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CONTENTS

Research Article

Presence of *Salmonella* spp., *Listeria monocytogenes* and *Staphylococcus aureus* in halloumi sold in Northern Cyprus

Fatma Işın MAHAN[1,a], Beyza Hatice ULUSOY[2,3,b], Fatma Kaya YILDIRIM[2,3,c,✉], Canan HECER[4,d]

[1]Turkish Repuclic of Northern Cyprus Ministry of Agrıculture and Natural Resources Veterinary Department, Cyprus; [2]Near East University, Faculty of Veterinary Medicine, Department of Food Hygiene and Technology, Nicosia, Cyprus; [3]DESAM Research Institute, Near East University, Nicosia, Cyprus; [4]Cyprus West University, Faculty of Health Sciences, Department of Nutrition and Dietetics, Famagusta, Cyprus.

[a]ORCID: 0009-0003-2124-088X; [b]ORCID: 0000-0001-9278-2537; [c]ORCID: 0000-0003-1281-846X; [d]ORCID: 0000-0003-1156-9510

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✉**Corresponding author**
fatma.kaya@neu.edu.tr

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ABSTRACT

Halloumi is an important part of the Cyprus dairy sector and consumed by a large volume of public. The microbiologic safety of the cheese is important in terms of public health. In this study, the presence of *Salmonella* spp., *Listeria monocytogenes* and *Staphylococcus aureus* on 1072 samples that collected for two years, their distribution according to the seasons and the effect of the seasons on the microbial load were investigated. As the result of the study, *Salmonella* spp. and *L. monocytogenes* could not be detected in any of the halloumi samples, while *S. aureus* was detected. It was determined that 39 (3.64%) of the halloumi samples contained *S. aureus* above $1x10^3$ cfu/g, 43 of them between $1x10^1$ and $1x10^3$ cfu/g, and 990 of them below $1x10^1$ cfu/g. It has been observed that the most intense contamination above $1x10^3$ cfu/g is formed in the spring season. In order to eliminate the food safety problem caused by *S. aureus*, first of all, the development of good manufacturing practices in farms, making the cold milk application cover all farms should be provided. On the other hand, within the framework of food safety from farm to fork, the end-product should be delivered to the consumer without breaking the cold chain.

Introduction

Halloumi is an important part of the Cyprus dairy sector. Historical documents showed us that this cheese has been produced in Cyprus since 1554 (24). The best-known characteristic of this cheese is to be produced the from raw milk without using starter culture. Halloumi is a type of cheese that can be consumed fresh or matured in brine. While industrial halloumi sold in cities is marketed in plastic vacuum packaging, it is preserved in brine in rural areas (1, 14, 15, 18, 19).

In the Northern Cyprus, approximately 164.250 tons of milk were produced in 2018, and a total of 144.345 tons in 2019, and 308.595 tons of milk was produced in a two-year period (9). Nowadays, implementation of Commission Regulation (EU) 2021/591of 12 April 2021 entering a name in the register of protected designations of origin (PDO) and protected geographical indications (PDI) with both Greek and Turkish names as Χαλλούμι-Halloumi/Hellim and Commission Decision (EU) 2021/586 of 12 April 2021 amending Decision 2007/330/EC lifting prohibitions on the movement of certain animal products on the island of Cyprus under Council Regulation (EC) No 866/2004 and laying down conditions for the movement of those products with regard to halloumi PDO have big importance so optimizing the food safety/quality properties (4).

It is reported that the shelf-life and quality of halloumi is affected by several factors such as the milk quality and the hygienic practices during manufacturing. Although halloumi is produced by boiling the curds in whey, several studies have reported the presence of contaminated microorganisms in the end product due to

poor hygiene during the production process and the survival of thermoduric microorganisms (22). The presence of *L. monocytogenes*, *Salmonella* spp. and *S. aureus* in ready-to-eat products is taken into account in the Turkish Food Codex Microbiological Criteria within the scope of food safety criteria in commercially available cheeses (3) and also with Commission Regulation (EC) No 2073/2005 on microbiological criteria for foodstuffs (2). In the Turkish Republic of Northern Cyprus (TRNC) legislation, it is stated that "there will be no pathogen harmful to health" (1). It has been observed that no study has been found for cheese produced in the north of Cyprus, especially considering the incidence of *Salmonella* spp., *L. monocytogenes*, and *S. aureus*, which are also included in the food safety criteria for halloumi. In addition, considering that the Cyprus exhibits a hot and dry climate starting from the spring months due to the climate zone in which it is located seasonal temperature differences can be effective on *S. aureus*, *Salmonella* spp., and *L. monocytogenes* presence in halloumi produced in Northern Cyprus.

In addition to being a commercial product of Cyprus, halloumi also plays an important role in paving the way for the export of animal foods to the EU market in accordance with Regulation (EC) No 866/2004, also known as the green line regulation and the commission implementing decision (EU) 2021/586. Under the Commission Implementing Regulation (EU) 2021/591, Halloumi is included in the scope of protected designations of origin. In this scope, studies are carried out in the north and south of the island for export to the EU. Following the completion of both compliances with EU food safety criteria and compliance with PDO criteria both in the north and south of the island, halloumi will be able to export to the EU within the scope of PDO.

The aim of this study is to evaluate the microbiological quality of halloumi sold in markets in Northern Cyprus, using a large sample size to ensure robust results. Given the hot climate of the region, the study also examines the impact of seasonal variations on microbial contamination, with a focus on ensuring food safety.

Materials and Methods

Sample Collection: In order to investigate the presence of *Salmonella* spp., *L. monocytogenes* and *S. aureus* in halloumi marketed in Northern Cyprus, halloumi samples were collected for 2 years from market shelves. 1072 halloumi samples were delivered to the Near East University Veterinary Medicine Food Hygiene and Technology Food Laboratory in their original packaging and by maintaining the cold chain.

Microbiological Analysis: AOAC 2013.01 bioMerueux Vidas UP SPT kit protocol was used for the isolation and identification of *Salmonella* spp. (6) and AOAC 2013.11 bioMerueux LMX kit protocol was used for the isolation and identification of *L. monocytogenes* (7). The details of both protocols are as follows: 25g of halloumi sample is mixed with 225 mL of Buffered Peptone Water in a blender for 2 minutes, then 1 mL of Salmonella supplement is added to the mixture and incubated at 42±1°C or 41.5±1°C for 18-24 hours. 2-3 mL of sample is boiled (5 minutes at 95-100°C) and VIDAS SPT results can be monitored in 48 minutes. 25g of halloumi sample is mixed with 225 mL of LMX Broth (with 0.5 mL of LMX supplement) in a blender for 2 minutes, then incubated at 37±1°C for 26-30 hours. 2-3 mL of sample is boiled (5 minutes at 95-100°C), and then VIDAS LMX results are determined (6, 7).

Isolation and identification of coagulase positive *S. aureus* was performed according to TS 6582-1 EN ISO 6888-1 standard by observing coagulase positive staphylococcal colonies after aerobic incubation at 34°C to 38°C in Baird-Parker solid medium (23).

Statistical Analysis: Statistically, the SPSS package program was used, and the existence of a significant relationship between seasons and values was determined by the chi-square independence test. Percentages were calculated with a cross tabulation table.

Results

As a result of this study, *Salmonella* spp. and *L. monocytogenes* could not be detected in any of the halloumi samples, while *S. aureus* was detected in different amounts: 39 (3.64%). The seasonal distribution of *S. aureus* presence in halloumi and its microbial loads and the percentages were shown in Table 1.

In this study, it was observed that the contamination of halloumi samples with *S. aureus* over $1x10^3$ cfu/g was most common in spring months (15 samples), followed by summer (12 samples). It was determined that the season where halloumi was least contaminated with *S. aureus* above $1x10^3$ cfu/g (3 samples) was winter (P=0.15). Totally, 92.35% of all positive samples were detected to contain *S. aureus* below $1x10^1$ cfu/g.

Discussion and Conclusion

In our study, 1072 halloumi samples were analyzed, and no samples were found to contain *Salmonella* spp. or *L. monocytogenes*, and this result complies with both the Turkish Food Codex Communiqué on Microbiological Criteria for food safety (3) and the EU microbiological criteria regulation for food safety criteria (2).

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Table 1. Microbial load and seasonal distribution of *S. aureus* in halloumi (P=0.15).

Season	<1x10^1 cfu/g n (%)	1x10^1-1x10^3 cfu/g n (%)	>1x10^3 cfu/g n (%)	Total
Winter (December January February)	249 (93.96%)	13 (4.91%)	3 (1.13%)	265
Spring (March April May)	309 (92.80%)	9 (2.70%)	15 (4.50%)	333
Summer (June July August)	205 (90.71%)	9 (3.98%)	12 (5.31%)	226
Autumn (September October November)	227 (91.53%)	12 (4.84%)	9 (3.63%)	248
Total	990 (92.35%)	43 (4.01%)	39 (3.64%)	1072

The survey studies for halloumi within the borders of island are limited. However, similar results were obtained in the past out of Cyprus. Regarding *S. aureus* incidence, Değirmencioğlu (10) found *S. aureus* in 2 of 34 halloumi samples (6%) and reported that only one of them (3%) contained more than ≥1x10^3 *S. aureus*, similar to our study. The researchers concluded that microbiological load and profile in the end product may originate from different sources (milk, starters, and contaminating microorganisms), and the growth of the microorganisms may be affected by factors such as raw milk usage and the maturating conditions (24). Usca and Erol (25) reported that they detected coagulase-positive staphylococci at the level of 10^3 cfu/g in 26% (13 samples) of 50 halloumi samples. With the study carried out by Eleftheriadou et al. (12), 21% of the dairy samples (Hellim, Flavuna, and Anari) obtained to contain *Salmonella* spp. and *L. monocytogenes*. In the same study, 12.415 cheese samples were analyzed for *S. aureus* and 132 of samples (1.1%) contain between 10^3 and 10^4 cfu/g, 90 (0.7%) of the samples contain 10^4 cfu/g, a total of 222 (1.8%) of the samples contain *S. aureus*. The possibility that the halloumi produced by boiling the curd may have been contaminated with *S. aureus* in the last step from food handlers and equipment before and/or during packaging.

A variety of raw milk cheeses purchased over the internet was investigated and 108 purchases from seven European countries were examined for the prevalence of *Salmonella* spp., *L. monocytogenes*, *Escherichia coli*, and coagulase positive staphylococci. In this study, *L. monocytogenes* was detected in 1.9% of all samples, one of which had counts of 9.5 x10^3 cfu/g. *Salmonella* spp. could not be detected in any of the samples. *E. coli* and *S. aureus* could be detected in a total of 29.6% (≥10 cfu/g; 32x10^8) and 8.3% (≥100 cfu/g; 9x10^8) of samples, respectively, indicating poor conditions of hygiene (20). Unlike many cheeses generally produced from raw milk, halloumi is a curd-cooked cheese. The cooking phase of the curd is a practice that allows the elimination of pathogens originating from raw milk.

Önganer et al. (17) reported that 30 pieces of cottage cheese sold unpackaged in Diyarbakir were contaminated with 7.80±0.64 log cfu/g *Salmonella* spp. and an average of 7.53 ±1.12 log cfu/g *S. aureus* and that this might be due to non-compliance with hygienic rules in the process from production to consumption expressed. *L. monocytogenes* was detected in 2 of 85 white cheese samples produced and/or sold in Antakya region, *Listeria* spp. in 7, *L. ivanovii* in 3, *L. innocua* in 3, and *L. seeligeri* in 2 (5). Cokal et al. (8) reported that 100 Mihaliç cheeses did not contain *Salmonella* spp., 5 samples contained *L. monocytogenes* and all of them were contaminated with *S. aureus* and contained an average of 2.69 log cfu/g *S. aureus*. As a result of a study on the microbiological quality of soft, ripened soft, and semi-hard cheeses obtained from raw, terminated or pasteurized milk and sold on the market in England, it was reported that *Salmonella* was not found in any of the cheeses. In the same study, out of a total of 1819 cheeses produced from raw and thermized milk, 1 of them was ≥ 10^2 cfu/g, 16 of them <10^2 cfu/g *L. monocytogenes*, 13 of them more than 10^5 cfu/g, 13 of them between 10^3<10^4 cfu/g and backwards. While the rest contained *S. aureus* less than 10^3 cfu/g, 4 of 2618 cheese samples obtained using pasteurized milk contained *L. monocytogenes* less than 10^2 cfu/g, while the remaining 2614 samples did not contain *L. monocytogenes* and 2 of them contained 1x10^4 cfu/g (1.9x10^4 cfu/g, 4x10^4 cfu/g) and 1 of them was found to be between 10^2 <10^3 cfu/g and the remaining samples were found to contain less than 10^2 cfu/g *S. aureus* (16). In a study on the presence of *S. aureus* and other staphylococci in cheese samples sold in the Bologna region that were examined, *S. aureus* was found mostly during the hot months, while the other common species were found mostly in the period October–March (11). Teymori et al. (21) reported that *S. aureus* was found in 2

($4.75x10^2$ and $2.8x10^2$ cfu/g) of 30 cheese samples in a study conducted in West Azerbaijan region.

In the studies on the bacterial contamination of raw milk according to the seasons, Fadaei (13) stated that coliform, *E. coli*, and *S. aureus* contamination is obtained mostly in summer in 29 (96.66%) of the samples. Vahedi et al. (26), in their study, found that the highest rate of contamination of raw milk with *E. coli*, coliform, and *S. aureus* in different seasons was observed in summer and that the samples were 24 (57.1%) *E. coli*, 19 (52.8%) coliform, and 10 (45.4%) reported that it was contaminated with *S. aureus*. In current study we performed; totally 39 samples were obtained to contain *S. aureus* above $1x10^3$ cfu/g and 15 of these results were obtained in spring season which is a rainy and warm period for Cyprus.

As a conclusion of this study, absence of *Salmonella* spp. and *L. monocytogenes* hazard in halloumi make us think of a positive result in terms of food safety. On the other hand, further studies should be performed on the staphylococcal enterotoxin incidence. The boiling stage of curd makes decontamination of *S. aureus*, but the toxin which leads to food poisoning may still be presence. *S. aureus* can also contaminate to halloumi at the folding stage, at which the food handlers are in direct contact with cheese. We believe the fact that dairy product manufacturers in Northern Cyprus have largely adopted the principle of "Good Production Practices" and that they have knowledge of the principles of the Hazard Analysis and Critical Control Point (HACCP) system which based on monitoring and catching hazards from beginning to the end of manufacturing process. The most intense detection of *S. aureus* was at levels above $1x10^3$ cfu/g. This may also be because of the high temperatures in the summer months of Cyprus and the abuse of cold-chain.

The development of hygiene conditions, the application of cold milk application on the basis of all farms, the adoption of good production practices at every stage of the product, the systematic training of the food handlers on food hygiene and safety, the well-determined and monitoring of critical control points during the process flow and the selection of raw materials are accepted as important parameters to obtain a safe product. We are of the opinion that more serious implementation of the storage conditions until the end of the shelf life of the final product, especially taking all the necessary measures to prevent the cold chain and ensuring traceability, will contribute significantly to the competitiveness of the product in the foreign market by increasing the safety of the product.

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Ethical Statement

This study does not present any ethical concerns.

Conflict of Interest

The authors confirm that they have no conflicts of interest with respect to the work described in this manuscript.

Author Contributions

FIM was responsible for the planning and execution of the study. FKY edited the article. BHU performed the analysis, drafted the article, and conducted a critical revision. CH was responsible for the final revision.

Data Availability Statement

The data supporting this study's findings are available from the corresponding author upon reasonable request.

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The willingness of Turkish consumers in different sociodemographic groups to try and consume in-vitro meat

Ayse Gulin ESER[1,a,✉], P. Dilara KECİCİ[2,b], Funda YILMAZ EKER[3,c], Bülent EKİZ[2,d]

[1]Çanakkale Onsekiz Mart University, Biga Vocational School, Department of Food Technology, Çanakkale, Türkiye; [2]Istanbul University-Cerrahpaşa, Faculty of Veterinary Medicine, Department of Animal Breeding and Husbandry, Istanbul, Türkiye; [3]Istanbul University-Cerrahpaşa, Faculty of Veterinary Medicine, Department of Food Hygiene and Technology, Istanbul, Türkiye.

[a]ORCID: 0000-0001-8799-3073; [b]ORCID: 0000-0003-1151-179X; [c]ORCID: 0000-0003-4315-5363; [d]ORCID: 0000-0001-6458-5747

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✉**Corresponding author**
gsezen@comu.edu.tr

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ABSTRACT

This study was conducted to determine the willingness of Turkish consumers in various socio-demographic groups to try, consume, and pay for in-vitro meat. The study was applied to potential participants through social media via Google Forms in the form of a questionnaire, and 989 responses were collected. Males were more willing than females to try in-vitro meat, consume it regularly, and try it when recommended. People who have master's and doctoral degrees are more willing to try in-vitro meat compared to those with other education levels. Related professionals, students, and health workers are more willing to try in-vitro meat directly and even more willing to try it, if recommended, than other occupational groups. The willingness to try, to consume regularly, and to try on recommendations were higher in the Mediterranean, Aegean, and Central Anatolia regions compared to others. Compared to individuals with one or two children, those without children and those with three or more children were found to be more willing to try in-vitro meat and to try if recommended. In-vitro meat is not yet commercially marketed in TürkiyeTurkey, and this study addresses the perceptions and opinions of consumer groups in different socio-economic statuses about in-vitro meat.

Introduction

Due to environmental, animal welfare, food safety, and public health issues, the rapid increase of the world population, and limited arable land and water resources, it is claimed that it will not be possible to meet the increasing demand for meat in the future by conventional meat production which is obtained from livestock (1, 12, 21, 39).

Therefore, despite advances in traditional breeding and production systems, researchers and private companies have been driven to develop alternatives for vegetarian meat substitutes (18).

In this context, researchers have focused on a new meat alternative derived from the living stem cells of farm animals (26). Various names have been used for this new product, such as cell-based meat or, more commonly, artificial meat, cultured meat, in-vitro meat, slaughter-free meat, or lab-grown meat (6). It has been hypothesised that conventional meat production will not be sufficient to solve issues such as ethics, environment, health, and hunger. Based on this hypothesis, the issue of in-vitro meat tends to be a current issue in industrial, political, social, and scientific terms (7). This new food product is also called clean meat because it is claimed to use fewer hormones and fewer resources, as well as being less harmful and less polluting to the environment. It is also proposed that this product will contribute to meeting the daily protein requirement (21).

Bioartificial muscles are produced from skeletal muscle stem cells, also known as satellite cells (10, 33). In-vitro meat is obtained from embryonic stem cells or embryonic myoblasts taken by biopsy from a living animal. These cells are developed in a culture medium with appropriate laboratory conditions for their

proliferation. In this context, Chriki and Hocquette (9), Hocquette et al. (17) and Post (27) reported that many cells proliferate, fuse and form muscle fibre clusters. After that, it's ready to be eaten like a burger (9, 23). This biotechnology came out in 2013 for the first time, and it awakened a strong interest in both scientific and mediatic areas (8, 13). Many surveys have been conducted in various countries to investigate consumers' attitudes on this issue and to assess their perceptions and consent to purchase and consume such a product. The common view in these surveys is that while many consumers have shown a willingness to taste "in-vitro meat" once, they are not yet ready to consume it regularly; however, a large section of them do not really know anything about in-vitro meat (16, 17, 22, 36, 37).

