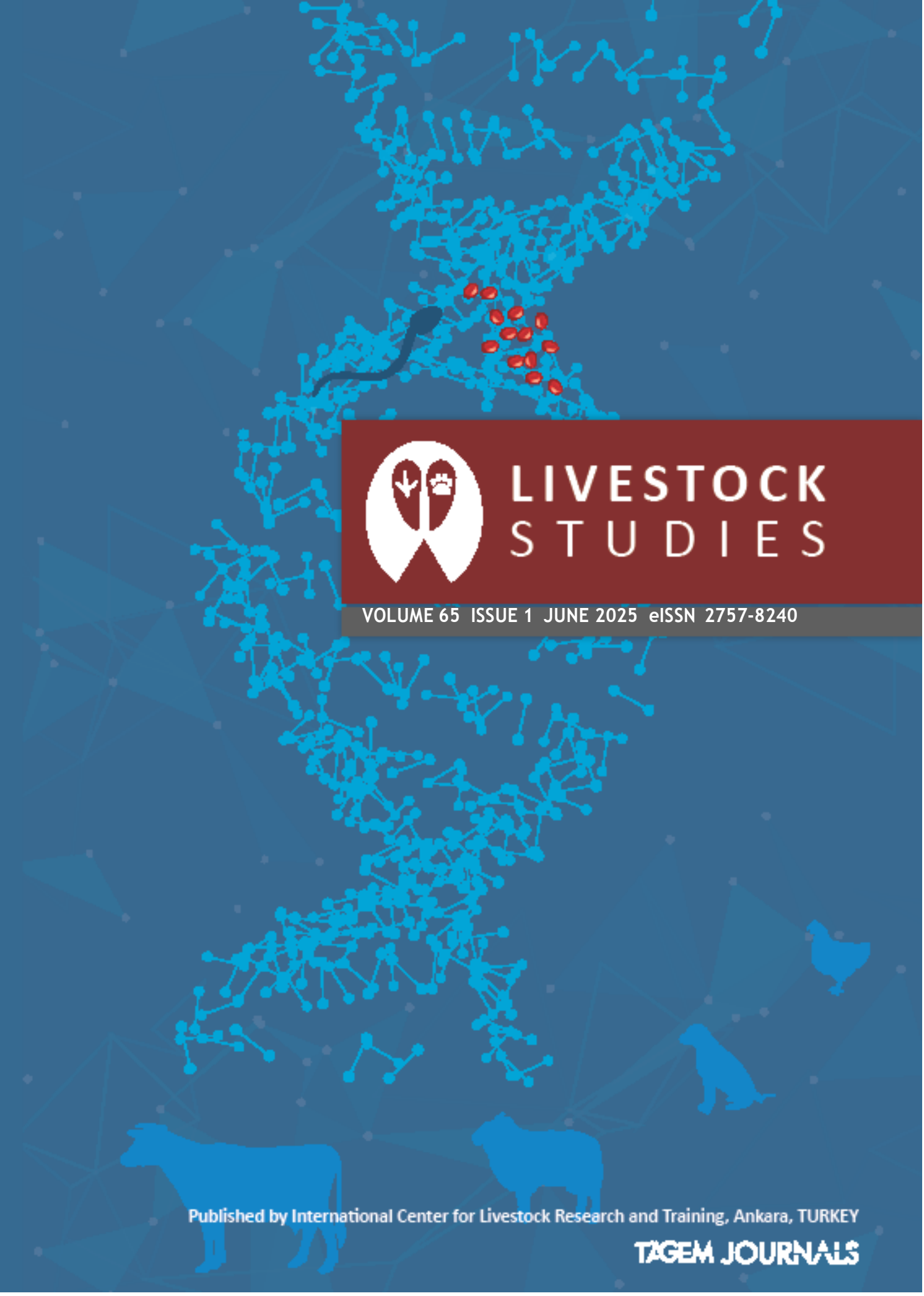

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REVIEW PAPER

Sperm DNA Damages and Damage Detection Methods: Current Approachs

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Abstract

There are many factors affecting male fertility, whose causes are still largely unknown. DNA damage in spermatozoa, in particular, contributes significantly to infertility. Maintaining DNA integrity in sperm is essential for successful fertilization and embryo development. Sources of DNA damage in sperm include errors during chromatin packaging, DNA breaks caused by defective apoptosis, and oxidative stress. These DNA damages are critical for male fertility and lead to issues such as reduced fertilization rates, poor embryo quality, and lower pregnancy rates. While routine examination methods provide a general overview of male fertility, they are often insufficient for a definitive diagnosis of infertility and sterility. For instance, DNA damage has been detected in 15% of spermatozoa with normal values in standard sperm analyses. Additionally, assessing DNA damage in sperm along with functional parameters provides insight into fertilization ability and embryonic development. The goal here is to emphasize the importance of examining sperm DNA to assess male fertility and identify DNA damage and its sources. Common tests used to detect DNA damage include Aniline Blue, Toluidine Blue, Chromomycin A3 (CMA3), Sperm Chromatin Dispersion Test (SCD), TUNEL, Single Cell Gel Electrophoresis (COMET), Sperm Chromatin Structure Assay (SCSA), and Acridine

Introduction

Infertility refers to reduced reproductive capacity in living organisms. Over 15% of infertility cases worldwide are female-related, while 50% are caused solely by male factors or a combination of male and female factors (Choy and Eisenberg, 2018). For infertile men, sperm analysis is still based on traditional techniques (motility, viability, morphology, density, etc.). However, these techniques alone do not accurately reflect male fertility or the success of assisted reproductive technologies (such as In Vitro Fertilization (IVF) and Intracytoplasmic Sperm Injection (ICSI), etc.), as 15% of infertile men show normal sperm analysis results (Varghese et al., 2011). In addition to routine examinations, sperm DNA damage detection methods should also be used for a definitive diagnosis of male infertility.

DNA Structure in Spermatozoa

Sperm DNA integrity is essential for embryo development (Fatehi et al., 2006). Spermatozoa with impaired DNA integrity can fertilize oocytes; however, healthy embryo development may not occur (Aitken, 2017). A morphologically mature spermatozoon forms in the seminiferous tubules through molecular and chemical changes known as spermatogenesis. This process, which includes meiotic and mitotic divisions, occurs in three main stages: (1) spermatocytogenesis, where spermatogonia undergo mitotic divisions to form primary spermatocytes; (2) meiosis, where primary spermatocytes (2n) undergo meiotic divisions to form spermatids (n); and finally, (3) spermiogenesis, where spermatids mature into spermatozoa, completing cytoplasmic and nuclear transformations (Zini and Agarwal, 2011). A major change in the sperm nucleus is the repackaging of chromatin. The nucleus of the spermatozoon has transcriptional activity containing condensed DNA in repressed chromatin. Sperm DNA is in a compact structure, which forms during sperm maturation as histones, the DNA-binding proteins in somatic cells, are replaced by transition proteins (TP1-TP2). TPs are crucial because they reduce DNA damage (Balhorn, 1982). At the end of spermiogenesis, TPs are replaced by protamines

specific to spermatozoa, further compacting the structure. Sperm DNA is divided into three regions as illustrated in Figure 1: toroid loops formed by tightly bound protamines, promoter regions containing histones that cover 12-20% of the DNA, and MAR (Matrix Attachment Regions) responsible for DNA replication and gene expression (Vilfan et al., 2004; Simon et al., 2017). Protamines are half the size of histones, compacting sperm DNA six times more than somatic cell DNA. The arginine-binding regions in protamines neutralize the phosphodiester bonds of DNA. Cysteine within protamines forms disulfide bonds that stabilize the chromatin structure, providing maximum protection to sperm DNA against damage (Ward, 2009). In the histone-containing regions of sperm DNA, genes essential for spermiogenesis and post-fertilization development are located; these areas remain unchanged by protamination. During spermatogenesis, DNA topoisomerase physiologically breaks and re-ligates DNA, facilitating compaction. Physiological DNA breaks in sperm DNA reduce chromatin torsion, supporting the protamination process. These endogenous DNA breaks should not persist in mature spermatozoa (McPherson and Longo, 1993) and must be repaired by the cell before protamination is complete (Andrabi, 2007; Balhorn, 2007). Since mature spermatozoa lack a DNA repair mechanism, cells with DNA damage may still enter the ejaculate (Lewis and Agbaje, 2008). In the early stages of fertilization, oocytes and embryos can repair sperm DNA damage, but this capacity is limited and cannot fully repair double-strand breaks or breaks in histone-bound regions (Menezo et al., 2010). These specific histone-containing and MAR regions in sperm are crucial for embryonic development after fertilization. Therefore, when evaluating sperm quality parameters, it is essential to use sperm DNA damage detection methods alongside conventional methods to ensure a comprehensive approach (Ahmadi and Ng, 1999).

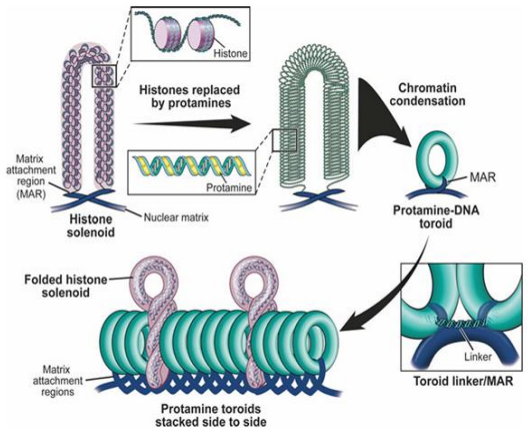

Figure 1. Parts of spermatozoon DNA (Agarwal and Singh, 2012)

DNA Damage Formation in Spermatozoa

There are three possible causes of damage to sperm DNA. These are: (1) abnormal or irregular chromatin packaging due to protamination errors, (2) abnormal apoptosis (programmed cell death), and (3) oxidative stress caused by various ROS/RON sources (Sotolongo et al., 2005). DNA damage resulting from these factors includes base mismatches, base loss (abasic sites), base modifications, DNA insertions and cross-links, pyrimidine dimers, single-strand breaks (SSBs), and double-strand breaks (DSBs) in the sperm nucleus. The increase in DNA damage can be induced by various factors such as lifestyle, diseases, medications, aging, infections, and exposure to chemicals (Figure 2) (Chatterjee and Walker, 2017).

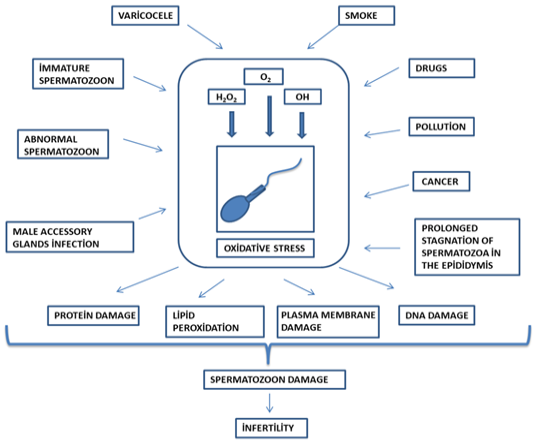

Figure 2. Factors causing damage to spermatozoa

Abnormal or Disordered Chromatin Packaging

The replacement of histones with sperm DNA proteins called protamines leads to the reorganization of sperm DNA and the tight binding of chromatin structures, making spermatozoa more resistant to potential damages. In other words, during spermatogenesis, chromatin is compacted through the exchange of histones with transition proteins and protamines (O'Donnell, 2014). The DNA topoisomerase II enzyme is responsible for repairing DNA breaks during the protamination process. When this process fails, spermatozoa with DNA breaks are produced. In a study conducted on infertile men, damage to protamines and sperm protamination was found to be associated with translational damage (Aoki et al., 2006). The amount and ratio of sperm-specific proteins Protamine 1 (P1) and Protamine 2 (P2) (in humans and mice) present in the sperm nucleus are crucial. The P1/P2 ratio should be around 1 on average. If this ratio is too high or too low, it is associated with an increase in sperm DNA damage, a decrease in fertilization rates, a decline in embryo quality, and ultimately lower pregnancy rates. Additionally, an increase in the histone protein ratio

(by 15%) causes abnormal DNA packaging, making the DNA more vulnerable to damage (Boekelheide, 2005).

Apoptosis (programmed cell death)

Apoptosis is the term used for programmed cell death, which normally occurs in many physiological processes. There are two apoptotic functions in spermatogenesis (Shukla et al., 2012). The first apoptotic function is to eliminate abnormal spermatozoa. The second is to limit the number of germ cells supported by Sertoli cells (Simon et al., 2013). In this way, apoptosis in the testis balances the ratio between germ and Sertoli cells by removing defective germ cells and controlling sperm production (abortive apoptotic process) (Singh and Stephens, 1998). The normal apoptosis of sperm cells plays a critical role in regulating sperm count, rapidly removing sperm with chromosomal abnormalities from the body, and maintaining sperm quality. During spermatogenesis, 25% to 75% of sperm cells are eliminated by apoptosis. Excessive formation of the apoptosis mechanism reduces sperm count in the ejaculate, while insufficient formation increases the number of defective spermatozoa (Agarwal and Singh, 2012).

Oxidative Stress

Oxidative stress (OS) in the testes results from an imbalance between reactive oxygen species (ROS), such as hydroxyl ions (OH), superoxide radicals (O2-), and hydrogen peroxide (H2O2), and antioxidant defense systems. This imbalance occurs due to a decrease in antioxidant defense or an increase in oxidant levels (Newsholme et al., 2016). In 1979, Jones et al. first proposed that human sperm is highly sensitive to OS and reported that it has a significant impact on male infertility (Jones et al., 1979). The primary sources of ROS in the ejaculate are immature spermatozoa and leukocytes. ROS are molecules required in reproductive processes, such as capacitation, hyperactivation, and the acrosome reaction, and are produced due to mitochondrial activation in normal testicular physiology. However, excessive production of ROS leads to sperm DNA breaks, lipid peroxidation, protein denaturation, and plasma membrane damage (Agarwal and Allameneni, 2005). Muratori et al. (2019) stated that sperm DNA fragmentation is induced by defective maturation and abortive apoptosis in the testis, or by ROS produced along the male reproductive system. ROS have been shown to cause various forms of DNA damage, including single and double-strand breaks, base modifications and deletions, cross-linking, and mutations (Agarwal and Allameneni, 2005).

DNA Damage in Spermatozoa

When sperm DNA integrity is studied by many researchers, it has been concluded that nuclear DNA damage negatively affects parameters commonly used, such as motility, viability, and morphology. A study has concluded that DNA damage in spermatozoa has a detrimental effect on reproduction (Saleh et al., 2002). Structural abnormalities in sperm DNA, such as chromatin anomalies, chromatin degradation, oxidation of DNA bases, inhibition of tubulin polymerization, DNA strand breaks, DNA-DNA and DNA-protein crosslinking, mispairing, and mutations, are significant factors affecting fertility (Türk et al., 2006). There are three possible outcomes for spermatozoa with DNA damage. The first is the activation of the apoptotic pathway, leading to the programmed death of the cell. The second is the tolerance of the damage, but this leads to mutations in future generations. Lastly, the repair mechanism of the cell can maintain genomic integrity, allowing the formation of healthy DNA-containing cells. Spermatozoa lack their own repair mechanisms. However, the necessary repair is provided by the oocyte after fertilization. If the damage is irreparable, the cell inevitably undergoes apoptosis and dies (Çevik, 2019).

Spermatozoa DNA Damage Detection Methods

Sperm DNA fragmentation (SDF) can result from failures in protamination, apoptosis, and oxidative stress. Therefore, when selecting the most suitable test to assess SDF, the underlying cause of the SDF should be taken into consideration (Jones et al., 1979).

Aniline Blue (AB)

Aniline blue is an acidic dye that has a high affinity for histones rich in lysine, which are not replaced by protamines during spermatogenesis. This dye is used to determine chromatin condensation. It does not affect protamines that are rich in cysteine/arginine. Therefore, it stains immature spermatozoa with histone-rich nuclei that have not completed protamination. The AB staining technique was first used by Terquem and Dadoune (1983). Spermatozoa with DNA damage are stained blue, while healthy cells do not take up the dye (Hammadeh et al., 2001).

Toluidine Blue (TB)

TB, a basic dye, tends to bind to phosphate residues found in the DNA of spermatozoa with immature or poorly packaged nuclei (Erenpreiss et al., 2001; Marchesi et al., 2010). Staining protocol: Sperm samples are spread on slides and air-dried. Cells are fixed on the slide using 96% ethanol-acetone (1:1) for 30 minutes at +4°C and hydrolyzed with 0.1N HCl for 5 minutes at +4°C. The samples on the slide are washed three times for 2 minutes each. Then, the sperm samples are stained and washed with 0.05% TB and 50% McIlvain buffer (pH 3.5) for 5 minutes. The samples are dried twice for 3 minutes in tertiary butanol and treated with xylene. Spermatozoa with

normal DNA integrity are stained light blue, while spermatozoa with damaged DNA are stained purple (Erenpreiss et al., 2001).

Chromomycin A3 (CMA3) test

CMA3 is an anthraquinone glycoside produced by the bacterium Streptomyces griseus that binds to DNA in the presence of magnesium and detects protamine deficiency (Lolis et al., 1996). Staining protocol: Sperm samples are fixed on slides with a methanol-glacial acetic acid (3:1, v/v) mixture at +4 °C for 20 minutes. Then, they are treated with CMA3 solution (10 mmol/L $MgCl_2$ in McIlvaine buffer) for 20 minutes, washed, and the cells are fixed with PBS-glycerol (1:1, v/v). CMA3-positive sperm cells (bright yellow or bright green staining) indicate insufficient DNA protamination, whereas CMA3-negative sperm cells (pale yellow or dull green staining) show high DNA protamination (Kazerooni, 2009; Marchiani et al., 2021).

