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Tel: +90 272 214 9309 Fax: +90 272 214 9309 E-mail: kvj@aku.edu.tr
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RESEARCH ARTICLES

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RESEARCH ARTICLE

Evaluation of Erythrocytes Indices Platelet Indices and Complete Blood Count in Feline Mammary Carcinomas

İbrahim KURBAN[1]*, Zeynep GÜNAY UÇMAK[2]

[1]*Vocational School of Veterinary Medicine, İstanbul University-Cerrahpaşa, İstanbul, Türkiye*
[2]*Department of Obstetrics and Gynaecology, Faculty of Veterinary Medicine, İstanbul University-Cerrahpaşa, İstanbul, Türkiye*

ABSTRACT

The aim of this study was to evaluate the differences in the platelet (PLT) indices, erythrocytes indices and complete blood count (CBC) parameters between the cats with mammary tumor and healthy queens. Also, the differences in above mentioned parameters are going to be investigated in cats with mammary tumors with regard to clinicopathological characteristics of the primary tumor. A total of 44 queens were included in the study. The study groups consisted of cats with mammary tumors (Group MT, n=22) and healthy cats presented for neutering (Group H, n=22). The ages of the cats were ranged between 5-15 years. The mean ages of the cats in Group MT was 10.77±0.80 and in Group H was 6.75±1.21 years. All data on CBC, thoracic radiography, clinical examination findings and histopathology were obtained from the the patient archive in the animal hospital automation system. While the highest red blood cell (RBC) and hemoglobulin (HGB) levels were observed in Group H, Group MT had the highest white blood cell (WBC), mean platelet volume (MPV) and plateletcrit (PCT) levels. The mean corpuscular volume (MCV) level in T1 was significantly lower than in T2-T3. The MCV level in G1-G2 tended to be higher compared to G3.In Group MT, CBC parameters were not significantly different related to the presence of metastasis, presence of ulceration and inflammation on the tumor. It was concluded that evaluation of CBC and PLT indices in cats with mammary tumors would be useful in understanding the hematological effects of the disease and tumor characteristics (tumor size and histological grade).
Keywords: Erythrocytes indices, Female cat, Mammary carcinoma, Platelet indices

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Kedi Meme Karsinomlarında Eritrosit İndeksleri, Trombosit İndeksleri ve Tam Kan Sayımının Değerlendirilmesi

ÖZ

Bu çalışmanın amacı, meme tümörlü kediler ile sağlıklı dişi kediler arasındaki trombosit (PLT) indeksleri, eritrosit indeksleri ve tam kan sayımı (CBC) parametrelerindeki farklılıkları değerlendirmektir. Ayrıca meme tümörlü kedilerde yukarıda belirtilen parametrelerdeki farklılıklar, primer tümörün klinikopatolojik özellikleri açısından araştırılacaktır. Çalışmaya toplam 44 dişi kedi dahil edildi. Çalışma grupları, meme tümörlü kediler (Grup MT, n=22) ve kısırlaştırma için getirilen sağlıklı kedilerden (Grup H, n=22) oluşturuldu. Kedilerin yaşları 5-15 arasında değişiyordu. Grup MT'deki kedilerin yaş ortalaması 10,77±0,80, Grup H'deki kedilerin yaş ortalaması ise 6,75±1,21 yıldı. Tam kan sayımı, toraks radyografisi, klinik muayene bulguları ve histopatolojik incelemeye ilişkin tüm veriler, hayvan hastanesi otomasyon sistemindeki hasta arşivinden elde edildi. En yüksek kırmızı kan hücresi (RBC) ve hemoglobin (HGB) düzeyleri Grup H'de görülürken, Grup MT en yüksek beyaz kan hücresi (WBC), ortalama trombosit hacmi (MPV) ve platelekrit (PCT) düzeylerine sahipti. T1'deki ortalama korpüsküler hacim (MCV) seviyesi T2-T3'e göre anlamlı derecede düşüktü. G1-G2'deki MCV seviyesi G3'e kıyasla daha yüksek olma eğilimindeydi. Grup MT'de CBC parametreleri tümörde metastaz varlığı, ülserasyon ve inflamasyon varlığına bağlı olarak anlamlı farklılık göstermedi. Meme tümörlü kedilerde CBC ve PLT indekslerinin değerlendirilmesinin hastalığın ve tümör özelliklerinin (tümör boyutu ve histolojik derecesi) hematolojik etkilerinin anlaşılmasında faydalı olacağı sonucuna varıldı.
Anahtar kelimeler: Dişi kedi, Eritrosit indeksleri, Meme karsinomu, Trombosit indeksleri

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ORCID ID; *İK: 0000-0002-8391-905X, ZGU: 0000-0003-2530-1291*
*Corresponding author e-mail: ibrahim.kurban@iuc.edu.tr

INTRODUCTION

Mammary tumors are the third frequent cancer type in queens (Thomas 2015). Feline mammary tumors are mostly malignant (80%-90%) and tend to metastasize (Lana et al. 2007; Goldschmidt et al. 2017). Histopathological examination is very important to evaluate the course of the disease (Klopfleisch 2017). Histological classification of malignant mammary tumors in cats have 3 origins as epithelial (carcinomas), mesenchimal (sarcomas) and, carcinosarcomas (Zappulli et al. 2008). Feline mammary carcinomas are biologically aggressive and usually associated with poor outcome (Ito et al. 1996; Dagher et al. 2019). Middle-aged, intact and fed with high-fat diet cats have high incidence of the mammary tumor development. Persian and Siamese cats are breeds that are predisposed to mammary tumors (Günay Uçmak and Kırşan 2021).

Mammary tumors in humans and cats exhibit similar pathophysiological structures, epidemiological and clinicopathological patterns (Nascimento and Ferreira 2021; Uçmak et al. 2023). Malignant mammary tumors can cause hematological disorders such as anemia, erythrocytosis, thrombocytosis, hyperproteinemia, and leucopenia both in humans (Mantas et al. 2016) and dogs (Günay Uçmak and Güvenç 2019; Uçmak et al. 2021). Da Silva Soares et al. (2023) reported that monocytes, platelets, mean corpuscular hemoglobin concentration and creatinine may be important noninvasive presurgical prognostic markers for the median overall survival in feline mammary carcinomas. Hristov and Binev (2018) reported that dogs which were over 8 years old and with mammary carcinomas had mean corpuscular volume (MCV, fL), mean corpuscular haemoglobin (MCH, pg) and mean corpuscular haemoglobin concentration (MCHC, g/L) within narrow limits. Platelets (PLT) are cytoplasmatic fragments of bone marrow megakaryocytes, which have an important role in the blood coagulation mechanism (Üstündağ Budak et al. 2016). PLT indices which are used to measure the total amount of PLT, PLT morphology and proliferation kinetics, are Mean Platelet Volume (MPV), Platelet Distribution Width (PDW) and Plateletcrit (PCT) (Koenhemsi et al. 2020). It has been reported that neutrophils and platelets contribute to cancer development and progression by directly affecting tumor growth, angiogenesis and metastasis (Nash et al. 2001; Coffelt et al. 2016; Contursi et al. 2018). The presence of high amounts of platelets increases the risk of metastasis, the prognosis becomes poor, and the incidence of multiple tumor types increases in humans (Buergy et al. 2012; Ji et al. 2015). The evaluation of leukocyte and platelet amounts in cancer patients may be useful in determining the potential for thrombosis formation (Naess et al. 2007). It has been reported that in tumor hypoxia or necrosis, an imbalance between neutrophils and lymphocytes may occur and this may be associated with antiapoptotic effects (Avcı et al. 2017). The absolute leukocyte count and the neutrophil/lymphocyte ratio (NLR) provide prognostic information in cats with mammary carcinoma (Petrucci et al. 2021).

Complete blood count parameters are routinely evaluated in clinical examination. However, limited information are presented about the alterations in hematological parameters in cats with mammary tumors. The aim of this study is to evaluate the differences in the PLT indices, erythrocytes indices and complete blood count parameters between the cats with mammary tumor and healthy queens. Also, the differences in above mentioned parameters are going to be investigated in cats with mammary tumors with regard to clinicopathological characteristics of the primary tumor.

MATERIALS and METHODS

Animals and Experimental Design

A total of 44 queens were included in the study. The study groups consisted of cats with mammary tumors (Group MT, n=22) and healthy cats presented for neutering (Group H, n=22). The ages of the cats were ranged between 5-15 years. The ages of the cats in Group MT were between 8 and 15 years, while the ages of the cats in Group H were between 5 and 9 years. The races of the cats were mostly mixed breed (n=32) and the rest of 12 cats were Iranian (n=6), Sphenx (n=2) and Siamese (n=4). Complete blood count (Procyte Dx Hematology Analyzer, Idexx, USA) and three-view thoracic radiographs (Orex PcCR 1417 and Viztekdiagnostic imaging program, USA) were performed to avoid anesthesia risk and to detect the possible metastasis. Red blood cell (RBC), hemoglobin (HGB), hematocrit (HCT), white blood cell (WBC), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), platelet (PLT), mean platelet volume (MPV), plateletcrit (PCT) results were incorporated into the study. In clinical examination, mass size, localisation of the mass/masses, presence of metastasis, presence of ulceration and inflammation on the tumor were noted. As a treatment, total mastectomy was performed for the cats in Group MT. Histopathology was performed as the researchers' reported (Günay Uçmak et al. 2023). In histopathological examination, tumor type and histological grades of the tumor were determined in cats belong to Group MT and the histological tumor type of the primary mass was taken into account in this study. All operative interventions (both ovariohysterectomy and total mastectomy) were performed under general anesthesia. Initially, the cats were premedicated with atropine sulfate (Atropin®, Teknovet, Turkiye) (0.03 mg/kg, sc). For the induction of anesthesia, propofol (Pofol ampoule, Dongkook Pharm, Korea) was given at a dose of 4 mg/kg/iv, and

then the anesthesia was continued by applying inhalation anesthesia with 3% isofluorane (Foran liquid, Abbott Laboratories, England) and 1% oxygen (Küçükbekir et al. 2020). All data on complete blood count, thoracic radiography, clinical examination findings and histopathology were obtained from the the patient archive in the animal hospital automation system.

Statistical Analysis

All statistical analysis were perfomed by SPSS 23.0 program (SPSS 23.0, IBM, USA). Normal distribution of the data was checked with Saphiro Wilk test. The differences of the data belong to the complete blood count parameters between the study groups was evaluated by t-test and Mann Whitney U test. In Group MT, differences in complete blood count parameters with regard to the clinicopathological parameters were checked with t-test and Mann Whitney U test. Statistical significance was accepted as $p<0.05$.

RESULTS

The mean ages of the cats in Group MT was 10.77±0.80 and in Group H was 6.75±1.21 years ($p<0.05$). The mean of the complete blood count result belong to the groups and their significancies were presented in Table 1. In Group MT, the mean HCT level tended to be lower than in Group H ($p=0.069$) while the mean MCV level in Group MT tended to be higher than in Group H ($p=0.070$) but they both could not reach the significance.

Table 1. The mean of the complete blood count result belong to the groups and their significancies.

	Group MT	Group H	Significance (p)
RBC (M/μL)	8.89±0.40[a]	10.10±0.27[b]	**0.017**
HGB (g/dL)	12.61±0.47[a]	13.8±0.29[b]	**0.011**
HCT (%)	37.63±1.43	41.02±1.10	0.069
MCV (fL)	42.31±0.72	40.77±0.67	0.070
MCH (pg)	14.31±0.29	13.74±0.31	0.198
MCHC (g/dL)	33.56±0.39	33.83±0.44	0.661
WBC (10^9/L)	15.16±2.02[a]	8.30±0.53[b]	**0.003**
PLT (10^9/L)	298.27±43.86	272±18	0.742
MPV (fL)	15.61±0.46[a]	10.6±0.32[b]	**0.000**
PCT (%)	0.55±0.05[a]	0.2±0.00[b]	**0.000**

[a,b] Different letters in the same line indicate the significance.

In Group MT, all cats have multiple tumoral masses which were all belong to malignant epithelial tumor type. Clinicopathological characteristics of the primary tumor belong to the cats in Group MT were presented in Table 2. Differences in complete blood count parameters were also evaluated related to the clinicopathological characteristics of the primary tumor. Due to the lack of statistically sufficient numbers in tumor sizes of T2 and T3, they were evaluated as a single group (T2-T3). The mean MCV level in T1 was significantly lower than in T2-T3 (41.19±0.99 vs 44.04±0.89, $p<0.046$). The mean WBC level in T1 was tended to be lower compared to T2-T3 (11.03±1.43 vs 19.29±3.42, $p=0.056$). Also the mean PCT level in T1 was tended to be lower than in T2-T3 (0.47±0.06 vs 0.63±0.08, $p=0.076$). However, the rest of the evaluated blood parameters did not exhibit a significance in terms of the tumor sizes ($p>0.05$). Due to the lack of statistically sufficient numbers in histological gades of G1 and G2, they were also evaluated as a single group (G1-G2). The mean MCV level in G1-G2 tended to be higher compared to G3 (43.86±0.85 vs 41.37±1.08, $p=0.085$). However, the rest of the evaluated blood parameters did not exhibit a significance in terms of the histological grades ($p>0.05$). In Group MT, complete blood count parameters were not significantly different related to the presence of metastasis, presence of ulceration and inflammation on the tumor ($p>0.05$).

Table 2. Clinicopathological characteristics of the primary tumor belong to the cats in Group MT.

Clinicopathological Parameters		Affected Numbers
Localization of the primary tumor	Axillar lobes	5
	Abdominal lobes	8
	Inguinal lobes	9
Tumor size (T)	T1 (0-3 cm)	11
	T2 (3-5 cm)	5
	T3 (>5 cm)	6
Histological grades	Grade 1	5
	Grade 2	6
	Grade 3	11
Presence of distant metastasis	(+)	10
	(-)	12
Presence of ulceration on tumor	(+)	11
	(-)	11
Presence of inflammation on tumor	(+)	12
	(-)	10

DISCUSSION

Complete blood count is a routinely available test which is usually performed as part of standard clinical care (Petrucci et al. 2021). Mammary carcinomas in cats have aggressive biological behavior (Hassan et al. 2017). Tumor progression and prognosis depend on tumor microenvironment, inflammation, and immune response (Fridman et al. 2017). Leukocytes which include lymphocytes, monocytes and neutrophils play important roles on proliferation, inflammation and immunity in various cancer types both in humans and animals (Nascimento and Ferreira 2021; Petrucci et al. 2021; Alan et al. 2022; Köse et al. 2023). Petrucci et al. (2021) investigated the association between the prognosis and neutrophil-to-lymphocyte ratio, neutrophil and WBC amounts in feline mammary carcinoma cases. Da Silva Soares et al. (2023) searched the changes in the blood count and serum biochemical profiles prior to mastectomy and their relation with the median overall survival and the disease-free survival in cats with mammary carcinoma. In our study, the differences in complete blood count parameters, which have been determined to have prognostic importance in previous studies (Petrucci et al. 2021; da Silva Soares et al. 2023), between feline mammary carcinoma cases and healthy queens, and the changes of these parameters according to clinical and pathological features in cats with mammary tumors were investigated.

Paraneoplastic syndromes (PNS) usually occur as a result of indirect effects of neoplasia in the body (Elliott 2014). Anemia which is the most common PNS in veterinary oncology, is strongly associated with tumor hypoxia (Elliott 2014; Gaspar et al. 2015). The most common in cancer patients with disseminated or metastatic tumors is anemia of chronic disease. As the disease progresses, iron metabolism and storage are disrupted. Anemia occurs due to the shortening of the lifespan of red blood cells and the decrease in the bone marrow's response to it. As a result of miscellaneous chronic infections, systemic diseases and oncology cases, normocytic normochromic anemia is usually observed (Elliott 2014; Tvedten 2022). Decrease in HGB levels are usually associated with both tumor hypoxia and poor prognosis in cancer patients (Varlotto and Stevenson 2005). Lallo et al. (2016) observed anemia and erythrocytosis in bitches with malignant mammary tumors. Uçmak et al. (2021) reported that HCT, HGB and MCHC levels in bitches with epitelial mammary tumors regardless of the tumor subtypes were lower than healthy ones. Even though the animal species that constituted our study were different, RBC, HGB and HCT levels were lower in feline mammary carcinoma cases compared to healthy ones, similar with the researchers (Lallo et al. 2016; Uçmak et al. 2021). As Gaspar et al. (2015) stated, it is thought that the decrease in RBC, HGB and HCT levels may lead to tumor hypoxia in cases of feline mammary carcinoma.

Erythrocytes indices which are MCV, MCH and MCHC elucidate the prognostic value of feline mammary carcinomas (da Silva Soares et al. 2023). In dogs with mammary tumors, Hristov and Binev (2018) observed the mean values of red blood cell indices within the reference ranges while MCV and MCHC levels were significantly higher than in healthy controls. Similarly, MCV levels in feline mammary carcinoma cases were tended to be higher compared to healthy queens in this study. Viste et al. (2002) indicated that tumor size in feline mammary adenocarcinomas is a prognostic indicator and tumors greater than 3 cm have poor prognosis. In this study, the highest MCV levels were determined in the increased tumor sizes (T2 and T3) which may reflect the worsening prognosis as the researchers' reported (Viste et al. 2002; da Silva Soares et al. 2023).

The most common hematological abnormalities described in animal oncology are anemia, leukocytosis, thrombocytopenia, and coagulopathies (Bailey 2020). However, Lallo et al. (2016) reported that leukocytosis and thrombocytopenia were less hematologic changes in dogs bearing mammary tumors. It has been reported that in tumor hypoxia or necrosis, an imbalance between neutrophils and lymphocytes may occur and this may be associated with antiapoptotic effects (Avcı et al. 2017). Also, pathologies in genital system such as vagina (Köse et al. 2023), ovary (Uçmak et al. 2018) and uterus (Hirota et al. 2014) causes leukocytosis in dogs and cats. In line with the previous reports, elevated levels of WBC in feline mammary carcinoma cases were detected compared to healthy queens in this study. Leukocytosis with neutrophilia could occur as a consequence of inflammation that results from the presence of the tumor as Lallo et al (2016) reported. Olivera et al. (2022) investigated hematological and biochemical alterations in female dogs with mammary cancer according to clinical staging and they determined the lowest WBC levels in dogs with early clinical stage ($T_{1,2,3}N_0M_0$). Similary, WBC levels in cats with T_1 sized tumor were tended to be lower compared to T_2 and T_3 in this study.

Platelet indices such as MPV, PDW, PCT are recognized as surrogate markers of platelet activation. Mean platelet volume (MPV) is the average platelet size. The variability in the size of the platelet is defined by PDW and the percentage of the blood volume that consists of platelets is expressed by PCT (Saran et al. 2022). In humans, increased levels of MPV have been a predictive and prognostic factor for invasive breast cancer (Gu et al. 2016). Alan et al. (2022) determined increased MPV levels in cats diagnosed with lymphoma compared to healthy queens. However, Uçmak et al. (2021) did not determine the significant changes between the dogs with epithelial mammary tumor and healthy ones. Contradictory with previous report, MPV levels in cats with mammary carcinoma were significantly higher than in control group in this study. The contradictory results can be obtained due to the differences in species (dogs vs cats) of the study material. Similar with Gu et al. (2016) reported, it is thought that increased MPV levels may be a predictive factor for feline mammary carcinoma cases. In humans, elevated PCT levels in ovarian and endometrial cancers were detected (Ma et al. 2014; Karateke et al. 2015). It has been reported that PCT levels in dogs with transmissible vaginal tumors tend to increase compared to healthy controls (Köse et al. 2023). Similar with the previous reports (Ma et al. 2014; Karateke et al. 2015; Köse et al. 2023), PCT levels in feline mammary carcinoma group were significantly higher than in control group in this study. Zhao et al. (2024) investigated the potential predictive value of PCT for early-stage breast cancer and they stated that PCT can't be neglected in humans. In this study, PCT levels in cats with mammary tumor less than 3 cm were tended to decrease compared to the cats with mammary carcinoma more than 3 cm. As Zhao et al. (2024) reported in breast cancer patients, it is thought that PCT levels may also be predictor for early stage feline mammary carcinomas. It further studies, it should be investigated with large number of patients.

CONCLUSION

It was concluded that the concentrations of RBC, HGB, MCV, WBC, MPV and PCT could exhibit the significant difference in the presence of tumor and in regard to the characteristic of the primary tumor such as tumor size and histological grades. It will be useful to evaluate the CBC and PLT indices in cats with mammary tumor to understand the hematological effect of this pathology.

Conflict of interest: The authors have no conflicts of interest to report.

Authors' Contributions: İbrahim Kurban and Zeynep Günay Uçmak contributed to the project idea, design and execution of the study. İbrahim Kurban and Zeynep Günay Uçmak contributed to the acquisition of and analysed the data, drafted, wrote and reviewed the manuscript critically. All authors have read and approved the finalized manuscript.

Ethical approval: All animal-related procedures were approved by the local ethic committee (approval İU-C HADYEK 2024/28).

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RESEARCH ARTICLE

Investigation of *Blastocystis* Prevalence in Rural Areas: A Field Study Example

Eylem AKDUR ÖZTÜRK[1]*, Funda DOĞRUMAN AL[2]

[1]*Department of Parasitology, Faculty of Medicine, Çukurova University, Adana, Türkiye*
[2]*Division of Medical Parasitology, Department of Medical Microbiology, Faculty of Medicine, Gazi University, Ankara, Türkiye*

ABSTRACT

Blastocystis is a zoonotic protist that is commonly found in humans and other mammals, as well as birds, reptiles, fish and insects. More epidemiological studies are needed to identify risk factors for *Blastocystis* transmission and to shape control programs. The aim of this study was to investigate the prevalence of *Blastocystis* among the people living in Kırıklı village in Karaisali district of Adana province. Stool samples of 115 participants were examined for the presence of *Blastocystis* by direct microscopy (DM) and the culture method. DM positivity was 40% (46/115) and culture positivity was 70.4% (81/115), and this difference was statistically significant (p = 0.006). It was determined that the use of the culture method in the diagnosis was advantageous in the detection of *Blastocystis*. While no statistically significant correlation was found between *Blastocystis* positivity and sociodemographic factors like age, gender, and body mass index (BMI), it is crucial to highlight the elevated prevalence of *Blastocystis* in areas where livestock farming is prevalent and the zoonotic cycle in the transmission of the parasite.
Keywords: *Blastocystis*, Culture, Field Study, Prevalence

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Kırsal Alanda *Blastocystis* Prevalansının Araştırılması: Saha Çalışma Örneği

ÖZ

Blastocystis, insanlar ile diğer memelilerin yanı sıra kuş, sürüngen, balık, böcek gibi pek çok canlıda yaygın olarak bulunan zoonotik bir protisttir. *Blastocystis* bulaşındaki risk faktörlerini belirlemek ve kontrol programlarını şekillendirmek için daha fazla epidemiyolojik çalışmaya ihtiyaç duyulmaktadır. Çalışmamızda Adana ilinin Karaisali ilçesine bağlı Kırıklı köyünde yaşayanlarda *Blastocystis* prevalansının araştırılması amaçlanmıştır. Çalışmaya dahil edilen 115 katılımcının dışkı örneği direkt mikroskobi (DM) ve kültür yöntemiyle *Blastocystis* varlığı açısından incelenmiştir. Yöntemlerden DM pozitifliği %40 (46/115), kültür pozitifliği ise %70,4 (81/115) olarak tespit edilmiş, bu fark istatistiksel olarak anlamlılık göstermiştir (p = 0,006). Tanıda kültür yönteminin kullanılmasının *Blastocystis* belirlenmesinde avantaj sağladığı tespit edilmiştir. *Blastocystis* varlığına ilişkin yaş, cinsiyet, vücut kitle indeksi (VKİ) gibi sosyodemografik faktörler ile *Blastocystis* pozitifliği arasında istatistiksel olarak anlamlı bir ilişki görülmemiş olsa da hayvancılık yapılan bölgelerde *Blastocystis* pozitifliğinin yüksek olduğunun gösterilmesi ve parazitin bulaşında zoonotik döngünün üzerinde durulması gerektiğine vurgu açısından önemli bulunmuştur.
Anahtar Kelimeler: *Blastocystis*, Kültür, Prevalans, Saha Çalışması

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ORCID ID; *EAÖ: 0000-0002-4886-6175, FDA: 0000-0002-9118-3935*
*Corresponding author e-mail: akdureylem@gmail.com

GİRİŞ

Blastocystis, insanların ve kuş, sürüngen, balık, böcek, memeli hayvanlar gibi pek çok hayvanın gastrointestinal sisteminde yaygın olarak bulunan bir protisttir. Aynı zamanda insanlarda yaşadığı bilinen tek Stramenofil olarak rapor edilmiştir (Hublin ve ark. 2021; Nguyen ve ark. 2023).

Hastalık Kontrol ve Korunma Merkezi (Centers for Disease Control and Prevention, (CDC) USA) tarafından biyolojisinin ve diğer organizmalarla ilişkilerinin tam olarak anlaşılamamasından dolayı aktif bir araştırma alanı olarak bildirilen *Blastocystis*, Dünya Sağlık Örgütü (World Health Organization)'nün "Su Sanitasyonu ve Sağlık Programı" başlıklı bildirisinde de patojen olarak tanımlanmıştır (World Health Organization[WHO] 2011; Centers for Disease Control and Prevention[CDC] 2023).

Parazitin yaşam döngüsünde kist, vakuolar, granüler, amoeboid ve kist formları bulunmaktadır. Bulaş, enfekte konağın dışkısıyla atılan kist formlarının fekal-oral yolla alınması sonucu gerçekleşmektedir (Hublin ve ark. 2021).

Blastocystis enfeksiyonu karın ağrısı, ishal, bulantı, kusma, şişkinlik ve anoreksi gibi semptomlarla ilişkilendirilmiştir. Spesifik olmayan gastrointestinal semptomlara ilave olarak ürtiker ve şiddetli kaşıntı gibi daha az görülen dermatolojik semptomlarla da ilişkili olduğu gösterilmiştir (Tan 2008; Kurt ve ark. 2016; Bahrami 2020; Aykur ve ark. 2022).

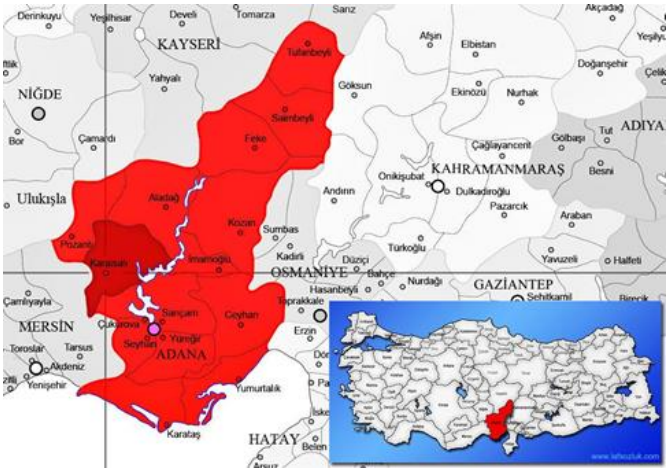
Gelişmiş ülkelerde *Blastocystis* prevalansının %5-20, gelişmekte olan ülkelerde ise %30-100 arasında değiştiği ve bu oranların coğrafi konum, çalışma grubunun demografik özellikleri, konağın bağışıklık sistemi gibi çeşitli faktörlerden etkilendiği bildirilmiştir (Tan ve ark. 2008; El Safadi ve ark. 2014; Khorshidvand ve ark. 2021). Ülkemizde *Blastocystis* pozitifliği %0,7 ile %63,6 arasında değişmekte olup veriler çoğunlukla hastanelere başvuran hastalardan elde edilmiştir (Maçin ve ark. 2019; Ergüden Gürbüz ve ark. 2020; Sarzhanov ve ark. 2021; Beyhan ve ark. 2023). Türkiye'de *Blastocystis* prevalansı ile ilgili yapılan saha çalışmaları oldukça kısıtlı olup ilkokul çocuklarında (Çelik ve ark. 2006), İzmir ilinde yaşayanlarda (Dagcı ve ark. 2008) ve gıda işçilerinde (Şahin ve ark. 2023) yapılan bağırsak parazitlerinin araştırıldığı çalışmalarından elde edilen saha verileri bulunmaktadır. *Blastocystis* bulaşındaki risk faktörlerini belirlemek ve kontrol programlarını şekillendirmek için daha fazla epidemiyolojik çalışmalara ihtiyaç duyulmaktadır.

Çalışmamızda Adana ilinin Karaisali ilçesine bağlı Kırıklı köyünde yaşayanlarda *Blastocystis* prevalansının araştırılması amaçlanmıştır. Kırıklı köyü coğrafi konumu, iklim koşulları, Seyhan Baraj gölü sınırlarında yerleşim alanının bulunması, hayvan popülasyonunun yoğunluğu, bölge haklının geçimini tarım ve/veya hayvancılıkla sağlaması, zengin meralarının bulunması nedeniyle *Blastocystis* bulaş döngüsü için uygun bir yerleşim yeridir.

MATERYAL ve METOT

Çalışma Alanı

Çalışmamız, analitik kesitsel çalışma olarak Adana ilinin Karaisalı ilçesine bağlı 582 nüfuslu Kırıklı köyünde (37°10'K, 35°14'D) gerçekleştirilmiştir. Bölge Akdeniz iklim kuşağındadır ve şehir merkezine 35 km uzaklıkta bulunmaktadır. Kırıklı köyü; Seyhan baraj gölüne sınırları bulunan ve burada yaşayanların geçimini çiftçilik ve/veya hayvancılıkla sağladığı kırsal bir yerleşim yeridir (Şekil 1). Seyhan baraj gölü suları Kasım- Aralık aylarında çekilmekte ve bu bölgeler insan aktivitelerine (oyun, gezi, piknik, kamp vb.) ve hayvanların otlayabilmesine uygun hale gelmektedir. Suların çekildiği bu dönemde bu alanların insan ve hayvan aktiviteleri sırasında bağırsak parazitleri ile kontamine olabileceği öngörülmektedir. Toroslardaki karların erimeye başlaması ile Nisan-Mayıs aylarında sular tekrar yükselmektedir.



Şekil 1: Adana ili Karaisali ilçesi haritası
Figure 1: Map of Adana province, Karaisalı district

Örneklerin Toplanması

Çalışama izni Çukurova Üniversitesi Tıp Fakültesi Etik Kurulu tarafından (No./Yıl: 49/14.07.2023) onaylanmıştır. Çalışmaya katılan olgular yapılacak araştırma hakkında bilgilendirildikten sonra imzalı onamları alınmıştır. Aynı zamanda katılımcılar bağırsak parazitleri enfeksiyonundan korunma konusunda sözel olarak bilgilendirilmiş ve konuyla ilgili broşür hazırlanarak dağıtılmıştır.

Çalışma; %95 güven aralığı ve %80 güç ile yapılan güç analizleri sonucu önerilen sayı ile uyumlu olacak şekilde tasarlanmış ve çalışmada 115 kişiden dışkı örneği toplanmıştır. Her katılımcıya etiketli, steril bir dışkı toplama kabı verilmiştir. Katılımcılar çalışmaya herhangi bir semptom veya belirti bulunmasından bağımsız olarak dahil edilmiştir. Dışkı örnekleri günlük olarak mümkün olan en kısa sürede buz aküleri içeren

köpük karton içerisinde laboratuvara ulaştırılmıştır (Tavur ve Onder 2022).

Direkt Mikroskopik (DM) İnceleme

Her bir katılımcıya ait dışkı örneklerinden serum fizyolojik ve Lugol solüsyonu (distile su ile 1:5 oranında seyreltilmiş) ile hazırlanan preparatlar ışık mikroskobunun (CX31, Olympus, Japan) 100x ve 400x objektifleriyle incelenmiştir (Gureser ve ark. 2023).

***Blastocystis* kültürü**

Tüm dışkı örneklerden bezelye büyüklüğünde (yaklaşık 50 mg) alınarak %10 at serumu içeren 2 ml Jones' besiyerine aktarılmıştır (Stensvold ve ark. 2007; Sarzhanov ve ark. 2021). Kültür örnekleri 37◦C etüvde 48–72 saat inkübe edilmiş ve sonrasında ışık mikroskobu kullanarak *Blastocystis* varlığı açısından değerlendirilmiştir.

Kültür yöntemi, direkt bakıda tek dışkı örneği ile oluşabilecek yanlış negatif sonuçların önlenmesi açısından prevalans çalışmalarında önerilmektedir (Ruang-Areerate ve ark. 2021).

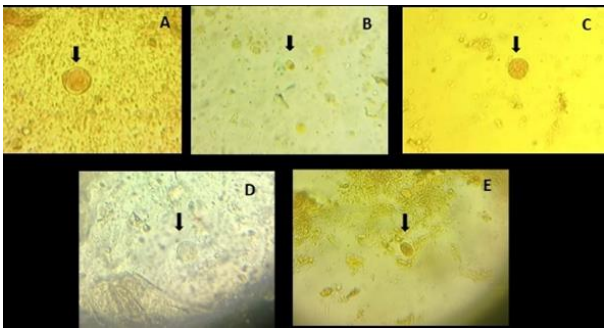
İstatistiksel Analiz

Çalışmada elde edilen veriler istatistiksel olarak IBM Statistical Package for the Social Sciences (SPSS) versiyon 29 yazılımı kullanılarak analiz edilmiştir. Verilerin cinsiyet, yaş gibi farklı gruplara göre dağılımlarının değerlendirilmesi için ki-kare testi kullanılmış ve $p < 0.05$ değeri istatistiksel olarak anlamlı kabul edilmiştir.

BULGULAR

Çalışmaya katılan 115 olgunun 54 (%47)'ü kadın, 61 (%53)'i erkek olup yaş ortalaması 43.5 (yaş aralığı 6-82) olarak hesaplanmıştır. Katılımcıların %70,4 (81/115)'ü hayvancılıkla uğraşırken %29,6 (34/115)'sı en az son altı aydır hayvanlarının olmadığını beyan etmiştir.

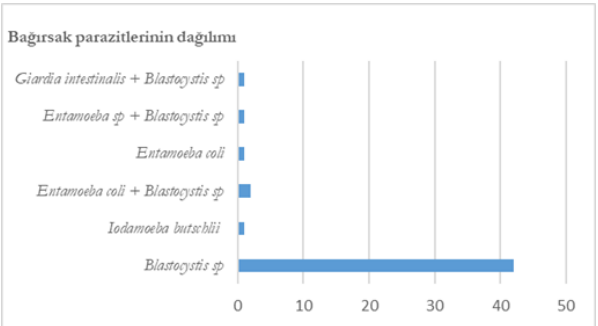
Bu çalışmada, direkt mikroskopik inceleme sonucu paraziter enfeksiyonların toplam prevalansı %41,7 (48/115) olarak tespit edilmiştir. Katılımcıların %40'ında (46/115) en sık tespit edilen parazit *Blastocystis* olarak belirlenmiş, bunu sırasıyla %2,6 (3/115) *Entamoeba coli*, %0,9 (1/115) *Giardia intestinalis*, %0,9(1/115) *Iodamoeba bütschlii* ve %0,9 (1/115) *Entamoeba histolytica/dispar* takip etmiştir (Şekil 2).



Şekil 2: Direkt mikroskobi sonucu tespit edilen parazitler (400x); (A) *Blastocystis* vakuoler form, (B) *Iodamoabe bütschlii*, (C) *Entamoeba coli* kisti, (D)*Entamoeba histolytica/dispar* kisti, (E) *Giardia intestinalis* kisti

Figure 2: Parasites detected by direct microscopy (400x); (A) *Blastocystis vacuolar* form, (B) *Iodamoabe bütschlii*, (C) *Entamoeba coli* cyst, (D) *Entamoeba histolytica/dispar* cyst, (E) *Giardia intestinalis* cyst

Parazit tespit edilen örneklerin %91,7'sinde (44/48) tek parazit, %8,3'ünden (4/48) ise iki farklı parazit tespit edilmiştir. Ayrıca 48 dışkı örneğinden 42'sinde (%87,5) yalnızca *Blastocystis* saptanırken, dördünde (%8,3) diğer parazitlerle *Blastocystis* birlikteliği gözlenmiştir. Çalışmamızda saptanan bağırsak parazitlerinin dağılımı Şekil 3 'de verilmiştir.



Şekil 3: Çalışmamızda saptanan bağırsak parazitlerinin dağılımı

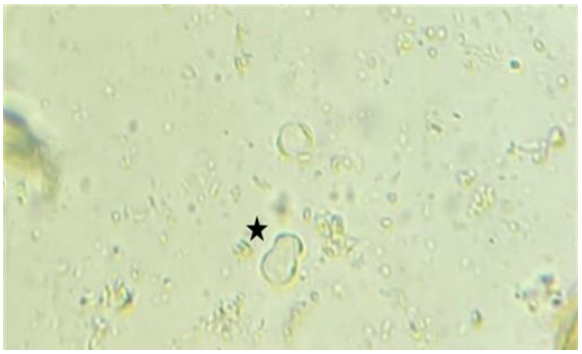
Figure 3: Distribution of intestinal parasites detected in the study

Tüm insan dışkı örneklerinde DM ve kültür yöntemi kullanılarak *Blastocystis* prevalansı araştırılmış ve bu iki yöntem karşılaştırılmıştır (Tablo 1). Yöntemlerden DM pozitifliği %40 (46/115) olarak saptanırken kültür

pozitifliği %70,4 (81/115) olarak tespit edilmiştir. Kültür pozitif bir örneğin mikroskobik görüntüsü Şekil4 'de verilmiştir.

Tablo 1. *Blastocystis* tanısında DM ve kültür yöntemlerinin karşılaştırılması
Table 1. Comparison of DM and culture methods in the diagnosis of *Blastocystis*

		Kültür		Toplam
		+	-	
DM	+	39	7	46
	-	42	27	69
Toplam		81	34	115



Şekil 4: Kültür pozitif örneklerden birinin mikroskobik görüntüsü, * Bölünen *Blastocystis*
Figure 4: Microscopic image of the culture-positive sample, *Dividing *Blastocystis*

Kültür yöntemiyle 81 *Blastocystis* pozitif örnek saptanmıştır. Bu örneklerden 39 tanesi DM ile de pozitif olarak tespit edilmiştir. DM pozitif olan yedi örneğin kültür yönteminde *Blastocystis* üremesi gözlenmemiştir. Kültür yöntemi ile elde edilen pozitiflik oranı DM' ye göre istatistiksel olarak anlamlı farklılık göstermiştir (p= 0,006).

Blastocystis varlığına ilişkin yaş, cinsiyet, vücut kitle indeksi (VKİ) gibi sosyodemografik faktörlerin istatistiksel analiz sonuçları Tablo 2'de verilmiştir. İncelenen bu faktörler ile *Blastocystis* pozitifliği arasında istatistiksel olarak anlamlı bir ilişki görülmemiştir. Ancak *Blastocystis* pozitifliğinde yaş ve VKİ ile orantısal olarak artış olduğu gözlenmiştir.

Tablo 2. *Blastocystis* varlığına göre katılımcıların sosyodemografik özelliklerinin dağılımı
Table 2. Distribution of sociodemographic characteristics of participants according to the presence of *Blastocystis*

	Pozitif (n%)	Negatif (n%)	Toplam (%)	P değeri
Cinsiyet				
Kadın	36 (%44,4)	18 (%53)	54 (%47)	0,405
Erkek	45 (%55,6)	16 (%47)	61(%53)	
Yaş				
0-18 yaş	11(%13,6)	4 (%11,7)	15(%13)	
19-39 yaş	19(%23,5)	7 (%20,6)	26 (%22,6)	0,917
40-59 yaş	37(%45,7)	18 (%53)	55(%47,2)	
60 ve üstü	14(%17,3)	5 (%14,7)	19(%16,2)	
Vücut Kitle İndeksi *				
18.5 – 24.9	12(%17,4)	1(%3,3)	13(%13,1)	0.057
25 ve üzeri	57(%82,6)	29(%96,7)	86(%86,9)	
Hayvancılık				
Var	54(%66,7)	27(%79,4)	81(%70,4)	0,172
Yok	27(%33,3)	7(%20,6)	34(%20,6)	

***Vücut kitle imdeksi 20 yaş ve üzerinde hesaplanmıştır.

TARTIŞMA

Adana ilinin kırsal kesiminde Kırıklı köyünde yapılan saha çalışmamızda dışkı örneklerinin DM incelemesi sonucunda %41,7 (48/115) oranında bağırsak paraziti tespit edilmiş, bu sonucun, Etiyopya'da (% 46,3), Fas'da (%44) ve Brezilya'da (%38,2) yürütülen çalışmaların sonuçlarıyla benzerlik gösterdiği belirlenmiştir (Takizawa ve ark. 2009; Hajare ve ark. 2021; Boutahar ve ark. 2023). Buna karşılık, İtalya (%13,24) (Peruzzi ve ark 2006) ve Fransa (%17) (Menu ve ark. 2019) gibi Avrupa ülkelerinde yapılan çalışmalarda bağırsak parazitleri daha düşük oranlarda bulunurken, Mısır'da (%72) (Hamdy ve ark. 2020), Lübnan' da (%85) (Osman ve ark. 2016) ve Nijerya' da (%97) (Idowu ve ark. 2006) ise çok daha yüksek oranlarda pozitiflik saptandığı bildirilmiştir.

Ülkemizde ise 2010 yılı sonrası bağırsak parazitleri üzerine yapılan çalışmalarda, parazit saptama oranının %1,84 – %31,6 arasında değiştiği gösterilmiştir (Tanrıverdi Çaycı ve ark. 2017; Öncel ve ark. 2018; Polat ve ark. 2020; Güler ve ark. 2021). Bu çalışmaların çoğunluğu, hastanelere başvuran ve belirli bir kısmı şehirlerde yaşaması muhtemel olan hastalardan bağırsak parazitlerinin araştırıldığı çalışmalardır. Çalışmamız ise Kırıklı köyünde yaşayan ve geçimini çiftçilik ve/veya hayvancılıkla sağlayan insanlarda bağırsak parazitleri araştırıldığı için pozitiflik oranı (%41,7) yüksek saptanmıştır. Çalışmamızda en sık tespit edilen parazit, yapılan benzer çalışmalarla (Polat ve ark. 2020; Güler ve ark. 2021) uyumlu şekilde *Blastocystis (*%40) olarak tespit edilmiştir.

Blastocystis'in prevalansının; coğrafi konum, çalışma grubunun demografik özellikleri, konağın bağışıklık sistemi, yaşı ve beslenme alışkanlıkları gibi çeşitli faktörlerden etkilendiği ve gelişmiş ülkelerde %5-20, gelişmekte olan ülkelerde ise %30-100 arasında değiştiği bildirilmiştir (Tan ve ark. 2008; El Safadi ve ark. 2014; Khorshidvand ve ark. 2021). Ülkemizde *Blastocystis* pozitifliğinin araştırıldığı çalışmalarda bu aralık %0,7 - %63,6 olarak kaydedilmiştir (Ergüden Gürbüz ve ark. 2020; Maçin ve ark. 2019; Sarzhanov ve ark. 2021; Beyhan ve ark. 2023). Çalışmamızda kültür yöntemiyle bu oran %70,4 (81/115) olarak tespit edilmiştir. Yapılan çalışmalarda tanıda en yaygın kullanılan yöntemin dışkının DM inceleme yöntemi olduğu dikkat çekmektedir. *Blastocystis*'in farklı morfolojik formlarının bulunmasının yanı sıra maya, lökosit, *Dientamoeba fragilis* ve *Cyclospor*a ile karıştırılması mikroskobik tanıyı zorlaştıran ve testin duyarlılığını düşüren faktörler arasında belirtilmektedir (Santos ve Rivera 2013).

Direkt mikroskobik inceleme yönteminin duyarlılığının düşük olması nedeniyle hem epidemiyolojik çalışmalarda hem de rutin tanıda *Blastocystis*'in tanısı için kültür yönteminin kullanılması önerilmektedir (Sarzhanov ve ark. 2021; Beyhan ve ark. 2023). Kültür yönteminin; DM, çoklaştıma yöntemi, kalıcı boyama gibi konvansiyonel yöntemlerle karşılaştırıldığı çalışmalarda kültür yönteminin daha yüksek duyarlılığa sahip olduğunu gösterilmiştir (Elghareeb ve ark. 2015; Mohemmi ve ark. 2015). Çalışmamızda da kültür yöntemi ile elde edilen pozitiflik oranı DM' ye göre istatistiksel olarak anlamlı derecede farklılık göstermiştir (p= 0,006).

Moleküler yöntemlerin son yıllarda *Blastocystis* tanımlanmasında ve alt tiplendirilmesinde yaygın şekilde kullanıldığı bildirilmiştir (Yakoob ve ark. 2010; Santos ve Rivera 2013; Sarzhanov ve ark. 2021, Ruang-Areerate ve ark. 2021; Aykur ve ark. 2023). *Blastocystis* tanısında farklı yöntemlerin karşılaştırıldığı çalışmalarda moleküler yöntemlerin diğer yöntemlerle kıyaslandığında daha yüksek duyarlılığa sahip olduğunu bildiren çok sayıda çalışma bulunmaktadır. Bu çalışmalardan, 81 irritabl bağırsak sendromu (IBS) hastasında *Blastocystis* varlığının araştırıldığı çalışmada hastaların %30,1'inin DM, %41'inin kültür yöntemi ve %44,6'sının polimeraz zincir reaksiyonu (PCR) yöntemiyle ile pozitif bulunduğu bildirilmiştir (Eida ve Eida 2008). Avustralya' da 513 hastaya ait dışkı örneklerinin kalıcı boyama, kültür ve PCR yöntemiyle incelendiği başka bir çalışmada ise %94 oranında *Blastocystis* pozitifliğini saptayan en duyarlı testin PCR olduğu bildirilmiştir (Robert 2011).

Moleküler yöntemlerin yüksek maliyeti nedeniyle araştırma alt yapı imkanlarının kısıtlı olduğu laboratuvarlarda kullanılamaması, zaman alıcı olması ve özel ekipman gerektirmesi, bu yöntemlerin sınırlılığı olarak belirtilmektedir (Boutahar ve ark 2023).

Ayrıca dışkıda bulunan inhibitör faktörlerin, moleküler yöntemlerle hatalı sonuçlar elde edilmesine neden olabileceği bildirilmiştir (El Safadi ve ark. 2013; Beyhan ve ark. 2023). Souppart ve ark.larının yaptığı çalışmada *Blastocystis* kültür pozitif örneklerin %2'sinin PCR yöntemiyle tespit edilememiştir (Souppart ve ark. 2009). IBS hastalarında yapılan bir başka çalışmada da PCR yönteminin dışkı kültürüne oranla daha düşük duyarlılık gösterdiği bulunmuştur (Yakoob ve ark. 2010). Zamani ve ark.larının DM, çoklaştırma, kalıcı boyama, kültür ve PCR yöntemlerini karşılaştırdığı çalışmalarında sırasıyla %16,3, %16,7, %17,4, %22,4 ve %22 oranında *Blastocystis* pozitifliği tespit edilmiş, yazarlar kültür yöntemini altın standart olarak kullandıklarını bildirmişlerdir (Zamani ve ark. 2021). Santos ve ark.ları 110 insan dışkı örneğinde DM ile %8,2, kültür yöntemiyle %32,7 oranında pozitiflik tespit etmişler, dışkı örneğinden DNA izolasyonuyla yapılan PCR ile % 9,1 oranında pozitiflik tespit edilirken kültür materyalinden elde edilen DNA ile yapılan PCR ile örneklerin % 23,6'sında pozitiflik elde etmişlerdir . Kültür ve PCR yöntemleri arasındaki bu farkın dışkı izolasyonunda yaşanan zorluklardan ve çalışmada kullanılan primerlerin, dışkı örneklerinde bulunan *Blastocystis* alt tiplerine ait hedef bölgeleri amplifiye edememiş olmasından kaynaklı olabileceği bildirilmiştir (Santos ve ark. 2013).

Blastocystis yaygınlığının araştırıldığı çalışmaların çoğunda *Blastocystis* görülme oranı ile cinsiyet arasındaki ilişki de incelenmiştir. Bu ilişkinin istatiksel

olarak anlamlı bulunduğu çalışmalar (Abdulsalam ve ark. 2013; Xu ve ark. 2021; Viesy ve ark. 2022) bildirilse de birçok çalışmada bu parazitin her iki cinsiyette de yakın oranlarda tespit edildiği ve bu ilişkinin istatiksel olarak anlamlı bulunmadığı bildirilmiştir (Dogruman ve ark. 2009; Piubelli ve ark. 2019; Sarzhanov ve ark. 2021; Ruang-Areerate ve ark. 2021; Aykur ve ark 2023; Boutahar ve ark 2023; Gureser ve ark 2023). Çalışmamızda ise *Blastocystis* saptanan hastaların %44,4'ü kadın, %55,6'sı erkek hastadan oluşmakta olup bu fark istatistiksel olarak anlamlı bulunmamıştır (p=0,405).

Blastocystis pozitiflik oranı ile yaş arasında ilişkinin incelendiği çalışmalarda farklı yaş gruplarında farklı pozitiflik oranları bildirilmiştir. Li ve ark.larının yaptığı çalışmada en yüksek *Blastocystis* pozitifliğinin 60 yaş ve üzeri bireylerde olduğu gösterilmiştir (Li ve ark. 2007). Rebolla ve ark.larının yaptığı çalışmada 11 ay- 6 yaş aralığında bulunan 172 çocukta %55,8 oranında *Blastocystis* pozitifliği tespit edilirken 19-58 yaş aralığında bulunan 33 kişide bu oran % 60.6 olarak bulunmuştur (Rebolla ve ark. 2016). Ancak yaş grupları ve *Blastocystis* pozitifliğinin karşılaştırıldığı birçok çalışmada 20–60 yaş aralığında diğer yaş gruplarına oranla daha yüksek *Blastocystis* pozitifliği gösterilmiştir (Seyer ve ark. 2017; Ruang-Areerate ve ark. 2021; Xu ve ark. 2022; Viesy ve ark. 2022; Boutahar ve ark. 2023). Çalışmamızda benzer şekilde, 19-39 yaş (%23,5) ve 40-59 yaş (%45,7) gruplarında *Blastocystis* pozitifliğin orantısal olarak diğer gruplardan yüksek olduğu görülmüştür (Tablo 2). Bu durumun belirtilen yaş gruplarının tarım ve hayvancılıkla aktif olarak uğraşmasından ve parazite maruz kalma ihtimalinin yaşla birlikte artmasından kaynaklı olabileceği şeklinde yorumlanmıştır.

Son yıllarda *Blastocystis*'in zoonotik bulaş potansiyelini araştırmaya yönelik çalışmalar artmaya başlamıştır. Mısır'da 136 insan ve 190 sığırın dışkı örneğinde yapılan çalışmada sırayla %38 ve %19 *Blastocystis* pozitifliği gösterilmiş ve sığırların zoonotik döngü için potansiyel bir rezervuar olabileceğine vurgu yapılmıştır (Abdo ve ark. 2021). Iran'da yapılan bir çalışmada ise 395 hayvanın dışkı örneğinde (220 kümes hayvanı, 100 koyun ve 75 sığır) % 29,1 oranında *Blastocystis* pozitifliği gösterilmiştir. Pozitif örnekler arasında en yüksek oran % 50,6 ile sığırlarda gösterilmiş olup, bunu koyun (%32,0) ve kümes hayvanları (%20,4) izlemiştir (Salehi ve ark. 2022). Shaker ve ark. larının yaptığı çalışmada kırsal kesimde yaşayanlarda *Blastocystis* pozitifliğinin (%59,3) kentte yaşayanlara (%40,7) oranla daha yüksek olduğu ve bu durumun köyde yaşayan insanların hayvanlarla doğrudan temas halinde olmasından ve sanitasyon eksikliğinden kaynaklanabileceği bildirilmiştir (Shaker ve ark. 2017). Çalışmamızda Kırıklı köyünde yaşayan ve geçimini çiftçilik ve/veya hayvancılıkla sağlayan olgularda, yukarıda belirtilen benzer çalışmalarla uyumlu olarak *Blastocystis* pozitifliği (%70,4) yüksek saptanmıştır. Ayrıca çalışmamızda katılımcıların %70,4'ü (81/115) hayvancılıkla uğraşırken %29,6'sı (34/115) en az son altı aydır hayvanlarının olmadığını beyan etmesine rağmen bu iki grup arasında *Blastocystis* pozitifliği açısından istatistiksel olarak anlamlı fark görülmemiştir. Bu durumun Kırıklı köyünün coğrafi konumundan kaynaklı olabileceği düşünülmektedir. Kırıklı Köyü, Seyhan baraj gölüne kıyısı bulunan bir yerleşim yeridir. Seyhan baraj gölü suları kış aylarında çekilmekte ve bu bölgelerde insan aktivitelerine ve hayvan otlatılmasına elverişli geniş meralar oluşmaktadır. Suların çekildiği dönemde bu alanların insan ve hayvan aktiviteleri sırasında bağırsak parazitleri ile kontamine olabileceği, Toroslardaki karların erimesiyle Nisan-Mayıs aylarında bu bölgelerde tekrar yükselen baraj gölü sularının bağırsak parazitleri ile kontamine olabileceği ön görülmektedir. Baraj gölünün köyün içinden geçen dallarının olması, bu suların ortak yaşam alanlarında, hayvanların ve tarlaların sulanmasında kullanılması *Blastocystis* pozitiliğinin, hayvancılıkla uğraşılmasından bağımsız olarak, sürekli temas nedeniyle yüksek oranda görülmesini açıklamaktadır.

Ruang-Areerate ve ark.larının yaptığı çalışmada domuz yetiştiriciliğinin yapıldığı bölgede yaşayan insanların *Blastocystis* enfeksiyonuna yakalanma riskinin, aynı bölgede ikamet etmeyenlere göre 5,4 kat daha yüksek olduğununun gösterilmesi, çalışmamız sonucuyla uyumlu şekilde hayvancılıkla uğraşılan bölgelerde yaşayanlarda *Blastocystis* pozitifliğinin daha yüksek oranda tespit edilebileceğini göstermektedir (Ruang-Areerate ve ark. 2021).

SONUÇ

Çalışmamızda *Blastocystis* pozitifliği ile risk faktörleri arasında istatistiksel olarak anlamlı bir ilişki bulunmasa da hayvancılık yapılan bölgelerde *Blastocystis* pozitifliğinin yüksek olduğunun gösterilmesi ve parazitin bulaşında zoonotik döngünün üzerinde durulması gerektiğine vurgu açısından önemli veriler elde edilmiştir. Bu çalışmanın güçlü yanlarından biri de bu bölgede ilk kez kırsal kesimde gastrointestinal semptomlardan bağımsız, kesitsel olarak *Blastocystis* prevalansının araştırılmış olmasıdır. Çalışmamızda aynı zamanda *Blastocystis* tespitinde kültür yönteminin güvenilir ve hassas bir yöntem olarak önemini ve direkt mikroskobiden üstünlüğünü vurgulayacak sonuçlar elde edilmiştir.

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FDA analysed the data. EAO drafted and wrote the manuscript. FDA reviewed the manuscript critically. All authors have read and approved the finalized manuscript.

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RESEARCH ARTICLE

Serum Progesterone and PAG Levels in Pregnant Karya Ewes

Çiğdem ÇEBİ[1*], Mehmet AKKÖSE[2]

[1]Department of Reproduction and Artificial Insemination, Faculty of Veterinary Medicine, Harran University, Sanliurfa, Türkiye
[2] Department of Livestock, Dalaman Agricultural Enterprise, General Directorate of Agricultural Enterprices, Dalaman, Muğla, Türkiye

ABSTRACT

The present study was aimed the determination of serum PAG concentrations during pregnancy and the first month postpartum in Karya ewes and the investigation of correlations between the serum concentrations of the steroid hormone progesterone, upon which the continuation of pregnancy depends, and PAG levels throughout pregnancy. Blood samples were collected from each of the ewes at weeks 1, 2, 3, 4, 5, 6, 7, 8, 9, 11, 13, 15, 17, 18, 19, 20, and 21 after the day of mating (day 0), at the time of parturition, and at weeks 1, 2, 3 and 4 postpartum. PAG level and progesterone level during pregnancy and serum PAG level in the first postpartum month were determined. Plasma progesterone concentrations were lowest on the day of mating, but progressively increased as of week 9 of pregnancy and peaked at gestational week 18. Progesterone levels were observed to decrease after week 18. The mean PAG concentrations of the pregnant animals were observed to be at the basal level until the second week of pregnancy, but increased as of week 3 and peaked at week 19. PAG concentrations displayed a dramatic decrease during the first 4 weeks postpartum. Based on this finding, in the Karya ewes, the increase in the progesterone levels at gestational weeks 8 and 11 were associated with an increase in the PAG levels. Differences in the results reported for the correlation between PAG and progesterone levels could be due to less frequent sampling, species-specific differences in placental function and PAG cross-reactivity, as well as species- or subspecies-specific differences in PAG and progesterone dynamics during pregnancy.

