experiment is consistent with the above relationship. In the present study, we found that TG levels were significantly higher in the Gly7% group, suggesting that dietary supplementation with 7% glycerol promotes fat synthesis, according to the report of Cheng et al. [24]. However, elevated TG leads to associated metabolic disturbances such as decreased HDL-C and increased LDL-C [25]. We found that as TG increased, HDL-C instead increased and LDL-C instead decreased, thus inferring that glycerol supplementation does not lead to metabolic disorders in animals. Serum TP and ALB reflect the ability of the liver to synthesize proteins. The TP and ALB levels increased after glycerol supplementation, and the TP levels were all lower than 60 g/L and the ALB levels were all higher than 20 g/L, which were in line with the healthy range reported by Braun et al.[26]. This suggests that glycerol supplementation does not affect the health of sheep and is beneficial in improving protein synthesis.

The nutritional level of the diet is a key factor influencing fat deposition in animals [27]. Glycerol had a significant effect on perirenal fat, caudal fat and caudal fat deposition in this study. Song et al.[28] found that tail fat and perirenal fat decreased the most when diets were energy restricted. Differently, we found that tail fat and perirenal fat in the CON group were significantly lower than those in the Gly1% and Gly7% groups, but the difference with the Gly12% group was not significant. This suggests that glycerol has a certain promotion effect on fat deposition in sheep, and the fat deposition was optimal when the supplementation amount was 7%. The pattern of change in fat deposition was similar to that of ADG and TG, which was also in line with our speculation above the energy provided by the Gly12% group exceeded the demand of sheep, and their growth and metabolism were inhibited in all aspects.

Diet energy levels are closely related to carcass traits [29]. We found that HCW, CCW and HCD were significantly higher in the Gly1% and Gly7% groups than in the CON group. This suggests that the supplementation of glycerol at appropriate levels in the diet has a positive effect on increasing the carcass dressing percentage of sheep. However, Chanjula et al.[30] found that the addition of 5%, 10% and 20% glycerol to the diet did not significantly affect the above indexes. This may be because the food-grade glycerol added in this experiment had a higher glycerol concentration than crude glycerol. In addition, LEA was not significantly different between groups, but LEA was lower in both glycerol groups than in the CON group, which is not by the idea that the greater the carcass weight, the greater the area of the loin and eye muscles [31]. It is hypothesized that the possible reason for this is the positive effect of glycerol on the deposition of fat and muscle in animals. The weight of visceral organs increased significantly with increasing nutritional levels [32,33]. However, in the present study, glycerol was found to have no significant effect and only increased the weight of the liver and kidneys. This may be related to the age of the animals; the organs of the adult ewes in the present study were fully developed and glycerol supplementation could only promote further development of organs involved in energy balance.

The pH is an important indicator of muscle glycolysis rate and the increase in lactate content after slaughter leads to a decrease in pH values to 5.3-5.8 [34]. The results of the present study were similar with pH values within the reported normal range [22]. The a* and b* of muscle decreased significantly with an increase in glycerol, suggesting a possible negative correlation between glycerol levels and flesh color. The L* of the muscle decreases with increasing energy [18], but this is not the case in our results, which may be related to the pH of the muscle, where L* increases as the final pH decreases [35]. In addition, we found that the muscles in each glycerol group had lower SF and higher EE content, with the Gly7% group having the highest EE content and the lowest SF. Our results are in line with the conclusion of Bezerra et al.[31] that there is a negative correlation between SF and EE. This suggests that glycerol promotes intramuscular fat deposition, which in turn improves muscle tenderness.

In conclusion, it was determined that glycerol supplementation was effective in increasing FBW, ADG, HCW, CCW, HCD and serum concentrations of glycolipid metabolites, and was effective in decreasing FCR and ADFI in Kazakh sheep. In addition, glycerol supplementation promotes fat deposition, increases intramuscular fat content, reduces SF and improves meat quality. Therefore, it is feasible to supplement glycerol in the diet of empty ewes for fattening to improve body condition and provide energy for reproductive performance. We suggest that optimal fattening can be achieved with a 7% glycerol supplementation in the diet of empty ewes.

Declarations

Availability of Data and Materials: The data that support the findings of this study are available on request from the corresponding author (Z. Zhao). The data are not publicly available due to privacy or ethical restrictions.

Ethical Statement: This study was approved by the Bioethics Committee of Shihezi University (Approval no: A2021-38). All sheeps were kept experimentally and euthanized in strict accordance with the committee's guidelines. During the experiment, every effort was made to minimize suffering by the animals.

Conflict of Interest: The authors have declared that no competing interests exist.

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References

1. Akhatayeva Z, Kozhakhmet A, Kapassuly T, Orazymbetova Z, Yergali K, Khamzin K, Iskakov K, Dossybayev K: Distribution of runs of homozygosity and their relationship with candidate genes for productivity in Kazakh meat-wool sheep breed. *Genes*, 14 (11): 1988, 2023. DOI: 10.3390/genes14111988

2. Lin MY, Diao QY, Tu Y: Application of glycerol in ruminant production. *Chin J Anim Nutr,* 33 (7): 3608-3621, 2021. DOI: 10.3969/j.issn.1006-267x.2021.07.002

3. Chanjula P, Cherdthong A: Effects of crude glycerin from waste vegetable oil in diets on performance and carcass characteristics of feedlot goats. *Asian Australas J Anim Sci,* 31 (4): 514-521, 2018. DOI: 10.5713/ajas.17.0194

4. Sehu A, Kucukersan S, Coskun B, Koksal BH: Effects of graded levels of crude glycerine addition to diets on growth performance, carcass traits and economic efficiency in broiler chickens. *Kafkas Univ Vet Fak Derg,* 19 (4): 569-574, 2013. DOI: 10.9775/kvfd.2012.8369

5. Cheng YP, Huang ZB: Effects of biodiesel by-products on growth performance, carcass traits, meat quality and economic benefits of beef cattle. *China Feed* (16): 110-113, 2021. DOI: 10.15906/j.cnki.cn11-2975/s.20211628

6. Barros MCC, Marques JD, da Silva FF, Silva RR, Guimaraes GS, da Silva LL, de Araujo FL: Crude glycerin in the diet of confined sheep: consumption, digestibility, performance, carcass morphometric measurements and meat characteristics. *Semin-Cienc Agrar,* 36 (1): 453-466, 2015. DOI: 10.5433/1679-0359.2015v36n1p453

7. Van Cleef E, Uwituze S, Alvarado-Gilis CA, Miller KA, Van Bibber-Krueger CL, Aperce CC, Drouillard JS: Elevated concentrations of crude glycerin in diets for beef cattle: Feedlot performance, carcass traits, and ruminal metabolism. *J Anim Sci,* 97 (10): 4341-4348, 2019. DOI: 10.1093/jas/skz281

8. Orrico MAP, Bottini Filho FDE, de Vargas Junior FM, Orrico ACA, Osorio JCS: Crude glycerin in the diets of confined lambs: Performance, carcass traits and economic feasibility. *Biosci J,* 31 (4): 1152-1158, 2015. DOI: 10.14393/BJ-v31n4a2015-26226

9. Ribeiro RDX, Oliveira RL, de Carvalho GGP, Silva TM, Costa JB, Oliveira VD, Freitas LS, Ribeiro CVD, Bezerra LR: Dietary inclusion of purified crude glycerin improves bodyweight at slaughter and affects the fatty acid profile of lamb meat. *Anim Prod Sci,* 62 (4): 358-367, 2022. DOI: 10.1071/an20020

10. Brant LMS, de Freitas JE, Pereira FM, Pina DD, Santos SA, Leite LC, Cirne LGA, Alba HDR, de Araujo M, Pimentel PRS, de Carvalho GGP: Effects of alternative energy and protein sources on performance, carcass characteristics, and meat quality of feedlot lambs. *Livest Sci,* 251, 251:104611 2021. DOI: 10.1016/j.livsci.2021.104611

11. Zheng ML, Li XL, Yan QX, Zhou CS, Tan ZL, Zhang PH: Effects of different zinc sources on growth performance, serum biochemical indexes and zinc metabolism of pregnant goats and their offspring. *Kafkas Univ Vet Fak Derg,* 27 (2): 141-149, 2021. DOI: 10.9775/kvfd.2020.24858

12. Coria MS, Reineri PS, Pighin D, Barrionuevo MG, Carranza PG, Grigioni G, Palma GA: Feeding strategies alter gene expression of the calpain system and meat quality in the *longissimus* muscle of Braford steers. *Asian Australas J Anim Sci,* 33 (5): 753-762, 2020. DOI: 10.5713/ajas.19.0163

13. Rao SJ, Cui ZJ, Zhang LM, Ma S, Huang SB, Feng L, Chen YL, Luo JX, Li JF, Qian SY, Liu HT, Liu YZ, Yang LF, Yin YL, Tan CQ: Effects of dietary adenosine and adenosine 5′-monophosphate supplementation on carcass characteristics, meat quality, and lipid metabolism in adipose tissues of finishing pigs. *Meat Sci,* 201, 2023. DOI: 10.1016/j.meatsci.2023.109174

14. Vallejo G, Moris J, Conejo NM: A SAS/IML program for implementing the modified Brown-Forsythe procedure in repeated measures designs. *Comput Meth Programs Biomed,* 83 (3): 169-177, 2006. DOI: 10.1016/j.cmpb.2006.06.006

15. Wang Z, Goonewardene LA: The use of MIXED models in the analysis of animal experiments with repeated measures data. *Can J Anim Sci,* 84 (1): 1-11, 2004. DOI: 10.4141/a03-123