Spermatozoon Chromatin Dissociation Test (SCD) / HALO Test

Sperm Chromatin Dispersion (SCD) was proposed by Fernández in 2003. This test, which is used to evaluate sperm DNA breaks, is based on the principle that when sperm samples are treated with an acid solution before the lysis buffer, nuclear proteins are removed, and DNA fragments in the sperm nucleus are separated. These fragments either form halos or do not. While little or no halos are observed in sperm cells with DNA breaks, large halos are formed in spermatozoa with intact DNA (Fernández et al., 2003). For examination, sperm samples are diluted with PBS to a concentration of 5–10 million/ml. The samples are mixed with a low- density liquid agarose gel (0.65% standard agarose dissolved in PBS at 80°C) and smeared onto a 50 µL slide. A coverslip is then placed on top. The slide is kept in a horizontal position at +4°C for 4 minutes to allow the gel to solidify. The samples are then immersed in 0.08N HCl in the dark at 22°C for 7 minutes for denaturation. Next, to neutralize and lyse the sperm samples, they are soaked in a solution containing 0.4 mol/L Tris, 0.8 mol/L DTT, 1% SDS, and 50 mmol/L EDTA (pH 7.5) for 10 minutes. The samples are then transferred to another neutralizing solution containing 0.4 mol/L Tris, 2 mol/L NaCl, and 1% SDS (pH 7.5) for 5 minutes. Afterward, the slides are carefully washed with Tris-borate EDTA buffer (0.09 mol/L Tris-borate and 0.002 mol/L EDTA, pH 7.5) for 2 minutes to remove ethanol, and the slides are air-dried. If evaluation is to be done using a light microscope, the samples are stained with Wright's stain; if a fluorescence microscope is used, they are stained with DAPI (4',6-diamidino-2-phenylindole). Additionally, Haloperm kits have been developed for evaluation using the tail (Fernández et al., 2003; Chohan et al., 2006). While the protocol is standardized, the analysis has some disadvantages due to its lack of full standardization. Moreover, it has been reported that this method, which can analyze even a low number of sperm cells, cannot detect DNA breaks related to the sperm nuclear matrix (MAR region) (Ribas-Maynou and Benet, 2019).

TUNEL [The Terminal Deoxynucleotidyl Transferase-Mediated Deoxyuridine (TdT) Triphosphate (dUTP) Nick End Labeling Assay]

The TUNEL method can directly measure both single- and double-stranded DNA breaks. The principle of the test is based on the detection of open 3’-OH ends in the broken DNA. These ends are first catalyzed by Terminal deoxynucleotidyl transferase (TdT) and then labeled with the biomarker deoxyuridine triphosphate (dUTP). In other words, the more open 3’-OH ends (nicks) present in the DNA, the more FITC-dUTP will bind, resulting in stronger fluorescence signals in more cells [44]. This method directly measures the unevenly broken DNA ends (open 3’-OH) without the need for denaturation, using light microscopy, fluorescence microscopy, or flow cytometry (Simon et al., 2017; Javed et al., 2019). While the TUNEL method is more sensitive and reliable compared to other techniques, its procedure is more complex and expensive.

Single Cell Gel Electrophoresis (COMET)

The comet assay, also known as single-cell gel electrophoresis, was first proposed by Ostling and Johanson in 1984. This technique is based on the principle that fragmented DNA of different sizes exhibits varying levels of permeability within an electrophoretic field. Fragmented DNA strands are separated from the nucleus in the electrophoretic field according to their size. Single- and double-strand DNA breaks produce a characteristic comet appearance, with the size of the tail depending on the amount of DNA damage. In contrast, intact DNA remains confined within the nucleus. The displacement between the nuclear genetic material (comet head) and the migrated, unwound DNA tail (i.e., the length of the tail) serves as an index of sperm DNA damage (Shukla et al., 2012). The comet assay is a sensitive and simple method for evaluating DNA damage, requiring only a small number of spermatozoa (Sharma, 2013).

Sperm Chromatin Structure Assay (SCSA)

SCSA (Sperm Chromatin Structure Assay) is based on the metachromatic properties of acridine orange and is a flow cytometric adaptation of the acridine orange test. Unlike the tight binding of normal double-stranded DNA, which provides stability and acid resistance, the chromatin structure of damaged sperm DNA is relatively loose and can be easily denatured into a single strand by the action of an acidic substance (Evenson and Wixon, 2006). Due to the metachromatic staining property, damaged DNA is separated into single strands and appears red,

while intact double-stranded DNA strands appear green. Additionally, DNA fragmentation levels are measured as the DNA Fragmentation Index (DFI). Since SCSA is a standardized test with a fixed protocol and provides consistent results over a long period, it is widely used in the andrological evaluation of male infertility by many reproductive medicine units (Evenson et al., 1980; Evenson and Wixon, 2006).

Acridine Orange Test (AOT)

The Acridine Orange Test was first conducted by Evenson et al. in 1980. The Acridine Orange Test (AOT) is a simple microscopic procedure based on the treatment of DNA with acid, followed by staining with acridine orange. AOT evaluates the degree of DNA denaturation by the metachromatic shift of AO fluorescence from green (intact DNA) to red (denatured DNA), which is similar to the method used in SCSA (Wang and Swerdloff, 2014).

Conclusion

In the evolving scientific world, spermatozoa are continuously exposed to internal and external harmful factors due to faulty manipulations during assisted reproductive techniques and throughout spermatogenesis. Maintaining the compact structure of sperm DNA is crucial for the proper transmission of male genetic information to future generations. Additionally, the resulting damage adversely affects normal fertilization, embryo development, and the success of assisted reproductive techniques. In other words, changes in sperm DNA structure can be responsible for abnormal embryo development and, consequently, the abnormal development of the offspring. Therefore, methods for detecting sperm DNA damage are important and should be used in conjunction with conventional methods for evaluating male infertility and predicting the development of a healthy embryo.

Conflicts of Interest

The authors declare that they have no known competing financial or non-financial, professional, or personal conflicts that could have appeared to influence the work reported in this paper.

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LIVESTOCK STUDIES

RESEARCH ARTICLE

Genetic Variation in HIAT1 and IGF1R Genes in Some Goat Populations Reared in Türkiye

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Abstract

This study aimed to assess whether genetic variants of the *HIAT1* and *IGF1R* genes could be integrated into future marker-assisted selection (MAS) studies in three native Turkish goat populations known as Hair (HAI), Honamlı (HNM), and Kabakulak (KBK) to improve meat traits. In terms of the *HIAT1* polymorphism, the frequency of the I allele ranged from 0.06 (KBK) to 0.35 (HNM), while the D allele frequency ranged from 0.65 (HNM) to 0.94 (KBK). No animals with the II genotype for the *HIAT1* gene polymorphism were identified in KBK goats, while one animal carried this genotype in HAI goats. Regarding the *IGFR1* gene polymorphism, the frequency of the A allele ranged from 0.72 (HAI) to 0.92 (HNM), while the T allele frequency varied between 0.08 (HNM) and 0.28 (HAI). Genetic distance-based phylogenetic tree separated HNM goats from KBK and HAI. The literature pointed out that animals with II and AT genotypes for the *HIAT1* and *IGF1R* genes, respectively, are superior in terms of meat traits. Therefore, this study implies that the *HIAT1* gene is promising for MAS studies in KBK goats, while *IGF1R* variation could be utilized to improve meat traits in all goat populations in the future.

Introduction

The domestication process of the goat (*Capra hircus*) is believed to have been achieved approximately 10,000 years ago in the Fertile Crescent, which includes the eastern part of Türkiye (Zeder and Hesse, 2000). Following domestication, goats have been an important livestock species for farmers to produce meat, milk, and leather (Degen, 2007). Compared to other ruminants, goats are able to effectively utilize marginal areas such as mountainous, hilly, and forested regions. Indeed, intensive goat breeding has become a way of life known as nomadic culture in the Taurus Mountains and their extensions located between the Mediterranean and Southeastern Anatolia (Alkan and Ugur, 2015; Daskiran *et al*., 2018).

Up-to-date statistics of the Food and Agriculture Organization of the United Nations (FAO) confirm that Türkiye ranks first in Europe in terms of population size by owning approximately 10.3 million goats (FAO, 2023). Angora, Hair (HAI), Honamlı (HNM), Kilis, and Norduz are native Turkish goat breeds in which HAI is the most widely distributed and common breed, due to constituting about 90% of the country's goat population (Demir, 2024). It is noteworthy that HAI goats possess several subgroups, such as Çandır, Kabakulak (KBK), and Pavga, which differ significantly regarding body size, fertility, and milk yield. The breeding area of the KBK genotype extends to the Fethiye district of Muğla province, including the Elmalı and Kaş districts of Antalya province (Karslı *et al*., 2020; Aslan *et al*., 2022. The HNM breed, which is distinguished by its characteristic convex nose structure (where the lower jaw is longer than the upper jaw), is bred in the Teke region, which includes the Isparta, Burdur, and Antalya provinces, areas where nomadic populations are dense (Elmaz *et al.* 2012; Daskiran *et al*., 2018; Aslan *et al*., 2022).

In Türkiye, goat breeding is primarily practiced to produce meat, while no systematic selection studies focused on improving meat yield are carried out by farmers. On the other hand, traditional breeding studies relying on breeding values calculated from pedigree records and phenotypic data have been utilized to improve economically important traits worldwide. Thanks to this kind of breeding efforts over the past 80 years, significant increases in economic yields have been achieved in livestock breeds. However, contemporary approaches such as marker-assisted selection (MAS) and genomic selection offer new opportunities to enhance various yield traits in livestock. MAS, which involves the study of one or a few major genes in addition to traditional breeding methods, can complement these practices. By integrating MAS with classical breeding, the success of selection and genetic progress can be enhanced.

Numerous candidate genes, including insulin-like growth factor receptor (*IGFR*) and hippocampus abundant transcript 1 (*HIAT1*), have been reported to be integrated into MAS studies since they were directly associated with growth and body weight in numerous goat breeds (Pehlivan, 2019). Indeed, during myogenesis, *IGF*s control and stimulate protein synthesis, cell division, hypertrophy, and proliferation (Mohammadabadi *et al*., 2021). *IGF* and *IGFR* genes also play crucial roles in growth, development, muscle formation, and metabolic processes in livestock. The *HIAT1* gene, on the other hand, is involved in both the molecular function of transporter activity and transmembrane transport. Indel mutations on the *HIAT1* gene have been reported to be associated with growth traits in goats and sheep (Gao *et al*., 2020; Luo *et al*., 2023). Numerous studies have linked mutations in these genes to growth traits and meat yield in livestock species (De la Rosa Reyna, *et al*., 2010; Anh *et al*., 2015; Ding *et al*., 2022; Alex *et al.,* 2023).

Recently, the protocols to investigate a 15-base pair (bp) insertion of *HIAT1* and an A>T (179170) mutation of *IGF1R* genes were designed by Gao *et al*. (2020) and Alex *et al*. (2023), respectively. Although these protocols seem easy to apply and cost-efficient, they have not been utilized to genotype native Turkish goat populations. In this context, this study aims i) to identify polymorphisms in the *HIAT1* and *IGF1R* genes, which have been reported to be associated with growth traits, and ii) to evaluate their potential use in (MAS) programs in HAI, HNM, and KBK goats to improve meat traits.

Material and Methods

Sample Collection and DNA Isolation

A total of 222 animals belonging to KBK (n = 70), HAI (n = 76), and HNM (n = 76) goats were sampled from at least three different farms located in Antalya province based on oral interviews with farmers to minimize kinship. A representative image of the studied goat populations is illustrated in Figure 1. Blood samples were subjected to a salting-out method described by Miller *et al*. (1988) to isolate genomic DNA. The success of DNA isolation was assessed via 1% agarose gel electrophoresis (Figure 2), while the quality and quantity measurements of the extracted DNA were evaluated using a spectrophotometer (Allsheng Nano-400A). DNA samples were then diluted to a final concentration of 50 ng/µL before polymerase chain reaction (PCR) was performed.

Detection of Polymorphisms in *HIAT1* and I*GF1R* Genes

In this study, the primer sets and PCR protocols reported by Gao *et al*. (2020) and Alex *et al*. (2023) were followed to genotype three native Turkish goat populations in terms of the polymorphisms of *HIAT1* and *IGF1R* genes, respectively. Since the polymorphism in the *HIAT1* gene occurs due to a 15 bp insertion, a standard PCR procedure was employed. In contrast, as the polymorphism in the *IGF1R* gene is a

Figure 1. The representative image of a) HNM, b) KBK, and c) HAI goats

single-nucleotide polymorphism (SNP; 179170 A>T), the tetra-primer ARMS-PCR (T-ARMS-PCR) method was utilized. The primer sets and the expected PCR product sizes for the *HIAT*1 and *IGF1R* polymorphisms are given in Table 1.

The PCR reaction used to detect the *HIAT1* gene polymorphism in this study was carried out in a total reaction volume of 50 µL [H_2O: 32,5, $MgCl_2$: 5, 10X buffer 4, dNTPs: 4 (2.5 mM), each primer: 0.5 (10 pmol), Taq: 0.5 (5U) and, DNA: 3 (50 ng)]. PCR amplification was carried out as follows: the first denaturation at 95°C for 5 minutes, followed by 35 cycles of denaturation at 95°C for 30 seconds, annealing at 60 °C for 45 seconds, extension at 72°C for 30 seconds, and the last extension at 72°C for 10 minutes. T-ARMS PCR used to determine *IGF1R* gene polymorphism was performed in a total volume of 50 µL [H_2O: 30,5, $MgCl_2$: 5, 10X buffer 4, dNTPs: 5 (2.5 mM), each primer 0.5 (10 pmol), Taq: 0.5 (5U) and, DNA: 3 (50 ng)]. Finally, the PCR products of the *HIAT1* and *IGF1R* genes were separated on 3.5% and 2.5% agarose gel electrophoresis, respectively, at 75 V for 120 minutes to genotype each animal.

Statistical Analyses

Popgene v.1.32 software (Yeh *et al.,* 1997) was utilized i) to calculate allele and genotype frequencies, ii) to estimate observed (H_O) and expected (H_E) heterozygosity, and iii) to assess Hardy-Weinberg equilibrium (HWE) via chi-square test. This software was also used to estimate genetic distance values among studied goat populations, which were further processed in MEGA 11 software (Tamura *et al*., 2021) to draw an unweighted pair-group with arithmetic average (UPGMA) dendrogram.

Results

Agarose gel electrophoresis-based genotyping revealed that three native Turkish goat populations showed polymorphisms regarding *HIAT1* (Figure 2) and *IGF1R* (Figure 3) variations.

The allele and genotype frequencies, genetic diversity parameters, and HWE test for the studied polymorphisms across three goat populations are summarised in Table 2. Regarding the *HIAT1* gene

Table 1. Some descriptive information for the studied gene regions.

Gene	Method	Primer Sequences (5'-3')	Genotype Sizes (bp)	References
***HIAT1* (Insertion 15 bp)**	PCR	F: AGAGCCTCAGTTTCGCTTATT R: GAGTTTATGAATCCAGCAGTTGT	II = 198 DD = 183 ID = 198, 183	Gao *et al*. (2020)
***IGF1R* (179170 A>T)**	T-ARMS-PCR	IR: TGTCCACATCTTACCTAAGGCTGCTG OF: TAGGTGGTTAGATGGTCGGAATGAGC OR: GCCTCATTCAGGTGTCTGGAACTCTT IF: TGGTACCGCAACTGTTCAGACTGGTA	AA= 323, 200 AT = 323, 200, 176 TT = 323, 176	Alex *et al*. (2023)

Table 2. Gene, genotype frequencies, genetic diversity, and chi-square test results for *HIAT1* and *IGF1R* gene regions across the studied goat populations.

			Gene Frequencie		Genotype Frequencies			Genetic Diversity Parameters			HWE
Gene	**Breed**	**n**	**I**	**D**	**II**	**ID**	**DD**	H_O	H_E	***Ne***	χ^2
HIAT1	HNM	73	0.35	0.65	0.16(12)	0.37 (27)	0.47 (34)	0.63	0.54	1.83	2,54[a]
	HAI	76	0.18	0.82	0.01(1)	0.34 (26)	0.65 (49)	0.66	0.70	1.42	1.45[a]
	KBK	70	0.06	0.94	0.00	0.13(9)	0.87 (61)	0.87	0.88	1.13	0.33[a]
Gene	**Breed**	**n**	**A**	**T**	**AA**	**AT**	**TT**	H_O	H_E	***Ne***	χ^2
IGF1R	HNM	73	0.92	0.08	0.85(62)	0.15 (11)	0.00 (0)	0.74	0.70	1.16	0.49[a]
	HAI	76	0.72	0.28	0.51(39)	0.41 (31)	0.08 (6)	0.60	0.58	1.70	0.02[a]
	KBK	70	0.79	0.21	0.70(49)	0.19 (13)	0.11 (8)	0.81	0.67	1.48	13,22[b]

H_O: Observed heterozygosity, H_E: Expected heterozygosity; *Ne*: number of effective alleles, HWE: Hardy-Weinberg equilibrium;
χ^2: chi-square value χ^2 ($_{0.05;1}$: 3.84); a: Non-significant deviation from HWE test, b: Significant deviation from HWE test

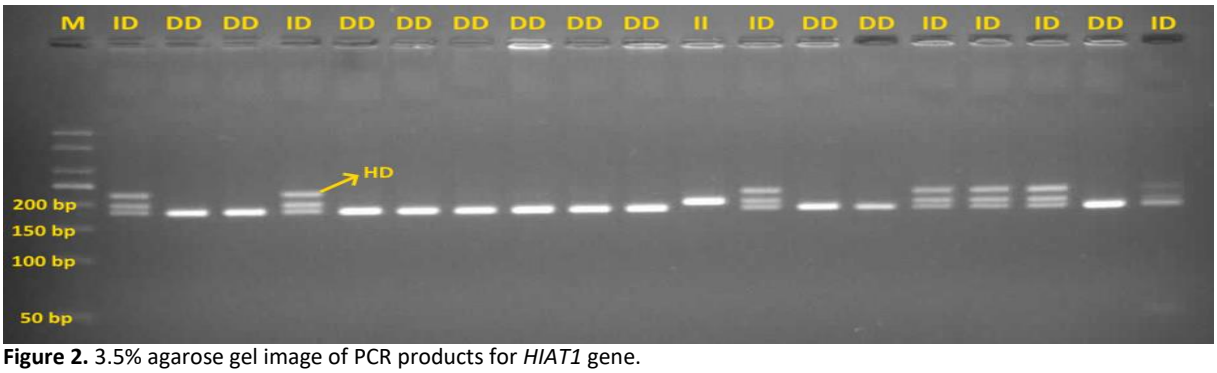

Figure 2. 3.5% agarose gel image of PCR products for *HIAT1* gene.
M: 50 bp Marker (softec, Kat. No: ZT-50BP-1); HD: heteroduplex

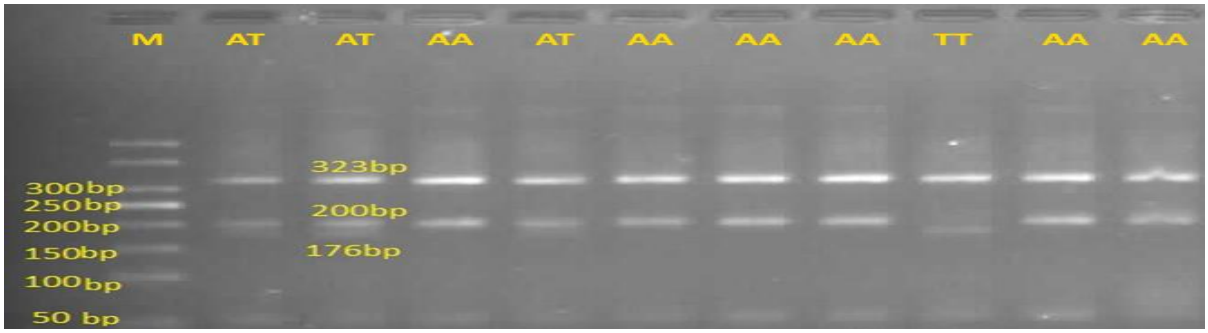

Figure 3. 3.5% agarose gel image of T-ARMS PCR products for *IGFR1* gene.
M: 50 bp Marker (softec, Kat. No: ZT-50BP-1)

polymorphism, the frequency of the I allele ranged from 0.06 (KBK) to 0.35 (HNM), while the D allele frequency ranged from 0.65 (HNM) to 0.94 (KBK). The II genotype frequency ranged from 0.00 (HNM) to 0.16 (KBK), the ID genotype frequency ranged from 0.13 (HNM) to 0.37 (KBK), and the DD genotype frequency ranged from 0.47 (HNM) to 0.87 (KBK). No significant deviation from HWE was detected in the *HIAT1* gene polymorphism across the studied goat populations.