Animal origined food sources are valuable for nutrition because of their high protein contents. A large proportion (85%) of the daily calories consumed per capita in Türkiye are herbal. In recent years, the demand for red meat has increased. In 2022, red meat consumption per capita was 23.9 kg for Türkiye. Of this consumption, 18.44 kg was met from cattle and 5.5 kg from small ruminants. On the other hand, 4413 tonnes of red meat were imported in 2023 because the red meat production in Türkiye had not been enough to meet the need (14). There is no production or sale of artificial meat in Türkiye. Meat consumption preferences may differ in various countries depending on socioeconomic factors, ethics, religious beliefs, or traditions (11).

Similarly, it is observed that there are differences between the results of studies conducted in various countries in terms of the approach to in-vitro meat (17). Considering this information, this study aimed to determine the willingness of Turkish consumers of various socio-demographic groups to try, consume, and pay for in-vitro meat.

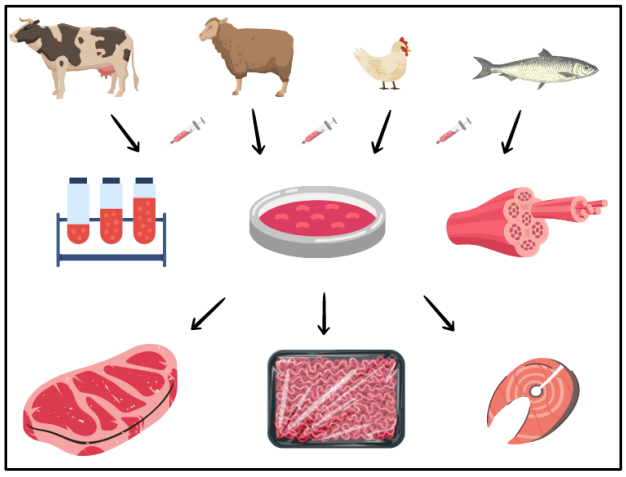
Materials and Methods

The procedures of the study were submitted to Çanakkale Onsekiz Mart University Graduate Education Institute Ethics Committee, and the project was approved by the Scientific Research Ethics Committee (Approval No: 2023-YÖNP-0498, Acceptance date: 21/06/2023, Decision number: 08/07).

Design of the Questionnaire: This study was conducted from July to September 2023. The target group of the questionnaire was people over the age of 18 years living in Türkiye. The questionnaire was administered in Turkish. The questionnaire consisted of 4 parts. The main headings of these sections are i. Sociodemographic information, ii. A brief introduction to in-vitro meat, iii. Questions about the willingness to try, consume, and purchase in-vitro meat, and iv. The extent of which respondents agree with various opinions presented about in-vitro meat.

The first part of the questionnaire is about the sociodemographic information of the participants. In this section, the participants were asked about their age, gender, education level, province of residence, number of children, income status, meat consumption habits, frequency of meat consumption, and whether they had heard of the concept of in-vitro meat before. The professions of the participants were grouped as follows: civil servants, healthcare professionals, housewives, related professions, retired, self-employed, skilled worker/service professionals, small business owners, students, unemployed, and white collars. Veterinarians, agricultural engineers, and food engineers who have received training on the biochemical properties of meat are categorised under "Related Professions". While determining the subgroups of household monthly income, the minimum wage in Türkiye was used as a base for the period when the survey was conducted.

Since in-vitro meat is not available in markets in Türkiye and considering that consumers may have limited knowledge about this product, the definition and visual expression of in-vitro meat were included in the second part of the questionnaire (Figure 1). In the second part of the questionnaire, a security question (Please tick "yes" in this question so that we can evaluate data security) was also asked to determine its security. Those who did not answer “yes” were excluded from the evaluation.



Artificial meat is a meat product produced in a laboratory using muscle stem cells that are never part of a living animal. This product is also called "cultured meat, cell-based meat, in-vitro meat, laboratory meat, clean meat, or synthetic meat." Stem cells are taken without causing any discomfort to living animals and turned into tissue in a controlled laboratory environment. Artificial meat is bioidentical to meat tissue derived from animals. Artificial meat products are not yet available for retail sale in Turkey.

Figure 1. Brief introductory information about artificial meat presented to participants.

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There were two parts in the third section of the questionnaire. In the first part, participants were asked about their willingness to try in-vitro meat, whether they would consider consuming it regularly, and whether they would be willing to try it if it was recommended. Participants were asked to answer this section on a Likert scale of 1-5 (1: Absoutely no, 2: No, 3: Not sure, 4: Yes, 5: Absolutely yes). In the second part, they were asked how much they would be willing to pay for in-vitro meat compared with farmed meat. Respondents were asked to answer 1: Much less, 2: Somewhat less, 3: Similar, 4: Somewhat more, 5: Much more

In the fourth part of the questionnaire, 32 different opinions about in-vitro meat were presented, and the participants marked this section, which was prepared to determine the perception levels, with a Likert scale of 1-5 (1: Strongly disagree, 2: Disagree, 3: Undecided, 4: Agree, 5: Strongly agree). Since the perceptions of the participants and their willingness to consume are intended to be discussed in different articles, the part of the study related to the fourth section is not interpreted and evaluated in this article.

Before the questionnaire was applied, a pilot survey was conducted to evaluate the comprehensibility, applicability, and usability of the questions, and 27 people participated in this survey online. As a result of the pilot survey, corrections were made to the Turkish wording and the fourth part of the questionnaire in line with the evaluations and comments of the participants.

Participants and Data Collection: The questionnaire form was prepared via Google Forms and delivered to potential participants via social media. "Informed voluntary consent text" was presented to the participants to inform them before participating in the survey and the consent text was expressed as follows, “We invite you to the research titled "Determination of Perceptions and Attitudes of Consumers in Türkiye on Artificial Meat (In-vitro Meat). The aim of this research is to analyse the knowledge, attitudes, and approaches of consumers in Türkiye regarding the concept of in-vitro meat and their perspectives on in-vitro meat. Participation in this study is completely voluntary. For the study to achieve its purpose, you are expected to answer all the questions completely, without being under any pressure or suggestion from anyone, and sincerely give the answers that suit you best. Reading and approving this form will mean that you agree to participate in the study. However, you also have the right not to participate in the study or to leave the study at any time after participation. The information obtained from this study will be used entirely for research." In addition, in the informed consent text, the e-mail address of the project coordinator was also provided so that the participants could reach the project coordinator.

A total of 1009 people participated in the questionnaire. Of these people, six people under the age of 18 were excluded from the evaluation. 9 people clicked on the link and answered "no" to the question "I agree to participate in the research". 5 people did not answer the security question. After cleaning, the data of 989 participants were evaluated.

***Statistical Analyses*:** Distribution of participants according to subgroups of demographic characteristics and descriptive statistics were determined using the Jamovi 2.3.21 programme (32). The Kruskall - Wallis test was applied in the Jamovi 2.3.21 programme to determine the effect of demographic characteristics on the participants' willingness to try in-vitro meat, to consume it regularly, to consume it instead of conventional meat, and to consume it if it is recommended by a friend and how much they are willing to pay compared to meat from farm animals. In case of significance for a sociodemographic characteristic, the Dwass-Steel-Critchlow-Fligner pairwise comparison test was performed (32).

Results

In the study, 989 responses were collected through an online survey. Regardless of sociodemographic characteristics, the general results related to the willingness of the participants to try, consume and pay for in-vitro meat are presented in Table 1. The mean scores given by participants for willingness to try in-vitro meat, consume it regularly, consume it instead of farmed animal meat, try it if recommended by friends, and pay for it compared to farmed animal meat were 2.44, 1.96, 2.03, 2.52, and 1.49, respectively.

Table 1. Mean, standard deviation (SD) and median values for the willingness of the respondents to try, consume, and pay for in-vitro meat.

Question	Mean	SD	Median
Would you be willing to try "in-vitro meat"?	2.44	1.27	2
Would you be willing to consume "in-vitro meat" regularly?	1.96	0.95	2
Would you be willing to consume "in-vitro meat" instead of meat from farmed animals?	2.03	1.09	2
Would you be willing to try "in-vitro meat" if it was recommended by your friends?	2.52	1.26	2
How much would you be willing to pay for in-vitro meat compared to meat from farmed animals?	1.49	0.82	1

The significance of the sociodemographic factors affecting the participants' willingness to try, consume, and pay for in-vitro meat is given in Table 2. Except for the frequency of meat consumption and familiarity with in-vitro meat, the effect of other factors on the willingness of the participants to try in-vitro meat and to try if recommended by their friends was found to be significant. The effect of education level on the willingness to regularly consume in-vitro meat was found to be insignificant (P>0.05). Sociodemographic factors other than gender, geographical region, and familiarity with in-vitro meat were also reported to have a significant effect on the willingness to consume in-vitro meat. On the other hand, the effects of age group (P<0.001), geographical region (P<0.01), number of children (P<0.001), dietary habits (P<0.001) and frequency of meat consumption (P<0.001) on how much participants were willing to pay for in-vitro meat were found to be important when compared with conventional meat.

The effect of gender, age group, and education level of the participants on their willingness to try, consume, and pay for in-vitro meat is presented in Table 3. In general, males were more willing than females to try in-vitro meat, consume it regularly, and try it when recommended. The differences between male and female participants in terms of willingness to consume and pay for in-vitro meat compared with meat from farm animals were found to be insignificant (P>0.05). The difference between the 18-25 and 26-35 age groups in terms of participants' willingness to try in-vitro meat, consume it regularly, consume it instead of traditional meat and willingness to pay was found to be insignificant. However, it was determined that the willingness of the participants aged 36-45 years and above to consume, try, and pay for in-vitro meat decreased (Table 3).

It is seen that people who have master's and doctoral degrees are more willing to try in-vitro meat compared to those ğ with other education levels. Those with technical school and bachelor's degrees were more willing to try in-vitro meat than those with primary and high school degrees. The willingness to try in-vitro meat if recommended by a friend was lower in primary, secondary, and high school graduates than in the other groups. In terms of willingness to consume in-vitro meat instead of meat obtained from farm animals, the ones with master's and doctoral degrees gave highest scores, yet the difference between them and highschool and technical school graduates were not significant. However, primary and middle school graduates had lower willingness to consume the in vitro meat when compared to ones which has higher educatation degrees (Table 3).

The effects of occupational groups and geographical regions on the willingness to try in-vitro meat, consume it regularly, consume it instead of farm animal meat, consume it if recommended, and willingness to pay are presented in Table 4. When compared with the occupational groups, it is seen that related professionals, students, and health workers are more willing to try in-vitro meat directly and even more willing to try it if it is recommended than other occupational groups. In terms of regular consumption and willingness to consume instead of conventional meat, it is seen that professionals and students are more willing. In terms of willingness to pay, the difference between occupational groups was found to be insignificant (Table 4). Regionally, it was found that the willingness to try, to consume regularly, and to try on recommendations were higher in the Mediterranean, Aegean, and Central Anatolia regions compared to other regions. There was no significant difference between the regions in terms of willingness to consume instead of livestock meat.

The effects of having children and monthly income on the willingness to try in-vitro meat, consume it regularly, consume it as a substitute for farm animal meat, consume it if recommended, and willingness to pay are presented in Table 5. Compared to people with one or two children, those without children and those with 3 or more children were found to be more willing to try in-vitro meat and to try it if recommended. People without children were found to be more willing to consume in-vitro meat instead of conventional meat (Table 5). People with a monthly income of 80.501 TL and above, 69.001-80.500 TL, and 57.501-69.000 TL had a higher willingness to try in-vitro meat, consume it regularly, consume it instead of conventional meat, and try it if recommended compared with other groups. There was no significant difference between the income groups in terms of willingness to pay (Table 5).

The effects of meat consumption habits, frequency of meat consumption, and familiarity with in-vitro meat on the willingness to try in-vitro meat, consume it regularly, consume it as a substitute for farm animal meat, consume it if recommended, and pay for it are presented in Table 6. White meat and seafood consumers and vegans were found to be more willing to try, to consume instead of traditional farmed meat, and to try if recommended compared with other groups. The willingness to consume regularly was higher in white meat and seafood consumers than in the other groups, while the willingness to pay was higher in vegans than in the other groups (Table 6).

In terms of frequency of meat consumption, the willingness to consume and pay for in-vitro meat instead of farm animal meat was found to be higher in those who never consumed meat. The effect of familiarity with in-vitro meat on the willingness to try, consume, and pay for in-vitro meat was found to be insignificant (P>0.05; Table 6).

Table 2. Significance levels of investigated factors on the willingness of the respondents to try, consume and pay for in-vitro meat (IVM).

Factor		Willing to try IVM		Willing to consume IVM regularly		Willing to consume IVM instead of meat from farmed animals		Willing to try IVM if it was recommended by friends		Willing to pay for IVM compared to meat from farmed animals	
	df	χ^2	P	χ^2	P	χ^2	P	χ^2	P	χ^2	P
Gender	2	14.81	<0.001	9.24	0.010	1.66	0.436	8.13	0.017	5.68	0.058
Age group	4	41.20	<0.001	24.60	<0.001	24.60	<0.001	46.50	<0.001	22.00	<0.001
Education Level	3	29.41	<0.001	7.11	0.068	7.90	0.048	19.79	<0.001	4.14	0.246
Occupation	10	74.90	<0.001	45.10	<0.001	36.20	<0.001	69.70	<0.001	16.20	0.093
Geographic Region	5	14.02	0.015	12.52	0.028	9.15	0.103	14.59	0.012	18.96	0.002
Number of Children	3	23.52	<0.001	8.27	0.041	22.89	<0.001	21.83	<0.001	25.59	<0.001
Household monthly income	7	30.30	<0.001	23.80	0.001	20.80	0.004	27.00	<0.001	10.10	0.183
Eating habits	3	16.40	<0.001	12.60	0.006	33.10	<0.001	15.10	0.002	98.20	<0.001
Meat consumption frequency	3	0.925	0.819	3.122	0.373	17.12	<0.001	0.838	0.840	88.37	<0.001
Familiarity with "in-vitro meat"	2	2.04	0.361	4.37	0.112	1.07	0.584	2.86	0.239	3.61	0.165

Table 3. The effects of gender, age group and education level of the respondents on willingness to try, consume and pay for in-vitro meat.

Factor		Willing to try		Willing to consume regularly		Willing to consume instead of conventional meats		Willing to try if recommended		Willing to pay	
	N	Mean (SD)	Med	Mean (SD)	Med	Mean (SD)	Med	Mean (SD)	Med	Mean (SD)	Med
Gender											
Female	557	2.30 ^{b} (1.19)	2	1.88 ^{b} (0.92)	2	2.02 (1.13)	2	2.43 ^{b} (1.24)	2	1.54 (0.86)	1
Male	419	2.64 ^{a} (1.35)	2	2.07 ^{a} (0.99)	2	2.06 (1.05)	2	2.65 ^{a} (1.29)	3	1.43 (0.74)	1
No wish to answer	13	2.31 ^{ab} (1.11)	2	1.77 ^{ab} (0.60)	2	1.85 (0.80)	2	2.08 ^{ab} (0.95)	3	1.23 (0.83)	1
Age group (years)											
18-25	163	2.85 ^{a} (1.25)	3	2.13 ^{a} (0.91)	2	2.18 ^{a} (1.05)	2	2.91 ^{a} (1.16)	3	1.62 ^{a} (0.85)	1
26-35	213	2.69 ^{a} (1.35)	3	2.15 ^{ab} (1.08)	2	2.27 ^{a} (1.20)	2	2.80 ^{a} (1.33)	3	1.61 ^{ab} (0.89)	1
36-45	274	2.30 ^{b} (1.27)	2	1.90 ^{bc} (0.94)	2	2.01 ^{ab} (1.20)	2	2.42 ^{b} (1.28)	2	1.47^{abc} (0.86)	1
46-55	210	2.27 ^{b} (1.16)	2	1.82 ^{c} (0.84)	2	1.86 ^{b} (0.95)	2	2.29 ^{b} (1.18)	2	1.40 ^{bc} (0.71)	1
>56	129	2.09 ^{b} (1.11)	2	1.77 ^{c} (0.87)	2	1.78 ^{b} (0.92)	2	2.14 ^{b} (1.14)	2	1.29 ^{c} (0.64)	1
Education Level											
Primary & Middle School	31	1.74 ^{c} (0.73)	2	1.71 (0.64)	2	1.68 ^{b} (0.70)	2	2.06 ^{b} (0.89)	2	1.26 (0.58)	1
High School	99	2.00 ^{c} (1.01)	2	1.77 (0.78)	2	1.80 ^{ab} (0.87)	2	2.08 ^{b} (1.08)	2	1.40 (0.71)	1
Technical college & Undergraduate degree	587	2.44 ^{b} (1.27)	2	1.95 (0.95)	2	2.03 ^{ab} (1.09)	2	2.54 ^{a} (1.25)	3	1.49 (0.83)	1
Master's & PhD degrees	272	2.69 ^{a} (1.33)	3	2.07 (1.01)	2	2.16 ^{a} (1.17)	2	2.69 ^{a} (1.33)	3	1.53 (0.85)	1

^{a,b,c} The differences between subgroups that do not have a common letter in the same column are significant (P<0.05). SD: Standard deviation, Med: Median.

Table 4. The effects of occupation and geographic region of the respondents on willingness to try, consume and pay for in-vitro meat.