16. Piao MY, Jung DJS, Kang HJ, Park SJ, Lee JO, Kim M, Kim HJ, Kim DH, Seo JK, Jo C, Haque MN, Baik M: Effects of dietary glycerol inclusion on growth performance, carcass and meat quality characteristics, glycogen content, and meat volatile compounds in Korean cattle steers. *Anim Biosci,* 34 (4): 603-612, 2021. DOI: 10.5713/ajas.20.0186

17. Almeida MTC, Ezequiel JMB, Paschoaloto JR, Perez HL, Carvalho VB, Castro ES, van Cleef E: Rumen and liver measurements of lambs fed with high inclusions of crude glycerin in adaptation and finishing period of feedlot. *Small Ruminant Res,* 167, 1-5, 2018. DOI: 10.1016/j.smallrumres.2018.08.001

18. Wang YC, Wang QY, Dai CP, Li JZ, Huang PF, Li YL, Ding XQ, Huang J, Hussain T, Yang HS: Effects of dietary energy on growth performance, carcass characteristics, serum biochemical index, and meat quality of female Hu lambs. *Anim Nutr,* 6 (4): 499-506, 2020. DOI: 10.1016/j.aninu.2020.05.008

19. de Andrade GP, de Carvalho FFR, Batista AMV, Pessoa RAS, da Costa CA, Cardoso DB, Maciel MD: Evaluation of crude glycerin as a partial substitute of corn grain in growing diets for lambs. *Small Ruminant Res,* 165, 41-47, 2018. DOI: 10.1016/j.smallrumres.2018.06.002

20. Ding H, Liu WJ, Erdene K, Du HX, Ao CJ: Effects of dietary supplementation with *Allium mongolicum* Regel extracts on growth performance, serum metabolites, immune responses, antioxidant status, and meat quality of lambs. *Anim Nutr,* 7 (2): 530-538, 2021. DOI: 10.1016/j.aninu.2021.04.001

21. Xiao HY, Qu JT, Lin H, Zhang ZY, He YK, Liu YH, Zhang PH: Effects of tea polyphenols on permance, blood indexes and antioxidant of dairy goats. *Chin J Anim Nutr,* 33 (8): 4533-4540, 2021. DOI: 10.3969/j.issn.1006-267x.2021.08.003

22. Zhang ZY, Liang CN, Yiao XX, Dingkao RQ, Ma XM, Chu M, Wu XY, Bao PJ, Yan P: Effects of different nutrition level of diets on production performance, slaughter indexes and serum biochemical indexes of Yak. *Acta Vet Zootech Sin,* 52 (1): 135-143, 2021. DOI: 10.11843/j.issn.0366-6964.2021.01.014

23. Melanson KJ, Westerterp-Plantenga MS, Campfield LA, Saris WHM: Blood glucose and meal patterns in time-blinded males, after aspartame, carbohydrate, and fat consumption, in relation to sweetness perception. *Br J Nutr,* 82 (6): 437-446, 1999. DOI: 10.1017/s0007114599001695

24. Cheng HB, Lv SY, Liu Y: Endoplasmic reticulum stress in hepatic glucose and lipid metabolism and metabolic liver diseases. *Chin J Cell Biol,* 41 (11): 2209-2219, 2019. DOI: 10.11844/cjcb.2019.11.0018

25. Tian SQ, Li YL, Pei HL, Tian YY, Zuo ZP, Zhao XY, Liu C, Wang ZB: The pathogenesis and drug therapy of hyperlipidemia. *Chem of Life,* 42 (12): 2237-2247, 2022. DOI: 10.13488/j.smhx.20220704

26. Braun JP, Trumel C, Bezille P: Clinical biochemistry in sheep: A selected review. *Small Rumin Res,* 92 (1-3): 10-18, 2010. DOI: 10.1016/j.smallrumres.2010.04.002

27. Li XS, Cui K, Fang W, Chen Q, Xu D, Mai KS, Zhang YJ, Ai QH: High level of dietary olive oil decreased growth, increased liver lipid deposition and induced inflammation by activating the p38 MAPK and JNK Cheek for pathways in large yellow croaker *(Larimichthys crocea). Fish Shellfish Immunol,* 94, 157-165, 2019. DOI: 10.1016/j.fsi.2019.08.062

28. Song SZ, Wu JP, Zhao SG, Casper DP, He B, Liu T, Lang X, Gong XY, Liu LS: The effect of energy restriction on fatty acid profiles of longissimus dorsi and tissue adipose depots in sheep. *J Anim Sci,* 95 (9): 3940-3948, 2017. DOI: 10.2527/jas.2016.1235

29. McCurdy MP, Krehbiel CR, Horn GW, Lancaster PA, Wagner JJ: Effects of winter growing program on visceral organ mass, composition, and oxygen consumption of beef steers during growing and finishing. *J Anim Sci,* 88 (4): 1554-1563, 2010. DOI: 10.2527/jas.2009-2415

30. Chanjula P, Pakdeechanuan P, Wattanasit S: Effects of feeding crude glycerin on feedlot performance and carcass characteristics in finishing goats. *Small Ruminant Res,* 123 (1): 95-102, 2015. DOI: 10.1016/j.smallrumres.2014.11.011

31. Bezerra HFC, Santos EM, de Carvalho GGP, de Oliveira JS, Zanine AD, Pinho RMA, de Araujo M, Perazzo AF, Ferreira DD: Effect of crude glycerin levels on meat quality and carcass characteristics of crossbred Boer goats. *Food Sci Nutr,* 10 (7): 2312-2317, 2022. DOI: 10.1002/fsn3.2839

32. Xu GS, Diao QY, Ji SK, Deng KD, Jiang CG, Liu J, Zhao YG, Ma T, Lou C: Effects of different feeding levels on growth performance, slaughter performance and organ indexes of Mutton sheep. *Chin J Anim Nutr,* 24 (05): 953-960, 2012.

33. Tekce E, Bayraktar B, Aksakal V, Dertli E, Kamiloglu A, Karaalp M, Timurkaan S, Gül M: Response of probiotics and yeast added in different doses to rations of Anatolian Merino lambs on fattening performance, meat quality, duodenum and rumen histology. *Kafkas Univ Vet Fak Derg,* 27 (1): 57-65, 2021. DOI: 10.9775/kvfd.2020.24747

34. Savell JW, Mueller SL, Baird BE: The chilling of carcasses. *Meat Sci,* 70 (3): 449-459, 2005. DOI: 10.1016/j.meatsci.2004.06.027

35. Muchenje V, Dzama K, Chimonyo M, Strydom PE, Hugo A, Raats JG: Some biochemical aspects pertaining to beef eating quality and consumer health: A review. *Food Chem,* 112 (2): 279-289, 2009. DOI: 10.1016/j.foodchem.2008.05.103

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Short Communication

Initial Seroprevalence Records of Infectious Agents Implicated in Reproductive Issues in High-altitude Cattle from Two Districts in Cajamarca, Peru

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Abstract

In high Andean regions with subsistence livestock farming, diagnostic studies are rarely conducted for reproductive issues in extensively bred cows. This research aimed to determine the seroprevalence of *Neospora caninum*, bovine viral diarrhea virus (BVDV), and bovine herpesvirus (BHV-1) in cattle above 3300 meters in Peru. Blood from 292 cattle across categories and breeds was collected. Antibody prevalence was 13.70±3.94% for *N. caninum*, 30.14±5.26% for BVDV, and 2.74±1.87% for BHV-1. District-wise differences were not significant (P>0.05). Cow and Creole breed were the most affected, but without statistical variance (P>0.05). These results confirm *N. caninum*, BVDV, and BHV-1 presence in reproductive issues in high altitudes cattle in Cajamarca and Celendín provinces.

Keywords: Cattle, High altitude, BHV-1, BVDB, *Neospora caninum*, Reproductive problems, Serology

Introduction

The management of bovine reproduction is the cornerstone of herd health provision and the success in modern veterinary practice for large animals. However, over the last decades, fertility has steadily declined, despite increasing veterinary intervention [1]. The primary factors negatively affecting cattle reproduction are attributed to pathogenic microorganisms.

Protozoan parasites such as *Neospora caninum* directly impinge upon bovine reproductive health [2]. In cattle, they manifests considerable global economic losses attributable to reproductive disorders including abortion, retention of fetal membranes, metritis, estrus repetitions, and temporary anestrus [3]. Furthermore, viruses such as bovine viral diarrhea virus (BVDV) and bovine herpesvirus-1 (BHV-1) exert significant adverse reproductive impacts on cattle health. Within the reproductive tract, BVDV induces suboptimal fertility, abortions, and fetal deformities during gestation [4]. Conversely, BHV-1 precipitates systemic infections, fever, anorexia, nasal mucosa reddening, cough, and conjunctivitis, often culminating in abortions occurring. Early embryonic demise can also ensue as a consequence of the infection [5].

Moreover, climatic conditions exert a discernible influence on animal reproduction. Productive efficiency of dairy cows is diminished at higher altitudes compared to lower altitude areas [6]. Extreme weather conditions disrupt the energy exchange between the animal and its environment,

negatively impacting reproduction. Conception rates decline under heat and cold stress, with endocrine functions being perturbed by climatic extremes [7].

The raising of dairy cattle in the highlands of the Peruvian mountains is centered on an extensive system, with an average of six to seven animals per farm and distinct health and reproductive management practices compared to valleys or intensive systems, including the use of dogs for herding and livestock care. A study conducted at 3200 meters above sea level in hamlets of the provinces of Cajamarca, Celendín, and Hualgayoc evaluated the effectiveness of estrus synchronization and artificial insemination, achieving a pregnancy rate of 42.82% (301/703) [8]. However, after the study, producers frequently reported cases of infertility, abortions, and retained placentas.