Key Words: PAG, Pregnancy, Progesteron, Sheep

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Gebe Karya Koyunlarında Serum Progesteron ve PAG Düzeyleri

ÖZ

Var olan çalışmanın amacı Karya koyunlarında gebelik süresince PAG düzeyi ve progesteron seviyesi ile postpartum ilk aydaki serum PAG düzeyini belirleyerek progesteron ile PAG arasındaki korelasyonların gebelik süresince saptanması amaçlandı. Tüm koyunlardan, koçların aşım yaptığı günden sonraki 1, 2, 3, 4, 5, 6, 7, 8, 9, 11, 13, 15, 17, 18, 19, 20, 21'inci haftalar ile doğum sonrası 1, 2, 3 ve 4'üncü haftalarda kan örnekleri toplandı ve gebelik süresince PAG düzeyi ve progesteron seviyesi ile postpartum ilk aydaki serum PAG düzeyi belirlendi. Plazma progesteron konsantrasyonu gebeliğin 9. haftasından itibaren artış göstermiş olup, 18. haftada pik düzeye ulaşdı ve 18. haftadan sonra progesteron düzeylerinde düşüş gözlendi. Gebe hayvanlarda ortalama PAG konsantrasyonları ise gebeliğin ikinci haftasına kadar bazal düzeyde iken gebeliğin 3. haftasından itibaren yükselmeye başladı, gebeliğin 19. haftasında pik yaparak gebelik sonuna kadar da artış gösterdi. Doğumdan sonraki ilk 4 hafta süresince ise PAG konsantrasyonu dramatik bir şekilde bir düşüş gösterdi. Karya koyunlarında gebeliğin 8. ve 11. haftalarında progesteron seviyesi arttıkça PAG düzeyi de artış gösterdi. Bu bulgulara göre PAG ve progesteron arasında bildirilen korelasyonlardaki farklılıklar, daha az sıklıkta örnekleme yapılmasına, plasental fonksiyondaki türe özgü farklılıklara veya PAG çapraz reaktivitesindeki farklılıklara ve gebelik sırasında PAG ve progesteron dinamiklerindeki tür veya alt tür farklılıklarından kaynaklanıyor olabilir.

Anahtar Kelimeler: Gebelik, Koyun, PAG, Progesteron

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ORCID ID; *ÇÇ: 0000-0001-6876-2069 MA: 0000-0001-7517-4927*
*Corresponding author e-mail: cigdemcebi@harran.edu.tr

INTRODUCTION

The Karya breed is known to have emerged from the ungoverned crossbreeding performed by Turkish sheep breeders in Western Anatolia between Chios, Kıvırcık and Chios X Kıvırcık rams and ewes of several local fat-tailed breeds, including the Ödemiş, Çine Çaparı and Dağlıç. The Karya was named by the Breed Registration Commission of the Ministry of Agriculture and Forestry after the Carian civilization, known to have reigned in the region during antiquity, and was registered as an indigenous breed by the same Commission in 2013 (Karaca and Cemal 1998, Karaca and Cemal 2005; Yücel 2022). Elite flocks of the prolific Karya ewes show a twin pregnancy rate of almost 100% and sometimes present with triplet and quadruplet pregnancies. Early pregnancy diagnosis is very important for the economics of sheep production (Akdeniz 2022). The detection of pregnant and non-pregnant animals in the flock not only avoids feed waste on infertile animals, but also enables formulating rations according to the energy requirements of pregnant and nursing ewes, both of which contribute to preventing the development of metabolic diseases and reducing the occurrence of reproductive health problems. Moreover, distinguishing between singleton and multiple pregnancies by means of early pregnancy diagnosis enables producers to plan the lambing method in advance, such that the dependence on labor force is minimized and lambing efficiency is maximized (Akdeniz 2022; De Carolis et al. 2020; Kaplan Bilmez 2018). The timely and accurate diagnosis of pregnancy also contributes to the prevention of fertility losses (Kaplan Bilmez 2018). Despite the availability of a broad array of options for pregnancy diagnosis today (i.e. ultrasonography, measurement of pregnancy-associated glycoprotein levels, radiography, abdominal palpation, vaginal biopsy, the cervical mucus test, vaginal smear examination, serum progesterone analysis), the methods commonly used for early pregnancy diagnosis are ultrasonographic examination, measurement of blood plasma/serum progesterone levels and the detection of pregnancy-specific proteins (Akdeniz 2022; Kaplan Bilmez 2018). While ultrasonography offers a high level of diagnostic accuracy, its time-consuming and labour-intensive implementation, which requires the skills of an experienced practitioner and can be challenging for large flocks, is disadvantageous (Erdem and Sarıbay 2015; Lucy et al. 2013). One of the most popular methods used for pregnancy diagnosis on sheep farms is the measurement of the post-mating blood levels of the progesterone hormone, as proof of the presence of a functional corpus luteum (Anghel et al. 2011; Zamfirescu et al. 2011). In ewes, progesterone concentrations are detectable in the blood as of the 18th day after mating and progressively increase up to a level of 2-3 ng/ml on day 50 of pregnancy, displaying a rapid increase after the first three months of gestation to reach a peak level of 12-20 ng/ml (Kaplan Bilmez 2018). While a high level of success is achieved in the detection of pregnant ewes with progesterone measurement, cases of early embryonic death, corpus luteum persistence, the prolongation or shortening of the oestrus cycle, faulty insemination, and uterine and ovarian pathologies may cause misdiagnosis. Furthermore, the high progesterone levels observed during certain phases of the oestrus cycle may reduce the success of detecting non-pregnant ewes (Karen et al. 2001; Karen et al. 2004). An alternative to the mentioned pregnancy diagnosis methods is the detection of pregnancy-associated glycoproteins (PAGs) (Rovani et al. 2016; Steckeler et al. 2018). Pregancy-specific protein B (PSPB) and other PAGs are synthesized during pregnancy in ewes (and other female ruminants), and thus, are used as indicators of pregnancy. PSPB, which is a form of PAG, is also referred to as pregnancy-serum protein 60 kDa (PSP60) and SBU-3 antigen (Adeyeye et al. 2021; El Amiri et al. 2007). Given the role of PAGs as pregnancy indicators, in the past 30 years, commercial test kits have been developed for early pregnancy diagnosis based on the detection of PAGs belonging to the aspartic proteinase family (Akköse 2020; Szenci 2021). These PAG ELISA kits, originally developed for use in cattle, also produce successful results for early pregnancy diagnosis in sheep and goats (Akdeniz 2022; Akköse 2020).

Pregnancy, which is defined as the period in-between fertile mating and parturition, starts immediately after the fertilization of the ovum and lasts for 147-152 days in ewes. During this period, several biomarkers, including among others, enzymes, growth factors, inhibitors, hormones and proteins, either appear or increase in the maternal blood circulation (Adeyeye et al. 2021). The majority of the biomarker proteins are of fetoplacental origin and are produced by mono- and binucleate trophoblast-derived cells in the ruminant placenta, from which binucleate giant cells migrate into the maternal circulation through the foetal chorion (Adeyeye et al. 2021; Ranilla et al. 1994). Various cDNAs encoding different PAG molecules during different phases of pregnancy have been identified in the ovine placenta, similar to the placenta of other ruminant species, and it has been reported that more than 100 PAG genes are involved in the production of glycoproteins in large and small ruminants (De Carolis et al. 2020; Ledezma-Torres 2009). The production of PAG molecules starts with the implantation of the embryo, and during placentation, these molecules enter the maternal circulation, such that their detection in the ovine maternal blood plasma is possible as early as 18 to 20 days after conception with the radioimmunoassay (RIA) and enzyme immunoassay (EIA) techniques (De Carolis et al. 2020). While the detection of

placental proteins in the maternal blood is a tool most commonly used for pregnancy diagnosis (El Amiri et al. 2007), the plasma and milk levels of PAG/PSPB molecules can also be used for the assessment of the fetal number, and the determination of pregnancy losses and obstetric diseases (Adeyeye et al. 2021; Roberts et al. 2017). Furthermore, the measurement of the levels of PAG/PSPB molecules in ovine peripheral blood samples may provide valuable input for developing feeding strategies for pregnant animals as well as for implementing measures to prevent the occurrence of metabolic disorders in the dam and enable the growth of the fetus (El Amiri et al. 2007). However, PSPB concentrations may vary in ruminants during pregnancy, in association with several parameters, including the genus or subspecies, number of previous births of the dam (parity), fetal number, fetal sex, birth weight, nutritional status of the dam, environmental conditions and method used for the measurement of PSPB concentrations (Adeyeye et al. 2021; De Carolis et al. 2020, Roberts et al. 2017). The present study was aimed at the determination of serum PAG concentrations during pregnancy and the first month postpartum in Karya ewes, one of the major local sheep breeds of Türkiye, and at the investigation of any correlation between the serum concentrations of the steroid hormone progesterone, upon which the continuation of pregnancy depends, and PAG levels throughout pregnancy.

MATERIALS and METHODS

Animals

This study was conducted pursuant to the approval of the Local Ethics Board for Animal Experiments of Pamukkale University. The study was carried out between December 2023 and February 2024 at a sheep farm located in the Serinhisar (Kızılhisar) district of the Denizli province in Türkiye. The study animals were comprised of ten 2- to 5-year-old Karya ewes, which weighed 40-60 kg and were confirmed to have no health problem. Firstly, the ewes were synchronized for oestrus by the insertion of progesterone-impregnated intravaginal sponges (Esponjavet, Hipra, Spain) for a period of 12 days. Immediately after the removal of the sponges, the ewes were administered with 400 IU of equine chorionic gonadotropin (eCG) (Folligon, MSD, Spain). All ewes were allowed to mate naturally. The ewes that had mated were recognized by the paint on their dorsum after the introduction of teaser rams with marking harnesses. Pregnancy diagnosis was made by abdominal ultrasonography (Siui 838, China) on day 35 post-mating (Tekin and Köse, 2022), such that the empty ewes were synchronized and prepared for mating for a second time. Pregnancy was confirmed based on the observation of fetal fluids, placentomes and fetal structures at ultrasonographic examination. Blood samples were collected from each of the ewes into gel-coated vacutainers (Hematube, Ankara, Türkiye) at weeks 1, 2, 3, 4, 5, 6, 7, 8, 9, 11, 13, 15, 17, 18, 19, 20, and 21 after the day of mating (day 0), at the time of parturition, and at weeks 1, 2, 3 and 4 postpartum. Pregnancy was diagnosed by transabdominal ultrasonography on day 35 post-mating. Samples taken during the first 5 weeks from the non-pregnant ewes were excluded from the study, and after the animals were examined for pregnancy, only those confirmed to be pregnant were sampled for blood. The blood samples were firstly allowed to coagulate at room temperature, and were later centrifuged at 3000 rpm for 5 min for the extraction of sera. Until being used for laboratory analyses, the blood serum samples were stored at -20 0C. PAG level and progesterone level during pregnancy and serum PAG level in the first postpartum month were determined. Serum PAG levels were measured using a commercial ELISA kit originally developed for pregnancy diagnosis in cattle (IDEXX Laboratories, Westbrook, ME). The ELISA test was performed by experienced technical staff in accordance with the manufacturer's instructions. The test results were read on a spectrophotometer at a wavelength of 450 nm. The S-N (sample minus negative) values of the samples were calculated by subtracting the optical density (OD) of the negative control from the OD of the sample. Based on the correlation of the PAG concentrations of the samples with the OD values measured, the S-N values were used as the serum PAG levels of the samples. The results of the plasma samples of the ewes were assessed in accordance with the manufacturer's instructions such that values, which were ≥0.3, were considered positive and indicated pregnancy, whilst those, which were <0.3, were considered negative and indicated the absence of pregnancy. At the end of the study, the stored serum samples were quantitatively analyzed for progesterone concentrations at the MG Veterinary Diagnosis Laboratory using chemiluminescence assay (CLIA) kits. The CLIA method is based on the measurement of the light emission resulting from the antigen-antibody reaction with the aid of a calibrated luminometer. The sensitivity and specificity of the CLIA method were 100% and 95.5%, respectively. The specificity of the assay for progesterone and the assay range have been reported as 0.21-60 ng/mL (0.67-190.8 nmol/L). Based on the results of the analyses, the ewes, which were determined to have serum progesterone concentrations of >1.0 ng/ml, were considered to be pregnant.

Statistics

The study data were statistically evaluated using the 'IBM SPSS Statistics 26' software package. Prior to data analysis, the normality distribution of the data was evaluated with the Shapiro-Wilk test. The results are given as mean ± standard deviation. The differences between the two groups were assessed with the Mann-Whitney U test. The correlation

between the variables was evaluated with Spearman's rho correlation coefficient. Statistical significance was set at $p<0.05$.

RESULTS

Progesterone concentrations of the pregnant animals

As can be seen in Figure 1, the plasma P4 concentrations were lowest on the day of mating, but progressively increased as of week 9 of pregnancy and peaked at gestational week (GW) 18. Progesterone levels were observed to decrease after GW 18.

PAG concentrations of the pregnant animals

The mean PAG concentrations of the pregnant animals were observed to be at the basal level until the second week of pregnancy, but increased as of GW 3 and peaked at week 19. PAG concentrations displayed a dramatic decrease during the first 4 weeks postpartum. The PAG levels of the Karya ewes during pregnancy and during the postpartum period are shown in Figure 1 and Figure 2.

The correlation between PAG and P4 concentrations

As can be seen in Table 1, the PAG and progesterone concentrations were determined to be positively correlated with each other at GW 8 (r: 0.699) and GW 11 (r: 0.736) ($p<0.05$). Based on this finding, in the Karya ewes, the increase in the progesterone levels at gestational weeks 8 and 11 were associated with an increase in the PAG levels.

PAG and P4 concentrations in single and multiple pregnancies

As shown in Table 2, the mean PAG level at GW 7 was 0.50±0.11 in the ewes with singleton pregnancy and 0.67±0.09 in the ewes with twin pregnancy, and the Mann-Whitney U test revealed that the difference between the two groups was statistically significant (Z: -2.009; $p<0.05$). Furthermore, the mean PAG level at GW 11 was 0.93±0.21 in the ewes with singleton pregnancy and 1.62±0.20 in the ewes with twin pregnancy, and statistical analysis showed that the two groups significantly differed from each other (Z: -2.514; $p<0.05$). At week 13 of pregnancy, the mean PAG level was 1.04±0.37 in the singleton-pregnant ewes and 1.81±.28 in the twin-pregnant ewes, and it was ascertained that the two groups significantly differed from each other (Z: -2.402; $p<.005$). The mean PAG levels determined at GW 15 were 1.16±.26 and 2.04±.15 in the singleton-pregnant and twin-pregnant ewes, respectively, and the difference between the two groups was found to be statistically significant (Z: -2.611; $p<0.05$). The mean PAG levels of the ewes with singleton and twin pregnancies at GW 17 were 1.38±.31 and 2.22±.24, respectively, and statistical analysis revealed that the difference between the two groups was significant (Z: -2.611; $p<0.05$). During the remaining weeks of pregnancy, namely, at weeks 1, 2, 3, 4, 5, 6, 8, 9, 18, 19, 20 and 21, no statistically significant difference was observed between the mean PAG levels of the ewes with singleton and twin pregnancies ($p>0.05$). Based on the findings obtained in the present study, it was determined that the PAG levels of the twin-pregnant ewes were higher than those of the singleton-pregnant ewes at GWs 7, 11, 13, 15 and 17.

As shown in Table 3, the mean progesterone levels of the ewes with singleton and twin pregnancies at week 11 of pregnancy were 3.22±1.11 and 6.65±2.90, respectively, and the Mann-Whitney U test revealed that the two groups significantly differed from each other (Z: -2.193; $p<0.05$). During the remaining weeks of pregnancy, no such statistically significant difference was determined between the progesterone levels of the singleton-pregnant and twin-pregnant ewes ($p>0.05$). Based on the findings of the present study, it was ascertained that at GW 11, the progesterone levels of the twin-pregnant ewes were higher than those of the singleton-pregnant ewes. No significant difference was determined during the remaining weeks of pregnancy.

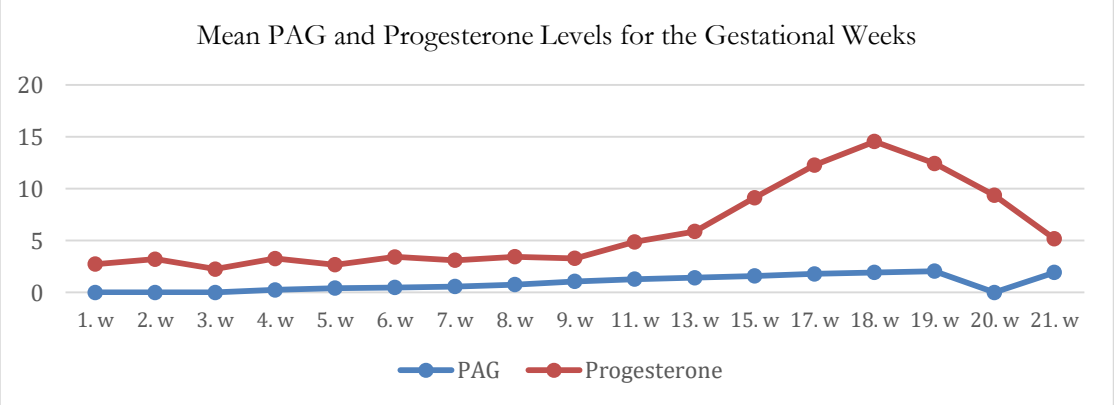

Figure 1. Mean PAG and Progesterone Levels for the Gestational Weeks

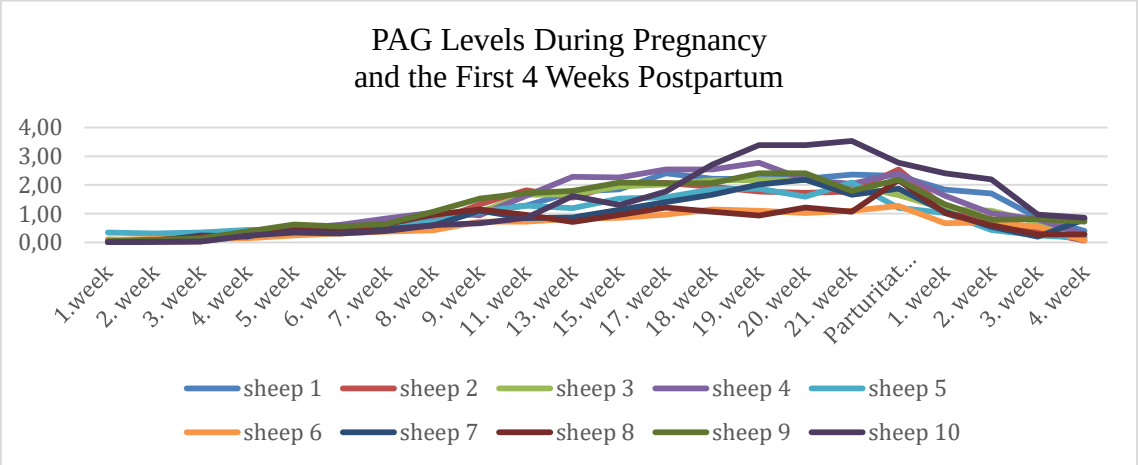

Figure 2. PAG levels during pregnancy and the first 4 weeks postpartum

Table 1. The correlation between PAG and progsterone levels for the gestational weeks (n:10)

Gestational Week	r	p
Week 1	0.228	0.527
Week 2	0.350	0.321
Week 3	-0.463	0.178
Week 4	-0.177	0.625
Week 5	-0.213	0.554
Week 6	0.469	0.172
Week 7	0.278	0.437
Week 8	**0.699**	**0.024***
Week 9	0.576	0.082
Week 11	**0.736**	**0.015***
Week 13	0.261	0.467
Week 15	0.321	0.365
Week 17	0.511	0.132
Week 18	0.285	0.425
Week 19	0.073	0.841
Week 20	-0.103	0.776
Week 21	0.188	0.603

Table 2. PAG differences for singleton and twin pregnancies

Gestational Week	Singleton (n:5) x±ss	Twin (n:5) x±ss	Test	p
Week 1	0.1±0.15	0.04±0.04	Z: -0.106	p: 0.916
Week 2	0.1±0.12	0.04±0.03	Z: -1.178	p: 0.239
Week 3	0.19±0.12	0.06±0.04	Z: -1.471	p: 0.141
Week 4	0.25±0.11	0.26±0.07	Z: -0.420	p: 0.674
Week 5	0.40±0.11	0.44±0.11	Z: -0.525	p: 0.599
Week 6	0.43±0.11	0.55±0.06	Z: -1.946	p: 0.052
Week 7	0.50±0.11	0.67±0.09	**Z: -2.009**	**p: 0.045**
Week 8	0.66±0.20	0.87±0.17	Z: -1.776	p: 0.076
Week 9	0.94±0.23	1.19±0.23	Z: -1.149	p: 0.251
Week 11	0.93±0.21	1.62±0.20	**Z: -2.514**	**p: 0.012**
Week 13	1.04±0.37	1.81±0.28	**Z: -2.402**	**p: 0.016**
Week 15	1.16±0.26	2.04±0.15	**Z: -2.611**	**p: 0.009**
Week 17	1.38±0.31	2.22±0.24	**Z: -2.611**	**p: 0.009**
Week 18	1.69±0.66	2.18±0.23	Z: -1.567	p: 0.117
Week 19	1.86±0.97	2.26±0.37	Z: -1.149	p: 0.251
Week 20	1.88±0.95	2.14±0.25	Z: -0.943	p: 0.346
Week 21	1.89±1	1.99±0.24	Z: -0.731	p: 0.465

Table 3. Differences between the progesterone levels of the ewes with singleton and twin pregnancies for the gestational weeks

Gestational week	Singleton (n:5) x±ss	Twin (n:5) x±ss	Test	p
Week 1	2.60±1.24	2.88±0.82	Z: -0.522	p: 0.602
Week 2	2.52±1	3.92±1.69	Z: -1.576	p: 0.115
Week 3	2±.96	2.50±1.21	Z: -0.736	p: 0.462
Week 4	2.28±1.54	4.28±3.38	Z: -1.149	p: 0.251
Week 5	1.96±0.70	3.40±2.16	Z: -1.358	p: 0.175
Week 6	2.22±0.68	4.64±3.48	Z: -1.781	p: 0.075
Week 7	2.34±0.31	3.86±1.95	Z: -1.681	p: 0.093
Week 8	2.48±0.69	4.40±2.92	Z: -1.467	p: 0.142
Week 9	2.70±0.74	3.88±2.68	Z: -0.940	p: 0.347
Week 11	3.22±1.11	6.65±2.90	**Z: -2.193**	**p: 0.028**
Week 13	4.60±1.73	7.16±4.20	Z: -1.567	p: 0.117
Week 15	7.36±1.24	10.92±4.26	Z: -1.567	p: 0.117
Week 17	8.02±2.05	16.51±7.93	Z: -1.991	p: 0.052
Week 18	8.18±2.78	20.92±14.1	Z: -1.567	p: 0.117
Week 19	8.32±3.97	16.54±10.33	Z: -1.257	p: 0.209
Week 20	6.72±3.14	12.02±9.88	Z: -0.522	p: 0.602
Week 21	3.88±0.98	6.46±4.95	Z: -0.522	p: 0.602

DISCUSSION

The present study was aimed at the investigation of the impact of pregnancy and the postpartum period on the serum PAG levels of ewes and the determination of any correlation between the levels of progesterone, a steroid hormone upon which the maintenance of pregnancy depends, and the levels of PAGs throughout the course of gestation. Previously, our research team had investigated the early pregnancy serum PAG profiles of ewes of the Karya breed, a major and highly prolific sheep breed indigenous to Türkiye (Akköse et al. 2024). To the best of our knowledge, there is no previous study on prepartum and postpartum plasma PAG profiles or the investigation of any possible correlation between these profiles and progesterone levels. In sheep production, pregnancy diagnosis is particularly significant and valuable during the early period, and early pregnancy diagnosis can be made by means of several methods, including among others, transrectal USG, the progesterone (P4) test and the measurement of PAG levels. Among the available clinical methods, USG offers a high level of accuracy for pregnancy diagnosis, but is labor-intensive and time-consuming, especially when performed in large flocks, and thus, efforts continue to develop more practical methods for use on the field. As a well-established alternative to ultrasonographic examination, the detection of high progesterone levels secreted from the ovarian corpus luteum throughout gestation is a reliable determinant for pregnancy diagnosis. While low progesterone concentrations are accurately indicative of oestrus or the absence of conception/pregnancy, the corpus luteum becoming a permanent structure, embryonic death, samples being collected either too early or too late, and some other conditions irrelevant to pregnancy may also lead to the detection of elevated blood progesterone levels and rule out the determinative quality of this parameter. Despite the progesterone hormone being required for the continuation of pregnancy in ewes, the high prevalence of false positive results hinders its use as a specific indicator for pregnancy diagnosis in sheep. On the other hand, PAGs, owing to the specific temporal detection they allow during the attachment of the conceptus, are considered as ideal bioimarkers for pregnancy and are commonly used for the diagnosis of pregnancy in ruminants (Akdeniz 2022; Roberts et al., 2017). In recent years, several studies have been conducted on the use of the measurement of the blood levels of pregnancy-associated glycoproteins (PAGs) as an alternative to ultrasonography. Although pregnancy diagnosis based on the detection of PAG levels requires laboratory equipment, the use of the right test kits have shortened the time required for the laboratory diagnosis of pregnancy. Furthermore, the most recently designed visual inspection kits have enabled both the clinical and on-the-field diagnosis of pregnancy (Chavez et al. 2017; Chavez et al. 2020). The molecular structure of PAGs being well-conserved across ruminant species allows for the use of the less costly and more easily accessible bovine test kits in sheep as well, and thus, a PAG kit originally developed for cattle was used for Karya ewes in the present study. While various research has been carried out to individually assess the aforementioned pregnancy diagnosis methods, only a limited number of previous studies provide a comparative assessment of the three referred methods for their accuracy in pregnancy diagnosis, based on their concurrent use in the same animals. This is the first study investigating the correlation between PAG and progesterone levels in the blood of pregnant Karya ewes throughout gestation, as well as PAG concentrations during the first month postpartum.

Placentation, which starts with implantation, is usually completed by days 50-60 of gestation in sheep and goats (Guillomot 1995). In sheep, corpus luteum-derived progesterone is required only until day 50 of gestation for the maintenance of pregnancy. Later, placental progesterone comes into play, and the placenta becomes the main source of progesterone (Özdemir Salcı 2015). The progesterone level being higher than the threshold value (>1 ng.ml-1) indicates either the presence of a pregnancy-associated functional corpus luteum, a natural oestrus cycle or a pathological condition of the ovaries or uterus (Karen et al. 2003). The findings of our study showed that the mean progesterone concentration was lowest on the day of mating, increased as of the 9th week of pregnancy, and reached its peak level at GW 18. Progesterone levels were observed to decrease after GW 18. In agreement with our study, it has been reported that in both Churra and Merino ewes, progesterone levels increased throughout pregnancy, peaked at weeks 19-20, and decreased two weeks prior to parturition (Ranilla et al.1994). In a previous study on pregnant Sarda and Lacaune ewes, the progesterone levels were determined as 5.26±0.7 ng/mL and 10.9±1 ng/mL, respectively, on gestational day 18, and as 6.05±0.5 ng/mL and 12.0±0.7 ng/mL, respectively, on gestational day 60, and thus, were reported at levels higher than those determined in the present study. In Barbari goats, progesterone concentrations, which were lowest on the day of mating were reported to significantly increase as of the second week of pregnancy, peak between gestational weeks 10-14, and decrease thereafter, reaching the basal level just before parturition (Ujjawala et al. 2013l). On the other hand, different from our study, Humblot et al. (1990) reported no significant increase or decrease in the mean progesterone concentrations of pregnant Alpine goats as of the 21st day of pregnancy.

Humblot et al. (1988) attributed the increase observed in progesterone levels during the first 3 weeks of gestation followed by relatively stabile levels throughout the remaining period of pregnancy to the possibility of progesterone production being regulated by factors other than those involved in antiluteolytic mechanisms. The luteotrophic factors found in the allantoic fluid may also show effect on the production of progesterone by the corpus luteum. As shown in Table 3, the mean progesterone concentrations of the ewes with singleton and twin pregnancies at week 11 of gestation were determined as 3.22±1.11 and 6.65±2.90, respectively, and the difference between the two groups was found to be statistically significant (Z: -2.193; $p<0.05$). During the other weeks of gestation, no statistically significant difference was observed between the progesterone levels of the ewes with singleton and twin pregnancies ($p>0.05$). Different from the present study, there are literature reports indicating a positive correlation between the mean progesterone concentrations of ewes with singleton pregnancy and the mean progesterone concentrations of ewes with twin pregnancy ($r=0.782$; $P<0.01$) (Hall et al. 1992; Ranilla et al. 1997; Ranilla et al. 1994). The mean progesterone concentration of twin-pregnant ewes has been reported to be higher than that of singleton-pregnant ewes between GWs 12-20 (Ranilla et al. 1997). Meshref et al. (2022) reported the level of progesterone measured on the day of mating in singleton- and multiple-pregnant Osseimi ewes as 0.23±0.01 ng/ml and 0.4±0.01 ng/ml, respectively. Progesterone levels measured in singleton- and multiple-pregnant Osseimi ewes have been reported as 2.9±0.01 ng/ml and 3.07±0.01 ng/ml, respectively, on day 10 of pregnancy and 8.35±0.22 and 9.52±0.175 ng/ml, respectively, on day 90 of pregnancy, and it has been indicated that the progesterone concentration increases with a greater number of fetuses (Meshref et al. 2022). Furthermore, Ranilla et al. (1994) and Hall et al. (1992) suggested that the level of progesterone required for the maintenance of pregnancy may vary with the size of the fetus. In parallel with our study, Mukasa-Mugerwa and Viviani (1992) reported that there was no consistent correlation between blood progesterone level and fetal size. The measurement of progesterone levels being costly, requiring a well-equipped laboratory, and depending on the collection of blood samples on a certain day, as well as on the availability of accurate knowledge on the day of mating or artificial insemination reduces the preference of this method for pregnancy diagnosis, when compared to ultrasonography (Medan et al., 2004).

PAG concentrations have been indicated to produce more reliable results for early pregnancy diagnosis than progesterone concentrations, as PAGs enable the differentiation of cases of pregnancy and prolonged oestrus. The earliest day of pregnancy on which PAGs have been detected in sheep in previous studies has been reported as day 20 post-mating (Karen et al. 2003). The present study demonstrated that the mean PAG concentration was at the basal level until the second week of pregnancy and progressively increased as of the third gestational week until reaching the peak at GW 19 (Figure 1 and Figure 2). Ledezma-Torres et al. (2006) and Sousa et al. (2006) described the determination of PAG levels as a reliable method for the on-farm early diagnosis of pregnancy in sheep as of the fourth gestational week. Ledezma-Torres et al. (2006) reported the PAG levels of non-pregnant ewes to be five-fold lower than the levels of pregnant ewes. Similarly, Anghel et al. (2011) confirmed that the PAG concentrations of non-pregnant Merino ewes were below 1.5 ng/ml. In previous studies by Rovani et al. (2016) and Uçar (2017), it was reported that increased PAG OD values were detectable as of days 33 and 35 post-mating. The findings of the present study agree with the reports of these researchers in that the pregnancy-positive values progressively increased as of the third week of pregnancy. Willard et al. (1995) suggested the primary mechanism underlying the passage of PAGs into the maternal circulation as the migration of binucleate cells from the placenta to the maternal endometrium as of the 18th day of pregnancy. Thus, the measurement of PAG levels for early pregnancy diagnosis in sheep before GW 3 may cause false negative results. The present study having demonstrated the increase of PAG concentrations as of the 3rd week post-mating is in agreement with previous reports made for the Karpat goat (Zamfirescu et al. 2011), Awassi and Merino sheep (Anghel et al. 2011; Karen et al. 2003), Sarda sheep (Barbato et al. 2009), a heterogenous group of sheep (Ledezma-Torres et al., 2006), Karya and Merino sheep (Akköse et al. 2024) and the Sirohi goat (Salve et al. 2016). In our study, we determined that PAG levels peaked at gestational week 19, progressively increased throughout pregnancy, and showed a dramatic decrease after parturition. In agreement with our study, Roberts et al. (2017) reported that PAG1 levels steadily increased from day 3 to day 120 of gestation. Moreover, De Carolis et al. (2020) and Gajewski et al. (1999) indicated that PAG concentrations increased until day 60 of pregnancy in sheep, and thereafter, decreased until day 120, reaching a peak level at the time of parturition. The initial tendency of increase followed by a decrease to the initial levels at mid-pregnancy reported for PAG levels in Sarda and Lacaune ewes (De Carolis et al. 2020), Berrichon ewes (Gajewski et al. 1999), Churra ewes (Ranilla et al. 1997), various sheep breeds (Ledezma-Torres et al. 2006), and Osseimi ewes (Meshref et al. 2022) was not observed in the present study in Karya ewes and has not been reported in Merino sheep either. While PAG concentrations between gestational weeks 9-17 vary among sheep breeds, the concentrations detected from GW 17 to lambing have been observed to increase in all breeds

(Karen et al. 2001). This difference could be related to the structure of the placenta and the decrease that occurs in the number of binucleate cells in sheep (De Carolis et al. 2020). Ovine PAG concentrations fluctuate throughout pregnancy in relation to breed, sex and fetal number (Gajewski et al. 1999; Ranilla et al. 1997). In the present study, the PAG levels of the twin-pregnant ewes at gestational weeks 7, 11, 13, 15 and 17 were found to be higher than those of the singleton-pregnant ewes. The demonstration of higher PAG concentrations in cases of multiple pregnancy, when compared to singleton-pregnancy, at the indicated time points, suggests a positive correlation between PAG concentration and fetal number. This finding is in agreement with a previous study reporting higher PAG concentrations in multiple pregnancies, compared to singleton pregnancies, in Sarda ewes (De Carolis et al. 2020). In a study by Ranilla et al. (1997), similarly, it was determined that the mean PAG concentrations of twin-pregnant ewes were higher than the levels of singleton-pregnant ewes, during the period from the GW 12 to lambing. However, this difference was statistically significant only at GW 21 (Karen et al. 2001). Furthermore, Roberts et al. (2017) reported that maternal serum PAG1 concentrations were higher during the last month of pregnancy in twin-pregnant cattle.. The higher PAG concentrations determined in cases of multiple pregnancy, when compared to singleton pregnancy, have been attributed to the larger placental mass, and therefore, the greater number of binucleate giant cells, which are the main source of PAGs, and the greater secretory activity of these cells (Ranilla et al. 1997). The prediction of the fetal number would be of benefit for sheep breeders as it would enable them to feed pregnant ewes accordingly in the prepartum period. Thus, the best option would be to use PAG concentrations as an indicator of multiple fetuses. However, it should be noted that multiple fetuses may not always be determined by measuring PAG concentrations (Roberts et al. 2017).

In the present study, PAG levels showed a rapid decrease after lambing and fell to the basal level by the 4th week postpartum. It was ascertained that the decrease in serum PAG levels occurred more rapidly, and thus, PAG clearance from the blood circulation occurred within a shorter time period in sheep, when compared to cattle (Mukasa-Mugerwa ve Viviani 1992). In parallel with our study, several literature reports have indicated that serum PAG levels, which show a continuous increase from week 17 of pregnancy to lambing, rapidly decrease after parturition and fall to the lowest level by week 4 postpartum (Ranilla et al. 1994; Ranilla et al. 1997; Szenci et al. 1998; Gonzalez et al. 1999; Gajewski et al. 1999; Roberts et al. 2017). It has been reported that PAG concentrations show a progressive decrease in the postpartum period and fall to a level of 0 ng/mL by day 28 postpartum (De Carolis et al. 2020). The decrease determined at the time of parturition could be related to the expulsion of the fetal membranes from ewes within 6 to 12 h after lambing or to a delayed postpartum sampling (Roberts et al. 2017). Differently, in cattle, PAG concentrations decrease slowly in the postpartum period, such that they are still detectable by day 100 after parturition. Therefore, contrary to the case in cattle, the rapid decrease of PAG levels in the postpartum period in ewes is critical to the use of PAGs for pregnancy diagnosis in sheep (De Carolis et al. 2020). However, the use of PAG1 for pregnancy diagnosis may cause false positive results in the event of remating after early pregnancy loss due to residual PAG1 levels remaining from the ended pregnancy (Roberts et al. 2017).

In the present study, as shown in Table 1, a positive correlation was determined between PAG and progesterone levels at GW 8 (r: 0.699) and GW 11 (r: 0.736) ($p<0.05$). Based on this finding, at weeks 8 and 11 of pregnancy, increased progesterone levels were associated with increased PAG levels in the Karya ewes. The present study has determined, for the first time, the correlation between maternal serum progesterone levels and PAG levels throughout pregnancy in Karya ewes. Previous studies in cattle and goats differ in their results, such that while some researchers have reported a positive correlation between progesterone and PAG levels throughout pregnancy (Mercadante et al. 2013; Roberts et al. 2017; Tandiya et al. 2013), some other have reported no such correlation (Lobago et al. 2009). In parallel with our investigation, Roberts et al. (2017) reported a strong correlation between PAG1 and progesterone levels as of mid-pregnancy, and thus, suggested that PAG1 levels could be effectively used as an indicator of placental function throughout gestation in sheep. Furthermore, in a study on Sirohi goats, the correlation coefficient between PAG and progesterone concentrations was found to be statistically significant ($p<0.05$) and positive ($r=0.98$) from day 4 before oestrus to day 28 of pregnancy (Salve et al. 2016). Moreover, it has been reported that, in sheep, PAG and progesterone profiles differ, such that while PSPB displays a bimodal pattern of secretion and peaks twice during pregnancy on gestational days 60 and 120, progesterone concentrations show a steady increase throughout pregnancy (Roberts et al. 2017). Several studies have demonstrated that both progesterone and PAG levels are generally higher in cases of multiple pregnancy, in comparison to singleton pregnancy (Barbato et al. 2009), and that a strong positive correlation exists between progesterone and PAG levels (Roberts et al. 2017). These results differ from the report of Ranilla et al. (1997), which indicated an insignificant correlation between the progesterone and PAG concentrations of twin- and singleton-pregnant ewes. In fact, in the present study, excluding gestational weeks 8 and 11, no correlation was detected between

the progesterone and PAG levels. Similar results have been previously reported for Churra and Merino ewes, suggesting no correlation to exist between progesterone and PAG concentrations throughout gestation (Ranilla et al. 1994).

CONCLUSION

Differences in the results reported for the correlation between PAG and progesterone levels could be due to less frequent sampling, species-specific differences in placental function and PAG cross-reactivity, as well as species- or subspecies-specific differences in PAG and progesterone dynamics during pregnancy. There is need for furthermore comprehensive studies on a greater number of ewes to more specifically determine the plasma PAG and progesterone profiles of Karya ewes throughout pregnancy. Based on our results, we conclude that the concurrent measurement of progesterone and PAG levels at a defined time point of pregnancy could serve as a predictive method for the diagnosis and differentiation of singleton and multiple pregnancies.

Conflict of interest: The authors have no conflicts of interest to report.

Authors' Contributions: CC and MA contributed to the project idea, design and execution of the study. CC and RF contributed to the acquisition of data. CC and MA analysed the data. CC and MA drafted and wrote the manuscript. CC and MA reviewed the manuscript critically. All authors have read and approved the finalized manuscript.

Ethical approval: This research was conducted with the approval of Pamukkale University, Animal Research The Ethics Committee (**PAUHDEK-2023/38).**

Explanation: We have no presented as a oral, poster, abstract vs.

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RESEARCH ARTICLE

Effects of Organic Acid Treatments and Modified Atmosphere Packaging on the Presence of *Staphylococcus aureus* and *Escherichia coli* O157:H7 and Shelf-life in Meatballs

Funda YILMAZ EKER[1], Esra AKKAYA[1*], Enver Barış BİNGÖL[1]

[1]*Department of Food Hygiene and Technology, Faculty of Veterinary Medicine, İstanbul University-Cerrahpaşa, İstanbul, Türkiye*

ABSTRACT

This study was carried out to determine the effects of modified atmosphere packaging (ambient air, 80:20/O_2:CO_2 - MAP-O_2, 0.4:30:69.60/CO:CO_2:N_2 - MAP-CO) combined with organic acid (1% and 2% sodium lactate, 0.5% potassium sorbate, 0.5% sodium citrate and 1% sodium acetate) addition on the presence of *Staphylococcus aureus* and *Escherichia coli* O157:H7 in meatballs and the quality changes during cold storage. In this context, meatball samples experimentally contaminated with *S. aureus* and *E. coli* O157:H7 strains were treated with the relevant organic acids and then subjected to modified atmosphere packaging process. The samples were evaluated in terms of microbiological, physicochemical and sensory parameters during the 15-day storage period. The addition of 2% sodium lactate provided the highest inhibition on the number of *S. aureus* and *E. coli* O157:H7 in meatballs among the organic acids, while the combined CO packaging was the highest inhibition among the treatments. It was determined that off-odour formation was decreased in meatballs added with organic acids and packaged with CO, while red colour stability was achieved in sodium lactate-treated samples, the highest red colour values were observed in meatball samples packaged with CO. In conclusion, the application of organic acid treatment in combination with modified atmosphere packaging inhibits pathogenic microorganisms while prolonging the shelf-life and preserving the sensory properties of meatballs.

Keywords: *Escherichia coli* O157:H7, Meatballs, Modified Atmosphere Packaging, Organic acid, Shelf-life, *Staphylococcus aureus*

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Organik Asit Uygulamalarının ve Modifiye Atmosfer Paketlemenin Köftelerde *Staphylococcus aureus* ve *Escherichia coli* O157:H7 Varlığı ve Raf Ömrü Üzerine Etkileri

ÖZ

Bu çalışma, organik asit (%1 ve %2 sodyum laktat, %0,5 potasyum sorbat, %0,5 sodyum sitrat ve %1 sodyum asetat) ilavesi ile kombine modifiye atmosfer paketleme uygulamalarının (ortam havası, 80:20/O_2:CO_2 - MAP-O_2, 0,4:30:69,60/CO:CO_2:N_2 - MAP-CO) köftelerde *Staphylococcus aureus* ile *Escherichia coli* O157:H7 varlığı üzerine etkilerini belirlemek ve soğuk muhafaza süresince kalite değişimlerini ortaya koymak amacıyla gerçekleştirilmiştir. Bu kapsamda, deneysel olarak *S. aureus* ve *E. coli* O157:H7 suşları ile kontamine edilen köfte örnekleri ilgili organik asitler ile muamele edildikten sonra modifiye atmosfer paketleme işlemine tabi tutulmuştur. Örnekler 15 günlük muhafaza süresi boyunca mikrobiyolojik, fizikokimyasal ve duyusal parametreler yönünden değerlendirilmiştir. %2 sodyum laktat ilavesi köftelerdeki *S. aureus* ve *E. coli* O157:H7 sayısı üzerinde organik asitler içerisinde en yüksek inhibisyonu sağlarken, kombine olarak CO ile paketleme uygulamalar arasında en yüksek inhibisyon kaydedilen işlem olmuştur. Organik asit ilave edilen ve CO ile paketlenen köftelerde kötü koku oluşumunun azaldığı, sodyum laktat ile muamele edilen örneklerde kırmızı renk stabilitesi sağlanırken en yüksek kırmızı renk değerlerinin CO ile paketli köfte örneklerinde gözlendiği belirlenmiştir. Sonuç olarak, organik asit muamelesinin modifiye atmosfer paketlemeyle birlikte uygulanması patojen mikroorganizmalar üzerinde inhibisyon oluştururken, köftelerin raf ömrünün uzamasına ve duyusal özelliklerinin de korunmasına olanak sağlamaktadır.

Anahtar Kelimeler: *Escherichia coli* O157:H7, Köfte, Modifiye Atmosfer Paketleme, Organik Asit, Raf Ömrü. *Staphylococcus aureus*

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ORCID ID; *FYE: 0000-0003-4315-5363, EA: 0000-0002-2665-4788, EBB: 0000-0002-6452-4706*

*Corresponding author e-mail: esra.akkaya@iuc.edu.tr

GİRİŞ

Kırmızı et, değerli proteinleri yüksek düzeyde içermesi sebebiyle elzem bir besin kaynağıdır. Aynı zamanda pek çok farklı ürüne dönüştürülerek tüketime sunulan kırmızı et, bozulma yapıcı ve patojen mikroorganizmaların üremesi için riskli gıdalar arasındadır (Djenane ve ark. 2016; Djenane ve Roncalés 2018). Özellikle kıyma ve köfte gibi et ürünleri, yapısal özellikleri nedeniyle mikrobiyal kontaminasyon açısından yüksek risk taşıyan ve bu nedenle koruyucu müdahaleler gerektiren gıdalar olarak değerlendirilmektedir. Bunun yanı sıra; köftenin hijyenik kalitesi üretim yöntemine, çiğ kıymaya eklenen baharat ve diğer bileşenlerin kalitesine ve üreticilerin kişisel hijyenine bağlı olarak değişebilmektedir (Bingol ve ark. 2012; Bingol ve ark. 2014; Meng ve ark. 2022). Belirli bir mikroorganizma yüküne sahip köfte gibi et ürünlerinde saklama koşullarının bozulması, paketleme hataları, yetersiz ısı uygulamaları gibi dış faktörler de gıda zehirlenmesi riskini arttırarak halk sağlığını olumsuz yönde etkileyebilmektedir. Bu bağlamda, et ürünlerinde söz konusu risk durumunu bertaraf etmek için çeşitli muhafaza yöntemleri uygulanmaktadır. Soğutma ve dondurma işlemi, kıyma ve köftelerin muhafazası için en çok tercih edilen geleneksel yöntemlerdendir (Ozturk ve ark. 2017). Bunlara ilaveten, antimikrobiyal maddelerin kullanımı da bozulma yapıcı ve patojen mikroorganizmaların gelişimini önleyerek veya inhibisyonuna neden olarak gıda maddesinin hijyenik kalitesini sağlamak ve raf ömrünü uzatmak amacıyla tercih edilen başarılı muhafaza yöntemlerinden biridir (Pegg ve Shahidi 2000; Theron ve Lues 2007). Bu antimikrobiyal maddeler arasında organik asitler ve tuzları; erişilebilirlikleri, toksik olmamaları ve yüksek etkinlikleri nedeniyle et endüstrisinde yaygın olarak kullanılan doğal bileşiklerdir (Coban 2020). Et ürünlerinde kullanılan bu asitler GRAS (genel olarak güvenli kabul edilen) listesinde yer almakta olup, FDA (U.S. Food and Drug Administration, Amerikan Gıda ve İlaç Dairesi) tarafından onaylı maddelerdir (Mir ve Masoodi 2018).

Laktatlar, asetatlar, sitratlar ve sorbatlar gıda endüstrisinde ürünün yapısal özelliklerini geliştirmek ve mikrobiyal büyümeyi yavaşlatmak için kullanılan organik katkı maddeleridir. Laktatlar (L(+)-laktik asit tuzları) mikrobiyal güvenliği sağlamak, raf ömrünü uzatmak, renk stabilitesi sağlamak ve lipid oksidasyonunu en aza indirmek için yaygın olarak kullanılan antimikrobiyal ajanlardır (Kim ve ark. 2006; Mancini ve ark. 2009; Mancini ve ark. 2010). Asetatlar, asetik asidin düşük pKa (asidik iyonlaşma sabiti; 4,76) değeri ve asetatların büyük bir kısmının ayrışmamış formda kalması nedeniyle et ve et ürünlerinde kullanılan güçlü antimikrobiyal maddeler olarak bilinmektedir. Asetatların antioksidan etkileri et ürünlerinin rengini ve lipid oksidasyonunu iyileştirmektedir (Lee ve ark. 2005; Mir ve Masoodi 2018). Sitratlar, et ürünlerinin görünümünü, lezzetini ve raf ömrünü iyileştirebilen potansiyel antioksidan ve antimikrobiyal koruyuculardır (Igwegbe ve ark. 2019). Diğer bir koruyucu ajan olan sorbatlar ise hem antifungal özellikleri hem de zayıf asitlikleri (pKa = 4.76) ve ayrışmamış asit formları nedeniyle antimikrobiyal amaçla kullanılmaktadır (Stopforth ve Kudron 2020).

Ürün formülasyonuna eklenerek gıda güvenliğinin sağlanmasına yardımcı olan organik asit ve tuzlarının yanı sıra, ürünün albenisini arttırması ve raf ömrünü uzatması nedeniyle modern paketleme teknikleri de sıklıkla tercih edilen uygulamalar olmuştur. Modifiye atmosfer paketleme (MAP), et ürünlerinin raf ömrünü uzatmayı, mikrobiyal ve duyusal kalitesini korumayı amaçlayan modern paketleme tekniklerinin başında gelmektedir (Bingol ve Ergun 2011; Jaspal ve ark. 2021). Karbondioksit (CO_2), oksijen (O_2) ve nitrojen (N_2) MAP'de yaygın olarak kullanılan gazlardandır (Jaspal ve ark. 2021). Ayrıca, kırmızı rengi stabilize ederek, mikrobiyal gelişmeyi baskılayarak, oksidasyonu ve kemik kararmasını önleyerek, lezzet kabul edilebilirliğini arttırarak etin istenen özelliklerinin korunması için et endüstrilerinde düşük seviyelerde karbonmonoksit (CO) kullanılmasına da izin verilmektedir (Cornforth ve Hunt 2008; Djenane ve Roncalés 2018; Mortazavi ve ark. 2023).

Bu kapsamda, çalışmamızda farklı organik asit uygulaması ile kombine olarak modifiye atmosfer paketlemenin köftelerdeki *Staphylococcus aureus* ve *Escherichia coli* O157:H7 gelişimi ile kalite parametreleri üzerindeki etkilerinin incelenmesi amaçlanmıştır.

MATERYAL ve METOT

Deneysel Köfte Üretimi

Köfte örneklerinin hazırlanmasında kullanılan kıyma, galeta unu, kuru soğan ve baharatlar İstanbul'daki yerel bir marketten temin edilmiş olup, üretim sürecine kadar İstanbul Üniversitesi-Cerrahpaşa Veteriner Fakültesi Besin Hijyeni ve Teknolojisi Bölümü laboratuvarlarında uygun koşullar altında muhafaza edilmiştir.

Deneysel köfte üretimi için %10 yağ içeren dana kıyma (%84), galeta unu (%8,3), ince doğranmış soğan (%3), tuz (%2,2), kimyon (%1), karabiber (%0,3), sarımsak (%0,5) ve maydanoz (%0,7) karıştırılarak köfte hamuru elde edilmiştir. Hazırlanan köfte hamuru üç eşit parçaya ayrılarak biri kalite analizlerinde kullanılmak, diğerleri ise ilgili bakteri solüsyonları (*Staphylococcus aureus* ve *Escherichia coli* O157:H7) ile ayrı ayrı kontamine edilmek üzere ayrılmıştır.

Kontamine edilen ve edilmeyen her bir köfte hamuru biri kontrol grubu olmak üzere, beş eşit gruba ayrılmış ve Tablo 1'de belirtilen organik asit çözeltileri ile muamele edilmiştir. Bunun akabinde, organik asitlerle muamele edilen her bir gruptaki köfte hamuru farklı gaz karışımları ile paketleme uygulamaları için üçer alt gruba ayrılarak paketlenmiştir.

Tablo 1. Deneysel köfte üretiminde kullanılan organik asit tuzları
Table 1. Organic acid salts used in experimental meatball production

Grup	Potasyum sorbat (%)	Sodyum sitrat (%)	Sodyum asetat (%)	Sodyum laktat (%)
K (Kontrol)	-	-	-	-
NaL_1	-	-	-	1,0
NaL_2				2,0
A Sol. (A Solüsyonu)	0,5	0,5	1,0	-
B Sol. (B Solüsyonu)	0,5	0,5	-	1,0

Kültürlerin Hazırlanması ve İnokülasyonu
Staphylococcus aureus (ATCC 29213) ve *Escherichia coli* O157:H7 (ATCC 700927) suşları Microbiologics® (Minnesota, ABD) firmasından temin edilmiştir. Suşlar Tryptone Soy Agar'a (Oxoid CM131, UK) ekilerek 35°C'de 24 saat inkübe edilmiştir. İnkübasyonun ardından, *Staphylococcus aureus* kolonileri Brain Heart Infusion Broth'a (Oxoid CM1135, UK), *Escherichia coli* O157:H7 kolonileri ise Tryptone Soy Broth'a (Oxoid CM0129, UK) geçilerek 37°C'de 18 saat inkübasyona bırakılmıştır.
Kültürler kullanılmadan önce, her bakteriye ait süspansiyonun optik yoğunluğu spektrofotometrede (Shimadzu UV-1202 UV-VIS, Japonya) 620 nm dalga boyunda ölçülmüştür. Süspansiyon, McFarland Densitometre DEN-1 (Biosan) cihazı ile 1 McFarland standart değerine (3×10^8 kob/ml) ulaşıncaya kadar TSB içinde sulandırılmıştır.
Konsantrasyonlar daha sonra 10^6 kob/ml düzeyine ulaşıncaya kadar peptonlu su (Oxoid CM0733, UK) içerisinde seyreltilmiştir ve gruplara ayrılan köfte hamurları her bir bakteri için ayrı olarak hazırlanan sıvı süspansiyon ile karıştırılmıştır. Ortam sıcaklığında 10-15 dakika süreyle bekletilen köfte hamurları porsiyonlara ayrılana kadar 4°C'de bekletilmiştir.

Köfte Örneklerinin Paketlenmesi
Deneysel olarak kontamine edilen ve kontamine edilmemiş köfte hamurları, organik asit muamelelerinin ardından (Tablo 1) 3,5 cm yarıçaplı ve 25±2 g'lık yuvarlak köfteler haline getirilerek, düşük O_2 geçirgenliğine (8–12 $cm^3/m^2/24$ sa) sahip polietilen kaplar içerisine (yaklaşık 300 g olarak) yerleştirilmiştir. Köfte örnekleri ortam havası ve modifiye atmosfer (MAP-O_2: 80:20/O_2:CO_2 ve MAP-CO: 0,4:30:69,60/CO:CO_2:N_2) ile vakumlu kapama makinası (VTK 40 SC, Ponapack, İstanbul, Türkiye) kullanılarak paketlenmiştir.
Paketlenen köfteler 15 gün süreyle buzdolabı sıcaklığında (4±1°C) muhafaza edilmiş ve depolamanın 1., 3., 6., 9., 12. ve 15. günlerinde ilgili analizlere tabi tutulmuştur. Deneysel köfte üretimi farklı tarihlerde üç kere tekrarlanmıştır.

Mikrobiyolojik Analizler
Her bir grup için aseptik koşullar altında steril poşetlere 25 g olacak şekilde alınan köfte örnekleri üzerine 225 ml steril peptonlu su (Oxoid, CM0061) eklenerek, stomacher cihazında (Interscience, Fransa) homojenize edilmiştir (ISO 6887-1 2017). Ana dilüsyondan sulandırıcı kullanılarak hazırlanan seri dilüsyonlar ile *Staphylococcus aureus* ve *Escherichia coli* O157:H7 sayımı gerçekleştirilmiştir.

***Staphylococcus aureus* sayımı**
Staphylococcus aureus sayısının saptanması amacıyla Baird–Parker Agar (BPA - Oxoid CM0275) besiyerine yayma plak ekim yöntemi ile ekim yapılmış ve 35°C'de 24-48 saat inkübasyondan sonra üreyen bütün tipik koloniler değerlendirilmiştir. *S. aureus* şüpheli görülen koloniler ise doğrulama amacıyla DNAse, koagulaz ve katalaz testlerine tabi tutulmuştur (ISO 6888-1 2021).

***Escherichia coli* O157:H7 sayımı**
Escherichia coli O157:H7 sayısının belirlenmesi için Cefixime-Tellurite Supplement (Oxoid, SR0172) ilave edilen Sorbitol MacConkey (SMAC) Agar'a (Oxoid, CM0813) ekim yapılmıştır. 35°C'de 20-24 saatlik inkübasyondan sonra tipik koloniler O157 ve H7 antiserumları ile doğrulama işlemine tabi tutularak değerlendirilmiştir (ISO 16654 2001/Amd 2 2023).

Fiziko-kimyasal Analizler
Kontamine edilmemiş köfte örneklerinin pH değerleri, dijital bir pH metre (Hanna HI 9321) kullanılarak 10 g örneğin 100 ml saf su içerisinde homojenize edilmesini takiben saptanmıştır (AOAC 2005).
Küçük parçalar halinde kıyılan köfte örneklerinin su aktivitesi (a_w) değerleri, su aktivitesi ölçüm cihazı (Decagon AquaLab Series 4TE) kullanılarak belirlenmiştir (ISO 18787 2017).

Duyusal Analiz
Köfte örneklerinin duyusal özellikleri 12 kişilik eğitimli panelist (28-47 yaşları arasında, 5 kadın ve 7 erkek) grubu tarafından değerlendirilmiştir (ISO 8586 2023). Duyusal değerlendirmeden önce panelistlere seçilen özelliklerin her biri için iki ayrı oturumda standartlaştırılmış bir prosedür (ISO 13299 2016) kullanarak bilgi verilmiştir. Her bir numune çiğ olarak servis edilmiş ve panel soğuk depolamanın 3., 9. ve

15. günlerinde iki oturumda üç tekrar olarak gerçekleştirilmiştir.
Panelistler köfte örneklerini renk ve koku kriterleri yönünden vizüel olarak 9 puanlık iki yönlü-bipolar skala ile değerlendirmiş ve örneklerin puanlamaları sonrasında elde edilen sonuçların aritmetik ortalamalar alınarak hesaplamalar yapılmıştır.