The result of the T-ARMS PCR analysis indicated that all populations were polymorphic in terms of *IGFR1* gene polymorphism. The frequency of the A allele for the *IGF1R* gene in the studied breeds ranged from 0.72 (HAI) to 0.92 (HNM), while the T allele frequency varied between 0.08 (HNM) and 0.28 (HAI). The AA genotype frequency ranged from 0.51 (HAI) to 0.85 (HNM), the AT genotype frequency ranged from 0.19 (HAI) to 0.41 (KBK), and the TT genotype frequency ranged from 0.00 (HNM) to 0.11 (KBK). A significant deviation from HWE was detected only in the KBK population regarding the *IGFR1* gene polymorphism.

Among the studied populations, the lowest and highest *Ho* value for the *HIAT1* gene were estimated in the HNM (0.63) and KBK (0.87) goats, respectively. The effective allele numbers (*Ne*) for the *HIAT1* gene were determined as 1.83, 1.42, and 1.13 for the HNM, HAI, and KBK goats, respectively. The lowest observed heterozygosity for the *IGF1R* gene was found in the HAI breed (0.58), while the highest was observed in the KBK breed (0.81). The *Ne* for the *IGF1R* gene were determined as 1.16, 0.05, and 1.48 for the HNM, HAI, and KBK breeds, respectively.

The UPGMA dendrogram based on the *HIAT1* and *IGF1* genes is shown in Figure 4. According to the UPGMA dendrogram constructed from the

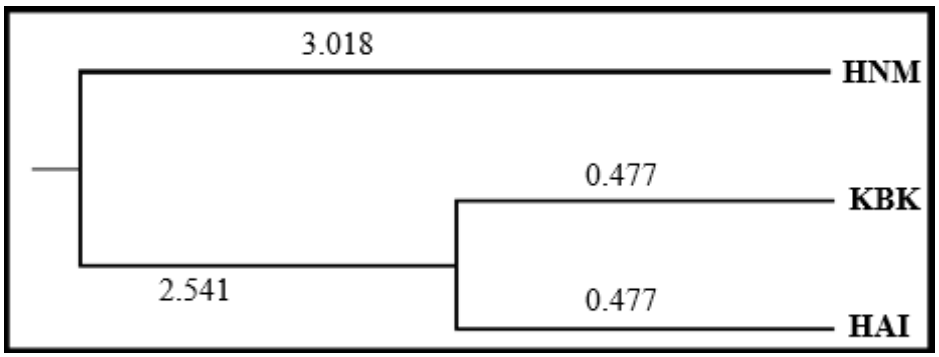

Figure 4. UPGMA cluster analysis based on *HIAT1* and *IGF1R* gene polymorphisms.

genetic distance values for both genes, the KBK and HAI populations formed a cluster, while the KBK goats were placed in a separate branch.

Discussion

Recently, several molecular-based studies have been conducted to identify candidate genes associated with meat traits such as body weight and growth in various goat breeds (Gao *et al*., 2020; Wei *et al*., 2021; Moaeen-ud-Din *et al*. 2022; Alex *et al*., 2023; Dai *et al*., 2024; Xu *et al*., 2024). Among these studies, Gao *et al*. (2020) examined the association between a 15-bp indel polymorphism in the *HIAT1* gene and some growth traits in Shaanbei White Cashmere goats. Researchers reported that the frequency of II, ID, and DD genotypes was 0.044, 0.317, and 0.639, respectively, while animals with II genotypes showed superior meat traits compared to animals with ID and DD genotypes. In the present study, the II genotype was not detected in KBK goats, whereas its frequency was 0.01 and 0.16 in HAI and HNM goats, respectively. Since the absence of II genotype in KBK could be explained by the small sample size. The frequency of the II genotype observed in the HNM goats was higher than that found in HAI goats and the value reported by Gao *et al*. (2020) in the Shaanbei White Cashmere goats. The differences in genotype frequencies across native Turkish goats and Shaanbei White Cashmere may arise from variations in ecological, demographic, and genetic background.

Another candidate gene reported to be associated with growth traits in goats is the *IGF1R* gene (Luo *et al*., 2019; Lestari *et al*., 2020; Alex *et al*., 2023). Alex *et al*. (2023) reported that, among the genotypes (AA, AT, and TT) resulting from a point mutation (179170 A>T) in the second intron of this gene, goats with the AT genotype had significantly higher body weights ($p < 0.05$) compared to those with the AA and TT genotypes. Alex *et al*. (2023) reported the frequencies of the AA, AT, and TT genotypes of the *IGF1R* gene as 0.40, 0.44, and 0.16, respectively, in the Malabari goat breed, and 0.32, 0.40, and 0.28, in the Attappady Black goat breed. In the current study, the frequencies of the AT genotype in HNM, HAI, and KBK goats were 0.15, 0.41, and 0.19, respectively. While the AT genotype frequency observed in the HAI breed was comparable to the values reported by Alex *et al*. (2023) for the Attappady Black and Malabari breeds, the frequencies in the KBK and HNM breeds were lower.

Regarding the *IGF1R* gene, the presence of all genotypes with varying frequencies across the three goat populations (particularly the favorable AT genotype associated with growth) and the observed heterozygosity of higher than expected heterozygosity values suggests that this gene region holds potential for MAS studies. However, II, which is the desired genotype for growth in the *HIAT1* gene, was detected at higher frequency in the HNM goats compared to other populations. The presence of high genetic variation for this gene region in the HNM goats and the presence of the HWE indicate that this gene region can be used in MAS studies in HNM goats. Türkiye contributes to global animal genetic resources with five officially registered native goat breeds (Angora, HNM, HAI, Kilis, and Norduz). Among these, HAI goats are the most commonly reared, accounting for approximately 90% of the country's total goat population (Demir, 2024). KBK goats, still classified as a variety of Hair goats, exhibit notable phenotypic differences (such as longer ear length and a larger body size) (Gezer, 2018; Karslı *et al*., 2020). Despite these distinctions, morphological and molecular studies on this variety remain limited. In some phylogenetic analyses performed at the molecular level in recent years, it has been reported that HAI and KBK breeds are clustered differently (Karslı *et al*., 2020; Karslı and Demir, 2024; Karslı and Atmaca 2025), while other studies assigned HAI and KBK into the same genetic cluster (Aslan *et al*., 2022; Aktaş *et al*., 2024). In the present study, HAI and KBK goats were found to be genetically close to each other. Since the analysis was conducted using two gene regions (*HIAT1* and *IGF1R*), the results seem preliminary, which should be further confirmed by various studies. Among the studies reviewed, the most comprehensive appears to be that of Karslı *et al*. (2020), which utilized 20 microsatellite loci. However, to more accurately differentiate between HAI and KBK goats, further research at the whole-genome level (such as studies employing SNP chips or whole-genome sequencing) is required.

Conclusions

In this study, polymorphisms in the *HIAT1* and *IGF1R* genes associated with growth traits were evaluated for the first time via cost-efficient molecular genotyping methods in Turkish indigenous goat populations. No animals with the II genotype for the *HIAT1* gene polymorphism were identified in KBK goats, while one animal carried this genotype in HAI goats. It is noteworthy that the absence of II genotype in KBK may occur due to the small sample size, which requires further studies focusing on a better sampling strategy. Still, the current findings indicate that the *HIAT1* gene polymorphism could be utilized in MAS studies only for HNM goats. On the other hand, the desired genotypes (AT) for meat traits regarding the *IGF1R* gene polymorphism were detected at sufficient frequencies among all goat populations. This finding

implies that the *IGF1R* gene is promising to improve meat production in native Turkish goat populations via future MAS programs. However, it should be noted that this study was limited to the identification of polymorphisms, and further association analyses with phenotypic data are necessary before implementing MAS. Additionally, KBK goats, which are considered a subtype of the HAI breed, were found to be genetically similar to HAI goats based on the two examined gene regions.

Author Contributions

First Author: Laboratory analyses, funding acquisition, manuscript drafting; Second Author: Laboratory analyses, manuscript drafting; Third Author: Laboratory analyses, manuscript drafting; Fourth Author: Laboratory analyses, manuscript drafting; Five Author: Laboratory analyses, manuscript drafting; Six Author: Manuscript drafting, critical review; Seven Author: Supervision, Methodology, funding acquisition, manuscript drafting, critical review.

Conflict of Interest

The author(s) declare that they have no known competing financial or non-financial, professional, or personal conflicts that could have appeared to influence the work reported in this paper.

Ethical Statement:

This study was approved by Eskisehir Osmangazi University Animal Experiments Local Ethics Committee (Protokol No: HAYDEK-967/2023).

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LIVESTOCK STUDIES

RESEARCH PAPER

The Significance of Animal Husbandry in Agricultural Output and Exportation within Gaziantep Province

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Abstract

The present study investigates the importance of animal husbandry in agricultural activities and exportation in Gaziantep province. For this purpose, the data obtained from the following institutions was subjected to evaluation: Presidency of the Republic of Türkiye Strategy and Budget Directorate, Turkish Statistical Institute, Ministry of Agriculture and Forestry, Provincial Organisations, and Gaziantep Chamber of Commerce and Industry. The study revealed that the Gaziantep province has 17.727 agricultural enterprises. Furthermore, it was determined that 1.56% of ovine animals and 1.18% of bovine animals in Türkiye are raised in Gaziantep province. In the context of Türkiye's total exports, it is noteworthy that the Gaziantep province plays a significant role in the live animal trade, with a percentage of 10.91%, followed by meat and offal exports, accounting for 0.94%. The province also stands out in the export of dairy and egg products, contributing 2.05%, as well as animal and vegetable fats, with a notable 19.71% share. The share of animal products in agricultural exports made on a provincial basis was calculated as 11% in live animal exports, 15% in feed and additives, 5% in poultry meat and products, and 47% in eggs. In conclusion, animal husbandry plays a pivotal role in enhancing agricultural productivity and contributes significantly to the export potential of Gaziantep province, underscoring its importance in both local economic development and national agricultural strategies.

Introduction

The distribution of income and welfare levels across different countries worldwide is influenced by a variety of geographical, economic and labour force factors. It is important to note that agricultural earnings are subject to variation depending on the climatic, topographical and soil structural characteristics of the region. The evaluation of development in a nation-state in a single direction will result in an incomplete definition of the role of other sectors in economic development. However, when economic development is analysed in many aspects, the role and importance of agriculture will be better understood. Economic development is a dynamic structure in the international dimension and is a holistic formation that includes service, industry, agriculture and industry sectors.

An examination of the economic development process of countries readily reveals that income derived from agriculture is often utilised as a source of capital in the industrial and commercial sectors. Türkiye, with its diverse geography and substantial agricultural product market, is among the countries with strong economies and is frequently mentioned in international markets. The country's rich geographical characteristics and the resulting agricultural genetic diversity provide a significant advantage in contributing to regional economic development (Arzık *et al*., 2023; Kizilaslan *et al*., 2024). The presence of different climate types and

ecological conditions allows for the cultivation of diverse crop patterns and the expansion of livestock activities (Ertan *et al*., 2025); this, in turn, diversifies income sources for the local population and supports the balanced and sustainable growth of the national economy (Arzık *et al*., 2025). In the contemporary era, the challenges associated with meeting the nutritional requirements of individuals through agricultural products, as well as the difficulties encountered during the procurement process, underscore the strategic importance of this sector. From this standpoint, it is imperative to ascertain the characteristics of a province, including its geographical situation, soil structure, climatic data, and the diversity and quantity of agricultural products produced.

Gaziantep, which is known as the Upper Mesopotamian Plain in history and is part of the Fertile Crescent, is one of the provinces with the highest potential to become a leader in agricultural production in Türkiye. The province has achieved renowned for its industrial prowess and contributes substantially to the national economy through agricultural production. As is well documented, Pistachio, almond, walnut, grape, red lentil, chickpea, cotton, sesame, wheat, garlic, olive and fig cultivation is widespread in Gaziantep, in addition to livestock breeding activities. The production branches in question include the textile, metal, machinery, chemical, plastic, food, footwear, leather, wood products and furniture sectors (Anonymous, 2019; Yanar and Savrun, 2023; Ağazade, 2023). The economic contribution of livestock production in Gaziantep extends beyond primary production, encompassing a wide range of sectors such as feed manufacturing, veterinary services, processing industries, and logistics, thereby promoting employment across multiple domains. Animal products are increasingly becoming a significant part of the province's export portfolio, particularly through value-added exports of processed meat, dairy products, and traditional items such as sausages and cheese. This economic diversification aligns with national agricultural policies aimed at strengthening rural economies and enhancing international competitiveness. Nevertheless, the livestock sector in Gaziantep faces several challenges, including limited feed resources, climate variability, inadequate pasture management, and market fluctuations. Addressing these issues requires the integration of traditional knowledge with modern practices, sustainable management of natural resources, and the development of supportive policy frameworks. In this context, a comprehensive analysis of the province’s unique livestock dynamics is crucial for designing targeted interventions that can enhance both productivity and long-term sustainability.

The present study was conducted for the purpose of analysing the economic contribution of animal husbandry to the agricultural sector in Gaziantep province. In addition, it was investigated how income and employment opportunities are created by animal husbandry activities. Finally, the effects on the local, regional and national economy were evaluated.

Material and Method

The material of the study consists of the economic data obtained from Gaziantep province and the share of animal husbandry in these data. The geographical location of the province in question is such that it is situated along the historical Silk Road, which was a major commercial and cultural route between the Southeast and the Mediterranean regions. Gaziantep, constituting approximately 1% of Türkiye's territory, is also bordered by the provinces of Kahramanmaraş, Adıyaman, Şanlıurfa, Osmaniye, Hatay and Kilis (Anoymous, 2016).

In this study, data obtained from institutions and organisations operating in different fields in our country were analysed. In this context, data from the Presidency of the Republic of Türkiye Strategy and Budget Directorate, the Turkish Statistical Institute, the Ministry of Agriculture and Forestry and its Provincial Organisations, and the Gaziantep Chamber of Commerce and Industry were subjected to evaluation. Furthermore, the information has been expanded upon in several academic studies.

Results and Discussion

The Place and Importance of the Agricultural Sector in the Turkish Economy

While the share of agriculture in the Gross National Product was approximately 40 % in the early years of the Republic, it reached approximately 47 % in 1929 (Karluk, 2007). The agricultural sector, a dominant economic force throughout the country, experienced a significant shift in its share. In the 1980s, the sector's contribution to the economy stood at 25.8%, a figure that declined to 5.54% in 2021. However, the sector witnessed a recovery, with its share increasing to 6.5% in 2022. The agricultural sector has been experiencing a gradual decline in its overall strength and this ratio in GNP was determined as 4.4% in the second quarter of 2023. The employment rate was determined as 15.8% (about 4.9 million people) among all sectors (about 30.8 million people) (Anonymous, 2023a).

The total arable land in Türkiye is 38.482.000 hectares, of which 20.194.000 hectares were under production as of 2022 (Anonymous, 2023a). A total area of 16.510.000 hectares is dedicated to cereal and other vegetable production, 718.000 hectares are allocated for vegetable production, 6,000 hectares are designated for ornamental plant production, and 2.960.000 hectares of land remains fallow. Concurrently, 14.617.000 ha of land is utilised for

meadow pasture, while 23.110.000 ha is designated for forestry.

Furthermore, sectoral-based GDP amounts are enumerated in Table 1. As demonstrated in the table 1, there has been a substantial increase of 141.28% in the agricultural sector between 2021 and 2022, contingent on the implementation of production and price policies across all sectors.

In comparison to other sectors, the agricultural sector in 2022 accounted for 23.86 % of the income from industry and 11.53 % of the service sector (excluding taxes and subsidies). Agricultural products of Turkish provenance are exported to numerous regions across the globe. According to data from the Turkish Statistical Institute, the agricultural sector constituted 701 million USD (3.99%) of the total exports of 17.554 billion USD in 2022 (Anonymous, 2023a).

In the statement made by the International Trade Centre, the share of agriculture in total exports in the world at the end of 2022 was 2.021 trillion dollars, accounting for 7.94% of total exports. The rate was documented as 7.35% in 2003 and 9.87% in 2020. Türkiye's agricultural products export value was reported as 3.7 billion dollars in 2002, 15.2 billion dollars in 2012, and 20.7 billion dollars in 2020. With a total agricultural product export volume of 29.9 billion dollars, Türkiye ranked 21st among the world countries in 2022. Among the agricultural products exported worldwide, 8% of meat and meat products are supplied from our country. While Germany ranked first among agricultural export countries in 2008, Iraq rose to first place in 2011. As of the close of 2022, Iraq has the highest ranking with a 17% share of 5.2 billion USD, followed by Russia with a 7% share of 2.1 billion USD, the USA with a 6.3% share of 1.9 billion USD, Germany with a 6% share of 1.8 billion USD, Syria with a 3.3% share of 1 billion USD, and 22% of agricultural products exports to EU countries.

Table 1. GDP amounts by sector over the years, Thousand TL (Anonymous, 2023b).