Given the background of reproductive problems in cows from the mentioned provinces and with the aim of pinpointing whether the causes could be of infectious origin, this study determines the seroprevalence of three etiological agents involved in reproductive issues: *N. caninum*, BVDV, BHV-1, in extensively raised dairy cattle located above 3300 meters above sea level in hamlets of two districts of the provinces of Cajamarca and Celendín.

Material and Methods

Ethical Considerations

The owners of the cattle taken for consultation were informed and agreed to the participation of their animal in the study. Sampling was performed taking all biosafety and animal welfare measures, according to the guidelines of the Ley de Protección y Bienestar Animal - N° 30407, of the Peruvian State.

Study and Sampling Area

This is an exploratory study in which a total of 292 cattle were evaluated. Cattle of varying age categories and breeds were randomly sampled, situated at altitudes ranging from 3311 to 3979 meters above sea level, and distributed across hamlets within the La Encañada and Sorochuco districts of the Cajamarca and Celendín provinces, respectively. Among the total sampled animals, six of them had a history of abortion (n=5) and infertility (n=1), as per information provided by the livestock producers. The study area exhibits an average annual temperature of 7.85°C and a relative humidity of 83.93%. GPS technology (GPSMAP® 66i, USA) was employed to ascertain the altitude of each hamlet.

The hamlets under investigation in this study are situated within mining-influenced zones that promote livestock husbandry improvement through cattle acquisition and the implementation of reproductive biotechnology programs. These areas boast a diverse array of wildlife, including Andean foxes, Andean deer, and others. The cattle were reared in an extensive husbandry system, with interactions occurring among ovine, caprine, equine, porcine, and, in some instances, alpacas. All livestock keepers had dogs without health controls, which they employed for both companionship and assistance in livestock management. On the other hand, it was observed that the pasture intended for cattle consumption exhibited poor quality and limited quantity *(Fig. 1)*.

Fig 1. Presentation of cattle farming at high altitudes. Buena Vecindad village: Extensive cattle farming is observed alongside alpacas and donkeys (A, D). El Porvenir village: Cattle are seen in fields with low vegetation coverage and small-sized pastures (B, C)

Sampling and Diagnostics

The animals were identified using information provided by the livestock owner (names and ear tag numbers). These animals had not been vaccinated at any point against BVDB or BHV-1. From each animal, 5 mL of blood was collected by coccygeal venipuncture using the vacuum tube system (Vacutainer) into additive-free tubes. The tubes were placed in a rack and transported in a thermal box to the Laboratory of the Fondo de Crédito para el Desarrollo Agroforestal (FONCREAGRO), Cajamarca.

The tubes were centrifuged at 2500 rpm for 10 min. The serum was transferred to 2 mL Eppendorf tubes, frozen at -8°C, and subsequently transported by air to the Microbiology and Parasitology Laboratory of the Faculty of Veterinary Medicine (FMV) at the National University of San Marcos (UNMSM) in Lima, Peru.

Antibodies against *N. caninum* were detected using a commercial competitive ELISA kit *(N. caninum* Antibody Test Kit, cELISA, VMRD, USA). The procedure was conducted according to the manufacturer's instructions. Serum samples with an inhibition percentage of 30% or higher were classified as positive. Antibodies against Bovine Viral Diarrhea virus (BVD) and Bovine Herpesvirus

(BHV-1) were detected using viral neutralization. Fetal bovine nasal turbinate cell cultures, free of BVD virus, were employed as indicator systems in both cases. The cells were cultured using Minimum Essential Medium (MEM) and Leibovitz (L-15) Medium (SIGMA, USA), in a 50:50 ratio supplemented with 10% BVD-free fetal bovine serum and antibiotics (SIGMA, USA).

The technique described by the OIE [9] and the protocol of the Virology Laboratory of the FMV, UNMSM, were followed. For the detection of antibodies against BVD, the NADL strain, CP biotype genotype I, with a titer of 10^{-5} DI50 CC/50 µL, was employed as the antigen. The Cooper strain (Ames, USA), prototype strain of BHV-1 with a titer of 10^{-5} DI_{50} CC/50 mL, was used as the antigen in the diagnosis of antibodies against BHV-1.

Samples were considered positive for BVD and BHV-1 antibodies when the serum titers were equal to or greater than 1:2, as evidenced by the absence of cytopathic effects on the indicator cells.

Statistical Analysis

The results were organized in MS Excel 2019, and seroprevalence along with a 95% confidence interval was calculated. Using the SPSS Statistics 27.0.1 software, the independence of results between districts was assessed using the Mann-Whitney U test, and P-values were adjusted using the Bonferroni correction. Age category and breed were analyzed using the non-parametric Kruskal-Wallis test, and in cases where statistical differences were identified, the Mann-Whitney U test was employed to pinpoint the differing group.

Results

Calf (0 - 12 months), heifer (>12 months - up to before pregnancy), and cow (≥parturitions) of Brown Swiss, Holstein, and Creole breeds were sampled, distributed across fifteen hamlets located between altitudes of 3311 to 3979 meters above sea level, all of which had not been previously vaccinated. Antibodies against *N. caninum* (13.70 [95% CI, 9.75-17.64]), Bovine Viral Diarrhea Virus - BVD (30.14 [95% CI, 24.87 - 35.40]), and Bovine Herpesvirus - BHV-1 (2.74 [95% CI, 0.87-4.61]) were detected in the districts *(Table 1, Fig. 2)*.

With the exception of the calf age group, which did not show cases of BHV-1, all other categories exhibited antibodies against *N. caninum*, BVD, and BHV-1 *(Table*

Table 1. *Prevalence (%) of seropositive animals for Neospora caninum, BVD, and BHV-1, by provinces in extensively raised dairy cattle located above 3300 meters above sea level*

Province: District	Village	Altitude (masl)	Nº	*N. caninum*		BVD		BHV-1	
				Positive	Prevalence (95% CI)	Positive	Prevalence (95% CI)	Positive	Prevalence (95% CI)
Cajamarca: La Encañada	El Porvenir	3791	26	4	15.38 (1.52-29.25)	8	30.77 (13.03-48.51)	0	0.00 (0.00-0.00)
	Buena Vecindad	3476	11	1	9.09 (0.00-26.08)	0	0.00 (0.00-0.00)	0	0.00 (0.00-0.00)
	El Valle	3311	5	2	40.00 (0.00-82.94)	0	0.00 (0.00-0.00)	0	0.00 (0.00-0.00)
	Yerba Buena Chica	3719	44	4	9.09 (0.60-17.59)	16	36.36 (22.15-50.58)	4	9.09 (0.60-17.59)
	Lagunas de Combayo	3979	23	2	8.79 (0.00-20.21)	9	39.13 (19.18-59.08)	2	8.70 (0.00-20.21)
	Quinuapampa	3828	24	1	4.17 (0.00-12.16)	15	62.50 (43.13-81.87)	1	4.17 (0.00-12.16)
	San Juan de Yerba Buena	3561	28	5	17.86 (3.67-32.04)	15	53.57 (35.10-72.04)	0	0.00 (0.00-0.00)
Subtotal			161	19	11.80 (6.82-16.78)[a]	63	39.13 (31.59-46.67)[a]	7	4.35 (1.20-7.50)[a]
Celendín: Sorochuco	San Nicolás de Challuagón	3705	10	1	10.00 (0.00-28.59)	2	20.00 (0.00-44.79)	0	0.00 (0.00-0.00)
	Chugurmayo	3713	18	3	16.67 (0.00-33.88)	5	27.78 (7.09-48.47)	0	0.00 (0.00-0.00)
	Alto Cruz Pampa	3547	10	0	0.00 (0.00-0.00)	0	0.00 (0.00-0.00)	0	0.00 (0.00-0.00)
	Agua Blanca	3667	62	11	17.74 (8.23-27.25)	13	20.97 (10.83-31.10)	1	1.61 (0.00-4.75)
	El Tingo	3414	3	1	33.33 (0.00-86.68)	1	33.33 (0.00-86.68)	0	0.00 (0.00-0.00)
	La Chorrera	3688	5	1	20.00 (0.00-55.06)	1	20.00 (0.00-55.06)	0	0.00 (0.00-0.00)
	Uñigan Pululo	3744	23	4	17.39 (1.90-32.88)	3	13.04 (0.00-26.81)	0	0.00 (0.00-0.00)
Subtotal			131	21	16.03 (9.75-22.31)[b]	25	19.08 (12.35-25.81)[b]	1	0.76 (0.00-2.25)[b]
Total			**292**	**40**	**13.70 (9.75-17.64)**	**88**	**30.14 (24.87-35.40)**	**8**	**2.74 (0.87-4.61)**

[a,b]*Different letters indicate statistical differences between districts for each disease agent (Mann-Whitney U test + post hoc test [Bonferroni correction], P<0.05)*

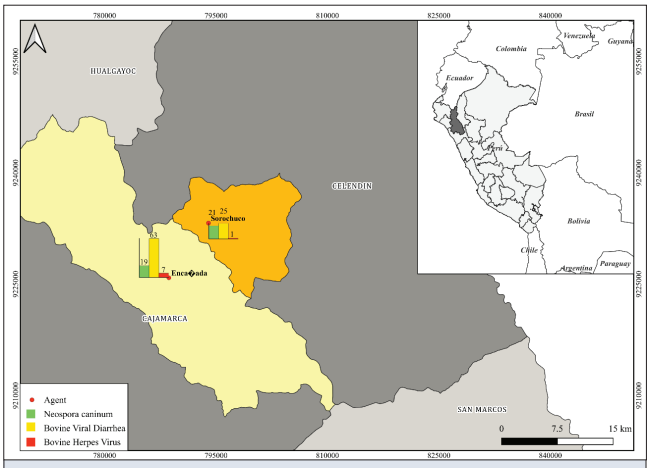

Fig 2. Distribution of seropositive cases in the villages of the two districts, La Encañada and Sorochuco