İstatistiksel Analiz
Uygulama grupları arasındaki istatistiksel karşılaştırma zaman periyoduna göre SPSS programının General Linear Model (GLM) prosedürü kullanılarak hesaplanmıştır (SPSS 21.00). İnteraksiyonun önemli bulunduğu durumlarda gruplar arasındaki farkın önem kontrolü için Duncan testi uygulanmıştır.

BULGULAR

İnoküle edilmiş köfte örneklerine ait *Staphylococcus aureus* sayısındaki değişimler
Deneysel olarak kontamine edilen köftelerin muhafaza süresi boyunca *Staphylococcus aureus* sayısındaki değişimler Tablo 2'te gösterilmiştir. Organik asitlerle muamele edilen köfte örneklerindeki *S. aureus* sayısı 15 günlük muhafaza süresince azalma gösterirken, kontrol grubu örneklerindeki bakteri sayısında artış kaydedilmiş ve bu değişim, muhafazanın 6. gününden itibaren önemli bulunmuştur ($p<0,05$). Bunun yanı sıra, başlangıç yükü 5,462 log kob/g düzeyinde olan köfte örneklerinden MAP uygulamasına tabi tutulanların *S. aureus* sayıları muhafaza süresinin sonunda ortam havası ile paketlenenlerden 0,2-0,5 log kob/g daha düşük olarak kaydedilmiştir (Tablo 2). Ortam havasıyla paketlenmiş organik asit gruplarında muhafaza süresinin sonunda 0,3-0,9 log kob/g düzeyinde azalma gözlenirken; yüksek O_2 ile paketlenen köftelerde 0,4-1 log kob/g, CO ile paketlenen örneklerde ise 0,7-1,3 log kob/g inhibisyon belirlenmiştir (Şekil 1). Farklı gaz karışımları ile modifiye atmosfer paketleme uygulaması, muhafazanın 3. gününden itibaren gruplar arasında istatistiksel olarak önemli bir farklılık oluşturmuştur ($p<0,05$).
Organik asitler içerisinde %2 NaL ilavesi köftelerdeki *S. aureus* sayısı üzerinde en yüksek inhibisyonu sağlarken, bunu B solüsyonu ile muamele edilen örnekler izlemiştir. Muhafaza süresince en yüksek *S. aureus* inhibisyonu, %2 NaL ilavesiyle üretilen ve CO ile paketlenmiş köfte örneklerinde kaydedilmiştir (Şekil 1).

Tablo 2. Organik asit ve paketleme uygulamalarının soğuk muhafaza altındaki köftelerin *Staphylococcus aureus* sayıları üzerine etkisi (Log kob/g)
Table 2. Effect of organic acid and packaging treatments on *Staphylococcus aureus* counts of meatballs under cold storage (Log cfu/g)

Özellik	Grup	Muhafaza süresi (4°C)					
		1. gün	3. gün	6. gün	9. gün	12. gün	15. gün
Uygulama	K	5,544	5,648	6,011^{a}	6,181^{a}	6,241^{a}	6,059^{a}
	NaL_1	5,363	5,337	5,544^{b}	5,438^{b}	5,219^{b}	4,944^{b}
	NaL_2	5,300	5,227	4,974^{c}	4,877^{c}	4,579^{c}	4,409^{c}
	A Sol.	5,412	5,498	5,710^{ab}	5,529^{b}	5,323^{b}	5,014^{b}
	B Sol.	5,350	5,270	5,465^{b}	5,285^{b}	4,961^{b}	4,704^{bc}
	SE	0,043	0,121	0,146	0,084	0,183	0,121
	P	0,675	0,111	**0,000**	**0,000**	**0,000**	**0,000**
Paketleme	Hava	5,573	5,638^{a}	5,717^{a}	5,662^{a}	5,545^{a}	5,242^{a}
	MAP-O_2	5,343	5,338^{b}	5,537^{ab}	5,472^{ab}	5,267^{b}	5,096^{a}
	MAP-CO	5,266	5,211^{b}	5,370^{b}	5,252^{b}	4,981^{c}	4,740^{b}
	SE	0,094	0,044	0,026	0,028	0,094	0,056
	P	0,072	**0,010**	**0,047**	**0,016**	**0,001**	**0,002**
Uygulama × Paketleme		1,000	1,000	1,000	1,000	0,998	0,997

K: Kontrol, NaL_1: %1 Sodyum laktat, NaL_2: %2 Sodyum laktat,
A Sol.: %0,5 Potasyum sorbat + %0,5 Sodyum sitrat + %1,0 Sodyum asetat
B Sol.: %0,5 Potasyum sorbat + %0,5 Sodyum sitrat + %1,0 Sodyum laktat
Hava: Ortam havası ile paketleme, MAP-O_2: 80:20/O_2:CO_2 gaz karışımları ile paketleme, MAP-CO: 0,4:30:69,60/CO:CO_2:N_2 gaz karışımları ile paketleme
SE: Standart hata ^{a,b,c}: Aynı sütundaki farklı harfler istatistiksel açıdan birbirinden anlamlı olarak farklıdır ($p<0,05$).

İnoküle edilmiş köfte örneklerine ait *Escherichia coli* O157:H7 sayısındaki değişimler

Deneysel olarak kontamine edilen köfte örneklerinin muhafaza süresi boyunca *Escherichia coli* O157:H7 sayısındaki değişimler Tablo 3'de gösterilmiştir. Başlangıç *E. coli* O157:H7 sayısı 5,491 log kob/g olan örneklerin bakteri sayıları bütün uygulama gruplarında zaman içerisinde artış göstermiştir. Organik asitlerle muamele edilen köfte örneklerine ait *E. coli* O157:H7 sayısında muhafaza süresi boyunca ortam havası ile paketlenenlerde 0,7-1,4 log kob/g, yüksek O_2 içerenlerde ise 0,2-1 log kob/g düzeyinde artış gösterirken; CO içeren paketlerde 0,2-0,5 log kob/g düzeyinde azalma gözlenmiştir. Organik asit uygulamalarına bağlı olarak *E. coli* O157:H7 sayısında gözlenen değişimler muhafazanın 9. gününden itibaren önemli bulunmuştur ($p<0,05$).

Ortam havası ile paketlenen kontrol grubu örneklerindeki *E. coli* O157:H7 sayısı muhafazanın sonunda 1,9 log kob/g düzeyinde artış gösterirken, organik asit içeren gruplarda bu değişim 1,2-1,7 log kob/g olarak belirlenmiştir. Yüksek O_2 ile paketlenen kontrol grubu örneklerinin bakteri sayısındaki artış muhafaza süresinin sonunda 1,5 log kob/g olarak tespit edilirken, organik asit ile muamele edilen gruplarda 0,7-1 log kob/g düzeyinde artış gözlenmiştir. CO ile paketlenen kontrol grubu örneklerindeki bakteri sayısı ise 0,5 log kob/g düzeyinde artarken, organik asit gruplarında 0,04-0,4 log kob/g artış kaydedilmiştir (Şekil 1). Farklı gaz karışımları ile modifiye atmosfer paketleme muhafazanın 3. gününden itibaren gruplar arasında önemli bir farklılık oluşturmuştur ($p<0,05$).

Organik asitler içerisinde %2 NaL ilavesi köftelerdeki *E. coli* O157:H7 sayısı üzerinde en yüksek inhibisyonu sağlarken, bunu sırasıyla B solüsyonu, A solüsyonu ve %1 NaL ilavesi izlemiştir. Muhafaza süresince en yüksek *E. coli* O157:H7 inhibisyonu %2 NaL ilavesiyle üretilen CO ile paketli köfte örneklerinde kaydedilmiştir (Şekil 1).

Tablo 3. Organik asit ve paketleme uygulamalarının soğuk muhafaza altındaki köftelerin *Escherichia coli* O157:H7 sayıları üzerine etkisi (Log kob/g)

Table 3. Effect of organic acid and packaging treatments on *Escherichia coli* O157:H7 counts of meatballs under cold storage (Log cfu/g)

Özellik	Grup	Muhafaza süresi (4°C)					
		1. gün	3. gün	6. gün	9. gün	12. gün	15. gün
Uygulama	K	5,573	5,561	5,804	6,117^{a}	6,565^{a}	6,798^{a}
	NaL_1	5,477	5,494	5,575	5,895^{ab}	6,230^{ab}	6,530^{ab}
	NaL_2	5,357	5,360	5,439	5,569^{b}	5,798^{c}	6,133^{c}
	A Sol.	5,440	5,465	5,623	5,851^{ab}	6,103^{bc}	6,397^{bc}
	B Sol.	5,386	5,446	5,580	5,722^{b}	5,923^{bc}	6,249^{bc}
	SE	0,043	0,121	0,086	0,042	0,063	0,242
	p	0,746	0,834	0,345	**0,042**	**0,001**	**0,006**
Paketleme	Hava	5,553	5,702^{a}	5,986^{a}	6,359^{a}	6,694^{a}	7,019^{a}
	MAP-O_2	5,430	5,531^{a}	5,750^{a}	5,869^{b}	6,180^{b}	6,501^{b}
	MAP-CO	5,357	5,163^{b}	5,077^{b}	5,263^{c}	5,497^{c}	5,744^{c}
	SE	0,054	0,062	0,094	0,086	0,022	0,094
	p	0,346	**0,001**	**0,000**	**0,000**	**0,000**	**0,000**
Uygulama × Paketleme		1,000	1,000	0,964	0,984	0,991	0,997

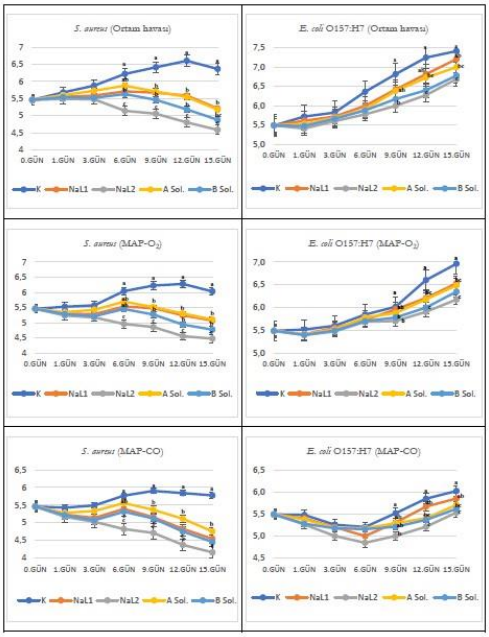
K: Kontrol, NaL_1: %1 Sodyum laktat, NaL_2: %2 Sodyum laktat,

A Sol.: %0,5 Potasyum sorbat + %0,5 Sodyum sitrat + %1,0 Sodyum asetat

B Sol.: %0,5 Potasyum sorbat + %0,5 Sodyum sitrat + %1,0 Sodyum laktat

Hava: Ortam havası ile paketleme, MAP-O_2: 80:20/O_2:CO_2 gaz karışımları ile paketleme, MAP-CO: 0,4:30:69,60/CO:CO_2:N_2 gaz karışımları ile paketleme, SE: Standart hata

^{a,b,c}: Aynı sütundaki farklı harfler istatistiksel açıdan birbirinden anlamlı olarak farklıdır ($p<0,05$).



Şekil 1: Organik asit ilavesi ile üretilen modifiye atmosfer paketli köftelerin soğuk muhafaza süresince *Staphylococcus aureus* ve *Escherichia coli* O157:H7 sayılarındaki değişimler (Log kob/g)
Fig 1: Changes in *Staphylococcus aureus* and *Escherichia coli* O157:H7 counts during cold storage of modified atmosphere packaged meatballs produced with organic acid addition (Log cfu/g)

Bakteri inoküle edilmemiş köftelerin fiziko-kimyasal özelliklerindeki değişimler

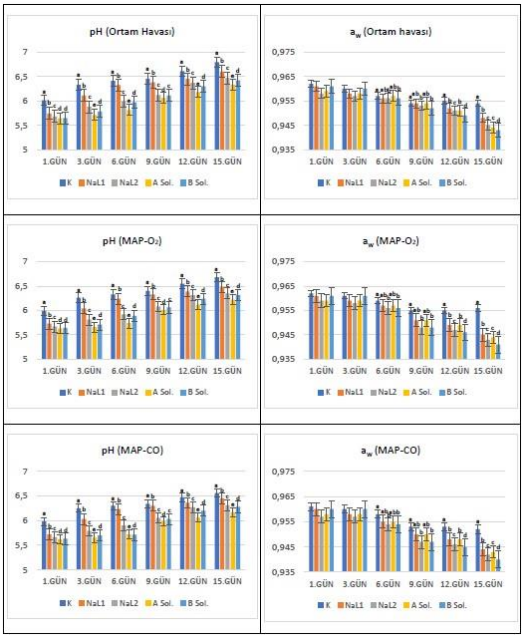
Bütün uygulama gruplarındaki köftelerin pH değerleri depolama süresi boyunca artış göstermiştir. Hem organik asit uygulanan gruplar arasındaki hem de modifiye atmosfer paketleme uygulamalarındaki farklılıklar muhafaza süresi boyunca istatistiksel olarak önemli bulunmuştur (Şekil 2, $p<0,05$).

Başlangıç pH değeri 5,77 olan köftelerin muhafaza süresi sonunda kontrol grubundaki pH değeri 6,5'i geçerken, bu gruba ait en düşük değerler CO ile paketli örneklerde gözlenmiştir. Organik asit ilave edilen köftelerin pH değerleri, bütün paketleme gruplarında kontrol örneklerinden daha düşük seyretmiştir. Ortam havası ile paketli kontrol grubu köftelerin pH değeri muhafazanın 3. gününden itibaren 6,3'ün üzerindeyken; %1 NaL ilave edilen köfteler 6. günden, %2 NaL ve B solüsyonu ilave edilen köfteler 12. günden, A solüsyonu ilave edilen köftelerin pH değerleri ise 15. günden itibaren bu değerin üzerinde belirlenmiştir.

CO ile paketlenen kontrol grubu örneklerinin pH değeri muhafazanın 6. gününde 6,3'e ulaşırken; %1 NaL ilave edilen köfteler 9. günde, %2 NaL ilave edilen köfteler 15. günde bu değere ulaşmıştır. Buna karşın, A ve B solüsyonları ilave edilerek üretilen köfteler muhafazanın 15. gününde bile bu değere ulaşmamıştır. Organik asit ilavesinin yanı sıra modifiye atmosfer paketlemede CO kullanımı, köftelerin pH değerlerindeki artışın daha düşük seviyelerde kalmasını sağlamıştır (Şekil 2, $p<0,05$).

Köftelerin su aktivitesi değerleri ise muhafaza süresi boyunca azalma göstermiştir (Şekil 2). Farklı organik asit ilavesiyle üretilen köfte örneklerinin a_w değerleri arasındaki farklılık, muhafazanın 6. gününden itibaren istatistiksel olarak önemli bulunurken; benzer şekilde farklı gaz karışımları ile modifiye atmosfer paketlenen gruplar arasında da muhafazanın 6. gününden itibaren önemli bir farklılık belirlenmiştir ($p<0,05$).

Başlangıçta 0,965 olarak ölçülen köfte örneklerinin a_w değeri, 15 günlük muhafazanın sonunda 0,940-0,956 değerlerine düşmüştür. Kontrol grubu köftelerinin a_w değerleri, organik asit ilave edilen gruplara göre nispeten daha yüksek seyretmiştir. Organik asit ilavesine bağlı olarak a_w değerlerinde azalma tespit edilirken, muhafaza süresinin sonunda en düşük a_w değerleri B solüsyonu ve %2 NaL ilave edilen köftelerde ölçülmüştür. Ayrıca, ortam havası ile paketlenen köfte örneklerinin a_w değerleri, diğer paketleme gruplarına göre nispeten daha yüksek seyrederken, en düşük a_w değerleri CO ile paketlenen köfte örneklerinde saptanmıştır.



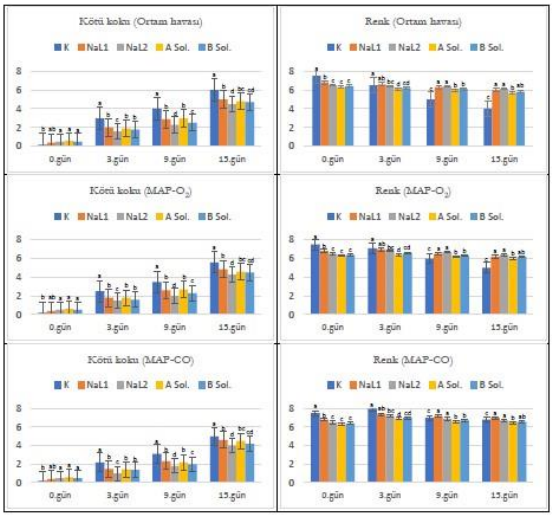
Şekil 2: Organik asit ilavesi ile üretilen modifiye atmosfer paketli köftelerin soğuk muhafaza süresince pH ve a_w değerlerindeki değişimler
Fig 2: Changes in pH and a_w values of modified atmosphere packaged meatballs produced with organic acid addition during cold storage

Bakteri inoküle edilmemiş köftelerin duyusal özelliklerindeki değişimler

Farklı organik asit ilavesi ile üretilen modifiye atmosfer paketli köftelerin muhafaza süresi boyunca koku ve renk özelliklerindeki değişimler Şekil 3'te gösterilmiştir. Farklı organik asitler ve kombinasyonları ile muamele edilen uygulama gruplarına ait koku değerleri muhafaza süresi boyunca önemli bir farklılık göstermiştir ($p<0,05$). Kötü koku oluşumu 15 günlük muhafaza süresi boyunca artış gösterirken, organik asit ilave edilen gruplardaki kötü koku oluşumu kontrol gruplarına göre daha az olarak

belirlenmiştir. Depolama sürecinde paketlerde oluşan kötü koku teşekkülü en az %2 NaL ilave edilen köftelerde tespit edilirken, bunu sırasıyla B ve A solüsyonları ilave edilen örnekler takip etmiştir. Modifiye atmosfer paketleme uygulamaları arasındaki farklılık muhafazanın 3. gününden itibaren istatistiksel olarak önemli bulunmuştur ($p<0,05$). Paketleme grupları içerisinde en fazla kötü koku oluşumu ortam havası ile paketlenen örneklerde gözlenirken, CO ile paketlenen köftelerde kötü koku oluşumu en az olarak belirlenmiştir.

Organik asit uygulanan gruplara ait renk değerleri muhafaza süresi boyunca azalırken, bu değişim istatistiksel olarak önemli bulunmuştur ($p<0,05$). Muhafaza periyodunun başlangıcında organik asit ilave edilen gruplardaki köftelere ait kırmızı renk kontrol grubu köftelerine göre daha az olarak belirlenirken, bu durum muhafazanın 3. gününden sonra değişiklik göstermiş ve muhafazanın 9. gününde yapılan değerlendirmede kontrol grubu köftelerindeki kırmızı rengin (CO ile paketlenen örnekler hariç) organik asit ilave edilen örneklerden daha az olduğu belirlenmiştir. Kırmızı renk stabilitesi en yüksek NaL ilave edilen köftelerde tespit edilirken, en düşük olarak A solüsyonu eklenen köftelerde saptanmıştır. Modifiye atmosfer paketleme uygulamaları arasındaki farklılık muhafazanın 3. gününden itibaren istatistiksel olarak önemli bulunurken ($p<0,05$); en yüksek kırmızı renk değeri CO ile paketlenen köfte örneklerinde belirgin olarak gözlenmiştir.



Şekil 3: Organik asit ilavesi ile üretilen modifiye atmosfer paketli köftelerin soğuk muhafaza süresince duyusal değerlerindeki (koku ve renk) değişimler

Fig 3: Changes in sensory values (odour and colour) of modified atmosphere packaged meatballs produced with organic acid addition during cold storage

TARTIŞMA

Köfte gibi et ürünleri mikrobiyal bozulmaya ve lipid oksidasyona eğilimli, kolay bozulabilir gıda ürünleri olmaları sebebiyle raf ömürlerini arttırmak için farklı muhafaza yöntemlerine ihtiyaç duyulmaktadır. Bu sebeple son yıllarda, doğal koruyucular ile paketleme koşullarının birlikte uygulandığı muhafaza tekniklerinin kullanımı önem kazanmıştır. Organik asitler ve tuzları ile kombine uygulanan kimyasal, fiziksel veya biyolojik kaynaklı kontrol önlemlerinin temel amacı; antimikrobiyal etkinin artırılması, duyusal kalitenin iyileştirilmesi ve raf ömrünün uzatılması ile et endüstrisinde ekonomik kazanç sağlamaktır. Bu yönde yapılan çalışmalarda organik asitler ve paketleme yöntemlerinin sinerjik etkilerinin ürünlerde oluşturduğu olumlu değişimler vurgulanmıştır (Jaspal ve ark. 2021; Meng ve ark. 2022).

Farklı organik asit ilavesi ile kombine olarak uygulanan modifiye atmosfer paketleme uygulamalarının köftelerdeki *S. aureus* ve *E. coli* O157:H7 gelişimi ile kalite parametreleri üzerindeki etkilerinin incelendiği bu çalışmada, hem ilgili mikroorganizmalarda istatistiksel olarak önemli bir inhibisyon sağlandığı hem de ürünlerin raf ömrünün uzatıldığı gözlemlenmiştir. Bunun yanı sıra, soğuk zincir altında depolama süresince köfte örneklerinin duyusal özelliklerinde de olumlu etkiler oluştuğu kaydedilmiştir.

Farklı gıda matrislerinde uygulanan organik asit ilavesi ve/veya paketleme tekniklerinin ürünlerdeki antimikrobiyal etkinliğinin araştırıldığı çalışmalarda, Lim ve Mustapha (2004) potasyum sorbatın tek başına veya diğer asitlerle kombinasyon şeklinde kullanılmasının sığır etlerindeki patojen bakterilerin, özellikle de *S. aureus*'un inhibisyonunda etkin bir rol oynayabilmesi için >%0,1 konsantrasyonlarda ürüne ilave edilmesi gerektiğini bildirmiştir. Abu-Ghazaleh (2013), %0,05'lik potasyum sorbat ve %0,03'lük sitrik asit bileşiminin farklı ortamlara inoküle edilen *S. aureus* sayısında büyük oranda azalmaya neden olduğunu tespit etmiştir. Ayrıca, %0,09'luk potasyum sorbatın 24 saatlik inkübasyon süresi sonunda mevcut *E. coli* sayısında %18 oranında inhibisyon sağladığı bildirilirken, kekik ekstraktı (%0,3) ile %0,05'lik potasyum sorbat kombinasyonunun *S. aureus* düzeyini belirgin bir şekilde düşürdüğü gözlemlenmiştir ($p<0,05$). Aynı konsantrasyondaki potasyum sorbatın sitrik asitle (%0,03) birlikte kullanımı ise mevcut bakteri yükünde total inhibisyona neden olmuştur.

Organik asitlerin (Monolaurin, sorbik asit ve potasyum sorbat) *S. aureus*'un ve *E. coli* O157:H7'nin gelişimi üzerindeki antimikrobiyal etkinliklerinin incelendiği bir diğer çalışmada, uygulanan asitlerin maksimum konsantrasyonlarda dahi *E. coli* O157:H7 üzerinde inhibitör etki göstermediği, sadece potasyum sorbatın

S. aureus'u 2500 MIC seviyelerinde tamamen inhibe ettiği bildirilmiştir (Amin Zare ve ark., 2014).

Hwang ve Juneja (2011), kıyma örneklerine katmış oldukları laktat içeren tuz kombinasyonunun *E. coli* O157:H7'nin gelişimini geciktirebildiğini hatta baskılayabildiğini ifade etmiştir. Araştırmacılar laktatın bu inhibitör mekanizmasını, bakteri hücrelerinin sitoplazmasını asitleştirmesine ve dissosiye olmayan halinin hücre metabolik faaliyetleri üzerindeki etkisine

bağlamıştır. Ponrajan ve ark. (2011), sığır etlerinde sodyum sitrat ve sodyum diasetat ilavesinin vakum paketleme ile kombine olarak uygulandığı çalışmalarında, *E. coli* O157:H7 seviyesini örneklerde 6 log kob/cm² olacak şekilde inoküle etmişler ve bir kısmı çiğ olarak diğer yarısı ise iç sıcaklık 60°C'ye ulaşıncaya kadar pişirildikten sonra 4°C'de 10 gün boyunca depolamışlardır. Bu süre sonunda çiğ numunelerde *E. coli* O157:H7 sayısında 0,6 kob/g'a kadar önemli bir azalma gözlenirken, pişirilen numunelerin hiçbirinde *E. coli* O157:H7 tespit edilememiştir.

S. aureus'un üreme potansiyeli üzerinde farklı paketleme metotlarının etkisinin araştırıldığı bir çalışmada, yüksek sıcaklıkların üremeyi arttırmaya yönelik etkisi ve düşük sıcaklıkların mevcut mikroorganizma sayısındaki inhibisyon etkisi dikkat çekici bulunmuştur (Yu ve ark. 2020). Buna ek olarak, Farber (1991) et örneklerinde uyguladığı modifiye atmosfer paketleme tekniğinin *S. aureus* gelişimini engellemede oldukça başarılı olduğunu, bunun paketleme bileşimindeki yüksek CO_2 konsantrasyonuna bağlı olarak gerçekleştiğini ifade etmiştir.

Uyttendaele ve ark. (2001), deneysel olarak *E. coli* O157:H7 inoküle ettikleri sığır eti örneklerine uyguladıkları modifiye atmosfer paketleme (%40 CO_2/%60 N_2) işleminin ilgili mikroorganizma sayısını önemli ölçüde etkilemediğini bildirmiştir ($p>0,05$). Ayrıca, sığır eti kompozisyonuna yakın içerikteki bir besiyerine yapılan ekimde modifiye atmosfer paketleme uygulanan örneklerdeki mikroorganizma sayısında 1,6 log düzeyinde azalma saptandığı görülmüş olup, bu durumun et suyunun olduğu ortamda rekabetçi floranın bulunmamasından kaynaklandığı ifade edilmiştir. Benzer olarak, Beterams ve ark. (2023) paketleme uyguladıkları gıda örneklerindeki *E. coli* sayılarının muhafaza süresi boyunca anlamlı bir değişime uğramadığını, mevcut mikroorganizma sayısının inokülasyon seviyesinde kaldığını bildirmişlerdir.

Gıda maddelerinin muhafazası sırasında pH değerlerinde değişimler meydana gelmektedir. Et ve et ürünleri hafif asidik yapıya sahip olup, ürün çeşidine göre değişiklik göstermekle birlikte bu değer ortalama 5,3-6,5 arasındadır. Ürünün tazeliğiyle ve hijyenik kalitesi ile alakalı olan bu parametre, patojen mikroorganizmaların gıdalarda üremesiyle açığa çıkan alkali maddelerden dolayı artış gösterebilmektedir. Çalışmamızda da köfte örneklerinin pH değerlerinde muhafaza süresince anlamlı artışlar kaydedilmiştir (Şekil 2; $p<0,05$). Suman ve ark. (2010), laktat ile muamele edilmiş köfte örneklerinin pH değerini 5,79 olarak tespit ederken, muamele edilmemiş kontrol grubu örneklerinin pH değerini 5,69 olarak belirlemişlerdir. Tenderis ve ark. (2020) pişmiş sığır kıymasının pH değerlerinin 4°C ve 10°C'de depolamanın başlangıcında sırasıyla 5,49-6,09 ve 5,52-6,27 arasında değiştiğini ve depolama sırasında sodyum laktat ilavesinin pH üzerinde önemli bir etkisi olmadığını bildirmişlerdir. Djordjević ve ark. (2018) modifiye atmosfer paketleme uygulanan kıyma örneklerinin pH değerinin depolama süresi boyunca artış gösterdiğini ifade etmişlerdir. Başlangıç pH değeri ortalama 5,85 olan örnekler 15. günün sonunda 6,43-6,49 aralığına ulaşmış olup, bu durum istatistiksel olarak önemli ($p<0,05$) bulunmuştur. Bunun yanı sıra, Byrne ve ark. (2002) sodyum laktatın sığır burgerlerinde pH değerini düşürdüğünü belirtmişlerdir. Sallam ve Samejima (2004) da kıymaya sodyum laktat ilavesinin pH değişimlerini sabit seviyede tuttuğunu vurgulamıştır. Amin Zare ve ark. (2014) ise organik asitlerin pH seviyesi ile ters orantılı olarak aktivite gösterdiklerini belirtmiştir ($p<0,05$).

Gıda maddelerinde mikrobiyal üreme ile doğru orantılı olan su aktivitesi değeri, taze etlerde 0,98-1,00 arasındadır. Nem içeriği yüksek gıda maddelerinden olan köfte örneklerine ait a_w değerleri muhafaza süresince düzenli bir azalma eğilimi göstermiştir. Buna uygun olarak, Tenderis ve ark. (2020) pişmiş kıymada a_w değerlerinin 4°C ve 10°C'de depolamanın ilk gününde sırasıyla 0,92-0,96 arasında değiştiğini vurgulamıştır. Aynı şekilde María ve ark. (2015) sodyum laktatın ürünlerin su aktivitesi üzerinde baskılayıcı bir etkisi olduğu fikrini desteklemiştir.

Et ürünlerinin duyusal özellikleri tüketiciler tarafından kabul edilmesinde ve seçilmesinde önemli parametrelerin başında gelmektedir. Yapılan pek çok araştırmada duyusal özelliklerin kalite üzerine olan etkisi gösterilmiştir. Bu bağlamda, Mir ve Masoodi (2018) depolama süresi boyunca sodyum asetat ilavesiyle köfte kalitesinin belirli bir ölçüde iyileştirilebileceğini belirtmiştir. İşlem görmüş örneklerin lezzet, sululuk ve genel kabul edilebilirlik puanları, depolama boyunca işlem görmemiş köftelerden daha yüksek olduğu vurgulanmıştır. Buna uygun olarak, Hoffman ve ark. (2008) ürünlerin duyusal özelliklerini arttırmak için fosfat ve laktatların sığır eti kaslarına enjeksiyon karışımları olarak uygulanmasının kabul edilebilir olduğunu belirtmiştir. Quilo ve ark. (2009), %3 potasyum laktat ile muamele edilen kıyma örneklerinin genel renk özellikleri için 1-3. günlerde muamele edilmeyenlere göre daha yüksek puanlar aldığını ve antimikrobiyal ajanlar kullanıldığında daha düşük renk değişikliği gözlendiğini bildirmiştir. Antimikrobiyal maddelerin eklenmesinden sonra kıymanın duyusal özelliklerinde teşhirin ilk gününde tespit edilebilir bir farklılık olmamasına rağmen, depolamanın sonunda işlem görmemiş örneklere kıyasla daha düşük renk değişikliği görüldüğü bildirilmiştir (Quilo ve ark. 2010). Öte yandan, Suman ve ark. (2010) laktatın kıymanın yüzey renk değişikliği üzerindeki etkisinin paketleme sistemlerinden etkilendiğini belirtmiştir. PVC ve vakum ambalajlardaki laktat eklenmiş köfteler, işlem görmemiş kıymaya kıyasla daha düşük renk değişikliği gösterirken, CO ve yüksek O_2 ile paketlenen örneklerde laktat işlemleri için herhangi bir fark bulunmamıştır.

SONUÇ

Laktat, asetat ve sitrat gibi organik asitlerin ve tuzlarının farklı kombinasyonlar şeklinde ilavesinin köftelerin raf ömrünü önemli ölçüde arttırdığı belirlenmiştir. Konsantrasyona bağlı olarak organik asitlerin hem Gram (+) hem de Gram (-) bakterilerin gelişimini inhibe ettiği, ürünün duyusal özelliklerinde istenmeyen bir değişikliğe neden olmadığı ve hatta taze ürüne özgü rengin korunarak köftenin kalite özelliklerinin iyileştirildiği tespit edilmiştir.

Farklı gaz oranları kullanılarak uygulanan modifiye atmosfer paketlemenin köftelerin raf ömrünü önemli ölçüde etkilediği; düşük oranda CO içeren modifiye atmosfer paketleme ile bakteriler üzerinde daha fazla inhibisyon sağlarken, köftelerin kırmızı renginin korunduğu belirlenmiştir.

Sonuç olarak, sodyum sitrat, potasyum sorbat ve sodyum laktat solüsyonu ile tek başına sodyum laktatın %2'lik konsantrasyonda köftelere ilavesi, %0,4 CO paketleme ile kombine olarak uygulandığında soğuk muhafaza altında depolanan köftelerin duyusal özelliklerinin 12 güne kadar korunmasında etkili olduğu saptanmıştır.

Çıkar çatışması: Yazarların bildirecekleri çıkar çatışması yoktur.

Yazarların Katkıları: EBB, projenin fikrine, tasarımına ve çalışmanın yürütülmesine katkıda bulunmuştur. FYE ve EA, verilerin elde edilmesine katkıda bulunmuştur. FYE, EBB ve EA, verileri analiz etmiştir. FYE ve EA, taslağı hazırlamış ve yazmıştır. FYE, EA ve EBB, taslağı eleştirel bir şekilde incelemiştir. Tüm yazarlar, son halini almış taslağı okumuş ve onaylamıştır.

Etik onay: Bu çalışma, "Hayvan Deneyleri Etik Kurullarının Çalışma Usul ve Esasları Hakkında Yönetmelik" 8 (k) uyarınca HADYEK iznine tabi değildir. Bu makalede sunulan veriler, bilgiler ve belgeler, akademik ve etik kurallar çerçevesinde elde edilmiştir.

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RESEARCH ARTICLE

Global Analysis of Research on Opisthorchisasis Infection Caused by *Opisthorchis* spp.

Milad AFŞAR[1]*, Rahmi YILDIZ[2], Sadi ELASAN[3]

[1]*Department of Medical Parasitology, Faculty of Medicine, Van Yüzüncü Yıl University, Van, Türkiye.*
[2]*Department of Veterinary Medicine, Gevaş Vocational School, Van Yüzüncü Yıl University, Van, Türkiye.*
[3]*Department of Biostatistics, Faculty of Medicine, Van Yüzüncü Yıl University, Van, Türkiye.*

ABSTRACT

This study investigated the existence of infections caused by opisthorchiasis and conducted a bibliometric analysis of its global trends. The main objective of this study is to analyse trends and cluster in this field among researchers in developed and developing countries by reviewing publications on opisthorchiasis around the world in order to develop effective control strategies. We searched the Web of Science (WOS) databases for studies published between 1980 and 2024 using the keywords '*Opisthorchis* spp and *Opisthorchis viverrini* and *Opisthorchis felineus* and opisthorchiasis." Information like title, author names, year of publication, journal names and number of citations to journals were used for data collection. In the study, the software VOSviewer (ver.1.6.20) was used to visualize literature data on a global level. This study aimed to make the results more understandable through analysis using text mining and data visualisation methods (bubble maps and graphs). In this study, information was provided on 1957 articles from the WOS databases and the references to these articles. The H-index is 90. It was then observed that the number of studies conducted after 2005 increased. It was reported that most of the published articles (40%) were in the field of parasitology. Thailand (50%), USA (13%) and Japan (10%) are the countries that publish the most articles on this topic. Most published articles are in the international SCI-Expanded category (93%). The results of this bibliometric study reveal global trends in opisthorchiasis and provide important information for future research directions in this field. This study is the first bibliometric analysis of opisthorchiasis.

Keywords: Bibliometrics, *opisthorchis felineu*, *opisthorchis viverrini*

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Opisthorchis spp'nin Neden Olduğu Opisthorchisiasis Enfeksiyonuna İlişkin Araştırmaların Küresel Analizi

ÖZ

Bu çalışma, opisthorchiasis'in neden olduğu enfeksiyonların varlığını araştırmış ve küresel eğilimlerinin bibliyometrik bir analizini yapmıştır. Bu çalışmanın temel amacı, etkili kontrol stratejileri geliştirmek, dünya çapında opisthorchiasis ile ilgili yayınları gözden geçirerek gelişmiş ve gelişmekte olan ülkelerdeki araştırmacılar arasında bu alandaki eğilimleri ve grupları analiz etmektir. Bu amaçla 1980-2024 yılları arasında yayınlanmış çalışmaları Web of Science (WOS) veri tabanlarında '*Opisthorchis* spp., *Opisthorchis viverrini*, *Opisthorchis felineus* ve opisthorchiasis' anahtar sözcüklerini kullanarak arama yapılmıştır. Verilerin toplanmasında başlık, yazar isimleri, yayın yılı, dergi adı ve atıf sayısı gibi bilgiler kullanılmıştır. Çalışmada, literatür verilerini küresel düzeyde görselleştirmek için VOSviewer (ver:1.6.20) yazılımı kullanılmıştır. Bu çalışma, metin madenciliği ve veri görselleştirme yöntemlerini (kabarcık haritaları ve grafikler) kullanarak analiz yoluyla sonuçları daha anlaşılır hale getirmeyi amaçlamıştır. Bu çalışmada, WOS veri tabanlarından 1957 makale ve bu makalelere yapılan atıflar hakkında bilgi verilmiştir. H-index'i 90'dır. 2005 yılından sonra yapılan çalışmaların sayısının arttığı gözlemlenmiştir. Yayınlanan makalelerin çoğunun (%40) parazitoloji alanında olduğu bildirilmiştir. Tayland (%50), ABD (%13) ve Japonya (%10) bu konuda en çok makale yayınlayan ülkelerdir. Yayınlanan makalelerin çoğu uluslararası SCI-Expanded kategorisindedir (%93). Bu bibliyometrik çalışmanın sonuçları opisthorchiasis konusundaki küresel eğilimleri ortaya koymakta ve bu alanda gelecekte yapılacak araştırmalar için önemli bilgiler sağlamaktadır. Bu çalışma opisthorchiasis'in ilk bibliyometrik analizidir.

Anahtar kelime: Bibliometrik, *opisthorchis felineu, opisthorchis viverrini*

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ORCID ID; *MA: 0000-0003-1978-4127, RY: 0000-0003-2089-0664, SE: 0000-0002-3149-6462*
*Corresponding author e-mail: mtamilad@gmail.com

INTRODUCTION

Opisthorchiasis caused by *Opisthorchis viverrini* (*Distomum sibiricum*) and *Opisthorchis felineus* (*Distoma felineum*), which have an important place in the list of food-borne trematodes, is a common zoonotic infection among dogs in endemic areas and many other fish-eating mammals, including humans. Infections caused by *O. felineus* are particularly prevalent in Siberia, East and South-East Asian countries and some European countries, and maintain their place among the trematodes that affect public health on a daily basis (Petney et al., 2013; FAO/WHO, 2014; Pakharukova and Mordvinov, 2022).

It has been reported that infections caused by opisthorchiasis are common in people of low socio-economic status. More than 700 million people are currently at risk from these fish-borne trematodes, while up to 40 million people are at direct risk of infection. Opisthorchiasis infections have been reported in people in Eastern European countries and in countries in Asia (particularly East and South-East Asia) where it is common. While the adult worms of *Opisthorchis* spp. cause serious complications in the bile ducts and hepatobiliary system, these trematodes (*Opisthorchis* spp.) are known to be a major cause of liver and bile duct cancer (Hotez et al., 2007; IARC, 2012; Petney et al., 2013; FAO/WHO, 2023).

Two intermediate hosts are required in the parasite's life cycle. The first is the water snail *Bithynia leachi*, and the second is the intermediate host of various species of freshwater fish in the family *Cyprinidae*. Eggs excreted with the faeces of the final host develop in the body of the intermediate host and become cercariae. The cercariae leave the first intermediate host, then the cercariae penetrate the skin of the second intermediate host, the freshwater fish, settle in the muscles and become metacercariae, the infective form of their final host. Infection begins when fish with these metacercariae are consumed raw or undercooked (Crellen et al., 2021; Grundy et al. 2012).

The aim of this analysis is to review the policies aimed at controlling the incidence of the disease in humans in countries where opisthorchiasis infection, which is of zoonotic importance, is widespread. When selecting bibliographic sources, we first evaluated studies aimed at finding differences in opisthorchiasis, especially climate, geographical location, life cycle, intermediate host range, disease morbidity, carcinogenicity, genome and transcriptome (Pakharukova and Mordvinov, 2022).

The WOS database was searched using the terms *Opisthorchis viverrini*, *Opisthorchis felineus*, *Opisthorchis* spp. and Opisthorchiasis. A total of 1957 studies were listed from 1980 to 2024.

MATERIALS and METHODS

Data Collection

In this bibliometric study, the Web of Science Core Collection (WOS) databases were used to search for studies on the analysis of global trends in opisthorchiasis published between 1980 and 2024. Searches of the database using the keywords "*Opisthorchis* spp., *Opisthorchis viverrini*, *Opisthorchis felineus* and opisthorchiasis" yielded 1957 studies. The necessary analyses were carried out using information such as the articles in the database, the title of the article, the names of the authors, the year of publication, the name of the journal in which it was published and the number of citations. Entries and references were exported as plain text files and saved in download txt format. The data were obtained by using the online library and digital resources of Van Yüzüncü Yıl University. The search language is English.

Data Analysis

In this bibliometric study, "Collaboration Network, Highlights and Future Trends" was analysed using VOSviewer to determine the global trends in the presence of opisthorchiasis and the major research topics in the field. VOSviewer places great importance on the graphical presentation of bibliometrics in general. It is especially useful for presenting large bibliometrics in an comprehensible way (Van E. 2010). Web of Science databases were used for systematic data collection, and all textual data of the publications included in the study were picking and analysed using VOSviewer software. We used VOSviewer software to prove country/region and institutional collaborations, author supports, and keyword analysis. We also used Microsoft Office Excel 2019 to assess trends. These analyses were performed using text mining and data visualisation (bubble maps and other graphical methods) to demonstrate the correctness and credibility of the study.

RESULTS

A total 1957 published articles were inclusive in the search of the WOS database. The articles had a total of 27166 citations (23670 citations excluding self-citations). The H-index is 90. In particular, since 2005 there has been an upward trend in both the number of citations to articles and the number of articles. The distribution of publications and citations is shown in Figure 1. When examined based on the bibliometric network across research fields, it is evident that publications in the field of parasitology are among the largest and most interconnected research areas (Figure 2).

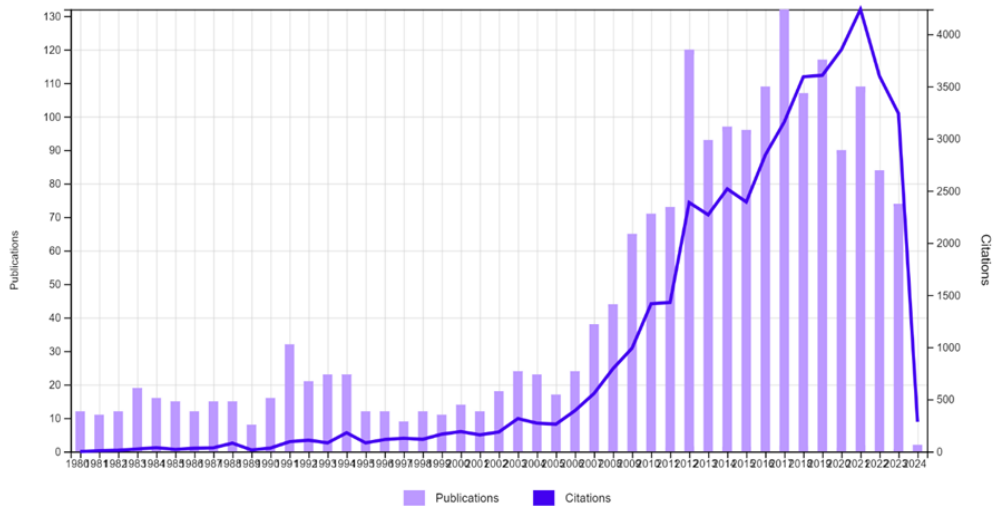

Figure 1: Frequency of publications and quotes by year

The highest number of articles were published in the fields of Parasitology (40.6%), Tropical Medicine (20.3%), Infectious Diseases (10.5%) and Oncology (10.3%), respectively. The distribution of the top ten publications according to the research field is shown in Table 1.

Table 1. Categories of publication areas that are related to *Opisthorchis* spp.

Research Areas	**Record Count**	**% of 1.957**
Parasitology	796	40.6
Tropical Medicine	398	20.3
İnfectious Diseases	206	10.5
Oncology	202	10.3
Public Environmental Occupational Health	200	10.2
Biochemistry Molecular Biology	95	4.8
General İnternal Medicine	91	4.6
Gastroenterology Hepatology	90	4.6
Veterinary Sciences	79	4.0
İmmunology	76	3.9

Showing 10 out of 75 entries

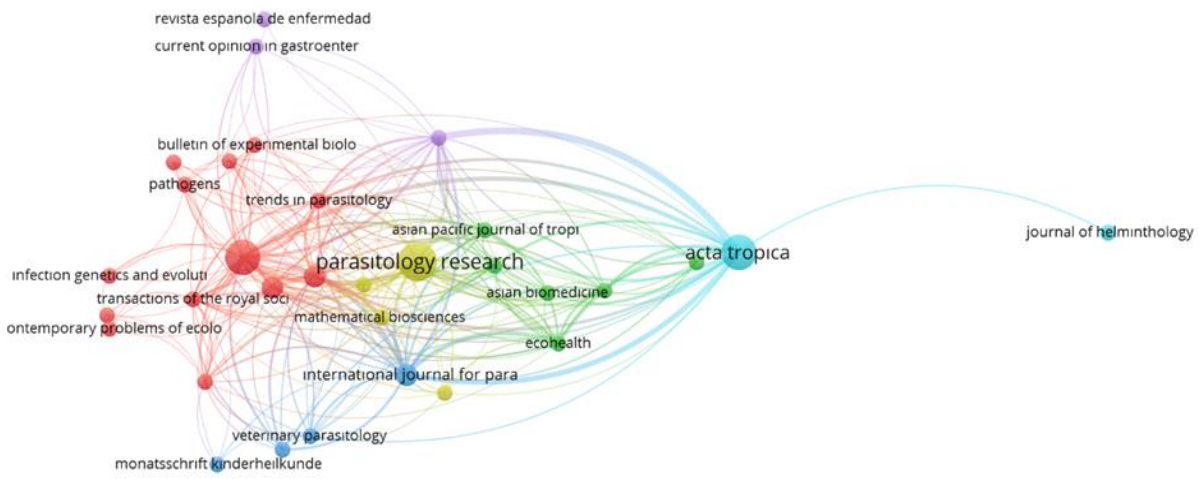

Figure2: Bibliometric network by research fields.

Thailand ranked first in terms of number of articles published (n=1028; 52.5%), followed by the USA (n=260; 13.3%), Japan (n=204; 10.4%) and Russia (n=204; 10.4%). The top 25 countries in this ranking are listed in Table 2.

Table 2. Countries with at least 30 publications

Countries/Regions	Record Count	% of 1.957
Thaıland	1028	52.5
Usa	260	13.3
Japan	204	10.4
Russıa	204	10.4
Australıa	201	10.3
Germany	127	6.5
England	123	6.3
Peoples R Chına	120	6.1
South Korea	113	5.8
Swıtzerland	105	5.4
Laos	103	5.3
Ussr	64	3.3
Vıetnam	59	3.0
Canada	44	2.3
Sıngapore	39	2.0
France	37	2.0
Spaın	36	1.8
Italy	33	1.7
Denmark	32	1.6
İndıa	28	1.4
Cambodıa	22	1.1
Netherlands	22	1.1
Scotland	17	0.9
Belgıum	16	0.8
Czech Republıc	14	0.7

When Bibliographic coupling is evaluated by country, it is seen that Thailand, the USA, Japan and Russia have a greater representation among the prominent countries in this field (Figure 3).

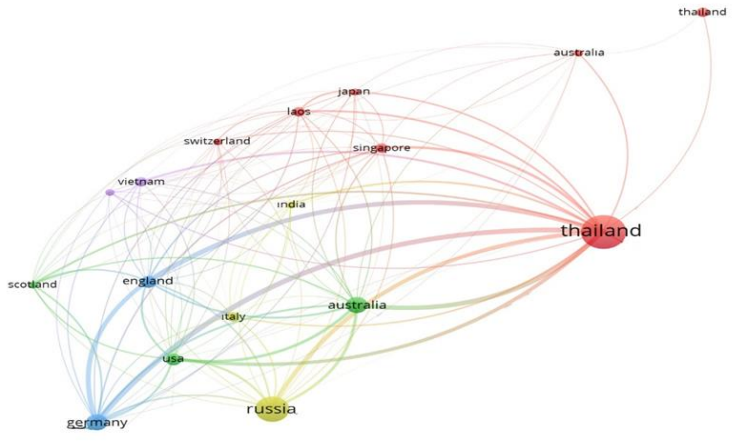

Figure 3: Citation network visualisation map among countries with publications on *Opisthorchis* spp. (The relatedness of items was determined based on the number of references the share)

During the evaluation of the priority status of the universities and research institutions in terms of the number of publications, the leading institutions were Khon Kaen University (37.6%), Mahidol University (11.6%) and the Russian Academy of Sciences (7.0%). Accordingly, the top 10 leading institutions are shown in Table 3 according to their affiliations (Table 3).

Table 3. List of the top affiliations

Affiliations	Record Count	% of 1.957
Khon Kaen University	737	37.6
Mahidol University	227	11.6
Russian Academy Of Sciences	138	7.0
George Washington University	104	5.3
University Of Basel	95	4.8
Swiss Tropical Public Health İnstitute	94	4.8
Mahasarakham University	83	4.2
İnstitute of Cytology and Genetics of SB RAS	82	4.2
Siberian State Medical University	76	3.9
James Cook University	69	3.5

When we look at the Web of Science indexes, it is seen that the majority of the articles are in the Science Citation Index Expanded (SCI-Expanded) category (93%), followed by Emerging Sources Citation Index (ESCI) (4.9%) and Conference Proceedings Citation Index. (CPCI-S) (3.0%) (Table 4).

Table 4. Web of Science Categories İndex

Web of Science Index	Record Count	% of 1.957
SCI-EXPANDED	1822	93.0
ESCI	95	4.9
CPCI-S	59	3.0
BKCI-S	57	2.9
SSCI	28	1.4
A&HCI	4	0.2
İndex Chemicus (IC)	3	0.15
CPCI-SSH	1	0.05

When analysing the selected keywords, keywords such as *Opisthoris viverrini* and *Opisthoris felineus* are among the largest and most linked topic areas (Figure 4).

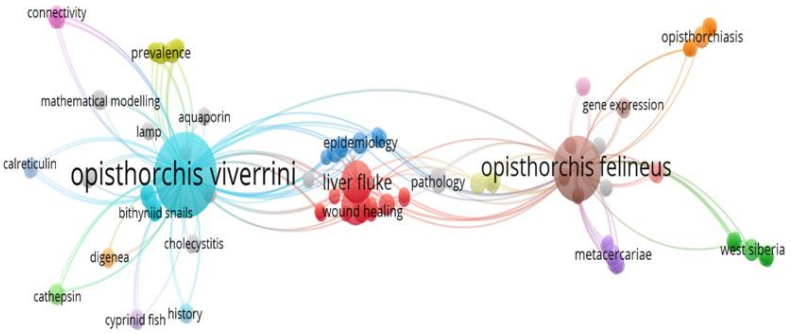

Figure 4: Keyword analysis (Shows which keywords the topic is associated with and how often those keywords are used).

DISCUSSION

These food-borne zoonotic trematode infections are a major public health problem in Asian countries, including Vietnam, Thailand, China and Korea. Fish-borne trematodes, in particular, cause significant morbidity in local populations and serious damage to the aquaculture industry. Almost all fish-borne trematode infections in humans are due to the habit of eating raw fish that comprise infective larvae (Andrews va ake. 2008; Chai et al., 2014; Sakamoto et al., 2023).

These infections are particularly common in riverine areas and in communities where raw fish is consumed. Riparian areas in Southeast Asia, particularly the Mekong River basin in Vietnam, China and Thailand, are known to have a very high incidence of fish-borne trematode infections (Suwannatrai et al., 2018; Sripa et al., 2021; Sakamoto et al., 2023). This trematode is most prevalent in Thailand, which therefore has one of the highest incidences of cholangiocarcinoma (CCA), a cancer associated with opisthorchiasis, in the world (Rachprakhon et al., 2021; Sripa et al., 2021).

The purpose of bibliometric studies is both to guide scientific research and to show the current state of a scientific subject. There are many valuable studies on Opisthorchis in the scientific world. It will guide scientists who want to do more research on these topics (Maciver et al. 2020).

The target of this bibliometric study is to investigate the role of *Opisthorchis viverrini and Opisthorchis felineus* in opisthorchiasis infection and to show the global increase in the prevalence of scientific publications on the studies performed. The aim of the studies is to identify global trends and clusters in the investigation of the presence of Opisthorchis spp in patients diagnosed with opisthorchiasis, and to publish them in the form of a report on the areas in which research in this field is focused and in which countries it is most prevalent. In addition, important journals, authors and studies in this field were identified, and it was pointed out that they can take into account the existing deficiencies in this field and lead to future studies.

The search using the terms "*Opisthorchis viverrini* and *Opisthorchis felineus* and pisthorchiasis" in the WOS database yielded a total of 1957 studies. The articles had a total of 27166 citations (23670 citations excluding self-citations). The H-index is 90. Specially since 2005, both the number of citations and the number of articles show an increasing trend (Figure 1).

Search the WOS database search engine for 'Opisthorchis viverrini, opisthorchis felineus, opisthorchis spp.' By typing the terms 'and opisthorchiasis', studies conducted from 1975 to 2023 were scanned, and as a result of the scanning, 1957 articles were reached. It was determined that very few studies were conducted on opisthorchiasis from 1975 to 2000, and more publications were made after 2005, and the most studies were conducted after 2017. It has been reported that almost half (40%) of the articles published on Opisthorchiasis are in the field of Parasitology. The countries with the most publications were determined to be Thailand (50%), USA (13%) and Japan (10%), respectively (Table 1.Figure 2, Table 2).

The analysis of the most searched keywords in the subject areas showed that Opisthoris viverrini and Opisthoris felineus were the most common. Among the universities and research institutions that researched the prevalence of this trematode, the most researched were Khon Kaen University (37.62%) and Mahidol University (11.58%), and the most researched institution was the Russian Academy of Sciences (47.04%) (Figure 3, Table 3).

When analysing the keywords selected for detailed scanning in the WOS database, keywords such as Opisthoris viverrini and Opisthoris felineus are among the largest and most linked subject areas (Figure 3).

CONCLUSION

The most commonly used keywords for opisthorchiasis are *Opisthorchis viverrini* and *Opisthorchis felineus*, the most studied field is parasitology and the most studied country is Thailand. It has been reported that one of the reasons for its prevalence in this country is the consumption of raw freshwater fish. In order to improve effectual control strategies in the fight against opisthorchiasis, it would be useful for researchers and scientists in developed countries to establish research collaborations that will ensure the success of control and eradication programmes against this infection in Siberia, East and South-East Asia and some European countries where the disease is found.

Conflict of interest: The authors have no conflicts of interest to report.

Authors' Contributions: MA and SA contributed to the project idea, design and execution of the study. RY and MA contributed to the acquisition of data. RY and SA analysed the data. MA and RY drafted and wrote the manuscript. SA and MA reviewed the manuscript critically. All authors have read and approved the finalized manuscript.

Ethical approval: Since it was a bibliometric study, no ethical report was received. This study is not subject to the approval of HADYEK in according to the Regulation on Working Procedures and Principles of Animal Experiments Ethics Committees 8 (k). The data, information and documents presented in this

article were obtained within the framework of academic and ethical rules.

Limitations: Our study is the first bibliometric analysis of published studies of people with opisthorchiasis infection. As WOS is one of the most commonly used databases for bibliometric analysis, the data for our study were only obtained from WOS. Therefore, it is possible that publications found in other search engines but not in the Scopus database may have been overlooked. Our results must be interpreted with this limitation in mind.

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RESEARCH ARTICLE

Effect of Freezing Rate on the Freezability of Ram Semen Diluted with Tris Extenders of Different Properties

Mustafa SÖNMEZ[1], Abdoul Bhassit BONKOUNGOU[1], İbrahim Halil GÜNGÖR[1]*, Aslıhan ÇAKIR CİHANGİROĞLU[2], Tutku Can ACISU[1]

[1] Fırat Üniversitesi, Veteriner Fakültesi, Dölerme ve Suni Tohumlama Anabilim Dalı, Elazığ, Türkiye
[2] Siirt Üniversitesi, Veteriner Fakültesi, Dölerme ve Suni Tohumlama Anabilim Dalı, Siirt, Türkiye

ABSTRACT

The objective of this study was to investigate the effects of different freezing rate on the motility rates and kinematic velocity parameter values of ram semen diluted with tris diluents prepared using standard and ultra-specific chemicals. Semen obtained from six different rams by the artificial vagina method was used in the study. The semen was individually divided into 2 equal parts. Then each group was diluted with one of the two tris diluents. The diluted semen was drawn into 0.25 ml straws and cooled to 4 °C. After equilibration, they were frozen in an automatic freezing device according to fast and slow freezing methods. After freezing, the straws were stored in liquid nitrogen tanks at -196 °C. Motility and kinematic parameter values were determined in thawed semen samples using CASA system. It was determined that the freezing of ram semen by dilution with Tris diluents of varying chemical composition did not affect the spermatological quality following thawing. On the other hand, the results indicated that the slow freezing method provided a significant increase in the rates of rapid spermatozoon, total motility and progressive motility after thawing in comparison to the fast freezing method for both diluents. In addition, when the results obtained following thawing were evaluated individually; it was observed that there were significant individual differences among the rams in terms of total and progressive motility rates. In conclusion, the use of the slow freezing method during the freezing of ram semen can provided more successful results in terms of post-thawing motility rates compared to the fast freezing method. Furthermore, it also appears that individual characteristics may be a crucial factor affecting the success of semen freezing in rams.