Sectors	2021	2022	2023
Agriculture, forestry and fishing	401.536,982	972.301,593	1.624.740,882
Industry	1.891.987,439	3.964.834,337	5.959.554,257
Manufacturing industry	1.613.624,455	3.318.900,769	5.099.529,147
Non-manufacturing industry	278.362,984	645.933,568	860.025,110
Services	4.195.020,291	8.499.650,864	15.668.237,817
Construction	367.052,709	732.915,929	1.466.467,835
Trade, transport and accommodation	1.773.981,389	4.010.484,772	7.009.908,056
Information and communication	202.927,171	355.572,981	643.801,086
Financial and insurance activities	211.257,040	494.575,554	867.450,349
Real estate activities	358.675,898	556.821,344	1.018.322,943
Professional, administrative and support service activities	342.342,371	685.846,287	1.349.518,484
Public administration, education, human health and social work activities	771.799,917	1.360.100,775	2.732.208,111
Other service activities	166.983,795	303.333,221	580.560,952
Total sectors (1+2+3)	6.488.544,712	13.436.786,794	23.252.532,956
Taxes-subsidies	767.597,025	1.574.989,184	3.023.774,417
GDP at purchasers' prices (4+5)	7.256.141,737	15.011.775,979	26.276.307,373

An examination of the composition of export products (Figure 1) reveals that in 2022, the top three exports were cereals and pulses (44.5%), aquaculture products and animal products (15.8%), and fresh fruits and vegetables (11.4%) (Anonymous, 2023a: 2023c). Erhalim *et al*. (2011) and Demir *et al*. (2023) emphasized in their studies that agriculture plays a vital role in the national economy, and they highlighted substantial fluctuations in its contribution to the GDP over the years.

Türkiye's total imports in 2022 were $364.4 billion, of which 6.4% ($23.2 billion) were agricultural products. $5.7 billion (25%) of these imports were realized under the inward processing regime. These imported products were leaf tobacco ($149 million), palm oil ($249 million), corn ($210 million), red lentils ($413 million), crude sunflower oil ($948 million) and bread wheat ($2 billion), respectively. When analysing the importation of agricultural products according to chapters, it was found that 4.6% of the total consisted of fruits and vegetables, 10.6% comprised animal feeds meal and bran, 14.2% constituted oilseeds, 17.7% consisted of animal and vegetable oils, and 23.1% were cereals (Anonymous, 2023a: 2023c). Aksoylu and

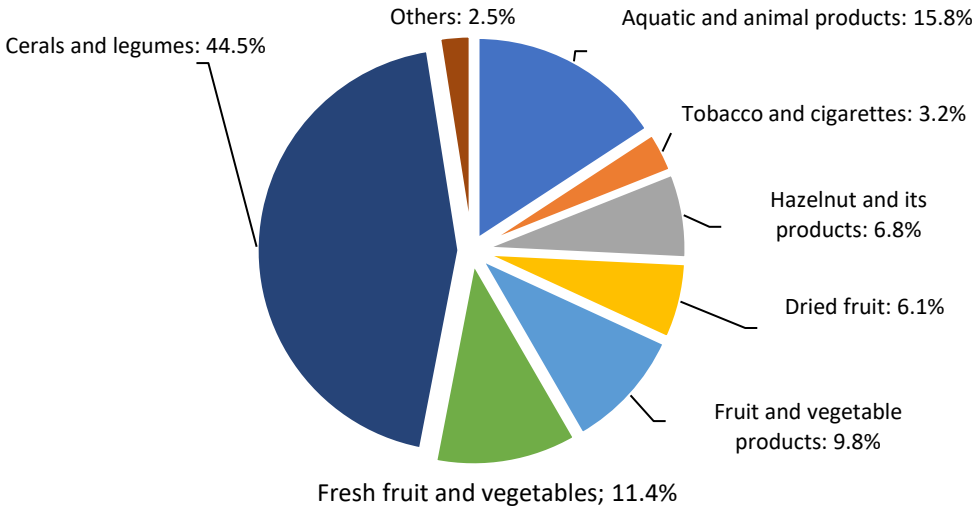

Figure 1: Export rates by product (for 2022; Anonymous, 2023a).

Karaalp Orhan (2022) and İstikbal (2022) examined the year-by-year fluctuations in Türkiye's agricultural export and import shares, underscoring the critical importance of agriculture's contribution to the overall national income.

Demographic Structure of Gaziantep Province

The population of Gaziantep, which has a total surface area of 6887 km2, is 2.164.134 of which 1.091.830 (50.45%) are male and 1.072.304 (49.55%) are female, with an average age of 26.6. The annual population increase compared to the previous year was calculated as 4.7%. The city of Gaziantep has a population of 560.349 households. The ratio of people forming a household was determined as 3.8. Gaziantep is administratively divided into a total of nine districts, of which two are considered central and the remaining seven are districts affiliated to the centre (Anonymous, 2023a). The central districts of Gaziantep province are Şahinbey and Şehitkamil. The central districts constitute 83.48% of the total provincial population. This phenomenon is further compounded by the province's status as an industrial city and the ongoing migration from rural areas to urban centres. Consequently, the young population is increasingly migrating from rural to urban areas, thereby disincentivising engagement in agricultural activities. The recent trend of converting agricultural and pasture lands into urban construction sites, the escalating costs of inputs, and the challenges in selling agricultural produce at optimal prices have been identified as the primary factors driving rural-to-urban migration.

As illustrated in Table 2, the educational attainment of the Gaziantep province population is shown according to gender. As demonstrated in the chart, the number of illiterate women in our province is significantly higher than that of men.

Table 2. Educational attainment in Gaziantep province (Anonymous, 2023c).

Education level (age 7+)	Male	Female	Total
Illiterate	6.121	44.978	51.099
Literate	111.315	135.228	246.543
Primary school graduate	280.049	310.108	590.157
Middle school graduate	225.278	185.388	410.566
High school graduate	198.064	146.150	344.214
Associate and bachelor's degree	160.670	98.281	209.101
Postgraduate degree	11.001	11.410	27.477
Total	**992.498**	**931.543**	**1.879.157**

Recent studies have indicated that the proportion of high school graduates is higher among males than among females. It has been observed that there may be minor discrepancies between males and females regarding individuals in possession of associate and bachelor's degrees (Erhalim, 2011; Semerci, 2018; Kuzu, 2022).

Employment in Gaziantep Province

According to the 2022 data of the Turkish Statistical Institute, the population of Gaziantep province was determined to be 2.154.051 people. The labour force participation rate in our province was reported as 49.7%, the employment rate as 44.4% and the unemployment rate as 10.7%. When the distribution of the labour force by sector is examined, it is reported that 51.2% of individuals work in the service sector, 33.6% in the industry sector and 15.2% in the agriculture sector. The GDP per capita in Gaziantep province was calculated as 70.228 TL (Sarışık ve ark., 2023).

Land Assets in Gaziantep Province

Gaziantep province has 6,807,419 da of land, including forest, agricultural, pasture and non-agricultural land. Of this land, 51% is agricultural land, 17% is forest, 8% is pasture and 24% is non-agricultural land. Within the usable agricultural land in Gaziantep, 135,409 da are used for vegetable cultivation, 2.189.908 da for-fruit cultivation, 1,126,982 da for arable farming and 15,803 da for other agricultural activities. Pistachios, olives, vines, grapes and other agricultural products account for 65%, 21%, 8%, 8% and 6% respectively of the fruit-growing area. Wheat (57%), barley (20%), chickpeas (8%), other crops (15%), red peppers (36%), garlic (26%), mint (7%) and other crops (31%) are cultivated as vegetables. While 1,517,340 ha of agricultural land is available for irrigation in Gaziantep, only 688,950 ha of this area is currently irrigated. The main reason for this is the high cost of irrigation and energy. While the proportion of irrigable land in Türkiye is 24 per cent, this is below the national average (20 per cent). In Gaziantep province, the transition from field to crop agriculture has increased significantly in recent years, and the cultivation of strategic crops such as wheat, barley, lentils and chickpeas has decreased significantly. In this context, fruit production increased from 41% in 2004 to 63% in 2002 (Anonymous, 2023d).

Crop Production in Gaziantep Province

Gaziantep province comprises 25% of the Southeastern Anatolia Project (GAP) (Anonymous, 2023e). This fertile, high quality and productive land, irrigated by the Euphrates River, is home to many crop production activities. In this province, there are 38.625 enterprises engaged in crop production. Gaziantep province, an important agricultural centre, is the leading producer of garlic in Türkiye, a title it has held since 2019. Gaziantep was responsible for 33% of the country's garlic production in 2022. Gaziantep has been identified as a leading producer of mints, with a significant proportion of Türkiye’s mint production (72%) taking place in this city. In the context of pistachio production, which derives its name from the province, Gaziantep attained second position in Türkiye, accounting for 23% of the total production in 2022. In 2022, a total of 273.846 tonnes of red pepper were produced in our country, with 79,993 tonnes (29.21%) being produced in Gaziantep province. Our province ranked second in terms of production. In 2022, the province contributed 150.677 tonnes of the total 4.165.000 tonnes of fresh grape production across the country, thereby ranking eighth. Gaziantep is positioned 9th in Türkiye in terms of red lentil production, 12th in chickpea production, 13th in olive production, 29th in wheat production and 45th in barley production (Anonymous, 2023d).

Animal Production in Gaziantep Province

The number of livestock enterprises in Gaziantep province decreased in 2023 compared to the previous year. While this number was 19.658 in 2022, it decreased to 17.727 in 2023. In this area, the number of aquaculture enterprises remained constant in both years and was determined as 22 units. In our country, 821.853 heads (1.56%) of the total 52.363.410 heads of small ruminants and 196.534 heads (1.18%) of the total 16.583.005 heads of bovine animals are raised in our province. When we detail our animal existence based on species, there are 97.438 heads of culture, 94.360 heads of culture hybrid, 4.186 heads of domestic cattle and 280 heads of buffalo in terms of cattle in our province. As for small ruminants, there are 3.190 heads of Merino sheep, 587.352 heads of Awassi sheep and their hybrids, and 231.311 heads of Hair goats as goats. In Gaziantep province, the projects named Public Hand Breeding of Kilis Goat I and II" and "Public Hand Breeding of Awassi Sheep I and II" supported by the Ministry of Agriculture and Forestry have been successfully carried out since 2011 (Anonymous, 2023e). Bee breeding is carried out in 315 villages in our province, and it has been reported that the number of new hives is 24.054, the number of old type hives is 5.784, a total of 29.838 hives, 209 tonnes of honey production, 3 tonnes of beeswax production (Burucu, 2022). When honey production in our province is compared to the total amount of honey produced in the country, it is seen that it has a share of 0.18% (Anonymous, 2023d).

Exports of Agricultural Products in Gaziantep

Gaziantep, which is the gateway of the historical Silk Road to Anatolia, is a city that has made a name for itself in every field with its commercial ability and productivity and has made serious contributions to the national economy. Since the establishment of the Republic, it has made significant investments and built facilities in the field of industry. Türkiye was divided into11 industrial zones and 7 provinces (Bitlis, Diyarbakır, Elazığ, Malatya, Siirt, Şanlıurfa and Mardin) were connected to Gaziantep province in 1925. Of the 2008 small industrial facilities in our city in 1927, 55%were based on agriculture, 21% on weaving, 10% on wood products and metal goods. Today, it has the largest organised industry in Türkiye with approximately 4500 hectares of land (Anonymous, 2021). The products manufactured in both small industrial sites and organised industrial zones are sold both domestically and internationally in Gaziantep. In this province, which is predominantly agricultural, the export values of animal products are outlined in Table 3.

As illustrated in the accompanying table, the quantity of income derived from livestock sales and related products varies across different years within the province. As indicated by the data, the export of live animals has been identified as a priority area for consideration. However, a significant decrease was observed in 2023 compared to previous years. This decline is concomitant with the number of live animals in our country. Despite the decline in the quantity of live animals, there has been a substantial increase in the exportation of carcass meat and offal.

A decline in sales has been observed in dairy products, animal fat, eggs, honey, silk, leather and products derived from animals. In the context of the present study, an evaluation of the year 2023 reveals that it was a suboptimal year regarding provincial exports. A comparison of the year 2023 with previous years reveals that it is a suboptimal period regarding the provincial economy. This phenomenon may be associated with the global economic crisis and the challenges faced in the agricultural sector, particularly within our nation. An analysis of the export values derived from animal husbandry activities reveals that, in 2023, the products exported in this sector in Türkiye and the total sales obtained in our province hold significant importance in our country, which is comprised of 81 provinces and accounts for a 7.22% share. Once more, when proportioned to the aggregate provincial agricultural product export values, it was ascertained that a considerable income was obtained, with a percentage share of 28.84%, according to the same year data (Anonymous, 2023g).

Table 4 shows the plant-based export products exported from our province. It is seen that the most remarkable items among these products are cereals and milling products. Especially the export of processed products such as flour and starch has a large share in total sales. Mundan *et al*. (2017), in their study conducted in the Southeastern Anatolia Region, and Semerci (2018), in research carried out in the province of Hatay, highlighted the significance of livestock production within the overall agricultural sector. Their findings indicate that the share of animal husbandry in total agricultural output is substantial and cannot be overlooked.

Table 3. Animal-Origin products exported from Gaziantep (Thousand USD) (Anonymous, 2023f).

Export Items	**2021**	**2022**	**2023**
Live animals	16.660,924	18.800,138	7.057,734
Meat and offal	4.477,137	5.760,540	7.937,754
Dairy products, eggs, honey, other edible animal products	42.780,747	46.854,059	38.712,517
Other animal products (bones, horns, intestines, etc,)	2.721,443	2.268,068	2.058,448
Animal and vegetable fats and oils	625.956,842	769.756,200	633.750,700
Fishery products	4.029,655	7.243,930	5.215,616
Raw hides and skins (except fur) and leather	234,042	64,851	31,518
Leather products (bags, home and decorative items, etc,)	955,504	1.501,960	1.948,605
Silk	23,542	83,038	48,997
Wool, hair, horsehair yarn and woven fabrics	5.218,817	3.788,418	4.630,580
Total	703.058,653	856.121,202	701.392,469
Animal-origin exports from Türkiye	4.974.340,476	7.028.679,693	7.174.952,001
Ratio to plant-origin exports (%)	33.80	35.24	28.84
Share in total exports (%)	7.17	8.41	7.22

Table 4. Plant-Origin products exported from Gaziantep (Thousand USD) (Anonymous, 2023f).

Export Items	**2021**	**2022**	**2023**
Edible vegetables and certain roots and tubers	20.854,495	17.936,142	16.376,109
Edible fruits and nuts	101.891,842	90.314.234	65.442,108
Coffee, tea, maté and spices	7.486,232	8.107.308	8.935,456
Cereals	15.237,723	8.825,217	462.101,966
Milling products	321.597,740	362.970,534	362.300,526
Oil seeds and oleaginous fruits	79.971,3	100.271,366	57.900,297
Vegetable plaiting materials	282,603	1.020,868	1.535,516
Cereal, flour, starch or milk preparations; pastry products	880.524,162	1.102.161,034	1.077.270,987
Preparations of vegetables and fruits	259.797,167	393.539,629	218.052,854
Miscellaneous edible preparations	101.624,139	118.541,710	13.648,688
Cotton, cotton yarn and cotton fabrics	277.401,566	213.731,351	137.811,166
Other vegetable textile fibres, paper yarn and fabrics	13.619,166	11.829,460	10.385,313
Total	2.080.508,675	2.429.254,588	2.431.812,996
Plant-origin exports from Türkiye	18.077.985,210	19.880.114,210	21.729.953,611
Ratio to animal-origin exports (%)	295.89	283.75	346.70
Share in total exports (%)	21.23	23.86	25.03

It is seen that fresh fruits and vegetables, which are other remarkable products, are exported from our province and foreign currency inflow to our country. Again, it is seen that significant revenues are obtained in the export of cotton and products obtained from cotton in our province, which has an important production in our province and where the important textile industry of our country is located. In the export of some chapters of plant products, it is seen that a similar situation occurred in the export of animal products in terms of export values in 2023. Although the monetary value is close to the previous year, it has increased from 23.86% to 25.03% in total export share. When a general evaluation on the export values of Gaziantep province is made, our province is an important centre in terms of organised industrial zone and is the largest industrial zone in Türkiye. In particular, the fact that many leading textile companies with high brand value and important food factories operate in this region explains why the export data are high (Anonymous, 2023f).

Agricultural products exported from Türkiye and their monetary values are given in Table 5. In this table, animal and vegetable fats and oils are the most prominent export item among animal chapters. It is not clear what is the most exported product within this chapter, since official statistical data give this item. Considering that Türkiye is a sunflower, cotton and olive paradise, this situation can be easily explained. Other attractive products in the same group are meat, offal, milk, eggs, honey and other products of animal origin. Raw leather and its products are also among the products with a significant share in exports. When we look at the share of animal products in total agricultural exports, it is seen that it is 23.03% in 2023, although it has been changing over the years. This value can be considered as a very serious economic indicator of how much importance should be given to this chapter only in terms of animal products. It is seen that the most striking item among the plant chapters is fruit and nutshell products. It is reported that cereals, vegetables, milling products, cotton and cotton products are among the most export items. The fact that the textile industry is developed in our country explains the exports of cotton and cotton products, and the fact that the food industry is developed explains the exports of other foodstuffs. The share of agricultural exports in the total exports of our country is calculated as 11.26 per cent in 2023 with an increase compared to previous years (Anonymous, 2023f).

The values of the share of exports of agricultural products produced in Gaziantep province in Türkiye's total exports are given in Table 6. When analyse the table, the most striking export product among animal products is animal and vegetable oils. This is followed by fleece and its products, milk, oil, honey and foods of animal origin. When we consider the export products of vegetable origin, it is seen that the most exported products are cereal products and milling products. As mentioned above, the food factories in our province are known as important trade centres at national and international level. This item is among the most exported products in the export of agricultural products of our country. Again, this situation is explained by the fact that our province stands out in animal husbandry activities and the industrial establishments it has within it. In addition to all this information, according to the data of Gaziantep Provincial Directorate of Agriculture

and Forestry, in 2023, 157.244.060 eggs were exported to Syria, United Arab Emirates, Qatar, Gambia, Liberia, Somalia and Taiwan; 2.738,111 kg of chicken meat and products were exported to Iraq, Libya, Canada, Somalia and Mauritius; 1.218.012 kg of milk and dairy products, 6.996 kg of honey and beekeeping products to the United Kingdom and Cape Verde, 575.806 kg of fishery and aquaculture products to Iraq, 120 breeding sheep and goats to Azerbaijan, 1.798.780 poultry (chicks) to Syria and Georgia.

Again, the distribution of total animal-based exports is shown in Figure 2. The highest product among animal export items in Gaziantep province is eggs with 47%, followed by feed and additives 250 with 15%, milk and dairy products with 12%, and live animal exports with 11%.

The GDP values for 2021 in our province were realised as 5.53 billion TL in crop production and 3.06 billion TL in animal production, and their ratios to the country production were 1.81% and 1.28% in the same

Table 5. Products of animal origin exported from Türkiye (Thousand US$) (Anonymous, 2023f).