Table 2. *Prevalence (%) of seropositive animals for Neospora caninum, BVD, and BHV-1, by age group and breed in extensively raised dairy cattle located above 3300 meters above sea level*

Classification	Category	Nº	*N. caninum*		BVD		BHV-1	
			Positive	Prevalence (95% CI)	Positive	Prevalence (95% CI)	Positive	Prevalence (95% CI)
Age group	Calf	15	1	6.67 (0.00-19.29)[a]	3	20.00 (0.00-40.24)[a]	0	0.00 (0.00-0.00)[a]
	Heifer	22	5	22.73 (5.22-40.23)[a]	10	45.45 (24.65-66.26)[a]	1	4.55 (0.00-13.25)[a]
	Cow	255	34	13.33 (9.16-17.51)[a]	75	29.41 (23.82-35.00)[a]	7	2.75 (0.74-4.75)[a]
Breed	Brown Swiss	148	11	7.43 (3.21-11.66)[b]	46	31.08 (23.62-38.54)[a]	6	4.05 (0.88-7.23)[a]
	Creole	128	25	19.53 (12.66-26.40)[a]	39	30.47 (22.49-38.44)[a]	2	1.56 (0.00-3.71)[a]
	Holstein	16	4	25.00 (3.78-46.22)[a]	3	18.75 (0.00-37.88)[a]	0	0.00 (0.00-0.00)[a]
Total		**292**	**40**	**13.70 (9.75-17.64)**	**88**	**30.14 (24.87 - 35.40)**	**8**	**2.74 (0.87-4.61)**

[a,b]*Different letters indicate statistical differences between categories within each column (Kruskal-Wallis + post hoc test [Mann-Whitney U test], P<0.05)*

2). Similarly, the only group that did not show antibodies against BHV-1 was the one composed of Holstein breed animals *(Table 2)*. However, it is necessary to note that the number of individuals in both of these categories (calves and Holstein breed) was smaller compared to the heifer and cow categories, and to the Brown Swiss and Creole breeds.

Out of the five animals with a history of abortion, four were seropositive for BVD, and one was seropositive for both *N. caninum* and BVD. The remaining animal with a history of infertility was seropositive for BHV-1. Among the positive animals, 5.14% exhibited antibodies against both *N. caninum* and BVD simultaneously. Similarly, 1.71% showed antibodies against both BVD and BHV-1.

Discussion

A global seroprevalence of 13.70±3.94% for *Neospora caninum*, 30.14±5.26% for BVD, and 2.74±1.87% for BHV-1 was found across the two districts. In Sorochuco, a higher number of seropositive cases were observed for *N. caninum*, unlike BVD and BHV-1, which were more prevalent in La Encañada. Numerically, the group of cows exhibited the highest presence of antibodies against the evaluated infectious agents. Creole animals showed the highest reactivity against *N. caninum*, while Brown Swiss exhibited higher reactivity against BVD and BHV-1.

Since all the cattle owners had dogs for companionship and cattle care, these dogs could be one of the causes contributing to the spread and latency of neosporosis in the area. Studies have determined that the presence of dogs on the farm is a determining factor for *N. caninum* infection in cattle [10,11].

A common practice among livestock farmers in the assessed areas is to introduce animals by purchasing them from local, regional, and national herds to improve genetics. This practice may introduce pathogens due to the lack of sanitary control before acquiring the

animals. Furthermore, various reproductive techniques, such as artificial insemination, have been employed to optimize cattle reproduction, achieving a pregnancy rate effectiveness of 42.82% (301/703) in a previous study using estrus synchronization and artificial insemination. However, before and after the study, producers frequently reported cases of infertility, abortions, and retained placentas [8]. It is possible that these actions have introduced and disseminated BVDV, as this virus can be transmitted directly to the reproductive tract through semen or through insemination or embryo transfer materials. Additionally, most producers, especially those who disagree with artificial insemination or embryo transfer programs, rely on natural mating in their herds. In such cases, infected bulls transmit and spread BVDV and BHV-1 to the cows [12]. Consequently, the virus can remain in constant transmission from mother to fetus [4].

It is plausible that altitude may not serve as a predisposing factor for the presence of BHV-1 and BVD, given that both diseases have been reported across various altitudinal ranges [13,14]. Generally, in more remote areas far from major cities, there is limited animal movement due to poor road development and access routes, which might prevent disease dissemination.

An important factor to consider is the effect of pasture quality on the normal physiology of animals. *Fig. 1* illustrates agricultural conditions with underdeveloped and poor-quality pastures. It has been demonstrated that poor food quality adversely affects the fertility of cows [15]. Therefore, in addition to *N. caninum*, BVD, and BHV-1, the impact of low-quality pastures on the reproductive performance of cows in the evaluated districts should also be taken into account. On the other hand, extensive breeding of different animal species could potentially contribute to the conservation and dissemination of neosporosis, BVD, and BHV-1 in the livestock of the La Encañada and Sorochuco districts.

In conclusion, the presence of antibodies against *N. caninum*, Bovine Viral Diarrhea Virus, and Bovine Infectious Rhinotracheitis Virus type 1 was evidenced for the first time in extensively reared cattle in the high-altitude areas (>3300 meters above sea level) of two districts in the provinces of Cajamarca and Celendín. These agents could be contributing to the occurrence of reproductive issues in the cattle population of the region.

Declerations

Availability of Data and Materials: All relevant data are contained in this manuscript. However, for detailed information, please contact the corresponding author (L. Vargas-Rocha).

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Ethical Considerations: The owners of the cattle taken for consultation were informed and agreed to the participation of their animal in the study. Sampling was performed taking all biosafety and animal welfare measures, according to the guidelines of the Ley de Protección y Bienestar Animal - N° 30407, of the Peruvian State.

Competing of Interests: The authors declare the absence of any known competing financial interests or personal relationships that might have seemed to influence the work reported in this paper.

Author Contributions: JEME: Conceptualization, Methodology, Investigation, Data Curation & Visualization. GBM: Funding Acquisition, Allocation of Resources, Project Administration, Supervision & Validation. LVR: Formal Analysis, Managed Software, Writing Original Draft, Writing - Review & Editing. All authors read and approved the final version of the manuscript.

References

1. Sheldon IM, Wathes DC, Dobson H: The management of bovine reproduction in elite herds. *Vet J*, 171 (1): 70-78, 2006. DOI: 10.1016/j.tvjl.2004.06.008

2. Irehan B, Sonmez A, Atalay MM, Ekinci AI, Celik F, Durmus N, Ciftci AT, Simsek S: Investigation of *Toxoplasma gondii*, *Neospora caninum* and *Tritrichomonas foetus* in abortions of cattle, sheep and goats in Turkey: Analysis by real-time PCR, conventional PCR and histopathological methods. *Comp Immunol Microbiol Infect Dis*, 89:101867, 2022. DOI: 10.1016/j.cimid.2022.101867

3. Reichel MP, Ayanegui-Alcérreca MA, Gondim LFP, Ellis JT: What is the global economic impact of *Neospora caninum* in cattle - The billion dollar question. *Int J Parasitol*, 43 (2): 133-142, 2013. DOI: 10.1016/j.ijpara.2012.10.022

4. Peterhans E, Schweizer M: Pestiviruses: How to outmaneuver your hosts. *Vet Microbiol*, 142 (1-2): 18-25, 2010. DOI: 10.1016/j.vetmic.2009.09.038

5. Miller JM, Van Der Maaten MJ: Experimentally induced infectious bovine rhinotracheitis virus infection during early pregnancy: Effect on the bovine corpus luteum and conceptus. *Am J Vet Res*, 47 (2): 223-228, 1986.

6. Qiao GH, Shao T, Yu CQ, Wang XL, Yang X, Zhu XQ, Lu Y: A comparative study at two different altitudes with two dietary nutrition levels on rumen fermentation and energy metabolism in Chinese Holstein cows. *J Anim Physiol Anim Nutr*, 97 (5): 933-941, 2013. DOI: 10.1111/j.1439-0396.2012.01339.x

7. Gwazdauskas FC: Effects of climate on reproduction in cattle. *J Dairy Sci*, 68 (6): 1568-1578, 1985. DOI: 10.3168/jds.s0022-0302(85)80995-4

8. Mendoza J, Lanatta R, López J, Narvaez O, Sangay F, Rodriguez A, Cerquín G, De la Cruz R: Efficacy of an estrous synchronization protocol in cows breeding at 3200 masl in the provinces of Cajamarca, Celendin and Hualgayoc. *Spermova*, 3 (1): 49-50, 2013.

9. Office International des Epizooties: Bovine Viral Diarrhoea. **In,** World Organization for Animal Health (Ed): Manual of Standards Diagnostic Tests and Vaccines. 698-711, Paris, 2009.