Keywords: Ram, Semen, Tris, Chemical Properties, Freezing Rate, Individuality.

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Farklı Özellikteki Tris Sulandırıcıları ile Sulandırılan Koç Spermasının Dondurularak Saklanabilirliği Üzerine Dondurma Hızının Etkisi

ÖZ

Bu çalışmanın amacı, standart ve ultra özellikteki kimyasal maddeler kullanılarak hazırlanan tris sulandırıcıları ile sulandırılan koç spermasının motilite oranları ve kinematik hız parametre değerleri üzerine farklı dondurma hızlarının etkilerini araştırmaktı. Çalışmada altı farklı koçtan suni vajen yöntemi ile alınan spermalar kullanıldı. Alınan spermalar bireysel olarak 2 eşit kısma ayrıldı. Ardından her bir grup, iki tris sulandırıcısından biri ile sulandırıldı. Sulandırılan spermalar 0.25 ml payetlere çekilip 4°C'ye soğutuldu. Ekilibrasyondan sonra otomatik dondurma cihazında hızlı dondurma ve yavaş dondurma yöntemlerine göre donduruldu. Dondurma sonrası payetler -196 °C'deki sıvı azot tanklarında saklandı. Çözdürülen sperma örneklerinde CASA sistemi kullanılarak motilite ve kinematik parametre değerleri belirlendi. Koç spermasının farklı kimyasal içerikli Tris sulandırıcıları ile sulandırılarak dondurulmasının çözdürme sonrası spermatolojik kaliteyi etkilemediği tespit edildi. Diğer taraftan, her iki sulandırıcı için hızlı dondurma yöntemiyle karşılaştırıldığında yavaş dondurma yönteminin çözdürme sonrası total motilite, progresif motilite ve hızlı hareket eden spermatozoon oranlarında önemli derecede bir artış sağladığı gözlendi. Ek olarak çözdürme sonrası elde edilen sonuçlar bireysel olarak değerlendirildiğinde; koçlar arasında total ve progresif motilite yönünden önemli bireysel farklılıkların olduğu belirlendi. Sonuç olarak, koç spermasının dondurulması sırasında yavaş dondurma yönteminin kullanılması, hızlı dondurma yöntemine kıyasla çözdürme sonrası hareketlilik oranları açısından daha başarılı sonuçlar sağlayabilir. Ayrıca, bireysel özelliklerin koçlarda sperma dondurma işleminin başarısını etkileyen önemli bir faktör olabileceği de görülmektedir.

Anahtar Kelimeler: Koç, Sperma, Tris, Kimyasal Özellikler, Dondurma Hızı, Bireysellik.

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ORCID ID; *MS: 0000-0003-0281-7228, ABB: 0009-0008-5354-792X, İHG: 0000-0002-5250-1478, AÇC: 0000-0003-3365-8960, TCA: 0000-0002-0882-937X*

*Corresponding author e-mail: ihgungor@firat.edu.tr

GİRİŞ

Erkek bir damızlık hayvandan uygun metot ve tekniklerle alınıp suni tohumlama uygulamalarında kullanılacak bir spermanın, muayenesi ve kontrolü yapıldıktan sonra mutlaka belirli işlemlerden geçirilerek sulandırılması ve uygun koşullarda soğutulup kısa süreli ya da dondurulup uzun süreli olarak saklanması gerekir. Spermanın dondurularak saklanabilmesi, özellikle spermanın uzun mesafeler arasında güvenilir bir şekilde transferini kolaylaştırmış ve uzun yıllar boyunca saklanabilen spermaların istenildiği zaman çözdürülerek kullanılması imkânını sağlamıştır. Bu durum, spermanın daha etkin bir şekilde kullanımını sağlayarak suni tohumlama uygulamalarının saha koşullarında uygulanabilirliğini ve yaygınlaşmasını artırmıştır (Hafez ve ark. 2000; Sönmez 2022; Salamon ve Maxwell 2000). Bununla birlikte, dondurulmuş bir spermanın çözdürme sonrası spermatolojik özellikler yönünden belirli kalite standartlarını sürdürmesi, elde edilen fertilite oranlarının istenilen düzeyde sağlanması açısından oldukça önemlidir (Yanez-Ortiz ve ark. 2022; Layek ve ark. 2022).

Birçok hayvan türünde spermanın başarılı bir şekilde dondurulmasında uygun sperma sulandırıcılarının geliştirilmesi oldukça önemli bir role sahip olup dondurma işlemleri öncesinde spermanın sulandırılmasında kullanılan sulandırıcının kimyasal içeriği, spermanın çözdürülmesi sonrası elde edilen motilite oranlarını etkileyebilen önemli faktörlerden birisi olarak kabul edilmektedir (Gil ve ark. 2003; Bearden ve ark. 2004). Günümüzde, Tris - Sitrik asit – Fruktoz - Yumurta sarısı - Gliserol içeren sperma sulandırıcısı, özellikle koç spermasının dondurulması amacıyla yapılan bilimsel çalışmalarda (Ustuner ve ark. 2014; Güngör ve ark. 2022; Zhang ve ark. 2024) yaygın olarak kullanılmakta olup güncelliğini devam ettirmektedir.

Belirli şirketlerce üretilip ticari olarak satışa sunulan kimyasal maddelerin, her ne kadar kimyasal formülleri aynı olsa da, üretim teknolojilerine göre molekül büyüklükleri ve saflık oranları önemli düzeyde değişkenlik gösterebilmektedir. Hatta ticari firmalar kendi ürün kataloglarında bir kimyasal maddenin aynı formüle sahip olmasına rağmen saflık derecesine göre farklı ürün seçeneklerini sunabilmektedir. Bu durum, kullanım amaçlarına göre kimyasal maddeler için bir tercih seçeneği oluşturmakla birlikte etkinlik ve saflık düzeylerinde göre satış fiyatlarının önemli derecede artmasına neden olmaktadır. Spermanın dondurulması amacıyla hazırlanan sperma sulandırıcıları düşünüldüğünde; bu sulandırıcılar hazırlanırken kullanılacak kimyasal maddelerin daha hassas teknoloji ile ultra saflıkta üretilmiş serilerinin tercih edilmesinin, standart teknoloji ile üretilmiş muadillerine göre dondurulup çözdürülme sonrası sperma kalitesini nasıl etkileyeceği merak konusudur.

Dondurulmuş bir spermanın çözdürme sonrası spermatolojik kalitesini etkileyen bir diğer faktör ise spermanın dondurulması işlemi sırasında uygulanan dondurma hızıdır (Kulíková ve ark. 2018; Vozaf ve ark. 2021). Donma anında gerçekleşen en önemli olay hücrenin kristalleşmesidir. Bu olay neticesinde oluşan önemli pH değişiklikleri; enzimatik reaksiyonların bozulmasına, hücrede oluşan dehidrasyon; hücre membran geçirgenliğinin değişmesine ve şekillenen geniş buz kristalleri ise; hücre membranında yırtılmalara sebep olmaktadır. Bununla birlikte, reaksiyon sırasında şekillenen buz kristalleri ne kadar küçük hacimli olursa, meydana gelen zarar da o kadar az olmaktadır (Watson 2000; Saha ve ark. 2022). Spermanın dondurulması sırasında spermatozoa membranındaki hasarlar, özellikle buz kristallerinin şekillenme devresi olarak kabul edilen 0 °C ile –20 °C arasındaki sıcaklıklarda meydana gelmekte olup bu aralığın çok hızlı geçilmesi buz kristallerinin şekillenme hızını artırmaktadır. Bu nedenle dondurma işleminin ilk aşaması olan 5 °C'den –20 °C'ye sıcaklığın düşürülmesi sırasındaki geçiş hızının oldukça iyi ayarlanması gereklidir (Watson 2000; Zamiri 2020).

Bu düşüncelerden yola çıkarak yürütülen bu çalışma; iki farklı üretim teknolojisi ile üretilmiş farklı saflık derecelerine sahip kimyasal maddeler kullanılarak hazırlanan Tris - Sitrik asit – Fruktoz - Yumurta sarısı - Gliserol içeren sperma sulandırıcıları ile sulandırılan koç spermasının dondurulması aşamasında uygulanan hızlı ve yavaş dondurma hızı yöntemlerinin çözdürme sonrası motilite ve hareket hızı oranları ile bazı kinematik parametre değerler üzerine etkisini belirlemek amacıyla yapıldı.

MATERYAL METOT

Araştırmanın Yeri

Araştırmada kullanılacak hayvanlar; Fırat Üniversitesi, Tarım ve Hayvancılık Araştırma ve Uygulama Merkezinden (TAHAM) temin edildi. Hayvanlardan spermanın alınması işlemleri Fırat Üniversitesi, Hayvan Hastanesi, Hospitalizasyon birimindeki padoklarda gerçekleştirilirken, spermanın muayeneleri ise Fırat Üniversitesi, Veteriner Fakültesi, Dölerme ve Suni Tohumlama Anabilim Dalı Androloji Laboratuvarında yapıldı. Çalışma için Fırat Üniversitesi Hayvan Deneyleri Yerel Etik Kurulu (FÜHADYEK)'ndan onay alındı (Tarih: 16.11.2022 - Protokol No: 2022/18-09).

Hayvan Materyali

Araştırmada hayvan materyali olarak 2 yaşlarında, klinik olarak sağlıklı, fertilitesi bilinen 65-70 kg canlı ağırlığında olan 6 baş Akkaraman ırkı koç kullanıldı. Uygulamalar süresince hayvanlara konsantre ve kaliteli kaba yem verildi. İçme suyu ad libitum olarak sağlandı.

Deneysel Aşama

Spermanın Alınması

Deneysel aşamalar sırasında koçlar ve koyun ayrı padoklarda tutuldu. Çalışma üreme mevsiminde gerçekleştirildi. Sperma alma döneminde koyunda seksüel uyarımın devamlılığı için 5 mg / 2mL dozunda

17-β-östradiol susam yağı içerisinde çözdürülerek uygulandı. Yapılan hormonal uygulamalarla kızgınlığa getirilen koyuna seksüel stimülasyon sağlanmış koçların atlaması sağlanarak suni vajen yöntemiyle sperma alındı. Bu işlem, haftada iki kez ve günün aynı saatlerinde (09.00-11.00) gerçekleştirildi. Çalışma boyunca, en az 0,8 ml hacme sahip, yoğunluğu 3 milyar/ml'nin üzerinde olan, başlangıç total motilitesi %80'in üzeri ve progresif motilite değeri ise %40'ın üzeri olan sperma örnekleri kullanıldı. Araştırmanın deneysel aşaması 6 tekrar olarak gerçekleştirildi.

Spermanın Sulandırılması, Paketlenmesi ve Soğutulması

Her bir koçtan alınan ejakülat, hacmi kaydedildikten sonra 37 °C'de su içeren bir beherglassa konularak hızlı bir şekilde laboratuvara taşındı. Laboratuvara getirilen ve ön muayeneleri yapılan sperma örnekleri otomatik pipet yardımıyla iki eşit kısma ayrılıp falkon tüplerine aktarıldı. Bu işlemin ardından her bir grup sperma; 37°C'deki etüvde tutulan iki farklı teknoloji ile üretilmiş (hassas teknoloji; TRS-H ya da standart teknoloji; TRS-S) kimyasal maddeler kullanılarak aynı oranlarda hazırlanmış Tris - Sitrik asit – Fruktoz - Yumurta sarısı - Gliserol içerikli sperma sulandırıcılarından biri ile (Tablo 1) 1:2 oranında sulandırılıp ön sulandırmaları yapıldı. Ön sulandırılması yapılmış her bir spermanın yoğunluğu ve motilite oranı belirlenerek son hacimlerinde mililitrede 400 milyon motil spermatozoa olacak şekilde aynı sperma sulandırıcısı ile sulandırılma işlemleri tamamlandı.

Sulandırılmış tüm sperma örnekleri; ayarlanabilir özel soğutma kabinine yerleştirildikten sonra sıcaklıkları 30 dakika içerisinde (2 dakikada 1 °C olacak şekilde) 37 °C'den 22 °C'ye (oda sıcaklığına) düşürüldü. Bu sıcaklıkta, sperma örnekleri 0.25 ml plastik payetlere çekilip uçları kapatılarak metal dondurma raflarına dizildi. Bu raflar üzerinde ayarlanabilir özel soğutma kabinine yerleştirilen payetlerin sıcaklığı ise yaklaşık 90 dakika içerisinde (5 dakikada 1 °C olacak şekilde) 23 °C'den 5 °C'ye düşürüldü. Soğutma işleminin ardından 5 °C'de tüm sulandırılmış sperma örnekleri 3 saat süreyle ekilibrasyona tabi tutuldu.

Spermanın Dondurulması

Ekilibrasyon süresinin tamamlanmasının ardından her bir grup sperma örneği; iki alt gruba ayrıldı (toplamda 4 grup). Bilgisayar sistemli otomatik dondurma kabinine (Micro-Digitcool™, PAF, IMV, Fransa) yerleştirilen payetler sırasıyla sıvı azot buharı yardımıyla hızlı (8 dakikalık program) ve yavaş (24 dakikalık program) dondurma prosedürlerine göre (Tablo 2) dondurulduu (Byrne ve ark. 2000; Güngör ve ark. 2022). Dondurma işleminin tamamlanmasının ardından dondurulmuş sperma payetleri gruplarına göre hızlı bir şekilde plastik gobletlere konulup sıvı azot tanklarına aktarılarak gerekli muayeneler yapılıncaya kadar bu tanklarda -196 °C'deki sıvı azot içerisinde saklandı.

Dondurulmuş Spermanın Çözdürülmesi

Dondurma işleminden en az 24 saat sonra dondurulmuş sperma içeren payetler sıvı azot tankından hızlı bir şekilde alınıp 37 °C'deki su banyosu içinde 25 saniye süreyle çözdürüldü. Daha sonra, payetin uç kısmı kesilerek bir ependorf tüpe aktarılan çözdürülmüş sperma örnekleri, muayeneler tamamlanıncaya kadar sıcaklığı 37 °C'ye ayarlanmış özel ısıtma ünitesinde muhafaza edildi.

CASA Sistemi Yardımıyla Çözdürme Sonrası Motilite ve Hareket Hızı Oranları ile Kinematik Parametre Değerlerinin Belirlenmesi

Sperma örneklerine ait motilite oranları ile kinematik parametre değerleri; Bilgisayar Destekli Sperm Analiz (CASA) cihazıyla (ISASv1.2, Proiser ® İspanya) belirlendi. Muayene sırasında, her bir sperma örneğinden alınan numune, görüntü alanına düşen spermatozoon sayısı 100-150 adet olacak şekilde tris buffer solüsyonu ile 1:10 oranında sulandırıldı. Daha sonra CASA sistemine bağlı faz-kontrast mikroskobun 37 °C'ye ayarlanmış ısıtma tablalı sehpasına konulmuş olan lam (Spermtrack-20 μm®) üzerine sulandırılmış örnekten 3 μL aktarılarak üzerine özel lameli kapatıldı. Ardından bilgisayarlı görüntü sistemi üzerinden en az 5 farklı saha incelenerek ortalama değerler kaydedildi (Güngör ve ark. 2022; Sönmez ve Fırat 2022). Bu muayene esnasında total ve progresif motilite değerleri ile spermatozoonların hız değerleri dikkate alınarak belirlenen; çok hızlı hareket eden spermatozoonlar (rapid), orta hızda hareket eden spermatozoonlar (medium), yavaş hareket eden spermatozoonlar (slow) ve hareket etmeyen spermatozoonlar (statik) şeklinde bir sınıflandırma yapılarak bunların oraları "%" şeklinde ifade edildi. Buna ilaveten, spermatozoonların hareket özelliklerine bağlı olarak oluşan kinematik parametrelerine ait değerlerde [VCL-eğrisel yol hızı (μm/s); VAP-ortalama yol hızı (μm/s); VSL-doğrusal yol hızı (μm/s); LIN-eğrisel yolun doğrusallığı (%); STR-ortalama yolun doğrusallığı (%), WOB-kararsızlık ölçüsü (%) belirlendi (Şekil 1). Yapılan analizler sırasında koç sperması için standart prosedür olarak görüntü süresi; 10 sn, görüntü hızı; 25 kare/sn, hız ölçütleri; 10 < slow < 45 < medium <75 < rapid ve STR değeri; %80 olarak uygulandı (İnanç ve ark. 2017; Özer Kaya ve ark. 2021).

İstatistiki Analiz

İstatistiki analizler için SPSS (Version 22.0) istatistik programı kullanıldı. Elde edilen veriler ortalama ± standart hata olarak sunuldu. Ham verilerin normal dağılım gösterip göstermediklerini tespit etmek için Kolmogorow-Smirnow normallik analizi yapıldı. Parametrik dağılım gösteren değerler arasındaki farklılıkların değerlendirilmesi için Varyans analizi (One-Way ANOVA) ve ikili karşılaştırmalar için ise post-hoc Tukey-HSD testi kullanıldı. Tüm analizlerde p değerinin 0.05'den daha az olduğu durumlar istatistiksel açıdan önemli olarak kabul edildi.

Tablo 1. Hazırlanan sulandırıcıların kimyasal içerikleri.

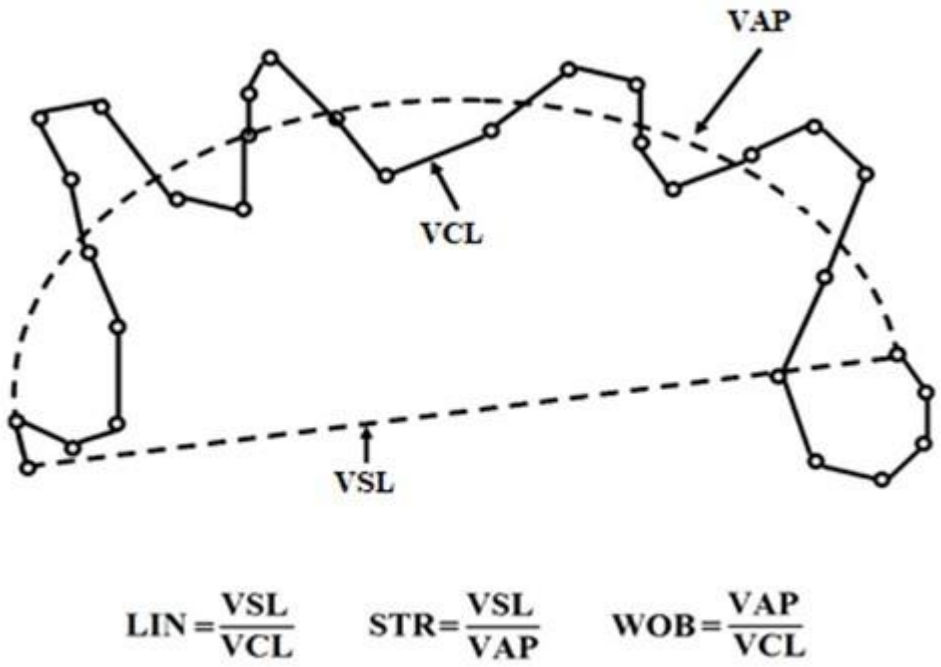
Table 1. Chemical contents of the prepared diluents.

Kimyasal Madde	**Miktarı**	**Standart İçerikli TRİS Sulandırıcı (TRS-S)**	**Hassas İçerikli TRİS Sulandırıcı (TRS-H)**
Tris (hidroksimetilen) aminomethane	3,63 gr	Trizma® Base Standard (Sigma, T1503)	Trizma® Base Bioxtra (Sigma, T6791)
Sitrik Asit	1,99 gr	Citric Acid (Sigma, C7129)	Citric Acid Bioxtra (Sigma, C0706)
Fruktoz	0,25 gr	Fructose (Sigma, F0127)	Fructose Bioxtra (Sigma, F2543)
Yumurta Sarısı	%16	Günlük taze tavuk yumurtasından filtrelenerek elde edilmiş ve her iki sulandırıcı için ortak kullanılmıştır.	
Gliserol	%5	Glycerol Bioxtra (Sigma, G6279) (her iki sulandırıcı için ortak kullanılmıştır.)	

Tablo 2. Otomatik dondurma kabininde koç spermasının dondurulması için dondurulma hızı yöntemleri.

Table 2. Freezing rate methods for freezing ram semen in the automatic freezer cabinet

Hızlı Dondurma Yöntemi				**Yavaş Dondurma Yöntemi**		
Sıcaklık Değişimi	**Soğutma Hızı**	**Süresi**		**Sıcaklık Değişimi**	**Soğutma Hızı**	**Süresi**
+5°C'den -10°C'ye	Dakikada 5°C	3,0 dk		+5°C'den -10°C'ye	Dakikada 1°C	15,0 dk
-10°C'den -20°C'ye	Dakikada 10°C	1,0 dk		-10°C'den -20°C'ye	Dakikada 2°C	5,0 dk
-20°C'den -100°C'ye	Dakikada 40°C	2,0 dk		-20°C'den -100°C'ye	Dakikada 40°C	2,0 dk
-100°C'den -140°C'ye	Dakikada 20°C	2,0 dk		-100°C'den -140°C'ye	Dakikada 20°C	2,0 dk
	Toplam süre	**8,0 dk**			**Toplam süre**	**24,0 dk**



Şekil 1: Spermatozoonun kinematik hız parametrelerinin şematik görünümü.

Figure 1: Schematic view of kinematic speed parameters of spermatozoon.

BULGULAR

Farklı özellikteki kimyasal maddeler kullanılarak hazırlanan sperma sulandırıcıları ile sulandırılıp hızlı ve yavaş dondurma hızı yöntemleri kullanılarak dondurulan koç spermasının çözdürme sonrası belirlenen total motilite, progresif motilite ve hareketsiz (statik) spermatozoon oranları Tablo 3'de sunuldu. Sunulan değerler incelendiğinde; farklı özellikteki kimyasal maddeler kullanılarak hazırlanan sulandırıcılar ile spermaların sulandırılmasının; her iki dondurma yöntemi için de çözdürme sonrası total motilite ve progresif motilite oranlarında kısmi bir sayısal artış; hareketsiz (statik) spermatozoon oranında ise kısmi bir sayısal azalma sağlamasına rağmen bu farklılıkların istatistiki yönden önemli olmadığı ($p>0,05$) tespit edildi. Diğer taraftan belirlenen değerler dondurma hızlarına göre değerlendirildiğinde ise; kullanılan her iki sulandırıcı için de uzun süreli dondurma yönteminin kısa süreli dondurma yöntemine göre çözdürme sonrası total ve progresif motilite oranlarında önemli derecede bir artış ($p<0,05$) ve hareketsiz (statik) spermatozoon oranında ise önemli derecede bir azalma sağladığı ($p<0,05$) belirlendi.

Farklı özellikteki kimyasal maddeler kullanılarak hazırlanan sperma sulandırıcıları ile sulandırılıp hızlı ve yavaş dondurma hızı yöntemleri kullanılarak dondurulan koç spermasının çözdürme sonrası belirlenen spermatozoon hız oranları Tablo 4'de sunuldu. Sunulan bu değerler incelendiğinde; farklı özellikteki kimyasal maddeler kullanılarak hazırlanan sulandırıcılar ile spermaların sulandırılmasının; her iki dondurma yöntemi için de çözdürme sonrası hızlı (rapid) spermatozoon oranın da kısmi bir sayısal artış sağlamasına rağmen bu farklılıkların istatistiki yönden önemli olmadığı ($p>0,05$) tespit edildi. Diğer taraftan belirlenen değerler dondurma hızlarına göre değerlendirildiğinde ise; kullanılan her iki sulandırıcı için de uzun süreli dondurma yönteminin kısa süreli dondurma yöntemine göre çözdürme sonrası hızlı hareket eden (rapid) spermatozoon oranın da önemli derecede bir artış ($p<0,05$) sağladığı belirlendi. Buna ilaveten, hem sulandırıcı özelliği hem de dondurma yöntemleri açısından yapılan değerlendirmede çözdürme sonrası orta hızda hareket eden (medium) ve yavaş hareket eden (slow) spermatozoon oranları yönünden önemli bir farklılık ($p>0,05$) gözlenmedi.

Farklı özellikteki kimyasal maddeler kullanılarak hazırlanan sperma sulandırıcıları ile sulandırılıp hızlı ve yavaş dondurma hızı yöntemleri kullanılarak dondurulan koç spermasının çözdürme sonrası spermatozoanın hızlarına ve doğrusallıklarına göre belirlenen kinematik parametre değerleri Tablo 5 ve Tablo 6'da gösterildi. Sunulan değerler incelendiğinde; hem sulandırıcı özelliği hem de dondurma yöntemleri açısından çözdürme sonrası VCL, VAP ve VSL yol hızı değerlerinde ve LIN, STR ve WOB doğrusallık oranlarında istatistiki yönden önemli bir farklılığın olmadığı ($p>0,05$) tespit edildi.

Çözdürme sonrası elde edilen sonuçlar koçlar arasında bireysel olarak değerlendirildiğinde ise; total motilite, progresif motilite, hızlı hareket eden ve hareketsiz (statik) spermatozoon oranı yönünden koçlar arasında önemli bireysel farklılıkların olduğu ($p<0,05$) göze çarptı.

Tablo 3. Farklı özellikteki tris sulandırıcılarıyla sulandırılıp hızlı ve yavaş dondurma hızı uygulanarak dondurulan koç spermalarının çözdürme sonrası total motilite, progresif motilite ve hareketsiz spermatozoon oranları.

Table 3. Total motility, progressive motility and immotile spermatozoon rates after thawing of ram semen diluted with Tris extenders of different properties and frozen at fast and slow freezing rates.

	Total Motilite Oranı (%)				**Progresif Motilite Oranı (%)**				**Hareketsiz (Statik) Spermatozoon Oranı (%)**			
	Kısa Süreli Dondurma		**Uzun Süreli Dondurma**		**Kısa Süreli Dondurma**		**Uzun Süreli Dondurma**		**Kısa Süreli Dondurma**		**Uzun Süreli Dondurma**	
	Tris-S	**Tris-U**	**Tris-S**	**Tris-U**	**Tris-S**	**Tris-U**	**Tris-S**	**Tris-U**	**Tris-S**	**Tris-U**	**Tris-S**	**Tris-U**
K-1	$35,4\pm5,7^{ABa}$	$40,8\pm4,6^{ABa}$	$42,6\pm8,2^{ABCa}$	$53,2\pm3,4^{BCa}$	$16,9\pm5,7^{Ba}$	$23,5\pm2,1^{Ca}$	$20,0\pm5,5^{ABa}$	$25,6\pm2,7^{BCa}$	$64,6\pm5,7^{ABa}$	$59,2\pm4,6^{ABa}$	$57,4\pm8,2^{ABCa}$	$46,8\pm3,4^{BCa}$
K-2	$21,1\pm3,5^{Aa}$	$26,8\pm2,8^{Aab}$	$33,4\pm2,3^{ABab}$	$39,5\pm3,9^{ABb}$	$9,8\pm1,8^{ABa}$	$12,5\pm0,7^{ABab}$	$16,3\pm1,3^{ABb}$	$17,5\pm1,4^{ABb}$	$78,9\pm3,5^{Aa}$	$73,2\pm2,8^{Aab}$	$66,6\pm2,3^{ABab}$	$60,5\pm3,9^{ABb}$
K-3	$30,7\pm2,5^{ABa}$	$36,3\pm1,4^{Aa}$	$58,2\pm1,5^{Cb}$	$61,1\pm2,8^{Cb}$	$11,8\pm1,5^{ABa}$	$15,5\pm0,9^{ABCa}$	$26,1\pm2,0^{Bb}$	$26,7\pm2,2^{BCb}$	$69,3\pm2,5^{ABa}$	$63,7\pm1,4^{Aa}$	$41,8\pm1,5^{Cb}$	$38,9\pm2,8^{Cb}$
K-4	$32,3\pm2,6^{ABa}$	$31,7\pm2,6^{Aa}$	$58,5\pm2,6^{Cb}$	$58,1\pm2,1^{Cb}$	$17,6\pm0,5^{Ba}$	$19,3\pm0,9^{ABCa}$	$26,6\pm1,8^{Bb}$	$28,5\pm2,2^{Cb}$	$67,7\pm2,6^{ABa}$	$68,3\pm2,6^{Aa}$	$41,5\pm2,6^{Cb}$	$41,9\pm2,1^{Cb}$
K-5	$43,3\pm6,6^{Ba}$	$53,2\pm4,2^{Bab}$	$51,5\pm1,8^{BCab}$	$58,8\pm3,8^{Cb}$	$16,9\pm1,6^{Ba}$	$21,3\pm3,4^{BCab}$	$19,9\pm1,6^{ABab}$	$29,0\pm3,3^{Cb}$	$56,7\pm6,6^{Ba}$	$46,8\pm4,2^{Bab}$	$48,5\pm1,8^{BCab}$	$41,2\pm3,8^{Ca}$
K-6	$18,2\pm1,7^{Aa}$	$26,7\pm4,1^{Aab}$	$26,8\pm5,4^{Aab}$	$35,1\pm2,7^{Aa}$	$6,9\pm1,4^{Aa}$	$10,3\pm2,6^{Aab}$	$12,0\pm2,3^{Aab}$	$15,4\pm1,0^{Ab}$	$81,8\pm1,7^{Aa}$	$73,3\pm4,1^{Aab}$	$73,2\pm5,4^{Aab}$	$64,9\pm2,7^{Ab}$
GNL	**$30,1\pm2,3^{a}$**	**$35,9\pm2,3^{ab}$**	**$45,2\pm3,0^{bc}$**	**$51,0\pm2,4^{c}$**	**$13,3\pm1,3^{a}$**	**$17,1\pm1,2^{ab}$**	**$20,2\pm1,5^{bc}$**	**$23,8\pm1,4^{c}$**	**$69,9\pm2,3^{a}$**	**$64,1\pm2,3^{ab}$**	**$54,8\pm3,0^{bc}$**	**$49,0\pm2,4^{c}$**

$^{A, B, C}$: Her bir parametre için aynı sütun içinde farklı harf taşıyan ortalama değerler arasındaki farklılıklar istatistiki açıdan önemlidir ($p<0,05$).
$^{a, b, c}$: Her bir parametre için aynı satır içinde farklı harf taşıyan ortalama değerler arasındaki farklılıklar istatistiki açıdan önemlidir ($p<0,05$).
Tris-S: standart teknoloji ile üretilmiş kimyasal maddeler kullanılarak hazırlanmış tris-sitrik asit-fruktoz-yumurta sarısı-gliserol içerikli sperma sulandırıcısı
Tris-H: hassas teknoloji ile üretilmiş kimyasal maddeler kullanılarak hazırlanmış tris-sitrik asit-fruktoz-yumurta sarısı-gliserol içerikli sperma sulandırıcısı
$^{A, B, C}$: Differences between mean values with different letters in the same column for each parameter are statistically significant ($p<0.05$).
$^{a, b, c}$: Differences between mean values with different letters in the same row for each parameter are statistically significant ($p<0.05$).
Tris-S: semen extender containing tris-citric acid-fructose-egg yolk-glycerol prepared using chemical substances produced with standard technology
Tris-H: semen extender containing tris-citric acid-fructose-egg yolk-glycerol prepared using chemicals produced with sensitive technology.

Tablo 4. Farklı özellikteki tris sulandırıcılarıyla sulandırılıp hızlı ve yavaş dondurma hızı uygulanarak dondurulan koç spermalarının çözdürme sonrası hızlı, orta hızda ve yavaş hareket eden spermatozoon oranları

Table 4. The rates of rapid, medium and slow moving spermatozoon after thawing of ram semen diluted with Tris extenders of different properties and frozen at fast and slow freezing rates.

	Hızlı Hareket Eden (Rapid) Spermatozoon Oranı (%)				**Orta Hızda Hareket Eden (Medium) Spermatozoon Oranı (%)**				**Yavaş Hareket Eden (Slow) Spermatozoon Oranı (%)**			
	Kısa Süreli Dondurma		**Uzun Süreli Dondurma**		**Kısa Süreli Dondurma**		**Uzun Süreli Dondurma**		**Kısa Süreli Dondurma**		**Uzun Süreli Dondurma**	
	Tris-S	**Tris-U**	**Tris-S**	**Tris-U**	**Tris-S**	**Tris-U**	**Tris-S**	**Tris-U**	**Tris-S**	**Tris-U**	**Tris-S**	**Tris-U**
K-1	19,8±4,7^{ABa}	29,0±3,8^{Ba}	26,6±7,6^{ABa}	32,0±5,6^{ABCa}	6,4±1,8^{Aa}	4,3±1,0^{ABa}	4,5±1,0^{Aa}	10,1±2,0^{Aa}	9,2±3,5^{Aa}	7,5±1,6^{Aa}	11,6±2,9^{Aa}	11,1±2,4^{Aa}
K-2	12,9±2,2^{ABa}	16,6±1,9^{Aab}	19,7±1,9^{ABab}	23,5±2,6^{ABb}	2,5±0,4^{Aa}	3,0±1,0^{Aa}	4,9±0,9^{Aa}	5,6±1,3^{Aa}	5,7±1,4^{Aa}	7,2±1,9^{Aa}	8,8±0,5^{Aa}	10,4±0,8^{Aa}
K-3	16,9±2,5^{ABa}	22,1±0,7^{ABa}	38,3±4,6^{Bb}	42,0±3,3^{Cb}	4,6±1,1^{Aa}	4,6±1,5^{ABa}	6,9±1,0^{Aa}	4,8±1,6^{Aa}	9,1±2,0^{Aa}	9,6±1,3^{ABa}	13,0±4,1^{Aa}	14,3±3,0^{Aa}
K-4	21,7±1,6^{ABa}	23,4±2,1^{ABa}	34,3±3,1^{Bb}	39,5±1,4^{BCb}	4,2±1,1^{Aa}	2,5±0,5^{Aa}	6,6±0,9^{Aa}	6,2±1,2^{Aa}	6,3±1,1^{Aa}	5,8±0,8^{Aa}	17,6±3,9^{Ab}	12,4±2,1^{Aab}
K-5	24,0±1,2^{Ba}	28,1±4,7^{Ba}	32,1±3,3^{Ba}	35,6±6,1^{ABCa}	5,2±1,1^{Aa}	8,1±1,6^{Ba}	8,1±0,9^{Aa}	8,7±2,6^{Aa}	14,1±5,1^{Aa}	17,0±3,0^{Ba}	11,3±3,1^{Aa}	14,6±2,9^{Aa}
K-6	10,4±0,6^{Aa}	16,7±3,0^{Aab}	12,8±2,8^{Aab}	21,5±1,6^{Aa}	2,0±0,4^{Aa}	3,0±0,3^{Aa}	4,0±1,2^{Aa}	4,2±0,8^{Aa}	5,8±1,7^{Aa}	7,0±1,2^{Aa}	10,0±2,4^{Aa}	9,4±3,5^{Aa}
GNL	**17,6±1,3^{a}**	**22,6±1,5^{ab}**	**27,3±2,4^{bc}**	**32,3±2,1^{c}**	**4,1±0,5^{a}**	**4,3±0,6^{a}**	**5,8±0,5^{a}**	**6,6±0,8^{a}**	**8,4±1,2^{a}**	**9,0±1,0^{a}**	**12,0±1,2^{a}**	**12,0±1,0^{a}**

$^{A, B, C}$: Her bir parametre için aynı sütun içinde farklı harf taşıyan ortalama değerler arasındaki farklılıklar istatistiki açıdan önemlidir ($p<0,05$).
$^{a, b, c}$: Her bir parametre için aynı satır içinde farklı harf taşıyan ortalama değerler arasındaki farklılıklar istatistiki açıdan önemlidir ($p<0,05$).
Tris-S: standart teknoloji ile üretilmiş kimyasal maddeler kullanılarak hazırlanmış tris-sitrik asit-fruktoz-yumurta sarısı-gliserol içerikli sperma sulandırıcısı
Tris-H: hassas teknoloji ile üretilmiş kimyasal maddeler kullanılarak hazırlanmış tris-sitrik asit-fruktoz-yumurta sarısı-gliserol içerikli sperma sulandırıcısı
$^{A, B, C}$: Differences between mean values with different letters in the same column for each parameter are statistically significant ($p<0.05$).
$^{a, b, c}$: Differences between mean values with different letters in the same row for each parameter are statistically significant ($p<0.05$).
Tris-S: semen extender containing tris-citric acid-fructose-egg yolk-glycerol prepared using chemical substances produced with standard technology
Tris-H: semen extender containing tris-citric acid-fructose-egg yolk-glycerol prepared using chemicals produced with sensitive technology.

Tablo 5. Farklı özellikteki tris sulandırıcılarıyla sulandırılıp hızlı ve yavaş dondurma hızı uygulanarak dondurulan koç spermalarının çözdürme sonrası spermatozoonların hızlarına ait kinematik parametre değerleri

Table 5. Kinematic parameters of spermatozoa velocities after thawing of ram semen diluted with Tris extenders of different properties and frozen at fast and slow freezing rates.

	VCL				**VAP**				**VSL**			
	Kısa Süreli Dondurma		**Uzun Süreli Dondurma**		**Kısa Süreli Dondurma**		**Uzun Süreli Dondurma**		**Kısa Süreli Dondurma**		**Uzun Süreli Dondurma**	
	Tris-S	**Tris-U**	**Tris-S**	**Tris-U**	**Tris-S**	**Tris-U**	**Tris-S**	**Tris-U**	**Tris-S**	**Tris-U**	**Tris-S**	**Tris-U**
K-1	100,3±5,4^{Aa}	124,7±9,3^{Aa}	111,7±5,0^{ABa}	114,9±4,5^{ABa}	65,4±7,5^{Aa}	96,3±14,9^{Aa}	75,2±6,3^{ABa}	75,6±6,2^{ABa}	53,8±8,5^{Aa}	87,4±16,1^{Aa}	63,5±6,4^{ABa}	61,7±3,9^{ABa}
K-2	107,5±5,0^{Aa}	113,6±7,5^{Aa}	117,6±4,8^{ABa}	103,8±5,4^{Aa}	74,9±7,2^{Aa}	80,3±10,5^{ABa}	92,2±4,6^{ABa}	68,5±8,5^{Aa}	61,0±6,2^{Aa}	69,0±9,4^{Aa}	78,7±4,8^{ABa}	54,4±5,6^{Aa}
K-3	99,7±3,5^{Aa}	108,3±9,9^{Aab}	119,7±3,3^{ABab}	131,6±6,0^{Bb}	69,3±8,4^{Aa}	73,0±12,9^{Aa}	80,7±6,0A^{Ba}	92,7±11,1^{ABa}	55,5±8,9^{Aa}	60,5±11,5^{Aa}	64,7±4,9^{ABa}	75,6±11,1^{ABa}
K-4	115,6±9,9^{Aa}	114,7±5,1^{Aa}	126,4±10,6^{Ba}	125,9±6,1^{ABa}	88,5±13,2^{Aa}	80,2±3,8^{Aa}	100,3±11,9^{Ba}	97,6±7,6^{Ba}	79,2±12,6^{Aa}	71,4±5,1^{Aa}	86,2±11,5^{Ba}	78,5±5,7^{Ba}
K-5	119,1±7,6^{Aa}	105,1±5,7^{Aa}	110,4±3,7^{ABa}	111,5±9,9^{ABa}	83,2±10,7^{Aa}	72,1±5,9^{Aa}	62,4±4,9^{Aa}	81,0±11,2^{ABa}	67,1±11,0^{Aa}	57,7±5,2^{Aa}	47,1±5,0^{Aa}	67,1±9,4^{ABa}
K-6	102,8±6,9^{Aa}	117,5±7,6^{Aa}	98,3±7,9^{Aa}	116,7±8,7^{ABa}	61,4±8,4^{Aa}	72,7±13,9^{Aa}	68,8±13,7^{Aa}	68,1±2,6^{Aa}	51,6±9,6^{Aa}	54,5±11,5^{Aa}	58,6±13,2^{ABa}	56,3±2,3^{ABa}
GNL	**107,5±2,9^{a}**	**113,9±3,1^{a}**	**114,0±3,0^{a}**	**117,4±3,2^{a}**	**73,8±4,0^{a}**	**79,1±4,4^{a}**	**79,9±4,1^{a}**	**80,6±3,8^{a}**	**61,4±4,0^{a}**	**66,8±4,4^{a}**	**66,5±4,0^{a}**	**65,6±3,2^{a}**

$^{A, B, C}$: Her bir parametre için aynı sütun içinde farklı harf taşıyan ortalama değerler arasındaki farklılıklar istatistiki açıdan önemlidir ($p<0,05$).
$^{a, b, c}$: Her bir parametre için aynı satır içinde farklı harf taşıyan ortalama değerler arasındaki farklılıklar istatistiki açıdan önemlidir ($p<0,05$).
Tris-S: standart teknoloji ile üretilmiş kimyasal maddeler kullanılarak hazırlanmış tris-sitrik asit-fruktoz-yumurta sarısı-gliserol içerikli sperma sulandırıcısı
Tris-H: hassas teknoloji ile üretilmiş kimyasal maddeler kullanılarak hazırlanmış tris-sitrik asit-fruktoz-yumurta sarısı-gliserol içerikli sperma sulandırıcısı
VCL: Eğrisel (Gerçek) Yol Hızı, VAP Ortalama Yol Hızı, VSL: Doğrusal Yol Hızı
$^{A, B, C}$: Differences between mean values with different letters in the same column for each parameter are statistically significant ($p<0.05$).
$^{a, b, c}$: Differences between mean values with different letters in the same row for each parameter are statistically significant ($p<0.05$).
Tris-S: semen extender containing tris-citric acid-fructose-egg yolk-glycerol prepared using chemical substances produced with standard technology
Tris-H: semen extender containing tris-citric acid-fructose-egg yolk-glycerol prepared using chemicals produced with sensitive technology.
VCL: Curvilinear velocity, VAP Average path velocity, VSL: Straight line velocity

Tablo 6. Farklı özellikteki tris sulandırıcılarıyla sulandırılıp hızlı ve yavaş dondurma hızı uygulanarak dondurulan koç spermalarının çözdürme sonrası spermatozoonların doğrusallıklarına ait kinematik parametre değerleri

Table 6. Kinematic parameters of spermatozoa linearity after thawing of ram semen diluted with Tris-based semen extenders prepared with chemical substances of different purity and frozen at fast and slow freezing rates.

	LIN				**STR**				**WOB**			
	Kısa Süreli Dondurma		**Uzun Süreli Dondurma**		**Kısa Süreli Dondurma**		**Uzun Süreli Dondurma**		**Kısa Süreli Dondurma**		**Uzun Süreli Dondurma**	
	Tris-S	**Tris-U**	**Tris-S**	**Tris-U**	**Tris-S**	**Tris-U**	**Tris-S**	**Tris-U**	**Tris-S**	**Tris-U**	**Tris-S**	**Tris-U**
K-1	53,5±7,4^{Aa}	68,5±8,1^{Aa}	57,1±6,2^{Aa}	53,5±1,6^{Aa}	80,9±5,5^{Aa}	89,5±3,0^{Ba}	84,1±2,0^{ABa}	82,0±3,0^{Aa}	65,0±5,7^{Aa}	76,0±6,7^{Aa}	67,7±6,2^{ABa}	65,6±3,5^{ABa}
K-2	56,8±5,4^{Aa}	60,0±4,3^{Aa}	66,9±3,3^{Aa}	52,1±3,3^{Aa}	81,5±3,8^{Aa}	85,9±2,6^{Ba}	85,2±1,6^{Ba}	79,9±2,4^{Aa}	69,4±4,6^{Aa}	69,8±4,4^{Aa}	78,4±2,3^{ABa}	65,4±4,8^{ABa}
K-3	55,1±7,3^{Aa}	54,6±5,4^{Aa}	54,2±4,4^{Aa}	56,7±6,4^{Aa}	79,0±3,4^{Aa}	82,5±1,2^{ABa}	80,2±2,2^{ABa}	80,7±2,8^{Aa}	69,1±6,5^{Aa}	65,9±5,6^{Aa}	67,5±5,1^{ABa}	69,9±6,0^{ABa}
K-4	67,3±4,7^{Aa}	63,1±6,7^{Aa}	67,3±3,7^{Aa}	62,2±2,0^{Aa}	89,2±3,2^{Aa}	88,9±3,4^{Ba}	85,4±1,7^{Ba}	80,8±2,9^{Aa}	75,5±4,3^{Aa}	70,6±5,6^{Aa}	78,7±3,2^{Aa}	77,2±2,8^{Aa}
K-5	55,5±6,8^{Aa}	55,2±5,2^{Aa}	42,5±3,5^{Aa}	60,0±4,9^{Aa}	79,4±3,3^{Aa}	80,0±2,6^{ABa}	75,0±2,3^{Aa}	82,8±0,4^{Aa}	69,3±6,3^{Aa}	68,8±5,1^{Aa}	56,5±3,3^{Ba}	72,4±5,8^{ABa}
K-6	51,9±11,4^{Aa}	45,7±8,5^{Aa}	57,7±8,8^{Aa}	49,0±4,3^{Aa}	82,4±5,6^{Aa}	74,0±2,5^{Ba}	84,0±2,7^{ABa}	82,8±2,8^{Aa}	61,0±9,9^{Aa}	61,3±10,5^{Aa}	68,2±8,9^{ABa}	59,2±5,9^{Ba}
GNL	**56,7±2,9^{a}**	**57,8±2,8^{a}**	**57,6±2,6^{a}**	**55,6±1,8^{a}**	**82,1±1,7^{a}**	**83,4±1,5^{a}**	**82,3±1,1^{a}**	**81,5±1,0^{a}**	**68,2±2,5^{a}**	**68,7±2,6^{a}**	**69,5±2,5^{a}**	**68,3±2,1^{a}**

$^{A, B, C}$: Her bir parametre için aynı sütun içinde farklı harf taşıyan ortalama değerler arasındaki farklılıklar istatistiki açıdan önemlidir ($p<0,05$).
$^{a, b, c}$: Her bir parametre için aynı satır içinde farklı harf taşıyan ortalama değerler arasındaki farklılıklar istatistiki açıdan önemlidir ($p<0,05$).
Tris-S: standart teknoloji ile üretilmiş kimyasal maddeler kullanılarak hazırlanmış tris-sitrik asit-fruktoz-yumurta sarısı-gliserol içerikli sperma sulandırıcısı
Tris-H: hassas teknoloji ile üretilmiş kimyasal maddeler kullanılarak hazırlanmış tris-sitrik asit-fruktoz-yumurta sarısı-gliserol içerikli sperma sulandırıcısı
LIN: Gerçek Yolun Doğrusallığı, STR: Ortalama Yolun Doğrusallığı, WOB: Yalpalama
$^{A, B, C}$: Differences between mean values with different letters in the same column for each parameter are statistically significant ($p<0.05$).
$^{a, b, c}$: Differences between mean values with different letters in the same row for each parameter are statistically significant ($p<0.05$).
Tris-S: semen extender containing tris-citric acid-fructose-egg yolk-glycerol prepared using chemical substances produced with standard technology
Tris-H: semen extender containing tris-citric acid-fructose-egg yolk-glycerol prepared using chemicals produced with sensitive technology.
LIN: Linearity, STR: Straightness, WOB: Wobble

TARTIŞMA

Spermanın sulandırılmasında kullanılan sulandırıcının bileşimi, sulandırma oranı, ilave edilen gliserol oranı, ekilibrasyon süresi, dondurma yöntemi, dondurma hızı ve çözme sıcaklığı; koç spermasının başarılı bir şekilde dondurulmasını etkileyen en önemli faktörler olarak sıralanabilir. Bu araştırmada, farklı yöntemlerle üretilmiş kimyasal maddelerle hazırlanan Tris temelli sulandırıcıların ve dondurma sırasında farklı dondurma hızı uygulanmasının koç spermasının çözdürme sonrası sperma kalitesi üzerindeki etkileri incelendi.

Yapılan birçok bilimsel çalışma (Ustuner ve ark. 2014; Güngör ve ark. 2022; Zhang ve ark. 2024), Tris - Sitrik asit – Fruktoz - Yumurta sarısı - Gliserol içeren sperma sulandırıcısının koç spermasının dondurulması amacıyla kullanılan temel sperma sulandırıcı olduğu görülmektedir. Tris (hidroksimetilen) aminomethane, sperm hücresinin içerisine girebilme ve pH değişimlerine karşı hücreler arası güçlü bir tamponlama özelliğine sahip olup uygun konsantrasyonlarda spermatozoa üzerine toksik etki göstermeyen ve izotonik bir ortam sağlanması amacıyla kullanılan bir maddedir. Hazırlanan tris buffer solüsyonunun istenilen pH ve osmatik basınç düzeyine ulaşması için ise genellikle sulandırıcıya sitrik asit ilavesi yapılmaktadır. Bunun yanında, bu solüsyona spermanın saklanması sırasında spermatozoa için gerekli olacak enerji rezervini sağlamak amacıyla da genellikle metabolize edilebilir bir şeker olan fruktoz ilave edilmektedir (Maxwell ve Salamon 1993; Gil ve ark. 2003). Diğer taraftan, spermanın sıcaklığının 37 °C'den 5 °C'ye düşürülerek soğutulması sırasında oluşan soğuk şokunun membran yapısı ve geçirgenliği üzerindeki olumsuz etkilerini en az düzeye indirmek amacıyla hazırlanan tris temelli sulandırıcılara yaygın olarak %15–20 oranında yumurta sarısı katılması tavsiye edilmektedir (Swelum ve ark. 2022; Sönmez 2022). Buna ilaveten, spermanın dondurma ve çözdürme işlemleri sırasında ortaya çıkan bu ani sıcaklık değişikliklerine karşı spermatozoayı korumak amacıyla kriyoprotektan bir madde olan gliserol de %5-7 oranında hazırlanan sperma sulandırıcılarına ilave edilmektedir (Salamon ve Maxwell 2000; Mehta ve ark. 2020). Bununla birlikte, tris temelli sperma sulandırıcıların hazırlanmasında kullanılan bu kimyasal maddelerin kimyasal formüleri aynı olmakla birlikte aynı şirket tarafından farklı üretim teknolojilerine göre değişebilen saflık-homojenite düzeylerinde üretilmektedir (Tablo 1). Yapılan çalışmamızda, iki farklı üretim teknoloji ile üretilmiş kimyasal maddeler kullanılarak aynı oranlarda hazırlanan tris temelli sperma sulandırıcılarıyla sulandırılan koç spermalarının bireysel olarak dondurulup çözdürmesi sonrası total motilite, progresif motilite oranları ile spermatozoa hız oranları ve kinematik parametre değerleri yönünden istatistiki olarak önemli bir farklılık oluşmadığı ($p>0,05$) tespit edilmiştir. Bu bulgulara göre; koç spermasının dondurulması amacıyla hazırlanan sperma sulandırıcılarında standart teknoloji ile üretilmiş kimyasal maddelerin kullanılmasının izotonik bir ortam oluşturulması ve pH dengesinin sağlanması açısından yeterli olduğu, diğer taraftan standart teknoloji yerine daha hassas teknoloji ile ultra saflıkta üretilmiş kimyasal maddelerin tercih edilmesinin ise toksitite düzeyi ve hassasiyet açısından önemli bir avantaj sağlamadığı söylenebilir. Bu sonuç, sperma sulandırıcıları hazırlanırken kullanılan kimyasal maddelerin ultra saflıkta üretilmiş daha pahalı serileri yerine aynı formüle sahip daha ekonomik standart üretimlerinin kullanılabileceğini göstermiştir.

Spermanın dondurulmasının amacı, spermanın sıcaklığının spermatozoonlara zarar vermeden kademeli olarak 5°C'den -196 °C'ye düşürmesidir. Spermanın dondurulması aşamasında kullanılacak dondurma hızı, çözdürme sonrası elde edilecek total ve progresif motilite oranları açısından tatmin edici değerlerin elde edilmesini etkileyen önemli bir faktördür (Byrne ve ark. 2000). Spermatozoonların biyokimyasal yapısı oldukça karışık ve sabit olmayan bir özellik taşır. Spermatozoon plazma membranının düzenli bir fonksiyon göstermesi açısından membran yapısındaki lipid ve protein bileşikleri arasında sıkı bir ilişki vardır. Spermatozoonların dondurulması, membran lipid katmanının iki tabakası arasındaki fosfolipit dağılımında değişikliğe yol açar. Membranda meydana gelen bu değişiklikler, belirli düzeyde membran yapısının bozulmasına neden olarak çözdürme sonrası spermatozoonların motilitesini ve fertilizasyon kabiliyetini sınırlayabilir (Parks ve Graham 1992; Hinkovska-Galcheva ve ark. 1998; Diaz ve ark. 2016). Bu açıdan, spermanın dondurulması sırasında özellikle 0 °C ile –20 °C arasındaki sıcaklıklar derecelerinin geçilme süreci oldukça önemli olup oldukça kritik bir aşama olarak değerlendirilir. Spermanın sıcaklığı -10 °C'ye yaklaştığında hücre içi su donar ve spermatozoonlar buz kristalleri oluşma riskiyle karşı karşıya bırakır. Diğer bir ifadeyle, bu aşamada çok hızlı bir soğutma yöntemi uygulandığında artan hücre içi buz kristallerinin oluşumu hücrelerin ölümü ile sonuçlanır (Salamon ve Maxwell 2000).

Nur ve ark. (2011), koç spermasının dondurulması için 5°C ile -20°C arasındaki sıcaklık geçişi sırasında hızlı (6°C/dak) ve yavaş (0,5°C/dak) dondurma hızı yöntemi uyguladıkları çalışmalarında, koç spermasının yavaş soğutma yöntemi ile dondurmasının hızlı dondurma yöntemine göre çözdürme sonrası motilite değerlerini olumlu yönde etkilediğini bildirmişlerdir. Benzer şekilde Vichas ve ark. (2018) ise, 5°C ile -8°C arasında hızlı (5°C/dak) ve yavaş (3°C/dak) dondurma hızı uyguladıkları çalışmalarında koç spermasında yavaş soğutma prosedürü ile dondurmasının hızlı dondurma yöntemine göre çözdürme sonrası daha iyi motilite sonuçları sağladığını bildirmişlerdir. Yürütülen çalışmamızda da, koç spermasının dondurulması aşamasında uygulanan yavaş dondurma yönteminin

hızlı dondurma yöntemine göre çözdürme sonrası total motilite, progresif motilite ve hızlı hareket eden (rapid) spermatozoon oranın da önemli derecede bir artış ($p<0,05$), hareketsiz (statik) spermatozoon oranında ise önemli derecede bir azalma sağladığı ($p<0,05$) belirlenmiştir. Bu sonuç yukarıdaki çalışmaların sonuçları ile benzerlik göstermektedir.

Byrne ve ark. (2000) dondurma işlemi sırasında koç spermatozoasında en fazla hasarın -10°C ile -25°C arasında meydana geldiğini ve yavaş dondurmaya eşlik eden hücre dehidrasyon sürecinin hücrenin hayatta kalması için potansiyel olarak daha faydalı olacağını, buna karşın hızlı dondurma oranlarının hücre ölümüne neden olma olasılığının daha yüksek olduğunu bildirmişlerdir. Bununla birlikte, araştırmacılar bu kritik sıcaklık derecelerini geçişte hızlı (5°C/dak) ve yavaş (0,5°C/dak) dondurma hızı uyguladıkları çalışmalarında çözülme sonrası spermatolojik özellikler açısından iki dondurma yöntemi arasında önemli bir farklılığın olmadığını bildirmişlerdir. Nitekim, Pontbriand ve ark. (1989) da koç spermatozoonlarının dondurulurken kullanılacak soğutma hızı açısından dakikada 6 ile 24 °C arasındaki sıcaklık değişimlerine dayanabildiğini, bu nedenle koç spermasının dondurulması sırasında geniş bir soğuma hızı aralığının kullanılabileceğini bildirmiştir. Genel olarak kabul edilen teoriye göre, en iyi dondurma hızı, suyun spermatozoonları terk etmesine izin verecek kadar yavaş olmalıdır. Bu durum, dondurma sırasında hücre içi su kristalleşmesini önler. Bununla birlikte, yavaş bir soğutma hızının uygulanması spermatozoonların uzun bir süre boyunca yüksek çözünen madde konsantrasyonlarına maruz kalmasına ve hücre dehidrasyonuna neden olabilir. Bu yüzden, soğutma hızı açısından uygulanan dondurma hızı protokollerinden hangisinin yeterince yavaş veya hangisinin yeterince hızlı olduğunu rakamlarla kategorize etmek basit bir iş olmayıp türlere ve bireylere göre önemli değişiklikler gösterebilir (Saha ve ark. 2022).