Factor		Willing to try		Willing to consume regularly		Willing to consume instead of conventional meats		Willing to try if recommended		Willing to pay	
	n	Mean (SD)	Med	Mean (SD)	Med	Mean (SD)	Med	Mean (SD)	Med	Mean (SD)	Med
Occupation											
Civil servants	154	2.14 ^{c} (1.20)	2	1.82 ^{cd} (0.92)	2	1.90 ^{bc} (1.06)	2	2.29 ^{cd} (1.28)	2	1.38 (0.75)	1
Healthcare professionals	66	2.50 ^{b} (1.18)	3	2.11 ^{ab} (0.90)	2	2.23 ^{ab} (1.06)	2	2.61 ^{bc} (1.20)	3	1.55 (0.79)	1
Housewifes	45	1.71 ^{d} (0.90)	1	1.62 ^{d} (0.81)	1	1.64 ^{c} (0.83)	1	1.93 ^{d} (1.03)	2	1.47 (0.87)	1
Related professions[1]	72	3.18 ^{a} (1.35)	3	2.38 ^{a} (1.07)	2	2.42 ^{a} (1.24)	2	3.21 ^{a} (1.22)	3	1.54 (0.77)	1
Retired people	65	2.05 ^{cd} (1.11)	2	1.71 ^{d} (0.81)	2	1.78 ^{c} (0.93)	2	1.92 ^{d} (1.01)	2	1.23 (0.52)	1
Self-employed	30	1.90 ^{cd} (1.09)	2	1.57 ^{d} (0.73)	1	1.77 ^{c} (0.90)	1.5	2.07 ^{cd} (1.17)	2	1.43 (0.77)	1
Skilled worker/service professionals	79	2.43 ^{bc} (1.25)	2	2.01 ^{bc} (0.90)	2	1.91 ^{bc} (0.93)	2	2.46 ^{c} (1.14)	2	1.44 (0.71)	1
Small business owners	40	2.27 ^{bc} (1.06)	2	1.95 ^{bc} (0.88)	2	1.93 ^{abc} (0.97)	2	2.40 ^{cd} (1.19)	2	1.50 (1.01)	1
Students	97	2.93 ^{a} (1.26)	3	2.21 ^{ab} (0.97)	2	2.31 ^{a} (1.08)	2	2.97 ^{ab} (1.16)	3	1.55 (0.76)	1
Unemployed people	15	2.27 ^{bc} (1.28)	2	1.53 ^{d} (0.52)	2	1.53 ^{c} (0.92)	1	2.60 ^{bc} (1.12)	3	1.40 (0.63)	1
White collars	326	2.52 ^{b} (1.29)	2	1.97 ^{bc} (0.99)	2	2.08 ^{b} (1.16)	2	2.60 ^{bc} (1.31)	3	1.56 (0.91)	1
Geographic Region											
Aegean	85	2.71 ^{a} (1.36)	3	2.07 ^{ab} (0.96)	2	2.18 (1.15)	2	2.80 ^{a} (1.28)	3	1.66 ^{a} (0.85)	1
Black Sea	57	2.28 ^{bc} (1.36)	2	1.77 ^{c} (1.00)	1	1.82 (1.07)	1	2.37 ^{bc} (1.35)	2	1.42 ^{bc} (0.89)	1
Central Anatolia	106	2.62 ^{ab} (1.24)	2	2.04 ^{ab} (0.89)	2	2.04 (1.03)	2	2.68 ^{ab} (1.22)	3	1.54 ^{ab} (0.71)	1
Eastern and Southeastern Anatolia	33	2.03 ^{c} (1.08)	2	1.73 ^{bc} (0.76)	2	1.82 (0.95)	2	2.03 ^{c} (0.95)	2	1.18 ^{c} (0.64)	1
Marmara	659	2.39 ^{bc} (1.25)	2	1.94 ^{bc} (0.96)	2	2.02 (1.09)	2	2.48 ^{bc} (1.27)	2	1.47 ^{bc} (0.83)	1
Mediterranean	49	2.73 ^{a} (1.29)	3	2.22 ^{a} (0.99)	2	2.31 (1.14)	2	2.76 ^{ab} (1.22)	3	1.59 ^{ab} (0.81)	1

^{a,b,c} The differences between subgroups that do not have a common letter in the same column are significant (P<0.05).
SD: Standard deviation, Med: Median.

Table 5. The effects of number of children and household monthly income of the respondents on willingness to try, consume and pay for in-vitro meat.

Factor		Willing to try		Willing to consume regularly		Willing to consume instead of conventional meats		Willing to try if recommended		Willing to pay	
	n	Mean (SD)	Med	Mean (SD)	Med	Mean (SD)	Med	Mean (SD)	Med	Mean (SD)	Med
Number of Children											
0	415	2.67 ^{a} (1.33)	3	2.06^{a} (1.12)	2	2.24 ^{a} (1.20)	2	2.74 ^{a} (1.31)	3	1.64 ^{a} (0.93)	1
1	270	2.32 ^{b} (1.20)	2	1.90^{ab} (0.88)	2	1.89 ^{b} (0.98)	2	2.40 ^{b} (1.19)	2	1.43 ^{b} (0.73)	1
2	229	2.20 ^{b} (1.239	2	1.84 ^{b} (0.93)	2	1.83 ^{b} (0.98)	2	2.29 ^{b} (1.22)	2	1.34 ^{b} (0.69)	1
≥3	75	2.35 ^{ab} (1.11)	2	1.96^{ab} (0.83)	2	2.01 ^{ab} (0.99)	2	2.41 ^{ab} (1.19)	2	1.27 ^{b} (0.58)	1
Household monthly income (TL)											
≤11 500 TL	105	2.37 ^{c} (1.33)	2	1.96 ^{bc} (1.01)	2	2.10 ^{ab} (1.19)	2	2.45^{c} (1.28)	2	1.53 (0.87)	1
11 501 – 23 000 TL	246	2.28 ^{c} (1.19)	2	1.87 ^{bc} (0.89)	2	1.90 ^{b} (0.99)	2	2.40 ^{c} (1.19)	2	1.42 (0.74)	1
23 001 – 34 500 TL	206	2.29 ^{c} (1.24)	2	1.83 ^{c} (0.93)	2	1.90 ^{b} (1.10)	2	2.34 ^{c} (1.22)	2	1.51 (0.78)	1
34 501 – 46 000 TL	155	2.52 ^{bc} (1.28)	2	2.01 ^{ab} (0.92)	2	2.09 ^{ab} (1.08)	2	2.59 ^{bc} (1.25)	3	1.45 (0.86)	1
46 001 – 57 500 TL	101	2.29 ^{c} (1.11)	2	1.86 ^{bc} (0.91)	2	1.97 ^{b} (1.01)	2	2.39 ^{c} (1.14)	2	1.44 (0.78)	1
57 501 – 69 000 TL	58	2.81 ^{ab} (1.36)	3	2.21 ^{a} (1.01)	2	2.16 ^{ab} (1.09)	2	2.83 ^{ab} (1.33)	3	1.41 (0.80)	1
69 001 – 80 500 TL	45	2.89 ^{ab} (1.50)	3	2.36 ^{a} (1.13)	2	2.49 ^{a} (1.27)	3	3.02 ^{ab} (1.56)	4	1.53 (0.79)	1
≥ 80 501 TL	73	2.99 ^{a} (1.26)	3	2.19 ^{a} (0.97)	2	2.32 ^{a} (1.15)	2	3.01 ^{a} (1.30)	3	1.73 (1.02)	1

^{a,b,c} The differences between subgroups that do not have a common letter in the same column are significant (P<0.05). SD: Standard deviation, Med: Median.

Table 6. The effects of eating habits, meat consumption frequency and familiarity with IVM of the respondents on willingness to try, consume and pay for in-vitro meat.

Factor		Willing to try		Willing to consume regularly		Willing to consume instead of conventional meats		Willing to try if recommended		Willing to pay	
	n	Mean (SD)	Med	Mean (SD)	Med	Mean (SD)	Med	Mean (SD)	Med	Mean (SD)	Med
Eating habits											
Meat eating	879	2.41^{b} (1.26)	2	1.94^{b} (0.94)	2	1.95^{b} (1.02)	2	2.48^{b} (1.25)	2	1.39^{c} (0.71)	1
White meat only & Pescatarian	38	3.05^{a} (1.25)	3	2.47^{a} (1.03)	2	2.68^{a} (1.28)	2	3.18^{a} (1.25)	3	1.84^{b} (0.89)	2
Vegan	44	2.86^{a} (1.37)	3	2.00^{b} (0.99)	2	2.98^{a} (1.49)	3	2.86^{ab} (1.39)	3	2.64^{a} (1.20)	3
Vegetarian	28	2.07^{b} (1.22)	2	1.75^{b} (0.93)	1.5	2.11^{b} (1.34)	2	2.25^{b} (1.24)	2	2.29^{ab} (1.18)	2.5
Meat consumption frequency											
Never	77	2.60 (1.41)	2	1.94 (1.02)	2	2.66^{a} (1.15)	2	2.66 (1.40)	3	2.47^{a} (1.18)	3
Rarely	305	2.39 (1.20)	2	1.98 (0.96)	2	2.06^{b} (1.08)	2	2.52 (1.21)	2	1.45^{b} (0.78)	1
Regularly	564	2.44 (1.28)	2	1.96 (0.93)	2	1.96^{bc} (1.01)	2	2.50 (1.27)	2	1.38^{b} (0.67)	1
Everyday	43	2.51 (1.37)	2	1.77 (0.97)	2	1.70^{c} (0.86)	2	2.53 (1.26	3	1.33^{b} (0.89)	1
Familiarity with “in-vitro meat”											
Heard and know	256	2.51 (1.49)	2	1.93 (1.11)	2	2.08 (1.30)	2	2.45 (1.46)	2	1.63 (1.01)	1
Heard but not know	530	2.47 (1.22)	2	1.96 (0.92)	2	2.05 (1.06)	2	2.56 (1.21)	3	1.44 (0.74)	1
Never heard before	203	2.29 (1.06)	2	1.98 (0.80)	2	1.94 (0.85)	2	2.49 (1.11)	2	1.42 (0.69)	1

^{a,b,c} The differences between subgroups that do not have a common letter in the same column are significant (P<0.05). SD: Standard deviation, Med: Median.

Discussion and Conclusion

In this study, participants responded to willingness questions on a scale of 1 (Definitely No) to 5 (Definitely Yes). The average scores were 2.44 for willingness to try in-vitro meat, 1.96 for willingness to consume in-vitro meat regularly, and 2.52 for willingness to try in-vitro meat if recommended by friends. In a study conducted in the UK with 1010 male and 1072 female participants, it was emphasised that the reaction to the idea of in-vitro meat was predominantly negative; half of the consumers clearly rejected it and only 16% stated that they would buy it, while 33% were not sure (35). Baybars et al. (5), in a similar study, reported that the participants did not have a positive attitude towards regular consumption of in-vitro meat, experimentation, and recommendations. Zhang et al. (40) reported that 84.72% of 1004 participants were willing to try in-vitro meat. These reports indicate that there are differences among countries in approach to in-vitro meat. However, the results obtained in our study show that Turkish consumers are more distant from in-vitro meat than the studies listed above.

Another notable finding of the study is that nearly all participants responded with 'Much less' or 'Somewhat less' to the question, 'How much would you be willing to pay for in-vitro meat compared to meat from farmed animals?' The average score for this question was 1.49. Van Loo et al. (34), in a survey of more than 1800 consumers in the USA, presented the choice between farm-raised beef and in-vitro meat, holding the price constant, and reported that 72% of the respondents chose beef and 5% chose in-vitro meat, and that even if the price of in-vitro meat decreased, the market shares of farm-raised beef was higher. In a study conducted in India, it was reported that participants tended to pay less for in-vitro meat compared with conventional meat (3). Liu et al. (20) reported that 94% of the participants in their study stated that they were not willing to pay more for in-vitro meat than for conventional meat.

In our study, compared with women, men were more willing to try in-vitro meat (mean score: 2.64 vs. 2.30), to consume it regularly (mean score: 2.07 vs. 1.88) and to try it if recommended (mean score: 2.65 vs. 2.43). In a similar study, Shaw and Iomaire (29) reported that 63% of men reported that they were willing to try cultured meat, whereas this rate was only 46% for women. Also, in a study conducted in the USA, it was stated that men are more willing to consume in-vitro meat (37). According to the studies, it can be concluded that men are more open to trying in-vitro meat. While women tend to turn to vegetables and fruits as meat alternatives, it is also seen that men may prefer in-vitro meat with high similarity to meat as a meat alternative.

In our study, when the relationship between the willingness to try in-vitro meat and age groups was

examined, it was determined that the willingness of the participants to try in-vitro meat, to consume it regularly, to consume it instead of traditional meat, to try it if recommended, and to pay was generally higher in participants aged 18-25 and 26-35 years, whereas the willingness decreased in participants older than this age. Shaw and Iomaire (29) reported that there was a statistically significant relationship between the age of the participants and their willingness to try cultured meat, and that those under the age of 35 were more willing to try cultured meat than those over the age of 55. In a study conducted in the UK, it was reported that consumers over the age of 55 were the least likely to purchase in-vitro meat (35). In a study conducted in Italy, it was reported that those who wanted to try in-vitro meat were 71% of participants under the age of 25, 56% of participants between the ages of 25 and 45, 47% of participants between the ages of 46 and 65, and 40% of participants over the age of 65 (22). The finding obtained in our study that "Younger age participants are more willing to try, consume, and pay for in-vitro meat, and as the age of the participants increases, the level of willingness to try and consume decreases" is generally compatible with the results previously reported in other countries (22, 29, 35).

When evaluating education levels in our study, it was determined that people with master's and doctorate degrees were more willing to try in-vitro meat and consume it instead of conventional meat compared with people with other education levels. In a study conducted in Italy, it was reported that participants with a higher level of education had a more favourable attitude towards in-vitro meat, with 62% of highly educated participants willing to try in-vitro meat when compared with less educated participants**,** who were only 36% (22). Supporting the results of the present study, it has been reported in many previous studies that highly educated individuals have a more positive view of in-vitro meat (22, 31, 38, 40). Sikora and Rzymski (30) stated that education level did not play a role in accepting of in-vitro meat. On the other hand, the difference between educational level groups in terms of willingness to consume in-vitro meat regularly and willingness to pay for in-vitro meat was found to be insignificant. This result indicates that although highly educated Turkish consumers are more willing to try in-vitro meat than other education groups, they have not yet adopted the idea of regular consumption and purchase. Weinrich et al. (36) reported that highly educated people have a more positive attitude towards the willingness to try in-vitro meat, to consume it regularly, and to recommend it. Kombolo et al. (19) stated that higher education level has a significant effect on the willingness to consume in-vitro meat regularly and willingness to pay. Hocquette et al. (16) reported that educated consumers were skeptical about in-vitro meat consumption. In our study, it is seen that professional workers, students, health workers, and white-collar workers who have knowledge about meat biochemistry are more willing to try in-vitro meat compared with other occupational groups. The more favourable view of students towards in-vitro meat may be related to the fact that most of them are also young. In a previous study, non-scientists and scientists reported the lowest level of willingness to consume compared with participants who were not familiar with the meat sector (17). In another study, it was reported that scientists not working in the meat sector had a higher willingness to try and consume in-vitro meat than non-scientists and people working in the meat sector; also, the participants who were dealing with meat science were 1.6 times more likely to consume in-vitro meat than non-scientists but working in the meat sector, while non-scientists and people not working in the meat sector were 2.7 times more likely to consume in-vitro meat (20). In the study, as the monthly household income of the participants increased, their willingness to try in-vitro meat, consume it regularly, consume it instead of conventional meat, and try it when recommended by a friend increased. Wilks and Phillps (37) determined that low-income participants were more willing to try in-vitro meat than high-income participants. On the other hand, the effect of income level on willingness to pay for in-vitro meat was found to be insignificant.

When the eating habits of the participants were evaluated in our study, it was found that only white meat-fish consumers and vegans had a higher willingness to try in-vitro meat and consume it instead of conventional meat compared with meat consumers. In addition, it is seen that the willingness of white meat and fish consumers to consume in-vitro meat regularly is higher than that of other groups. It is seen that vegans are willing to pay more for in-vitro meat compared with individuals who consume red meat, white meat, and fish regularly. In a study conducted in Brazil with 408 participants, it was reported that 65.2% frequently consumed meat, 66.4% could try in-vitro meat, and 24% of vegetarians and vegans could eat in-vitro meat (19). Wilks and Phillips (37) similarly reported more favourable perceptions among vegetarians and vegans but a lower willingness to try. Except for a recent study by Anonymous (2), which is more accepted among vegans, it shows that individuals with high meat consumption compared to vegetarians are more open to trying cultured meat (19, 25, 28). In another study, it was reported that participants following a vegan or vegetarian diet were less likely to accept in-vitro meat compared with those consuming meat (15). This contradictory behaviour of non-meat eaters may be explained by the fact that they are not only against cultured meat but are also not interested in consuming it.

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In our study, in terms of frequency of meat consumption, people who never consumed meat were found to be more willing to consume and pay for in-vitro meat instead of conventional meat. In a study, it was found that participants under and over 31 years of age who ate meat every day had a lower willingness to try in-vitro meat than participants of the same age who rarely or never ate meat. In the same study, the willingness to try in-vitro meat was found to be lower in non-scientist participants who regularly/every day eat meat and know/do not know the meat sector than in scientists who do not know the meat sector or know the meat sector but never eat meat (17).

When our study was evaluated in terms of familiarity with in-vitro meat, it was found that 25.9% of the participants had heard of in-vitro meat and knew what it was, 53.6% had heard of it but had no knowledge, and 20.5% had never heard of it before. In a study conducted in two different regions of Brazil, it was reported that 81.6% and 82.6% of the participants had little or no knowledge about in-vitro meat (19). In another study, it was reported that 86.3% of the participants were familiar with in-vitro meat and 16.7% had not heard of it (17). Heidmeier and Teuber (15) stated that 62% of 526 participants had heard of in-vitro meat and 54% of those familiar with in-vitro meat were willing to purchase it. Asioli et al. (4) also reported that consumers who had heard of in-vitro meat were willing to pay more than those who had not heard of the term. Min et al. (24) reported that participants with knowledge about in-vitro meat had a higher willingness to try and consume in-vitro meat compared with other groups. In our study, the effect of familiarity with in-vitro meat on the willingness to try, consume, and pay for in-vitro meat was also found to be insignificant.

In this study, Turkish consumers' willingness to try, consume, and pay for in-vitro meat was analysed by matching the sociodemographic characteristics of the participants. The results of the study show that Turkish consumers are distant to in-vitro meat. The willingness to try, consume, and especially pay for in-vitro meat was found to be quite low for all sociodemographic groups in general. On the other hand, the fact that the vegan, vegetarian, only white meat, and pescatarian consumption groups are willing to pay more for in-vitro meat indicates that in-vitro meat can be considered as an alternative product for these people in Türkiye.

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Ethical Statement

This study was carried out after the project was approved by Çanakkale Onsekiz Mart University Graduate Education Institute Ethics Committee, Scientific Research Ethics Committee (Approval No: 2023-YÖNP-0498, Acceptance date: 21/06/2023, Decision number: 08/07).

Conflict of Interest

The authors declared that there is no conflict of interest.

Author Contributions

AGE; conceptualization, methodology, project administration, investigation, writing-original draft. PDK; investigation, formal analysis, writing-review & editing. FYE; investigation, writing-review & editing. BE: conceptualization, data curation, methodology, project administration, formal analysis, supervision, writing-review & editing.

Data Availability Statement

The data supporting this study's findings are available from the corresponding author upon reasonable request.

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Investigation of some neonicotinoids in honey by LC-MS/MS

Halil ERGÜN[1,a,✉], Levent ALTINTAŞ[2,b]

[1]Republic of Türkiye Ministry of Agriculture and Forestry Veterinary Control Central Research Institute, Department of Toxicology, Ankara, Türkiye.
[2]Ankara University, Faculty of Veterinary Medicine, Department of Pharmacology and Toxicology, Ankara, Türkiye.

[a]ORCID: 0000-0001-6460-3255; [b]ORCID: 0000-0002-5148-723X

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✉Corresponding author
hergun84@msn.com

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ABSTRACT

Honey is a natural substance that is susceptible to contamination by environmental pollutants. The presence of contaminants in honey is an indicator of environmental pollution. Furthermore, it may pose risks to consumer health. This research aimed to optimise a method for the detection of residual quantities of the pesticides acetamiprid (ACE), clothianidin (CLO), imidacloprid (IMI), thiamethoxam (TMX) and thiacloprid (THI) in honey, and subsequently to apply this optimised method to an investigation into the prevalence of neonicotinoidal contamination. The QuEChERS (quick, easy, cheap, effective, robust and safe) method, in conjunction with liquid chromatography-mass spectrometry/mass spectrometry (LC-MS/MS), was employed for the determination of five neonicotinoid in honey. The method was optimized and validated in accordance with European Commission guidelines (2002/657/EC). The method demonstrated a linear correlation with R^2 values exceeding 0.99 for all investigated compounds. Mean recoveries ranged between 70% and 110% (99% on average), and relative standard deviations (RSDs) were generally below 20%. The method's CCα (decision limit) and CCβ (detection capability) both ranged from 5 to 20 ng/g and 5-21 ng/g, respectively. Following method validation, the concentrations of IMI, ACE, TMX, CLO, and THI in all honey samples (flower, pine, and chestnut honey) available for retail in Ankara, Türkiye were determined to be <LOD based on the analytical results. It was concluded that the proposed method is usable and advantageous because it is effective, reliable, sensitive, and reproducible and can be used for the simultaneous analysis of more than one analyte in a short time using a few reagents.