Animal Chapters	**2021**	**2022**	**2023**
Live Animals	106.121,167	129.006,111	1.195.976,533
Meats and offal	878.930,223	1.148.986,167	358.424,752
Milk products, eggs, honey, other edible animal Origin products	858.579,585	977.568,721	171.689,751
Other animal Origin products (bone, horn, intestine, etc.)	98.416,503	102.998,345	69.938,904
Animal And vegetable thick and liquid oils	2.080.808,276	3.538.967,264	3.215.508,838
Seafood	191.289,471	267.589,947	297.523,531
Raw hides, skins (furs) excluding) and leathers	253.123,288	259.994,102	315.505,244
Leather products (bags, home and shush belongings etc.)	413.276,875	513.244,892	623.105,932
Silk	5.924,363	1.677,082	26,646,269
Fleece, wool, hair, horsehair thread and woven Textiles	87.870,725	88.647,062	350.774.11
Total	**4.974.340,476**	**7.028.679,693**	**6.625.093,864**
Total agricultural in exports share (%)	**21.69**	**26.26**	**23.03**
Vegetable Chapters			
Renewed vegetables and some coke and tubers	1.748.932.455	2.107,567,347	1,266,183,719
Renewed fruits and hard -shelled fruits	5.367.314,161	4.943,155,761	1.263.038.136
Coffee, tea, Paraguayan tea and spice	271.280,419	285.673,271	620.241,884
Cereals	416.431,868	688.542,028	5.075.073,645
Milling products	1.439.693,364	1.900.506,528	244.484,759
Fatty seed and fruits	579.045,559	701.423,33	2.990.011,379
To be knitted convenient vegetable substances	33.306,758	38.279,01	14.940,663
Cereals, flour, starch or milk preparations, pastry products	2.168.272,512	2.626.928,718	2.668.983,962
Vegetable and from fruits in hand said preparations	2.695,915,469	3.079.430,505	205.150,690
Renewed various food preparations (coffee and tea extracts, etc.)	877.811,679	1.040.755,627	887.893,957
Cotton, cotton thread and cotton Textiles	2.265.699,974	2.263.889,736	2.746.157,883
To weave convenient other vegetable fibres, paper thread and paper from the thread	65.394,285	65.713,807	290.980,917
Total	**17.929,098,503**	**19.741.865,668**	**21.594.727,329**
General Total	**22.903.438,979**	**26.770.545,361**	**28.769.679,330**
Türkiye export values	**225.214.458,038**	**254.169.747,663**	**255.437.723,543**
Agricultural of exports total in exports share (%)	**10.17**	**10.53**	**11.26**

Table 6. Share of Gaziantep province in animal products exported in Türkiye per chapter (%).

Chapters	2021	2022	2023
Live Animals	15.70	14.57	10.91
Meats and offal	0.51	0.50	0.94
Milk products, eggs, honey, other edible animal Origin products	4.98	4.79	2.05
Other animal Origin products (bone, horn, intestine, etc.)	2.77	2.20	2.94
Animal and vegetable thick and liquid oils	30.08	21.75	19.71
Seafood	2.11	2.71	1.75
Raw hides, skins (furs) excluding) and leathers	0.09	0.02	0.02
Leather products (bags, home and shush belongings etc.)	0.23	0.29	0.40
Silk	0.40	4.95	2.46
Fleece, wool, hair, horsehair thread and woven Textiles	5.94	4.27	4.70
Renewed vegetables and some coke and tubers	1.19	0.85	0.67
Renewed fruits and hard -shelled fruits	1.90	1.83	1.22
Coffee, tea, Paraguayan tea and spice	2.76	2.84	2.80
Cereals	3.66	1.28	26.50
Milling products	22.34	19.10	19.31
Fatty seed and fruits	13.81	14.30	9.05
To be knitted convenient vegetable substances	0.85	2.67	4.52
Cereals, flour, starch or milk preparations, pastry products	40.61	41.96	40.36
Vegetable And from fruits in hand said preparations	9.64	12.78	6.99
Renewed various food preparations (coffee and tea extracts, etc.)	11.58	11.39	1.12
Cotton, cotton thread and cotton textiles	12.24	9.44	6.56
To weave convenient other vegetable fibres, paper thread and paper thread	20.83	18.00	16.15

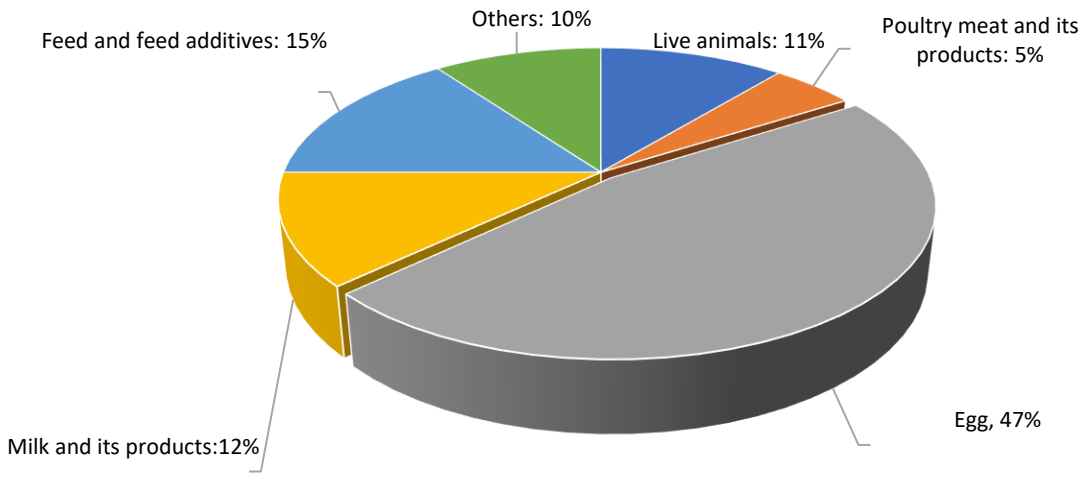

Figure 2. Distribution of export shares of animal products in Gaziantep province (%)

order, and the GDP per capita was reported as $8.725. Gaziantep, which boasts a rich cuisine that has been recognised by UNESCO, has undergone significant development in terms of industry, fertile soils, employment opportunities, and its contributions to the national economy. Strategically positioned as a trade gateway to Asian countries, Gaziantep is a frequently mentioned city. According to the Industrial Registry Information System (IRIS), 2.65% of all industrial enterprises, 4.82% of large-scale enterprises and 3.21% of medium-scale enterprises operate in our province. An examination of the sectoral distribution of enterprises operating within the province reveals that the manufacture of textile products occupies the pre-eminent position (21.75%), the manufacture of food products ranks second (18.09%), and the manufacture of leather and leather products is positioned third (Anonymous, 2023f).

In the context of academic research conducted within our nation, the contributions of agricultural activities to the national economy have been documented (Kayabaş, 2016; Semerci, 2019; Karakaya, 2023; Yanar and Savrun, 2023). It is evident that these contributions are commensurate with the impact of factors such as agricultural patterns, geographical location, and the industrial potential of the province or region. It is imperative that each study is addressed within its respective region. In this study, Gaziantep province was analysed. This province has a developed industry, large agricultural lands and high production potential. A comparison of the results with those obtained in other studies reveals numerous similarities (Mundan *et al*., 2017; Semerci, 2018; İstikbal, 2022; Kuzu, 2022).

Conclusion

Agriculture has been the main source of livelihood for societies throughout human history and has played a critical role in the development of civilisations. Even in modern economies, the agricultural sector is vital for economic growth, employment and sustainable development. In this study, which investigated animal husbandry in agricultural production in Gaziantep province, it can be said that the production potential is much higher than the current production. Decisions in favour of animal husbandry should be taken within agricultural policies to protect and develop the existing ones and to encourage new formations. It is important to establish the necessary standards for the expansion of trade in foreign sales and to make it widespread.

In conclusion, livestock production is an indispensable component to increase the productivity of agricultural production, improve the livelihoods of rural populations and achieve sustainable development goals. In the future, innovative approaches and the development of integrated agro-industrial systems will be essential to create an agricultural sector that is both economically and environmentally sustainable. In this framework, shaping agricultural policies with an understanding that emphasises and supports the importance of animal husbandry will contribute to ensuring food security and rural development throughout the country.

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Author Contributions

Sabri GÜL: Conceptualization, Data Curation, Formal Analysis, Investigation, Methodology, Visualization and Writing -original draft; Fatih ALTUNSES: Data Curation, Methodology, Visualization, Writing -review and editing.

Conflict of Interest

“The author(s) declare that they have no known competing financial or non-financial, professional, or personal conflicts that could have appeared to influence the work reported in this paper.”

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LIVESTOCK STUDIES

RESEARCH ARTICLE

Effects of Electrical Conductivity, Activity Level, Age and Lactation Number on Milk Yield in Dairy Cows during the Lactation Period

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Abstract

This study aimed to evaluate the relationships between milk yield and variables such as milk electrical conductivity, animal activity, age, and lactation number. The study was based on 2022 data from Holstein cows on a private dairy farm located in the Karapınar district of Konya, Türkiye. Cows were fed a total mixed ration with a roughage-to-concentrate ratio of 55:45 and 17.5% crude protein. Data were obtained from the farm's computerized herd management system, which recorded daily data on milk yield, milk conductivity, and animal activity for each cow. Pearson correlation analysis and one-way analysis of variance (ANOVA) were used to analyze the data. A significant positive correlation was found between milk yield and both age ($r = 0.353$, $P<0.01$) and lactation number ($r = 0.269$, $P<0.01$). However, the correlation between milk conductivity and milk yield was weak and not statistically significant ($r =-0.086$). ANOVA results revealed that milk yield differed significantly among age groups ($P<0.001$), with cows aged 4–5 years producing more milk than younger cows aged 1–3 years. These findings suggest that age and lactation number significantly affect milk yield, whereas milk conductivity and animal activity are not directly related to production. Although these factors may not directly influence milk yield, the results can provide a useful basis for developing herd management strategies that support animal health, economic outcomes, and farm efficiency. Future research involving different breeds or production systems could further validate these findings.

Introduction

Dairy cattle breeding plays a strategic role not only in contributing to global human nutrition but also in the sustainability of agricultural economies. Milk yield is shaped by the interaction of multifaceted factors such as genetic potential, nutritional levels, environmental conditions, and management practices. The genetic structure is associated with production traits like feed intake, body weight, and milk yield, and significant genetic correlations have been reported among these traits (Veerkamp and Brotherstone, 1997). Adequate and balanced provision of the nutrients required during lactation directly impacts the physiological performance of the animals (NRC, 2001). Particularly, heat stress caused by high environmental temperatures can lead to significant losses in milk yield and physiological imbalances in high-producing cows due to increased metabolic heat production (Kadzere *et al*., 2002). Furthermore, recent studies have highlighted the impact of global warming on herd health and productivity, stressing the need for adaptive management strategies to mitigate these effects (Arslan *et al*., 2024; Erzurum, 2024). In this context, it is essential to address these factors with a holistic approach to optimize milk production.

The effects of age and lactation number on milk yield have been widely studied in dairy cattle literature. It has been reported that milk yield peaks during the 3rd and 4th lactation periods, but declines are observed from the 5th lactation onwards (Vijayakumar *et al*., 2017). However, the relationship between age and milk

yield is complex, with some studies indicating an increase in yield with age, but a plateau or decline after a certain age (Kerslake *et al*., 2018). These findings emphasize the importance of considering age and lactation number to optimize milk yield.

The relationship between milk electrical conductivity, animal movement, and milk yield has not been clearly established in the literature, and different results have been obtained in various studies. Milk electrical conductivity is generally associated with udder health and is used as an indicator of infections such as mastitis (Norberg, 2005). The relationship between electrical conductivity and yield is uncertain due to the influence of non-mastitis factors such as temperature and milking intervals (Nielen *et al*., 1993). Studies on the relationship between animal movement and milk yield have yielded inconsistent results, as movement data produce inconsistent results due to both metabolic effects of activity increase and differences in measurement methodologies (Chapinal *et al*., 2011). This highlights the complexity of factors affecting milk yield, suggesting that a single biometric parameter may not be sufficient.

Electrical conductivity refers to the measurement of the electrical level between two electrolytes, typically expressed in mS/cm (miliSiemens/santimetre) (Hilerton and Walton, 1991; Johri *et al*., 2023). Milk electrical conductivity began to be used in the 1940s for mastitis evaluation (Davis, 1947). The electrical conductivity of milk is measured and recorded by most automatic milking systems together with data such as milking time and milk yield (Norberg, 2005). Such electronic measurement systems have an important place for precision technology applications (Ordolff, 2001). The desire to increase milk yield in dairy cows has led to an increase in udder problems and mastitis risk (Alaçam *et al*., 1983). Key anions and cations in milk, such as Na+ and Cl-, increase in the presence of intramammary infections (Nielen *et al.,* 1992; Bruckmaier *et al*., 2004; Norberg, 2004). Mastitis causes significant economic losses in terms of reduced milk production and quality, inability to use milk, treatment costs, and culling of cows (Vilas Boas *et al*., 2017; Guimarães *et al*., 2017; Samaraweera *et al*., 2022). The financial burden of mastitis, ranging from $100-228 per cow per year in Turkey, €240 in Germany, and €440 in Canada, has led to increased research in this area (Aghamohammadi *et al.*, 2018; Firth *et al.*, 2019; Sarıözkan, 2019). This impact has prompted the replacement of human observers with automatic milking systems (Inzaghi *et al*., 2021). As a result, a focus has shifted to milk electrical conductivity, a cheaper and more practical method (Mottram *et al*., 2007).

Electrical conductivity is also influenced by factors such as lactation number, udder lobe, milking intervals, milk composition, estrus, and nutritional levels (Nielen *et al*., 1992; Norberg, 2005; Špauskas *et al*., 2006). Prolonged milking intervals increase conductivity, while increased milk fat reduces it (Atasever and Erdem, 2008). Furthermore, it has been reported that conductivity values in front udder lobes are lower than those in rear udder lobes (Cavero *et al*., 2006).

In a healthy cow, the electrical conductivity of milk at 25°C ranges from 4-5.5 mS/cm, values up to 6.1 mS/cm are considered normal, and values above 6.2 mS/cm are considered abnormal (Nielen *et al*., 1992; Špauskas *et al*., 2006; Çelik, 2020). However, it has been noted that milk with conductivity above 6.2 mS/cm is not always indicative of mastitis, as the value of colostrum may exceed 6.2 mS/cm (Çelik, 2020). High-temperature milk (38°C) has been shown to increase electrical conductivity levels (Norberg, 2005). Furthermore, when electrical conductivity exceeds 6.5 mS/cm, and the difference between udder lobes exceeds 1 mS/cm, it indicates the likelihood of disease (Janzekovic *et al*., 2009). An increase in conductivity by 1 mS/cm corresponds to a 0.88 kg/day decrease in milk yield (Nielen *et al*., 1993).

The aim of this study is to investigate the effects of factors such as age, lactation number, animal movement, and milk electrical conductivity on milk yield, as well as their interactions, using the 2022 data from a dairy cattle farm in Konya province. The study aims to provide new insights on how these factors can be managed to enhance productivity in dairy farming.

Materials and Methods

This study was carried out at a private dairy cattle farm in the Karapınar district of Konya province. The material of the study consisted of the 2022 lactation period data obtained from the computer-based herd management program for 170 Holstein cows of different ages at the farm. The dataset included individual records of milk yield, age, lactation number, milk conductivity, and animal movement for all animals. All animals included in the study were subjected to the same feeding and management protocol. The cows were housed in modern free-stall barns and fed twice daily, in the morning and evening, ad libitum with a total mixed ration (TMR) diet. Access to clean drinking water was provided continuously through automatic float-controlled waterers.

The composition of the ration used in the feeding included wheat straw, dry alfalfa, alfalfa silage, corn silage, barley, canola, carrots, urea, molasses, yeast, vitamin-mineral premix, salt, marble powder, bypass fat, toxin binder, and concentrated milk feed. The ration was formulated according to NRC (2001), with a roughage/concentrate ratio of 55%/45% and a protein level of 17.5%.

There were no restrictions based on age and lactation number for the animals included in the study. The age distribution varied from 1 to 9 years. Lactation numbers ranged from the 1st to the 7th lactation. Milk yield, milk conductivity, and animal movement data were measured and recorded daily via transponders attached to the animals and the computerised milking

system. These data were periodically transferred to the farm management software for analysis. In the study, 65 cows underwent two milkings per day, and 105 cows were milked three times daily. The sample size for statistical analysis was determined after excluding any outlier data.

Statistical analyses

In this study, the relationships between milk yield and age, lactation number, milk conductivity, and animal movement were investigated using correlation and variance analysis. The data were analyzed using IBM Corp. (2012) SPSS Statistics software (v.21). The normality of the data was tested using the Shapiro-Wilk test. Data that did not show normal distribution were excluded from the study. For data that followed a normal distribution, the relationships between milk yield and age, lactation number, milk conductivity, and animal movement were determined using Pearson's correlation coefficient (r). Differences in milk yield among groups were evaluated using one-way analysis of variance (ANOVA). Statistical significance of the differences between groups was tested using the Duncan test. Statistical significance was considered at $P < 0.05$.

Ethical approval for the study was granted by the Ethics Committee of the Experimental Animal Production and Research Center (SUVDAMEK), Faculty of Veterinary Medicine, Selcuk University (Decision No: 2025/99).

Results and Discussion

The descriptive statistics for the dairy cows evaluated in this study are presented in Table 1. The average age of the cows was 3 years, with an average lactation number of 2, a daily average step count of 115 steps, an average milk yield of 10.836 kg throughout the lactation period, and an average milk electrical conductivity of 6.5 mS/cm. Additionally, the incidence of mastitis cases confirmed by the farm's veterinarian was calculated as 9.38%. In the study by Dağ and Zülkadir (2024), milk electrical conductivity was reported to be lower than in this study (6.12±0.097 mS/cm).

The findings of the study reveal the relationships between milk yield and age, lactation order, animal movement and milk conductivity (Table 2). It was observed that these findings were largely consistent with previous studies, but presented some new findings. According to the results of the correlation analysis, a positive and significant relationship was found between age and milk yield ($r = 0.353$, $P<0.01$). Particularly, cows aged between 4 and 5 years exhibited higher milk yields (Table 3). This finding

Table 1. Descriptive statistics of the traits in the dairy cows used in the study (n=170).

Variables	Mean	Std. Error	Minimum	Maximum	Std. Deviation
Age (years)	3.14	0.10	1	9	1.31
Lactation number	1.95	0.85	1	7	1.10
Activity (steps/day)	114.69	2.56	61	334	33.42
MEC (mS/cm)	6.5	0.09	4.5	10.5	1.13
Lactation milk yield (kg)	10836.04	202.36	4642	19759	2630.70

Abbreviations: MEC (mS/cm); milk electrical conductivity (miliSiemens/santimetre).

Table 2. Correlations among dependent variables in the dairy cows used in the study (n=170).