Rickard ve ark.'ları (2016) yaptıkları çalışmada, koç spermatozoonlarının donma direncindeki tolerans düzeyinin, dondurma öncesi sperma kalitesinden bağımsız olarak bireyler arasında değişkenlik gösterebileceğini bildirmişlerdir. Yürütülen araştırmamızda da, oluşturulan dört gruptan elde edilen sonuçlar değerlendirildiğinde; çözdürme sonrası total motilite, progresif motilite, hızlı hareket eden ve hareketsiz (statik) spermatozoon oranları yönünden koçlar arasında bireysel olarak önemli farklılıkların olduğu ($p<0,05$) göze çarpmaktadır.

SONUÇ

Sonuç olarak, koç spermasının dondurulması aşamasında özellikle 5°C ile -20°C arasındaki sıcaklık geçişi sırasında yavaş dondurma (1°C/dak) yöntemi kullanılması; hızlı dondurma (5°C/dak) yöntemine göre çözdürme sonrası motilite oranları yönünden daha başarılı sonuçlar elde edilmesini sağlamıştır. Diğer taraftan, koç spermasının dondurulması amacıyla sperma sulandırıcıları hazırlanırken ultra saflıkta üretilmiş kimyasal maddelerin ya da aynı formüle sahip standart üretimlerinin kullanılmasının çözdürme sonrası sperma kalitesini yönünden önemli bir farklılık oluşturmadığı gözlenmiştir. Ayrıca bu çalışmanın sonuçları, koçlar arasında dondurulup çözdürülme işlemine karşı bireysel tolerans düzeylerinin önemli değişkenlik gösterdiğini ve bu durumun çözdürme sonrası sperma kalitesinde bireysel olarak önemli farklılıklar oluşturduğunu da göstermektedir.

Çıkar çatışması: Yazarların rapor edecekleri herhangi bir çıkar çatışması yoktur.

Yazar Katkıları: MS ve ABB çalışmayı planlayıp projelendirme aşamalarını gerçekleştirdi. Deneysel aşamaları ABB, İHG, AÇC ve TCA gerçekleştirdi. İlk makale halini İHG yazdı. MS makaleyi okuyarak düzenledi.

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RESEARCH ARTICLE

The Relationship Between Individual Career Planning and Occupational Anxiety of Vocational School Students of Laboratory and Veterinary Health Department

Sibel KULA ÖLMEZ[1]*, Mustafa ÖZBEK[2]

[1]*Department of Human Research Management, Bayat Vocational School, Afyon Kocatepe University, Afyonkarahisar, Türkiye*
[2]*Department of Laborant and Veterinary Health, Bayat Vocational School, Afyon Kocatepe University, Afyonkarahisar, Türkiye*

ABSTRACT

Vocational schools are very important in providing qualified staff to businesses in Turkey. The aim of this study was to determine the individual career planning and future expectations of the students of Afyon Kocatepe University, Department of Laboratory and Veterinary Health Vocational School. The survey is performed on vocational schools resident in three districts, Bayat, Emirdağ and Şuhut, under the roof of Kocatepe University. In this context, although survey questions were sent to all students studying in these schools, 278 students who answered the survey constitute the sample of the study. As a result, no significant relationship was found between the education level, employment status and number of siblings of Vocational School students and their career planning decisions and occupational anxiety levels. This situation is accepted as an indicator that the career planning and anxiety levels of Generation Z students are low. It is thought that this study will shed light on future studies and it will be useful to repeat and further elaborate these studies with students studying in different departments.
Keywords: Future Concern, Personal Career Planning, Vocational Schools, Vocational School Issues, Z Gen

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Laborant ve Veteriner Sağlık Bölümü Meslek Yüksekokulu Öğrencilerinin Bireysel Kariyer Planlamaları ve Meslek Kaygıları Arasındaki İlişki

ÖZ

Türkiye'deki işletmelere kalifiye eleman sağlamada meslek yüksekokullarının çok önemli olduğu bilinmektedir. Bu çalışmanın amacı, Afyon Kocatepe Üniversitesi Laborant ve Veteriner Sağlık Bölümü Meslek Yüksekokulu öğrencilerinin bireysel kariyer planlamalarını ve gelecek beklentilerini belirlemektir. Söz konusu bölüm Kocatepe Üniversitesi çatısı altında Bayat, Emirdağ ve Şuhut olmak üzere üç ilçede bulunmaktadır. Bu bağlamda bu okullarda öğrenim gören tüm öğrencilere anket soruları gönderilmesine rağmen anketi cevaplayan 278 öğrenci araştırmanın örneklemini oluşturmaktadır. Sonuç olarak, Meslek Yüksekokulu öğrencilerinin eğitim düzeyi, çalışma durumu ve kardeş sayısı ile kariyer planlama kararları ve mesleki kaygı düzeyleri arasında anlamlı bir ilişki bulunmamıştır. Bu durum Z kuşağındaki öğrencilerin kariyer planlama ve kaygı düzeylerinin düşük olduğunun bir göstergesi olarak kabul edilmektedir. Bu çalışma ile gelecek çalışmalara ışık tutulacağı, aynı zamanda farklı bölümlerde eğitim gören öğrencilerle bu çalışmaların tekrarlanması ve daha da detaylandırılmasının faydalı olacağı düşünülmektedir.
Anahtar Kelimeler: Gelecek Kaygısı, Kişisel Kariyer Planlaması, Meslek Yüksek Okulları, Meslek Yüksek Okulları Sorunları, Z Kuşağı.

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ORCID ID; *SKÖ: 0000-0002-5116-9485, MÖ: 0000-0002-6736-2124*
*Corresponding author e-mail: skulaolmez@aku.edu.tr

INTRODUCTION

In vocational schools, which are part of universities, students receive two years of education and training to become intermediate staff members for both public and private corporations. In other words, vocational schools are educational institutions established to meet the increasing demand for higher education, expand educational opportunities, respond to globally changing qualified personnel needs, and develop society (Özhavalı et al. 2010). In Turkey, technician and high technician schools started to be established in 1954 (Kılıç 2008). In 1981, it was restructured and defined as institutions with the status of colleges that provide two-year associate degree education (Eşme 2007). In short, vocational colleges are institutions that work to meet the needs expected by society, such as providing education to a wider group of students, especially with the recognition of the right to pass without exams from vocational high schools, creating programs that will cover all of the constantly developing and changing new fields of knowledge, focusing on practice in addition to the production of knowledge, and supporting regional and national development (Günay and Özer 2016).

Individual career planning is the process of setting goals for one's professional life and organizing actions to reach these objectives while accounting for the unique circumstances of the individual (Erdoğmuş 2003). However, a certain decision on these goals leads to determining future vocational purposes and encourages necessary individual planning by accelerating personal development (Demirbilek 1994). Career development of individuals is a very long and complex process. In this process, the individual can be affected by many events. According to the results of the research analysing the three most important career theories and the main findings of these theories, demographic factors are among the factors that affect individuals most at the career stage (Adıgüzel and Erdoğan 2014).

The origin of the word anxiety is 'anxietas' in ancient Greek. Anxietas means curiosity, fear, worry (Köknel 2014). Mithat Enç (1980) defines the concept of anxiety as 'an uneasy feeling that arises when a strong desire or impulse seems to be unable to reach its goal'. And occupational anxiety is one of the comon problems in young generation to avoid them performing a proper career plan.

The purpose of this study was to determine whether there is a relationship between individual career planning and future and occupational concerns of students in Vocational Schools.

MATERIAL and METHODS

Purpose, Method and Sample of the Study, and Evaluation of the Obtained Data

In the research; for the Individual Career Planning variable, a scale with proven validity and reliability was used in the study prepared by Hasibe SEÇER (2013) with the title 'The Relationship Between Individual Career Planning and Personal Success Perception and a Research in Pamukkale University'.In addition, the scale used by Meryem Akoğlan Kozak and Tülin Dalkıranoğlu in the study titled 'Career Perceptions of Graduate Students: The Case of Anadolu University' was updated by the authors and included in the study. For the Future and Professional Anxiety variable, the scales used in three different studies were taken as basis. These researches were "Occupational Anxiety Scale for Prospective Teachers: Validity and Reliability Study" by Cabi and Yalçınalp (2013); "Development of Occupational Anxiety Scale for Emergency Health Workers" by Postacı et al. (2020) and "Investigation of Occupational Anxiety Levels of Police Candidates in Terms of Various Variables" by Uludağ et al. (2014). All three studies are important scales developed for difficult, exhausting, and stressful professions such as teaching, emergency health workers, and police work.

The hypotheses of the research are based on the relationship between the concepts of individual career planning and occupational anxiety, which were reached as a result of the studies in the literature:

H_1: There is a relationship between students' grade level and their career planning decisions and occupational anxiety levels.

H_2: There is a relationship between students' gender and their career planning decisions and vocational anxiety levels.

H_3: There is a relationship between students' age and their career planning decisions and vocational anxiety levels.

H_4: There is a relationship between the educational level, employment status and number of siblings of students' families and their career planning decisions and occupational anxiety levels.

H_5: The sector in which students want to work affects their career planning decisions and occupational anxiety.

H_6: Depending on the students' desire to continue their undergraduate education, career planning decisions and occupational anxiety levels are affected and vary.

In addition, with the survey study, students' thoughts on issues such as how many job applications they need to make in order to have a good job, how many years they need to work for a professional position, wage expectations, the methods they use for job applications, meaning of work-life to students were taken and their level of professional anxiety was tried to be measured with multiple-choice questions.

The survey results were analysed with IBM SPSS Statistics 22 software. Reliability analysis was performed to test the accuracy of the answers and

Cronbach's Alpha value was measured based on the 0.70 limit stipulated by Hair (2014). Accordingly, the result of the Reliability Analysis (Table 1) of the study was found to be 0.87, which is quite high.

Table 1. Reliability Analysis Result

Cronbach's Alpha	Cronbach's Alpha Based on Standardized Items	N of Items
.873	.874	40

This research was approved by the Ethics Commitee of Afyon Kocatepe University, Social Sciences and Humanities Scientific Research and Publication Ethics Committee (Ref No: 2024/174, Date: 15/05/2024). Vocational Schools in Bayat, Emirdağ and Şuhut districts were visited and school administrators, department lecturers and students were informed about the survey questions, and it was explained that the data to be obtained would be used for a scientific research and that the information provided would remain confidential. The survey was applied face-to-face to all students present in the schools on the day of the visit, and the students who were not in the schools were excluded from the research. A total of 278 students from three vocational schools participated in the study.

Demographic Structure of the Sample

Table 2. Demographic Characteristics of Participants

Grade	**f**	**%**
First Grade	144	51.8
Second Grade	134	48.2
Gender		
Female	156	56.1
Male	122	43.9
Age		
16-17	1	0.4
18-19	73	26.3
20-21	160	57.6
22 and older	44	15.8
TOTAL	**278**	**100.0**

The study starts with demographic questions (Table 2). Especially in the first question, it is asked which grade the students are in. Of the students participating in the survey, 144 were in the first year and 134 were in the second year. In the survey, the number of 3 or more years was even added for students with extension. However, it was observed that this option was not marked at all in the answers. The first year of university life is regarded as a period of orientation and getting used to a new way of life after high school. Students need a few more years to complete their career awareness. However, in institutions such as Vocational Schools, which provide two-year education, students who do not have such a period start to experience occupational anxiety and fear of finding a job in the second year of education. For this reason, with the first question, one of the hypotheses of the research, that there is a relationship between the class of the Vocational School students and their career planning and occupational anxiety levels, is tried to be measured. In order to test this hypothesis, it was first checked whether the data were normally distributed. According to the result of Shapiro-Wilk, the level of skewness and skewness is between -1.5 and +1.5 (Sig:0.510/Skewness:0.82-Kurtosis:0.21). Due to the normal distribution of the data, Independent Sample T test was used to examine whether there was a statistically significant relationship between the class of the students and their career planning and occupational anxiety levels.

Table 3. T Test Results of the relationship between career planning (CP) and occupational anxiety (OC) levels of Vocational School students and the class they are in

	N	**Mean CP / OA**	**Std. Deviation CP / OA**	**t CP / OA**	**df CP / OA**	**p CP / OA**
First Grade	144	3.9906/3.0358	0.56190	1.474/1.144	276	0.142/0.254
Second Grade	134	3.8922/3.1455	0.80152			

According to the result of the analysis (Hypothesis 1), it is seen that there is no significant difference between career planning and occupational anxiety levels of Vocational School students with the class they are in (Table 3: p=0.142/0.254>0.05). Especially when it is considered that second year students will have higher future and occupational anxiety levels when their graduation is approaching, it is understood that only second year students' occupational anxiety levels are slightly higher than first year students (X= 3.1455>3.0358).

When the relationship between the gender of the students and their career planning decisions and occupational anxiety levels was analysed;

Table 4. T test Results of the relationship between the gender of Vocational School students and their career planning (CP) and occupational anxiety (OA) levels

	N	Mean CP / OA	Std. Deviation CP / OA	t CP / OA	df CP / OA	p CP / OA
Female	156	3.9478/3.1410	0.55324/0.75476	0.155/1.235	276	0.877/0.218
Male	122	3.9373/3.0217	0.56571/0.85244			

Due to the normal distribution of the data, Independent Sample T test was used to examine whether the relationship between the gender of the students and their career planning and occupational anxiety levels was statistically significant (Table 4). According to the results of the analysis (Hypothesis 2), there is no significant difference between the gender of Vocational School students and their career planning and occupational anxiety levels (p=0.877/0.218>0.05). When the relationship between students' ages and their career planning decisions and occupational anxiety levels was analysed;

Table 5. Anova Test Results of the relationship between the age of Vocational School students and their career planning (CP) and occupational anxiety (OA) levels

	Sum Of Squares CP / OA	sd CP / OA	Mean Square CP / OA	F CP / OA	p CP / OA
Between Groups	0.455/3.839	3	0.152/1.280	0.485/0.022	0.693/0.111
Within Groups	85.717/173.358	274	0.313/0.633		
TOTAL	86.172/177.197	277			

According to Table 5, Anova test was applied to examine whether the relationship between students' ages and career planning decisions and occupational anxiety levels was statistically significant. According to the results of this analysis (Hypothesis 3), there was no significant difference between the ages of the students and their career planning decisions and occupational anxiety levels (p=0.693/0.111>0.05).

According to Table 6, when the percentages related to the education level, employment status and number of siblings of the students‘ families are examined, 208 of the students’ mothers are primary school graduates. Especially in a study conducted in 2024, it is quite thought-provoking that 74.8% of the students, i.e. 3 out of every 4 students' mothers are primary school graduates. In addition, when we look at the occupation of the mother, it is seen that a very high rate of nearly 70% of the mothers stay in the domestic sphere. Considering that mothers are role models especially for female students, the low level of education is regrettable. However, when we look at the father's education level, more than half (52.2%) of the fathers of Vocational School students are again primary school graduates. Since the father's salary is usually the only source of income in the family, the fact that 15% of the fathers' occupation is labelled as unemployed or not working creates a line parallel to the unemployment rates in our country.

Table 6. Demographic Information of Students' Families

	f	%		f	%
Mother Education Level			**Mother Working Status**		
Primary Education	208	74.8	Unemployed	193	69.4
High Education	54	19.4	Private Sector	48	17.3
University	16	5.8	Public Sector	12	4.3
TOTAL	**278**	**100.0**	Self-Employment	25	9.0
			Father Working Status		
Father Education Level			Unemployed	43	15.5
Primary Education	145	52.2	Private Sector	93	33.5
High Education	112	40.3	Public Sector	24	8.6
University	21	7.6	Self-Employment	118	42.4
			TOTAL	**278**	**100.0**
Number of Siblings					
None				28	10.1
One				86	30.9
Two				70	25.2
Three				51	18.3
Four				22	7.9
Five and more				21	7.6
TOTAL				**278**	**100.0**

When the relationship between the educational level, employment status and number of siblings of the students' families and their career planning decisions and vocational anxiety levels was analysed, Anova Test was applied separately for each qualitative variable. According to this;

Table 7. Anova Test Results of the relationship between career planning (CP) and occupational anxiety (OA) levels of Vocational School students with their mother's and father's education level, employment status and number of siblings

		Sum Of Squares CP / OA	sd CP / OA	Mean Square CP / OA	F CP / OA	p CP / OA
Education Level of Mother	Between Groups	0.974/2.340	2	0.487/1.170	1.572/1.840	0.210/0.161
	Within Groups	85.198/174.857	275	0.310/0.636		
Education Level of Father	Between Groups	0.753/0.591	2	0.376/0.296	1.212/0.460	0.299/0.632
	Within Groups	85.419/176.606	275	0.311/0.642		
Working Status of Mother	Between Groups	1.865/3.128	2	0.622/1.043	2.020/1.641	0.111/0.180
	Within Groups	84.307/174.069	275	0.308/0.635		
Working Status of Father	Between Groups	0.171/0.752	3	0.057/0.251	0.182/0.389	0.909/0.761
	Within Groups	86.001/176.445	277	0.314/0.644		
Number of Siblings	Between Groups	1.015/6.346	5	0.203/1.296	0.648/2.020	0,663/0.076
	Within Groups	85.157/170.851	272	0.313/0.628		
TOTAL		**177.197**	**277**			

As a result of the analyses (Table 7- Hypothesis 4), there is no significant difference between the career planning decisions and vocational anxiety levels of Vocational School Students and their families' education level, working status and number of siblings (for mother education level p=0.210/0.161>0.005, for father education level p=0.299/0.632>0.005, for mother working status p=0.111/0.180>0.05, for father working status p=0.909/0.761>0.005, for number of siblings p=0.663/0.076>0.05).

Regression analysis was performed to analyse the hypothesis that the sector in which students want to work affects their career planning decisions and occupational anxiety. The desired sector to work in, career planning variable and occupational anxiety variable were measured separately and presented in Table 8:

Table 8. Regression analyse results of the sector in which students want to work and its effect on their career planning decisions and occupational anxiety.

	R Square	Sum Of Squares	df	Mean Square	F	p
CP						
Regression	0,000	0.033	1	0.033	0.107	0.744
Residual		86.139	276	0.312		
OA						
Regression	0.023	4,077	1	4,077	6.499	0.011
Residual		173.120	276	0.627		
TOTAL		86.172/177.197	277			

The sector in which Vocational School students want to work was grouped under 3 groups as Public, Private and Independent. Regression Analysis was applied to examine whether it statistically affects career planning decisions and occupational anxiety levels. According to the results of this analysis (Hypothesis 6); the sector in which students want to work does not affect their career planning decisions and occupational anxiety levels

(p=0.744/0.011>0.05). 168 students (60.4%) answered yes and 110 students (39.6%) answered no to the statement given to measure whether Vocational School students consider two-year education sufficient and whether they want to use a special right such as Vertical Transfer Examination, which is especially

recognized for vocational school students. Although the option of completing bachelor's degree with open education was added to the survey form, no student marked this option.

Table 9. T test Results of the relationship between the desire of the Vocational School students willingness to continue their undergraduate studies and their career planning (CP) and occupational anxiety (OA) levels

	N	Mean	Std. Deviation	t	df	p
		CP / OA K	CP / OA	CP / OA	CP / OA	CP / OA
Yes	168	3.9595/3.0458	0.60727/0.83579	0.604/-1.104	276	0.547/0.271
No	110	3.0458/3.1541	0.47384/0.74045			

Since they are a bivariate group, Hypothesis 6: There is a relationship between career planning decisions and occupational anxiety levels of students depending on their willingness to continue their undergraduate education was analysed by T test and according to the result of the analysis, no relationship was found (Table 9: p=0.547/0.271>0.05).

In addition, the professional anxiety levels of the students were assessed by multiple-choice questions by taking their opinions on issues such as the number of job applications they should make in order to have a good job, how many years they should work for a professional position, their wage expectations, the methods they use for job applications, the meaning of working life for students. The data obtained by these expressions was analysed using frequency analysis, and the following results were obtained.

Table 10. Findings related to students' perspective on business life, job application and salary expectations.

	f	%
Number of job applications required to have a good job		
1	21	7.6
2	28	10.1
3	78	28.1
4	32	11.5
5 and More	119	42.8
Number of years to work in the first professional position		
1	15	5.4
2	43	15.5
3	82	29.5
4	39	14.0
5 and More	99	35.6
Expectation of salary (TRL)		
Minimum Salary	11	4.0
20.000-35.000	91	32.7
36.000-49.000	109	39.2
50.000 and more	67	24.1
Methods used for job applications		
Recommendations from friends/Acquaintances/References by Lecturers	111	39.9
Websites of employers	34	12.2
Career websites	54	19.4
Personal contact with employers	70	25.2
Job adverts in newspapers and magazines	1	0.4
Employer promotions organised at universities	8	2.9
Meaning of work life		
Long-term earnings	52	18.7
Experience and Specialisation	110	39.6
Regular work/life balance	91	32.7
Practical work training	6	2.2
Direct entry to work	5	1.8
A safe position	14	5.0
Most important issue in work life		
Equal opportunities	30	10.8
Location of the workplace	10	3.6
Value given to the employee	120	43.6
Starting point for a good career	71	25.5
Existence of corporate social responsibility projects	5	1.8
Work to be done	7	2.5
Management style of the employer	10	3.6
Personal development support	2	0.7
Starting salary	4	1.4
Work life balance	19	6.8
TOTAL	**278**	**100.0**

As presented in Table 10, 119 (42.8%) of students think that they need to apply for at least 5 or more jobs in order to have a good job, 99 (35.6%) think that they need to work for at least 5 years or more for a professional position, and 109 (39.2%) of the students have a salary expectation between 36,000-45,000 TRL. Again, 111 (39.9%) of the students participating in the research tend to use the recommendations of friends, acquaintances, and academic professors as a method for job application. When the meaning of work-life is asked, 110 (39.6%) students answered it as experience and specialization. This answer is in parallel with the expectations of the students of a department such as Laboratory and Veterinary Health, which has a curriculum with intensive practical courses, in terms of the fact that Veterinary Medicine is a sector where professional experience can be gained through practice. In addition, when the students were asked about the most important issue in their working lives, 120 (43.6%) of the students selected the answer 'Value Given to the Employee', which suggests that the commitment of the students to their jobs and the business they work for will vary depending on their perception of value.

DISCUSSION

The aim of this study is to determine whether there is a relationship between individual career planning and future and occupational concerns of students in Vocational Schools (Bayat, Şuhut and Emirdağ) in Afyon Kocatepe University. This department was subjected to research because it is both difficult to study and it concerns the health of living beings.

Vocational schools are institutions that are between high schools and faculties. They train personnel for career in their field and also allow them to further education through vertical transfer to obtain a bachelor's degree (Terim and Öztürk 2009). Most of these students use their two-year education to continue their business careers, even though some of them use vertical transfer to transfer to four-year faculties. Compared to undergraduate students, these students receive a shorter and more focused education, which makes it easier and faster for them to find employment after graduation. As their employment mostly depends on their education branch, they have to make long-term and extensive plans that involve their entire future (Günay and Özer 2016).

Only the individual knows the own career-related wishes and expectations, and these expectations may vary from person to person. Since career planning is genuinely something that each individual can handle on their own, no one else can arrange the professional path of another. Therefore, individuals who plan their own careers are more likely to be satisfied with their careers and jobs in the future. Because people who have a good career plan are aware of the pivotal points on their career path. If they reach their set point as a result, they are more likely to feel a sense of achievement (Byars and Leslie 2004). Five distinct stages become apparent when individual career planning is examined in different sections:

1. Self-Assesment: This stage involves gathering all of the individual's existing qualifications, including talent, knowledge, skill, desire, and knowledge, and creating an inventory (Palmer 1993).

2. Evaluation of Career Opportunities: The second stage in individual career planning is the evaluation of career opportunities, which is the step in which the person identifies the opportunities and potentials that can be evaluated within and outside the organization. At this stage, the individual also makes an evaluation about the job market and economic situation. In addition, the person tries to obtain information about the training and development opportunities that the organization can offer to the individual, such as different jobs or working in different departments (Daft 1991).

3. Setting a Goal: Individuals need to have their goals clearly defined and understood in order to achieve their career aspirations (Fındıkçı 2000). Individuals should strive to realize their goals by using the opportunities they have in accordance with the goals they have set (Aytaç 1997). In this process, individuals often act alone. For this reason, it is sometimes not possible for individuals to proceed correctly on the desired path. In such cases, it will be in the best interest of individuals to seek help from experts (Anafarta 2001).

4. Preparation and Implementation of Career Plan: At this stage, the individual who has determined his goals will now enter the process of creating his career plan. In this planning step, the individual must be in an environment that supports self process and can activate it (Tunç and Uygur 2001). At this point, the individual's life should be taken into consideration when choosing a career, and acting in this direction should also help to create the career route that was properly planned (Ültanır and Ültanır 2005).

5. Feedback: The last step of individual career planning is the feedback process. During the feedback stage, the person's actions are reviewed and updated on a regular basis. In this step, the individual should make a self-assessment every six months. Issues such as the progress of the individual, the achieved goals, or whether the new goals that can be set will be better for the person should be among the issues that the individual can pay attention to when assessing at the feedback stage (Daft 1991). With the feedback to be obtained as a result of a good assessment of the person, the cons and pros of the individual can be better understood.

In this study, it was determined that Vocational School students were not very aware of individual career planning, they did not choose any goal when they made career plans; and their awareness levels did not reach sufficient maturity.

Anne Roe, a clinical psychologist, suggested that early childhood experiences affect career behaviour and argued that the relationship between early childhood experiences and later career behaviour is mediated by the structure of psychological needs that develops depending on the pattern of disappointments and satisfactions experienced in childhood. In another study, John L. Holland, a vocational consultant who has worked in educational institutions, military institutions, and psychiatric institutions, argues that people necessarily carry the characteristics of one of the six personality types defined and that they choose one of the occupations suitable for one of these types. Only when an individual chooses one of these occupations, it can lead to reaching fulfillment. Besides, Edgar Schein stated that the personality structures that guide individuals in determining their careers are formed in childhood and that the behaviours of individuals as a result of their needs and motives are effective in their career choices. Career value, as defined by Schein, is a self-concept that guides the individual, restricts him/her, enables him/her to act consistently, shapes his/her individual career, and reflects the abilities, attitudes, motives, and core values perceived by the individual's self. Therefore, personality traits are an important factor affecting career plans (Erdoğan 2009).

According to previous studies, there are different factors such as environmental, economic, social, cultural, future, occupational concerns and anxieties that affect the individual career planning of university students (Keskin and Korkut 2016). Furthermore, in individual career planning, the families of individuals can also be effective in their career choices. The structure, culture, income level and orientation of the family is an important factor affecting the career of the individual. Even if individuals are sometimes interested in some professions and are capable of doing this profession, their families may not want individuals to work in that job and problems may arise between the individual and the family (Seçer 2013).

In this study, it was determined that the income levels of the families of the students were quite low and the household budget was mostly dependent to the father. Besides, the majority of students' mothers did not work, and the education levels of the parents were low. In addition, although the occupational and future anxiety of the students participating in the questionnaire was low, it was determined that they mostly chose that they would help their families in their answers to the survey questions. This situation is thought to be caused by the fact that although the students know the importance of the economy, since they have not received adequate career planning training and their families have not been able to support them consciously.

The first studies on the concept of anxiety in the field of psychology started in the late 1940s. The word anxiety was used for the first time by Freud and its definition was made as a function of the ego. While it was previously accepted that anxiety was a biological concept, later it entered the psychological literatüre (Mert 2017; Manav 2011). According to Freud, anxiety contributes to the function of warning the person against threats from the physical and social environment and taking the necessary measures to sustain life (Gençtan 1997). In general, anxiety is the emotional reaction of the individual to environmental and psychological events. In a narrow sense, anxiety can be defined as a disorder of the emotional state in which the onset and source of anxiety are not recognised but consciously felt (Kaya and Varol 2004). Anxiety is the feeling of distress that one feels as if bad things will happen.

Tendency of the individuals to experience anxiety may depend on the situation they are in. Therefore, an individual who is anxious in one situation may not be anxious in another situation. In other words, anxiety can vary according to situations and conditions. From this point of view, the issue of anxiety is also evaluated in the field of occupational psychology (Savickas 2000). It is known that there are significant relationships between occupational and career anxiety, economic perceptions and career planning. In the study conducted by Akoğlan Kozak and Dalkıran (2013), it was concluded that economic perception is effective in career planning, individuals make career planning with traditional structure, and economic conjuncture is perceived as more important than career planning. In the study conducted by Ulaş and Özdemir (2018) to determine the factors that students perceive as career barriers, it was concluded that self-efficacy and trait anxiety in career decision-making were perceived as career barriers by students, and that family was not seen as an obstacle. Merve Gerçek (2018) examined the relationship between adaptation-centered anxiety and course-centered anxiety with career adaptability in a sample of pre-service teachers studying at the faculty of education and found that pre-service teachers' anxiety about the teaching profession increased, their beliefs about their ability to overcome the problems they may encounter in finding a job and advancing in their careers in the future and to adapt to unexpected changes gradually decreased. As a result of this study, although the students did not have any concerns about their future, they stated that they were worried especially because of dealinng with the life of a living creature due to their profession and that they were worried about paying compensation by damaging an animal during their work. It has been determined that they have reached a conclusion that this profession is difficult and that these students are willing to continue their careers by transferring to faculties with Vertical Transfer Examination with the education they receive.

CONCLUSION

As a result, according to the results of the survey applied to 278 students studying in the Department of Laboratory and Veterinary Health in three Vocational

Schools located in Bayat, Emirdağ and Şuhut districts of Afyon Kocatepe University, no significant relationship was found between the students' grade, gender and age and their career planning decisions and occupational anxiety levels. There is no significant relationship between the educational level, employment status and number of siblings of the Vocational School students and their career planning decisions and vocational anxiety levels. This situation corresponds with the information stated in the literature section and is accepted as an indication that the career planning and anxiety levels of the students in Generation Z are low. As a suggestion for future studies, it would be useful to complete and deepen such studies by conducting interviews with students.

Conflict of interest: The authors have no conflicts of interest to report.

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RESEARCH ARTICLE

The Effect of Atmospheric Cold Plasma (ACP) Application on the Physicochemical and Microbiological Quality of Steak Obtained from Beef *Musculus Longissimus Dorsi* Muscle

Gökhan AKARCA[1], Azize ATİK[2], İlker ATİK[2]*, Ayşe Janseli DENİZKARA[1]

[1]*Department of Food Engineering, Faculty of Engineering, Afyon Kocatepe University, Afyonkarahisar, Türkiye*
[2]*Food Technology Program, Afyon Vocational School, Afyon Kocatepe University, Afyonkarahisar, Türkiye*

ABSTRACT

This study examined the effect of atmospheric cold plasma (ACP) application on the microbiological and physicochemical properties of steaks obtained from beef *Musculus longissimus dorsi* muscle. In the ACP application, O_2 and Ar gases were applied to the samples separately and as a mixture for 15 and 30 minutes, respectively. While the application decreased the pH and aw of the samples, it caused an increase in TBARS values. In addition, there was a decrease in the L*, a*, and b* values of the samples in other ACP applications, except for applications using O_2 gas. Apart from this, the application was effective in the positive development of the textural properties of the samples. In steak samples obtained from the *M. longissimus dorsi* muscle, ACP application reduced the total mesophilic aerobic bacteria, total psychrophilic aerobic bacteria, total coliform, and total yeast/mold counts by an average of 2 log cfu/g.

Key words: : Cold plasma, *M. longissimus dorsi*, Meat, Quality

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Dana *Musculus Longissimus Dorsi* Kasından Elde Edilen Bifteklerin, Fizikokimyasal ve Mikrobiyolojik Kalitesi Üzerine Atmosferik Soğuk Plazma (ASP) Uygulamasının Etkisi

ÖZ

Bu çalışmada atmosferik soğuk plazma (ASP) uygulamasının sığır *Musculus longissimus dorsi* kasından elde edilen bifteklerin mikrobiyolojik ve fizikokimyasal özellikleri üzerine etkisi incelenmiştir. ASP uygulamasında O_2 ve Ar gazları ayrı ayrı ve karışım halinde, 15 ve 30 dk süreyle örneklere uygulanmıştır. Uygulama örneklerin pH ve a_w düşürürken, TBARS değerlerinde artışa neden olmuştur. Ayrıca, O_2 gazı kullanılan uygulamalar hariç diğer ASP uygulamalarında örneklerin L*, a* ve b* değerlerinde azalma meydana gelmiştir. Ek olarak uygulama örneklerin tekstürel özelliklerin olumlu gelişmesinde etkili olmuştur. *M. longissimus dorsi* kasından elde edilen biftek örneklerine ASP uygulaması toplam mezofilik aerobik bakteri, toplam psikrofilik aerobik bakteri, toplam koliform ve toplam maya/küf sayılarında ortalama 2 log kob/g oranında azalma sağlamıştır.

Anahtar Kelimeler: Soğuk plazma, *M. longissimus dorsi*, Biftek, Kalite

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ORCID ID; *GA: 0000-0002-5055-2722, AA: 0000-0002-3294-380X, İA: 0000-0001-8049-0465, AJD: 0000-0002-3078-8914*
*Corresponding author e-mail: ilkeratik@hotmail.com

INTRODUCTION

Meat and meat products are an essential food category that can provide energy and nutrition for people (Wang et al. 2023). Meat is an indispensable part of the daily diet due to the valuable nutritional components it contains. Meat consumption increases daily depending on the increasing world population and purchasing power. Meat and meat products are ideal environments for the growth and spread of microorganisms that cause meat spoilage and common foodborne pathogens. This is because they have high-quality protein content, essential amino acids, B-group vitamins, minerals, and other vital nutritional elements (Ji et al. 2023). Therefore, ensuring microbial decontamination and inactivation in meat and meat products is important for food safety. Thermal methods are commonly used for microbial inactivation. However, studies have shown that traditional thermal processes such as pasteurization, sterilization, cooking, drying, etc., can neutralize foodborne pathogens but may have a negative effect on the nutritional value and sensory qualities of the food (Jayasena et al. 2015). Meat, on the one hand, can quickly spoil due to its structure, and on the other hand, it reacts very sensitively to decontamination processes (Fröhling et al. 2012). Studies on alternative decontamination methods to heat treatment have gained momentum in recent years. Atmospheric cold plasma (ACP) is an emerging non-thermal decontamination technology (Gao et al. 2021). Plasma is literally an ionized gas defined as the fourth state of matter, along with solids, liquids, and gases (Jung et al., 2017) Cold plasma can be produced by a variety of electrical discharges, such as DC glow discharge, radio frequency (RF) discharge, dielectric barrier discharge (DBD), atmospheric pressure plasma jet (APPJ), microwave, and pulsed power discharge (Albertos et al. 2017). ACP production provides a rich mixture of reactive neutral species, energetically charged particles, UV photons, and intense transient electric fields that can interact simultaneously and synergistically on the food surface (Bauer et al. 2017). The resulting antimicrobial effects caused by reactive species such as radicals, ions, and other chemical compounds enable application for food surface decontamination (Fröhling et al. 2012).

Non-thermal cold plasma technology is a green technique for many food industries, such as inactivating microorganisms and enzymes, removing toxins, and degrading pesticides (Asl et al. 2022).

This study aimed to investigate the effect of the use of ACP, a new green technology, with different gases on the physicochemical and microbiological properties of steaks obtained from beef *M. longissimus dorsi* muscle during the storage period.

MATERIALS and METHODS

Material

The beef *Musculus longissimus dorsi* used in the study was obtained from a local slaughterhouse operating in Afyonkarahisar. The raw material was brought to Afyon Kocatepe University Food Engineering Department laboratory under the cold chain. Before the application, the meat was cut into steaks measuring 15 x 15 x 5 mm (length x width x thickness) with the help of a sterile knife.

Atmospheric Cold Plasma (ACP) Application

ACP application was made according to the method of Akarca et al. (2023). The gases used in the application were supplied from Afyonkarahisar province (Kocaşaban Gases Corp., Afyonkarahisar, Turkey). The prepared samples were exposed to ACP application with 100% O_2, 100% Ar, and 50% O_2 - 50% Ar gas mixture for 15 and 30 minutes. The application was made in a semicircular glass chamber with a radius of 28 mm. The power supply used was 25 kV, 42 kHz frequency, and the system was operated in continuous mode. Each application was carried out in two recurrences and two parallels.

Physicochemical Analysis

pH values of meat samples exposed to ACP were determined using a pH meter (Hanna, HI 2215 pH/ORP meter) while water activity (a_w) of the samples were determined using a water activity device (Novasina LabTouch-aw, Lachen, Switzerland) (AOAC 2016). TBARS value, as an indicator of lipid oxidation, was determined according to Gao et al. (2021). For this, 5 g of sample was used, and absorbance was measured at 531 nm. TBARS values were calculated from the standard curve and expressed as mg malondialdehyde (MDA)/kg meat.

Color values (L^*, a^* and b^*) of meat samples were measured using a colorimeter (Minolta Chroma Meter CR-400, Osaka) (AOAC, 2016).

Texture profile analysis (TPA) measurements of the samples were performed with Stable Micro Systems Texture Analyzer (Stable Micro Systems, Surrey, England). Measurements were performed using a TA-XT2 texture analyzer equipped with a 25 kg load cell, cylindrical aluminum probe (aluminum cylinder probe P/50 (50 mm diameter) Stable Micro Systems Ltd., Godalming, UK). Hardness, adhesiveness, springiness, cohesiveness, gumminess, chewiness, resilience, and shear force values were determined by compressing the samples to 50% of their original height for 5 seconds between two compressions (Akarca et al. 2023), and the cutting force was determined according to Kim et al. (2014) using the TA-XT2 texture analyzer.

Microbiological Analysis

To determine the microbiological quality of the samples exposed to ACP application during the

storage period, total aerobic mesophilic bacteria (TAMB), total aerobic psychrophilic bacteria (TAPB), total coliform group bacteria (TCGB), and total yeast mold (TYM) counts were determined.

For this purpose, 10 g of weighed meat sample was taken into a sterile stomacher bag (BagMixer® 400 P-080921247), and 90 mL of Ringer's solution (Merck, 115525, Germany) was added and homogenized for 2 minutes, then serial dilutions were prepared, and the samples were prepared for analysis (Anonymous 2001).

To determine the TAMB and TAPB numbers, 0.1 mL of sample from each of the prepared dilutions was taken with the help of a sterile pipette (Eppendorf, Research plus, Germany) and cultivated on plate count agar (Oxoid, CM0325) using the spread plate technique. The cultivated petri dishes were incubated at 30°C for 72 hours to determine the number of TAMB under aerobic conditions, and at 4°C for 5-7 days to determine the number of TAPB, and the colonies that developed at the end of the incubation were counted and the counts of TAMB and TAPB were determined (ISO 2013a; ISO 2013b, Halkman 2005). Violet Red Bile (VRB) Agar (Merck, Germany, 1.01406) medium was used for TCGB count. Petri dishes were incubated in an incubator (MM Incucell 55, Germany) for 24-48 hours at 30°C under anaerobic conditions, and the TCGB number was determined at the end of the period (ISO 1991). Potato Dextrose Agar (PDA) (Merck, Germany, 1.10130) medium was used for TYM count and the colonies were counted by incubating the cultivated petri dishes in an incubator (MM Incucell 55, Germany) at 22°C for 5-7 days under aerobic conditions. (ISO 2008).

Statistical analysis

The results obtained in the study were made in two parallels and SPSS software program V 23.0.0 was used for the variance analysis. A significant difference was determined by Duncan's multiple range tests (*$p<0.05$).

RESULTS

The changes in pH, aw, and TBARS values of the samples stored after ACP application during storage are given in Table 1. According to the variation analysis results, it was revealed that the effects of both sample type and storage time interactions were very highly significant on pH, aw and TBARS values ($p<0.0001$). According to the correlation analysis results, sample type and storage time had a negative correlative effect on pH and aw values and a very positive correlative effect on TBARS value.

It was determined that the lowest pH value was (5.05) in the ACP-applied samples using a mixture of 50% O_2 + 50% Ar gas for 30 minutes. Additionally, it was determined that the pH values of the samples decreased during the storage period ($p<0.05$). On the last day of storage, it was determined that the lowest pH value was 5.05 in the samples with ACP-applied, which was also made using a 50% O_2 + 50% Ar gas mixture. On the other hand, it was revealed that the highest pH value was in the control sample (5.30).

Similarly, ACP application had a decreasing effect on the a_w values of the samples ($p<0.05$). After the application, it was determined that the lowest a_w value was in the ACP application using 100% Ar gas, whereas the highest a_w value was in the control samples. Additionally, a_w values decreased in all samples during storage ($p<0.05$). Similarly, it was determined that the lowest a_w value on the 7th day of storage was in the samples performed using 100% Ar gas (Table 1).

ACP application caused the TBARS values of the samples to increase ($p<0.05$). After the application, it was determined that the highest TBARS value among the samples was 1.97 mg MA/kg in the ACP application using 100% O_2 for 30 minutes. This value was followed by samples treated with ACP using a mixture of 50% O_2 + 50% Ar gas for 30 minutes at 1.58 mg MA/kg. Again, TBARS values increased during the storage period in all samples ($p<0.05$). Although the highest TBARS value on the last day of storage was in the samples with ACP-applied, which was performed using 100% O_2 for 30 minutes, with a value of 2.98 mg MA/kg, it was determined that the increase rate in the control sample was higher compared to the ACP-applied samples (Table 1).

Both sample type and storage time interactions had very highly significant effects on the *L**, *a**, and *b** values of steak samples obtained from *M. longissimus dorsi* muscles to which ACP was applied ($p<0.0001$). In addition, the interactions between sample type and storage time showed a very negative correlative effect on the color values of the samples (Table 2).

Although using O_2 gas and the application time increased the samples' *L**, *a**, and *b** values , the use of Ar gas and the extension of the application time caused the *L**, *a**, and *b** values to decrease ($p<0.05$). At the end of the application, the highest *L**, *a**, and *b** values (46.43, 34.07, and 11.52, respectively) were detected in ACP-applied samples using 100% O_2 gas for 30 minutes, and the lowest *L**, *a**, and *b** values (39.48, 18.18, and 6.30, respectively) were detected in ACP-applied samples using 100% Ar gas for 30 minutes. Additionally, 7 days of storage had a decreasing effect on the *L**, *a**, and *b** values of all samples ($p<0.05$). At the end of storage, the lowest L*, a*, and b* values were detected in the samples with ACP-applied using 100% Ar gas for 30 min (34.85, 11.71, and 2.11, respectively). These values were followed by the ACP-applied samples using 100% Ar gas for 15 min (37.41, 16.33, and 3.50, respectively) and the control samples (37.93, 19.98, and 4.76, respectively) (Figure 1.,2., and 3.).

Table 1. pH, aw, and TBARS values of ACP-applied meat samples

Analysis	Samples	Storage Time (Day)		
		1.	4.	7.
pH	Control	5.51±0.05^{aA}	5.33±0.04^{bcB}	5.30±0.02^{aB}
	15 min % 100 O_2	5.51±0.01^{aA}	5.46±0.02^{aA}	5.20±0.05^{bB}
	15 min % 100 Ar	5.51±0.02^{aA}	5.44±0.03^{aA}	5.31±0.04^{aB}
	15 min %50 O_2 +%50 Ar	5.44±0.01^{abA}	5.41±0.02^{abA}	5.08±0.04^{cB}
	30 min % 100 O_2	5.48±0.01^{aA}	5.23±0.04^{cdB}	5.12±0.06^{bBC}
	30 min % 100 Ar	5.46±0.01^{aA}	5.31±0.09^{bcA}	5.11±0.03^{bBC}
	30 min %50 O_2 +%50 Ar	5.36±0.04^{bA}	5.15±0.02^{dB}	5.05±0.02^{cB}
	Interactions		**P Value**	**r**
	Sample (S)		<0.0001	-0.417**
	Storage Time (ST)		<0.0001	-0.805**
	S x ST		<0.0001	--
a_w	Control	0.890±0.01^{aA}	0.867±0.01^{aB}	0.860±0.01^{aC}
	15 min % 100 O_2	0.864±0.01^{bA}	0.860±0.01^{bB}	0.851±0.01^{bC}
	15 min % 100 Ar	0.863±0.01^{bcA}	0.856±0.01^{cB}	0.847±0.01^{cC}
	15 min %50 O_2 +%50 Ar	0.865±0.01^{bA}	0.853±0.01^{deB}	0.841±0.01^{dC}
	30 min % 100 O_2	0.863±0.01^{bcA}	0.855±0.01^{cdB}	0.845±0.01^{cC}
	30 min % 100 Ar	0.861±0.01^{cA}	0.849±0.01^{fB}	0.841±0.01^{dC}
	30 min %50 O_2 +%50 Ar	0.863±0.01^{bcA}	0.851±0.01^{efB}	0.845±0.01^{cC}
	Interactions		**P Value**	**r**
	Sample (S)		<0.0001	-0.489**
	Storage Time (ST)		<0.0001	-0.746**
	S x ST		<0.0001	--
TBARS	Control	0.98±0,04^{eB}	1.16±0,03^{fB}	2.48±0,08^{bA}
	15 min % 100 O_2	1.28±0,06^{cC}	1.58±0,11^{cdB}	1.90±0,05^{dA}
	15 min % 100 Ar	1.04±0,07^{deC}	1.36±0,05^{eB}	1.62±0,08^{eA}
	15 min %50 O_2 +%50 Ar	1.10±0,03^{dC}	1.52±0,05^{dB}	1.74±0,02^{dcA}
	30 min % 100 O_2	1.97±0,05^{aC}	2.28±0,06^{aB}	2.89±0,04^{aA}
	30 min % 100 Ar	1.03±0,02^{deC}	1.68±0,06^{cB}	2.12±0,14^{cA}
	30 min %50 O_2 +%50 Ar	1.58±0,05^{bC}	2.09±0,05^{bB}	2.58±0,04^{bA}
	Interactions		**P Value**	**r**
	Sample (S)		<0.0001	0,363*
	Storage Time (ST)		<0.0001	0,692**
	S x ST		<0.0001	--

a - e (↓): Values with the same capital letters in the same column for each analysis differ significantly (P< 0.05), A(→)C: Values with the same capital letters in the same column for each analysis differ significantly (P<0.05) *P* < 0.0001: Statistically too much significant. **. Correlation is significant at the 0.01 level (2-tailed). *. Correlation is significant at the 0.05 level (2-tailed).

Table 2. Variation analysis of color values of ACP-applied meat samples

Interactions	L* Value		a* Value		b* Value	
	P Value	R	P Value	r	P Value	r
Sample (S)	<0.0001	-0.145	<0.0001	-0.250	<0.0001	-0.320*
Storage Time (ST)	<0.0001	-0.692**	<0.0001	-0.505**	<0.0001	-0.680**
S x ST	0.163	--	0.103	--	0.824	--

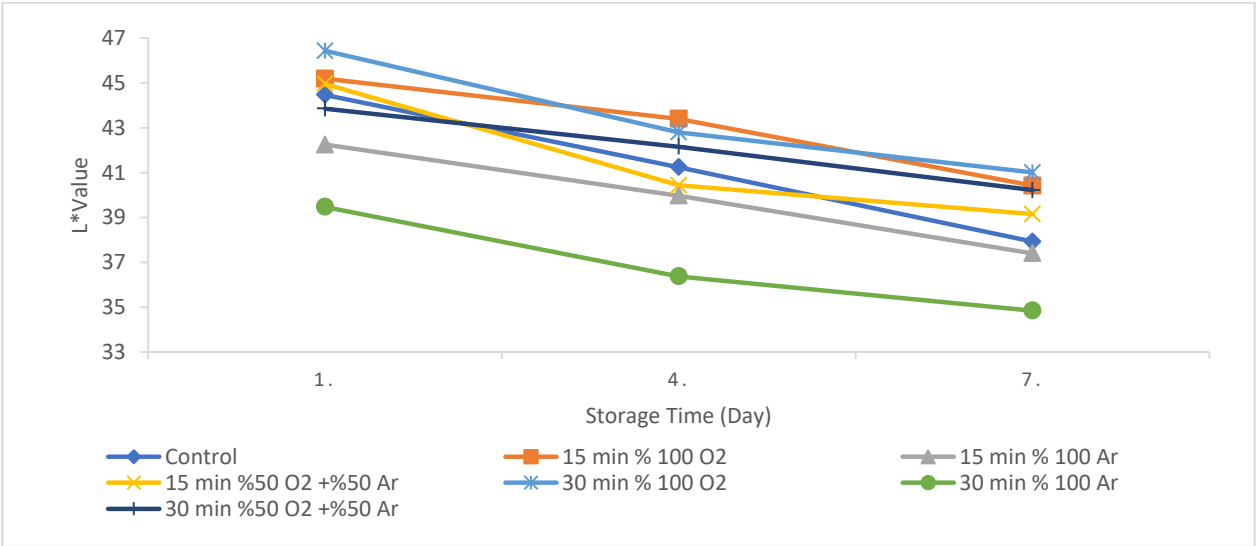

Figure 1: L* values of ACP-applied meat samples

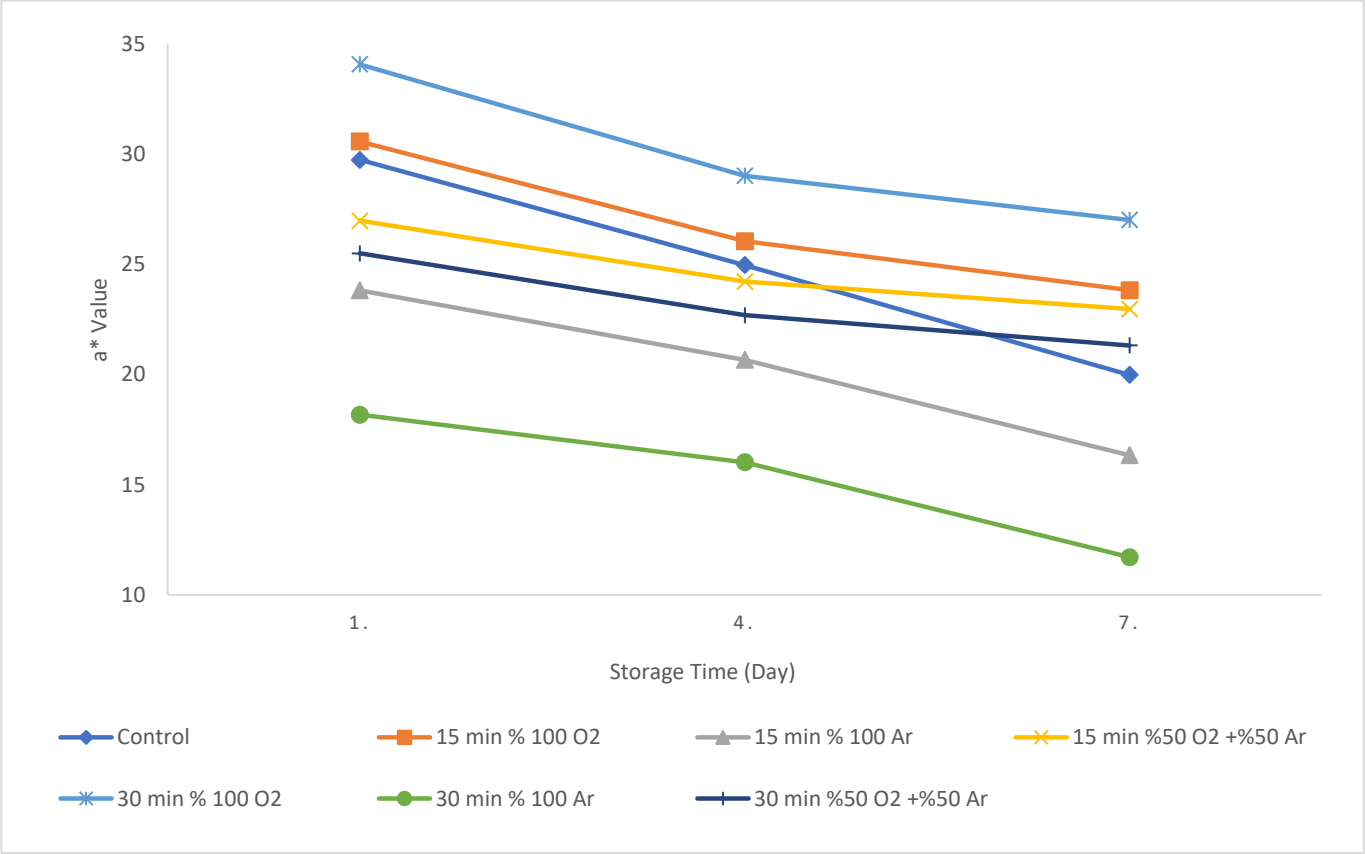

Figure 2: a* values of ACP-applied meat samples

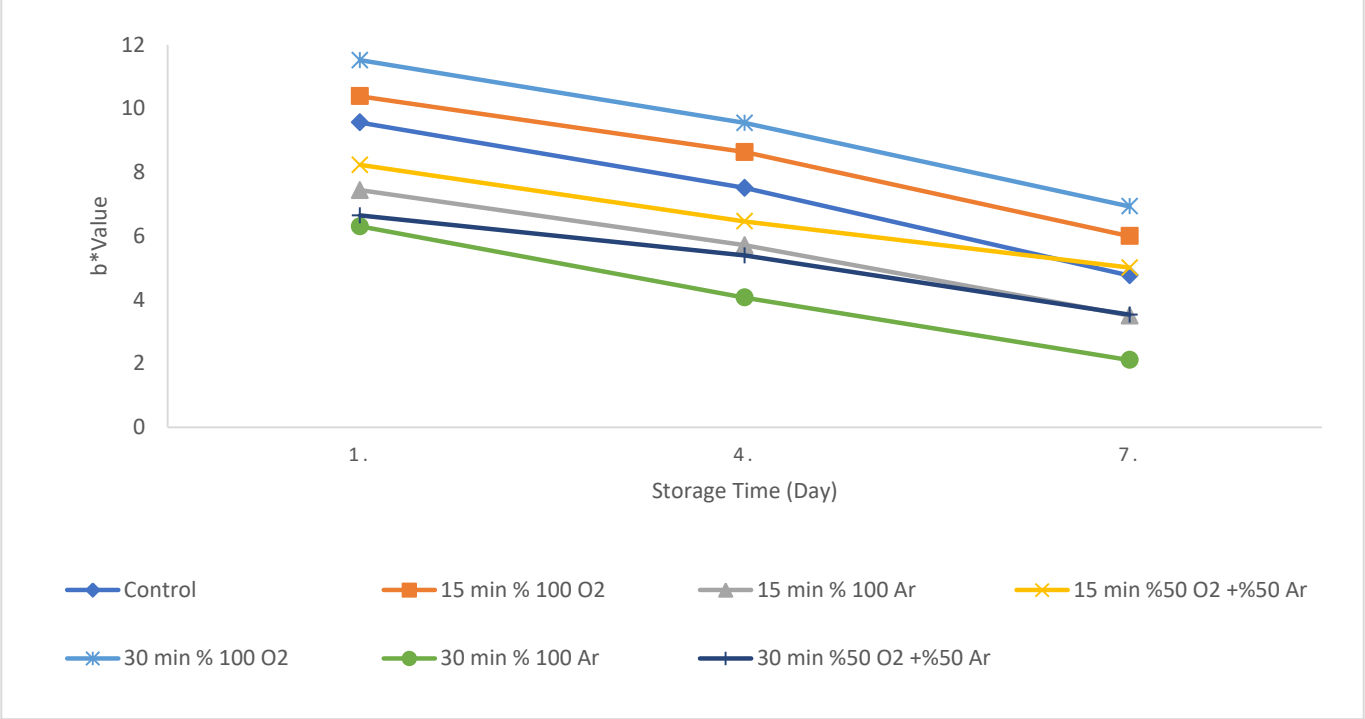

Figure 3: b* values of ACP-applied meat samples

ACP application affects the techno-functional properties of the protein depending on its natural structure (Pérez-Andrés et al. 2019). Therefore, the post-application and storage process caused the textural properties of the steak samples to change (Table 3).

Both sample type and storage time interactions had very highly significant effects on the texture values of the samples (except gumminess and chewiness) ($p<0.0001$). Only storage time interaction had very highly significant effect on gumminess and chewiness values ($p<0.0001$). According to the correlation analysis results, sample type and storage time interactions showed a positive correlative effect on hardness, gumminess, and shear force, and a very negative correlative effect on adhesiveness, and cohesiveness (Table 3).