Introduction

Honey has a long history as a natural product processed by bees throughout the world. Honey produced by bees is both a natural and healthy food and is nutritious (14, 30). In general, honey consists of 79% different sugars (38% fructose, 31% glucose, 8% disaccharides, and 2% other sugars) and 17% water. The remaining 4% of honey is a complex matrix containing more than 300 chemical compounds, including enzymes, vitamins, minerals, and amino acids. There are more than 300 monoclonal honey varieties worldwide (acacia, clover, eucalyptus, orange-flower, pine, etc.) that have a unique flavor or stand out with another characteristic (9, 10).

Türkiye is one of the world's richest honey-producing regions, thanks to its geography, climate, and the fact that it produces honey throughout the year. Türkiye is one of the countries with the richest flora in Europe with the presence of approximately 10,000 different plant species, as well as having approximately 75% of the nectar plant species that are important for beekeeping identified in the world. According to the FAO, world honey production report, China has the highest honey production, followed by Türkiye in second place. Almost all (90%) of the global pine honey is produced in Türkiye (3, 9, 17, 26, 28). The production of pine honey is unique among honey types. It is created by the pine cotton bollworm (*Marchalina hellenica*), which lives on the red pine tree (*Pinus brutia*). The pine cotton bollworm takes the protein in the sap that it sucks from the pine and excretes sugary juice from its body. This sweet secretion is collected by honey bees and is transformed into pine honey (1).

Neonicotinoids (NEOs) are crop protection products widely used around the world. Recent scientific studies have reported that they may present a potential health risk. Thus, understanding the amount of NEOs in food products for human consumption is essential. Recently, honey consumption has increased significantly because of its health benefits (4). It is therefore important to monitor NEOs in honey, not only in view of the potential risks to human health and as an indicator of environmental pollution (2). The European Commission (EC) maximum residue levels (MRLs) of neonicotinoid authorized in honey are 50 ng/g for acetamiprid (ACE), imidacloprid (IMI), clothianidin (CLO), and thiamethoxam (TMX) and 200 ng/g for thiacloprid (THI) (Table 1) (7, 12). In 2013, EC seriously limited the use of neonicotinoid pesticides and coated seeds (clothianidin, thiamethoxam and imidacloprid) (5). In April 2018, the European Commission banned these compounds for all outdoor activities (6). The use of CLO, IMI, and TMX was banned by the General Directorate of Food and Control in Türkiye on December 19, 2018 (21).

Several methods have been described for quantifying NEOs in honey using a variety of techniques (LC-MS/MS, GC-MS/MS, UPLC-UV, UPLC-DAD and LC-amperometric detector). Most neonicotinoids are not suitable for Gas chromatography (GC) because they are volatile and non-polar. GC is an analytical technique applicable to gas, liquid, and solid samples (components that are vaporized by heat) (12, 16). Therefore, in this study, LC-MS/MS was used to analyze the extracts obtained from the honey samples.

The complex nature of the honey matrix and the need for nanogram-per-gram measurement necessitates the inclusion of the sample preparation phase in the test procedure. For liquid chromatographic analyses of neonicotinoid pesticide residues, a number of pre-treatment procedures for honey samples have been described. The techniques commonly used as pretreatment procedures include conventional liquid–liquid extraction (LLE), modified QuEChERS, and dispersive liquid–liquid microextraction (DLLME). The QuEChERS method is currently the most universally used and accepted sample preparation method because it requires some chemicals compared with conventional methods, allowing the simultaneous determination of many pesticides (4, 12, 18, 24). In the extraction phase of the honey samples, the QuEChERS method was selected.

To date, a considerable number of studies have been conducted on neonicotinoid in honey on a global scale (22, 19). However, there is a paucity of research on neonicotinoid in honey produced in Türkiye. Given that 80% of Türkiye's honey exports are strained, primarily to the United States and European Union countries, addressing this issue is of particular importance. Furthermore, to the best of our knowledge, no other study on neonicotinoids in honey, either in terms of sample size or sample diversity (especially pine honey), has been published for Türkiye that is as comprehensive as this one.

The aim of this research is to establish a suitable procedure for the analysis of commonly used NEOs (ACE, IMI, THX, CLO and THI) in strained honey samples line with European requirements (Commission Decision 2002/657/EC). The method was subsequently used to examine honey samples from Türkiye, with the objective of evaluating the efficacy of the extant prohibition using LC-MS/MS. The present study on honey sourced from Türkiye represents a significant contribution to the global repository of data on neonicotinoid exposure.

Materials and Methods

Chemicals, Reagents, and Solutions: Imidacloprid (IMI), thiacloprid (THI), acetamiprid (ACE), thiamethoxam (TMX), clothianidin (CLO), and citric acid trisodium salt dihydrate were purchased from Sigma Aldrich (Germany). All standards had a purity greater than 98%. Acetonitrile (ACN) (LC Purity) and methanol (MeOH) (LC Purity) were obtained from Isolab (Germany). Formic acid was obtained from Merck (USA). Magnesium sulfate was obtained from Sigma-Aldrich (Japan). Sodium chloride (NaCl) was purchased from Sigma-Aldrich (Denmark). Ammonium formate was purchased from Sigma-Aldrich (India). Primary and secondary amino acid (PSA) was obtained from Agilent (USA). Sodium hydrogen citrate dehydrate was purchased from Sigma-Aldrich (Belgium). An Elga 664 (UK) water purification system was used to purify the water.

Standards: Primary standard dilutions (S_1) of all analytes were made in 1000 ng/µl of acetonitrile and kept in vials refrigerated at +4 ◦C. Intermediate standard solutions (S_2) were further diluted as required in ACN. S_2 100 ng/µl and 10 ng/µl standards were prepared. Working solutions (S_3) were obtained by diluting S_2 with acetonitrile. For IMI, ACE, TMX, and CLO with a maximum residue limit of 50 ng/g, a mixed S_3 of 10 ng/µl was prepared from the S_2 of 100 ng/µl. For THI with a maximum residue limit of 200 ng/g, an S_3 of 4 ng/µl was prepared from standard S_2. These S_3's was used to prepare positive (spike) samples at the 0.5, 1, 1.5, 2, and 5 MRL levels.

LC–MS/MS Conditions: LC-MS/MS analysis was performed using a Shimadzu HPLC instrument (Shimadzu Corporation, Kyoto, Japan). A Shimadzu (8040) triple-quadrupole mass spectrometer was used to link the system. The mass spectrometer (MS) was also fitted with an electrospray ion source. Analyte retention was conducted at 40°C on a Phenomenex Synergi (4 µm Max-RP 80 A 50 x 2mm) LC column. The flow rate and volume

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of injection were set to 0.4 ml min^{-1} and 10 µl respectively. The mobile-phase solvents used were water (0.1% formic acid) solvent A and methanol (MeOH, 5mM ammonium formate) solvent B. Gradient elution program was 0-1 min; 5% B; 1-6 min; 95% B; 6-6.50 (min); 5% B and a 3-min wash at 100% A. The entire chromatography run time was 10 min. The MS parameters were as follows: nebulizing gas stream, 3 L/min; DL heat, 250°C; heating block heat, 400°C; drying gas stream, 15 L/min; and column oven, 40°C.

Sample Collection: A sample of 60 honey (20 each of pine, blossom and chestnut) was obtained from wholesale, retail, and local outlets and offered for consumption in the central districts of Ankara province between June and December 2021. Honey samples were collected according to the sampling procedure outlined in the National Residue Monitoring Program (NRMP) of the Ministry of Agriculture and Forestry, Türkiye (29). Prior to analysis, all samples were stored at room temperature and in darkness.

Sample Extraction from the Analyses: Honey samples were analyzed using a partial modification of the QuEChERS method described by Mrzlikar et al. (23). For sample preparation, a 10-g test solution of honey was placed into a 50 ml centrifuge tube made of polypropylene (Falcon). Ultrapure water 10 mL and acetonitrile 10 mL were added. To this was added (4 g of anhydrous $MgSO_4$, 1 g of NaCl and 1 g of citric acid trisodium salt dihydrate ($C_6H_5Na_3O_7 \cdot {}_2H_2O$) and 0.5 g of disodium hydrogen citrate sesquihydrate ($C_6H_6Na_2O_{7.1.5}H_2O$). The sample was then shaken vigorously for 60 s. Centrifuged at 3000 rcf for 10 min. The supernatant (4 mL) was recovered, and 0.9 g of anhydrous MgSO4 and 0.15 g of primary secondary amino acid sorbent were added. The mixture was vortexed for 30 s and then centrifuged at 3000 rcf for 5 min. Dried under nitrogen (N_2) atmosphere at 40 °C. Diluted in 1000 µl methanol/water solvent (20/80). The final extracts were analyzed by LC MS-MS after they had been filtered by passing them through a size 0.22 µm PTFE filter.

Validation Parameters: European requirements (Commission Decision 2002/657/EC) for method performance were followed. The linearity, limit of detection (LOD), limit of quantification (LOQ), decision limit (CCα), detectability (CCβ), accuracy (recovery), precision (repeatability and within-laboratory reproducibility), selectivity, and robustness were evaluated for each NEO (8, 13, 20).

The linearity of the method was verified by constructing calibration curves using spiked blank honey (negative control) samples at concentrations between 25 and 1000 ng/g. The precision and accuracy of the analytical method were evaluated by analyzing spiked honey samples containing IMI, ACE, TMX, CLO, and THI at concentrations of 0.5, 1, and 1.5 times the permitted limit set forth by the European Commission. The method's selectivity was assessed by analyzing a blank honey matrix (n = 10) and verifying the absence of any overlap (signal, peak, etc.) at the point at which the target analytes were expected to elute. LOD and LOQ were calculated using the slope of the calibration curve and the standard deviation (Sd) of the response. European Decision 657/2002/EC recommends two analytical parameters: CCα (the critical alpha concentration at risk alpha, and CCβ, the critical beta concentration at risk) (8). These parameters facilitate the evaluation of the critical concentrations at which the technique can consistently distinguish and quantify a substance while simultaneously considering the inherent variability of the method and the statistical probability of erroneous determination. For substances with maximum residue limits, detection capacity refers to the concentration level at which the method can accurately detect the permissible limit concentrations with a confidence level of 95%. CCα was calculated as the mean measured concentration at the MRL level plus 1.64 times the variance of reproducibility (SR_{MRL}) at these concentrations. The calculation for CCβ is derived by summing CCα by 1.64 times the corresponding SR_{MRL}, assuming that the SR_{MRL} at the CCα level is equal to that at the MRL level (8).

Results

MS/MS Method Development: The initial phase involved optimising MS detection conditions. Each compound was injected at 100 ng/g to optimise MS conditions. The instrument was run in the multiple reaction monitoring (mrm) mode. For each analyte, two precursor-to-product ionic passes were monitored. All analytes were analyzed by optimization in ESI mode. IMI, ACE, TMX, CLO, and THI were positively identified. An overview of the precursor and product ions, collision energies (CE), and retention times of each analyte are given in Table 1.

Chromatographic Conditions Optimization: A Phenomenex Synergi (4 µm Max-RP 80 A 50 x 2mm) LC column was used for the chromatographic separation of neonicotinoid. The peak shapes, heights, and retention times of neonicotinoid were determined using this column. To achieve optimal separation of analytes in gradient flow and obtain satisfactory chromatographic separation, the gradient elution program was established as follows: 0-1 min; 5% B; 1-6 min; 95% B; 6-6.50 (min); 5% B and a 3-min wash at 100% A. To determine the flow gradients, the analysis times and peak heights of the chemicals were adjusted under the most favorable conditions. In the mobile phase study, neonicotinoid was

ionized, and the peak shapes, peak heights, and response values obtained were found to be best in mobile phase A (water, 0.1% formic acid) and mobile phase B (methanol, 5 mM ammonium formate). Separation of the five neonicotinoid was completed in a time period of less than 7 mins. The optimized analytical conditions of LC-MS/MS enabled the identification and effective separation of all investigated chemicals with good peak resolution (Figure 1).

Method Validation: Linearities were tested by establishing calibration curves using neonicotinoid-free honey samples (matrix-matched curves) at concentrations within the range allowed by the European Decision for IMI, ACE, TMX, CLO, and THI. Method linearity matrix-matched calibrations (mmc) with triplicate replicates showed that the method was linear, with R^2 values >0.99 for all investigated compounds (Figure 2).

Table 1. MRM values for neonicotinoids and Maximum residue levels (MRLs) for neonicotinoid pesticides in honey.

Analyte	Mass	Products Ion	Dwell Time	Polarity	Collision Energy	Retention Time	MRL (ng/g)
Acetamiprid	223	Q1 126	24	Positive	23	3.592	50
		Q2 56	24	Positive	17	3.592	
Clothianidin	249	Q1 169	38	Positive	12	3.349	50
		Q2 131	38	Positive	16	3.349	
Imidacloprid	256	Q1 209	24	Positive	19	3.336	50
		Q2 175	24	Positive	16	3.336	
Thiamethoxam	291	Q1 211	24	Positive	13	2.952	50
		Q2 181	24	Positive	24	2.952	
Thiacloprid	252	Q1 126	24	Positive	21	3.832	200
		Q2 90	24	Positive	40	3.832	

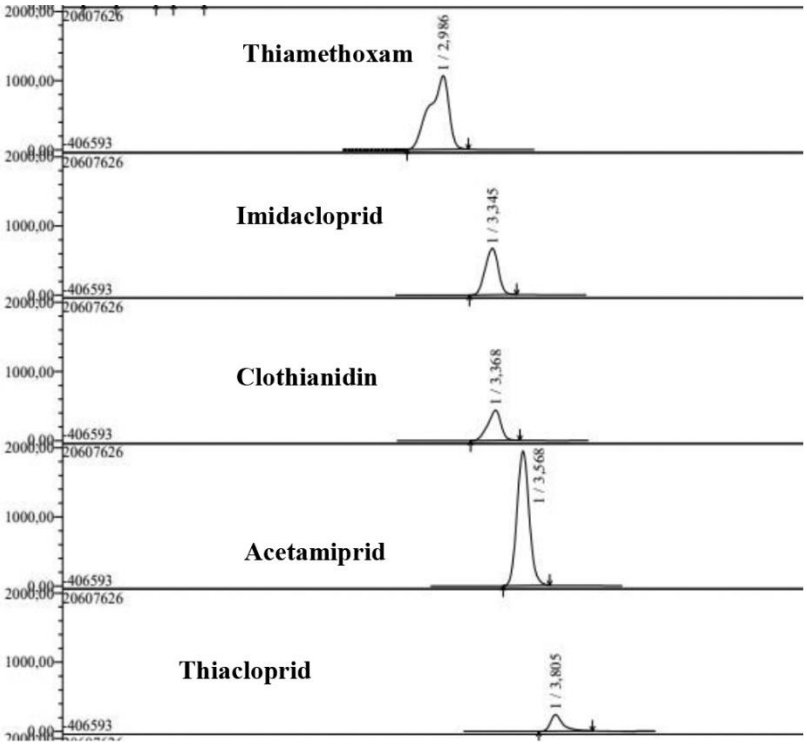
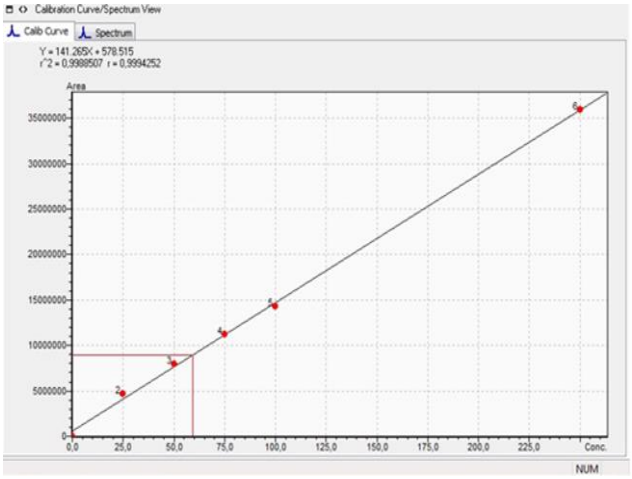
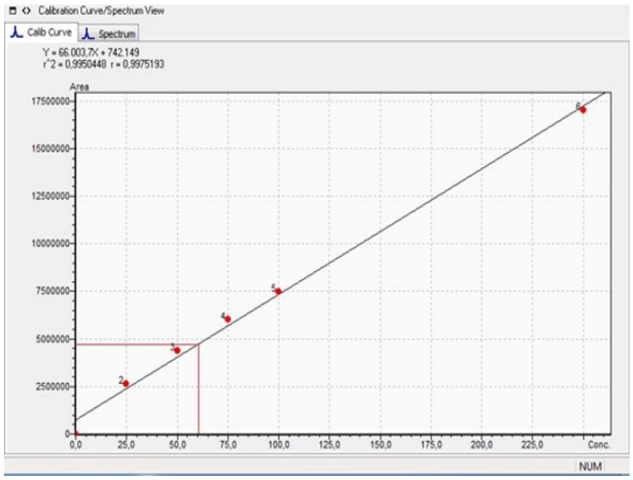


Figure 1. LC–MS/MS chromatograms of neonicotinoid.

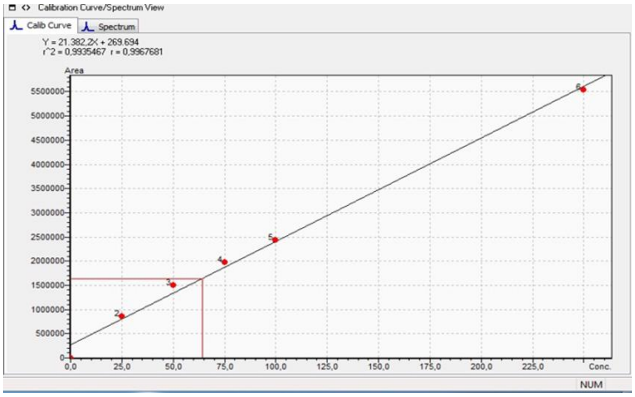
Acetamiprid; Correlation coefficient (R^2) = 0,9999



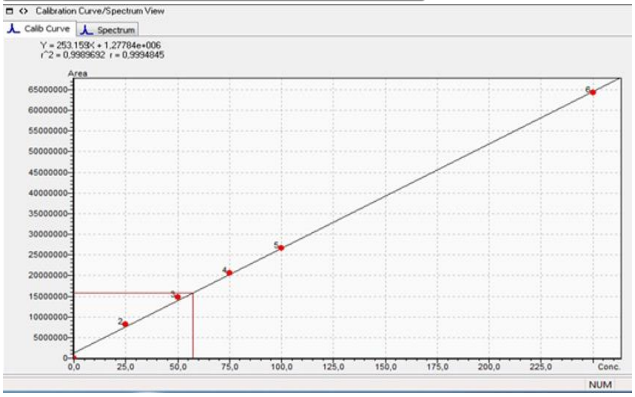
Imidacloprid; correlation coefficient (R^2) = 0.9950.