10. Fávero JF, Da Silva AS, Campigotto G, Machado G, De Barros LD, Garcia JL, Vogel FSF, Mendes RE, Stefani LM: Risk factors for *Neospora caninum* infection in dairy cattle and their possible cause-effect relation for disease. *Microb Pathog*, 110, 202-207, 2017. DOI: 10.1016/j.micpath.2017.06.042

11. Wouda W, Dijkstra T, Kramer AMH, Van Maneem C, Brinkhof JMA: Seroepidemiologic evidence for a relationship between *Neospora caninum* infections in dog and cattle. *Int J Parasitol*, 29 (10): 1677-1682, 1999. DOI: 10.1016/S0020-7519(99)00105-8

12. Givens MD, Riddell KP, Edmondson MA, Walz PH, Gard JA, Zhang Y, Galik PK, Brodersen BW, Carson RL, Stringfellow DA: Epidemiology of prolonged testicular infections with bovine viral diarrhea virus. *Vet Microbiol*, 139 (1-2): 42-51, 2009. DOI: 10.1016/j.vetmic.2009.04.029

13. Sánchez G, Benito A, Rivera H: Seroprevalencia del virus de la rinotraqueitis infecciosa bovina en ganado lechero del valle de Lima. *Rev Inv Vet Perú*, 14 (1): 54-60, 2003. DOI: 10.15381/rivep.v14i1.1604

14. Quispe R, Ccama A, Rivera H, Araínga M: Bovine viral diarrea virus in criollo cattle of the province of Melgar, Puno. *Rev Inv Vet Perú*, 19 (2): 176-182, 2008. DOI: 10.15381/rivep.v19i2.1165

15. McClure TJ: An experimental study of the causes of a nutritional and lactational stress infertility of pasture-fed cows, associated with loss of bodyweight at about the time of mating. *Res Vet Sci*, 11 (3): 247-254, 1970. DOI: 10.1016/S0034-5288(18)34329-7

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Case Report

Duplication of Caudal Vena Cava in a Cat

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Abstract

Caudal vena cava (CVC) may develop abnormally due to its complex embryogenesis. Understanding congenital variants such as duplication of CVC is essential for clinical interventions, especially performed by surgeons and radiologists. In this context, we summarize the imaging and clinical characteristics of CVC duplication and accompanying portosystemic shunt diagnosed in a six-month-old cat using computed tomography angiography. In our patient, the CVC branched into two vessels on either side of the abdominal aorta and merged into a single vessel at the level where the renal veins emerge. The duplication of the CVC, along with portosystemic shunts and ureter anomalies, can increase the risk of thromboembolism, especially in cats with heart disease. Due to the evolving nature of computed tomography technology in animals, the number of diagnoses made using this method is still relatively low. It is anticipated that the rate of CVC duplication in cats and dogs will increase as diagnoses become more frequent.

Keywords: Duplicated caudal vena cava, Venous anomaly, Diagnosis, Computed tomography, Angiography

Introduction

In mammals, the caudal vena cava (CVC) forms as a single vessel on the right side of the aorta as a result of a complex process that includes the development, regression, anastomoses and displacement of three pairs of embryonic vessels: supracardinal, subcardinal and vitelline vessels [1,2]. The presence of the Duplicated Caudal Vena Cava (dCVC) in the abdomen is most commonly due to the persistence of two embryonic supracardinal veins. The dCVC is physiological in whales and dolphins, but has been reported as a rare variant in humans and domestic animals. The prevalence of inferior vena cava duplication in humans has been reported at 0.2-3%. The frequency of dCVC occurrence varies between 3% and 27%, influenced by factors such as the species or breed of the animal and the type of diagnostic equipment employed [3]. It has been reported that awareness of abnormal retroperitoneal vessels is important to avoid diagnostic pitfalls and intraoperative complications during surgical or interventional procedures. The dCVC has rarely been reported in small animals. In reported cases, dCVC was primarily identified as an incidental finding during imaging for other reasons and was associated with other congenital anomalies [2]. When the collateral system ensures sufficient venous return, the majority of congenital venous anomalies, such as dCVC, typically remain undetected and produce no symptoms [2,4,5].

Reflecting on the findings from retrospective veterinary research on domestic animals, it has been observed that numerous variations of the CVC exist. The occurrence of these variants is closely associated with an increased risk of concurrent ureteric anomalies or the development of portosystemic shunts [1]. In this report, we present the symptoms and diagnosis of a rare anatomical variation of the CVC, known as dCVC, using computed tomography. The diagnosis of dCVC is important for recognizing clinical symptoms, preventing diagnostic errors during surgical interventions, and reducing intraoperative complications. Additionally, being aware of congenital anomalies that may accompany this anatomical variation is crucial for a comprehensive patient assessment and treatment planning. The purpose of this case report is to contribute to the enhancement of surgical and diagnostic approaches by addressing the clinical implications of dCVC.

Case History

Ethical Approval

Since this article is categorized as a case report, it is not subject to ethical committee approval. Informed consent was obtained from the animal owner to use the data obtained from the clinical examination.

Clinical Examination of the Cat

A 6-month-old Siamese male cat was admitted to Istanbul University-Cerrahpaşa Veterinary Faculty Animal Hospital with complaints of incoordination and seizures. The cat was reported to undergo daily seizures, particularly occurring after meals. The patient's rectal temperature was 38.5°C and heart rhythm was 160 beats per minute. Neurological examination showed increased tendon reflexes in the front and hind legs. Proprioception and cranial nerve examination were normal. It was reported that the patient had a decreased appetite recently. The patient underwent a blood sampling after a twelve-hour fasting period, during which fasting bile acids and ammonia levels were measured. Subsequently, the patient was fed, and two hours later, another blood sample was taken to analyze postprandial bile acid levels. At presentation, the patient's hemogram values were within normal limits *(Table 1)*, while biochemical parameters showed significant changes. Elevated levels of ammonia, fasting and postprandial bile acids, ALT, and ALP were noted. In contrast, a decrease in BUN levels was observed *(Table 2)*. The patient reported not using any medications.

Table 1. *Heamatological values of cat at presentation*

Test	Reference Value	Result
RBC, x10^6/µL	6.61	5.65-8.87
Hct, %	38.9	37.3-61.7
Hb, g/dL	9.9	13.1-20.5
MCV, fL	65.3	61.6-73.5
MCH, pg	15.0	11.8-17.3
MCHC, g/dL	36.0	32.0-37.9
RDW, %	25.6	13.6-21.7
Retic, %	0.6	
Retic, x10^3/µL	30.8	10.0-110.0
WBC, x10^3/µL	12.80	5.05-16.76
Neu, x10^3/µL	6.46	2.95-11.64
Lym, x10^3/µL	5.31	0.92-6.88

RBC: *red blood cell,* ***Hct:*** *haematocrit,* ***Hb:*** *hemoglobin* ***MCV:*** *mean corpuscular volume,* ***MCH:*** *mean corpuscular hemoglobin,* ***MCHC:*** *mean corpuscular hemoglobin concentration,* ***RDW:*** *red cell distribution width,* ***Retic:*** *reticulocyte,* ***WBC:*** *white blood cell,* ***Neu:*** *neutrophil* ***Lym:*** *lymphocyte*

Table 2. *Serum biochemical panel of cat at presentation*

Test	Reference Value	Result
Bile acids (fasting), µg mol/L	0.0-3.0	21.98
Bile acids (postprandial), µg mol/L	0.0-11.0	22.54
Ammonia, µg/dL	23.00-78.00	160
ALT, U/L	12-130	194
ALP, U/L	14-111	158
BUN, mg/dL	7.03-26.98	5.03
Glucose, mg/dL	85	74-143
CK, mg/dL	0,7	0.5-1.8
Calcium, mg/dL	8.6	7.9-11.3
TP, g/dL	5.8	5.2-8.2
Albumin, g/dL	2.5	2.3-4.0
Globulin, g/dL	3.9	2.8-4.8

ALT: *Alanine Aminotransferase,* ***ALP:*** *Alkaline Phosphatase* ***BUN:*** *Blood Urea Nitrogen,* ***CK:*** *Creatin,* ***TP:*** *Total Protein*

Computed Tomography (CT) Examination

CT, Siemens SOMATOM go. Now CT scanner with 32 detectors was used. The patient was given Propofol (Propofol-PF®) at 5 mg/kg for induction and Isoflurane (2%) in oxygen (Isoflurane USP®) for maintenance of anesthesia through the cuffed endotracheal tube. The patient was imaged in the supine position. To determine the scanning area, the topogram image was first taken, and then Opaxol™ contrast agent at a dose of 640 mg/kg was administered in the cephalic vein with a 20 gauge catheter at a speed of 3 mL/s using high-pressure syringe pump. The aortic lumen was manually marked, the predicted threshold contrast enhancement level (100 HU) was set and scanning was initiated. Repeated images taken from a single section in which the aortic lumen was marked were obtained using 'bolus tracking' technique. When the prescribed threshold contrast enhancement level was reached, the scanning device was automatically activated. Sections from the caudal thorax to the pelvis were taken and arterial phase images (0.70 mm collimation, 1 mm section thickness, 1.5 mm table speed per gantry rotation) were obtained. The case was also imaged in portal phase (30 sec after contrast) at the same collimation and section thickness. Then, dorsal, sagittal, maximum intensity projection (MIP), Multiplanar Reformat (MPR) and volume rendering images were created from the transversal images.