Table 3. Texture profile analysis values of ACP-applied meat samples

Analysis	Samples	Storage Time (Day)		
		1.	**4.**	**7.**
Hardness	Control	615,10±32,83^{bC}	1804,92±90,11^{bcB}	2616,22±66,60^{aA}
	15 min % 100 O_2	671,53±55,88^{bC}	1646,39±60,71^{dB}	1944,43±57,82^{dA}
	15 min % 100 Ar	669,85±26,18^{bC}	1766,45±45,12^{cB}	1976,78±41,77^{dA}
	15 min %50 O_2 +%50 Ar	674,97±37,22^{bC}	1537,69±9,43^{dB}	1983,17±25,25^{cdA}
	30 min % 100 O_2	804,76±49,34^{aC}	1899,25±30,96^{abB}	2097,30±12,85^{cA}
	30 min % 100 Ar	879,54±34,18^{aC}	1933,28±45,30^{aB}	2322,78±64,25^{bA}
	30 min %50 O_2 +%50 Ar	834,33±45,26^{aC}	1923,68±25,91^{abB}	2102,30±47,96^{cA}
	Interactions	**P Value**		**r**
	Sample (S)	<0.0001		0,062
	Storage Time (ST)	<0.0001		0.928**
	S x ST	<0.0001		--
Adhesiveness	Control	-15,89±0,81^{eA}	-56,93±2,18^{dB}	-74,04±0,23^{fC}
	15 min % 100 O_2	-12,61±1,47^{dA}	-35,90±0,77^{cB}	-56,65±2,21^{eC}
	15 min % 100 Ar	-9,90±1,07^{bcA}	-23,66±0,69^{bB}	-38,24±1,27^{dC}
	15 min %50 O_2 +%50 Ar	-10,85±0,30^{cdA}	-26,18±1,20^{bB}	-32,93±0,86^{dC}
	30 min % 100 O_2	-8,89±0,33^{bA}	-12,53±1,67^{aB}	-28,75±0,79^{cC}
	30 min % 100 Ar	-6,90±0,04^{aA}	-10,05±1,25^{aB}	-19,87±0,76^{aC}
	30 min %50 O_2 +%50 Ar	-8,23±0,29^{abA}	-11,33±0,96^{aB}	-24,95±0,42^{bC}
	Interactions	**P Value**		**r**
	Sample (S)	<0.0001		-0.234
	Storage Time (ST)	<0.0001		-0.900**
	S x ST	<0.0001		--
Springiness	Control	14,52±0,33^{aA}	9,20±0,22^{bB}	6,35±0,19^{dC}
	15 min % 100 O_2	13,53±0,16^{bA}	11,62±0,21^{aB}	7,38±0,09^{acdC}
	15 min % 100 Ar	12,92±0,13^{cA}	11,25±0,18^{aB}	7,13±0,10^{abC}
	15 min %50 O_2 +%50 Ar	13,06±0,07^{cA}	11,40±0,19^{aB}	7,21±0,15^{abC}
	30 min % 100 O_2	11,25±0,13^{eA}	9,36±0,17^{bB}	6,93±0,16^{bcC}
	30 min % 100 Ar	9,98±0,14f^{A}	8,84±0,27^{bB}	6,67±0,11^{cdC}
	30 min %50 O_2 +%50 Ar	11,79±0,24^{dA}	9,29±0,51^{bB}	6,71±0,23^{cdC}
	Interactions	**P Value**		**r**
	Sample (S)	<0.0001		0.610**
	Storage Time (ST)	<0.0001		-0.649**
	S x ST	<0.0001		--

…Continues Table 3. Texture profile analysis values of ACP-applied meat samples

Analysis	Samples	Storage Time (Day)		
		1.	4.	7.
Cohesiveness	Control	0,925±0,01^{abA}	0,825±0,02^{aB}	0,695±0,02^{cC}
	15 min % 100 O_2	0,950±0,01^{aA}	0,865±0,02^{aB}	0,810±0,01^{aC}
	15 min % 100 Ar	0,915±0,01^{bA}	0,840±0,01^{aB}	0,780±0,01^{abC}
	15 min %50 O_2 +%50 Ar	0,930±0,01^{abA}	0,865±0,02^{aB}	0,790±0,01^{abC}
	30 min % 100 O_2	0,870±0,01^{cA}	0,835±0,02^{aAB}	0,785±0,04^{abB}
	30 min % 100 Ar	0,820±0,01^{dA}	0,775±0,02^{bAB}	0,740±0,01^{bcB}
	30 min %50 O_2 +%50 Ar	0,845±0,01^{cdA}	0,820±0,02^{abA}	0,755±0,01^{bB}
	Interactions	**P Value**		**r**
	Sample (S)	<0.0001		-0,255
	Storage Time (ST)	<0.0001		-0.802**
	S x ST	0.001		--
Gumminess	Control	569,08±34,72^{cC}	1490,01±112,63^{abB}	1817,56±9,21^{aA}
	15 min % 100 O_2	638,35±62,58^{abcB}	1424,77±87,43^{abA}	1575,40±74,33^{bcA}
	15 min % 100 Ar	612,82±19,22^{cB}	1484,14±69,88^{abA}	1542,18±60,54^{cA}
	15 min %50 O_2 +%50 Ar	627,46±25,07^{bcC}	1330,20±40,78^{bB}	1566,88±47,99^{bcA}
	30 min % 100 O_2	699,79±31,54^{abB}	1586,20±67,14^{aA}	1646,61±84,24^{bcA}
	30 min % 100 Ar	720,98±15,59^{aC}	1497,81±5,89^{aB}	1719,31±80,39^{abA}
	30 min %50 O_2 +%50 Ar	704,85±32,34^{abB}	1577,23±5,94^{aA}	1587,74±80,80^{bcA}
	Interactions	**P Value**		**r**
	Sample (S)	0.001		0.047
	Storage Time (ST)	<0.0001		0.910**
	S x ST	0.007		--
Chewiness	Control	8269,03±697,37^{abC}	13703,25±710,16^{cdA}	11551,53±405,51^{aB}
	15 min % 100 O_2	8631,56±738,47^{aC}	16554,08±724,30^{aA}	11623,30±414,91^{aB}
	15 min % 100 Ar	7919,42±166,09^{abC}	16690,82±434,63^{aA}	11000,72±290,19^{aB}
	15 min %50 O_2 +%50 Ar	8193,81±283,12^{abC}	15167,12±211,15^{bA}	11301,53±113,61^{aB}
	30 min % 100 O_2	7874,08±261,03^{abC}	14841,24±349,93^{bcA}	11426,11±852,03^{aB}
	30 min % 100 Ar	7197,84±48,62^{bC}	13241,46±454,58^{dA}	11472,15±354,27^{aB}
	30 min %50 O_2 +%50 Ar	8306,29±211,94^{aC}	14658,90±758,80^{bcA}	10671,14±913,11^{aB}
	Interactions	**P Value**		**r**
	Sample (S)	0.001		-0.088
	Storage Time (ST)	<0.0001		0.448**
	S x ST	0.001		--

…Continues Table 3. Texture profile analysis values of ACP-applied meat samples

Analysis	Samples	Storage Time (Day)		
		1.	4.	7.
Resilience	Control	0,80±0,01^{cA}	0,77±0,01^{dA}	0,62±0,02^{eB}
	15 min % 100 O_2	0,84±0,01^{bcA}	0,80±0,01^{cdA}	0,69±0,03^{dB}
	15 min % 100 Ar	0,86±0,01^{bA}	0,82±0,02^{bcA}	0,73±0,03^{cdB}
	15 min %50 O_2 +%50 Ar	0,84±0,01^{bcA}	0,80±0,01^{cdAB}	0,76±0,01^{bcB}
	30 min % 100 O_2	0,91±0,01^{aA}	0,86±0,01^{abAB}	0,82±0,04^{abB}
	30 min % 100 Ar	0,93±0,03^{aA}	0,90±0,03^{aAB}	0,85±0,02^{aB}
	30 min %50 O_2 +%50 Ar	0,87±0,01^{bA}	0,83±0,01^{bcB}	0,78±0,01^{bcC}
	Interactions	**P Value**		**r**
	Sample (S)	<0.0001		0.569**
	Storage Time (ST)	<0.0001		-0.648**
	S x ST	<0.0001		--
Shear Force	Control	179,34±2,85^{eC}	242,57±8,19^{bB}	488,70±4,81^{bA}
	15 min % 100 O_2	189,05±4,80^{dC}	255,56±13,93^{bB}	470,69±5,49^{cA}
	15 min % 100 Ar	201,75±5,07^{cC}	257,07±4,15^{bB}	467,78±6,45^{cA}
	15 min %50 O_2 +%50 Ar	195,61±2,74^{cdC}	248,97±7,45^{bB}	471,76±11,72^{cA}
	30 min % 100 O_2	232,42±4,73^{bC}	301,78±8,98^{aB}	496,09±6,04^{abA}
	30 min % 100 Ar	245,94±3,06^{aC}	319,27±22,43^{aB}	506,76±6,29^{aA}
	30 min %50 O_2 +%50 Ar	238,23±3,93^{abC}	292,25±11,37^{aB}	501,29±4,17^{abA}
	Interactions	**P Value**		**r**
	Sample (S)	<0.0001		0.155
	Storage Time (ST)	<0.0001		0.935**
	S x ST	0.006		--

a - e (↓): Values with the same capital letters in the same column for each analysis differ significantly (P< 0.05), A(→)C: Values with the same capital letters in the same column for each analysis differ significantly (P<0.05) *P* < 0.0001: Statistically too much significant. **. Correlation is significant at the 0.01 level (2-tailed). *. Correlation is significant at the 0.05 level (2-tailed).

ACP application caused the hardness value of the samples to increase ($p<0.05$). Among the samples, the highest increase in hardness value was detected in the ACP-applied samples using 100% Ar gas for 30 minutes with 879.54 N. This sample was followed by ACP-applied samples using 50% O_2 and 50% Ar gas for 30 minutes (834.33 N), and 100% O_2 gas for 30 minutes (804.76 N). Similarly, there was an increase in the hardness values of all samples during 7 days of storage ($p<0.05$).

Although the adhesiveness, springiness, and cohesiveness values of the samples increased with ACP application ($p<0.05$), they decreased during storage (Table 3; $p<0.05$).

ACP application and storage time caused an increase on the gumminess value ($p<0.05$). On the last day of storage, the highest gumminess value was detected in the control samples with 1817.56 N, and the lowest value was detected in ACP-applied samples using 100% Ar gas for 15 minutes with 1542.18 N.

Although the chewiness value increased in the first 7 days of storage, it decreased in later periods ($p<0.05$). It was revealed that the lowest difference between the first and last days of storage was 2364.85 N in the application using 50% O_2 and 50% Ar gas for 30 minutes, and the highest difference was 4274.31 N in the ACP-applied samples using 50% O_2 and 50% Ar gas for 15 minutes.

ACP application increased resilience and shear force values ($p<0.05$). It was revealed that the highest resilience and shear force values at the beginning of the application were 0.93 and 245.93, respectively, in the ACP-applied samples using 50% O_2 and 50% Ar gas for 30 minutes. Although the cutting force values of the steak samples decreased during the 7-day storage period, it was determined that there was an increase in the shear force values ($p<0.05$). At the end of storage, the lowest resilience value was determined as 0.62 in the control, and the highest resilience value was determined as 0.85 in the ACP-applied samples using 50% O_2 and 50% Ar gas for 30 minutes. Similarly, on the 7th storage day, the highest shear force was determined to be 506.76 in the samples using 50% O_2 and Ar gas mixtures for 30 minutes.

Sample type, storage time, and sample type x storage time interactions had very highly significant effects on all microbiological analysis results ($p<0.0001$). Sample type interaction had a negative correlative effect, while storage time interaction had a very positive correlative effect ($p<0.0001$) on the microbiological analysis results (Table 4).

ACP application showed a reducing effect on the counts of total aerophilic mesophilic bacteria (TAMB), total aerophilic psychrophilic bacteria (TAPB), total coliform group bacteria (TCGB), and total yeast & mold (TYM) ($p<0.01$). At the beginning of the application, it was determined that the applications using 50% O_2 and Ar gas mixtures for 30 minutes had the lowest TAMB, TAPB, TCGB, and TYM counts among the samples. TAMB, TAPB, TCGB and TYM counts increased in all steak samples obtained from beef *M. longissimus dorsi* muscles during the storage period ($p<0.05$). All microbiological analysis results on the last day of storage determined that the lowest microorganism counts were in the samples where 50% O_2 and Ar gas mixtures were used for 30 minutes, and the highest microorganism counts were in the control group samples (Table 3, Table 4).

Table 4. Microbiological analysis results of ACP-applied meat samples

Analysis	Samples	Storage Time (Day)		
		1.	4.	7.
TAMB	Control	4.48±0,04^{aC}	5.77±0,019^{aB}	7.11±0,03^{aA}
	15 min % 100 O_2	4.30±0,04^{bB}	4.64±0,17^{bB}	5.14±0,04^{bA}
	15 min % 100 Ar	4.24±0,03^{bC}	4.50±0,08^{bcB}	4.98±0,08^{cA}
	15 min %50 O_2 +%50 Ar	4.11±0,02^{cC}	4.32±0,01^{cdB}	4.61±0,09^{dA}
	30 min % 100 O_2	3.98±0,05^{dB}	4.13±0,06^{deB}	4.41±0,04^{eA}
	30 min % 100 Ar	3.90±0,08^{deB}	4.06±0,06^{deB}	4.29±0,04^{efA}
	30 min %50 O_2 +%50 Ar	3.79±0,03^{eB}	3.90±0,05^{eAB}	4.12±0,11^{fA}
	Interactions	**P Value**		**r**
	Sample (S)	<0.0001		-0,693**
	Storage Time (ST)	<0.0001		0.462**
	S x ST	<0.0001		--
TAPB	Control	4.15±0,04^{aC}	4.71±0,08^{aB}	6.07±0,07^{aA}
	15 min % 100 O_2	4.07±0,02^{abB}	4.33±0,07^{bB}	4.90±0,12^{bA}
	15 min % 100 Ar	4.05±0,03^{cC}	4.27±0,08^{bB}	4.77±0,06^{bcA}
	15 min %50 O_2 +%50 Ar	3.95±0,06^{dC}	4.19±0,06^{bB}	4.67±0,04^{cA}
	30 min % 100 O_2	3.60±0,03^{eC}	3.90±0,03^{cB}	4.40±0,07^{dA}
	30 min % 100 Ar	3.54±0,04^{efC}	3.79±0,04^{cdB}	4.12±0,06^{eA}
	30 min %50 O_2 +%50 Ar	3.49±0,01^{fC}	3.70±0,09^{dB}	3.93±0,04^{fA}
	Interactions	**P Value**		**r**
	Sample (S)	<0.0001		-0.678**
	Storage Time (ST)	<0.0001		0.614**
	S x ST	<0.0001		--
TCGB	Control	3,19±0,02^{aC}	4,06±0,04^{aB}	5,08±0,08^{aA}
	15 min % 100 O_2	3,08±0,02^{bC}	3,68±0,04^{bB}	4,15±0,01^{bA}
	15 min % 100 Ar	3,03±0,02^{bC}	3,57±0,04^{cB}	4,05±0,01^{cA}
	15 min %50 O_2 +%50 Ar	2,96±0,01^{cC}	3,52±0,02^{cB}	4,02±0,01^{cA}
	30 min % 100 O_2	2,86±0,02^{dC}	3,24±0,05^{dB}	3,88±0,04^{dA}
	30 min % 100 Ar	2,73±0,01^{eC}	3,21±0,04^{deB}	3,72±0,02^{eA}
	30 min %50 O_2 +%50 Ar	2,54±0,03^{fC}	3,14±0,04^{eB}	3,64±0,03^{fA}
	Interactions	**P Value**		**r**
	Sample (S)	<0.0001		-0,503**
	Storage Time (ST)	<0.0001		0.819**
	S x ST	<0.0001		--
TYM	Control	3,63±0,03^{aC}	4,86±0,02^{aB}	6,75±0,04^{aA}
	15 min % 100 O_2	3,60±0,03^{abC}	4,47±0,06^{bB}	5,32±0,02^{bA}
	15 min % 100 Ar	3,56±0,02^{abcC}	4,37±0,02^{cB}	5,11±0,04^{cA}
	15 min %50 O_2 +%50 Ar	3,55±0,01^{bcC}	3,90±0,02^{dB}	4,48±0,03^{dA}
	30 min % 100 O_2	3,49±0,02^{cdC}	3,81±0,03^{eB}	4,35±0,04^{eA}
	30 min % 100 Ar	3,45±0,02^{dC}	3,58±0,04^{fB}	4,30±0,04^{efA}
	30 min %50 O_2 +%50 Ar	3,45±0,04^{dC}	3,55±0,03^{fB}	4,25±0,03^{fA}
	Interactions	**P Value**		**r**
	Sample (S)	<0.0001		-0.522**
	Storage Time (ST)	<0.0001		0.716**
	S x ST	<0.0001		--

TAMB: Total aerobic mesophilic bacteria; TAMP: Total aerobic psychrophilic bacteria
TCGB: Total coliform group bacteria, TYM: Total yeast mold
a - f (↓): Values with the same capital letters in the same column for each analysis differ significantly (P< 0.05), A(→)C: Values with the same capital letters in the same column for each analysis differ significantly (P<0.05) *P* < 0.0001: Statistically too much significant. **. Correlation is significant at the 0.01 level (2-tailed). *. Correlation is significant at the 0.05 level (2-tailed).

DISCUSSION

ACP application showed a decreasing effect on the pH values of the samples (p<0.05). It was determined that the lowest pH value was (5.05) in the ACP-applied samples using a mixture of 50% O_2 + 50% Ar gas for 30 minutes. Additionally, it was determined that the pH values of the samples decreased during the storage period (p<0.05). On the last day of storage, it was determined that the lowest pH value was 5.05 in the samples with ACP-applied, which was also made using a 50% O_2 + 50% Ar gas mixture. On the other hand, it was revealed that the highest pH value was in the control sample (5.30).

The significantly higher drop in pH after ACP application can be attributed to acidogenic molecules such as NOx normally produced in air plasmas (Gao et al. 2021).

The decrease in a_w value is because the O_2 and Ar gases used in the application retain the free water molecules on the sample. (Lee et al. 2020). In addition, it is thought that the drying occurring on the

surface due to gas circulation during application may also be effective in the decrease in the a_w value.

Reactive oxygen and nitrogen species (RONS) produced by ACP are free to interact with intramuscular fat-containing surface tissue. Reactive oxygen species interact specifically with the unsaturated fatty acid fraction and can, therefore, cause lipid peroxidation. Secondary oxidation products can initiate conformational changes in myoglobin, causing both increased oxidation and brown discoloration (Bauer et al. 2017). Malondialdehyde (MDA) is a polyunsaturated fatty acid oxidation product and is considered an important marker of lipid oxidation (Gonz´alez-Gonz´alez et al. 2021). Therefore, an increase in TBARS values, which is an indicator of oxidation, is expected after the application of ACP.

The color of red meat is an important factor in meat quality and appeal. The reason for this is that the color of meat is perceived by the consumer as an indicator of freshness and quality. Therefore, the consumer's purchasing decision is directly affected (Fröhling et al. 2012).

Since the use of O_2 gas increases the formation of oxymyoglobin, which combines with myoglobin, gives the meat its red color and makes the meat appear brighter and redder, the *L**, *a**, and *b** values of the samples using O_2 gas were found to be higher. On the other hand, using Ar gas during application caused a decrease in the *L**, *a**, and *b** values, as it caused metmyoglobin formation due to the decrease in contact with O_2 on the surface (Jayasena et al.), and drying due to gas contact on the surface (Kim et al. 2013). Reactive species formed during the ACP application can enter into oxidative reactions with pigment compounds in the food product and change the color of the product (Lee et al. 2022).

Decreasing a_w, is one of the main reasons for the increase in hardness value. The airflow created by gas circulation during application causes drying on the surface. Therefore, both a_w decreased and hardness value increased. In the samples, adhesiveness, springiness and cohesiveness values decreased due to the decreasing a_w value and the increase in hardness.

ACP application reduced the water retention capacity of proteins in the *M. longissimus dorsi* muscle, resulting in a decrease in aw in the samples (Pérez-Andrés et al. 2019). It is thought that drying and decreasing moisture content on the surface may be the reason for the increase in shear force values.

Reactive species present in plasma have oxidative effects on the external surfaces of microbial cells (Asl et al. 2022). ACP application can generate specific types of ROS, such as oxygen atoms, ozone, metastable oxygen molecules, peroxide, superoxide, and hydroxyl radicals, all bactericidal (Kim et al. 2015). The gas mixture containing air or O_2 used in the ACP system produces (i) reactive species (RNS and ROS) that play an important inactivation role by attacking the microbial cell wall, leading to cell rupture and oxidation of peptidoglycan or lipopolysaccharides and (ii) intracellular components (Laroque et al. 2022).

In addition, ACP reactive species against fungal cells cause deformation of the micelle tip, cell apoptosis, cellular protein damage, disintegration, and release, causing loss of function and death of cells (Misra et al. 2019).

CONCLUSION

This research investigated the effect of ACP application with different gases and mixtures on the physicochemical and microbiological properties of beef steaks obtained from the *M. longissimus dorsi* muscle during the storage period. While ACP application decreased the pH and a_w of the samples, it caused an increase in TBARS values. In addition, there was a decrease in the *L**, *a**, and *b** values of the samples in other ACP applications, except for applications using O_2 gas. Besides, the application was effective in the positive development of the textural properties of the samples. There was a decrease in the counts of TAMB, TAPB, TCGB, and TYM in all samples where ACP was applied. Additionally, it was determined that there was a smaller increase (on average 2-3 logs) in the TAMB, TAPB, TCGB, and TYM counts of these samples compared to the control samples during the storage period.

Meat is a valuable food product in terms of nutrition due to its protein content, high biological value, and richness in essential amino acids, B-group vitamins, and various minerals. The high a_w value and rich nutritional content of meat are effective in providing a suitable environment for microbial growth. Therefore, microbial decontamination and inactivation of meat is mandatory for food safety.

Research findings revealed that ACP application can be used to extend the shelf life and increase the physicochemical quality of steaks obtained from beef *M. longissimus dorsi* muscles. These results will lead to future studies on non-thermal techniques considered to be applied in meat preservation.

Conflict of interest: The authors have no conflicts of interest to report**.**

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Ethical approval: "This study is not subject to the permission of HADYEK in accordance with the "Regulation on Working Procedures and Principles of Animal Experiments Ethics Committees" 8 (k). The data, information and documents presented in this article were obtained within the framework of academic and ethical rules."

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RESEARCH ARTICLE

Heavy Metal Levels In Octopos Caught From Iskenderun Bay

Alaeddin CİLLİ[1], Erdinç TÜRK[1*]

[1]Department of Pharmacology and Toxicology, Faculty of Veterinary Medicine, Hatay Mustafa Kemal University, Hatay, Türkiye

ABSTRACT

In recent years, in parallel with increasing industrialization and unregulated urbanization, there has been an increase in air, soil and water pollution. Iskenderun Bay and industrial establishments around it, pollution resulting from agricultural activities, domestic waste, industrial waste, waste gases released into the atmosphere from residences and vehicles cause the Gulf to be polluted. The port and maritime transportation in the Gulf of Iskenderun also contribute to this pollution and increase the accumulation of chemicals in marine organisms. This study aimed to determine heavy metal pollution in Iskenderun Bay and, accordingly, to evaluate the risks of octopus consumption on consumer health. In this context, 40 octopuses caught from Iskenderun Bay were used in the research. arsenic (As), mercury (Hg), cadmium (Cd), lead (Pb), nickel (Ni), copper (Cu), zinc (Zn), aluminum (Al), iron (Fe) and heavy metal levels such as manganese (Mn) were determined by ICP-OES (Inductively coupled plasma – Optical emission spectrophotometer) device. As a result of the study, metal levels in the edible parts of the octopus were found to be within acceptable limits. No accumulation of Hg or Pb was found in any of the octopus samples analyzed. Metal levels in the edible tissues of octopuses were determined in accordance with the Turkish Food Codex and European Food standards. As a result of the study, it was determined that heavy metal accumulation in octopus tissues obtained from Iskenderun Bay is not dangerous for human health and ecosystem.

Keywords: Heavy Metal, Iskenderun Bay, Octopus Octopus

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İskenderun Körfezi'nden Yakalanan Ahtapotlarda Ağır Metal Düzeyleri

ÖZ

Son yıllarda artan sanayileşme ve düzensiz kentleşmeye paralel olarak hava, toprak ve su kirliliğinde de artış görülmektedir. İskenderun Körfezi, çevresinde bulunan sanayi kuruluşları, tarımsal faaliyetlerden kaynaklanan kirlilik, evsel atıklar, endüstriyel atıklar, konutlar ve araçlardan atmosfere salınan atık gazlar Körfezi'nin kirlenmesine neden olmaktadır. İskenderun Körfezi'ndeki liman ve deniz ulaşımı da bu kirliliğe katkıda bulunmakta ve deniz canlılarındaki kimyasal madde birikimini artırmaktadır. Bu çalışmada, İskenderun Körfezi'ndeki ağır metal kirliliğinin belirlenmesi ve buna bağlı olarak ahtapot tüketiminin tüketici sağlığı üzerine risklerinin değerlendirilmesini amaçlanmıştır. Bu kapsamda, araştırmada İskenderun Körfezi'nden yakalanan 40 adet ahtapot kullanılmıştır. Ahtapotların kas ve mantle dokularında Arsenik (As), Cıva (Hg), Kadmiyum (Cd), Kurşun (Pb), Nikel (Ni), Bakır (Cu), Çinko (Zn), Alüminyum (Al), Demir (Fe) ve Manganez (Mn) gibi ağır metal seviyeleri ICP-OES (Endüktif olarak birleştirilmiş plazma – Optik emisyon spektrofotometresi) cihazı ile belirlenmiştir. Çalışma sonucunda ahtapotun yenilebilir kısımlarındaki metal seviyeleri kabul edilebilir sınırlar içerisinde bulunmuştur. Analiz edilen ahtapot numunelerinin hiçbirinde Hg ve Pb birikimine rastlanmamıştır. Ahtapotların yenilebilir dokularındaki metal seviyeleri Türk Gıda Kodeksine ve Avrupa Gıda standartlarına uygun olarak tespit edilmiştir. Çalışma sonucunda İskenderun Körfezi'nden elde edilen ahtapot dokularındaki ağır metal birikiminin insan sağlığı ve ekosistem için tehlikeli olmadığı tespit edilmiştir.

Anahtar Kelimeler: Ahtapot, Ağır Metal, İskenderun Körfezi

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ORCID ID; *AC: 0000-0003-4713-6506, ET: 0000-0003-1735-1774*

*Corresponding author e-mail: erdincturk48@mku.edu.tr

INTRODUCTION

Heavy metals are elements that can have toxic effects even at low concentrations. Heavy metals are examined in two groups, essential and non-essential, according to their degree of impact on biological events. Metals such as nickel (Ni), zinc (Zn), copper (Cu), iron (Fe), selenium (Se), which generally serve as co-factors in enzymatic reactions and must be present in a certain concentration in the organism structure, are classified as essential. Levels of these metals greater than 1-10 ppm may cause toxic effects.

Metals such as mercury (Hg), cadmium (Cd) and lead (Pb) are non-essential heavy metals that are not necessary for the organism and can cause toxic effects even at concentrations of 0.001-0.1 ppm. Heavy metal toxicity causes a decrease in energy level and deterioration in the functioning of the lungs, brain, liver, kidney, blood composition and other important organs. Long-term exposure to heavy metals can lead to gradually progressive physical and neurological degenerative damage that mimics diseases such as Multiple sclerosis, Parkinson's disease, Alzheimer's disease and muscular dystrophy. Long-term exposure to some metals and their compounds can also cause cancer. (Jarup 2003; Türk et al. 2020).

Heavy metals have been found naturally in the earth's crust since the formation of the planet. Due to the increase in the use of heavy metals, metallic substances have increased in both the terrestrial and aquatic environments (Gautam et al. 2016). Heavy metal pollution has arisen as a result of anthropogenic activity, which is the primary cause of pollution, primarily due to the leaching of metals from different sources such as metal mining, smelting, foundries and other metal-based industries, and landfills. Automobiles, road works, the use of heavy metals in agriculture, use of pesticides, insecticides and fertilizers have become secondary sources of heavy metal pollution. Natural causes are also other sources of heavy metal pollution such as volcanic activity, metal corrosion, metal evaporation from soil and water and resuspension of sediment, soil erosion, geological weathering (Shallari 1998; Herawati et al. 2000; He et al. 2005).

Water pollution increases with the discharge of industrial and domestic wastes into aquatic environments without treatment. Air and soil pollution are other factors that pollute water resources. Water pollution means a decrease in the quality of water that prevents its use. The effect of a radioactive, organic, inorganic or biological substance on water indicates that the water is polluted. Deterioration of water resources negatively affects living life. As a result of wastewater not being treated and being discharged into water resources in an uncontrolled manner; highly polluted bays, rivers and seas emerge (Anonymous 2004).

Octopus lives in 0-200 m depths of the sea, mostly on stony hard surfaces and bottom. It is mostly found in tropical seas around the world and in temperate regions such as the Marmara Sea, the Aegean Sea and mostly the Mediterranean coasts in Turkey. It is economically important that its consumption is high in these regions (Roper 1984; Katağan and Benli 1990; Katağan and Kocataş 1990; Salman et al. 1997). The nutritional importance of octopuses, a subclass of cephalopods, is due to their fat content, like many other marine creatures; It consists of unsaturated fatty acids omega-3 and omega-6 and glycerol. The presence of unsaturated fatty acids is important for many systems in the human body (Güner et al. 1998).

Iskenderun Bay is a region where industrial establishments, agricultural activities, population and maritime transportation are dense. As a result of these activities, environmental pollution increases. Many living creatures in the sea are used as bioindicators to determine pollution. Since octopuses have nutritional value, the pollution they accumulate is important for human health, which is the top link of the food chain (Sönmezateş and Türk 2023). This study aimed to determine the level of heavy metals in the edible tissues of octopuses caught from Iskenderun Bay, which is polluted with domestic, agricultural and industrial waste.

MATERIAL and METHOD

The samples used in the study were obtained from octopuses (Octopus vulgaris) caught in İskenderun Bay, Hatay province (Longitude: 36* 12' 16" E, Latitude: 36* 39' 54" N). The captured octopus samples were transported under cold chain to the Namık Kemal University Central laboratory, where analyzes were carried out. The samples were stored at -20 °C until analysis. This project was carried out with the approval of Hatay Mustafa Kemal University Animal Experiments Local Ethics Committee, decision numbered 2019/04-2 dated 25/04/2019.

Preparation of Samples

Before starting the heavy metal analysis, octopus samples frozen at -20 °C were thawed in the refrigerator at +4 °C for 12 hours. 25 grams of muscle and mantle tissue of octopuses were weighed and homogenized with a homogenizer, and 1 gram of it was placed in glass tubes. 3 ml of the solution prepared from HCl and HNO_3 in a 1:1 ratio was added to the glass tubes and left at room temperature for 1 hour. The volume of the mixture was completed to 5 ml with HNO_3 and kept in an oven at 95 °C for 2 hours. After being removed from the oven, the tubes were kept until they reached room temperature. After 2 ml of ultrapure water was added to the cooled tube, 3 ml of H_2O_2 was added and it was kept in the oven at 95 °C for two hours. After the tubes were removed from the oven and reached room temperature, 2 ml

of ultrapure water was added and filtered with Watman filter paper. The filtrate taken into Falkon tubes was centrifuged at 3000 rpm for 12 minutes. After centrifugation, the liquid remaining in the upper part was transferred to falcon tubes and 1% Triton X 100 mixture was added to the tube volume to 10 ml. The mixtures in falcon tubes with a final volume of 10 ml were prepared to be measured in ICP-OES (Alam et al. 2002; EPA Method 3052, 1996).

Preparation of blank solutions and calibration (standards)

Blank solution was used to adjust the optical settings of ICP-OES. Distilled water used to dilute the samples in the study was used blindly. Multi-element calibration standard solutions containing elements at different concentrations were prepared for metal analyses. 5 different calibration standards were prepared for the elements As, Hg, Cd, Pb, Ni, Cu, Zn, Al, Fe and Mn in the range of 25, 50, 250, 500 and 1000 μg kg^{-1}.

Measuring Samples

Residue levels of As, Hg, Cd, Pb, Ni, Cu, Zn, Al, Fe and Mn metals in the prepared samples were measured by Inductively Coupled Plasma Atomic Emission Spectroscopy (ICP-OES, Inductively Coupled Plasma Optical Emission Spectrometer). The plasma temperature varied between 6000-8000 °C. The samples were aerosolized with a nebulizer and given to plasma. The measurement was determined by repeating 3 times for each sample. These operations were carried out automatically by the device, and the results were interpreted by the computer system connected to the device and software specific to this system (EPA Method 3052, 1996).

Statistical analysis

SPSS package program (IBM SPSS Statistics 23, USA) was used for statistical analysis. Heavy metal levels in octopus samples were presented as arithmetic mean±standard error and minimum and maximum values.

RESULTS

Method Validation Findings

Calibration standards prepared for heavy metals Hg, Cd, Pb, Ni, Cu, Zn, Al, Fe and Mn in the range of 25, 50, 250, 500 and 1000 μg kg^{-1} were linear ($R^2 \geq 0.99$). The lowest detectable limit (LOD) values for the heavy metals Hg, Cd, Pb, Ni, Cu, Zn, Al, Fe and Mn are 3.51, 0.73, 17, 4.01, 2.01, 1.57, 0.48, 1.06 and 0.52 μg kg^{-1}, respectively. The lowest calculable limit (LOQ) values were determined as 25 μg kg^{-1} for all elements. Recovery values for Hg, Cd, Pb, Ni, Cu, Zn, Al, Fe and Mn elements are 90.40%, 109.21%, 104.10%, 106.60%, 100.66%, 110.18%, 115.20%, 107.16% and 105.70%, respectively. Intraday and interday coefficients of variation were found to be <1.45% and <7.33%.

Result in Octopus Samples

Heavy metal results obtained from 40 octopus samples caught from Iskenderun Bay, Hatay province, are presented in Table 1, and mean ± standard error in Table 2. While Cd, Cu, Zn, Al, Fe and Mn heavy metals were detected in all 40 samples analyzed, Ni element was detected in 11 of them. Hg and Pb could not be detected in any sample. Of these heavy metals, maximum residue limits are specified only for Hg, Cd and Pb. The maximum residue limits specified for Hg, Cd and Pb are 0.5, 1 and 1 mg kg^{-1}, respectively, according to the Turkish Food Codex, and 0.3, 1 and 0.3 mg kg^{-1}, respectively, according to the European Union. When these results were evaluated, it was determined that Hg, Cd and Pb levels in octopuses were below the maximum residue limits (TGK, 2011; EU, 2023)

Table 1. Heavy metal levels obtained from octopus samples

Number of samples	As µg kg^{-1}	Hg mg kg^{-1}	Cd µg kg^{-1}	Ni mg kg^{-1}	Pb mg kg^{-1}	Cu mg kg^{-1}	Zn mg kg^{-1}	Al µg kg^{-1}	Fe mg kg^{-1}	Mn mg kg^{-1}
1	37,68	0,00	1,97	0,00	0,00	10,27	221,23	11,79	8,67	1,14
2	61,34	0,00	7,20	0,00	0,00	21,08	94,10	8,70	2,72	0,81
3	105,70	0,00	2,02	0,00	0,00	10,30	75,94	6,72	2,71	0,77
4	118,64	0,00	1,10	0,00	0,00	11,69	70,55	8,46	2,76	1,07
5	32,45	0,00	1,31	0,00	0,00	7,52	77,39	9,02	45,95	0,77
6	56,49	0,00	1,65	0,00	0,00	9,24	128,22	6,97	3,56	0,73
7	50,13	0,00	2,32	0,00	0,00	13,76	90,54	9,13	2,39	1,02
8	45,64	0,00	1,25	0,00	0,00	10,44	77,89	8,16	7,64	1,17
9	46,25	0,00	4,74	0,00	0,00	7,26	77,38	10,22	3,28	0,65
10	48,51	0,00	9,26	0,00	0,00	14,12	291,80	14,35	7,16	0,79
11	42,28	0,00	2,08	0,00	0,00	10,23	87,33	9,34	2,55	0,87
12	20,09	0,00	1,03	0,00	0,00	4,32	68,04	7,99	1,53	0,45
13	44,59	0,00	1,24	0,00	0,00	7,01	87,24	8,62	2,30	0,63
14	43,69	0,00	1,78	0,00	0,00	18,53	88,75	10,30	4,16	0,90
15	32,62	0,00	1,53	0,00	0,00	6,59	81,26	8,74	1,65	0,84
16	35,51	0,00	43,96	1,37	0,00	39,33	70,29	8,74	15,62	1,91
17	28,21	0,00	2,07	0,00	0,00	9,83	103,82	9,68	4,37	0,73
18	37,65	0,00	74,25	0,75	0,00	0,09	182,57	8,76	13,92	1,33
19	26,60	0,00	8,83	0,38	0,00	10,73	103,38	8,50	4,41	1,18
20	37,94	0,00	2,45	0,00	0,00	8,07	81,97	8,90	3,57	0,85
21	44,79	0,00	1,68	0,00	0,00	6,92	82,64	3,01	1,75	0,75
22	43,22	0,00	1,79	0,43	0,00	7,51	81,33	2,27	1,53	0,77
23	24,74	0,00	21,47	0,37	0,00	15,65	107,00	2,21	9,97	0,91
24	48,38	0,00	1,98	0,00	0,00	5,56	80,12	5,31	3,91	0,64
25	43,36	0,00	22,82	1,61	0,00	37,41	360,57	2,83	8,88	2,58
26	29,71	0,00	62,11	0,80	0,00	37,66	156,60	2,18	13,73	1,37
27	45,72	0,00	15,77	0,00	0,00	21,64	90,70	2,75	5,70	0,98
28	40,45	0,00	3,22	0,00	0,00	16,19	100,38	1,84	2,67	1,05
29	55,81	0,00	1,34	0,00	0,00	6,61	83,83	2,86	0,50	0,66
30	48,73	0,00	4,26	0,00	0,00	8,78	72,31	3,12	0,50	0,72
31	37,55	0,00	22,21	0,74	0,00	36,13	87,56	11,39	10,25	3,95
32	37,51	0,00	9,61	0,00	0,00	10,71	96,75	2,54	6,43	0,67
33	50,49	0,00	3,61	0,00	0,00	13,93	82,17	3,78	2,39	0,87
34	23,68	0,00	24,25	0,89	0,00	27,71	80,81	4,27	8,78	2,05
35	44,85	0,00	1,41	0,00	0,00	11,27	89,95	2,75	1,54	0,77
36	30,55	0,00	174,62	1,86	0,00	74,76	186,51	6,53	37,34	2,26
37	70,75	0,00	1,30	0,00	0,00	11,65	101,70	1,91	0,61	0,78
38	49,10	0,00	4,82	0,00	0,00	7,72	97,54	1,73	0,73	0,70
39	172,47	0,00	0,11	0,43	0,00	14,16	86,64	340,61	48,88	2,32
40	270,50	0,00	0,14	0,00	0,00	11,99	69,79	23,54	3,18	1,25

Arsenic (As), Mercury (Hg), Cadmium (Cd), Lead (Pb), Nickel (Ni), Copper (Cu), Zinc (Zn), Aluminum (Al), Iron(Fe) and Manganese (Mn).

Table 2. Mean (±SE) heavy metal levels obtained from octopus samples

Parameter	N	Mean	Minimum	Maximum
As (μg kg^{-1})	40	49,75±7,05	20,09	270,50
Hg (mg kg^{-1})	40	ND	0,00	0,00
Cd (μg kg^{-1})	40	12,56±4,87	0,11	174,62
Ni (mg kg^{-1})	40	0,24±0,08	0,00	1,86
Pb (mg kg^{-1})	40	ND	0,00	0,00
Cu (mg kg^{-1})	40	15,36±2,14	0,09	74,76
Zn (mg kg^{-1})	40	108,87±9,64	68,04	360,57
Al (μg kg^{-1})	40	15,26±8,37	1,73	340,61
Fe (mg kg^{-1})	40	7,76±1,78	0,50	48,88
Mn (mg kg^{-1})	40	1,12±0,11	0,45	3,95

Arsenic (As), Mercury (Hg), Cadmium (Cd), Lead (Pb), Nickel (Ni), Copper (Cu), Zinc (Zn), Aluminum (Al), Iron (Fe) and Manganese (Mn). ND: not detected.

DISCUSSION

Many industrial establishments (such as iron-steel, fertilizer, profile, sheet metal, pipe factories) located on the coast of Iskenderun Bay, pollution resulting from agricultural activities around the bay, domestic waste, industrial waste, waste gases released into the atmosphere from residences and vehicles increasingly cause the bay to be polluted. In addition, the port and maritime transportation in the bay also contribute to this pollution in the bay. As a result, all these reasons increase chemical accumulation in marine organisms.

This study examined the metal levels of Arsenic 49.75±7.05 μg.kg^{-1}, Cadmium 12.56±4.87 μg kg^{-1}, Nickel 0.24±0.08 mg kg^{-1}, Copper 15.36±2.14 mg kg^{-1}, Zinc 108.87±9.64 mg kg^{-1}, Aluminum 15.26±8 .37 μg kg^{-1}, Iron 7.76±1.78 mg kg^{-1} and Manganese 1.12±0.1 mg kg^{-1} in 40 octopuses caught from Iskenderun Bay. Hg and Pb were not found in any of the analyzed samples. Metal levels in the edible tissues of octopuses were determined to be below the residue limits determined by the Turkish Food Codex Contaminants Regulation and the European Union Regulation.

In the study conducted by Kosker (2020), Pb levels measured in the muscle and mantle tissues of 24 octopuses caught from Mersin Bay were above the limit level of 1 mg · kg^{-1} determined by the European Union (EC, 2023) and the Turkish Food Codex (TGK, 2011). It was reported that the Cd level was below the limit level. In our current study, Pb could not be detected in all samples. In our study, Cd values were determined below the maximum limits, consistent with the current study.

Raimundo et al. (2015) on 24 octopuses caught off the Portuguese coast, the average Fe, Cu, Zn and Cd values measured in muscle and mantle tissues were reported as 21.25, 23.4, 77.5, 0.67 mg kg^{-1}, respectively. Ariano et al. (2019), Cd and Hg levels in 38 octopuses caught from the southern Tyrrhenian Sea of Italy were found below the legal limits for human consumption. Fe, Cu, Zn, Cd and Hg levels in our current study were found to be compatible with these studies.

Ahdy et al. (2007) determined Ca, K, Cu, Fe, Se, Sr, Zn, Cr, Cd, Hg and Pb levels in the muscles of octopuses caught off the coast of Egypt. In this study, the values of essential metals such as Ca, K, Cu, Fe, Se, Sr, Zn and Cr were found to be 815, 133, 11.7, 97.9, 1.8, 12.4, 69 and 2.7 mg kg^{-1}, respectively. The values of non-essential substances such as Cd, Hg and Pb were determined as 1.7, 0.053 and 1.3 mg kg^{-1}. The values obtained in this study were higher than the values obtained in our current study. When these results are evaluated, it is understood that the accumulation of heavy metals and the potential environmental stress caused by octopuses caught from Iskenderun Bay are less than the octopuses from the compared regions.

Mok. et al. (2014), the levels of essential and non-essential metals in octopus tissues caught from the Korean coast were 71.72 mg kg^{-1} for Zn, 24.148 mg kg^{-1} for Cu, 1.568 mg kg^{-1} for Cd, 0.303 mg kg^{-1} for Ni. 1, 0.125 mg kg^{-1} for Ag, 0.045 mg kg^{-1} for Cr, 0.032 mg kg^{-1} for Pb and 0.029 mg kg^{-1} for Hg. In this study, Cd content was found to be above the legal limits set by Korea and the European Union. The main way of Cd uptake in octopuses is through food, and the digestive gland is the main retention organ. The probable cause of the high Cd level in the above study is octopuses; They feed on a wide variety of other marine animals, including crustaceans, molluscs, fish and other cephalopod species, and may result from bioaccumulation of pollutants through

the food chain. Compared to our current study, essential metals were consistent with our study, while non-essential metals were higher.

Neto et al. (2014), As, Se and Zn levels in 117 octopuses obtained from sales points in Brazil were determined as 5.67, 1.40 and 14.2 mg kg^{-1}, respectively. According to Brazilian authorities, the Maximum Tolerance Limit for arsenic in fish and fish products is 1.0 mg kg^{-1}, and in solid foods it is determined as 0.30 mg kg^{-1} for Se and 50.0 mg kg^{-1} for Zn. As and Se were above the specified legal limits. When we compare it with our current study, high Zn results were found to be similar.

Karim et al. (2016) in the muscle tissues of octopuses collected in Morocco, the Cd, Zn, Cu, Fe and Co values were 0.58, 8.95, 280.68, 171.12, 229.35 and 2.73 mg kg^{-1} in the summer months, and 0.24, 298.6, 133.04, 40.551.17 mg kg^{-1} in the winter months. and was determined. Compared to the maximum residue limit allowed by WHO, the concentrations of the studied metallic elements were generally detected within the permissible limits for human consumption. When we compare the results of octopuses caught in Iskenderun Bay, it can be said that non-essential elements are high and essential elements are similar.

Cd 0.68-9.51, Cu 5.97-324.1, Cr 0.48-8.75, Fe 7.23-131.35, Mn 0.52-52.46, Pb 1.49-3.10, Zn 9.13-85.19 mg kg^{-1} were determined between in 60 squid tissues caught from İskenderun Bay by Duysak and Dural (2015). In this study, Cd and Pb limit values were above the residue limits determined by the Turkish Food Codex Contaminants Regulation and the European Commission Regulation. In our current study in the same region, all values were found to be below acceptable levels.

Baş and Altındağ (2019) investigated heavy metal accumulations such as Pb, Cd, Fe, Ni, Cu, Zn, Cr, As, Al, and Mn in sardine (Sardina pilchardus) and horse mackerel (Trachurus trachurus) samples obtained from İskenderun Bay. It has been determined that iron is the most accumulated heavy metal and the highest accumulation is in the gill tissues. Metal values in all tissues were found to be in accordance with the Turkish Food Codex and European Union norms. The results were found to be compatible with our current study conducted in the same region.

In their study, Kaya and Türkoğlu (2017) conducted heavy metal levels in the muscle tissues of fish and shrimps caught from the bay of İskenderun: 0.103-4.988 mg kg^{-1} for Mn, 0.134-0.336 for Cr, 0.005-0.008 for Cd, 0.091-0.110 for Ni, It was determined between 0.026-0.228 for Hg, 1.741-29.254 for As, 0.087-0.110 for Pb, and <0.00050-0.027 mg kg^{-1} for Co. In this study, it was determined that the total arsenic level in shrimp muscle was at high concentrations and it was concluded that shrimp may pose a health risk because it exceeds the legal limits for both total arsenic and estimated inorganic arsenic amounts. When compared to our octopus study conducted in the same region, the values were found to be similar except for arsenic.

CONCLUSION

Industrialization and unregulated urbanization play a major role in deteriorating the ecological balance. In parallel with the increase in industrialization, water, soil and air pollution has also been gradually increasing. The environmental balance, which has been functioning spontaneously for centuries, has begun to deteriorate in a way that it can no longer fulfill this function. The important thing is that chemicals pass through the food chain to plant and animal bodies, and from there to humans, the last link of the food chain. Factory wastes, untreated material discharge, agricultural wastes, domestic wastes and maritime transportation cause an increase in chemical pollution in Iskenderun Bay. Heavy metal pollution, one of the chemical pollutions, has negative effects on marine creatures.

Heavy metal pollution, one of the chemical pollutions, has negative effects on marine organisms.

In the measurements made, the highest heavy metal level in octopus tissues was determined for Zn, and Hg and Pb could not be detected in any sample. It has been determined that the heavy metal levels in their edible tissues comply with the European Union norms and the values published in the Turkish Food Codex. As a result of the study, it was understood that the heavy metal accumulation detected in octopus tissues was not dangerous for human health and ecosystem.

It has been observed that the heavy metal levels found in the research conducted in the bay of Iskenderun increase every year, and in recent years, the levels of some heavy metals have exceeded the maximum residue amount specified in the Turkish Food Codex Contaminants Regulation. Future plans and programs should be made to reduce and prevent chemical pollution in Iskenderun Bay.

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RESEARCH ARTICLE

Estimation of Milk Yield Losses from Subclinical Mastitis in Dairy Cows

Murat TANDOĞAN[1], Özay GÜLEŞ[2]*

[1]*Department of Animal Health Economics and Management, Faculty of Veterinary Medicine, Afyon Kocatepe University, Afyonkarahisar, Türkiye*
[2]*Department of Histology and Embryology, Faculty of Veterinary Medicine, Aydın Adnan Menderes University, Aydın, Türkiye*

ABSTRACT

In order to estimate the prevalence of subclinical mastitis, somatic cell content in cow's milk is the main indicator. It was aimed at revealing the relationship between breed, age, number of lactations, lactation periods, average milk yield of cows, and somatic cell count (SCC) used in mastitis diagnosis. The material of the study consisted of milk from 300 mammary lobes belonging to 75 cows from different breeds (Holstein and Holstein Crossbred) aged between 3 and 8 years in dairy cattle enterprises in Efeler district of Aydın province between December 2020 and February 2021. To determine the association between SCC and milk yield, multiple regression analysis was used. There was no significant difference in SCC between crossbred Holstein and Holstein cows. It was determined that SCC increased with increasing age (≤4 and 5≥). In the study, it was determined that the daily milk yield of the cows was 21.1 kg, and it was found that the milk yield loss showed significant differences according to the SCC. In the analysis, there is a negative relationship between SCC and milk yield; an increase of 1 unit in SCC was estimated to result in a daily loss of 0.71 kg of milk output per cow.

Key words: Cow, milk yield loss, somatic cell count, subclinic mastitis

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Süt İneklerinde Subklinik Mastitisten Kaynaklanan Süt Verim Kayıplarının Tahmini

ÖZ

İnek sütündeki somatik hücre konsantrasyonu, subklinik mastitis prevalansının tahmin edilmesinin ana göstergedir. Bu çalışmada ineklerin ırk, yaş, laktasyon sayısı, laktasyon dönemleri ve ortalama süt verimi ile mastitis tanısında kullanılan somatik hücre sayısı (SHS) arasındaki ilişkiyi ortaya çıkarmak amaçlanmıştır. Araştırmanın materyalini, Aydın ili Efeler ilçesindeki süt sığırcılığı işletmelerinde, Aralık 2020 ile Şubat 2021 tarihleri arasında 3 ila 8 yaşları arasındaki farklı ırklardan (Holstein ve Holstein Melez) 75 ineğe ait her meme lobundan (toplam 300) alınan süt örnekleri oluşturmuştur. SHS ve süt verimi arasındaki ilişkiyi belirlemek için çoklu regresyon analizi kullanılmıştır. Melez Holstein ve Holstein inekleri arasında SHS bakımından anlamlı bir fark bulunmamıştır. SHS'nin yaş arttıkça (≤4 ve ≥5 yaş) arttığı belirlenmiştir. Çalışmada, ineklerin günlük süt veriminin 21.1 kg olduğu ve SHS'ye göre süt verim kaybının anlamlı farklılıklar gösterdiği bulunmuştur. Analizde, SHS ve süt verimi arasında negatif bir ilişki olduğu; SHS'deki 1 birimlik artışın, inek başına günlük 0.71 kg süt verimi kaybına neden olduğu tahmin edilmiştir.

Anahtar Kelimeler: İnek, somatik hücre sayısı, subklinik mastitis, süt verimi kaybı

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ORCID ID; *MT: 0000-0002-6467-3699, ÖG: 0000-0001-6170-1706*
*Corresponding author e-mail: ozaygules@adu.edu.tr

INTRODUCTION

For the majority of people in the world, milk and dairy products represent some of their most basic dietary sources. The average milk yield per cow is rising as a result of the rising demand for dairy products worldwide (Lucy 2001). The increase in milk yield has resulted from genetic selection as well as improved cow nutrition and herd management. One of the major problems affecting high milk yield is poor udder health, especially due to mastitis (De Vliegher et al. 2003). The onset of clinical mastitis and the development of intramammary infection (IMI) result in large financial losses for dairy farmers (El-Awady and Oudah, 2011).

Mastitis is an inflammatory disorder of the mammary gland that can be brought on by microorganisms, disease-related tissue damage, and chemical, physical, or traumatic incidents (Bae et al. 2017). As a result of mastitis disease, the disease has economic importance as significant losses occur due to decreased quantity and quality of milk produced, antibiotic treatment, and increased veterinary care costs (Jilo et al. 2017).

There are two basic types of mastitis: subclinical and clinical. It is commonly acknowledged that subclinical mastitis accounts for the majority of the economic costs associated with mastitis. From an economic point of view, for many cattle farms, subclinical mastitis is considered to be the most economically important type of mastitis due to its long-term impact on total milk yield (Halasa et al. 2007).

The disease-related decrease in product and the forfeiture of production benefits can be characterized as the economic losses resulting from mastitis. The first of these is represented by the milk that must be thrown out following antibiotic treatment, and the second is the benefit of the milk that this disease will prevent from ever being produced (Kossaibati and Esslemont 1997).

Direct and indirect expenses are the two categories of costs associated with mastitis. Veterinary services, diagnosis, treatment, extra labor costs, and discarded milk (during treatment) are all considered direct expenses. Known as hidden costs, indirect expenses are described as costs that are not always evident to the milk producer. Indirect losses due to subclinical mastitis (SCM) can be listed as decreased milk yield, early slaughter losses due to the disease, and poor milk quality (Nielsen 2009).

The detection of subclinical mastitis is a very difficult task for producers, but its detection is very important to save both producers and animals from many problems (Kabir et al. 2017).

Although subclinical mastitis cases cannot be diagnosed clinically because clinical symptoms are not observed, since the disease manifests itself through an increase in the number of somatic cells and bacteria in milk, it can be detected indirectly by looking at the level of somatic cell count (SCC) in milk. At the same time, milk yield losses occurring at different levels of subclinical mastitis can be determined by quantitative methods (Yalcin, et al. 1999a; Sumon, et al. 2020).

It has been observed that somatic cell concentration in cow's milk is the main indicator for estimating the prevalence of subclinical mastitis. Cows with subclinical mastitis have no visible signs but have a high somatic cell count (SCC, defined as the number of somatic cells per milliliter of milk). High SCC in milk indicates the presence of pathogens in the udder and is an indicator of intramammary infection (IMI) and also a measure of response to infection (Pyörälä 2003; Heringstad et al. 2006).

To estimate the possible milk yield losses caused by subclinical mastitis, it is important to define healthy or uninfected. The threshold for a healthy udder has been considered to be SCC ≤50 000 (Seegers et al. 2003) or about 70 000 (Djabri et al. 2002; Schukken et al. 2003). Some authors have defined a healthy animal as having a slightly higher SCC, i.e., ≤100 000 (Hand et al. 2012). SCC less than 100,000 is considered to be uninfected, and there is no significant milk yield loss due to subclinical mastitis. The new definition of subclinical mastitis assumes a new case if SCC reaches >100 000 after a test day with SCC <50 000 (Halasa et al. 2009). Therefore, the choice of an appropriate threshold to identify an uninfected mammary depends on the purpose. At a lower threshold, more cases of CBE (increased sensitivity and fewer false negatives) are identified, whereas using a higher threshold (increased specificity) may result in fewer false positive results (Pantoja et al. 2009).

The problem of subclinical mastitis is extremely complicated. So, the dairy industry is very interested in developing a simple, economical, and effective way to forecast the correlations between high SCC, subclinical mastitis, and possible loss of milk yield in dairy cows (Jeretina et al. 2017).

Furthermore, because harmful organisms like Staphylococcus aureus, Streptococcus agalactiae, Escherichia coli, Mycobacterium bovis, and Mycoplasma spp. can be found in the milk collected from the afflicted cow, subclinical mastitis has zoonotic significance. For this reason, it is critical that customers have early detection and treatment of subclinical mastitis (Dhakal et al. 2007).

In this study, the estimation results of milk yield losses due to subclinical mastitis on dairy cattle farms are evaluated. In addition, it was aimed at revealing the relationship between breed, age, number of lactations, lactation periods, average milk yield of cows, and somatic cell count used in mastitis diagnosis.

MATERIALS and METHODS

Experimental Design

The data and samples required for the study were obtained from dairy cattle farms operating in the Efeler District of Aydın Province between December 2020 and February 2021. In the study, 10-15 ml milk samples were taken from 300 udder lobes of 75 Holstein and Holstein Crossbred cows, aged between 3 and 8 years, which did not show clinical symptoms, and used in sterile plastic tubes. Information on daily milk yield, number of lactations, and lactation period was obtained from the farms visited for sample collection.

Taking Milk Samples

Somatic cell counts were determined in milk samples taken from each udder lobe of 75 animals. For this purpose, milk samples were taken from four udder lobes (300 milk samples in total) of each cow during evening milking and analyzed on the same day. Before taking the samples, the sampler disinfected his or her hands and the udders of the cows. After discarding the first 5 ml of milk to remove saprophytic bacteria from each teat, 10-15 ml milk samples were taken into sterile plastic tubes (Zajac et al. 2018; Kabir et al. 2019).

Preparation of Milk Films

Milk films were prepared within 1 hour after the milk samples were collected. For this purpose, 10-15 µl of milk were taken from each milk sample using a micropipette and spread on 1 cm^2 square areas on a clean microscope slide. After the milk films were prepared, they were dried at room temperature (Zajac et al. 2018; Kabir et al. 2019).

Dyeing of Milk Films

The prepared milk films were stained according to the Broadhurst-Paley staining method. For this purpose, the slides were immersed in xylene for 2 minutes and 95% ethyl alcohol for 2-5 minutes and filtered. Then the slides were immersed in Broadhurst-Paley stain for 30 seconds. Finally, the slides were rinsed by immersion in three separate distilled waters and dried at room temperature (Broadhurst and Paley 1939; Moraes et al. 2018).

Microscopic Somatic Cell Count

The stained milk films were examined under an immersion objective using a light microscope (Leica DMLB Meyer Instruments, Inc., Houston, TX). The diameter of the image area under the immersion objective was measured at 195 µm with a micrometer, and the working factor (WF) was calculated at 13400. For somatic cell counting, 25 fields were counted in each milk film. For reliability, somatic cells were counted on two milk films prepared from each milk sample and averaged. The average cell count was then multiplied by WF to calculate the number of cells in 1 ml of milk (Zajac et al. 2018).