Variables	Age	Lactation number	Activity	MEC	Lactation milk yield
Age	1				
Lactation number	.914**	1			
Activity	-.115	-.116	1		
MEC	.164*	.055	-.032	1	
Lactation milk yield	.353**	.269**	-.085	-.086	1

*Correlation is significant *P* <0.05 level

**Correlation is very significant *P* <0.01 level.

Abbreviations: MEC; milk electrical conductivity.

indicates that age has a significant impact on milk yield, which is a commonly emphasized factor in the literature. Wilmink (1987) similarly reported a noticeable increase in milk yield as age increased. Physiological changes that occur with age (e.g., more developed udders and more mature metabolic systems) are thought to positively affect milk yield. Moreover, the cows in this age group have a longer capacity for milk production, which is important for producers to optimize efficiency.

Another finding from the analysis was that there is a positive and significant relationship between age and milk electrical conductivity ($r = 0.164$, $P < 0.05$). Timurkan (2004) found no difference in electrical conductivity values of animals from the same breed, with values exceeding 5.6 mS/cm, and noted that electrical conductivity increased with age. However, Özdemir and Kaymaz (2013) found that the lactation period, age, and mammary lobe differences did not significantly affect electrical conductivity. Sheldrake *et al*. (1983) reported that as lactation number increases, there is a corresponding rise in milk conductivity and somatic cell count, which may be associated with intramammary infections such as mastitis. Similarly, Inzaghi *et al.* (2021) observed that differences in electrical conductivity between udder quarters become more evident after the third lactation, possibly due to a history of mastitis in older cows. When evaluated together, these findings suggest that the variations in milk conductivity observed with increasing lactation number may be linked to previous udder infections.

The effects of lactation number on milk yield are a debated topic in the literature. In this study, a positive relationship was found between lactation number and milk yield ($r = 0.269$, $P<0.01$). An increase in milk yield was observed as lactation number progressed. This finding is consistent with some studies (Özçelik and Arpacık, 2000; Erzurum and Kayar, 2024). As lactation progresses, cows are expected to become more mature and produce milk more efficiently. However, some studies have reported a decrease in milk yield after the 3rd to 5th lactations (Tekerli *et al*., 2000; Ekşi and Kurt, 2021). The absence of a decrease in milk yield after the 4th lactation in this study suggests that herd management and feeding strategies may influence this result. Bayrakdar *et al*. (2024) reported that lactation number affected electrical conductivity, daily milk yield, and milking duration, and similar to the present study, older cows had higher conductivity and milk yield.

The relationship between animal movement and milk yield has not been clearly established in the literature. In this study, no statistically significant relationship was found between animal movement and milk yield ($P >0.05$), and it was observed that animal movement had no direct effect on milk yield (Table 3). Results regarding the relationship between animal movement and milk yield in the literature may be contradictory. Some studies have reported a positive relationship between cow mobility and milk yield. For example, a study by Adamczyk *et al.* (2011) found a significant relationship between cows' 24-hour walking activity and milk yield. This study showed that more active cows had higher milk yields. However, in this presented study, no direct relationship between animal movement and milk yield was found. This could suggest that environmental factors may influence the cows' behavior, but these changes do not necessarily reflect in milk yield. Additionally, the mobility of animals could increase their stress levels, which could negatively affect milk yield. Therefore, further in-depth and long-term studies are needed to better understand the relationship between animal movement and productivity.

In this study, a weak and statistically insignificant relationship was found between milk conductivity and milk yield ($r =-0.086$). This suggests that there is no direct connection between milk conductivity and milk yield. Many studies have focused on the early detection of mastitis and udder health parameters rather than the direct relationship between electrical conductivity and milk yield (Hamann and Zecconi, 1998; Norberg *et al.,* 2004; Goodling *et al*., 2000; Rogers, 2002; Tatlısu and Zulkadir, 2024). Vilas Boas *et al.* (2017) reported a positive relationship between somatic cell count and electrical conductivity in dairy Zebu cows (Dairy Gyr), noting that somatic cell count increased for values between 4.81-5.00 mS/cm and that these values could indicate mastitis formation in dairy cows. In contrast, Kurt and Kaygısız (2024) found no significant difference in electrical conductivity among Red Holstein, Black Holstein, and Simmental breeds, and concluded that electrical conductivity cannot be used to detect mastitis. Another study by Timurkan (2014) also reported that there was no correlation between California Mastitis Test (CMT) results and electrical conductivity values.

The results of variance analysis showed that significant differences in milk yield were observed in age groups, with the 4-5 years and older groups having significantly higher milk yields compared to the 1-3 years group (12047-12301 kg; 10300 kg; $P<0.001$). Similarly, analyses based on lactation number showed that as lactation number increased, milk yield also increased. Milk conductivity and animal movement did not have significant effects on milk yield, in line with the correlation analyses (Table 3).

The findings also reveal the impact of environmental and management factors on milk yield. For example, environmental factors such as temperature and humidity can affect milk yield in cows. Additionally, herd management practices and feeding strategies can enhance milk yield in cows, particularly during lactation, which may explain the increases in milk yield observed in this study.

Table 3. Effects of fixed factors on lactation milk yield in dairy cows (Mean ± SE).

Fixed effects	N	Lactation Milk Yield (kg) Mean ± SE	*P*- value
Age (years)			
1 - 3	123	10300.919 ± 218.621 [b]	**0.000**
4 - 5	35	12047.500 ± 340.144 [a]	
≥6	12	12301.229 ± 1211.171[a]	
Lactation number			
1	72	9727.542 ± 260.940 [b]	**0.000**
2	56	11512.625 ± 323.467 [a]	
≥3	42	11814.191 ± 450.974 [a]	
Activity (steps/day)			
≤100	67	10808.060 ± 312.314	0.097
101 - 126	57	11009.421 ± 326.752	
126 - 151	24	11561.375 ± 583.497	
≥150	22	9680.727 ± 611.380	
MEC (mS/cm)			
4 - 5.6	33	10776.667 ± 522.027	0.989
5.6 - 6.2	42	10834.714 ± 412.747	
≥6.2	95	10857.242 ± 258.173	

Note. Different superscript letters (a, b) within a column indicate significant differences (P <0.05).

Conclusion

This study examined the relationships between milk yield and factors such as age, lactation number, animal movement, and milk conductivity. The findings indicate that age and lactation number have significant effects on milk yield. In particular, cows aged between 4 and 5 years were observed to produce higher milk yields. However, the results also show that milk conductivity and animal movement are not directly associated with milk yield. Nevertheless, although these factors do not have a direct impact on yield, they may serve as valuable tools for herd management and the development of producer strategies. In this context, implementing specialized care and feeding programs for cows aged 4–5 years could help optimize milk production. The lack of a direct relationship between milk conductivity and animal movement with milk yield suggests that these parameters should primarily be used for health monitoring purposes. Further studies involving different breeds and larger sample sizes are recommended to reassess the relationship between these parameters and milk yield. Additionally, the effects of animal welfare and environmental factors on milk production should be explored in more detail.

Conflict of Interest

The authors declare that there are no conflicts of interest related to this publication. No financial support that could have influenced the outcome of the study has been received. The manuscript has been read and approved by all listed authors, and there are no individuals who meet the authorship criteria but are not included.

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RESEARCH ARTICLE

LIVESTOCK STUDIES

Identification of Candidate Genes Associated with *Eimeria* spp. Oocyst Load in Central Anatolian Merino Sheep

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Abstract

Coccidiosis caused by *Eimeria* spp. is a significant protozoal disease impacting the health and productivity of sheep and other livestock species. Host resistance to coccidiosis exhibits considerable individual variation, suggesting a genetic basis for susceptibility and resilience. This study aimed to identify genomic regions associated with oocyst load of *Eimeria* spp. in sheep using a genome-wide association study (GWAS) approach. A total of 226 sheep were phenotyped for oocyst counts using a standardized flotation technique. Genotyping was performed using a 50 K high-density SNP array. Quality control measures included filtering for minor allele frequency, call rate, and Hardy-Weinberg equilibrium. GWAS analysis was conducted using a mixed linear model accounting for relatedness among individuals. Significant associations were identified on chromosomes 1, 8 and 20. Candidate genes mapped to these regions included *PARK2*, *PACRG*, *QKI*, *PDE10A*, *RAB44*, and *CDKN1A*, which are involved in mitochondrial quality control, cellular stress response, immune modulation, and epithelial integrity maintenance. These biological functions are critical for host defence mechanisms against protozoal infections such as coccidiosis. This study reveals novel candidate genes and biological pathways potentially influencing coccidial oocyst load in sheep. These findings contribute to the understanding of host genetic resistance to Eimeria infections and may inform future breeding strategies in sheep.

Introduction

Coccidiosis, caused by protozoan parasites of the genus Eimeria, remains a major health challenge in pasture-based sheep production systems, manifesting in weight loss, diarrhea, growth retardation, and increased mortality, ultimately compromising animal welfare and farm profitability (Reeg *et al.*, 2005; Arzik *et al.*, 2022). The disease incurs both direct economic losses, such as reduced productivity and increased mortality among young animals, and indirect costs arising from medical interventions and preventive treatments. Moreover, intensive reliance on anticoccidial agents has contributed to the emergence of drug-resistant Eimeria strains, urging the need for alternative, sustainable control strategies (Karshima, 2018; Liu *et al.*, 2024).

Genetic variation among individual sheep in susceptibility to coccidiosis has been widely reported, suggesting the possibility of improving resistance through selective breeding programs (Windon, Dineen and Wagland, 1987; Gul *et al.*, 2023). Recent breeding efforts have increasingly integrated health-related traits, including parasite resistance, into selection indices to enhance herd resilience and reduce dependence on chemical treatments. Understanding the genetic basis of resistance to Eimeria infections

could thus provide critical insights for developing sustainable control strategies (Gül *et al.*, 2020; Behrem and Gül, 2022; Hayward, 2022).

The immune response to Eimeria infection involves complex interactions between innate and adaptive immunity. Physical barriers, mucosal immune defences, and the activation of antigen-presenting cells play critical roles in mounting an effective defence (McGuckin *et al.*, 2011; Chen *et al.*, 2025). Genetic factors influencing immune cell signalling, epithelial integrity, and inflammation modulation are believed to contribute substantially to individual variation in resistance (Jäger *et al.*, 2014; Sabri *et al.*, 2018; Meningher *et al.*, 2020).

Advancements in high-throughput genotyping technologies and the availability of dense genome-wide SNP panels have enabled the use of Genome-Wide Association Studies (GWAS) to dissect the genetic architecture underlying complex traits such as parasite resistance (Zhu *et al.*, 2020; Arzik *et al.*, 2025). GWAS approaches allow the identification of candidate genomic regions and biological pathways associated with disease traits, offering opportunities for marker-assisted selection (Gül *et al.*, 2016; Arzik *et al.*, 2022).

In this context, the present study aimed to uncover the genetic basis of resistance to Eimeria infections in sheep by performing GWAS on naturally infected lambs under pasture conditions. By associating oocyst burden with genome-wide SNP data, we sought to identify candidate genes and molecular pathways involved in host resistance to coccidiosis.

Materials and Methods

Study Population and Phenotyping

The study was conducted in sheep flocks located in the outskirts of Ankara province, Türkiye, characterized by a continental climate with cold winters and hot, dry summers. The region experiences an average annual rainfall of 389 mm, an average temperature of 11.7 °C, and an altitude of approximately 938 meters. The flocks grazed on semi-arid pastures with limited nutritional quality.

A total of 226 lambs from the Central Anatolian Merino (CAM) breed, including both sexes, were randomly selected from three different farms participating in the National Community-based Small Ruminant Breeding Program. All lambs shared access to the same communal pastures without supplementary feeding. Animals were born between December 2022 and February 2023, weaned at approximately three months of age, and exposed to natural Eimeria infections during the grazing season.

Fecal samples were collected directly from the rectum of each animal between six and eight months of age (August 2023). Approximately 20-30 grams of faecal material was obtained per animal, ensuring minimal contamination. Sampling was performed at least 60 days after any antiparasitic treatment. The number of Eimeria oocysts per gram (OPG) was determined using the modified McMaster technique (MAFF, 1986) and treated as a continuous trait for subsequent analyses. Blood samples were simultaneously collected from the jugular vein for genotyping purposes.

Environmental covariates such as sex, farm, birth type, and age at sampling were recorded for inclusion in the statistical models.

DNA Extraction, Genotyping, and Quality Control

Genomic DNA was extracted from whole blood samples using an automated extraction system (Qiacube HT, Qiagen Blood kit, Hilden, Germany) following the manufacturer's protocol. DNA concentration and purity were assessed using spectrophotometry (A260/280 > 1.8; A260/230 > 1.5), and samples exceeding 70 ng/µl with high integrity were selected for genotyping.

Genotyping was conducted using the OvineSNP50 BeadChip array (Illumina Inc., San Diego, CA, USA) on the iScan platform. SNP quality control was performed by excluding markers with a minor allele frequency (MAF) < 0.05, call rate < 95%, or deviation from Hardy-Weinberg equilibrium (adjusted by Bonferroni correction at $P < 0.05$/number of SNPs). Samples with a genotype call rate < 90% or excessive relatedness (Identity-by-State [IBS] > 95%) were removed from the dataset.

Statistical Analyses

Genome-wide association analyses were performed using a Mixed Linear Model (MLM) approach implemented in the GenABEL R package (Aulchenko *et al.*, 2007). The model accounted for fixed environmental effects (sex, farm, age) and genetic relatedness among individuals using a genomic relationship matrix (Astle and Balding, 2009).

The statistical model was as follows:

$$Y = \mu + X\beta + Zu + e$$

Where y represents the vector of phenotypic observations (oocyst load), µ is the overall mean, β is the vector of fixed effects and SNP effects, u denotes the vector of random additive genetic effects ($u \sim N(0,\sigma^2_u G)$), and e is the vector of random residual errors $e \sim N(0,\sigma^2_e)$. X and Z are incidence matrices relating observations to fixed and random effects, respectively.

Quantile-Quantile (QQ) plots and Genomic Control (Devlin and Roeder, 1999) were used to assess the inflation of test statistics. Bonferroni-corrected

thresholds for genome-wide and chromosome-wide significance were applied to account for multiple tests.

Functional Gene Annotation

Significant SNPs were mapped to nearby genes using the Oar_v4.0 reference genome via the NCBI Genome Data Viewer (Rangwala *et al.*, 2021). Candidate genes were annotated using DAVID Bioinformatics Resources (Huang, Sherman and Lempicki, 2009), supplemented with orthologous information from cattle, goat, mouse, and human genomes when necessary. Gene Ontology (GO) terms were used to elucidate the biological processes and pathways associated with candidate genes.

Results

The outliers in the oocyst per gram (OPG) counts for *Eimeria spp*. were detected and eliminated from the dataset, yielding a mean oocyst count of 3,115 ± 263 per gram. To achieve normality and stabilize variance, oocyst counts were subjected to log10-transformation prior to statistical analyses. Comprehensive details regarding the phenotype data are provided in Table 1. Initially, the raw genotype dataset consisted of 61,465 SNPs for 227 animals. Following quality control procedures applied to the genotypic data, 44,871 SNPs and 226 animals were retained for further analysis.

Through genome-wide association analyses, 4 significant SNPs were identified, with 2 surpassing the chromosome-wide threshold and 2 suggestive significance. The names of these SNPs and the chromosomes they are located on, along with more detailed information, are presented in Table 2.

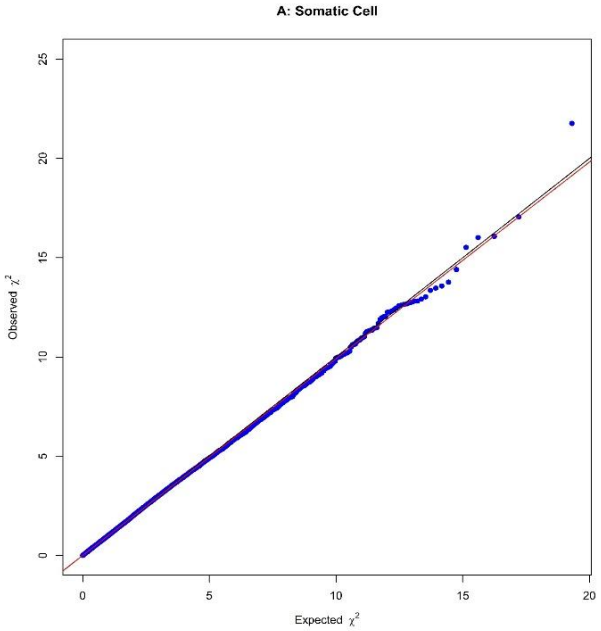

Figure 1 Quantile-quantile (Q-Q) plots of genome-wide association studies (GWAS) for the traits

Table 1. Descriptive statistics for animal age at recording (days), and fecal oocyte counts for the observations used.

Traits	N	Mean	SE Mean	Min[a]	Max[b]	SD[c]	C.V (%)[d]
Fecal oocyte count	226	3,115	263	0	28,957	3957	1,27
Age at fecal oocyte counting	227	200	2,59	124	274	39	0.19

aMin: minimum, b Max: maximum, cSD: standard deviation, dC.V: coefficient of variation.

Before the GWAS analysis, a linear mixed model was employed to assess the effects of fixed factors. Results indicated that sex, herd, and age (in days) significantly influenced log-transformed oocyst count. Consequently, these factors were incorporated into the GWAS models. Notably, herd and birth month were found to exert statistically significant effects, warranting their inclusion in subsequent analyses.

Quantile-quantile (Q-Q) plots of observed test statistics for each SNP were compared with those expected under the null hypothesis, as depicted in Supplementary Figure 1. The Q-Q plot and the estimated inflation factor lambda (λ) were obtained for the phenotype, with genomic control applied to normalize the data. Corrected p-values for each trait were derived from the GWAS, and Manhattan plots illustrating these results are provided in Figure 2.

In terms of functional annotation, based on positional information obtained from the NCBI Genome Data Viewer using the OAR_v4.0 assembly, all of these SNPs were directly associated with specific genes. Among these, several candidate genes of notable biological relevance were identified. *PARK2* (*Parkin RBR E3 Ubiquitin Protein Ligase*), involved in mitophagy and mitochondrial quality control, may contribute to cellular homeostasis under protozoan infection-induced stress. Similarly, *PACRG* (*Parkin Co-Regulated Gene*) plays a role in cytoskeletal organization and inflammatory responses, which are critical for maintaining epithelial integrity during parasite challenge.

QKI (*Quaking Homolog, KH Domain RNA Binding*), a regulator of RNA stabilization and epithelial barrier function, and *PDE10A* (*Phosphodiesterase 10A*), an enzyme controlling cAMP and cGMP intracellular

signalling pathways important for immune modulation, were also among the associated genes.