Duplicated caudal vena cava was detected incidentally during CT angiography scan. The portal vein diameter was measured as 1.8 mm and was relatively thin in calibration. It was determined that the cranial mesenteric vein was combined with the splenic vein and formed the portal vein. However, in cranial mesenteric vein, there was a varicose dilatation of 16 mm in length and 3.9 mm in width, located in the central abdomen, at the right lateral of the midline, approximately at the level of the caudal pole

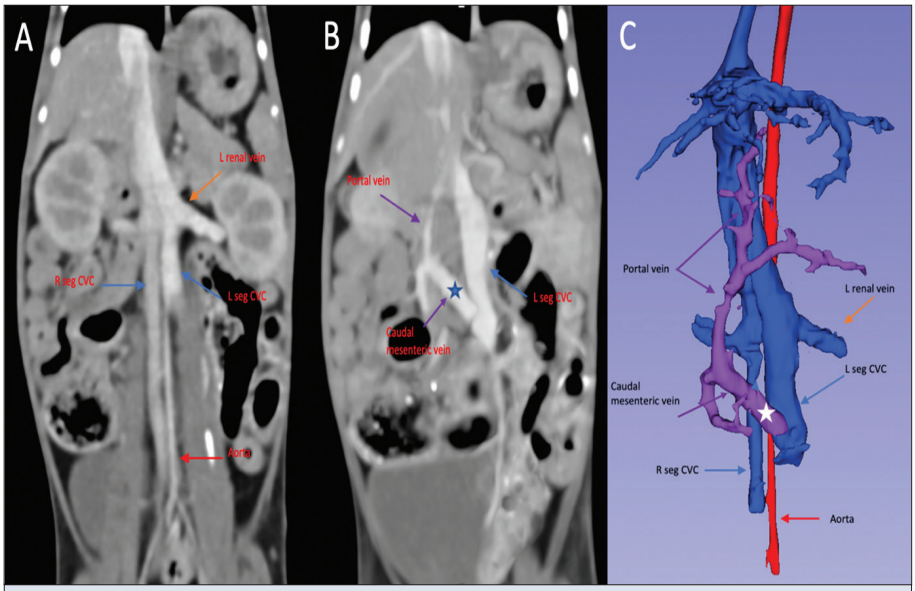

Fig 1. Duplication of the caudal vena cava and demonstration of a portosystemic shunt, **A, B:** Abdominal contrast-enhanced computed tomography image, ventro-dorsal section projection was used, **B:** Caudal mesenteric vein, in communication with the left segment of CVC (left subracardinal vein). CVC, vena cava. L, left. L seg CVC, left segment of the vena cava. R seg CVC, right segment of the vena cava. (star) Shows the direct communication between the caudal mesenteric vein and the CVC in CT image in portal phase, **C:** Image of dorsoventral 3D reconstruction showing the shunt vessel between the left segment of the CVC and the mesenteric vein

of the right kidney. A joint appearance with a diameter of 1.5 mm between the described vein and the left branch of the dCVC was found to be compatible with shunt. It was determined that the dCVC merged approximately at the level of the left renal vein and entered the hepatic hilus as a single caudal vena cava *(Fig. 1)*.

Discussion

Caudal Vena Cava duplication is among several congenital anomalies that may have potential clinical implications. However, this condition is usually asymptomatic and often discovered incidentally during the routine imaging techniques.

The development of the inferior/caudal vena cava in humans and domestic animals has not yet been fully elucidated, and various developmental theories have been suggested. Our case exhibited a rare instance of complete duplication among the variations of the caudal vena cava. In complete duplication, CVC takes a double form by dividing into two branches in the renal and pre-renal parts. Renal veins arise separately from both branches [1].

The presence of CVC variations significantly elevates the likelihood of concurrent development of ureter anomalies and portosystemic shunts [2,4,5]. In our patient, the cranial mesenteric vein was connected to the left branch of the dCVC through a shunt (cranial mesenteric-caval shunt). White et al.[6] found a shunt between the cranial mesenteric vein and CVC in three cats included, two of which having duplicated CVC. Especially in cats, the presence of dCVC may increase the likelihood of observing a cranial mesenteric-caval shunt.

Clinical symptoms that may result from this anomaly include deep vein thrombosis and pulmonary embolism in the lower extremities in humans [7]. In particular, Vena Cava anomalies have become a recognized risk factor for deep vein thrombosis of the lower extremities in young people [8]. One study found potential Vena Cava abnormalities in spontaneous, unprovoked deep vein thrombosis in 5% of young patients. In humans, the rate of Inferior Vena Cava (IVC) thrombosis in patients with Congenital Inferior Vena Cava (CIVC) anomaly is 60% to 80% [1]. IVC duplication may create a tendency to venous stasis and therefore venous thromboembolism due to insufficient blood circulation [9,10]. Myocardial heart diseases are common in cats. Thromboembolism is very common especially in these animals [11]. dCVC may be an additional factor to increase the risk of thromboembolism in these patients, as in humans.

dCVC is usually detected during surgery, necropsy or CT angiography [12]. This is thought to be due to the newly developing imaging methods in veterinary medicine. We predict that as the number of CT scans increases, the number of dCVC diagnosis will increase. The symptoms in our case were compatible with portosystemic shunt, however the effects of dCVC on the patient's clinical condition could not be evaluated.

In conclusion, it is important to evaluate patients for dCVC, especially those diagnosed with development of

ureter anomalies, congenital portosystemic shunts and thromboembolism. This case report provides significant insights into the diagnosis of dCVC. It offers valuable understanding in defining and comprehending this rare condition, thereby enriching the existing information in the literature. However, the scarcity of similar cases in the current literature limits the broader contextual evaluation of our findings.

Availability of Data and Materials

The data that support the findings of this case report are available from the corresponding author (Y. Kocak) upon reasonable request.

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We would like to thank VETMR for conducting the computed tomography scan.

Competing Interests

The authors declared that there is no conflict of interest.

Author Contributions

Clinical examinations were conducted by YK. The interpretation of the computed tomography scans was done by YK and ZM. YK wrote the article.

References

1. Ryu C, Choi S, Choi H, Lee Y, Lee K: CT variants of the caudal vena cava in 121 small breed dogs. *Vet Radiol Ultrasound*, 60 (6): 680-688, 2019. DOI: 10.1111/vru.12808

2. Bertolini G, Diana A, Cipone M, Drigo M, Caldin M: Multidetector row computed tomography and ultrasound characteristics of caudal vena cava duplication in dogs. *Vet Radiol Ultrasound*, 55 (5): 521-530, 2014. DOI: 10.1111/vru.12162

3. Korim F, Kuricová M, Eberlová L: Anatomical characteristics of duplicated caudal vena cava in cats - A case report. *Animals*, 13 (10):1585, 2023. DOI: 10.3390/ani13101585

4. Natsis K, Apostolidis S, Noussios G, Papathanasiou E, Kyriazidou A, Vyzas V: Duplication of the inferior vena cava: Anatomy, embryology and classification proposal. *Anat Sci Int*, 85, 56-60, 2010. DOI: 10.1007/s12565-009-0036-z

5. Ito T, Ikeda Y: A case of double inferior vena cava with renal, ovarian and iliac vein variation. *Anat Sci Int*, 93, 139-143, 2018. DOI: 10.1007/s12565-017-0397-7

6. White RN, Parry AT: Morphology of congenital portosystemic shunts involving the right gastric vein in dogs. *JSAP*, 56 (7): 430-440, 2015. DOI: 10.1111/jsap.12355

7. Li WR, Feng H, Jin L, Chen XM, Zhang ZW: Duplication of the inferior vena cava: A case series. *J Int Med Res*, 50 (5): 3000605221100771, 2022. DOI: 10.1177/03000605221100771

8. Alkhouli M, Morad M, Narins CR, Raza F, Bashir R: Inferior vena cava thrombosis. *JACC*, 9 (7): 629-643, 2016. DOI: 10.1016/j.jcin.2015.12.268

9. Mukai Y, Nozawa S, Suzuki T: Double venous compression due to duplicated inferior vena cava induced right common iliac vein thrombosis. *BMJ Case Rep*, 2018: bcr-2017, 2018. DOI: 10.1136/bcr-2017-223610

10. Chaijaroonkhanarak W, Pannangrong W, Welbat JU, Namking M, Khamanarong K, Prachaney P: Double inferior vena cava with three shunts: A rare anomaly with important implications for surgeons. *Folia Morphol*, 76 (2): 307-311, 2017. DOI: 10.5603/FM.a2016.0076

11. Payne JR, Borgeat K, Brodbelt DC, Connolly DJ, Fuentes VL: Risk factors associated with sudden death vs. congestive heart failure or arterial thromboembolism in cats with hypertrophic cardiomyopathy. *J Vet Cardiol*, 17, 318-S328, 2015. DOI: 10.1016/j.jvc.2015.09.008

12. Hunt GB, Bellenger CR, Borg R, Youmans KR, Tisdall PL, Malik R: Congenital interruption of the portal vein and caudal vena cava in dogs: Six case reports and a review of the literature. *Vet Surg*, 27 (3): 203-215, 1998. DOI: 10.1111/j.1532-950x.1998.tb00119.x

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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.

• Privacy

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.

• Authorship and Authors Rights

The authors of the manuscripts sent to Kafkas Universitesi Veteriner Fakultesi Dergisi must have contributed significantly to the design, execution or interpretation of the study. For example, in view of the research and publication ethics as well as authors rights, it is not acceptable to include those as authors who do not actively contribute to the research but just only help in writing or data collection processes, which may not require any scientific knowledge. All the authors in a publication should be in agreement of the names and the orders of the authors in the manuscript.

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".

• Originality of Research Findings

The authors should declare that the article they presented contained the original research results, that the study data were analyzed correctly, and that they were prepared for publication using adequate and appropriate references, in the "cover letter" section of the on-line system at the submission stage. Using expressions such as "it is the first study done", "there has been no previous study on this subject" and "there is a limited number of studies" to add originality and importance to the article is not acceptable and may cause prevention of the scientific evaluation of the article by the editor.

• Similarity

Articles submitted to the journal are subjected to similarity analysis using appropriate software (iThenticate by CrossCheck) at the beginning and at every required stage. If unethical similarities are detected regardless of the rate of similarity, this situation is reported to the authors and corrections are requested or articles containing excessive similarities are rejected at the first evaluation stage without being evaluated.