Statistical Analyses

Regression analysis was performed using the SPSS Statistics 18 package program for statistical calculations of the data obtained from the study. In the study, the number of lactations, average daily milk yield, age, breed, and lactation periods of the cows were determined. According to the data obtained, the cows;

Age = (years); 1st (≤ 4 years; 2nd (≥ 5 years).
Breed = 1. (Holstein), 2. (Holstein hybrid)
Average daily milk yield (kg) = 1st (1-19,4 kg), 2nd (19,5-23,1 kg), 3rd (23,2 kg ≥).
Number of lactations: 1st (first 3 lactations), 2nd (4th lactation and above)
Lactation period (months): 1st (1-3; early period), 2nd (4-6; middle period), 3rd (7-10; late period)
The somatic cell count was subdivided into 0 (below 200 000), 1 (200 001-500 000), 2 (500 001-1 million), 3 (over 1 million) and then subjected to the necessary analyses.
The quantitative relationship between milk yield and somatic cell count was estimated by multiple regression analysis. The model used was:

SVi = SCCi + LACNUMBERi + LACPERIODi + AGEi + RACEi

Equation:
SVi: Milk yield on the first visit day (kg/day/cow), SCCi: The number of somatic cells in each milliliter of milk on the day of the first visit, LACNUMBERi: Number of lactation of the cow on the first visit day, LACPERIODi: lactation period on the day of the first visit, AGEi: age of the cow on the day of the first visit, RACEi: breed of cow on the day of the first visit.

RESULTS

Model Estimation Results

The regression estimation results are presented in Table 1. There was a negative correlation between SCC and lactation number and a positive correlation between breed, age, and lactation period. The relationship between milk yield, SCC, and number of lactations was found to be statistically significant ($P<0.01$). The significant F statistic ($p<0.001$) indicated that the model was significant as a whole, and the adjusted R2 value of 0.464 indicated that the independent variables included in the model explained 46.4% of the variation in milk yield.
Durbin-Watson test results were analyzed for an autocorrelation problem, and it was concluded that there was no such problem (DW= 1.787). The multicollinearity problem (multicollinearity) was

investigated by analyzing the correlation matrix between the independent variables, and it was determined that there was no high correlation between any variables.

In the estimated model, the most important variable affecting the variation in milk yield was the increase in somatic cell count. According to the SCC result, the milk yield loss caused by the increase of l units was estimated to be 0.71 kg/cow/day.

According to 2019 data from the Turkish Statistical Institute (TurkStat), milk is obtained from 6.580.753 milking cows in Turkey. The loss of milk yield in our country due to SCC was estimated at 1.425.062 metric tons. As a result of the calculation made with the 2023 second-period raw milk current prices, the monetary equivalent of the yield loss was calculated as $ 602,286,428. When calculating the monetary equivalent of the loss of yield, the loss due to the decrease in milk quality caused by the disease and the loss in case of recurrence of the disease were not taken into account.

Table 1: Predicted milk yield regression estimation results

	b	SE	P
Fixed	2.471	0.640	0.000
SCC	-0.708	0,237	0.004
Race	0.089	0.192	0.644
Age	0.251	0.237	0.293
LacPeriod	0.114	0.120	0,345
LacNumber	-0.009	0.224	0.002

R=0.681 R2 =0.464F Value: 20.31 (P<0.001) Durbin-Watson= 1.787

DISCUSSION

One of the most important problems encountered on dairy cattle farms is mastitis. This disease causes millions of dollars of economic losses every year due to reasons such as the decrease in milk yield and quality, disposal of mastitic milk, medicine, and veterinary costs, removal of animals from the herd, decrease in the market value of animals, and protection and control practices in mastitis (Yalçın 2000).

Mastitis generally occurs in two forms: clinical and subclinical. Clinical mastitis has external signs that can be easily observed in the udder of the cow. However, subclinical mastitis is not recognized because the udder does not show a clinical picture and continues for a long time. Approximately 70-80% of milk yield losses due to mastitis are caused by subclinical mastitis (De Graves and Fetrow 1993; Yalçın 2000). Traumatic, bacterial, viral, parasitic, and chemical factors play a role in the occurrence of mastitis. Factors such as breed, age, milk yield level, lactation period and number, anatomical reasons, milking method, seasonal and climatic conditions, nutrition, barn and shelter conditions, metabolism, and hormonal balance of the animal play a role as predisposing factors (Contreras and Rodríguez 2011). Since the data obtained in this study were taken during the same period, and factors such as barn conditions, udder hygiene, and milking method were similar; other factors other than breed, age, milk yield, lactation period, and number were not taken into consideration in the study.

In previous scientific studies on mastitis in dairy cattle farms, the number of losses due to the disease varies between countries depending on factors such as calculation methods, loss items (milk, treatment, labor, reformation, etc.), disease form (clinical/subclinical) and severity (mild, severe), incidence rate, and prices/wages (veterinarian, medicine, milk, and labor) (Sarıözkan 2019). For example, losses of 22-31 € per cow per year in the USA (Kaneene and Hurd, 1990; Miller et al. 1993), 19-32 € in France (Fourichon et al. 2001), 3 € in Germany (Reinsch and Dempfle 1998), 102-279 € per case in the UK (Kossaibati and Esslemont 1997; McInerney et al. 1992), €240 in Germany (Clair et al. 2019), €440 in Canada (Aghamohammadi et al. 2018), and $80.09 in Iran (Sadeghi-Sefidmazgi et al. 2011). In a study conducted in 21 enterprises in Tunisia, it was estimated that there was an annual milk yield loss of 524 kg per cow (Mtaallah et al. 2002). Mungube et al. (2002) stated that the annual economic loss in milk yield per cow was between 29.1 and 66.6 USD. In Turkey, while an average loss of 315 TL per infected animal (equivalent to 271-1277 L milk) and 113 TL

per cow was reported in 2006 (Yalçın et al. 2010), an average loss of 244 TL per infected animal (equivalent to 158-1204 L milk) and 110 TL per cow was reported in 2014 (Yıldız and Yalçın 2014).
In this study, with the current prices of 2023, the amount of loss per infected animal in mastitis cases ($91.52) is equivalent to 216.55 L of milk. It is thought that the fact that the majority of the animals used in the study were cattle on family farms and that the traditional breeding model was applied to the farms was effective in the high rates of subclinical mastitis. To better determine the effect of the SCC increase, it may be useful to study more samples in large herds with standardized breeds, ages, number of lactations, and lactation periods raised in the same environmental conditions. In conclusion, in this study, the difference between the milk yield of cows and the SCC increase was found to be statistically significant, and it can be said that mastitis has a significant effect on decreasing milk yield. The average daily milk yield of the cows was determined to be 21.1 kg, and it was determined that milk yield loss varied significantly according to the SCC of the cows. Demir and Ekşi (2019) estimated that the milk yield loss caused by an increase of l units in CMT was 1.92 kg/cow/day. Dohoo et al. (1984) calculated 1.21-2.09 liters of yield loss per cow per day due to mastitis. Yalcin et al. (1999a) estimated the loss at 0.7 kg per cow. In another study, Yalcin et al. (1999b) calculated milk yield loss as 1.01 kg.

CONCLUSION and RECOMMENDATIONS

In conclusion, our statistical analyses identified a linear inverse relationship between somatic cell count (SCC) and milk yield. Milk production losses were estimated at 0.71 kg/cow/day per unit increase in SCC. As a result of the intensive polyculture production in the livestock sector in Turkey, producers cannot allocate enough time to dairy cattle breeding and cannot specialize in production, leading to yield loss. Moreover, their low level of technical and formal education prevents them from keeping up with the latest knowledge and advancements in their industry, which decreases their success. This situation results in a breakdown of enterprise controls and follow-up, a rise in illnesses, and ultimately a decrease in production. Ultimately, all these factors prevent the producers from working profitably and efficiently. It will be useful to inform the producers about the diseases and the extent of the losses they cause at the enterprise level, and some of these losses can be avoided by closing the technical knowledge deficits of the producers with the training programs to be carried out.
Since the clinical signs of subclinical mastitis are not visible in the field, it is important to determine the economic dimension of this disease, which is neglected by many producers in the sector today, and to take the necessary measures. To determine the economic weight of the disease, it would be more appropriate to extend the record-keeping system in enterprises and to carry out field research in different regions.

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Ethical Approval: This study is not subject to the permission of HADYEK in accordance with the "Regulation on Working Procedures and Principles of Animal Experiments Ethics Committees" 8 (k). The data, information and documents presented in this article were obtained with in the frame work of academic and ethical rules.

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RESEARCH ARTICLE

Evaluation of Relationship Between C-Reactive Protein, Leukocyte Count and Platelet Indices in Dogs with Leukocytosis

Serkan İrfan KÖSE[1*], Alper ERTÜRK[1], Gökhan UYANIK[1], Aliye SAĞKAN ÖZTÜRK[1]

[1]Department of Internal Medicine, Faculty of Veterinary Medicine, Hatay Mustafa Kemal University, 31060, Hatay, Türkiye.

ABSTRACT

A complete blood count and acute-phase protein analyses are the laboratory tests that are used in veterinary medicine practice. Leukocytes, also known as white blood cells (WBCs), are the primary biological components of inflammatory and immunological responses. C-reactive protein (CRP) is a powerful predictor of inflammation and/or infection in veterinary medicine. Starting at that point, the investigation of the alterations and relationship between WBC count, platelet indices, and blood serum CRP levels in dogs with leukocytosis was the aim of this study. A total of 135 blood analyses records, from January 2018 to December 2022, of dogs were analyzed. The inflammatory panel of complete blood count data, including WBC, PLT, MPV, PDW, PCT, and MPV/PLT, and CRP levels in blood serum chemistry analyses were investigated. The inflammatory panel of complete blood count data was categorized according to CRP levels in blood serum chemistry analyses. There was no significant difference in platelet indices according to the blood serum levels of CRP in the dogs' blood analyses records. The WBC count in dogs with an equal or above 10 mg.dL^{-1} CRP level was significantly higher than the other levels of CRP. A moderately positive correlation was detected between CRP and WBC in the blood analyses records of all dogs, undivided according to inflammatory status, and of dogs with inflammation status regardless of etiology. Consequently, in veterinary clinical practice, C-reactive protein measures in dogs may be more applicable in cases where an important rise in WBC over the cut-off value is observed in the complete blood count, commonly used to diagnose inflammatory or infectious status.

Keywords: Complete blood count; C-reactive protein; Dog; Leukocytosis

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Lökositozlu Köpeklerde C-Reaktif Protein, Lökosit Sayısı ve Trombosit İndeksleri Arasındaki İlişkinin Değerlendirilmesi

ÖZ

Tam kan sayımı ve akut faz protein analizleri veteriner hekimlik uygulamalarında kullanılan laboratuvar testleridir. Beyaz kan hücreleri (WBC) olarak da bilinen lökositler, enflamatuar ve immünolojik yanıtların birincil biyolojik bileşenleridir. C-reaktif protein (CRP) veteriner hekimlikte enflamasyon ve/veya enfeksiyonun güçlü bir belirleyicisidir. Bu noktadan hareketle, lökositozlu köpeklerde WBC sayısı, trombosit indeksleri ve kan serumu CRP seviyeleri arasındaki değişikliklerin ve ilişkinin araştırılması bu çalışmanın amacını oluşturmuştur. Ocak 2018'den Aralık 2022'ye kadar köpeklere ait toplam 135 kan tahlili kaydı analiz edilmiştir. WBC, PLT, MPV, PDW, PCT ve MPV/PLT dahil olmak üzere tam kan sayımı verilerinin enflamatuar paneli ve kan serumu kimyası analizlerinde CRP seviyeleri araştırıldı. Tam kan sayımı verilerinin enflamatuar paneli, kan serumu kimyası analizlerindeki CRP düzeylerine göre kategorize edildi. Köpeklerin kan serumu CRP seviyelerine göre trombosit indekslerinde anlamlı bir fark bulunmadı. CRP seviyesi 10 mg/dL ve üzerinde olan köpeklerde WBC sayısı diğer CRP seviyelerine göre anlamlı derecede yüksekti. Yangı durumuna göre ayrılmamış tüm köpeklerin ve etiyoloji gözetmeksizin yangı durumu olan köpeklerin kan tahlillerinde CRP ve WBC arasında orta düzeyde pozitif bir korelasyon tespit edilmiştir. Sonuç olarak, veteriner klinik uygulamalarında, köpeklerde C-reaktif protein ölçümleri, enflamatuar ya da enfeksiyöz durumu teşhis etmek için yaygın olarak kullanılan tam kan sayımında WBC'de kesme değerinin üzerinde önemli bir artış gözlendiği durumlarda daha uygulanabilir olabilir.

Anahtar Kelimeler: C-reaktif protein; Köpek; Lökositoz; Tam kan sayımı

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ORCID ID; *SİK: 0000-0003-3189-6690, AE: 0000-0002-4271-7953, GU: 0000-0003-4488-3055, ASÖ: 0000-0003-1037-6733*

*Corresponding author e-mail: serkanirfankose@mku.edu.tr

INTRODUCTION

A complete blood count is a supplementary laboratory test that determines the quantity of white and red blood cells circulating in the circulation. It is commonly requested by veterinarians and serves as a regular examination of healthy and ill animals (Moruzi et al. 2023). Leukocytes, also known as white blood cells (WBCs), are the primary biological components of inflammatory and immunological responses. They defend the body against infections and neoplasia and aid in the healing of injured tissue (Moruzi et al. 2023). The leukogram, which is part of the complete blood count (CBC), is an ordered tabulation of the total nucleated cell concentration as well as the concentrations of particular WBC types found in the blood sample, also known as the WBC differential (Kritsepi-Konstantinou and Oikonomidis 2016; Wood 2022b). Leukogram abnormalities include quantitative or numerical concentration abnormalities as well as morphologic abnormalities in WBCs (Wood 2022a). WBC concentrations are evaluated by comparing them to species-specific reference values (Wood 2022a). The reference values of WBC concentrations for dogs range approximately within 5.0-14.5×10^3.μL^{-1} (Kritsepi-Konstantinou and Oikonomidis 2016; Fielder 2022; McCourt and Rizzi 2022). Only absolute numbers should be considered when making interpretations (Wood, 2022a). Abnormalities in total WBC concentration only serve to alert the physician to search for and evaluate abnormalities in cell distributions in the differential (Wood, 2022a). Even if the overall WBC counts are normal, the differential may have one or more distributional abnormalities (Wood, 2022a). Leukocytosis is a rise in total WBC concentration, whereas leukopenia is a reduction in total WBC concentration (Wood, 2022a). Inflammatory, infectious, and immunologic responses can be interpreted by using a leukogram (McCourt and Rizzi 2022; Wood 2022b).

Acute-phase proteins (APP), being a response to release proinflammatory cytokines, are produced in situations ranging from infectious diseases to trauma (Murata et al. 2004; Kocaturk et al. 2010; Anziliero et al. 2013; Ok et al. 2015). In veterinary medicine, clinic-laboratory examinations have included the use of APPs to assess healthy and sick animals (Murata et al. 2004; Ok et al. 2015). C-Reactive Protein (CRP) is recognized as a powerful predictor of inflammation and/or infection in veterinary medicine (Anziliero et al. 2013). CRP activities rely on its ability to attach to bacteria and stimulate complement binding, allowing phagocytes to pick up bacteria (Alves et al. 2020). CRP also stimulates monocytes and macrophages to produce cytokines, inhibits chemotaxis, and modulates neutrophil activity (Alves et al. 2020; Malin and Witkowska-Pilaszewicz 2022). CRP is recognized to be a significant APP in dogs, and increased CRP values have been found in dogs with systemic inflammation (Anziliero et al. 2013; Christensen et al. 2014; Ok et al. 2015). Infectious or inflammatory conditions can cause CRP serum concentrations to rapidly rise from <1 mg.L^{-1} to >100 mg.L^{-1} in dogs (Eckersall and Bell 2010). Various studies have produced various upper-point values for the reference interval (RI) for CRP (Otabe et al. 1998; Martínez-Subiela et al. 2004; Kjelgaard-Hansen 2010; Casella et al. 2013; Malin and Witkowska-Pilaszewicz 2022). The technique, procedure, reagents and anticoagulants, and equipment may all be contributing factors to this difference between studies or laboratories (Malin and Witkowska-Pilaszewicz 2022).

Many automated hematology analyzers frequently used in veterinary clinics measure platelet number, size, and total platelet mass, which might offer clinically important information in some situations (Smith et al. 2014; Llewellyn et al. 2017). The platelet indices (PIs) include the following parameters: the number of platelets (PLT) that circulate in blood flow; the mean platelet volume (MPV), quantifying platelet size; the plateletcrit (PCT), which estimates platelet mass; and the platelet distribution width (PDW), revealing platelet size variability (Smith et al. 2014; Llewellyn et al. 2017). In many instances, elevated MPV and PDW indicate the presence of bigger, immature platelets released into the circulation in response to increased platelet synthesis (Llewellyn et al. 2017). The increase in plasma thrombopoietin concentration is caused by a decrease in platelet mass, which contributes to an increase in megakaryocytopoiesis. This condition may be further exacerbated when inflammatory cytokines are present (Yuri Gasparyan et al. 2011; Llewellyn et al. 2017). So, it is expressed that MPV serves as a marker of inflammation, disease activity, and the efficiency of anti-inflammatory therapy in various chronic inflammatory illnesses (Yuri Gasparyan et al. 2011). In addition, it is also stated that the size of circulating platelets may vary depending on the intensity of inflammation (Yuri Gasparyan et al. 2011). It is informed that the alterations for MPV, PCT, and PDW in PI were detected in dogs with inflammatory or infectious conditions (Smith et al. 2014).

The purpose of this study was to investigate the alterations and relationships between WBC count, platelet indices, and blood serum CRP levels in dogs with leukocytosis.

MATERIALS AND METHODS

Materials

The blood analyses data, including both complete blood counts and blood chemistry analyses of dogs, comprised the materials of the study. A total of 135 blood analyses records, from January 2018 to December 2022, of dogs admitted to the department of internal medicine clinic of Hatay Mustafa Kemal University, Veterinary Health, Practice, and Research center were obtained.

Methods

The blood analyses were performed via automated blood count device (MS4e, Melet Schloesing Laboratoires, France) and automated biochemistry analysis device (Chem 200vet, Gesan, Italy). The existence of both complete blood counts and blood serum chemistry analyses of each dog was used as inclusion criteria for blood analyses records. Another inclusion criteria was the existence of C-reactive protein (CRP) analysis in the blood serum chemistry analysis and the existence of white blood cells (WBC), platelet indices (PI) including platelets (PLT), platelet distrubution width (PDW), main platelet volume (MPV), and plateletcrit (PCT) counts in the complete blood count. The main platelet volume-to-platelet ratio (MPV/PLT) was also calculated. Data collection was conducted by using patient registries and patient monitoring software (EVET, Hasvet, Türkiye). The inflammatory panel of complete blood count data, including WBC, PLT, MPV, PDW, PCT, and MPV/PLT, were categorized according to CRP levels in blood serum chemistry analyses. The presence of infection or inflammation was considered when the WBC level in the complete blood count was above the upper value of the reference limit (Fielder 2022). The reference interval of CRP was accepted at 0.0–1 $mg.dL^{-1}$, as defined by the manufacturer of the reagents (Gesan, Italy) for the automated biochemistry analysis device.

Statistical Analysis

For categorical variables, descriptive statistics were presented as frequencies and percentages, and for continuous variables, as arithmetic means and standard errors. The normality assumption was checked with Shapiro Wilk Test. The blood analyses records were classified as normal or having an infection according to the WBC count, and the Mann-Whitney U test was used to detect the difference between platelet indices parameters in the complete blood count and blood serum CRP levels since the normality assumption was not fulfilled. The difference between inflammatory panel parameters of the complete blood count, categorized according to CRP levels, was determined with the Kruskal-Wallis test because the normality assumption was not held. One-way ANOVA test with Dunnett T3 post hoc test was used for the determination of difference within the categorized parameters. The accuracy of blood serum CRP level in predicting the presence of infection and the accuracy of WBC count in predicting CRP level were estimated by receiver operating characteristic (ROC) analysis by calculating Area Under the Curve (AUC) and 95% confidence intervals. The optimal cut-off point maximizing the Youden's J statistic of each parameter to predict the existence of infection and the inflammatory level of CRP was also determined [J=max (sensitivity+specificity-1)]. The relationships between complete blood count parameters and CRP in dogs without inflammatory status and those with inflammatory status according to CRP levels were specified with Spearman's rank correlation coefficient due to the normality assumption not holding. A p value of <0.05 was considered statistically significant. All statistical analyses were performed using SPSS Statistics for Windows, Version 26.0 (IBM Corp, Armonk, NY, USA).

RESULTS

The blood analyses records of dogs grouping having infection or not according to WBC count in CBC were given in Table 1. According to blood serum levels of CRP, alterations in CRP and blood parameters, including the inflammatory panel, were given in Table 2. There was no significant difference in PI according to blood serum levels of CRP of dogs' bloodwork records (Table 2). The WBC count in dogs having an equal or above 10 $mg.dL^{-1}$ CRP level was significantly higher than the other levels of CRP (Table 2). AUC results for CRP and WBC in blood analyses of dogs were given in Table 3, and were showed in Figure 1. The relationships between complete blood count parameters and CRP in dogs without inflammatory status and those have an inflammatory status according to CRP levels were given in Table 4, 5, and 6. A moderate positive correlation was detected between CRP and WBC in the bloodwork of all dogs, undivided according to inflammatory status (Table 4), and of dogs with an inflammatory status (Table 6).

Table 1. Descriptives of blood records of dogs grouping having leukocytosis or not according to WBC.

Parameters	Normal (N:83)	Having leukocytosis (N:52)	p[1]
White Blood Cells$\times 10^3.\mu L^{-1}$	13.148±0.781	21.809±1.927	0.000
CRP $mg.dL^{-1}$	5.028±0.544	14.270±1.821	0.000
Platelet$\times 10^3.\mu L^{-1}$	360.289±15.871	386.192±35.018	0.605
Main Platelet Volume fL	8.571±0.112	8.508±0.113	0.906
Platelet Distribution Width %	11.562±0.488	11.469±0.734	0.285
Plateletcrit	0.361±0.044	0.290±0.025	0.340
MPV to PLT ratio	0.033±0.004	0.032±0.003	0.603

[1] Mann-Whitney U test

Table 2. Changes in CRP and inflammatory blood parameters when blood data were grouped according to CRP levels (mean±SEM).

Parameters	Grouping in CRP levels (mg.dL^{-1})				p[1]
	0 – ≤1 (N:23)	1< – <5 (N:44)	5 – <10 (N:30)	≥10 (N:38)	
CRP mg.dL^{-1}	0.274±0.079^{d}	3.298± 0.149^{c}	6.747±0.232^{b}	21.198±1.784^{a}	0.000
White Blood Cells×10^{3}.µL^{-1}	13.743±2.120^{b}	12.827±0.966^{b}	14.483±1.631^{b}	23.958±2.238^{a}	0.000
Platelet×10^{3}.µL^{-1}	346.528±27.184	345.909±21.873	356.600±42.096	423.632±37.849	0.459
Main Platelet Volume fL	8.539±0.158	8.541±0.177	8.610±0.156	8.508±0.135	0.907
Platelet Distribution Width %	10.165±0.854	11.259±0.716	12.085±1.041	12.218±0.702	0.144
Plateletcrit	0.368±0.077	0.361±0.072	0.294±0.033	0.312±0.028	0.845
MPV to PLT ratio	0.029±0.003	0.032±0.004	0.038±0.006	0.030±0.007	0.456

[1] Kruskal Wallis,

[a,b,c,d] Superscripts in the same row define the difference at the 0.05 level between the columns

Table 3. AUC results for CRP and WBC in blood analyses of dogs.

	AUC (95%)	Cut-off	p	Sensitivity (%)	Specificity (%)
CRP levels[a]	0.736 (0.644-0.827)	5.45 mg.dL^{-1} 7.15 mg.dL^{-1}*	0.000	67.3 59.6*	65.1 80.7*
CRP levels ≤1 mg.dL^{-1}[b]	0.511 (0.211-0.811)		0.941		
CRP levels 1-5 mg.dL^{-1}[b]	0.547 (0.353-0.741)		0.645		
CRP levels 5-10 mg.dL^{-1}[b]	0.479 (0.228-0.729)		0.856		
CRP levels ≥10 mg.dL^{-1}[b]	0.805 (0.656-0.953)	16.75 mg.dL^{-1} 18.25 mg.dL^{-1}*	0.004	77.8 70.4*	81.8 90.9*
WBC counts[c]	0.665 (0.536-0.793)	11.55×10^{3}.µL^{-1} 10.65×10^{3}.µL^{-1}*	0.013	66.1 77.7*	65.2 65.2*
WBC counts[d]	0.731 (0.555-0.906)	15.90×10^{3}.µL^{-1} 14.40×10^{3}.µL^{-1}*	0.010	79.2 87.5*	64.3 57.1*

*The optimal cut-off point maximizing the Youden's J statistic

[a] AUC results for CRP in dogs with leukocytosis.

[b] AUC results for CRP in dogs with leukocytosis and divided to CRP levels (mg.dL^{-1}) in blood serum.

[c] AUC results for WBC in dogs having inflammation according to CRP levels greater than 1 mg.dL^{-1} in blood serum.

[d] AUC results for WBC in dogs having inflammation according to CRP levels ≥10 mg.dL^{-1} in blood serum.

Table 4. Correlations between CRP, WBC, and PIs parameters in blood records of dogs.

	CRP mg.dL^{-1}	White Blood Cells	Platelet	Main Platelet Volume	Platelet Distribution Width	Plateletcrit	MPV to PLT ratio
CRP mg.dL^{-1}	1	-0.305	0.029	0.254	0.320	-0.031	0.002
White Blood Cells		1	-0.448*	-0.047	0.138	-0.278	0.437*
Platelet			1	-0.027	-0.335	0.807**	-0.972**
Main Platelet Volume				1	0.154	0.187	0.190
Platelet Distribution Width					1	-0.292	0.391
Plateletcrit						1	-0.795**
MPV to PLT ratio							1

** Correlation is significant at the 0.01 level

* Correlation is significant at the 0.05 level

Table 5. Correlations between inflammatory parameters of the blood-works of dogs without leukocytosis.

	CRP mg.dL^{-1}	White Blood Cells	Platelet	Main Platelet Volume	Platelet Distribution Width	Plateletcrit	MPV to PLT ratio
CRP mg.dL^{-1}	1	0.450**	0.065	0.021	0.196*	0.026	-0.072
White Blood Cells		1	0.002	0.019	0.035	-0.047	0.005
Platelet			1	-0.190*	-0.140	0.903**	-0.981**
Main Platelet Volume				1	0.033	-0.023	0.343**
Platelet Distribution Width					1	-0.082	0.120
Plateletcrit						1	-0.853**
MPV to PLT ratio							1

** Correlation is significant at the 0.01 level
* Correlation is significant at the 0.05 level

Table 6. Correlations between inflammatory parameters of the blood-work records of dogs with leukocytosis.

	CRP mg.dL^{-1}	White Blood Cells	Platelet	Main Platelet Volume	Platelet Distribution Width	Plateletcrit	MPV to PLT ratio
CRP mg.dL^{-1}	1	0.477**	0.074	0.051	0.129	0.046	-0.070
White Blood Cells		1	0.093	0.053	-0.076	0.015	-0.077
Platelet			1	-0.225*	-0.113	0.915**	-0.982**
Main Platelet Volume				1	0.035	-0.072	0.372**
Platelet Distribution Width					1	-0.046	0.090
Plateletcrit						1	-0.869**
MPV to PLT ratio							1

** Correlation is significant at the 0.01 level
* Correlation is significant at the 0.05 level

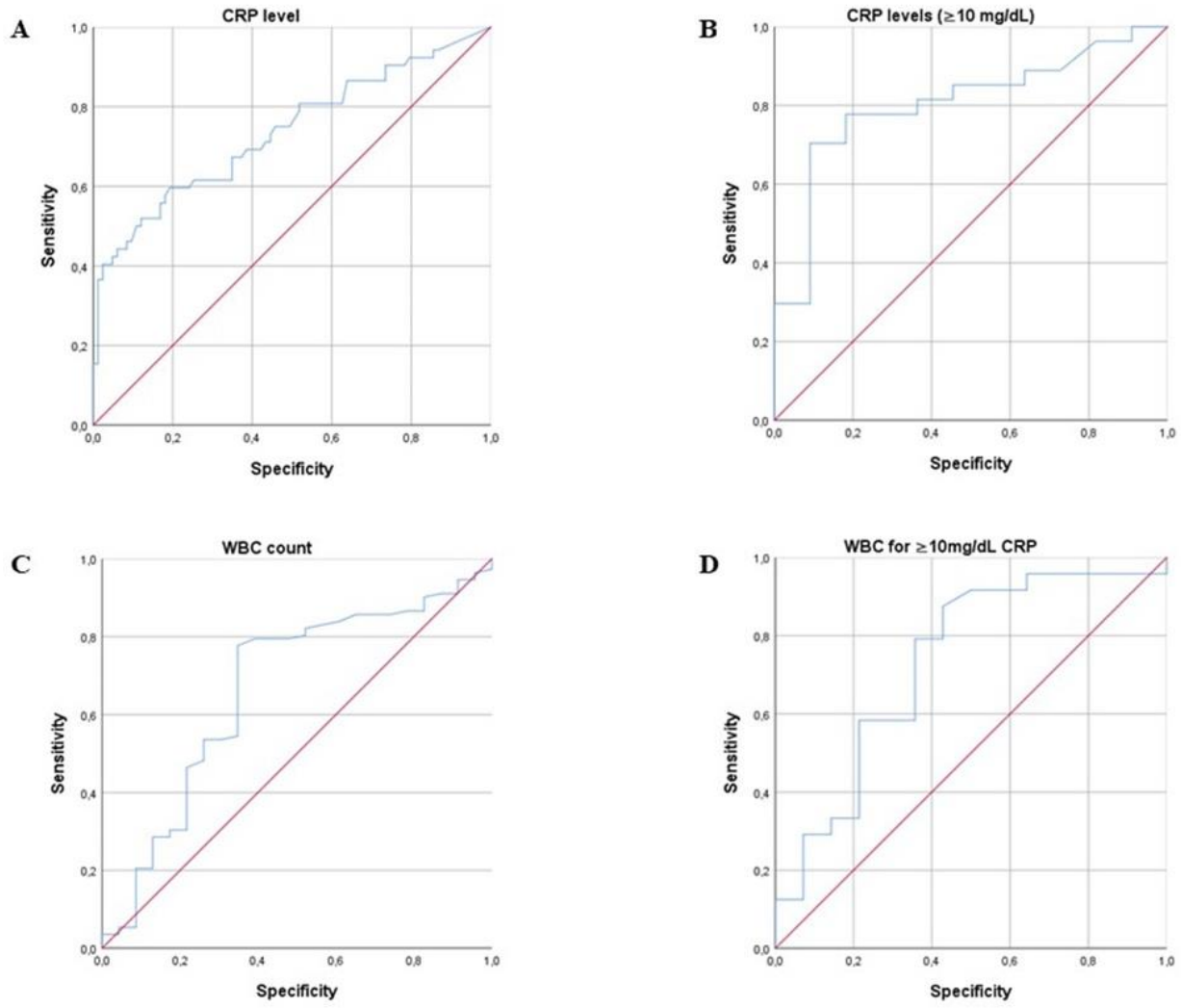

Figure 1: A: ROC curve of CRP in dogs with infection according to blood leucocyte count; **B**: ROC curve of CRP in dogs with infection according to blood leucocyte count and having CRP levels greater than 10 mg.dL^{-1} in blood serum; **C**: ROC curve of WBC in dogs having inflammation according to CRP levels greater than 1 mg.dL^{-1} in blood serum; **D**: ROC curve of WBC in dogs having inflammation according to CRP levels equal or greater than 10 mg.dL^{-1} in blood serum.

DISCUSSION

Diagnostic workups for veterinary patients include decision-making (Friedrichs et al. 2022). A detailed history and physical examination are used to make preliminary differential diagnosis (Friedrichs et al. 2022). Decisions to rule in or rule out differential diagnoses are then made based on the interpretation of laboratory tests such as hematology and other clinical pathological data (Friedrichs et al. 2022). For many regular hematological and biochemical tests, time-series data analysis provides a more accurate indication of the organ or cell's pathological status than data collected at a single time point (Honda et al. 2016). Furthermore, several standard tests must be utilized in conjunction to assess the patient's clinical condition. As a result, doing a single test at a particular time point is inadequate to provide a diagnosis (Honda et al. 2016).

A complete blood count is a laboratory analysis test that is highly demanded in the veterinary clinical routine due to its low budget and ease of application, as well as providing information about the instantaneous physiological or pathological state of the patient to which it is applied. The CBC analyzes blood components in the form of an erythrogram, a leukogram, and a platelet count, as well as the quantitative and qualitative outcomes of these parameters. (Rejec et al. 2017; Oliveira et al. 2020). Leukocytosis is defined as an elevated total leukocyte count in the circulatory blood flow (Weltan et al. 2008). Leukocytosis was reported in various clinical conditions such as diskospondylitis (Trub et al. 2021), canine demodicosis (Jaheen et al. 2022), sepsis (Ok et al. 2015), lower respiratory disease (Köse et al. 2021; Köse et al. 2023b). In this study, an increase in WBC level was identified in the blood analysis results of dogs, as in previous studies (Ok et al. 2015; Köse et al. 2021; Trub et al. 2021; Jaheen et al. 2022; Köse et al. 2023b), and was connected with inflammatory status (Table 2). In addition, according to the upper value of the WBC reference interval (5.0-14.5×103.µL-1), an increase of approximately 60% in the WBC count was observed in the blood analyses results of dogs with CRP levels greater than 10 mg.dL-1 (Table 2). On the other hands, it was found that WBC counts were within the reference range in the blood analyses results with CRP levels in the reference range and below 10 mg.dL-1 (Table 2). It is reported that serum CRP concentrations rise significantly 4 hours after the beginning of inflammation, while WBC counts do not rise at the same time (Cerón et al. 2005; Galezowski et al. 2010). Cut-off value of WBC in dogs with CRP levels greater than 10 mg.dL-1 was found as 14.40×103.µL-1 (Table 3). According to the results of the study, in the light of the literature, when a marked increase in WBC value (14.40×103.µL-1<) is determined in the blood analyses results, it is thought that the inflammatory process is older than four hours and the blood serum CRP value may be 10 mg.dL-1 or higher.

In many inflammatory and infectious situations, the liver produces acute phase proteins in response to the release of proinflammatory cytokines (De Laforcade et al. 2008; Ok et al. 2015). C-reactive protein is a valuable measure for identifying inflammation in humans and animals (Eckersall and Bell 2010; Christensen et al. 2014; Ok et al. 2015). The canine CRP reference values (<10–20 mg.L-1) (Klenner et al. 2010; Hillström et al. 2014; Hindenberg et al. 2018; Hindenberg et al. 2020) assayed by several methods, including ours (<1 mg.dL-1), seem to be approximately the same. In dogs with an inflammatory leukogram, it was firstly reported that CRP levels were increased (Burton et al. 1994). It is informed that serum CRP levels increased in the dogs with acute inflammatory disease (Tecles et al. 2005). The increase in CRP levels was reported in various clinical conditions such as demodicosis (Jaheen et al. 2022), parvoviral enteritis (Kubesy et al. 2019; Başbuğ et al. 2020), sepsis (Ok et al. 2015), systemic inflammatory response syndrome (SIRS)(Gebhardt et al. 2009). Similar to previous studies (Ok et al. 2015; Başbuğ et al. 2020; Jaheen et al. 2022), in this study, an increased CRP level was observed in the blood analyses results of dogs with leukocytosis. C-reactive protein cut-off value in dogs with parvoviral enteritis was reported as 120.5 mg.L-1 (Başbuğ et al. 2020), as well as 7.15 mg.dL-1 in the blood analyses with leukocytosis in this study (Table 3). In another study, it is reported that CRP levels were increased in dogs with SIRS (Gebhardt et al. 2009). On the other hand, in this study, the cut-off value for CRP was also detected as 16.75 mg.dL-1 in the blood analyses results with leukocytosis and including CRP levels ≥10 mg.dL-1 (Table 3). In a study conducted in dogs with acute abdominal syndrome, it was reported that there was no correlation between CRP level and WBC count at the time of admission to the clinic, but there was a positive correlation in these blood values 48–72 hours later, and both values showed a decrease (Galezowski et al. 2010). C-reactive protein, in this study, was positively correlated with WBC ($r=0.477$, $p<0.01$) (Table 6). The correlation coefficient was observed to be higher in this study than the firstly defined ($r=0.34$) by (Burton et al. 1994). The correlation coefficient ($r=0.44$) determined in another study (Nakamura et al. 2008) was quite similar to that in this study. It is thought that this increase in CRP level may be related to the inflammatory response in response to the condition causing leukocytosis. The correlation between CRP and WBC determined in this study also seems to support this idea.

In addition to their involvement in homeostasis, platelets play a crucial role in the inflammatory process by actively regulating host defenses and partnering in the occurrence of inflammation and tissue healing (Rejec et al. 2017). Platelet indices including mean platelet volume (MPV), platelet size distribution width (PDW), and plateletcrit (PCT) may now be measured

using automated blood cell analyzers (Moritz et al. 2005; Yilmaz et al. 2008). According to reports, the MPV/PLT ratio can be employed as a marker of the systemic inflammatory response, especially related to malignancy (Rejec et al. 2017; Köse et al. 2023a). It is informed that no differences could be determined in terms of platelet count and MPV/PLT ratio in dogs with periodontitis and oral malignancies (Rejec et al. 2017). Consistent with a previous study (Ok et al. 2015), there was no alteration in platelet count in this study. In the blood analyses of dogs with leukocytosis, platelet count was negatively correlated with main platelet volume ($r=-0.225$, $p<0.05$) and MPV/PLT ($r=0.982$, $p<0.01$), but it was positively correlated with plateletcrit ($r=0.915$, $p<0.01$) (Table 6).

CONCLUSION

It is obvious that the analysis of C-reactive protein in veterinary practice is a valuable supportive laboratory measure for identifying inflammation in dogs, as well as WBCs count. Considering the study results, it is concluded that in veterinary clinical practice, C-reactive protein measurements in dogs may be more useful in cases where a marked increase in WBC above the cut-off value is detected in the complete blood count routinely used to assess inflammatory or infectious status. The lack of etiology causing leukocytosis in the blood analyses of dogs may be the main limitation of this study. Therefore, it may be appropriate to conduct more comprehensive studies in this area, including etiology.

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Ethical approval: According to the legislation titled "Working Procedures and Ethics Committees of Animal Experiments" numbered 28914 published on February 15, 2014, in Turkey, the 8th article clearly suggested that clinical applications for diagnostic and therapeutic purposes are not subject to ethical committee approval. The data, information and documents presented in this article were obtained within the framework of academic and ethical rules.

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RESEARCH ARTICLE

Seasonal Variation in TLR4 Expression in The Testis and Epididymis of Anatolian Ground Squirrels *(Spermophilus xanthoprymnus)*: Insights From Non-Breeding Period of Pre-Hibernation and Hibernation

Mehmet ÖZBEK[1]

[1]*Department of Histology and Embryology, Faculty of Veterinary Medicine, Burdur Mehmet Akif Ersoy University, Burdur, Türkiye*

ABSTRACT

This study investigates the modulation of Toll-like receptor 4 (TLR4) expression within the testis and epididymis of Anatolian ground squirrels *(Spermophilus xanthoprymnus)* during the non-breeding period of pre-hibernation and hibernation. Immunohistochemical investigation showed that TLR4 was not detected in germ cells, Leydig cells, or Sertoli cells in the testicular tissue during the pre-hibernation period. Nevertheless, there was a presence of TLR4 in the vessel's walls and certain interstitial cells within the intertubular regions. Epithelial cells in the caput, corpus, and cauda regions of the epididymis showed no TLR4 expression. However, it was observed in the vessel walls, smooth muscle layers, and some interstitial cells. TLR4 expression was seen in spermatogonia and primary spermatocytes during hibernation, with strong labeling observed in the vessel walls of the intertubular area. In contrast, TLR4 was detected in the epididymal epithelium, as well as in the smooth muscle layers and vessel walls throughout all segments. A notable upregulation in the expression of TLR4 in the testis was identified through quantitative image analysis during hibernation as compared to pre-hibernation. During pre-hibernation, the cauda segment of the epididymis exhibited the highest expression of TLR4, whereas during hibernation, the corpus segment demonstrated the highest expression. These findings suggest a dynamic modulation of TLR4 in response to hibernation, highlighting its potential role in reproductive function and immune adaptation.
Keywords: Epididymis, *Spermophilus xanthoprymnus,* Testis, *TL*R4

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ÖZ

Anadolu Yer Sincaplarının (*Spermophilus xanthoprymnus)* Testis ve Epididimisinde TLR4 Ekspresyonundaki Dönemsel Değişiklikler: Üreme Dışı pre-Hibernasyon ve Hibernasyon Dönemlerinden Elde Edilen Bulgular

Bu çalışma, üreme dışı dönemde pre-hibernasyon ve hibernasyon süresince Anadolu yer sincaplarının *(Spermophilus xanthoprymnus)* testis ve epididimis dokularında Toll-like reseptör 4 (TLR4) ekspresyonunun regülasyonunu araştırmaktadır. İmmünohistokimyasal inceleme, TLR4'ün pre-hibernasyon döneminde testis dokusundaki germ hücreleri, Leydig hücreleri ve Sertoli hücrelerinde tespit edilmediğini göstermiştir. Bununla birlikte, intertubuler alandaki damar duvarlarında ve bazı interstisyel hücrelerde TLR4 mevcuttur. Epididimisin kaput, korpus ve kauda bölgelerindeki epitel hücrelerinde TLR4 ekspresyonu gözlenmemiştir. Ancak, damar duvarlarında, düz kas ve bazı interstisyel hücrelerde TLR4 mevcuttur. Hibernasyon sırasında, spermatogonyum ve primer spermatositlerde TLR4 ekspresyonu gözlenmiş olup, tübüller arası alanın damar duvarlarında güçlü bir immun reaksiyon görülmüştür. Buna karşılık, epididimisin bütün bölümlerindeki epitelinde, düz kas hücrelerinde ve damar duvarlarında TLR4 tespit edilmiştir. Hibernasyon döneminde, pre-hibernasyona kıyasla testiste TLR4 ekspresyonunda anlamlı bir artış, kantitatif görüntü analizi ile tespit edilmiştir. Pre-hibernasyon döneminde epididimisin kauda segmenti en yüksek TLR4 ekspresyonunu gösterirken, hibernasyon sırasında korpus segmenti en yüksek ekspresyonu göstermiştir. Bu bulgular, hibernasyona yanıt olarak TLR4'ün dinamik bir şekilde düzenlendiğini göstermekte olup, bu reseptörün üreme fonksiyonu ve bağışıklık adaptasyonundaki potansiyel rolüne işaret etmektedir.
Anahtar Kelimeler: Epididimis, *Spermophilus xanthoprymnus,* Testis, *TL*R4

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ORCID ID; *MÖ: 0000-0002-2274-5359* *Corresponding author e-mail: mehmetozbek2@gmail.com

INTRODUCTION

The innate immune system forms the body's first barrier against infections, utilizing pattern recognition receptors to detect characteristic molecular patterns known as pathogen-associated molecular patterns (Akira et al., 2006; Medzhitov, 2001). Toll-like receptors (TLRs) play a crucial role in identifying pathogen-associated molecular patterns and triggering immune responses (Takeuchi & Akira, 2010). TLRs are membrane-bound proteins that identify microbial components and initiate signaling pathways, resulting in the synthesis of cytokines and other critical mediators for the inflammatory response and subsequent activation of the adaptive immune system (Beutler, 2009; Kawai & Akira, 2010).

TLR4 is a well-researched receptor that plays a crucial role in detecting lipopolysaccharides (LPS). This receptor is essential for initiating the body's response to bacterial infections (Iwasaki & Medzhitov, 2004). TLR4 is extensively distributed across a multitude of tissues and cell types, from pivotal immune cells such as dendritic cells and macrophages to a diverse array of non-immune cells (Janeway & Medzhitov, 2002). The study of TLRs in several tissues, including reproductive organs, has attracted considerable attention due to their involvement in immunological privilege, inflammation, and tissue homeostasis (Kawasaki & Kawai, 2014; O'Neill & Bowie, 2007).

The testis and epididymis serve as fundamental organs in the male reproductive system, overseeing the critical processes of spermatogenesis, sperm maturation, and storage. These organs maintain a state of immunoprivilege, which protects germ cells from being attacked by the immune system. However, these organs also need strong mechanisms to defend against infections (N. Li et al., 2012; Meinhardt & Hedger, 2011). The expression and modulation of TLRs, namely TLR4, in the testis and epididymis play a vital role in maintaining a balance between immune protection and reproductive activities (Hargreaves & Medzhitov, 2005). The testis is considered immunoprivileged due to the presence of the blood-testis barrier and local immunosuppressive settings. These mechanisms are crucial for protecting developing germ cells from potential immune attacks (Hedger, 2011b).

Anatolian ground squirrels (*Spermophilus xanthoprymnus)* are a useful model for investigating hibernation and its impact on several physiological systems, such as the immunological and reproductive systems (Gür & Kart Gür, 2005; Kart Gür et al., 2009). Hibernation elicits significant alterations in metabolic, endocrine, and immune processes. Studying the presence of TLR4 in the testis and epididymis throughout the non-breeding periods of pre-hibernation and hibernation can offer valuable knowledge on the adaptive mechanisms that safeguard reproductive organs during these periods. Hibernation leads to a large decrease in metabolic activity, which can affect the immune system's ability to respond and require specific adjustments to preserve reproductive function and immune defense (Bouma et al., 2010; Giroud et al., 2020; Q. Li et al., 2015; Saeidi et al., 2014).

In this study, we aim to investigate the expression of TLR4 in the testis and epididymis of Anatolian ground squirrels during non-breeding period of pre-hibernation and hibernation. This research will enhance our understanding of the immune-regulatory mechanisms in the reproductive system during hibernation and contribute to the broader knowledge of TLR function in seasonal breeders.

MATERIALS and METHODS

Experiment conditions and an animal ethics statement

Regarding the treatment and utilization of animals, the experimental protocols were carried out in adherence to the standards established by the Ethical Committee of Erciyes University. The Erciyes University Local Ethics Committee for Animal Experiments (HADYEK), situated in Kayseri, granted approval for these protocols under the authorized number 15/140. Twelve male Anatolian ground squirrels *(Spermophilus xanthoprymnus)* weighing between 300 and 380 grams were uniformly distributed as a sample for the research. The exact chronological age of the animals is uncertain, given that they were procured from the steppes near Develi, Kayseri, Turkey in late August via Tomahawk Traps. Preceding their use in the experiments, this was performed to ensure that the rodents were in perfect health. The experimental groups were subsequently divided into two categories: pre-hibernation (comprising six male subjects) and hibernation (late torpor), which also comprised six male subjects. Following intraperitoneal administration of ketamine and xylazine, the pre-hibernation group was euthanized via cardiac puncture, and the hearts were immediately removed (Olson & McCabe, 1986). A series of alcohol solutions were used to dehydrate the testis and epididymis samples after they had been immersed in Bouin's solution for 12 hours. The samples were clarified with benzol and methyl benzoate subsequent to the water removal procedure. Subsequently, they were immersed in paraffin.

Hibernation group

Until their body lipid levels were at an optimal level for hibernation, the squirrels in the hibernation group were supplied with a standard rodent diet, sunflower seeds, fresh produce, and unrestricted access to water. Initially, a temperature of 21.1 ± 1 °C was maintained

in the laboratory. In order to replicate the natural photoperiod from September 2016 to December 2016, an artificial light-dark cycle was established, with values extending from 200 to 0 lx. In order to promote a smooth transition into torpor, the laboratory environment was subsequently adjusted to 6 °C, all food sources were eliminated from the enclosures, and the lights were entirely deactivated. A red safe light, ranging in intensity from 3 to 5 lumens, was employed upon entering the laboratory so as not to arouse dormant rodents. Three months after initially entering torpid and hypothermic conditions, the squirrels were rendered unconscious by intraperitoneal administration of ketamine and xylazine (11.1 mg of xylazine/ml and 88.9 mg of ketamine/ml, respectively) (Olson & McCabe, 1986). Tissue processing procedures were maintained for the experimental hibernation group subsequent to sampling.

Immunohistochemistry

A rotary microtome was employed to section paraffin-embedded tissue to a thickness of 5 µm. The sections were subsequently transferred to slides that had been coated with poly-L-lysine. For the purpose of immunohistochemistry staining, the streptavidin-biotin-peroxidase method was implemented, as described in a 2019 study by Özbek et al (Özbek et al., 2019). The sections were rehydrated through a succession of graduated alcohols following deparaffinization with xylene. Antigen retrieval was accomplished by subjecting the sample to a 20-minute microwave boil in citrate buffer with a pH of 6.0, subsequent to the washing phase in phosphate-buffered saline (PBS). For the purpose of inhibiting the activity of naturally occurring peroxidase, the sections were submerged in a solution containing 3% H_2O_2 in distilled water for-20 minutes. By incubating with Ultra V Block for a period of ten minutes, non-specific binding was averted. Then, they were treated overnight at 4 °C with an unconjugated monoclonal TLR4 primary antibody (Novus Biologicals, cat no: NB100-56566) subsequent to the removal of any surplus serum from the slides. After thirty minutes of exposure to a biotinylated secondary antibody at room temperature, the sections were subjected to the second wash in PBS. Following which, they underwent an additional 15-minute rinse with PBS. Using 3-amino-9-ethylcarbazole (AEC), the resulting signal was produced. Prior to being mounted in an aqueous medium, the slides were stained with Gill's hematoxylin. The primary antibody was substituted with PBS as the negative control; all other procedures were executed in accordance with the provided instructions. Colon tissue was employed as the positive control.

Qualitative Assessment of Immunohistochemical Staining Patterns

The results of the qualitative examination are meticulously documented in Table 1. Examination and imaging of the stained sections were performed with a BX51 microscope (Olympus, Tokyo, Japan). Utilizing a phenomenological intensity scoring system with four tiers, the immunostaining assessments were carried out. A 100x and 400x magnification, respectively, was applied to assess the intensity of TLR4 staining in the testis and epididymis. Öztop et al. (2019) outlined the scoring system employed to determine the intensity of TLR4 staining: "-" denoted the absence of staining, "+" indicated weak staining, "++" moderate staining, and "+++" intense staining (Öztop et al., 2019).

Image analysis

In accordance with the methodology described by Jensen (2017), the staining intensities of TLR4 in images of the epididymis and testes were quantified (Jensen et al., 2017). For the entire image, the intensity of immunostaining was measured. During the pre-hibernating and hibernating phases, images of the and epididymis were testes captured at a 400X magnification. Analyses of each tissue during each period comprised a total of twelve photographs. ImageJ 1.54 was utilized to import the pertinent photographs. The images were then processed using the 'color deconvolution' plug-in, which separated the hematoxylin and AEC stains into three distinct panels: one showcasing the hematoxylin, another the AEC, and the third serving as the background. Specific threshold values were set exclusively for the AEC images. For each image, the statistical analysis concentrated on the area and area fraction metrics, providing a detailed percentage-based assessment of the stained region's extent and staining intensity

Statistical Analysis

Utilizing GraphPad Prizm 8 for Windows, more precisely Version 8.0.2, the statistical analysis was conducted. The means, with or without the standard error of the means (SEM), were utilized to represent the data. In this study, a two-tailed Student's t test was employed to compare two distinct groups, namely the testis and epididymis. Before applying Bonferroni's multiple comparison test, a one-way factorial analysis of variance (ANOVA) was performed to compare the groups. We considered the data significant at a p-value of less than 0.05.

Table 1. Evaluation of TLR4 expression in the testicular and epididymal tissues of Anatolian ground squirrels *(Spermophilus xanthoprymnus)* during non-breeding period of pre-hibernation and hibernation

Tissue		Pre-hibernating	Hibernating
Testis	**Cell type**		
	Spermatogonium	-	+
	Spermatocyte/spermatid	-	+
	Sperm	No differentiation	No differentiation
	Leydig cells	-	
	Sertoli cells	-	-
	Peritubular myoid cells	-	-
	Vessel wall	+++	+++
Epididymis			
Caput epididymis	Principal cells	-	+
	Narrow cells	-	+
	Basal cells	-	+
	Apical cells	-	+
	Vessel wall	++	++
	Interstitial cell in connective tissue	+	+
	Muscle layer in the ductal wall	++	++
Corpus epididymis	Principal cells	-	+
	Narrow cells	-	+
	Basal cells	-	+
	Apical cells	-	+
	Vessel wall	++	++
	Interstitial cell in connective tissue	+	+
	Muscle layer in the ductal wall	+	++
Cauda epididymis	Principal cells	-	+
	Narrow cells	-	+
	Basal cells	-	+
	Apical cells	-	+
	Vessel wall	++	++
	Interstitial cell in connective tissue	+	+
	Muscle layer in the ductal wall	++	+++

RESULTS

Positive and negative control

Colon tissue was chosen as a positive control, and the reaction was detected in the smooth muscle cells in the vessels and mucosa. No reaction was observed from the testicular tissue used as negative control (Figure 1).

Non-breeding period of pre-hibernation

We did not observe TLR4 immunostaining in germ cells within the testicular tissue. Moreover, Leydig and Sertoli cells did not show TLR4 expression. However, we observed a positive immune reaction in vessel wall, and some interstitial cells in the intertubular areas. The epididymis was analyzed in three distinct sections: caput, corpus, and cauda. We did not observe TLR4 expression in the epithelium of all three epididymis segments. Nevertheless, we noticed a TLR4 immunoreaction in vessel wall, the smooth muscle layer in the ductal wall and some interstitial cells in all three segments of the epididymis (Figure 2).

Hibernation

Interestingly, TLR4 expression was observed in spermatogonia and primary spermatocytes. We did not detect TLR4 immunostaining in Sertoli cells, peritubular myoid cells, and Leydig cells in the intertubular area, but intense TLR4 staining was observed in the vessel walls in the intertubular area. In contrast to the pre-hibernation period, TLR4 immune labeling was observed in the epithelial cells of the epididymis. In addition, TLR4 labeling was detected in the smooth muscle layer in the ductal wall, some interstitial cells, and vessel walls in all three segments of the epididymis, similar to the pre-hibernation stage (Figure 3).

Quantitative image analysis of TLR4 immunostaining

Compared to the non-breeding period of pre-hibernation, TLR4 expression in the testis was significantly elevated during hibernation. TLR4 expression variations were identified in epididymis sections during the pre-hibernation. The cauda epididymis exhibited the highest expression of TLR4, whereas the corpus epididymis displayed the lowest TLR4 expression during the pre-hibernation. Curiously, the corpus epididymis had the most pronounced expression of TLR4 during the hibernation period. There was no significant variation observed in TLR4 expression between the caput and cauda epididymis (Figure 4).

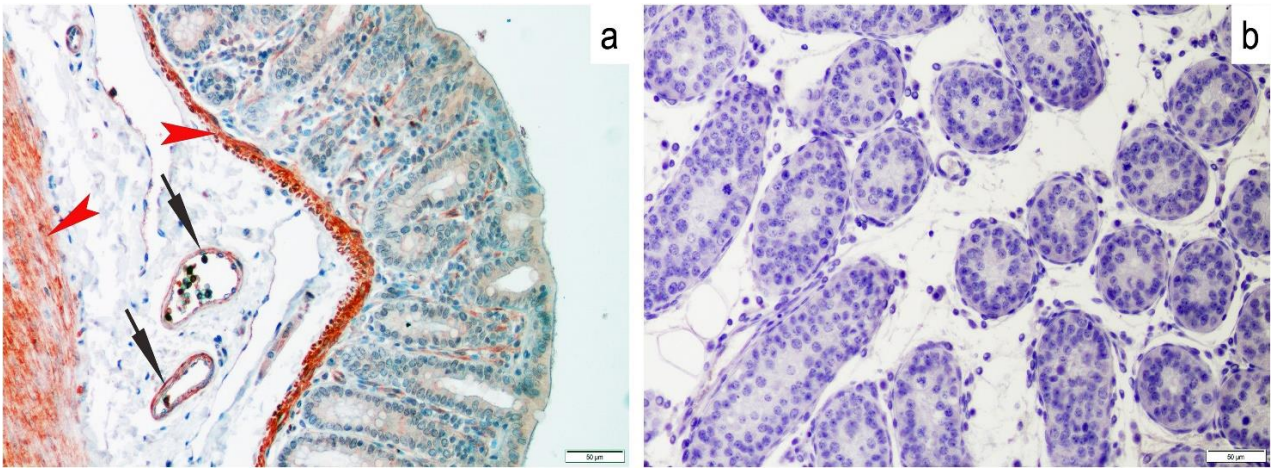

Figure 1: Colon tissue as a positive control for TLR4 (a) and testis as a negative control (b). Red arrowheads: smooth muscle cells. Black arrows: vessel walls.