Clothianidin; Correlation coefficients (R^2) =0,9935



Thiamethoxam; correlation coefficient (R^2) = 0.9994.



Thiacloprid; correlation coefficients (R^2) =0,9956

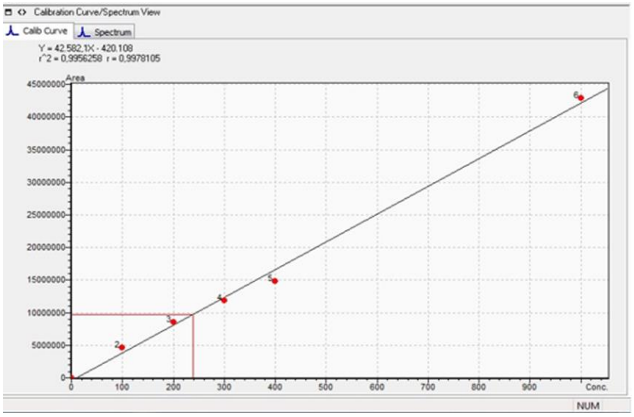


Figure 2. Calibration curves of the tested neonicotinoid.

For the purpose of determining LOD and LOQ, spike samples were prepared at concentrations of 5 ng/g for ACE, IMI, CLO, and TMX, and 10 ng/g for THI. These samples were applied to 10 separate empty honey samples using a 6-point calibration line and were measured ten times. The LOD was calculated by multiplying the Sd of the measurements by 3, whereas the LOQ was determined by multiplying the Sd by 10. The observations indicated that the LOD values for ACE, IMI, CLO, TMX, and THI were remarkably low, which can be attributed to the enrichment aspect of the method employed. The results of the analyses are presented in Table 3.

Table 2. Method validation data: Percentage recovery (R %), repeatability (RSDr %), and within-laboratory reproducibility (RSD_R %).

Analyte	Acetamiprid			Clothianidin			Imidacloprid			Thiamethoxam			Thiacloprid		
Spike Level (MRL)	0.5 MRL	1 MRL	1.5 MRL	0.5 MRL	1 MRL	1.5 MRL	0.5 MRL	1 MRL	1.5 MRL	0.5 MRL	1 MRL	1.5 MRL	0.5 MRL	1 MRL	1.5 MRL
R (%)	100.6	101.8	100.1	100.9	100.5	99.1	99.4	100.6	99.5	99.1	100.5	99.7	99.6	100.0	99.0
RSD_r (%)	3.1	3.5	3.3	3.3	2.8	2.2	3.3	1.8	1.9	3.5	2.0	1.4	3.3	2.1	1.7
RSD_R (%)	3.3	5.4	3.5	3.3	2.9	2.2	3.3	1.9	1.9	3.5	2.0	1.4	3.3	2.1	1.7

Table 3. Limit of detection (LOD), limit of quantification (LOQ), decision limit (CCα), and detection capability (CCβ) values (ng/g).

Analyte	Acetamiprid	Clothianidin	Imidacloprid	Thiamethoxam	Thiacloprid
LOD (Limit of Detection)	0.6	0.6	0.7	0.9	3.8
LOQ (Limit of Quantification)	2.1	2.2	2.6	3.2	12.7
CCα (Decision limit)	5.4	5.2	5.1	5.1	20.2
CCβ (Detection capability)	5.8	5.4	5.3	5.3	20.5

The precision and accuracy expressed in the recovery of honey samples was evaluated by analyzing samples containing IMI, ACE, TMX, CLO, and THI at concentrations of 0.5x, 1x, and 1.5x the European Decision limits. For the recovery assays, empty honey samples (10 g) were fortified with the corresponding levels of the S_3 mixture of the analytes. Six duplicates per spike stage were analyzed on the same day, and a matrix-matched calibration graph was generated. Each series was run on three different days (54 spiked samples in total) and consisted of one matrix calibration graph and 18 spiked samples. To determine the within-laboratory reproducibility, six replicates for each level of spiking and 3 spikes at each level were produced and analyzed on a different day (a total of 18 spiked samples). The formula was used to calculate the percentage recovery, R%: R% = (C1/C2)*100, where C1 is the level of the test substance in the fortified samples and C2 is the level of the analyte that is added to a "blank" honey. EC Decision 2002/657/EC recommends a recovery of 50-120% for <1 µg/kg, 70-110% for 1-10 µg/kg and 80-110% for >10 µg/kg. Table 2 presents the recoveries of the five analytes within the acceptable range of the precision criteria. Precision was based on two parameters: the repeatability and the within-laboratory reproducibility. From these experiments, the precision (repeatability and intra-laboratory reproducibility expressed as percentage relative standard deviations) was assessed. One-way analysis of variance (ANOVA) was also used to estimate method repeatability and within-laboratory reproducibility. In the ANOVA analysis of variance table, when the criterion Fh ($F_{\text{calculated, Table shows F value}}$) < Fk ($F_{\text{critical value, Table shows F measure value}}$) for each analyte was checked, it was found that the experimental Fh values were lower than the theoretical Fk in all analyses. It was seen that the results came from the same batch. Table 2 presents the data for repeatability (RSDr %) and within-laboratory reproducibility (RSDR %). These results indicate that the methods have good reproducibility.

To complete the validation procedure according to Decision 2002/657/EC, CCα and CCβ were calculated for honey. CCα and CCβ were calculated using the coefficient of variance of reproducibility (SR_{MRL}) at the MRL level determined using the ANOVA method in the precision section. The formula was used to calculate the $SR_{MRL} = \sqrt{S_r^2 + S_b^2}$, where S_r^2 is the square root of the within-group mean and S_b^2 is the between-group standard deviation. If S_b is negative, then it is taken as zero. Decision limits (CCα) were computed as the mean of the measured levels plus 1.64 times the corresponding SR_{MRL}. CCβ has been obtained as 1.64 times the decision limit (CCα) plus the relevant SR_{MRL}, as follows. The CCα and CCβ levels of the analytes at the MRL level are presented in Table 3.

For the selectivity and robustness parameters, 20 different samples (10 different blank honey matrices and 10 different blank honey mrl spiked matrices) were used**.** It was checked at the point where the objective analytes were expected to elute for any overlap (signal, peak, etc.). The chromatograms of each substance were not affected. In the robustness parameter of the method, matrix robustness and data from experiments performed over a long period by different analysts were evaluated. The specificity parameter data were checked for matrix robustness and reproducibility robustness in the context of in-laboratory reproducibility studies in which experiments were performed by different analysts.

Application to Real Samples: The developed method was used to analyze sixty honey samples. Analyses performed according to Directive 2002/657/EC the concentrations of IMI, ACE, TMX, CLO, and THI in all honey samples (flower, pine, and chestnut honey) available for retail in Ankara, Türkiye were determined to be <LOD based on the analytical results. An example of the chromatograms of the analyzed flower, pine, and chestnut honey is shown in Figure 3.

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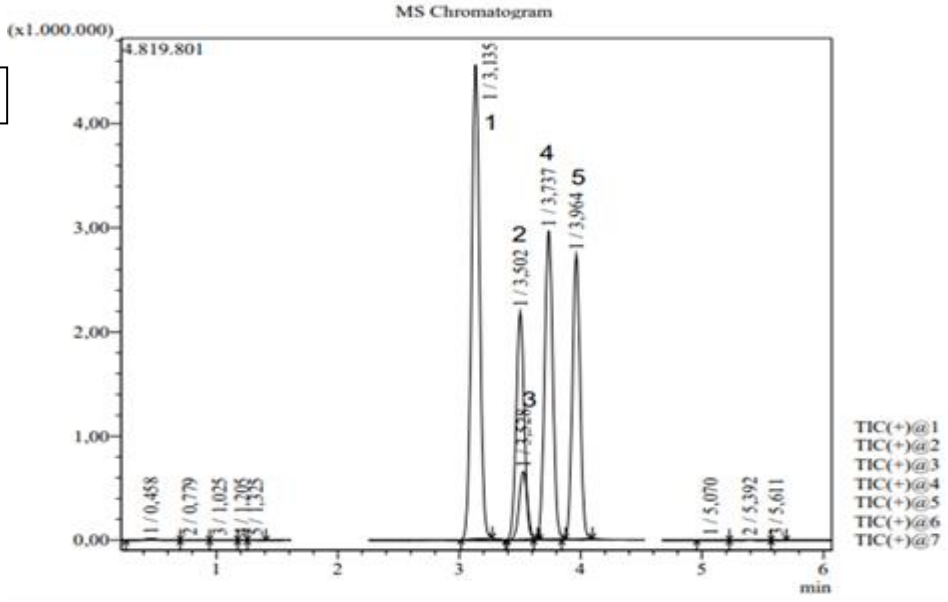
A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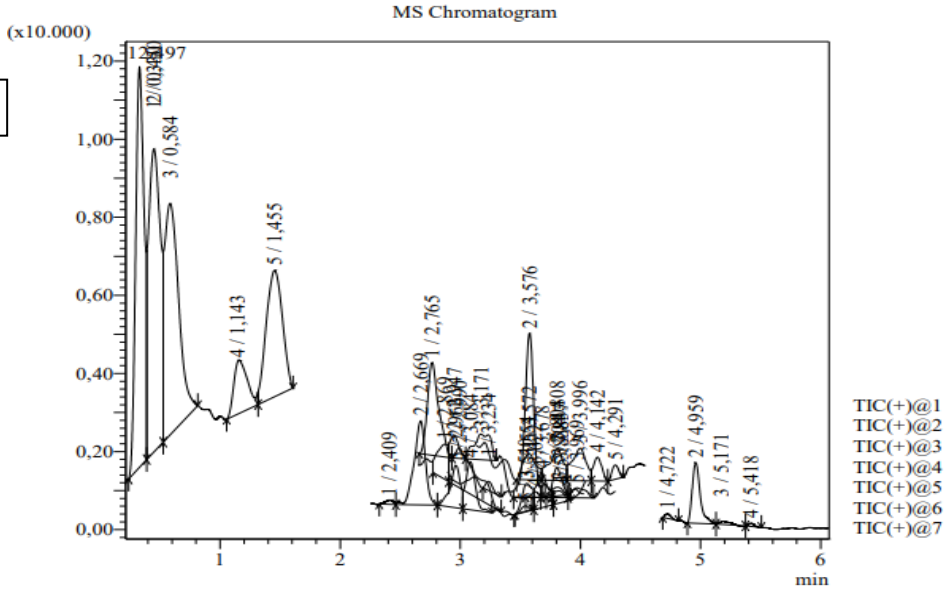
(x1.000.000)
4.819.801
1 / 3,135
2 / 3,502
1 / 3,737
1 / 3,964
1 / 0,458
2 / 0,779
3 / 1,025
1 / 5,070
2 / 5,392
3 / 5,611
TIC(+)@1
TIC(+)@2
TIC(+)@3
TIC(+)@4
TIC(+)@5
TIC(+)@6
TIC(+)@7
min

B


MS Chromatogram
(x10.000)
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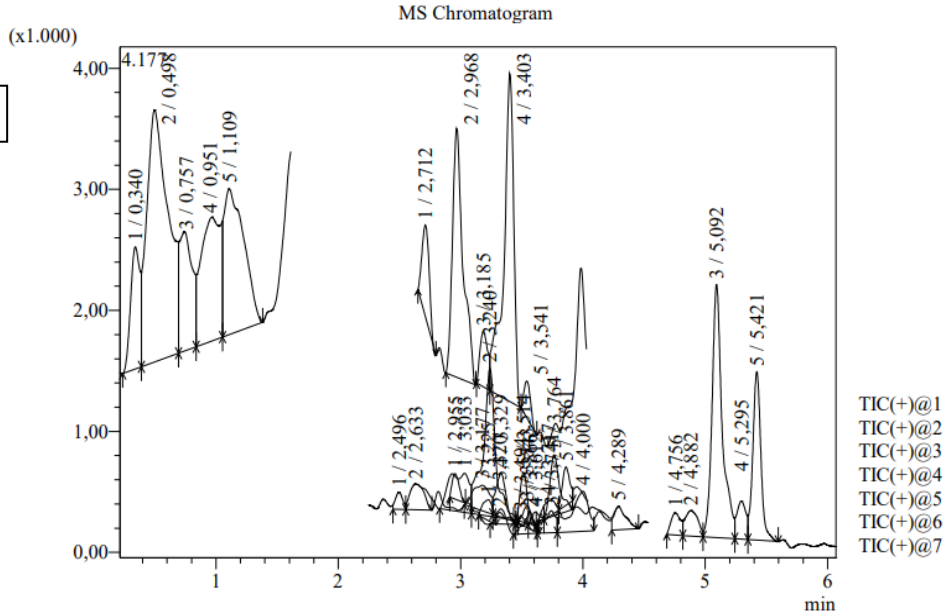
5 / 1,455
4 / 1,143
2 / 3,576
1 / 2,765
2 / 2,669
1 / 2,409
1 / 4,722
2 / 4,959
3 / 5,171
4 / 5,418
TIC(+)@1
TIC(+)@2
TIC(+)@3
TIC(+)@4
TIC(+)@5
TIC(+)@6
TIC(+)@7
min

C


MS Chromatogram
(x1.000)
2 / 0,498
1 / 0,340
3 / 0,757
4 / 0,951
5 / 1,109
2 / 2,968
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1 / 2,712
5 / 3,541
3 / 5,092
5 / 5,421
4 / 5,295
1 / 4,756
2 / 4,882
TIC(+)@1
TIC(+)@2
TIC(+)@3
TIC(+)@4
TIC(+)@5
TIC(+)@6
TIC(+)@7
min

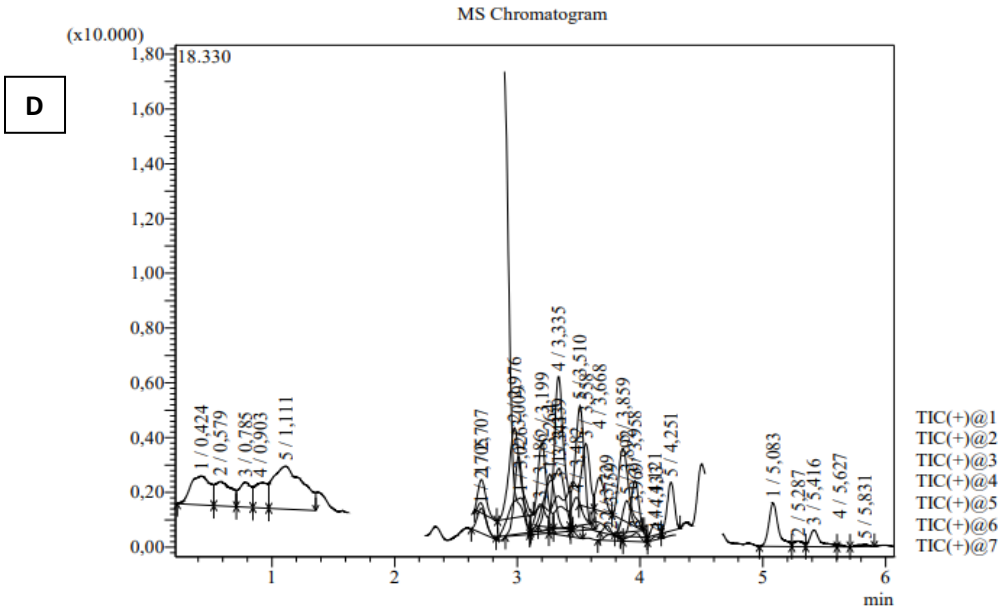

Figure 3. LC–MS/MS chromatograms of spiked and negative honey samples as Mass spectra and characteristic fragment pattern chromatograms. A: Honey samples spiked with 50 ng/g for ACE, IMI, CLO, TMX, and 200 ng/g for THI. B: negative pine honey chromatogram, C: negative flower honey chromatogram, D: negative chestnut honey chromatogram. Peaks 1: TMX, 2: IMI, 3: CLO, 4: ACE, 5: THI.

Discussion and Conclusion

Exposure of honey bees to NEOs can result in pollution of bee products, particularly honey, which is the most widely consumed bee product. This risks public health. Because of its health benefits, honey consumption has increased significantly in recent years (4). It is therefore important to be able to detect these substances in honey, not only because of the potential serious risks to public health, but also because their concentrations may indicate the danger they pose to the environment in general. Ensuring the safety and quality control of honey requires monitoring chemical contaminants in honey and ensuring the absence of toxic residues in the natural product at levels harmful to the consumer (11,27).

Tanner and Czerwenka (27) investigated the residues of three neonicotinoid in 41 honey samples in Austria by LC-MS/MS and revealed the presence of THI (27.4 µg/kg) in 18 samples (22%), ACE (15.2 µg/kg) in 2 samples (5%), and TMX in 1 sample. However, none of these residues exceeded the MRL, and on average, the floral honey samples contained more neo compounds than the wood honey. Although there are slight differences between these reported results and the results obtained from the study, the studies are similar in the sense that no samples exceeded the limit values in both studies. The differences between the analyses may be due to differences in regions and years of analysis and the fact that these products were used during the analysis period.

Song et al. (25), the residues of NEOs in 30 honey samples from various regions of China were investigated using LC MS-MS with anion exchange DPX and LC-MS/MS. They reported that the prevalence of neo pesticides in 30 honey samples ranged from 13% to 33%, and the residues were approximately 11–120 µg/kg, with the maximum levels of dinotefuran, CLO, IMI, and THX exceeding 102 µg/kg The difference between the results obtained in this study and the latest study could be due to the particular extraction methods used and the fact that the use of these products was permitted at the time of the study.

Mrzlikar et al. (23) investigated neonicotinoid residues in 51 honey samples of different plant origins (28 flowers, 15 forests, 5 acacia, 2 lindens, and 1 chestnut) obtained from particular geographical areas of Slovenia between 2014 and 2016 by LC-MS/MS revealed the presence of only THI and ACE. While ACE was detected in 6 samples honey (4 flowers, 1 forest, and 1 linden), it was reported to exceed the LOQ value (2 ng/g) in only one flower sample. THI was reported to be above the LOD in 30 honey samples. The highest level was observed in a flower honey sample (9.6 ng/g) Differences in regions and years of analysis may explain the discrepancy between this study and the results of the current study.

Iplikcioglu et al. (15) investigated the presence of neonicotinoid in 44 strained honey samples obtained from different areas of Türkiye using the LC-MS/Q-TOF method and reported that no neonicotinoid pesticide was found in any of the samples. In this study, analyses were performed by LC-MS/MS, and our findings are consistent with the literature. Although there are slight differences

between these reported results and the results obtained from the study, there is a similarity between the studies in terms of the absence of samples exceeding the limit values in all the study results. It is predicted that slight differences between analyses may be due to differences in regions and years of analysis and the methods and equipment used. Considering all these studies, it is also predicted that although the use of neo pesticides is widespread, the fact that the residue in honey samples is below the limit values may be due to the rapid metabolism of these products by bees.