• Plagiarism/Self-Plagiarism, Duplicate Publication

Kafkas Universitesi Veteriner Fakültesi Dergisi applies publication ethics and verifies the originality of content submitted before publication and checks all submitted manuscripts for plagiarism/self-plagiarism, similarity and duplication. All submitted manuscripts are meticilously screened by a similarity detection software (iThenticate by CrossCheck). Papers previously presented at scientific meetings and published only as an "abstract" should be indicated in the Title Page file as stated in the "Guidance for Authors". Authors do not have the right to use entire paragraphs from their previous publications into a new submission. These actions are also considered as a plagiarism. In any case, the manuscript should be original in terms of scientific contents and writing. In the event of alleged or suspected research misconduct, the Editorial Board will follow and act in accordance with "COPE Guidelines".

• Multi-part Publication (Piecemeal Publication)

Some authors may tend to divide study data into two or more articles and publish the results in different journals also having different authors names and orders. In principal, Kafkas Universitesi Veteriner Fakultesi Dergisi is against multi-part publication. When necessary, the ethical committee approval information of the study, project information, congress presentations, etc. are checked and such situations that will create an ethical problem are identified and reported to the authors.

Authors may think that their work should be published in multi parts that complement each other. For this, each part of the article should be titled "Part-I", "Part-II" and submitted to the journal "simultaneously". This issue can be evaluated by the editor-in-chief/subject editors/ referees who may suggest that the article can be published in parts or as a whole. In addition, rejection of a submission presented in parts means that all parts will be rejected.

• Animal Rights and Ethics

The authors are responsible for conducting experimental and clinical studies on animal experiments within the framework of existing international legislation on animal rights. Authors must also obtain permission from the Animal Experiment Ethics Committees and provide relevant information in the Material and Method section to experiment with animals. In clinical studies, as well as the approval of the ethics committee, an "informed consent form" should be obtained from the animal owners and the information related to it should be declared in the Material and Method section. Declaration of "informed consent form" is sufficient for the articles in the "Case report" and "Letter to the Editor" category.

Ethics committee permission taken for a study can only be used in one article. It is unacceptable to use the same ethics committee approval number in articles with different names and contents. The editor/subject editors can request from the corresponding author, if necessary, to send a copy of the ethics committee approval form to the journal (electronically or by post).

In cases of violation ethical rules, the article is not taken into consideration or if it is in the evaluation stage, the procedure is terminated and the article is rejected.

• Conflicts of Interest/Competing Interests

The editor-in-chief pays attention to whether there is a conflict of interest or union of interest between editors, reviewers and author (s) for an objective and unbiased evaluation of the article. In addition, the authors should disclose any financial interests or links or any conditions that may raise the bias issue in research and article under the above heading.

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The authors must fill in the "Copyright Agreement Form" and sign it with a wet signature. Authors who submit articles from abroad should scan the signed form and send it to the editor via the system or by e-mail. Original forms that are wet signed for articles sent domestically should be submitted to the journal via mail or cargo. The works of the authors who do not submit the Copyright Agreement Form on time are not published.

• Withdrawal of a Submission

In case of if the authors detect a significant error or deficiency in their article under review or if this error is reported to them by the editor/ subject editor/referees they can contact immediately to the editor-in-chief and ask the request to withdraw the article by stating the reason. The decision on this issue is up to the editorial board.

• Erratum

After an article has been published, the corresponding author may request the editor to publish "erratum" for any errors or inaccuracies noticed by the authors, editors or readers. In collaboration with the authors, the editor prepares and publishes the Erratum article in the first upcoming issue. These articles, like other publications, should contain the publication tag and DOI number.

• Retraction

If any ethical problem is detected about the article that cannot be compensated and cannot be eliminated with erratum after the article is published, the editor-in-chief and associate editors prepare a justification about the article and apply the retraction procedure to the article. The text file on the web page of a retracted article is blocked and the reason for retraction is added to the system as a file, ensuring that it is constantly in the archive.

• Advertising

Kafkas Universitesi Veteriner Fakultesi Dergisi do not accept advertising and sponsorships that are believed to create a potential conflict of interest. If the article sent to Kafkas Universitesi Veteriner Fakultesi Dergisi is for the promotion of a commercial product and/or the work carried out is directly supported by a company, it is rejected without consideration.

OPEN ACCESS STATEMENT

Kafkas Universitesi Veteriner Fakultesi Dergisi is an open access publication. The journal's publication model is based on Budapest Open Access Initiative (BOAI) declaration. Articles published in Kafkas Universitesi Veteriner Fakultesi Dergisi are available online, free of charge at https://vetdergikafkas.org/archive.php.

Except for commercial purposes, users are allowed to read, download, copy, print, search, or link to the full texts of the articles in this journal without asking prior permission from the publisher or the author. The open access articles in the journal are licensed under the terms of the Creative Commons Attribution-NonCommercial 4.0 International (CC BY-NC 4.0) licence.

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

Processing and publication are free of charge with the journal. There is no article processing char-ges, submission fees or any other fees for any submitted or accepted articles.

RESPONSIBILITIES OF THE PUBLISHER, EDITORS AND ASSOCIATE EDITORS

The publisher (Dean of the Faculty of Veterinary Medicine of Kafkas University) contributes to the execution of the journal's routine processes such as printing, archiving, and mailing, in line with requests from the editor.

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.

Editor-in-chief/editors/associate editors conduct the evaluation and decision process in the journal in coordination within the principles of confidentiality and have independent decision-making authority and responsibility without being affected by any internal or external factors.

Editor-in-chief/editors/associate editors make and implement all kinds of planning for the development of the journal and its international recognition. They also follow national and international meetings or events on the development of journals and article evaluation, and ensures that the journal is represented on these platforms.

The editor-in-chief/editors/associate editors make every effort to ensure that the journal's subject editors and referee pool have international qualifications. Likewise, it makes the necessary attempts to strengthen the author's profile.

Editor-in-chief/editors/associate editors make plans to improve the quality of the articles published in the journal and carry out the necessary process.

Editor-in-chief/editors/associate editors regularly conduct and control the initial evaluation, preliminary evaluation, peer review and acceptance-rejection decisions of articles submitted to the journal. While carrying out these procedures, features such as the suitability of the study for the aims and scope of the journal, its originality, the up-to-date and reliability of the scientific methods used, and the potential it will contribute to the development of the journal as well as its benefit to science/practice are taken into consideration.

Editor-in-chief/editors/associate editors systematically review, inspect and make decisions about the articles submitted to the journal in terms of features such as author rights, conflict of interest, observance and protection of animal rights, and compliance with research and publication ethics.

The editor-in-chief conducts the evaluation/revision process between the authors and subject editors and referees, and ensures that it is completed within the prescribed time.

ARCHIVE POLICY

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.

Even if the journal ceases to be published for any reason, the publisher (Dean's Office of the Faculty of Veterinary Medicine, Kafkas University) will continue to protect the journal content in the long term and provide convenient access to users. Electronic services of Kafkas University Information Technologies Department will be used for the journal to maintain this responsibility.

RESPONSIBILITIES OF SUBJECT EDITORS

Subject editors do reviews and evaluations in accordance with the main publication goals and policies of the journal and in line with the criteria that will contribute to the development of the journal.

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

Subject editors thoroughly examine the sections of the introduction, materials and methods, results, discussion and conclusion, in terms of journal publication policies, scope, originality and research ethics. Subject editor submits its decision (rejection, revision or peer-review) after evaluation to the chief editor in a reasoned report.

Subject editor may request additional information and documents related to the study from the authors, when necessary.

In multidisciplinary studies, the article can be submitted for the evaluation of multiple subject editors.

RESPONSIBILITIES OF REFEREES

Double-blinded peer-review procedure is applied in Kafkas Universitesi Veteriner Fakultesi Dergisi in order to evaluate the articles submitted to the journal in accordance with the principle of impartiality and in objective criteria; that is, referees and writers do not know about each other.

The referees submit their opinions and reports to the editor-in-chief to ensure the control and suitability of a submitted article, its scientific content, scientific consistency and compliance with the principles of the journal. When a referee makes a decision "reject" about an article, he/she prepares the reasons for the decision in accordance with the scientific norms and presents it to the editor.

The referee(s) also gives the authors the opportunity to improve the content of the article. Accordingly, the revisions requested from the authors should be of a quality that explains/questions specific issues rather than general statements.

Referees appointed for the evaluation of the articles agree that the articles are confidential documents and will not share any information about these documents with third parties, except for the editors participating in the evaluation.

Referees should place their criticism on scientific infrastructure and write their explanations based on scientific evidence. All comments made by the referees to improve the articles should be clear and direct, and should be written away from disturbing the feelings of the author. Insulting and derogatory statements should be avoided.

If a referee has an interest relationship with the author(s) on one or more issues, he/she must report the situation to the editor and ask his/her to withdraw from the referee position. The same is also applicable when the authors illegally obtain information about the referees of the article and try to influence them.

The editor-in-chief can share the comments and reports from the referees with the editors/associate editors and the relevant subject editor, as necessary, to ensure that the decision on the article is optimal. If necessary, the editor may share the critical decision and its grounds that a referee has sent about the article with the other referee(s) and present them to their attention.

Referee(s) may request revision many times for the article they evaluated.

The content of the referee reports is checked and evaluated by editor-in-chief/editors/associate editors. The final decision belongs to the editorial.

RESPONSIBILITIES OF AUTHOR(S)

It is not tolerable for the author (s) to send an article, which has been already sent to another journal, to Kafkas Universitesi Veteriner Fakultesi Dergisi within the scope of "which accepts" or "which publishes first" approach. If this is detected, the article is rejected at any stage of the evaluation. As a possible result of these actions, in the process following the previous acceptance of the article sent to another journal, the withdrawal request with this excuse that the authors submit for this article, the evaluation process of which is going on in our journal, is evaluated by the editors and associate editors of the journal and disciplinary action on the grounds of ethical violations about those responsible is started. This unethical action is also informed to the journal editor (if known) who accepted the article.