Furthermore, *RAB44* (*Member RAS Oncogene Family*) was implicated, a gene involved in vesicle trafficking and lysosomal function, which may be essential for intracellular parasite clearance. Finally, *CDKN1A* (*Cyclin-Dependent Kinase Inhibitor 1A, also known as p21*), a critical regulator of cell cycle arrest and cellular stress response, was associated with oocyst burden, suggesting a potential role in epithelial regeneration and immune defence during coccidial infection. In addition, a significant association was observed near *PCOLCE2* (*Procollagen C-Endopeptidase Enhancer 2*), a gene implicated in extracellular matrix remodelling and collagen maturation. Given the critical role of epithelial structural integrity in resistance against *Eimeria* infections, *PCOLCE2* may contribute to maintaining mucosal barrier function during the protozoan challenge. Further detailed information regarding the significant SNPs and associated genes is provided in Figure 2 and Table 2.

Experimental evidence from murine models demonstrates that Rab44 regulates granule exocytosis in mast cells and controls the release of lysosome-derived vesicles via interaction with vesicle-associated membrane protein 8 (VAMP8) (Kadowaki *et al.*, 2020). Given that *Eimeria* spp. heavily disrupt epithelial integrity and induce intracellular stress, the ability to efficiently manage lysosomal exocytosis may be critical for limiting epithelial damage and promoting the removal of infected or damaged cells. The functional localization of Rab44 to lysosomes and its role in promoting lysosomal secretion suggest that it may contribute to enhanced epithelial turnover and immune-mediated clearance during *Eimeria* infections (Noguromi *et al.*, 2023).

Moreover, recent reviews underscore the broader role of Rab44 and other Rab GTPases in immune cells, particularly in macrophages and lymphocytes, where they regulate phagocytosis, antigen presentation, and inflammatory responses (Moreno-Corona *et al.*, 2024). Notably, Rab44 expression has been associated with macrophage differentiation and cytokine production

Table 2. Significant SNPs associated with the *Eimeria* spp. fecal oocyte count.

SNP name	rs id	Chr.	Position (bp)[a]	P-value	Significance level	Associated genes	
						Name	Distance (bp)
s56894.1	rs429137901	8	84419597	$3.07x10^{-06}$	CW	*PARK2*	Within
						PACRG	~250 Kb
						QKI	~1000 Kb
OAR23_17139338.1	rs413019325	20	10643029	$3.63x10^{-05}$	*CW*	*CDKN1A*	Within
s43112.1	rs414685283	1	244063117	$6.09x10^{-05}$	*Suggestive*	*PCOLCE2*	Within
OAR6_116928489.1	rs407386965	8	87147762	$6.28x10^{-05}$	*Suggestive*	*PDE10A*	Within

[a] SNP position based on OAR_v4.0 assembly.

On chromosome 20, *RAB44* was associated with oocyst load. RAB44 plays a role in vesicle trafficking (GO:0016192) and lysosomal function (GO:0007040) (Stenmark, 2009). Efficient lysosomal degradation pathways are essential for the clearance of intracellular pathogens, implying that *RAB44* may contribute to the innate immune clearance of *Eimeria* parasites. The identification of *RAB44* as a candidate gene associated with oocyst load in *Eimeria* infections in sheep presents novel insights into the molecular mechanisms underlying host defence against protozoan parasites. Recent findings have highlighted Rab44 as an atypical member of the Rab GTPase family, characterized by its large size and additional domains such as EF-hand and coiled-coil motifs, which enable it to modulate intracellular trafficking and lysosomal Dynamics (Okuhira *et al.*, 2011).

in response to immune stimuli, implicating it as a key modulator of innate immunity. In the context of coccidiosis, where both the innate and adaptive immune responses are essential for controlling infection and minimizing pathology, Rab44-mediated vesicular trafficking and lysosomal activity could facilitate more effective antigen processing, pathogen elimination, and epithelial regeneration.

Collectively, these findings support the hypothesis that Rab44 plays a dual role in protozoan resistance: enhancing epithelial barrier protection via regulated lysosomal exocytosis and modulating immune cell functions that orchestrate the host defence against *Eimeria* spp. infections. Future functional studies specifically targeting Rab44 in ovine models will be crucial to fully elucidate its contribution to coccidiosis

resistance (Stenmark, 2009; Kadowaki *et al.*, 2020; Noguromi *et al.*, 2023; Moreno-Corona *et al.*, 2024).

PARK2 encodes an E3 ubiquitin ligase critically

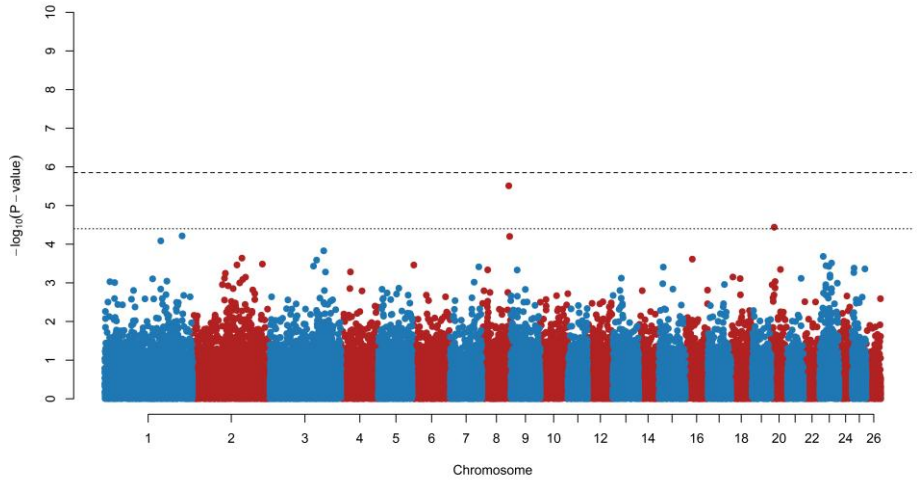

Figure 2. Manhattan plots of parasite resistance trait. The upper line indicates the genome-wide significance level, and the lower line indicates the chromosome-wide significance level. *Eimeria* spp. oocyst count at six months old.

damaged mitochondria. Protozoan infections, such as those caused by *Eimeria* spp., are known to induce cellular stress and mitochondrial dysfunction, suggesting that *PARK2* may facilitate the clearance of infected or damaged cells, thereby limiting parasite propagation and maintaining tissue homeostasis (Zilocchi *et al.*, 2020; Sun *et al.*, 2022).

Similarly, *PACRG* has been implicated in cytoskeletal organization (GO:0000226) and innate immune responses (Riva, 2024). The maintenance of intestinal epithelial barrier integrity is critical in resisting coccidial invasion, and PACRG may enhance epithelial resilience through stabilizing microtubular structures, supporting efficient repair mechanisms during *Eimeria* infection.

QKI, an RNA-binding protein that modulates mRNA stability (GO:0003723) and epithelial cell differentiation (GO:0030855), may limit the intestinal damage inflicted by *Eimeria* spp. by promoting epithelial renewal and regulating inflammatory responses (Herman and Autieri, 2017)

The identification of *PDE10A*, involved in cAMP and cGMP signalling pathways (GO:0006198; GO:0002376), underscores the importance of intracellular signalling in immune modulation. Alterations in cAMP levels can significantly affect immune cell activation and epithelial responses to infection, suggesting that PDE10A variants may modulate the host's susceptibility or resilience to coccidiosis (Koesling and Russwurm, 2015). Finally, *CDKN1A* regulates cell cycle arrest (GO:0045786) and cellular stress responses (GO:0033554) (Dutto *et al.*, 2015). During protozoan infections, the balance between epithelial cell proliferation and controlled immune activation is critical for tissue recovery, positioning *CDKN1A* as a potential modulator of epithelial regeneration during infection.

mitochondrial quality control, epithelial barrier maintenance, cellular stress responses, and immune modulation. These processes are highly relevant to the pathogenesis of coccidiosis, where epithelial disruption and inflammatory damage are hallmarks of the disease. The convergence of *PARK2*, *PACRG*, and *RAB44* in autophagy and vesicular trafficking pathways, and the involvement of *QKI*, *PDE10A*, and *CDKN1A* in inflammatory regulation, supports a multifaceted host response involving both epithelial resilience and immune defence.

Our findings are consistent with previous studies highlighting the genetic basis of resistance to gastrointestinal parasites in small ruminants (Bishop and Morris, 2007; Gül *et al.*, 2020; Arzik *et al.*, 2022), although to our knowledge, this is the first report linking these specific genomic regions and gene candidates to coccidial oocyst load in sheep.

Conclusion and Recommendations

This study performed a genome-wide association analysis to explore the genetic basis of oocyst load in sheep infected with Eimeria spp. Significant associations were identified on chromosomes 8 and 20, involving candidate genes such as *PARK2*, *PACRG*, *QKI*, *PDE10A*, *RAB44*, and *CDKN1A*. These genes are implicated in mitochondrial quality control, epithelial barrier maintenance, and immune regulation, suggesting potential mechanisms of host resistance to protozoan infections. Despite the valuable findings, the moderate sample size may have limited the ability to detect loci with small effects. Functional studies, including gene expression profiling and gene editing, are necessary to validate the roles of key genes like

PARK2, *QKI*, and *PACRG*. Furthermore, incorporating larger and genetically diverse populations, along with multi-omics approaches, would enhance our understanding of host-pathogen interactions in coccidiosis. Considering the role of humoral immunity, future research should also investigate B-cell-mediated responses. Serological assays, such as Western blot analyses using sera from naturally exposed sheep, could identify antibodies associated with resistance. These findings could support integrated breeding and vaccination strategies aimed at improving disease resilience in sheep. Overall, the study provides novel insights into the genetics of protozoan resistance and highlights opportunities for advancing selective breeding programs through functional and immunological research.

Data Availability Statement

The data presented in this study are available on a reasonable request from the corresponding author. The data are not publicly available due to the legal restriction of data deposition regarding indigenous breeds.

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Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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LIVESTOCK STUDIES

RESEARCH ARTICLE

Evaluation of Bull Semen Frozen with Different Antioxidants

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Abstract

In this project, ejaculates (n=34) were collected from three Brown Swiss bulls using the artificial vagina method, then divided into seven equal parts and diluted to 60×10^6 spermatozoa/mL using the following extenders: Control, Control+1mM carnosic acid (CR1), Control+3mM carnosic acid (CR3), Control+2.5mM glutamine (G2.5), Control+7.5mM glutamine (G7.5), Control+CR1+G7.5, and Control+CR3+G2.5. All diluted aliquots were frozen after 3 hours of equilibration. Thawed samples were analyzed for motility, plasma membrane and acrosome integrity (PMAI), mitochondrial membrane potential (MMP), and DNA integrity. The Control, G7.5, and G2.5 groups showed the highest total and progressive motility. The highest PMAI value was observed in the Control group, while the lowest was in the CR3 group. The PMAI values of G2.5, G7.5, and G7.5+CR1 were significantly higher than those of CR1, CR3, and CR3+G2.5. There were no statistically significant differences in MMP among the groups. The lowest DNA damage was observed in the Control group and the highest in CR3+G2.5. Control, G2.5, G7.5, and CR1+G7.5 groups had statistically similar kinematic values (VAP, VSL, VCL) and outperformed the others. Although 2.5 mM and 7.5 mM glutamine showed a synergistic interaction, they did not significantly improve spermatological parameters. Carnosic acid appeared to act as an antagonistic antioxidant.

Introduction

Cryopreserved bull semen in artificial insemination (AI) represents the most widely implemented biotechnological approach for genetically improving livestock worldwide. In recent years, the frozen semen industry has evolved into a globally competitive market, with intercontinental trade that surpasses even the economic potential of the meat and dairy industries.

The foundation of this industry lies in the genetic transmission potential of the bull, whose semen is to be cryopreserved, and the ability to freeze the semen under optimal conditions. Numerous studies have investigated various combinations of semen extenders and antioxidants to enhance the viability and functional quality of frozen-thawed spermatozoa. Initially, semen extenders were developed using animal-derived proteins such as egg yolk or milk powder. However, components poses significant challenges, and extenders containing such bases have a relatively limited shelf life. Moreover, semen extenders containing animal-derived proteins carry the risk of contamination with pathogenic agents, including E. coli, Staphylococcus, Streptococcus, Pseudomonas, Haemophilus, Salmonella, Avian influenza, Campylobacter, Listeria, and Mycoplasma. Therefore, semen extenders developed in recent years for cryopreservation purposes and now widely used commercially are primarily based on plant-derived protein sources (Bousseau et al., 1998; Thun et al., 2002; Crespilho et al., 2012; Layek et al., 2016; Ansari et al., 2017; Murphy et al., 2018; Gavin-Plagne et al., 2019).

During the processes of semen collection from bulls via the artificial vagina, macroscopic and microscopic evaluation, equilibration, straw filling, freezing, and thawing, exposure to oxygen and lipid

phase transitions in spermatozoon membranes may lead to osmotic and mechanical stress. These stressors contribute to an increase in reactive oxygen species (ROS), which can negatively impact the viability of frozen-thawed sperm, ultimately leading to reduced pregnancy rates (Arı and Öztürkler, 2015; Gürler et al., 2016).

Under normal physiological conditions, ROS are generated as byproducts of intracellular signal transduction and enzymatic reactions. In mammalian spermatozoa, ROS species such as hydrogen peroxide (H_2O_2), superoxide anion (O_2^-), hydroxyl radical (•OH), and hypochlorite radical (OHCl) can be generated. During cryopreservation, spermatozoa are exposed to various fluctuations in temperature, pH, and osmolarity. At low temperatures, phospholipids, proteins, and lipid molecules—abundantly present in the plasma membrane—tend to form tighter interactions. The formation of extracellular ice crystals creates a hyperosmotic environment, during which water efflux from the cell occurs through membrane macromolecules, leading to cell shrinkage. These structural disruptions also lead to the loss of cytoplasm, which is rich in endogenous antioxidants. Consequently, spermatozoa remain highly susceptible to oxidative stress during the freezing and thawing processes (Arı and Öztürkler, 2015; Gürler et al., 2016).

A physiological level of ROS in the composition of semen has been reported to contribute positively to sperm function by participating in the tyrosine-dependent cyclic adenosine monophosphate (cAMP) signaling pathway, thereby promoting hyperactivation, capacitation, and the acrosome reaction. However, excessive levels of ROS induce oxidative stress within the spermatozoon, leading to impaired motility and viability. Moreover, it has been demonstrated that elevated oxidative stress causes lipid peroxidation and damages sperm DNA integrity (Luconi et al., 2006; O'Flaherty et al., 2006; Gürler et al., 2016; Ugur et al., 2019).

Rosemary (Rosmarinus officinalis) is an evergreen shrub characterized by its needle-like leaves and distinctive purple flowers. Carnosic acid, a bioactive compound found in rosemary and extracted from its leaves, is recognized as a potent agent that inhibits tumour development in cancer therapy. Several studies have reported that carnosic acid has been added as an antioxidant to semen extenders in various animal species, including buffalo, bull, ram, deer, and pig, demonstrating beneficial effects on spermatozoon quality parameters (Malo et al., 2010; Zanganeh et al., 2013; Daghigh-Kia et al., 2014; Luño et al., 2014; Motlagh et al., 2014; Yeni et al., 2018; Gungor et al., 2019). In their cryopreservation study (Yeni et al., 2018) using epididymal buffalo semen, it was reported that the addition of carnosic acid at doses of 12.5 and 25 µg/mL to an egg yolk-based extender had a positive effect on spermatozoon motility and viability parameters.

Glutamine, a tripeptide, plays a regulatory role in numerous metabolic processes, including cell integrity (apoptosis and cell proliferation), protein synthesis and degradation, redox potential, gene expression, and extracellular matrix synthesis. During the cryopreservation process of sperm, glutamine has been reported to contribute to the preservation of the cytoskeletal structure of the plasma membrane. The addition of glutamine to semen extenders has been reported to contribute to preserving the cytoskeletal structure of the spermatozoon plasma membrane during the cryopreservation process, thereby exerting a protective effect against freezing-induced damage. Studies conducted on semen from Angora bucks, rams, stallions, and bulls have demonstrated this beneficial role (Khlifaoui et al., 2005; Amirat-Briand et al., 2009; Bucak et al., 2009a; Bucak et al., 2009b; Tuncer et al., 2011). Studies involving the supplementation of egg yolk-based extenders with varying concentrations of glutamine have shown that concentrations exceeding 10 mM negatively impact sperm motility (Amirat-Briand et al., 2009).

A review of current literature reveals that cryopreservation studies involve the addition of carnosic acid and glutamine to egg yolk-based semen extenders. However, no studies have been identified that investigate the addition of these compounds to plant protein-based, egg-yolk-free extenders, particularly in the context of extender optimization and synergistic effects. This study aimed to enhance the quality of frozen-thawed semen by using Andromed, a plant-based (soy lecithin) semen extender, in combination with varying concentrations of the antioxidants carnosic acid and glutamine, either individually or in combination. Furthermore, the project sought to determine the optimal antioxidant supplementation strategy for semen cryopreservation using plant-derived extenders.

Materials and Methods

Animals and Semen Collection

Semen samples were collected from three Brown Swiss bulls, each at least two years old, housed at the Artificial Insemination Unit of the International Center for Livestock Research and Training (Ankara, Turkey). Ejaculates were obtained twice weekly from each bull using an artificial vagina. A total of 34 ejaculates were collected during the study. Only samples with ≥ 75% progressive motility were used. Motility was assessed under a phase-contrast microscope at ×100 magnification, and sperm concentration was measured with a photometric device (Accucell, IMV). Each ejaculate was divided into seven equal parts, resulting in 238 aliquots. Dilution was performed using Andromed (Minitüb

GmbH), a soybean lecithin-based extender, to a final concentration of approximately 60 × 10^6 motile spermatozoa/mL. The extender was used alone or supplemented with antioxidants, as described below.

Semen Cryopreservation

The dilution process was carried out via a one-step method in a 37°C water bath. Samples were cooled to +4°C within 45–60 minutes using a digitally controlled cold chamber, followed by a 3-hour equilibration period. The semen was loaded into 0.25 mL straws using an automatic filler-sealer (MX4, IMV Technologies), frozen to approximately −100°C with a programmable freezer (Digital Cool 5300ZB 250; IMV), and stored in liquid nitrogen at −196°C.

Experimental Design

The experimental groups were designed by adding specific antioxidants to the control extender. The control group included only the commercial extender (Andromed) (C); 1 mM carnosic acid (C + CR1); 3 mM carnosic acid (C + CR3); 2.5 mM glutamine (C + G2.5); 7.5 mM glutamine (C + G7.5); 1 mM carnosic acid and 7.5 mM glutamine (C + CR1 + G7.5); and 3 mM carnosic acid and 2.5 mM glutamine (C + CR3 + G2.5).