In this study, adaptation and validation of the IMI, ACE, TMX, CLO, and THI test methods in strained honey were performed. Validation studies were carried out for all neonicotinoid in accordance with the requirements of Directive 2002/657/EC. The advantages of the QuEChERS (cheap, effective, fast, simple, robust and safe) method used in this study were assessed by the use of small amounts of reagents, which allows the analysis of multiple analytes simultaneously in a short time and at a lower cost. In addition, the fact that five different analytes could be analyzed using a single extraction method in this study also highlights the usability of the method. The results suggest that the method employed could be valuable for monitoring the analytes included in the EU residue limit. This was achieved through the use of a highly sensitive and specific LC-MS/MS analytical method developed for detecting neo pesticide residues in honey. The method's usefulness in terms of dissemination is also highlighted. Examination of the honey samples revealed the absence of any residues, indicating the success of the decree enacted in Türkiye in 2018, which prohibited the use of neonicotinoid.

Therefore, the non-detection of neo residues in honey samples is an important result for public health as well as for honey producers and consumers. However, because studies on neonicotinoid in our country are limited in number and region, it is necessary to perform these analyses within a traceable and sustainable plan to reach a definite conclusion in terms of public health.

Acknowledgments

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Financial Support

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Ethical Statement

This study does not present any ethical concerns. The study was submitted to the General Directorate of Food Control of the Ministry of Agriculture and Forestry of the Republic of Türkiye, and necessary permissions were obtained (27.12.2023/E-71037622-903.03.02.02-12602914).

Conflict of Interest

The authors declare that they have no conflict of interest.

Author Contributions

HE; conceptualization, methodology, writing original draft, writing-review & editing, visualization. LA; conceptualization, methodology, writing-review & editing, visualization.

Data Availability Statement

The data supporting this study's findings are available from the corresponding author upon reasonable request.

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Comparative study of immunocytological, immunohistochemical and in-situ hybridization methods in small ruminant neonatal mortality

Sevil Atalay VURAL[1,a,✉], Rıfkı HAZIROĞLU[1,b], Osman KUTSAL[1,c], Gözde YÜCEL TENEKECİ[1,d], Arda Selin TUNÇ[1,e], Yanad ABOU MONSEF[2,f], Ozan AHLAT[1,g], Kürşat FİLİKCİ[3,h], Özgür ÖZÖNER[4,i], Oya Burçin DEMİRTAŞ[1,j]

[1]Ankara University, Faculty of Veterinary Medicine, Department of Pathology, Ankara, Türkiye; [2]Université de Toulouse, Ecole Nationale Vétérinaire de Toulouse, Laboratory of Anatomic Pathology, Toulouse, France; [3]Harran University, Faculty of Veterinary Medicine, Department of Pathology, Şanlıurfa, Türkiye; [4]Siirt University, Faculty of Veterinary Medicine, Department of Pathology, Siirt, Türkiye.

[a]ORCID: 0000-0003-2111-3381; [b]ORCID: 0000-0002-1134-3581; [c]ORCID: 0000-0003-3599-6867; [d]ORCID: 0000-0002-2586-8346; [e]ORCID: 0000-0002-4813-7626; [f]ORCID: 0000-0002-4929-9395; [g]ORCID: 0000-0002-2580-8140; [h]ORCID: 0000-0001-9710-9480; [i]ORCID: 0000-0001-7354-0655; [j]ORCID: 0000-0003-0850-2374

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✉Corresponding author
sevilvural@yahoo.com

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ABSTRACT

It was aimed to identify diseases using immunocytochemical, immunohistochemical, and in-situ hybridization methods, to determine sensitivity and specificity among these techniques, and to highlight their advantages and disadvantages for certain respiratory (*Mycoplasma pneumonia, Pasteurella spp.,* Respiratory syncytial virus, Parainfluenza virus 3) and enteric (Coronavirus, Rotavirus, *Escherichia coli, Clostridium spp.*) agents. The obtained results were compared, and although the immunocytochemical method was found to be the fastest, immunohistochemistry was proved to be the most reliable method. Our other aim was to establish pathological diagnostic panels for neonatal infections. All antibodies tested were found to be positive except for *Pasteurella multocida*. The immunohistochemical findings of the study indicate that nearly all cases that result in death involved mixed infections.

Introduction

Breeding ruminants strongly contributes to the country's economy both in Türkiye and worldwide. These animals provide basic dietary needs with meat, milk, and their products, or complement the textile and leather industry with their skin and coat. Especially small ruminants play an important role in food diversity, mainly in rural areas, due to their contributions to the meat and dairy industry and their capability of reproducing. Having healthy offspring and protecting the high quality of the breed has importance in the continuity of such a substantial species. After birth, newborns transfer to an unprotected, risky environment from the warm and safe atmosphere of the uterus, being most vulnerable on the day of birth (10, 12, 17, 18).

Pathogens of the digestive and respiratory tracts are the foremost causes of neonatal deaths (4, 10, 12, 14, 16). Neonatal enteritis is one of the most common diseases in

small ruminants less than 3 weeks old, with a high morbidity and mortality rate (12). Being a syndrome known to be complex and multifactorial, the disease can occur due to a number of reasons, such as herd management system, hypothermia, hyperthermia, hunger, environmental factors, insufficient colostrum intake, and infectious agents (2). According to the study by Holmoy et al. (2017), 80% of neonates die within 2 days; 41% in 24 hours, and 27% in the first 3 hours. This lifespan is set as 5 weeks in some studies. In herds of sheep, the most common reason for neonatal deaths is infectious diseases, with a rate of 37%, with these being septicemia (48%), pneumonia (25%), gastroenteritis (22%), and other (5%). Lots of enteric pathogens are related to diarrhea in neonates, but agents most commonly seen are *Escherichia coli*, *Salmonella spp*., and *Clostridium perfringens* as bacteria; rotavirus, coronavirus, herpesvirus, and adenovirus as viruses; and *Cryptosporidium spp.* as protozoans (9, 10, 15, 18). Due to the fact that there are prophylactic methods and treatments developed for most of these pathogens, the etiological diagnosis of enteritis has significant importance (4, 8).

In this article, diagnosing diseases using immunocytochemical (ICC), immunohistochemical (IHC), and in-situ hybridization (ISH) methods, determining the sensitivity and specificity of these methods, and revealing their advantages and disadvantages in the diagnostic process, and by this, determining the quickest, most reliable, and most practical method for early diagnosis and updating information on the factors that cause death was aimed. Also, creating pathological diagnostic panels in newborns in our department, bringing them into active use in routine diagnosis, and preventing economic loss for the breeders was another important purpose.

Materials and Methods

Study material (n: 44) consisted of lungs, mediastinal/ mesenteric lymph nodes, and intestinal tissues of animals (kid n: 8 and lamb n: 36) of different breeds (n: 15 Merino, n: 5 Karaman, n: 1 Angora, Suffolk, and Sakız, n: 21 breed unknown) and ages (0-60 days) brought to the Department of Pathology for necropsy.

Cytochemical/Immunocytochemical Examinations: Cytological touch slides were prepared from lungs (affected areas and/or areas of bronchi) and mediastinal/mesenteric lymph nodes and stained with both Diff Quick and the indirect immunoperoxidase technique using primary antibodies simultaneously.

Immunohistochemical Examinations: Fixated tissues in 10% buffered formalin (pH: 7.2) were put in the routine tissue fixation device (Epredia/Thermo Scientific STP 120-2) and blocked in paraffin (Epredia/Thermo Scientific Histostar A81000001). 5 µm thick sections were prepared (Epredia/Thermo Scientific HM355S) and stained with hematoxylin eosin (HE) and avidin-biotin complex peroxidase (ABC-P) technique, according to the kit procedure. The primary antibodies used were *Mycoplasma pneumonia* (rabbit polyclonal, 1:100 NovusBio), *Pasteurella multocida* (pig, 1:200, ATCC), *Escherichia coli* (rabbit polyclonal, 1:1000, NovusBio), *Clostridium spp.* (rabbit polyclonal, 1:100, BioRAD), parainfluenza 3 (Mouse Ig1, 1:100, LsBio), respiratory syncytial virus (RSV) fusion protein (Mouse IgG2B, 1:200, Invitrogen), rotavirus (Mouse A2, 1:100, LsBio), and coronavirus (Mouse IPV3-70, 1:300, LsBio). Properties of the antibodies are given in Table 1. The immunohistochemical (IHC) presence of antigens was measured semiquantitatively using a x40 objective by at least two different experts and evaluated as follows: (+) 1-10 immunopositivity; (++) 10-50 immunopositivity; (+++) more than 50 immunopositivity in 10 different microscopical fields. As a negative control, mouse IgG serum was used. For IHC, 3-amino-9-ethylcarbazole (AEC) chromogen solution and Gill's hematoxylin were used, and slides were sealed with glycer gel.

Table 1. Antibodies used in the study and their properties.

Marker	Specie	Clone	Dilution	Brand	Antigen Retrieval
Mycoplasma pneumonia (M. pneumonia)	Rabbit	Polyclonal	1:100	NovusBio	Citrate buffer (pH:6.0)
Pasteurella multocida (P. multocida)	Pig	Polyclonal	1:200	ATCC	Citrate buffer (pH:6.0)
Escherichia coli (E. coli)	Rabbit	Polyclonal	1:1000	NovusBio	Citrate buffer (pH:6.0)
Clostridium spp.	Rabbit	Polyclonal	1:100	BioRAD	Citrate buffer (pH:6.0)
Parainfluenza 3	Mouse	IgG1	1:100	LsBio	Citrate buffer (pH:6.0)
RSV Fusion Protein	Mouse	IgG2B	1:200	Invitrogen	Citrate buffer (pH:6.0)
Rotavirus	Mouse	A2	1:100	LSBio	Citrate buffer (pH:6.0)
Coronavirus	Mouse	FIPV3-70	1:300	LSBio	Citrate buffer (pH:6.0)

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In-Situ Hybridization Examinations: The sections were stained according to the kit (ZytoDot 2C CISK Implement Kit, Zytovision) procedure. Probes labeled with digoxigenin (Table 2) matching the disease, and for control purposes, positive tissues and/or control probes were used.

Statistical Analysis: In 10 different areas at x40 (0.2 mm² per field) magnification, immunopositivities were counted [Leica Application Suite Version 4.9.0 (Build 129) Leica microsystem (Switzerland)]. The Kruskal-Wallis Test was used for statistical calculations between groups; the Dunn Test for testing between individual groups; and the IBM SPSS 21.0 (SPSS Inc., Chicago IL, USA) program to perform statistical calculations.

Results

Macroscopic Findings: A foamy to bloody exudate was seen in tracheal lumens and on the cut surface of lungs (n: 28). The pleura was thickened with fibrin (n: 13) (Figure 1a). Yellowish pus was observed on cut sections (n: 5), and an abscess (n: 1; 1 cm diameter). The lungs had crepitation (n: 9). Mediastinal lymph nodes were enlarged; their cut sections were moist (n: 18) and/or mottled grey/reddish (n: 4). Reddish color changes (n: 2) and hyperemia were observed in intestinal serosa (n: 2) (Figure 1b). Mucous content adhered to the mucosa, along with non-viscous, yellowish, or hard content in lumens. Adhesions along with intussusception were noted (n: 1). Mesenteric lymph nodes were enlarged, and the cut sections appeared moist (n: 27).

Table 2. Probes used in the study and their properties.

PROBE	Sequence	Oligo-base Type	Base Amount	Molecular Weight	GC Content	Ext.Cofficient (L / (mole.cm))
RSV (F)	5'- /5DigN/ TGA TAA GCT GCA GTC GAA TCC/ 3Dig_N/ -3'	DNA	21	7.907.9	47.6%	230.500
RSV (R)	5'- / 5DigN/ CTG AAC CAG ATC GTA ACG GC/3Dig_N/ -3'	DNA	20	7.588.7	55.0%	220.600
***E. coli* (F)**	5'-/5DigN/AAT AAA TCA TAA GTC AGT AGT AGA CCA TGT /3Dig_N/-3'	DNA	30	10.723.8	30.0%	343.600
***E. coli* (R)**	5'-/5DigN/AAT AAA TCA TAA TAA GCT GGT ATT GAT GCA /3Dig_N/ -3'	DNA	30	10.738.8	26.7%	340.600
***Rotavirus* (F)**	5-/5DigN/GAC GGV GCR ACT ACA TGG T/3Dig_N/ -3'	DNA	19	7.344.9	58.8%	213.113
***Rotavirus* (R)**	5'-/5DigN/GTC CAA TTC ATN CCT GGT G/3Dig_N/ -3'	DNA	19	7.252.3	50.0%	201.700
***Pasteurella multocida* (F)**	5'-5DigN/AGA AAG CAC ATG ACC AAA GGG /3Dig_N/-3'	DNA	21	7.984	47.6%	249.800
***Pasteurella multocida* (R)**	5'-/5DigN/GCA GCT ACT CGC AGA AGG TT/3Dig_N/ -3'	DNA	20	7.619.7	55.0%	218.800
***Mycoplasma pneumonia* (F)**	5'-/5DigN/ACT CCT ACG GGA GGC AGC AGT A/3Dig_N/-3'	DNA	22	8.247.2	59.1%	244.400
***Mycolasma pneumonia* (R)**	5'- /5DigN/TGC ACC ATC TGT CAC TCT GTT AAC CTC /3Dig_N/ -3'	DNA	27	9.599	48.1%	265.300

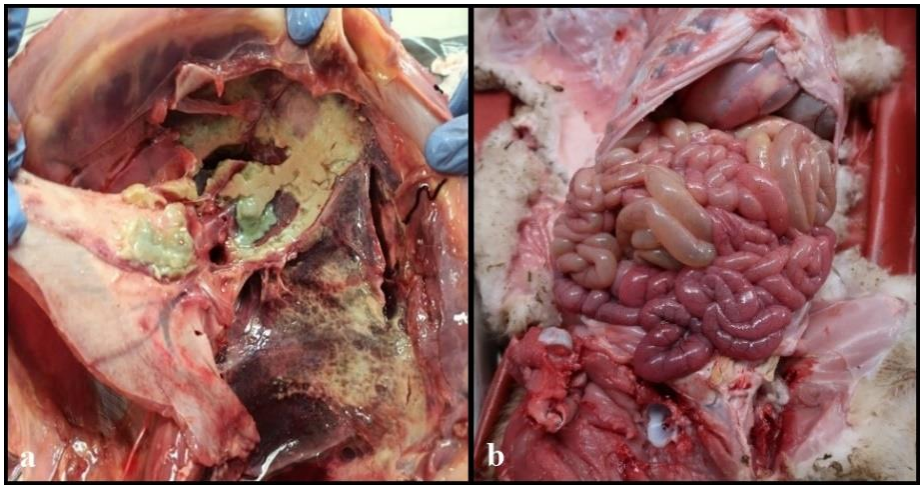

Figure 1. Macroscopic appearance: thickened pleura (a), intestines with reddish color changes in the serosa and gas-filled lumens (b).

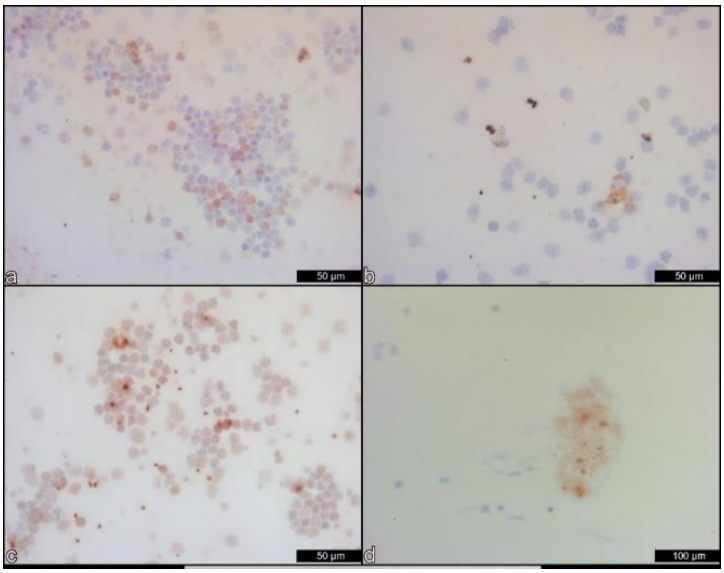

Figure 2. Rotavirus (a), *Clostridium spp*. in macrophages (b) and *E. coli* (c) immunopositivity in mesenterial lymph nodes; *M. pneumonia* (d) immunopositivity in mediastinal lymph nodes, ICC.

Cytochemical/Immunocytochemical Findings: In the examinations, inflammatory cells, especially lymphocytes and macrophages, were encountered. Rotavirus (Figure 2a), *Clostridium spp.* (Figure 2b), and *E. coli* (Figure 2c) were detected either free and/or within the cytoplasm of macrophages and lymphocytes. *M. pneumoniae* (Figure 2d) was found within the cytoplasms of macrophages and lymphocytes, while parainfluenza 3 was identified as a free agent in the lung exudates. In the mediastinal lymph nodes, no positivity was detected for coronavirus, RSV, or *P. multocida* antibodies.

Histopathological Findings: Microscopically, fewer or more neutrophils and leukocytes were seen only in the alveoli (n: 2; alveolitis); in the bronchi and bronchioli (n: 1; purulent bronchitis); or in both the alveoli and bronchi/bronchioli (n: 2; acute catarrhal bronchopneumonia, n: 2; subacute catarrhal bronchopneumonia; n: 4; subacute bronchopneumonia; n: 3; purulent bronchopneumonia) (Figure 3a, 3b). Also, necrosis was seen (n:2; necrotic bronchopneumonia). In addition, fibrin accumulations were found in both alveoli and bronchi/bronchioli lumens, along with pleura & interlobular septae (n: 5; fibrinonecrotic bronchopneumonia) and enlarged lymphatics. In some cases, bronchopneumonia accompanied only by fibrin was observed (n: 5). It was accompanied by purulent inflammation in a single case (n: 1; fibrinopurulent bronchopneumonia). In a few cases, although the alveoli were empty, cellular infiltrate with a majority of mononuclear cells was observed in the peribronchial/bronchiolar and perivascular areas. In some cases, the interalveolar region was thickened due to varying amounts of inflammatory cells (n: 6; interstitial pneumonia) (Figure 3c). In one case, granulomatous areas dominated the field (n:1; granulomatous pneumonia). Cases in which atelectasis, hyperemia, and/or edema (n: 6) were observed along with emphysema, where the inflammation had not started yet, were also encountered.

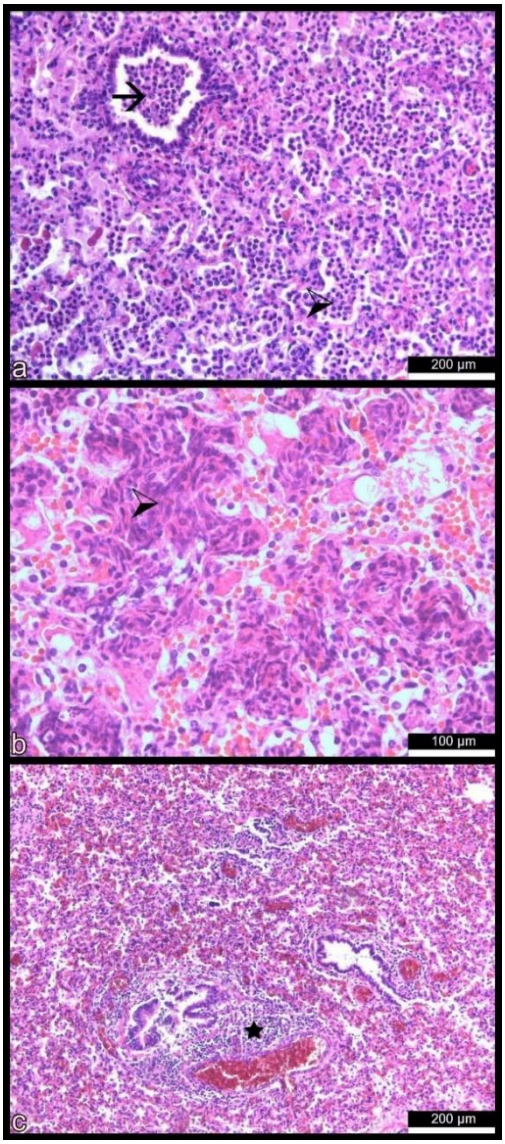

Figure 3. Purulent bronchopneumonia (a, b), neutrophil leukocytes in the bronchiolar lumen (arrow) and alveolar lumens (arrowhead), *M. pneumoniae*; interstitial pneumonia (c), peribronchiolar and perivascular mononuclear cell infiltration (stars), parainfluenza virus, HE.