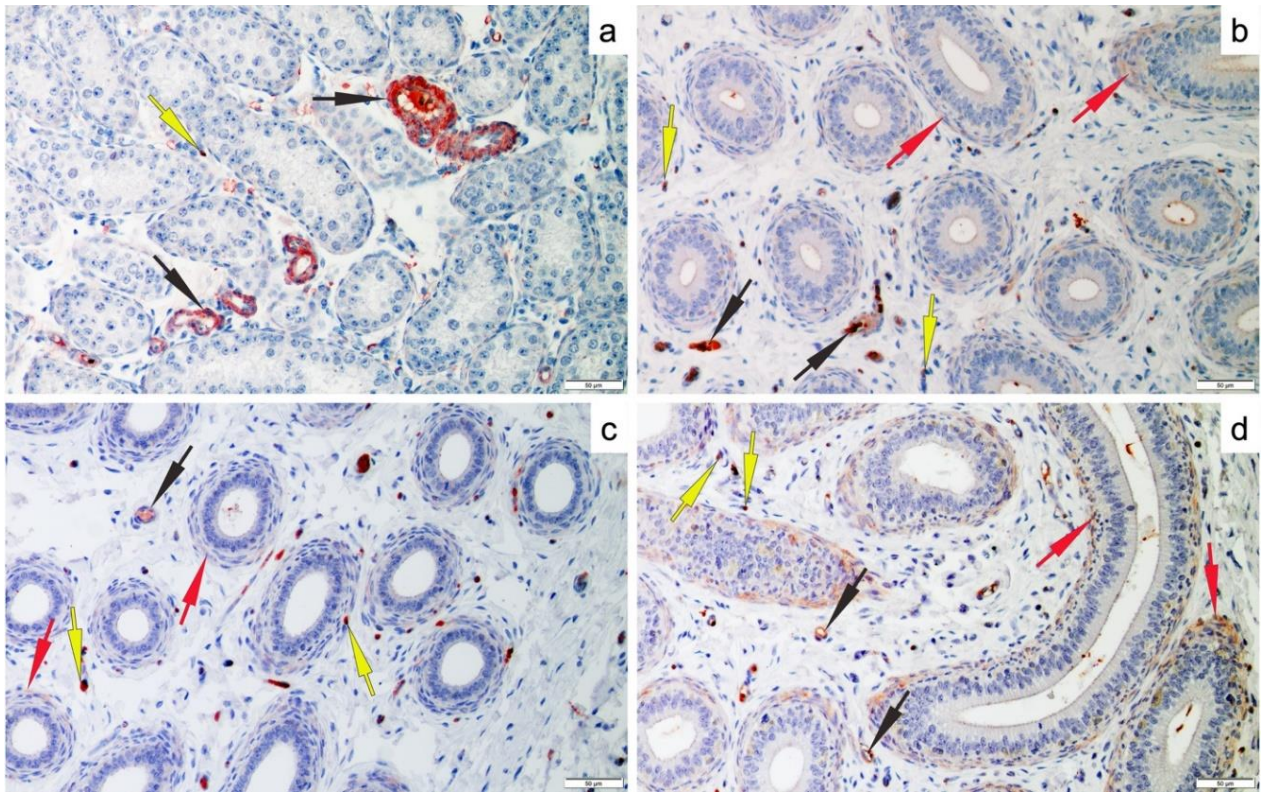

Figure 2: Immunohistochemical localization of TLR4 in the testis (a), and in the distinct segments of the epididymis: caput (b), corpus (c), and cauda (d) during the non-breeding period of pre-hibernation. Black arrows: Vessel walls. Yellow arrows: Interstitial cells. Red arrows: Muscle layer in the ductal wall of epididymis

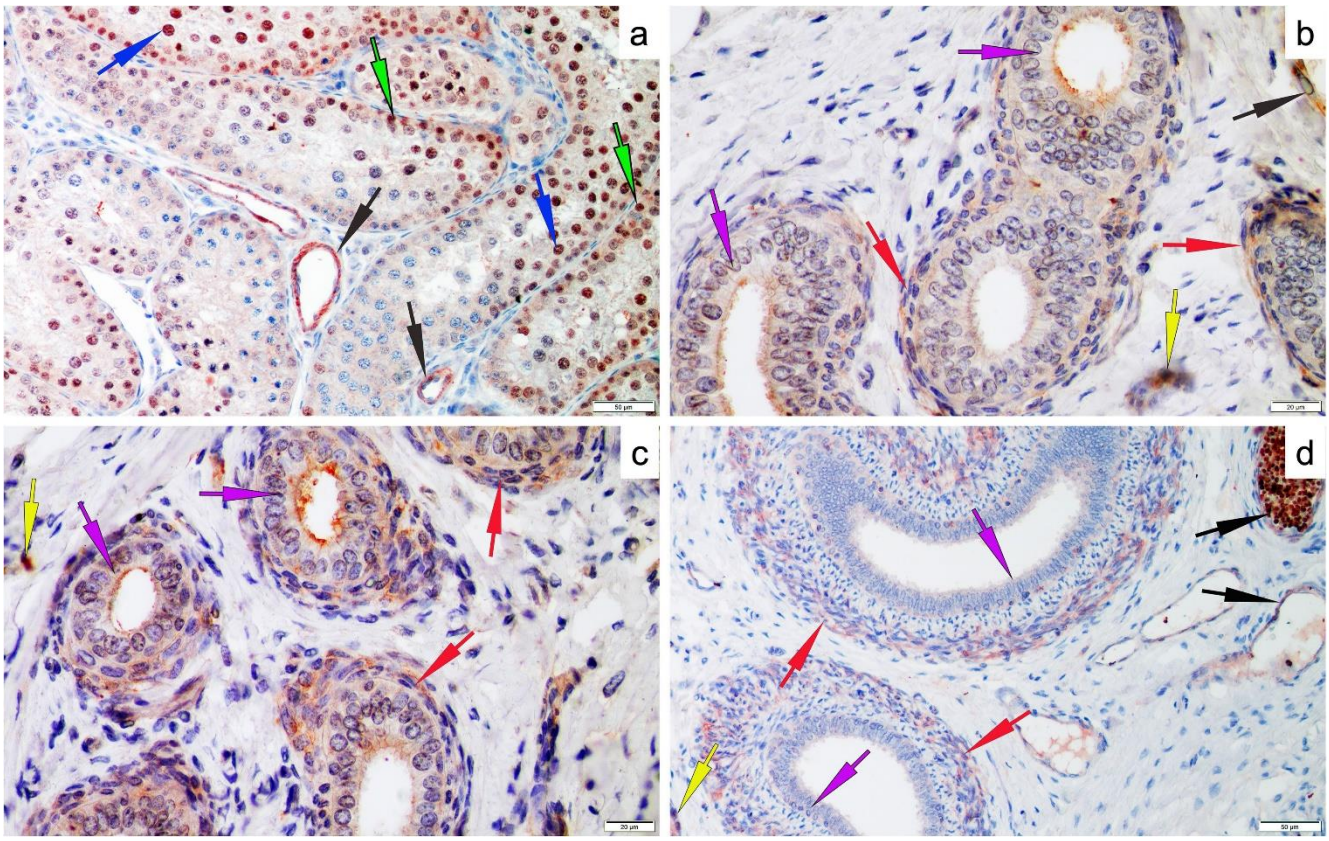

Figure 3: Immunohistochemical localization of TLR4 in the testis (a), and in the distinct segments of the epididymis: caput (b), corpus (c), and cauda (d) during the hibernation. Black arrows: Vessel walls. Yellow arrows: Interstitial cells. Red arrows: Muscle layer in the ductal wall of epididymis. Green arrows: Spermatogonium. Blue arrows: Primary spermatocytes. Purple arrows: Epididymal epithelium

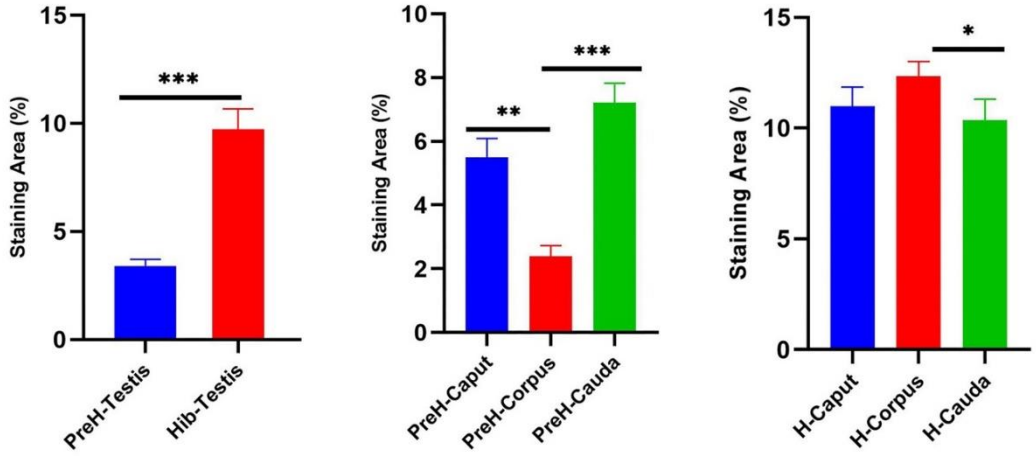

Figure 4: Quantitative evaluation of TLR4 immunostaining during the pre-hibernation (PreH) and hibernation (H) periods. Statistical significance is denoted as follows: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

DISCUSSION

The present study offers a comprehensive analysis of the dynamic expression patterns of TLR4 in the epididymis and testis of Anatolian ground squirrels *(Spermophilus xanthoprymnus)* throughout the pre-hibernation and hibernation periods, which are devoid of reproductive activity. Significant adaptive changes in immune regulation within the reproductive organs are revealed by the findings; these changes are crucial for comprehending how these animals maintain reproductive function and general health throughout these distinct physiological states.

The absence of TLR4 immunostaining in germ cells, Leydig cells, and Sertoli cells within the testicular tissue was conspicuous during the pre-hibernation period. This is consistent with the well-established notion that the testis functions as an immunoprivileged site, a

location where protective immune responses against pathogens are strictly regulated (Meinhardt & Hedger, 2011; Zhao et al., 2014). The blood-testis barrier and local immunosuppressive environments play a critical role in preserving the testis' immunoprivileged status. These mechanisms safeguard developing germ cells against potential immune assaults (Fijak & Meinhardt, 2006; Hedger, 2015).

Nevertheless, the identification of TLR4 in specific interstitial cells and vessel walls within the testicular intertubular regions indicates that these cells are involved in overseeing the local immune response and regulating inflammation. This aligns with prior research that emphasizes the contribution of interstitial and vascular cells to the regulation of the immune system in the testis Gu et al., 2022; Hedger, 2011a; Heinrich & DeFalco, 2020).

During pre-hibernation, the absence of TLR4 expression in the caput, corpus, and cauda segments of epithelial cells in the epididymis suggests the presence of a protective mechanism that aims to shield maturing sperm from potential immune attacks. On the contrary, TLR4 immunoreactivity was detected in certain interstitial cells, the smooth muscle layer of the ductal wall, and vessel walls, indicating that these anatomical components might participate in local immune reactions that regulate the immune environment in a way that promotes sperm maturation and storage (Hermo & Robaire, 2002; Hu et al., 2016). In contrast, TLR4 expression patterns underwent substantial alterations during the hibernation period. Observations of TLR4 expression in spermatogonia and primary spermatocytes indicate that germ cells enter hibernation with an enhanced immune readiness. Potential infections or stress may be evaded through this mechanism while in the metabolically dormant state of hibernation (Bouma et al., 2010; Carey et al., 2003; van Breukelen & Martin, 2015). The significance of vascular structures in immune regulation during hibernation is underscored by the lack of TLR4 in Sertoli cells, peritubular myoid cells, and Leydig cells, in conjunction with the prominent labeling of TLR4 on the vessel walls.

TLR4 immune labeling was notably observed in the epididymal epithelial cells throughout the hibernation phase, as opposed to the pre-hibernation phase. This implies that in order to safeguard spermatozoa during the susceptible hibernation phase, an adaptive immune mechanism is engaged. This concept is further substantiated by the consistent labeling of TLR4 in the smooth muscle layers, interstitial cells, and vessel walls throughout all segments of the epididymis while it is in hibernation. This suggests that the immune system places greater emphasis on protecting the reproductive tract from potential infections (Tung et al., 2022; Wang et al., 2019).

CONCLUSION

The intricate relationship between reproductive physiology and the immune system is highlighted by the dynamic expression of TLR4 in the testis and epididymis of Anatolian ground squirrels throughout the various seasonal phases. The alterations in TLR4 expression indicate that adaptive modulation of immune regulatory mechanisms safeguards reproductive health and guarantees effective reproduction, in spite of the physiological difficulties presented by hibernation. Future research should focus on clarifying the molecular processes that govern the modulation of TLR4 and its functional consequences in the testis and epididymis during hibernation. In addition, studying the interplay between TLR4 and other immune pathways could offer a more profound understanding of the immunological tactics utilized by hibernating animals to sustain reproductive function and overall well-being.

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Authors' Contributions: Idea, concept and design: MÖ Data collection and analysis: MÖ, Drafting of the manuscript: MÖ, Critical review: MÖ

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RESEARCH ARTICLE

Antibiotic Resistance Profile of Yogurt Bacteria Exposed to Various Stress Conditions

Gökhan AKARCA[1]*, Ayşe Janseli DENİZKARA[1]

[1]*Department of Food Engineering, Faculty of Engineering, Afyon Kocatepe University, Afyonkarahisar, Turkey*

ABSTRACT

This study aims to reveal the differences that may occur in the susceptibilities of 2 yogurt bacteria strains, *S. thermophilus* and *L. delbrueckii* subsp. *bulgaricus*, to antibiotics under 2 different durations and 3 different stress conditions. The study also introduces new approaches to reduce potential issues in the fermented milk industry containing antibiotics in milk. To this end, the bacteria were exposed to 2 different low-pressure, low-temperature, and magnetic field activities under 2 different durations. The research concluded that as the severity of the applied stress conditions and application period increase, the antibiotic susceptibility of the bacteria decreases, and resistance to certain antibiotics develops ($p<0,05$). In the conclusion of the 3 different stress applications, it was found that the bacteria had the highest resistance to antibiotics in the magnetic field applications. In these 3 different stress applications, *S. thermophilus* showed the highest resistance to lincomycin, cephalexin, and streptomycin; *L. delbrueckii* subsp. *bulgaricus* developed resistance to streptomycin, erythromycin, and chloramphenicol. Of the 2 yogurt bacteria, L. delbrueckii subsp. bulgaricus developed a resistance to more antibiotics than *S. thermophilus* after the stress applications; the developed resistance was also more substantial than that of *S. thermophilus*.

Keywords: Magnetic Field, Streptomycin, Stress, Yogurt Bacteria

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Çeşitli Stres Koşullarına Maruz Kalan Yoğurt Bakterilerinin Antibiyotik Direnç Profili

ÖZ

Bu araştırmada iki farklı süre ve üç farklı stres koşulları altında yoğurt bakterileri *S. thermophilus* ve L. delbrueckii subsp. *bulgaricus* suşlarının, antibiyotiklere karşı olan duyarlılıklarında meydana gelebilecek değişimlerin belirlenmesi ve fermente süt endüstrisinde antibiyotikli süt kullanımına bağlı ortaya çıkan sorunların azaltılmasında yeni yaklaşımların ortaya konulması amaçlanmıştır. Bu amaçla bakteriler iki farklı sürede olacak şekilde, iki farklı düşük basınç, düşük sıcaklık ve manyetik alan uygulamalarına tabi tutulmuşlardır. Araştırma sonucunda uygulanan stres koşullarının şiddeti ve uygulama zamanı arttıkça bakterilerde oluşan antibiyotik duyarlılığının azaldığı, bazı antibiyotik türlerine karşı ise direnç gelişiminin ortaya çıktığı tespit edilmiştir ($p<0,05$). Üç farklı stres uygulaması sonucunda bakterilerin antibiyotik türlerine karşı en fazla direnci manyetik alan uygulamalarında oluşturduğu tespit edilmiştir. Üç farklı stres uygulamasında *S. thermophilus* en fazla linkomisin, sefaleksin ve sitreptomisine, *L. delbrueckii* subsp. *bulgaricus* ise, Streptomisin, Erythromycin ve Chloramphenicole karşı direnç geliştirmiştir. İki farklı yoğurt bakterisinden L. delbrueckii subsp. bulgaricus bakterisinin, *S. thermophilus*'a kıyasla stres uygulamaları sonucunda daha fazla antibiyotiğe karşı direnç geliştirdiği, ayrıca gelişen direncin de *S. thermophilus*'dan daha yüksek olduğu araştırma sonucunda tespit edilmiştir.

Anahtar Kelimeler: Manyetik Alan, Streptomisin, Stres, Yoğurt Bakterileri

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ORCID ID; *GA: 0000-0002-5055-2722, AJD: 0000-0002-3078-8914*

*Corresponding author e-mail: gakarca@aku.edu.tr

INTRODUCTION

One of the most significant issues with fermented milk production recently has been the use of antibiotics in milk during the dairy process and the resulting loss in quality (Brady 1988). Specifically, a portion of the antibiotics (30–80%) used in the treatment of mastitis in dairy animals can pass into milk (Chiders and Jones 1985). Even if the antibiotic concentration passing into milk is insignificant, it slows down—and can even stop—starter culture/acid production in dairy products. This can cause problems in producing various dairy products, such as yogurt, ayran, and cheese and cause serious quality loss (Jones and Seymour 1988).

Lactobacillus delbrueckii subsp. *bulgaricus* and *Streptococcus thermophilus* are among the most used starter bacteria in the production of fermented dairy products (primarily yogurt and ayran) (Song and Aryana 2014). In the fermentation of milk, lactic acid bacteria (LAB) produce many products, such as lactic acid, other organic acids, bacteriocins, exopolysaccharides, and vitamins, and they are the foundation of the production and quality characteristics of fermented milk products (Devanthi et al. 2018). LAB can undergo various abiotic and biotic changes (e.g., acidic, thermal, osmotic, oxidative, and other stresses) that seriously affect metabolic activity and production efficiency (Papadimitriou et al. 2016).

As in other activities, LAB identify all of the surrounding inconvenient, physical, chemical, and biological conditions as stress. These stress factors can affect the cell wall and membrane of LAB (Lakhotia 2001). In response to these various stress conditions, LAB use multi-faceted strategies to resist the damage incurred by these challenging environments (Shin et al. 2018; Wei et al. 2019; Yang et al. 2023). LAB resist these stress environments using a system called cross-protection metabolism. The stress response developed against these stress factors enables LAB survival, and these factors can also cause changes in the bacteria's biological activities (acid formation, antibiotic resistance, etc.) (Chen et al. 2017; Shin et al. 2018; Kulkarni et al. 2018; Zhang et al. 2018).

Milk containing antibiotics used in the production of fermented dairy products leads to many substantial issues— from loss of quality in the end product to an inability to obtain a product in the first place. Previous studies have shown LAB's behaviors, metabolism, and the changes in the metabolites they produce under different stress conditions. This study aims to identify the changes that might occur in the susceptibilities of 2 yogurt bacteria to antibiotics under similar stress conditions and introduce new approaches to decrease issues related to using milk-containing antibiotics in the fermented milk industry.

MATERIALS and METHOD

Starter Cultures

S. thermophilus (DSM 20617, ATCC19258) and *L. delbrueckii* subsp. *bulgaricus* (DSM 20081, ATCC 11842) strains were used in this study.

The two different starter culture types were first incubated inside MRS broth (110661, Merck Millipore, Germany) at 37°C under anaerobic conditions for 48–72 hours. At the end of the incubation period, the bacteria developed inside the broth were inoculated into *Streptococcus* agar (11007, Merck Millipore, Germany) and *Lactobacillus bulgaricus* agar (17154, Merck Millipoe, Germany) growth mediums and incubated again at 37°C under anaerobic conditions for 48–72 hours.

Stress Applications

Low-pressure application

The incubated cultures were then subjected to a low-pressure application for 1 to 2 hours under 3 different pressures inside a cabin designed by Biosan (Konya, Türkiye). The environmental conditions in the application were as follows: temperature: 30°C; moisture: 55.7%; oxygen concentration: 0.06%; and carbon dioxide: 0.13 ppm.

Low-temperature application

The cultures were also subjected to a low-temperature application at 3 different temperatures and 2 different durations after the incubation process; a 0°C temperature application was carried out inside a refrigerator (Beko, D948ANEK, Türkiye), a –18°C temperature application was performed inside a deep freezer (Uğur ED 560 DS, Türkiye), and a –75 °C temperature application was implemented inside an ultra-freezer (Thermo Scientific Forma 900 Series, USA). The environmental conditions during the applications were as follows: temperature: 0°C; moisture: 65.1%; oxygen concentration: 14.3%; carbon dioxide: 0.733 ppm; temperature: –18 °C; moisture: 68.6%; oxygen concentration: 9.65%; carbon dioxide: 0.466 ppm; temperature: –75°C; moisture: 48.6%; oxygen concentration: 2.23%; and carbon dioxide: 0.33 ppm.

Magnetic field application

The study was conducted using a mechanism designed specifically for this purpose, which was made of aluminum materials (Figure 1). The rotating part of the device could move due to its rotor. However, there is a stable part inside the mechanism where the cultures are normally located. The rotating part is square prism-shaped, has 12 magnets (3 magnets on each side), and is connected to a rotator with an adjustable rotation speed. The rotation speed of the rotor was kept stable during the experiments and set to 80 rpm. The magnets were replaced after each trial; therefore, a magnetic field of 3 different strengths could be achieved. The strength of

themagnets was determined as 15, 20, and 25 μT. As a result, magnetic fields at strengths of 180, 240, and 300 μT were attained during the application. The magnetic field strength was constantly measured with the help of a Tesla meter during the research process. The environmental conditions during the applications were as follows: temperature: 30°C; moisture: 63.4%; oxygen concentration: 13.92%; and carbon dioxide: 0.652 ppm.

Antibiotic Discs

Antibiotic discs for penicillin, amoxicillin, cephalexin, erythromycin, chloramphenicol, streptomycin, lincomycin, and tetracycline (Oxoid) were used in the antibiotics susceptibility analysis.

Antibiotic Susceptibility Analysis

After the application procedures were completed, the starter bacteria in the Petri dishes were separately removed with a sterile loop and suspended in tubes containing 10 mL of sterile physiological saline (Merck, 115525, Germany) with the help of a densitometer until homogeneous turbidity was formed. The density of the obtained inoculum suspension was arranged to attain a 0.5 McFarland (8.17 Log kob/mL) standard with the help of a densitometer (Biosan, 1B, Türkiye). Subsequently, 0.1 mL of prepared inoculum was taken using a sterile pipette and inoculated into Mueller-Hinton agar (Merck, 1.05437, Germany) growth mediums. Later, the inoculum was homogenously separated on the Petri dish's surface using a sterile swap and, using sterile pliers, placed on different parts of the growth mediums at a distance, enabling the zones that would eventually grow antibiogram discs to not touch each other (Akarca et al. 2019). The Petri dishes were then placed into anaerobic jars (Merck, 113681, Germany); each jar was sealed after Anaerocult A was added to it (Merck, 113829, Germany), 3 mL of pure water was added to each section, and the samples were incubated at 42°C for 48–72 hours in a drying oven (Incucel, MMM, Germany) (Bracquart 1981). The anaerobicity of the incubation medium was checked with an Anaerotest (Merck, 132371, Germany), one piece of which was placed in each jar. The zones that formed around the disc following incubation were measured in mm in a sufficiently lighted environment using a digital caliper (Mitutoyo, IP67 0–150 mm, Japan).

Statistical Analyses

The study experiments were conducted as 2 parallel and 2 repetitions. The results were calculated using the SPSS V 27.0.0 (SPSS Inc., Chicago, IL, USA) statistical package program. The data obtained from the analyses were then evaluated using variance analysis. The significance level was determined according to Duncan's test ($p<0,05$), and the effect of the results was determined using the Pearson correlation coefficient.

RESULTS

The antibiotic susceptibility results for the *S. thermophilus* starter culture, at 2 different durations and after 3 different low-temperature applications, are given in Table 1. The interactions between antibiotic type, pressure, and interaction duration on antibiotic susceptibility were highly significant ($p<0,0001$). The interaction between antibiotic type × pressure interaction on antibiotic susceptibility was also found to be highly significant ($p<0,01$). The pressure interaction on antibiotic susceptibility also showed a very high negative correlative effect (Table 1).

As a result of the antibiotic susceptibility determined after 3 different low-pressure applications under 2 different durations on the *L. delbrueckii* subsp. *bulgaricus* starter culture, the interactions between antibiotic type, pressure, and antibiotic type × pressure on antibiotic susceptibility were found to be highly significant ($p<0,0001$). The interaction of pressure × interaction duration was also significant ($p<0,05$) on antibiotic susceptibility. In addition, the pressure interaction on antibiotic susceptibility showed a very high negative correlative effect, and antibiotic-type interaction on antibiotic susceptibility indicated a very high positive correlation (Table 2).

The antibiotic susceptibility results applied to the *S. thermophilus* starter culture at 2 different durations and 3 different temperatures are given in Table 3. It was determined that the interactions between antibiotic type, applied temperature, interaction duration, and the antibiotic type × degree of applied temperature were highly significant on antibiotic susceptibility ($p<0,0001$). Additionally, the interaction of temperature applied on antibiotic susceptibility had a high negative correlative effect (Table 3).

The interactions between antibiotic type, applied temperature, and antibiotic type × applied temperature × interaction duration on antibiotic susceptibility degree obtained after a low-temperature application to *L. delbrueckii* subsp. *bulgaricus* at 2 different durations and 3 different degrees were highly significant ($p<0,0001$). In addition, the low-temperature interaction showed a very high negative correlative effect on antibiotic susceptibility (Table 4).

The results of this study show that the interactions between antibiotic type, severity of the magnetic field, interaction duration, antibiotic type × severity of the magnetic field, and magnetic field × interaction duration on antibiotic susceptibility are highly significant ($p<0,0001$). In addition, the severity of the magnetic field interaction on antibiotic severity had a high negative correlative effect (Table 5).

The interactions between antibiotic type, magnetic field severity, and antibiotic type × applied temperature × magnetic field severity on antibiotic susceptibility degree identified after a magnetic field application to *L. delbrueckii* subsp. *bulgaricus* at 3 different durations and 3 different severities were highly significant ($p<0,0001$). Magnetic field severity

on antibiotic susceptibility also showed a high negative correlative effect (Table 6).

DISCUSSION

The susceptibility of the *S. thermophilus* starter culture to the antibiotic types used in the study differed ($p<0,05$). Before any application was initiated, it was determined that the antibiotic type that *S. thermophilus* was most susceptible to was tetracycline, with a 33.04-mm zone diameter, followed by erythromycin and penicillin, with 23.91 and 22.43-mm zone diameters, respectively. Before the applications, it was determined that the susceptibility of *S. thermophilus* to all of the antibiotic types used in the study was at an ultra-sensitive (≥18-mm zone diameter) level (Table 1).

As the severity of low pressure and application duration increased, the antibiotic susceptibilities of *S. thermophilus* decreased, but the resistance to antibiotic types increased ($p<0,05$). Especially after applying a pressure of –300 mbar for 2 hours, *S. thermophilus* showed resistance to 5 different antibiotic types, and its level of susceptibility to 3 different antibiotic types decreased from ultra-sensitive to moderately sensitive. In the conclusion of the application, after a –100 mbar pressure application for 1 hour, it was determined that the antibiotic type the bacterium was most susceptible to was tetracycline, with a zone diameter of 23.99 mm. Nonetheless, the bacteria showed the highest resistance to lincomycin, with a 9.15-mm zone diameter after a –300 mbar pressure application for 2 hours (Table 1).

The *L. delbrueckii* subsp. *bulgaricus* starter culture showed varying susceptibility to the antibiotic types used in the study ($p<0,05$). It was observed that before the applications, *L. delbrueckii* subsp. *bulgaricus* was most susceptible to the tetracycline antibiotic type (with a zone diameter of 30.14 mm) and most resistant to streptomycin (with a zone diameter of 17.59 mm). Similarly, at the beginning of the application, *L. delbrueckii* subsp. *bulgaricus* showed very high susceptibility (with a zone diameter ≥18 mm) to all antibiotic types used in the study (Table 2).

As the negative pressure and interaction duration increased, the susceptibility degree of *L. delbrueckii* subsp. *bulgaricus* to most of the antibiotic types decreased ($p<0,05$). This change mainly occurred during the –300 mbar application for 2 hours, followed by –300 mbar pressure for 1 hour and –200 mbar pressure for 2 hours. In addition, the susceptibility level (zone diameter ≥18 mm), which was very high in all antibiotics except for streptomycin at the beginning of the application, became susceptible to the 5 antibiotics used (10–14-mm zone diameter) after a –300 mbar pressure application for 2 hours. Although no difference was observed regarding the susceptibility level to tetracycline after the applications, the high susceptibility degree to streptomycin (14–16-mm zone diameter) reached the resistance (≤10-mm zone diameter) level (Table 2).

The physiological response that the different LABs exhibit to low-pressure stress varies. Most of the low-pressure stress LAB encounters impacts the cell wall (Silver 2003). However, LAB can change the nature of the cell wall and respond to this stress (Piuri et al. 2005; Koch et al. 2007); the cell wall responds by using a set of regulatory systems (Silver 2003).

The primary reaction of LAB to this stress is to produce or import tiny molecules called osmolytes (e.g., glycine betaine, choline, or proline) to balance the intracellular and extracellular difference (Molenaar et al. 1993; Glaasker et al. 1998).

The *S. thermophilus* starter culture exposed to different low temperatures showed varied susceptibility to antibiotic types used in the study ($p<0,05$). It was determined before initiating any applications that *S. thermophilus* had an ultra-sensitive (≥18-mm zone diameter) level of susceptibility to all of the antibiotic types used in the study; however, after the low-temperature applications, the susceptibility levels decreased drastically and turned into resistance in both samples (Table 3).

As the degree of low-temperature applied and application duration increased, the antibiotic susceptibilities of *S. thermophilus* decreased, but the resistance to antibiotic types increased ($p<0,05$). After the application at –75°C for 2 hours, *S. thermophilus* showed resistance to 2 different antibiotic types, and its susceptibility degree to the 5 different antibiotic types decreased from ultra-sensitive to moderately sensitive. In the conclusion of the applications, it was revealed that the antibiotic type the bacterium is most susceptible to was tetracycline, with a 28.38-mm zone diameter after the application at 0°C for 1 hour; however, the highest resistance was to cephalexin, resulting in a 9.13-mm zone diameter following an application at –65°C for 2 hours (Table 3).

L. delbrueckii subsp. *bulgaricus* exhibited different degrees of susceptibility to the antibiotic types used in the study ($p<0,05$). Before the applications began, *L. delbrueckii* subsp. *bulgaricus* showed very high susceptibility (≥18-mm zone diameter) to all antibiotic types except for streptomycin (Table 4). As the low-temperature application and interaction duration continued, the degree of susceptibility of *L. delbrueckii* subsp. *Bulgaricus* to the antibiotic types decreased ($p<0,05$). This change degree occurred the least on tetracycline; however, the most significant change was on streptomycin, followed by cephalexin and erythromycin, respectively. The bacteria developed resistance to 1 antibiotic after a –65°C low-temperature application for 1 hour and to 4 antibiotics following a –65°C low-temperature application for 2 hours (Table 4).

Exposure to low temperatures is common for LAB types in various contexts. Cold shock causes much less damage to cells compared to other stresses. LAB

produces the main reaction to cold shock with the help of cell walls, and this reaction occurs quickly in LAB by producing a subset of cell wall proteins, the heat shock proteins, or the molecular chaperons that help protein folding.

The negative effect of exposure to cold stress is primarily due to the physical impact of low temperature on cell structures and enzymatic reactions. Most LAB types react to cold shock through the temporary induction of specific protein sets called cold-induced proteins and by suppressing other proteins synthesized after exposure to stressful conditions. Such a reaction is assumed to help cells overcome the physiological stress caused by cold shock (Van de Guchte et al. 2002; Chastanet et al. 2003; Spano and Massa 2006; Fiocco et al. 2009).

The *S. thermophilus* starter culture showed different susceptibilities to the antibiotic types used in the study ($p<0,05$). Additionally, as the severity of the applied magnetic field and application duration increased, the high susceptibilities identified in the beginning decreased, and the resistance to these antibiotic types increased ($p<0,05$).

It was observed that especially after a 300-μT magnetic field application for 2 hours, the bacteria showed resistance to 6 different antibiotic types, and the susceptibility degree to 2 antibiotic types decreased from ultra-sensitive to moderately sensitive. In the different magnetic field applications, the antibiotic type the bacterium is most susceptible to was tetracycline, with a 19.99-mm zone diameter after a 180-μT magnetic field application for 1 hour. However, the bacteria showed its highest resistance to streptomycin, with an 8.01-mm zone diameter after a 300-μT magnetic field application for 2 hours (Table 5).

L. delbrueckii subsp. *bulgaricus* exhibited different degrees of susceptibility to the antibiotic types used in the study ($p<0,05$). As the magnetic field application and interaction duration continued, the susceptibility degree of the bacteria to antibiotic types decreased ($p<0,05$); this degree of change occurred the least with amoxicillin; however, the highest change was with streptomycin, followed by cephalexin and lincomycin, respectively. The bacteria developed resistance to 1 antibiotic after a 240-μT magnetic field application for 2 hours and 7 antibiotics after a 300-μT magnetic field application lasting 2 hours (Table 6).

A magnetic field causes biomolecular and chemical effects (that affect the electronic spin states of the reaction intermediates) in the cytoplasm of the lactic bacteria. This kind of effect can cause changes in intracellular ion homeostasis (Pei et al. 2006), enzyme activities (Dang et al. 2007), cell shape, cell growth (Wang et al. 2002), and cell division (Naruse 2002).

Previous research has shown that most biological tissues are diamagnetic (Liu et al. 2017). A diamagnetic response to an external magnetic effect striking it creates a magnetic induction in the opposite direction (Butler 2014). Specifically, a magnetic field of low severity and frequency affects the movement of ions along the cell membrane (Wang and Hladky 1994); the magnetic field also affects the conductance of K+ channels on cell membranes (Cecchetto et al. 2015).

In a study on the resistance of LAB to antibiotics, Aslım and Beyatlı (2004), found that 34 *S. thermophilus* strain isolates taken from yogurt samples from various villages and towns in Türkiye showed resistance to gentamicin and penicillin and that these strains were susceptible to tetracycline and chloramphenicol. Tatlı (2009), demonstrated that LAB strains isolated from traditionally produced dairy products were resistant to vancomycin, ciprofloxacin, gentamicin, erythromycin, and tetracycline. Similarly, research conducted by Kılıç (2014), found that isolates isolated from traditionally produced white cheese samples were susceptible to ampicillin, vancomycin, penicillin, gentamicin, chloramphenicol, and teicoplanin—and resistant to streptomycin and ciprofloxacin. Özteber (2013), found the highest resistance to lincomycin (25.59%) in the isolates isolated from fermented dairy products, followed by tetracycline, meropenem, ampicillin, gentamicin, erythromycin, ciprofloxacin, chloramphenicol, and vancomycin, respectively.

Table 1. Antibiotic sensitivity of *S. thermophilus* after different pressure and duration application

Type of Antibiotics	Control		Pressure											
			-100 mbar			-200 mbar				-300 mbar				
			1 Hour		2 Hour		1 Hour		2 Hour		1 Hour		2 Hour	
Penicilin	22.43±0.72^{Abc}	+++	21.24±1.17^{Aab}	+++	20.49±1.18^{Aa}	+++	15.86±0.26^{Bbc}	++	15.06±1.33^{Babc}	++	11.76±0.72^{Cb}	+	10.07±0.08^{Cb}	+
Amoxcicilin	20.66±0.80^{Abc}	+++	20.04±1.26^{ABb}	+++	19.04±0.53^{ABab}	+++	14.41±5.29^{BCbc}	++	12.32±3.93^{Cbc}	+	11.04±0.18^{Cbc}	+	9.91±0.44^{Cb}	-
Cephalexin	19.91±0.77^{Ac}	+++	18.73±1.28^{ABbc}	+++	15.70±1.18^{BCbc}	++	14.93±2.47^{Cbc}	++	12.97±1.90^{CDbc}	+	9.98±0.09^{DEc}	-	9.62±0.58^{Eb}	-
Erythromycin	23.91±2.19^{Ab}	+++	21.05±1.99^{ABab}	+++	19.17±2.05^{BCab}	+++	18.73±1.28^{BCab}	+++	16.11±0.33^{Cab}	++	11.80±0.54^{Db}	+	9.99±0.07^{Db}	-
Chloramphenicol	20.86±1.73^{Abc}	+++	18.24±0.59^{ABbc}	+++	16.53±0.99^{BCbc}	++	16.80±1.43^{BCbc}	++	14.90±1.64^{CDabc}	++	13.73±0.41^{CDa}	+	12.03±0.57^{Da}	+
Streptomycin	18.73±1.29^{Ac}	+++	14.93±2.46^{Bc}	++	13.10±2.16^{BCc}	+	11.41±1.09^{BCc}	+	10.05±0.58^{CDc}	+	10.57±0.62^{CDbc}	+	9.37±0.08^{Db}	-
Lincomycin	19.31±1.91^{Ac}	+++	17.56±1.20^{ABbc}	++	15.02±0.70^{BCc}	++	14.06±1.67^{BCbc}	++	11.49±2.92^{CDbc}	+	11.09±0.88^{CDbc}	+	9.15±0.39^{Db}	-
Tetracycline	33.04±2.34^{Aa}	+++	23.99±1.70^{Ba}	+++	20.97±2.19^{BCa}	+++	22.66±1.25^{BCa}	+++	19.31±1.26^{Ca}	+++	14.32±0.42^{Da}	++	12.47±1.48^{Da}	+
Variation					**P Value**						**r**			
Type of Antibotics(A)					<0.0001						0.111			
Pressure (P)					<0.0001						-0.784**			
Interaction Time (I)					<0.0001						-0.129			
A x P					0.01						--			
A x I					0.966						--			
P x I					0.042						--			
A x P x I					1.000						--			

a-c (→): Values shown with different letters on the same line differ from each other at the p<0.05 level, A-E (↓) : Values shown with different letters on the same column differ from each other at the p<0.05 level, ± Standard deviation,). *P* < 0.0001: Statistically too much significant, *P* =0.01: Statistically much significant, *P* =0.05: Statistically significant, **. Correlation is significant at the 0.01 level (2-tailed). ≤ 10(-): Resistant. 10- 14(+): Moderately Sensitive. 14-18(++): Sensitive. 18≥ (+++): Ultra-sensitive.

Table 2. Antibiotic sensitivity of *L. delbrueckii* subsp. *bulgaricus* after different pressure and duration application

Type of Antibiotics	Control		Pressure											
			-100 mbar			-200 mbar				-300 mbar				
			1 Hour		2 Hour		1 Hour		2 Hour		1 Hour		2 Hour	
Penicilin	23.26±0.24Abc	+++	17.05±1.35Bc	++	15.08±1.91BCcd	++	15.78±0.25BCb	++	13.13±1.01CDb	+	13.11±1.50CDcd	+	11.36±1.78Dbc	+
Amoxcicilin	21.84±0.13Ac	+++	19.36±1.85ABc	+++	17.00±1.89BCcd	++	15.82±1.02Cb	++	14.39±0.49CDb	++	11.85±2.54Dd	+	11.06±0.79Dbc	+
Cephalexin	25.93±2.06Ab	+++	23.25±1.52ABb	+++	22.17±0.63Bb	+++	18.47±0.85Cb	+++	16.52±1.37CDb	++	17.91±1.49Cab	++	13.75±0.41Db	++
Erythromycin	25.86±0.66Ab	+++	19.45±0.96Bc	+++	17.17±0.34Ccd	++	17.18±0.51Cb	++	13.85±0.83Db	+	15.81±0.37Cbc	++	11.97±0.93Ebc	+
Chloramphenicol	26.45±1.91Ab	+++	19.16±1.11Bc	+++	18.36±0.07BCc	+++	16.64±2.11BCb	++	15.52±1.70Cb	++	11.07±0.40Dd	+	10.17±0.41Dc	+
Streptomycin	17.59±0.50Ad	++	17.05±0.28Ac	++	14.42±1.83Bd	++	16.83±0.11Ab	++	13.63±1.40Bb	+	10.10±0.21Cd	+	9.61±0.69Cc	-
Lincomycin	22.97±0.27Abc	+++	16.23±1.28Bc	++	14.31±0.95BCDd	++	16.00±0.38BCb	++	13.97±0.77CDb	+	13.09±0.60DEcd	+	11.39±1.61Ebc	+
Tetracycline	30.14±3.15Aa	+++	26.45±1.91ABa	+++	26.45±1.91ABa	+++	21.91±3.03Ba	+++	21.91±3.03Ba	+++	20.22±2.15Ba	+++	20.22±2.15Ba	+++
Variation					**P Value**						**r**			
Type ofAntibotics(A)					<0.0001						0.194*			
Pressure (P)					<0.0001						-0.748**			
+Interaction Time (I)					<0.0001						-0.125			
A x P					<0.0001						--			
A x I					0.440						--			
P x I					0.033						--			
A x P x I					0.992						--			

a-c (→): Values shown with different letters on the same line differ from each other at the p<0.05 level, A-E (↓) : Values shown with different letters on the same column differ from each other at the p<0.05 level, ± Standard deviation,). *P* < 0.0001: Statistically too much significant, *P* =0.01: Statistically much significant, *P* =0.05: Statistically significant, **. Correlation is significant at the 0.01 level (2-tailed). ≤ 10(-): Resistant. 10- 14(+): Moderately Sensitive. 14-18 (++): Sensitive. 18≥ (+++): Ultra-sensitive. ≤ 10(-): Resistant. 10- 14 (+): Moderately Sensitive. 14-18 (++): Sensitive. 18≥ (+++): Ultra-sensitive.

Table 3. Antibiotic sensitivity of *S. thermophilus* after different temperature and duration application

Type of Antibiotics	Control		Temperature											
			0°C				-18°C				-75°C			
			1 Hour		2 Hour		1 Hour		2 Hour		1 Hour		2 Hour	
Penicilin	22.43±0.72^{Abc}	+++	21.65±0.42^{ABb}	+++	21.15±0.85^{ABb}	+++	20.68±0.55^{Bb}	+++	20.39±0.26^{Bb}	+++	18.10±0.77^{Cb}	+++	17.27±2.98^{Cbc}	++
Amoxcicilin	20.66±0.80^{Abc}	+++	20.49±0.44^{Ab}	+++	19.80±0.37^{ABbc}	+++	19.36±1.85^{ABbc}	+++	18.70±0.93^{ABbc}	+++	18.37±0.73^{ABb}	+++	17.47±1.50^{Bbc}	++
Cephalexin	19.91±0.77^{Ac}	+++	15.87±0.09^{Bc}	++	13.28±0.04^{CDd}	++	14.67±0.88^{BCde}	++	12.38±0.93^{DEef}	+	10.90±0.92^{EFc}	+	9.13±0.15^{Fd}	-
Erythromycin	23.91±2.19^{Ab}	+++	22.86±0.47^{ABb}	+++	20.50±0.06^{BCb}	+++	19.02±0.08^{CDbc}	+++	16.29±1.07^{DEcd}	++	17.36±0.51^{CDEb}	++	14.73±1.41^{Ec}	++
Chloramphenicol	20.86±1.73^{Abc}	+++	20.65±0.38^{Ab}	+++	18.89±1.08^{ABbc}	+++	19.26±1.35^{ABbc}	+++	17.16±1.40^{BCcd}	++	18.38±0.94^{ABb}	+++	14.79±0.65^{Cc}	++
Streptomycin	18.73±1.29^{Ac}	+++	15.21±0.68^{Bc}	++	14.27±1.32^{BCd}	++	12.22±0.37^{CDe}	+	10.79±0.57^{DEf}	+	10.28±0.15^{DEe}	+	9.28±0.21^{Ed}	-
Lincomycin	19.31±1.91^{Ac}	+++	17.60±0.35^{ABc}	++	16.10±1.34^{Bcd}	++	16.95±0.14^{ABcd}	++	14.88±0.78^{Bde}	++	16.80±0.05^{ABb}	++	14.79±0.92^{Bc}	++
Tetracycline	33.04±2.34^{Aa}	+++	28.38±1.92^{ABa}	+++	26.33±4.39^{BCa}	+++	26.45±1.91^{BCa}	+++	25.81±0.30^{BCa}	+++	20.89±0.56^{Ca}	+++	22.54±1.42^{BCa}	+++
Variation			**P Value**								**r**			
TypeofAntibotics(A)			<0.0001								0.168			
Temoerature (T)			<0.0001								-0.496**			
InteractionTime(I)			<0.0001								-0.114			
A x T			<0.0001								--			
A x I			0.409								--			
T x I			0.064								--			
A x T x I			0.992								--			

a-e (→): Values shown with different letters on the same line differ from each other at the p<0.05 level, A-E (↓) : Values shown with different letters on the same column differ from each other at the p<0.05 level, ± Standard deviation, *P* < 0.0001: Statistically too much significant, **. Correlation is significant at the 0.01 level (2-tailed). ≤ 10(-): Resistant. 10- 14(+): Moderately Sensitive. 14-18(++): Sensitive. 18≥ (+++): Ultra-sensitive. ≤ 10 (-): Resistant. 10- 14 (+): Moderately Sensitive. 14-18 (++): Sensitive. 18≥ (+++): Ultra-sensitive.

Table 4. Antibiotic sensitivity of *L. delbrueckii* subsp. *bulgaricus* after different temperature and duration application

Type of Antibiotics	Control		Temperature											
			0°C				-18°C				-65°C			
			1 Hour		2 Hour		1 Hour		2 Hour		1 Hour		2 Hour	
Penicilin	23.26±0.24^{Abc}	+++	22.88±0.33^{ABb}	+++	20.01±0.84^{Cab}	+++	21.04±0.53^{BCb}	+++	16.89±0.32^{Da}	++	16.36±1.62^{Db}	++	12.31±1.41^{Eab}	+
Amoxcicilin	21.84±0.13^{Ac}	+++	19.69±0.74^{ABc}	+++	16.39±0.93^{Ccd}	++	18.38±1.36^{BCbcd}	+++	13.03±1.83^{Dabc}	+	15.87±0.22^{Cb}	++	12.97±2.19^{Da}	+
Cephalexin	25.93±2.06^{Ab}	+++	22.30±1.11^{ABbc}	+++	19.89±0.91^{Bab}	+++	16.07±2.24^{Cd}	++	12.78±1.32^{CDbc}	+	14.13±1.35^{Cb}	++	9.58±1.49^{Dbc}	-
Erythromycin	25.86±0.66^{Ab}	+++	21.76±2.05^{Bbc}	+++	19.95±2.11^{BCab}	+++	17.78±0.76^{CDcd}	++	13.98±1.91^{Eabc}	+	14.82±2.26^{DEb}	++	9.91±0.18^{Fbc}	-
Chloramphenicol	26.45±1.91^{Ab}	+++	22.80±1.95^{ABb}	+++	19.60±1.49^{BCDab}	+++	20.62±0.64^{BCb}	+++	15.99±2.02^{Dab}	++	17.96±1.76^{CDab}	++	9.25±0.54^{Ec}	-
Streptomycin	17.59±0.50^{Ad}	++	16.10±0.40^{Ad}	++	13.62±1.40^{Be}	+	12.77±0.51^{Be}	+	10.70±0.93^{Cc}	+	9.92±0.88^{CDc}	-	8.85±0.32^{Dc}	-
Lincomycin	22.97±0.27^{Abc}	+++	20.37±0.37^{ABbc}	+++	17.50±1.45^{CDbc}	++	19.84±0.26^{BCbc}	+++	14.50±1.59^{Eabc}	++	16.26±1.95^{DEb}	++	10.86±0.92^{Fabc}	+
Tetracycline	30.14±3.15^{Aa}	+++	26.11±1.35^{ABa}	+++	22.28±0.78^{BCa}	+++	24.11±1.33^{BCa}	+++	16.48±1.64^{DEab}	++	20.63±1.37^{CDa}	+++	13.26±1.17^{Ea}	+
Variation			**P Value**								**r**			
Type ofAntibotics(A)			<0.0001								0.068			
Temoerature (T)			<0.0001								-0.758**			
Interaction Time(I)			0.251								-0.044			
A x T			0.501								--			
A x I			0.632								--			
T x I			0.398								--			
A x T x I			<0.0001								0.068			

a-d (→): Values shown with different letters on the same line differ from each other at the p<0.05 level, A-F (↓) : Values shown with different letters on the same column differ from each other at the p<0.05 level, ± Standard deviation, *P* < 0.0001: Statistically too much significant, **. Correlation is significant at the 0.01 level (2-tailed). ≤ 10(-): Resistant. 10- 14(+): Moderately Sensitive. 14-18(++): Sensitive. 18≥ (+++): Ultra-sensitive.

Table 5. Antibiotic sensitivity of *S. thermophilus* after different magnetic field and duration application

Type of Antibiotics	Control		Magnetic Field											
			180 µT				240 µT				300 µT			
			1 Hour		2 Hour		1 Hour		2 Hour		1 Hour		2 Hour	
Penicilin	22.43±0.72^{Abc}	+++	18.73±1.28^{Bab}	+++	14.94±0.26^{Cbc}	++	15.80±1.23^{Cabc}	++	12.83±0.56^{Dabc}	+	12.26±1.30^{Db}	+	10.01±0.25^{Eb}	+
Amoxcicilin	20.66±0.80^{Abc}	+++	17.16±1.52^{Bb}	++	14.98±0.95^{Cbc}	++	13.84±0.84^{CDcd}	+	12.01±0.65^{DEcd}	+	11.13±0.27^{EFbcd}	+	9.67±0.52^{Fbc}	-
Cephalexin	19.91±0.77^{Ac}	+++	14.24±1.12^{Bc}	++	12.29±1.29^{Ccd}	+	10.94±0.44^{CDe}	+	9.87±0.30^{DEde}	-	9.22±0.57^{DEe}	-	8.51±0.50^{Ebc}	-
Erythromycin	23.91±2.19^{Ab}	+++	19.45±0.96^{Bab}	+++	17.65±1.57^{BCab}	++	17.18±0.51^{BCa}	++	14.31±1.59^{CDab}	++	15.81±0.37^{BCDa}	++	12.37±1.53^{Da}	+
Chloramphenicol	20.86±1.73^{Abc}	+++	18.22±1.24^{ABab}	+++	16.56±1.39^{BCab}	++	14.49±1.20^{CDbcd}	++	12.29±0.47^{DEbc}	+	10.52±1.18^{Ebcd}	+	9.31±0.29^{Ebc}	-
Streptomycin	18.73±1.29^{Ac}	+++	13.34±1.00^{Bc}	+	11.36±1.66^{BCd}	+	10.80±0.99^{Ce}	+	9.04±0.39^{CDe}	-	9.98±0.15^{CDde}	-	8.01±0.33^{Dc}	-
Lincomycin	19.31±1.91^{Ac}	+++	13.76±0.54^{Bc}	+	11.85±0.52^{BCDd}	+	12.88±0.20^{BCde}	+	10.86±0.96^{CDcde}	+	11.04±0.44^{CDbcd}	+	9.35±0.44^{Dbc}	-
Tetracycline	33.04±2.34^{Aa}	+++	19.99±0.46^{Ba}	+++	17.88±0.63^{BCa}	++	16.38±1.51^{BCab}	++	14.61±1.48^{CDa}	++	11.63±1.41^{DEbc}	+	9.65±0.59^{Ebc}	-
Variation			**P Value**								**r**			
Type ofAntibotics(A)			<0.0001								0.059			
Magmetic Field (M)			<0.0001								-0.796**			
Interaction Time(I)			<0.0001								-0.141			
A x M			<0.0001								--			
A x I			0.800								--			
M x I			<0.0001								--			
A x M x I			1.000								--			

a-c (→): Values shown with different letters on the same line differ from each other at the p<0.05 level, A-F (↓) : Values shown with different letters on the same column differ from each other at the p<0.05 level, ± Standard deviation, *P* < 0.0001: Statistically too much significant, **. Correlation is significant at the 0.01 level (2-tailed). ≤ 10(-): Resistant. 10- 14(+): Moderately Sensitive. 14-18(++): Sensitive. 18≥ (+++): Ultra-sensitive.

Table 6. Antibiotic sensitivity of *L. delbrueckii* subsp. *bulgaricus* after different magnetic field and duration application

Type of Antibiotics	Control		Magnetic Field											
			180 µT				240 µT				300 µT			
			1 Hour		2 Hour		1 Hour		2 Hour		1 Hour		2 Hour	
Penicilin	23.26±0.24^{Abc}	+++	18.88±0.91^{Bb}	+++	15.88±2.21^{CDbc}	++	17.98±0.89^{BCab}	++	13.34±1.18^{Dabc}	+	13.37±1.04^{Da}	+	9.92±0.36^{Eab}	-
Amoxcicilin	21.84±0.13^{Ac}	+++	15.85±1.96^{Bb}	++	13.79±2.1^{BC4cd}	+	14.77±1.39^{Bbc}	+	11.11±0.61^{CDbc}	+	11.12±0.85^{CDa}	+	10.15±0.13^{Da}	+
Cephalexin	25.93±2.06^{Ab}	+++	23.47±1.32^{ABa}	+++	19.54±1.15^{BCab}	+++	17.75±2.24^{CDab}	++	14.60±3.14^{DEab}	++	10.72±0.77^{EFa}	+	8.84±0.51^{Fbc}	-
Erythromycin	25.86±0.66^{Ab}	+++	23.24±1.24^{Aa}	+++	18.94±0.69^{Bab}	+++	16.54±1.61^{Bb}	++	11.57±1.55^{Cbc}	++	11.60±2.20^{Ca}	+	9.70±0.46^{Cab}	-
Chloramphenicol	26.45±1.91^{Ab}	+++	25.39±1.72^{Aa}	+++	21.9±2.37^{ABa}	+++	21.72±0.62^{ABa}	+++	16.41±1.54^{BCa}	++	15.18±5.16^{CDa}	++	9.80±0.29^{Dab}	-
Streptomycin	17.59±0.50^{Ad}	++	14.94±2.23^{Ab}	++	10.93±0.53^{Bd}	+	11.60±2.13^{Bc}	+	9.97±0.15^{BCc}	-	10.79±1.01^{BCa}	+	7.94±0.66^{Cc}	-
Lincomycin	22.97±0.27^{Abc}	+++	19.13±1.91^{Bb}	+++	15.80±2.44^{BCbc}	++	14.74±2.36^{CDbc}	++	11.75±1.43^{DEbc}	+	11.56±1.12^{DEa}	+	9.38±0.38^{Eab}	-
Tetracycline	30.14±3.15^{Aa}	+++	24.02±2.19^{Ba}	+++	19.46±1.47^{Cab}	+++	16.61±1.01^{CDb}	++	13.74±0.80^{DEabc}	++	10.99±0.11^{Ea}	+	9.89±0.46^{Eab}	-
Variation			**P Value**								**r**			
Type ofAntibotics(A)			<0.0001								0.032			
Magmetic Field (M)			<0.0001								-0.829**			
Interaction Time(I)			0.912								0.004			
A x M			<0.0001								--			
A x I			0.684								--			
M x I			0.579								--			
A x M x I			0.699								--			

a-c (→): Values shown with different letters on the same line differ from each other at the p<0.05 level, A-F (↓) : Values shown with different letters on the same column differ from each other at the p<0.05 level, ± Standard deviation, *P* < 0.0001: Statistically too much significant, **. Correlation is significant at the 0.01 level (2-tailed). ≤ 10(-): Resistant. 10- 14(+): Moderately Sensitive. 14-18(++): Sensitive. 18≥ (+++): Ultra-sensitive.

CONCLUSION

This study aimed to reveal the changes that might occur in the susceptibilities of 2 yogurt bacteria types to antibiotics under 2 different durations and 3 different stress conditions and present new approaches to reduce issues arising from using milk with antibiotics in the fermented milk industry. The study found that as the severity and application duration of the stress conditions increased, the antibiotic susceptibility in the bacteria decreased, and resistance to certain antibiotic types developed. In the conclusion of the 3 different stress applications, it was determined that the bacteria had the highest resistance to the antibiotics in the magnetic field applications. In these 3 different stress applications, *S. thermophilus* showed the highest resistance to lincomycin, cephalexin, and streptomycin, and *L. delbrueckii* subsp. *bulgaricus* developed resistance to streptomycin, erythromycin, and chloramphenicol. Of the 2 yogurt bacteria, *L. delbrueckii* subsp. *bulgaricus* developed resistance to more antibiotics than *S. thermophilus* after the stress applications; in addition, the developed resistance was more substantial than that of *S. thermophilus*.

Beta-lactam antibiotics (penicillin, cephalosporins, etc.), mainly used in treating diseases such as mastitis, pass into milk and cause severe problems—from quality loss in the end-product to an inability to produce a product. Since the stress applications on the starter cultures used in production decrease the susceptibility of these bacteria to antibiotics, it is believed that potential issues that might arise from the antibiotic content can be drastically reduced. The relationship between stress applications and antibiotic residue in milk must be further examined in similar studies.

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Ethical approval: This study is not subject to the permission of HADYEK in accordance with the "Regulation on Working Procedures and Principles of Animal Experiments Ethics Committees" 8 (k). The data, information and documents presented in this article were obtained within the framework of academic and ethical rules."

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