Semen Evaluation

The effects of carnosic acid and glutamine applied individually or in combination with a soybean lecithin-based extender were evaluated in terms of post-thaw sperm motility and kinematic parameters, viability assessed through mitochondrial membrane potential (MMP) and plasma membrane and acrosome integrity (PMAI), and DNA integrity as a separate indicator of nuclear damage.

Sperm Motility Parameters Assessment

The motility and kinematic characteristics of individual frozen-thawed spermatozoa were analyzed using a computer-assisted sperm analysis (CASA) system (IVOS I, Hamilton Thorne, Beverly, MA, USA). Frozen semen samples were thawed in a water bath at 37°C for 30 seconds before sperm analysis. 3 µL aliquot of each sample was placed onto a pre-warmed four-chamber slide with a depth of 20 µm (Leja, IMV, France). Analyses were performed automatically by a CCD camera (30 frames/s, 60 Hz) integrated with a phase-contrast microscope at 37°C and 10X magnification. Images from at least five microscopic fields were captured and evaluated for each sample.

The CASA system recorded total motility (TM, %) and progressive motility (PM, %) as well as kinetic parameters, including average path velocity (VAP, µm/s), straight-line velocity (VSL, µm/s), curvilinear velocity (VCL, µm/s). Spermatozoa were classified as progressively motile if VSL was ≥70% and VAP was ≥50 µm/s. Based on VAP values, motility subcategories were defined as follows: static, slow (>40 µm/s), medium (>70 µm/s), and rapid (>100 µm/s).

Viability Parameters Assessment

Sperm viability was assessed using a flow cytometer (CytoFLEX System B4-R0-V0, Beckman Coulter, USA) with a 488 nm laser. Evaluations included plasma membrane and acrosome integrity and mitochondrial membrane potential.

Plasma Membrane and Acrosome Integrity (PMAI)

Plasma membrane and acrosome integrity of spermatozoa were assessed by flow cytometry using dual fluorescent staining with propidium iodide (PI) and fluorescein isothiocyanate–conjugated peanut agglutinin (FITC-PNA). For this analysis, 5 µL of semen was diluted in 241 µL of Tyrode's solution and stained with 1.5 µL of PI and 2.5 µL of FITC-PNA. After 15 minutes of incubation at 37°C, samples were analyzed. Spermatozoa were classified into four subpopulations according to their plasma membrane and acrosome status: cells with intact plasma membrane and acrosome, considered viable (PMAI, %) (PI- and FITC-PNA-negative); cells with damaged plasma membrane but intact acrosome (PI-positive, FITC-PNA-negative); cells with intact plasma membrane but damaged acrosome (PI-negative, FITC-PNA-positive); and cells with both damaged plasma membrane and acrosome (PI- and FITC-PNA-positive).

Mitochondrial Membrane Potential (MMP)

The mitochondrial membrane potential of spermatozoa was assessed by flow cytometry (CytoFLEX System B4-R0-V0, Beckman Coulter, USA) following fluorescent staining. For this purpose, 5 µL of semen was diluted in 241 µL of Tyrode's solution and stained with 1.5 µL of propidium iodide (PI) and 2.5 µL of JC-1. After 15 minutes of incubation at 37°C, samples were analyzed. After the exclusion of PI-positive cells, two subpopulations were identified among live spermatozoa according to JC-1 fluorescence: cells emitting green fluorescence, representing spermatozoa with low mitochondrial membrane potential (LMMP), and cells emitting orange fluorescence, representing spermatozoa with high mitochondrial membrane potential (HMMP).

DNA Integrity Assessment

DNA integrity was evaluated using acridine orange staining. For this purpose, 20 µL of sperm suspension was smeared onto pre-cleaned glass slides and air-dried. The dried samples were fixed with Carnoy's fixative for 1 hour, stained with acridine orange for 5 minutes in the dark, rinsed with distilled water, air-dried again, and examined under a fluorescence microscope. Sperm with green fluorescence were

considered to have intact DNA, while those with yellow-orange fluorescence were classified as having fragmented DNA. A minimum of 100 sperm per sample was counted to calculate the percentage of DNA fragmentation.

Statistical Analysis

All data were expressed as mean ± standard deviation (SD). Before statistical analysis, the data were tested for normality using the Shapiro–Wilk test and for homogeneity of variances using Levene's test. One-way analysis of variance (ANOVA) was used to compare more than two groups for spermatological parameters, and Tukey's multiple comparison test was applied to determine significant differences between groups. All statistical analyses were performed using SPSS version 22.0 for Windows (IBM, New York, USA), and differences were considered statistically significant at $P < 0.05$.

Results

The effects of carnosic acid and glutamine, used either individually or in various combinations, on sperm quality parameters are shown in Table 1. Total motility was highest in the Control, G7.5, G2.5, and CR1+G7.5 groups (50.6 ± 14.81; 48.79 ± 16.8; 48.23 ± 13.9; and 45.00 ± 13.28, respectively) ($P < 0.001$). Similarly, progressive motility was greatest in the Control (22.68 ± 6.00), G2.5 (21.02 ± 5.57), G7.5 (20.97 ± 6.40), and CR1+G7.5 (18.82 ± 5.47) groups ($P < 0.001$). The remaining groups, CR1 (17.13 ± 5.12), CR3 (16.30 ± 4.73), and CR3+G2.5 (13.48 ± 4.15), showed significantly lower values compared to the Control, G2.5, and G7.5 groups ($P < 0.001$). Although CR1+G7.5 differed significantly higher than CR3+G2.5 ($P < 0.001$), it did not differ statistically from the other groups ($P > 0.05$), indicating an intermediate pattern.

Linear kinematic parameters, including average path velocity (VAP), straight-line velocity (VSL), and curvilinear velocity (VCL), were evaluated to assess post-thaw sperm motion characteristics, and the results are presented in Table 2. These parameters were significantly elevated in the Control (68.59 ± 16.29; 56.25 ± 13.67; 96.15 ± 22.26 µm/s), G2.5 (66.26 ± 15.18; 53.77 ± 12.97; 92.74 ± 20.81 µm/s), G7.5 (66.37 ± 14.60; 53.44 ± 12.79; 94.53 ± 21.06 µm/s), and CR1+G7.5 (64.65 ± 14.55; 51.63 ± 12.55; 90.43 ± 19.60 µm/s) groups compared to the remaining groups *(P < 0.001)*.

Table 1. Total and progressive motility values of the experimental groups (Mean ± SD).

Group	n	Total Motility (%)	Progressive Motility (%)
Control (C)	34	50.6±14.81^{a}	22.68±6.28^{a}
C + Carnosic acid (CR) 1mM	34	35.35±11.9^{b}	17.08±5.68^{bc}
C + Carnosic acid (CR) 3 mM	34	31.29±8.79^{b}	15.88±4.47^{bc}
C + Glutamine 2.5 mM	34	48.23±13.9^{a}	21.02±5.57^{a}
C + CR 3 mM + Glutamine 2.5	34	30.58±8.29^{b}	15.20±3.58^{c}
C + Glutamine 7.5 mM mM	34	48.79±16.8^{a}	20.97±6.44^{a}
C + CR 1 mM + Glutamine 7.5 mM	34	45.00±13.28^{a}	19.5±4.73^{ab}

a,b,c: Different superscript letters within the same column indicate statistically significant differences *(P < 0.001).*

Table 2. CASA-based kinematic parameters of spermatozoa (Mean ± SD).

Group	n	VAP (µm/sn)	VSL (µm/sn)	VCL (µm/sn)
Control (C)	34	71.92±15.51^{a}	54.07±11.43^{a}	121.52±272.28^{a}
C + Carnosic acid (CR) 1mM	34	51.81±14.4^{b}	40.46±12.11^{b}	83.78±26.6^{b}
C + Carnosic acid (CR) 3 mM	34	45.94±15.8^{b}	34.92±13.06^{b}	73.77±30.42^{b}
C + Glutamine 2.5 mM	34	72.77±13.71^{a}	55.49±8,49^{a}	124.9±29.54^{a}
C + CR 3 mM + Glutamine 2.5	34	45.7±15.36^{b}	35.08±12.12^{b}	78.09±28.14^{b}
C + Glutamine 7.5 mM mM	34	71.72±20.56^{a}	53.99±14.09^{a}	123.39±34.45^{a}
C + CR 1 mM + Glutamine 7.5 mM	34	65.25±14.23^{a}	50.96±9.8^{a}	110.35±24.12^{a}

a,b,c: Different superscript letters within the same column indicate statistically significant differences ($P < 0.05$).

No significant differences were observed among these four groups.

Sperm viability was assessed by evaluating PMAI and MMP, with corresponding data summarized in Table 3. The Control group had the highest PMAI value (38.46 ± 12.97), which was significantly exceeding those of the CR1 (30.02 ± 8.91), CR3 (22.03 ± 8.71), and CR3+G2.5 (23.09 ± 7.60) groups (P < 0.001). However, the G2.5 (36.75 ± 11.86), G7.5 (35.75 ± 12.02), and CR1+G7.5 (32.32 ± 11.49) groups showed values statistically similar to those of the Control, as well as to CR1 (30.02 ± 8.91) (P > 0.05). Among all groups, the CR3 group (22.03 ± 8.71) exhibited the lowest PMAI value, which was significantly lower than those of all other groups except CR3+G2.5 (P < 0.001). No significant differences were found among groups for high mitochondrial membrane potential (P > 0.05).

DNA fragmentation results for the experimental groups are provided in Table 4. The Control group recorded the lowest level of DNA fragmentation (15.4 ± 2.14%) and differed significantly from all other groups (P < 0.001). The highest fragmentation rate was observed in the CR3+G2.5 group (19.31 ± 1.22%), which also showed significant differences from all other groups (P < 0.001). The G2.5 (16.25 ± 1.02%), CR3 (16.55 ± 0.5%), G7.5 (16.64 ± 0.58%), and CR1+G7.5 (16.91 ± 1.18%) groups shared statistically similar DNA fragmentation levels but were significantly different from both the Control and CR3+G2.5 groups (P > 0.05).

Table 3. Spermatozoon viability results (Mean ± SD).

Group	n	PMAI (%)	HMMP (%)
Control (C)	34	38.46±12.97^{a}	27.58±12.51
C + Carnosic acid (CR) 1mM	34	30.02±8.91b^{c}	21.79±11.19
C + Carnosic acid (CR) 3 mM	34	22.03±8.71^{d}	23.82±13.34
C + Glutamine 2.5 mM	34	36.75±11.86^{ab}	27.32±12.62
C + CR 3 mM + Glutamine 2.5	34	23.09±7.6^{cd}	22.03±13.73
C + Glutamine 7.5 mM mM	34	35.75±12.02^{ab}	27.38±11.77
C + CR 1 mM + Glutamine 7.5 mM	34	32.32±11.49^{ab}	22.0±11.78

a,b,c: Different superscript letters within the same column indicate statistically significant differences (P < 0.001).

Table 4. Spermatozoon DNA fragmentation results (Mean ± SD).

Group	n	DNA Damage (%)
Control (C)	34	15.4±2.14^{a}
C + Carnosic acid (CR) 1mM	34	17.13±0.4^{b}
C + Carnosic acid (CR) 3 mM	34	16.55±0.5^{c}
C + Glutamine 2.5 mM	34	16.25±1.02^{c}
C + CR 3 mM + Glutamine 2.5	34	19.31±1.22^{d}
C + Glutamine 7.5 mM mM	34	16.64±0.58^{c}
C + CR 1 mM + Glutamine 7.5 mM	34	16.91±1.18^{bc}

a,b,c: Different superscript letters within the same column indicate statistically

Discussion

Reproductive success in cattle is a multifaceted process influenced by various parameters, including animal care, nutrition, effective herd management, accurate estrus detection, the timing of artificial AI, the quality of frozen-thawed semen, and the expertise of inseminators (Gökçen, 2020). Among these factors, the production of high-quality frozen bull semen is the responsibility of bull stations and is regulated by national legal authorities. Frozen semen must be analyzed in authorized andrology laboratories as required by national legislation before its commercial use (HAYGEM, 2020).

In Türkiye, physical and morphological analyses of both imported and domestically produced frozen bull semen are conducted in laboratories authorized by the Ministry of Agriculture and Forestry for approval before use. According to national regulations, non-

sexed frozen semen must have at least 40% motility, a maximum of 30% abnormal spermatozoa, and a minimum concentration of 5 million motile spermatozoa per straw to be deemed acceptable for use. For sexed semen, the requirements include a minimum of 1 million motile spermatozoa per straw and ≤ 30% abnormal spermatozoa (HAYGEM, 2020). While these spermatological parameters may be adequate for intensive breeding, higher-quality semen may be more suitable for improving reproductive success in small-scale, family-operated farms that lack a structured reproductive management system.

Türkiye has approximately 14 million cattle, and the distribution of farms by herd size is as follows: 6.08% of farms have 200 or more animals, 16.45% have between 50 and 199 animals, and 77.47% have between 1 and 50 animals (Aytekin, 2011; TAGEM, 2019), as shown in Table 5.

Small-scale farms, also classified as family-based operations, are those that maintain between 1 and 50 head of cattle and collectively account for 77.47% of Türkiye's total cattle population. These farms generally operate under semi-intensive systems and are commonly populated with crossbreeds of native and dual-purpose cattle. Rather than aiming for high milk yields, the primary objective of these farms is often to produce animals for beef fattening. Most of these farms rely on services from private veterinarians to meet their herd health and AI needs. Estrus detection in such farms is typically performed through visual observation rather than systematic monitoring. In the absence of structured estrus detection systems, heat observation is usually carried out during morning and evening feeding or milking times, making AI procedures considerably more complicated (Önal and Özder, 2008; Roelofs et al., 2010; Aytekin, 2011; Aksoy and Yavuz, 2012; Pothmann et al., 2014; Tüzemen, 2015; Yener, 2017).

Due to the complex and multifactorial nature of reproductive success in family-type cattle farms, field veterinarians often demand high-quality frozen bull semen with superior motility and viability to be used in insemination procedures. Recently developed biotechnologies, such as microfluidic chip-based sperm separation and slow-release cryopreservation systems, have gained significant attention and support for this trend (Knowlton et al., 2015; Perteghella et al., 2017).

In line with these developments, various antioxidants or compounds with potential antioxidant properties have been added to semen extenders to reduce oxidative stress during both short-term and long-term storage, thereby improving post-thaw semen quality. Among these, glutamine and carnosic acid are commonly used in semen cryopreservation protocols (Arı and Öztürkler, 2015). The addition of 10 mM glutamine to a tris-egg yolk-based extender improved post-thaw sperm motility by approximately 5%, while higher doses (20, 30, 40, 80, 120 mM) adversely affected motility, as reported by (Amirat-Briand et al., 2009).

Similarly, a positive effect of 20 mM glutamine on boar sperm stored at 17°C for five days was also observed in a study by (Wang et al., 2018). Significant improvements in spermatological parameters were reported when 5 mM glutamine was added to a ram semen extender, according to (Bucak et al., 2009b). Furthermore, the addition of 60 mM glutamine was shown to have a beneficial effect on chimpanzee semen cryopreservation (Bottrel et al., 2018). In the present study, the addition of 2.5 mM, 7.5 mM glutamine or CR1+G7.5 to a soybean lecithin-based extender did not significantly affect total or progressive motility compared to the control group. This similarity was also reflected in linear kinematic parameters (VAP, VSL, VCL) and PMAI results. Although a positive correlation between intracellular glutamine concentration and sperm quality has been reported, the optimal exogenous glutamine dose may

Table 5. Number and percentage of cattle farms according to their scale

Farm Animal Capacity	Number of Farms (Units)	(%)	Number of Animals	Distribution by Animal Population (%)
01-09	1.155.958	75.77	4.251.049	31.35
10-49	339.306	22.24	6.252.878	46.12
50-199	28.580	1.87	2.231.149	16.45
≥200	1.757	0.11	823.466	6.08

vary by species and extender type, and remains unclear (Narud et al., 2020). Based on the present findings, neither 2.5 mM nor 7.5 mM glutamine appears to be ideal concentrations for soybean lecithin-based bull semen extenders.

Conversely, groups supplemented with 1 or 3 mM carnosic acid (CR1, CR3), or with 3 mM carnosic acid combined with 2.5 mM glutamine (CR3+G2.5), exhibited lower total and progressive motility, PMAI, and linear kinematic values. Similar adverse effects were observed in ram semen supplemented with 0.2 mM and 0.05 mM carnosic acid in tris-egg yolk extenders (Gungor et al., 2019). While positive impacts of 12.5 and 25 µg/mL carnosic acid on the short-term storage of Anatolian buffalo epididymal sperm at +4°C were reported (Yeni and Avdatek, 2017), another study found that rosmarinic acid containing 25–200 µg/mL of carnosic acid did not significantly improve motility or kinetic parameters in frozen bull semen (Yeni et al., 2018). Thus, the specified concentrations of carnosic acid are considered antagonistic when used in soybean lecithin-based bull semen extenders.

Mitochondrial membrane potential is an indicator of adenosine triphosphate production through oxidative phosphorylation in mitochondria and is associated with capacitation (Korkmaz and Çil, 2020). Due to the motile activity of spermatozoa, superoxide anions are generated within the mitochondria and may leak into the cytoplasm, potentially damaging both the plasma membrane and the DNA. (Aitken et al., 2014).

In this study, no significant differences in MMP were observed among groups. The lowest level of DNA damage was found in the control group; however, DNA damage in other groups did not correlate with other spermatological parameters. This may be explained by the fact that, despite the presence of oxidative stress during cryopreservation, the harmful effects of freezing itself on cells may be more severe than oxidative damage. Additionally, endogenous antioxidant mechanisms in semen may have mitigated oxidative damage during the process (Aitken and Baker, 2004; Arı and Öztürkler, 2015).

Although several studies have investigated the supplementation of semen extenders with various antioxidant compounds, there is no definitive evidence linking oxidative stress-related sperm damage to reduced fertilization or pregnancy rates. This may be due to the difficulty of optimizing experimental conditions in field settings (Arı and Öztürkler, 2015).

In conclusion, the addition of 2.5 mM and 7.5 mM glutamine to soybean lecithin-based extenders for the cryopreservation of Brown Swiss bull semen demonstrated a synergistic interaction; however, it did not result in significant improvements in motility, kinematic parameters, PMAI, HMMP, or DNA integrity compared to the control group. Moreover, the addition of 1 mM and 3 mM carnosic acid is considered to represent antagonistic doses when used in soybean lecithin-based extenders. The observation that some parameters in the CR1+G7.5 group were similar to those in the control may suggest that the antagonist effect of carnosic acid was partially compensated for by the presence of 7.5 mM glutamine.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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