It is essential that the articles to be sent to Kafkas Universitesi Veteriner Fakultesi Dergisi include studies that have up-to-date, original and important clinical/practical results and prepared in accordance with the journal's writing rules.

Authors should choose the references they use during the writing of the article in accordance with the ethical principles and cite them according to the rules.

The authors are obliged to revise the article in line with the issues conveyed to them during the initial evaluation, preliminary evaluation and peer-review phases of the article and to explain the changes they made/did not make sequentially in the "response to editor" and "response to reviewer comments" sections.

If information, documents or data regarding to the study are requested during the evaluation process, the corresponding author is obliged to submit them to the editorial.

Authors should know and take into account the issues listed in the "General Ethical Principles" section regarding scientific research and authors.

The authors do not have the right to simultaneously submit multiple articles to Kafkas Universitesi Veteriner Fakultesi Dergisi. It is more appropriate to submit them with acceptable time intervals for the journal's policy.

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INSTRUCTION FOR AUTHORS

1- Kafkas Universitesi Veteriner Fakultesi Dergisi (abbreviated title: Kafkas Univ Vet Fak Derg), published bi-monthly (ISSN: 1300-6045 and e-ISSN: 1309-2251). We follow a double-blind peer-review process, and therefore the authors should remove their name and any acknowledgment from the manuscript before submission. Author names, affiliations, present/permanent address etc. should be given on the title page only.

The journal publishes full-length research papers, short communications, preliminary scientific reports, case reports, observations, letters to the editor, and reviews. The scope of the journal includes all aspects of veterinary medicine and animal science.

Kafkas Universitesi Veteriner Fakultesi Dergisi is an Open Access journal, which means that all content is freely available without charge to the user or his/her institution. Users are allowed to read, download, copy, distribute, print, search, or link to the full texts of the articles, or use them for any other lawful purpose, without asking prior permission from the publisher or the author. This is in accordance with the BOAI definition of Open Access.

The official language of our journal is English. Additionally, all the manuscripts must also have Turkish title, keywords, and abstract (translation will be provided by our journal office for foreign authors).

2- The manuscripts submitted for publication should be prepared in the format of Times New Roman style, font size 12, A4 paper size, 1.5 line spacing, and 2.5 cm margins of all edges. The legend or caption of all illustrations such as figure and table and their appropriate position should be indicated in the text. Refer to tables and figures in the main text by their numbers. Also figure legends explanations should be given at the end of the text.

The figures should be at least 300 dpi resolution.

The manuscript and supplementary files (figure etc.) should be submitted by using online manuscript submission system at the address of http://vetdergi.kafkas.edu.tr/

During the submission process, the authors should upload the figures of the manuscript to the online manuscript submission system. If the manuscript is accepted for publication, the Copyright Agreement Form signed by all the authors should be sent to the editorial office.

3- The authors should indicate the name of the institute approves the necessary ethical commission report and the serial number of the approval in the material and methods section. If necessary, the editorial board may also request the official document of the ethical commission report. In case reports, a sentence stating that "informed consent" was received from the owner should be added to the main document. If an ethical problem is detected (not reporting project information, lack of ethical committee information, conflict of interest, etc.), the editorial board may reject the manuscript at any stage of the evaluation process.

4- Authors should know and take into account the issues listed in the "Ethical Principles and Publication Policy" section regarding scientific research and authors.

5- Types of Manuscripts

Original (full-length) manuscripts are original and proper scientific papers based on sufficient scientific investigations, observations and experiments.

Manuscripts consist of the title, abstract and keywords, introduction, material and methods, results, discussion, and references and it should not exceed 12 pages including text. The number of references should not exceed 50. The page limit does not include tables and illustrations. Abstract should contain 200±20 words.

Short communication manuscripts contain recent information and findings in the related topics; however, they are written with insufficient length to be a full-length original article. They should be prepared in the format of full-length original article but the abstract should not exceed 100 words, the reference numbers should not exceed 15 and the length of the text should be no longer than 6 pages in total. The page limit does not include tables and illustrations. Additionally, they should not contain more than 4 figures or tables.

Preliminary scientific reports are a short description of partially completed original research findings at an interpretable level. These should be prepared in the format of full-length original articles. The length of the text should be no longer than 4 pages in total.

Case reports describe rare significant findings encountered in the application, clinic, and laboratory of related fields. The title and abstract of these articles should be written in the format of full-length original articles (but the abstract should not exceed 100 words) and the remaining sections should be followed by the Introduction, Case History, Discussion and References. The reference numbers should not exceed 15 and the length of the text should be no longer than 4 pages in total. The page limit does not include tables and illustrations.

Letters to the editor are short and picture-documented presentations of subjects with scientific or practical benefits or interesting cases. The length of the text should be no longer than 3 pages in total. The page limit includes tables and illustrations.

Reviews are original manuscripts that gather the literature on the current and significant subject along with the commentary and findings of the author on a particular subject (It is essential that the author/s have international scientific publications on this subject). The title and summary of this manuscript should be prepared as described for the full-length original articles and the remaining sections should be followed by introduction, text (with appropriate titles), conclusion, and references.

"Invited review" articles requested from authors who have experience and recognition in international publishing in a particular field are primarily published in our journal.

Review articles submitted to our journal must be prepared in accordance with any of the three categories listed below.

Narrative reviews describe current published information on a scientific topic. However, it does not include a specific methodological process.

Systematic reviews include the search for original studies published in that field on a specific topic, the evaluation of validity, synthesis and interpretation within a systematic methodology.

Meta-analysis is a method of evaluating the results of many studies on a subject with the methods defined in this category and statistical analysis of the obtained findings.

6- The necessary descriptive information (thesis, projects, financial supports, etc.) scripted as an italic font style should be explained below the manuscript title after placing a superscript mark at the end of the title.

7- At least 30% of the references of any submitted manuscript (for all article categories) should include references published in the last five years.

References should be listed with numerical order as they appear in the text and the reference number should be indicated inside the parentheses at the cited text place. References should have the order of surnames and initial letters of the authors, title of the article, title of the journal (original abbreviated title), volume and issue numbers, page numbers and the year of publication and the text formatting should be performed as shown in the example below.

Example: Yang L, Liu B, Yan X, Zhang L, Gao F, Liu Z: Expression of ISG15 in bone marrow during early pregnancy in ewes. *Kafkas Univ Vet Fak Derg*, 23 (5): 767-772, 2017. DOI: 10.9775/kvfd.2017.17726

If the reference is a book, it should follow surnames and initial letters of the authors, title of the book, edition number, page numbers, name and location of publisher and year of publication. If a chapter in a book with an editor and several authors is used, names of chapter authors, name of chapter, editors, name of the book, edition number, page numbers, name and location of publisher and year of publication and the formatting should be performed as shown in the example below.

Example: Mcllwraith CW: Disease of joints, tendons, ligaments, and related structures. **In,** Stashak TS (Ed): Adam's Lameness in Horses. 4th ed., 339-447, Lea and Febiger, Philadelphia, 1988.

DOI number should be added to the end of the reference.

In the references can be reached online only, the web address and connection date should be added at the end of the reference information. The generally accepted scientific writing instructions must comply with the other references. Abbreviations, such as "et al" and "and friends" should not be used in the list of the references.

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8- Latin expression such as species names of bacteria, virus, parasite, and fungus and anatomical terms should be written in italic character, keeping their original forms.

9- The editorial board has the right to perform necessary modifications and a reduction in the manuscript submitted for publication and to express recommendations to the authors. The manuscripts sent to authors for correction should be returned to the editorial office within a month. After pre-evaluation and agreement of the submitted manuscripts by the editorial board, the article can only be published after the approval of the field editor and referee/s specialized in the particular field.

10- All responsibilities from published articles merely belong to the authors. According to the ethical policy of our journal, plagiarism/self-plagiarism will not be tolerated. All manuscripts received are checking by plagiarism checker software, which compares the content of the manuscript with a broad database of academic publications.

11- There is no copyright fee for the authors.

12- The authors are charged a fee on acceptance of the manuscript to cover printing costs and other expenses. This payment information can be found at http://vetdergi.kafkas.edu.tr/

SUBMISSION CHECKLIST

Please use below list to carry out a final check of your submission before you send it to the journal for review. Ensure that the following items are present in your submission:

- Cover letter

• Importance and acceptability of the submitted work for the journal have been discussed (Please avoid repeating information that is already present in the abstract and introduction).

• Other information has been added that should be known by the editorial board (e.g.; the manuscript or any part of it has not been published previously or is not under consideration for publication elsewhere.

- Title page

• Title, running title (should be a brief version of the title of your paper, no exceed 50 characters)

• The author's name, institutional affiliation, Open Researcher and Contributor ID (ORCID)

• Congress-symposium, project, thesis etc. information of the manuscript (if any)

• Corresponding author's address, phone, fax, and e-mail information

- Manuscript

• Title, abstract, keywords and main text

• All figures (include relevant captions)

• All tables (including titles, description, footnotes)

• Ensure all figure and table citations in the text match the files provided

• Indicate clearly if color should be used for any figures in print

- Availability of Data and Materials

- Acknowledgements

- Funding Support

- Competing Interests

- Authors' Contributions

Further considerations

• Journal policies detailed in this guide have been reviewed

• The manuscript has been "spell checked" and "grammar checked"

• Relevant declarations of interest have been made

• Statement of Author Contributions added to the text

• Acknowledgment and conflicts of interest statement provided