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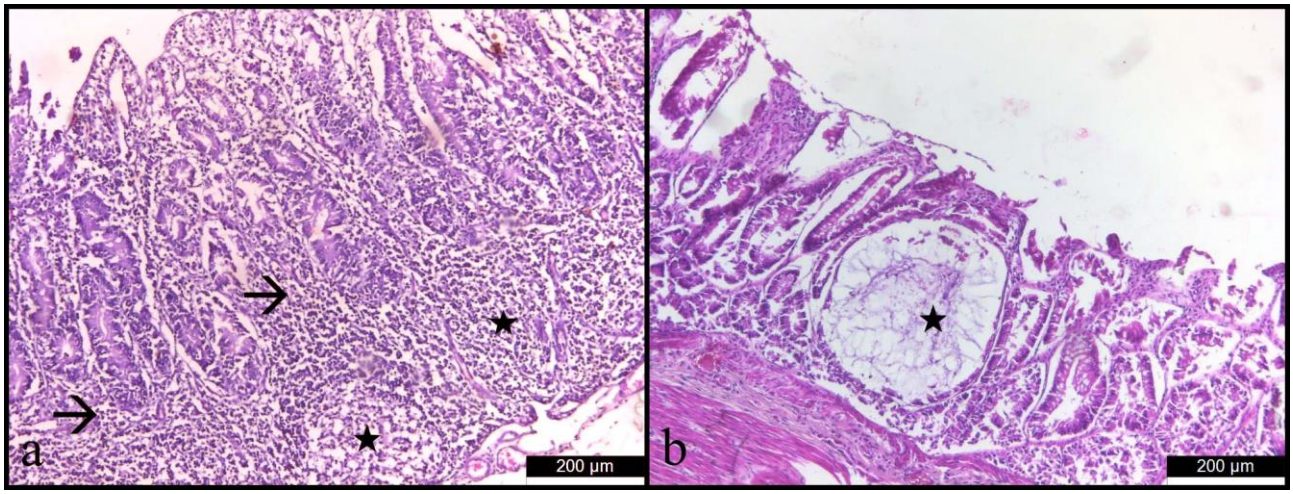

Figure 4. Subacute enteritis (a), *Clostridium spp*., inflammatory cell infiltrations (arrows) and hyperplastic lymphoid follicles (stars); cystic Lieberkühn glands (b), cystic structure (asterisk), rotavirus, HE.

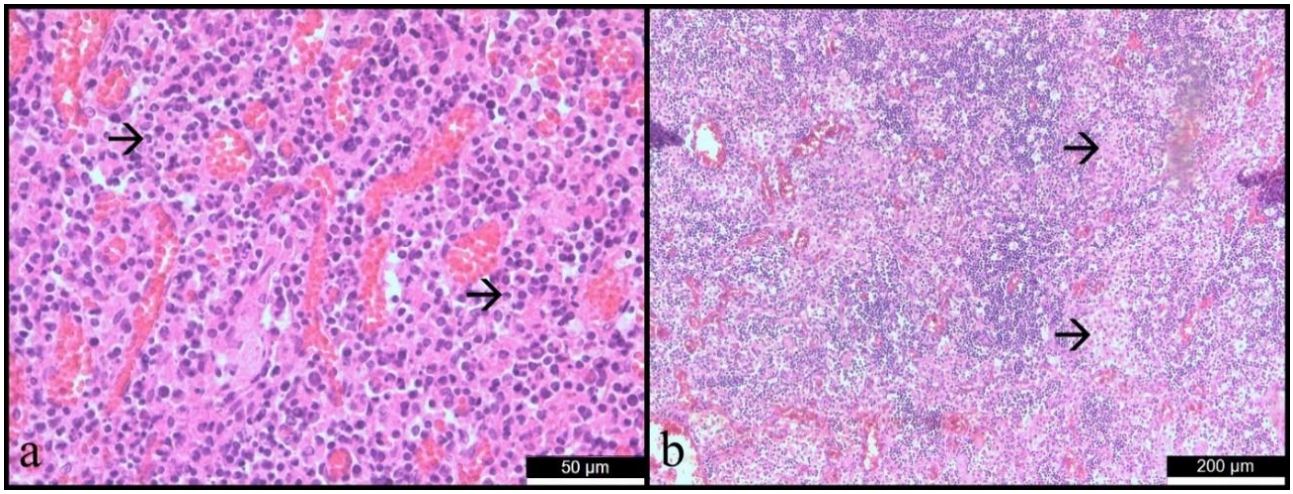

Figure 5. Sinus catarrh (a), neutrophils in sinuses (arrows), *M. pneumoniae*; necrotic lymphadenitis (b), necrotic lymphoid follicles (arrows), *E. coli*, HE.

In most of the intestines examined, inflammatory cell infiltrates consisting of a few neutrophils, leukocytes, a greater number of plasma cells, lymphocytes, and macrophages were observed between the glands in the lamina propria (n: 13). Changes in the ileum were accompanied by hyperplasia of aggregate lymph follicles (Figure 4a). Necrotic material, secretion, and a small number of neutrophil leukocytic cell infiltrations accompanied by cystic dilated changes in the crypts (n:1), inflammation only in the jejunum (n:1; jejunitis), and cystic changes only in the Lieberkühn glands (n:1) (Figure 4b) were observed. Parasites (n: 4; parasitic enteritis) were observed in intestinal tissues of 4 out of 16 animals. Lymphadenitis simplex (n: 14), sinus catarrh (n: 2) (Figure 5a), and necrotic lymphadenitis (n: 3) (Figure 5b) were seen in the mesenteric lymph nodes.

Immunohistochemical Findings: In all cases, lungs, intestinal sections, and existing mediastinal/mesenterial lymph nodes were stained with the relevant antibodies.

Lungs: *P. multocida* was negative for all lung tissues. *M. pneumonia* (n: 17, Figure 6a), parainfluenza 3 (n: 20, Figure 6b), RSV (n: 4, Figure 6c), and *E. coli* (n: 35, Figure 6d) were found to be positive.

Mediastinal Lymph Nodes: Immunohistochemical staining was performed (n: 20/22). No positivity was seen for *P. multocida* and RSV antibodies. *M. pneumoniae* (n: 1), parainfluenza 3 (n: 10, Figure 6e), and *E. coli* (n: 5, Figure 6f) were found positive in capsule, cortex, and/or medulla, free and/or in macrophages.

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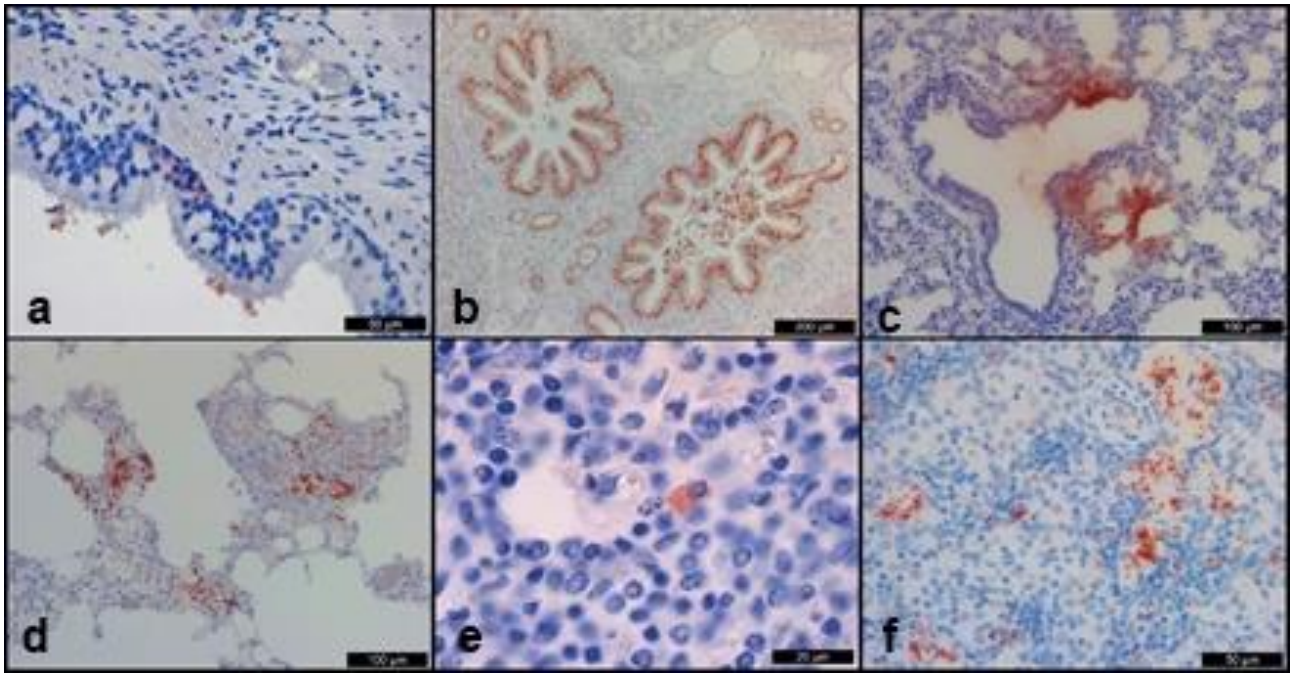

Figure 6. Immunopositivity of *M. pneumoniae* (a); parainfluenza 3 (b); RSV (c); and *E. coli* (d); parainfluenza 3 (e); and *E. coli* (f) seen free, in epithelial cells, and/or phagocytosed in macrophages, IHC.

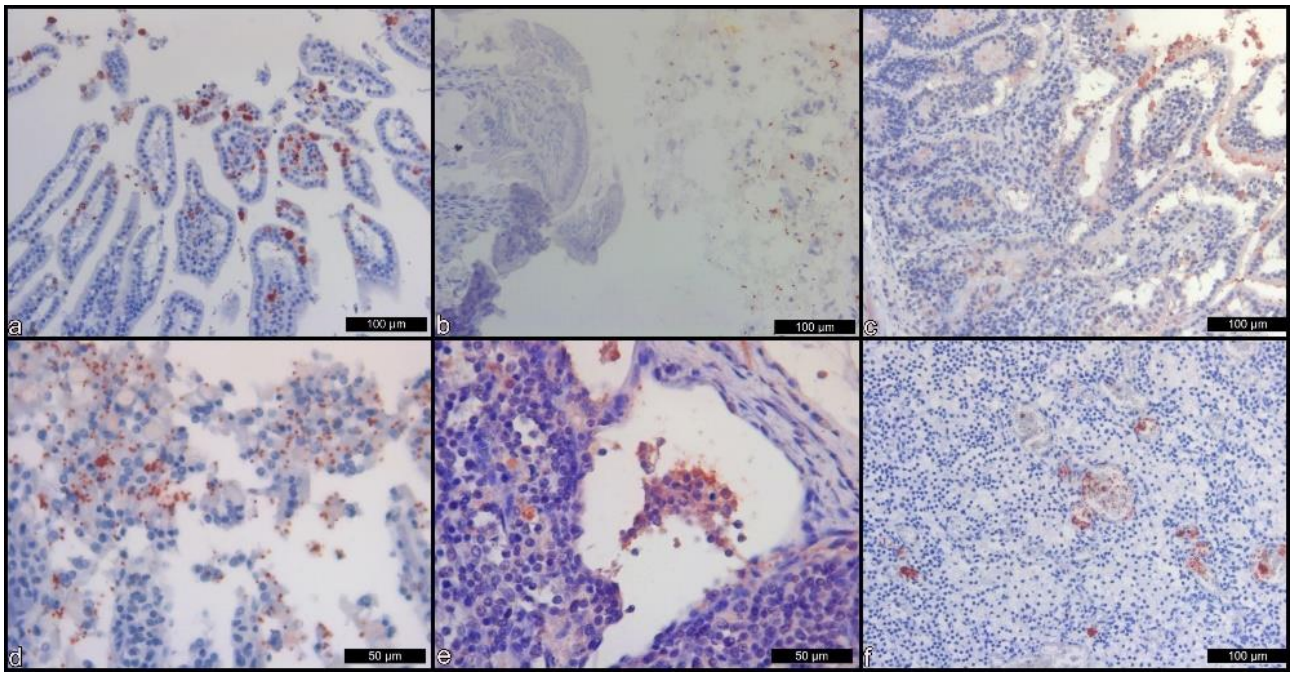

Figure 7. Rotavirus (a), coronavirus (b), *Clostridium spp.* (c), *E. coli* (d), and *Clostridium spp.* (e) immunopositivity in epithelial cells or macrophages, also *E. coli* positivity in the vascular lumens (f), IHC.

Intestines: Rotavirus (n: 11, Figure 7a) was found positive in the jejunum, ileum, colon, and cecum; coronavirus (n: 3, Figure 7b) in the duodenum, colon, and cecum; *Clostridium spp.* (n: 33, Figure 7c) in all intestines; and *E. coli* (n: 35, Figure 7d) in the duodenum, ileum, and cecum. The positivities were generally seen on villus surfaces, in the cytoplasm of epithelial cells, and in macrophages.

Mesenteric Lymph Nodes: Immunohistochemical staining was performed (n: 29/31). No positivity was seen for the coronavirus antibody, but rotavirus (n: 3), *Clostridium spp.* (n: 19, Figure 7e), and *E. coli* (n: 11, Figure 7f) positivities were seen in the cytoplasms of macrophages, in the cortex, and in the vascular lumens.

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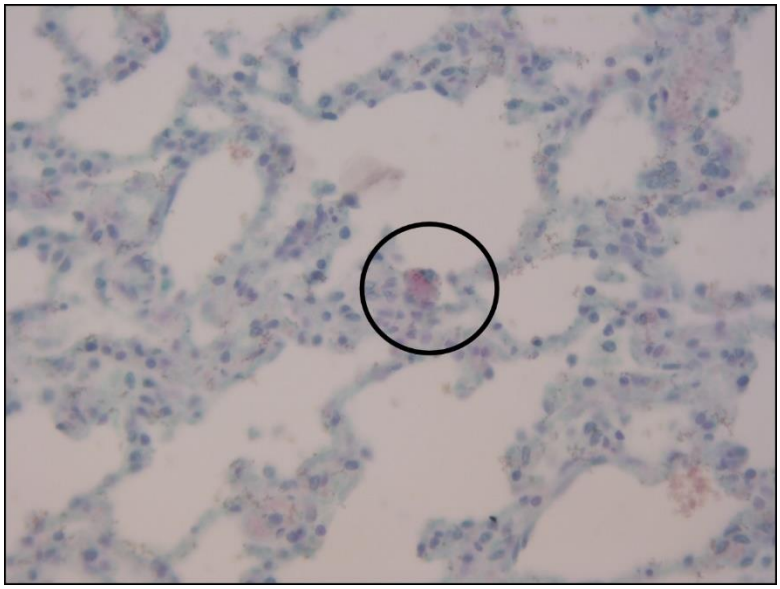

Figure 8. *E. coli* positivity in the lung (circle), CISH.

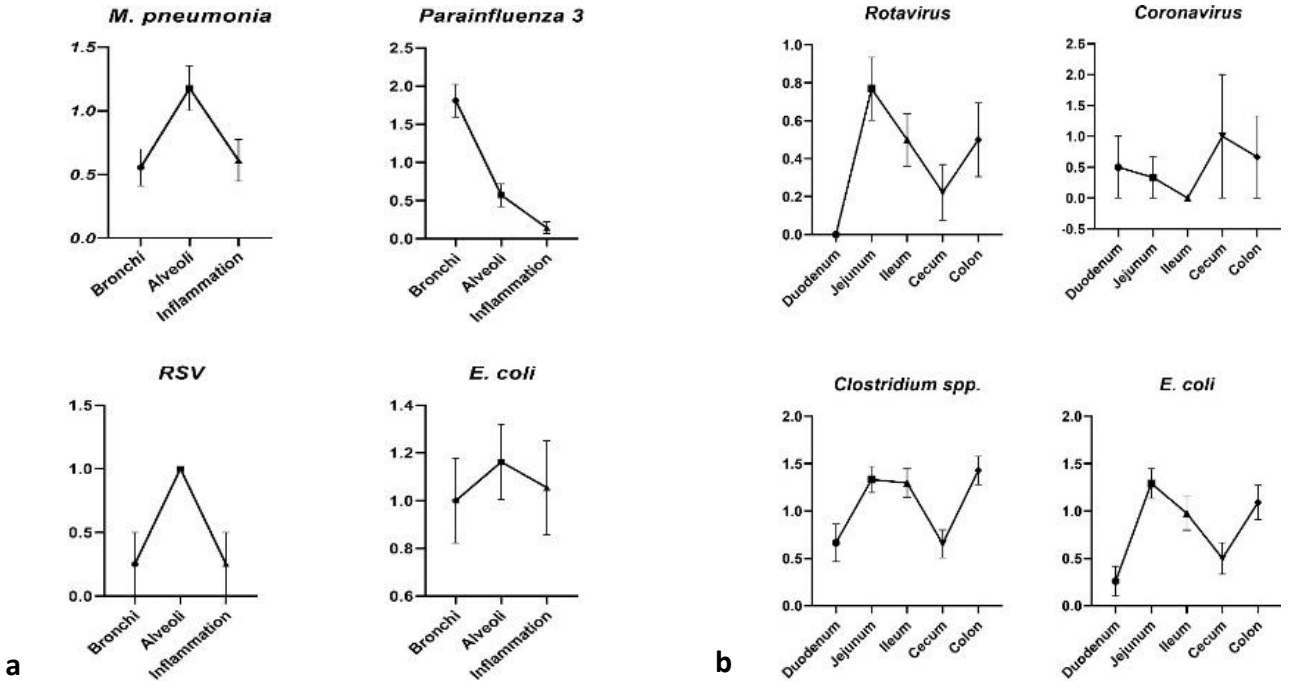

Figure 9. Statistical immunopositivity rates of the agents seen in the lungs (a) and intestines (b).

In Situ Hybridization Findings: In the staining results, *E. coli* (n: 4) (Figure 8) and parainfluenza 3 virus (n: 2) were detected in the lungs, while mild positivity was detected for *E. coli* (n: 4) and rotavirus (n: 2) in the intestines; no positivity was observed in any of the relevant regional lymph nodes.

Statistical Findings

Lungs: In statistical examinations of the regions, immunopositivity was seen, and a statistically significant difference was observed for *M. pneumoniae* (P=0.021). When the settlements are compared among themselves, a difference was observed between bronchi and alveoli, but none between bronchial inflammation and alveolar inflammation. For parainfluenza 3, a statistical difference was observed between settlements (P<0.001). When compared, although there was a statistical difference between the staining rate in the bronchi and both the alveoli and inflammation, no statistical difference was observed between the staining rates in the inflammation and alveoli. No statistically significant difference was observed for RSV (P=0.143) and *E. coli* (P=0.536) (Figure 9a). Statistical evaluation could not be made for the *P. multocida* due to a lack of samples.

Intestines: A statistically significant difference was observed for rotavirus (P=0.017). When the intestinal segments were compared, only a difference was seen between the duodenum and jejunum. A statistically significant difference was detected between locations and staining intensities for Clostridial factors (P<0.001). When the locations were compared, a statistically significant difference was seen between duodenum-jejunum, duodenum-colon, jejunum-cecum, and cecum-colon pairings. For E. coli, a statistically significant difference was observed when the staining intensities were compared (P<0.001). When the intestinal sections were compared, a statistically significant difference was detected between duodenum-jejunum, duodenum-ileum, duodenum-colon, and jejunum-cecum. There was no statistically significant difference for the coronavirus (P=0.964). Also, since the in situ hybridization staining results were few in number and mild in intensity, significant results could not be obtained. They were excluded from statistical evaluations (Figure 9b).

Discussion and Conclusion

The bacterial bronchopneumonias include *Mycoplasma spp.*, *Mannheimia haemolytica*, *Histophilus somni*, and *Pasteurella multocida*. *M. haemolytica* can cause septicemia without pneumonia in lambs younger than 2 months (3). In this study, *M. haemolytica* antigen was detected more prominently in cases diagnosed with fibrinous and/or fibrinonecrotic bronchopneumonia. When evaluated in general, it is concluded that, unlike the common approach, it would be appropriate to evaluate fibrinous pneumonias first for *M. haemolytica* and then for other fibrinous bronchopneumonia agents (*Mycoplasma spp.* and *Pasteurella spp.*). In the study of Yener et al. (2005), bacterial agents were detected in 32 of 42 pneumonia lung tissues. These are *P. haemolytica* (38.09%), *Mycoplasma spp.* (28.57%), *Staphylococcus aureus* (16.66%), *Klebsiella pneumoniae* (11.90%), *Moraxella spp.* (4.76%), *Bacillus spp.* (4.76%), and *P. multocida* (2.38%). The presence of parainfluenza 3 (PI3) viral antigen was found in 28 of these samples. However, no positivity was observed in tissues without pneumonia. In our study, in addition to lungs with pneumonia, mild PI3 positivity was also found in sections showing emphysema and atelectasis. The location of PI3 immunopositivity in the lung tissue in the study was mostly similar to other studies (1, 3, 6, 11, 19). In addition, it was noticed that it was found free or phagocytosed by macrophages in the interstitial area, interlobar septa, and vascular surroundings. In addition, this study detected the presence of PI3 viral antigen and *E. coli* immunopositivity in the mediastinal lymph nodes. It was noted that PI3 infection usually occurs as a mixed infection with other factors. These include mainly *M. pneumoniae* and *E. coli*, and to a lesser extent respiratory synctial virus (RSV) and *P. multocida*. Therefore, histopathological diagnoses vary. The most prominent findings of PI3 infection are bronchiolitis and mild bronchitis. In the acute period, eosinophilic, intracytoplasmic inclusion bodies are seen in the bronchus, bronchiole, alveolar epithelial cells, and alveolar macrophages. BRSV together with PI3 should also be considered in cases of necrotic bronchiolitis (3). In this study, similar to the study by Yener et al. (2005), no inclusion bodies were found, no matter how acute the infection was. Within the scope of this study, the incidence of PI3 comes second, especially *E. coli*. In future studies, it would be appropriate to investigate in detail which strains of *E. coli* are involved in both lung and intestinal infections in newborn small ruminants and similarly, which species in the genus *Clostridium* cause intestinal infections. The most striking finding of BRSV infection is bronchiole and alveolar epithelial syncytia formation along with bronchointerstitial pneumonia. Syncytia and intracytoplasmic eosinophilic inclusion bodies are evident in the early stages (3). In this study, syncytia formation and inclusion bodies were not noticed in RSV immunopositive cases. It has been determined that the localization of RSV is similar to PI3. In *Mannheimia haemolytica* infection, the appearance of multinucleated macrophages around the fibrin in the alveoli may lead to a false diagnosis of RSV infection (3). In this study, RSV viral antigen was found in bronchiolar and alveolar epithelial cells, alveolar macrophages, and macrophages located in the interstitium. Chronic bronchopneumonia (atypical or chronic nonprogressive pneumonia) is common in lambs and kids, and *Mycoplasma ovipneumoniae*, *M. haemolytica*, and PI3 have been detected in its etiology (17). In this study, similar to other studies, *M. pneumoniae* antigen was detected on the bronchial/bronchiolar/alveolar epithelial surface and/or free or in desquamated epithelial cells in their lumens and in alveolar macrophage cytoplasms. Although *M. pneumoniae*, *E. coli*, RSV, and PI3 immunopositivity were detected in some lung sections, it was noticed that the pneumonia had not yet formed. We can relate this situation to the amount of agent, pathogenicity, duration of infection, and individual factors. In this study, unlike others, immunocytochemical staining was performed on touch slides prepared from lung tissue, mediastinal and mesenterial lymph nodes, and successful results were obtained. Following immunocytochemical examinations, *M. pneumoniae* was found in mediastinal lymph nodes and PI3 in lung tissues; rotavirus and *Clostridium spp.* from mesenterial lymph nodes and *E. coli* immunopositivity drew attention. Thus, immunocytological and immunohistochemical methods have again shown their importance in rapid diagnosis.

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Enterotoxigenic *E. coli* (ETEC), rotavirus, *C. perfringens* type B, and *Cryptosporidium parvum* are the main etiologies of neonatal diarrhea in small ruminants (13, 18). In this study, rotavirus, *Clostridium spp.*, and *E. coli* immunopositivity were found in the intestinal sections, especially jejunum, ileum and colon and mesenterial lymph nodes. Enterotoxigenic *E. coli* can cause more severe infections together with rotavirus (18). Thus, it was concluded that *Clostridium spp.* and *E. coli* pathogens, alone or together, were responsible for neonatal deaths in small ruminants. It has been reported that coronavirus infection was seen older than three months (7). In this study, the presence of coronavirus antigen was noticed, especially in the intestinal content (colon) and duodenum, unlike the previous research (15).

In our ISH examinations, due to insufficient positivity, it was not foreseen to be evaluated as a method that should be used in the rapid and routine diagnosis of the disease (5). In the stainings performed, both positive controls and ICC and IHC positive samples were repeated, especially the staining method, to eliminate possible kit and/or method drawbacks. Considering all these results, it has been determined that *Mycoplasma pneumonia*, *Escherichia coli*, parainfluenza virus 3, and respiratory syncytial virus, which are respiratory agents, and *Escherichia coli*, *Clostridium spp.*, coronavirus, and rotavirus, which are enteric agents, are still active in our country, apart from Pasteurella multocida, which is one of the respiratory agents. It was determined that neonatal deaths were not caused by a single agent alone but mostly occurred after mixed infection. In addition, the creation of immunohistochemical diagnostic panels against neonatal infections specified in the aims of the study was achieved. In addition, immunohistochemical and molecular (PCR, ELISA, etc.) techniques are frequently used in literature, and immunocytochemical studies are almost non-existent. In this study, immunocytochemical, immunohistochemical, and in situ hybridization methods were used; comparisons were made between the methods according to the results obtained after these stainings, and although the immunocytochemical method was determined to be the fastest-yielding method, immunohistochemistry was the one that gave the most reliable results. In the presentation of this article, with the results obtained by using a limited number of antibodies in the respiratory and digestive systems, the expected targets were achieved, excluding ISH, and a relevant diagnostic panel (HP, ISC, and IHC) was created, which can be actively used in diagnosis in our laboratory in the future.

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Ethical Statement

This study does not present any ethical concerns.

Conflict of Interest

The authors declared no conflict of interest.

Author Contributions

SAV planned and conceived the idea of the research, along, with scientific support throughout the project. GYT, AST, YAM, OA, KF, ÖÖ, and OBD took part in collecting material, data and necessary stainings with pre-evaluation. RH and OK helped writing the article with their profound experience.

Data Availability Statement

The data supporting this study's findings are available from the corresponding author upon reasonable request.

Animal Welfare

The authors confirm that they have adhered to ARRIVE Guidelines to protect animals used for scientific purposes.

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Three-dimensional morphological variation and sexual dimorphism in the humerus of dromedary camels *(Camelus dromedarius)* from El Oued region: a geometric morphometric analysis

Mohamed Amine FARES[1,a,✉]

[1]University of Souk Ahras, Institute of Agriculture and Veterinary Sciences Taoura, Laboratory of Sciences and Technics of the Livings, Department of Veterinary Sciences, Souk Ahras, Algeria.

[a]ORCID: 0000-0003-4721-018X

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✉Corresponding author
m.fares@univ-soukahras.dz

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ABSTRACT

This study investigates the three-dimensional morphological variation and allometric relationships in the humerus of dromedary camels *(Camelus dromedarius)* from the El Oued region, with a focus on sex-based differences. The aim is to analyze the morphological diversity and sexual dimorphism in the humeri of dromedary camels using advanced geometric morphometric techniques. This includes discerning patterns of variation and covariation, particularly related to sexual dimorphism and size-related shape changes. A sample of 59 humeri (29 males and 30 females) was collected. High-resolution three-dimensional scans were used to capture detailed shapes, followed by Procrustes superimposition and Principal Component Analysis to analyze the data. The analysis revealed significant sexual dimorphism, with male camels having more robust and thicker humeri compared to the more slender and delicate humeri of female camels. The allometric analysis showed notable size-related shape changes, especially in the deltoid tuberosity and distal epiphysis regions. The study underscores the presence of sexual dimorphism and its impact on the functional morphology of camelid skeletal structures. The findings provide valuable comprehension into the adaptation pressures and functional demands shaping these bones, demonstrating the utility of geometric morphometrics as a powerful tool in skeletal morphology studies. This research sets a new standard for future studies by integrating high-resolution three-dimensional scanning with sophisticated morphometric analyses.

Introduction

The dromedary camel (*Camelus dromedarius*) is a keystone species in arid and semi-arid regions, particularly the Saharan desert, where it plays a crucial role in the socio-economic and cultural lives of nomadic and pastoralist communities. These animals are renowned for their remarkable adaptations to extreme environments, including their ability to survive long periods without water, as well as, their unique physiological and anatomical traits (1, 8). However, despite their importance, the detailed anatomical studies of their skeletal structures, particularly in the context of sexual dimorphism and regional morphological variations, remains underexplored.

The humerus, a primary bone of the forelimb, is integral to the locomotion and load-bearing functions of dromedaries. Understanding the morphological variations in the humerus is essential for clarity into the biomechanics, adaptations, and veterinary care of these animals. Prior studies on camelid bones have largely focused on the scapula and other bones (3), revealing significant sexual dimorphism and allometric patterns. However, comprehensive studies on the humerus,

especially in relation to sex-based differences and allometric relationships, are limited.

To address these gaps, geometricmorphometrics provides a powerful analytical framework for examining bone morphology. This analytical framework enables the precise quantification and comparison of complex shapes (4, 13, 15, 34). By employing geometric morphometric techniques, this study aims to elucidate the extent of sexual dimorphism and allometric relationships in the humerus of dromedary camels from the El Oued region.

Sexual dimorphism, defined as the systematic difference in form between individuals of different sex within the same species, is a well-documented phenomenon in many vertebrates, including mammals (26). In camelids, males and females often exhibit distinct morphological traits, likely due to differences in their roles and physical demands. Males typically engage in more physically demanding activities, such as territorial defense and mating competition, which may contribute to the development of more robust skeletal structures (25). In contrast, females may exhibit morphological adaptations associated with offspring care and foraging strategies.

Beyond sexual dimorphism, allometric relationships play a crucial role in shaping skeletal morphology, influencing both structural integrity and functional adaptations. That is allometry describes how variations in body size correlate with changes in shape, ensuring optimal biomechanical performance across different growth stages and ecological demands, the study of the relationship between size and shape, is another critical aspect of morphological research. Allometric patterns can provide insights into how size-related shape changes influence function and performance in skeletal elements (9, 10, 17). In this context, understanding the allometric relationships in the humerus of dromedaries can reveal how size variations impact bone morphology and, consequently, the biomechanical and functional capabilities of these animals.

Building on the advancements in geometric morphometrics, recent innovations in imaging and computational techniques have further refined the ability to analyze skeletal structures with greater precision. High-resolution 3D imaging, for instance, allows for the detailed reconstruction of bone surfaces, enabling more comprehensive assessments of shape variations (31). Such technologies have opened new avenues for studying the subtle morphological differences that may arise due to environmental pressures or genetic factors. In dromedary camels, these advanced methods can uncover previously unrecognized patterns of variation and adaptation, providing deeper insights into their evolutionary biology (21).

Applying these advanced analytical techniques in a geographically distinct population provides valuable insights into how regional factors influence skeletal morphology. Given this, the El Oued region, known for its distinct environmental conditions and traditional camel breeding practices, provides a unique context for studying the morphological variations in dromedaries. Camels in this region are adapted to specific ecological niches, which may influence their skeletal morphology (11). By focusing on a sample of humeri from this region, this study aims to provide a detailed analysis of sex-based morphological differences and allometric relationships, contributing to the broader understanding of camelid anatomy.

Building on the morphological analysis of dromedary humeri, This study has three primary objectives; the first is to document the three-dimensional morphological variation in the humerus of male and female dromedaries, the second is to analyze the allometric relationships between bone size and shape, and the third is to compare the findings with existing literature on camelid skeletal morphology. By addressing these objectives, this research aims to fill the existing gaps in our knowledge of dromedary humerus morphology and provide valuable insights for veterinary anatomists, biologists, and biomechanists. To accomplish this, geometric morphometric techniques are employed to investigate the three-dimensional morphological variation and allometric relationships in the humerus of dromedary camels from the El Oued region. The findings are expected to enhance our understanding of sexual dimorphism and size-related shape changes in camelid skeletal structures, contributing to improved veterinary care and deeper insights into the adaptations of these remarkable animals.

Materials and Methods

Sample Collection: Fifty nine adult dromedary camels (*Camelus dromedarius*) from the El Oued region (Figure 1) were selected for this study, comprising twenty nine males and thirty females. The animals were sourced from a regional slaughterhouse, ensuring ethical considerations were met, and permissions were obtained from the relevant authorities.

Following post-mortem examination, the humeri were carefully extracted and cleaned of soft tissues in preparation for detailed analysis. To remove residual soft tissues, the bones were subjected to a boiling method, in which they were immersed in hot water at approximately 80–90°C for several hours until all remaining soft tissues detached. Subsequently, the bones were manually cleaned to eliminate any residual tissues. No chemical bleaching agents were applied to preserve the natural bone structure and surface integrity.

To ensure that the sample accurately represented typical adult morphology and was free of skeletal abnormalities, the age and health status of the camels were documented.

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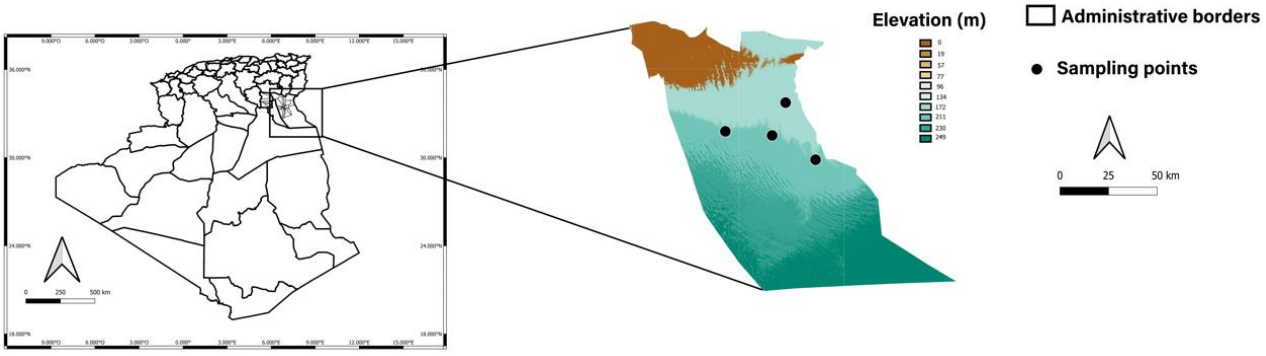

Figure 1. Study area: A digital elevation map of the El Oued region in southeast Algeria, showing sampling points.

Photogrammetry and 3D Modeling: High-resolution three-dimensional models of the humeri were created using photogrammetry. To ensure high image quality and precision, each humerus was photographed from approximately 80 to 120 different angles using a Canon EOS 90D DSLR camera equipped with an EF 50mm f/1.8 STM lens. The photographs were then processed using Meshroom software (Version 2023.3.0) (5) to generate 3D models of the bones. Subsequently, the generated models were refined and optimized using MeshLab software (Version 5.15.2).

Landmark Generation and Pseudolandmarks: In 3D Slicer (version 5.4.0), the PseudoLM Generator module in the GeoMorph extension was used to generate pseudo-landmarks on the humeri. A source landmark template was established using this plug-in, with a spacing tolerance of 3%. The 'Original Geometry' option was selected to derive a sampling pattern based on the model's geometry. The initial number of sampled points in the template was set to 15, and a template mesh was generated using the 'Generate Template' function. Subsequently, a 'Project points to surface' operation was performed, followed by enforcing a spatial sampling rate to exclude samples with a point-to-point distance lower than the defined threshold (Figure 2)(2, 7, 33).

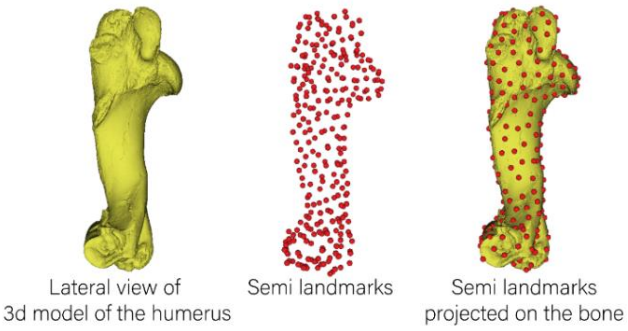

Figure 2. Lateral view of 3D humerus model showing semi-landmarks projection on the bone surface.

Landmark Transfer and Semi-landmarks: The ALPACA (Aligning Landmarks for Procrustes and Canonical Analyses) algorithm (24) tool was used for the efficient transfer of landmarks from the draft pseudo-landmark template to the target 3D models. A batch processing approach was implemented to apply the draft pseudo-landmark across all samples using the "Single Template (ALPACA)" option. The identical mesh model served as both the source and the target, with the draft pseudo-landmark prepared for each specific sample acting as the source landmark. The process was finalized with the execution of the 'Run-auto landmarking' function, resulting in the recording of 285 semi-landmark sets (28), each documented separately for all samples (Figure 3).

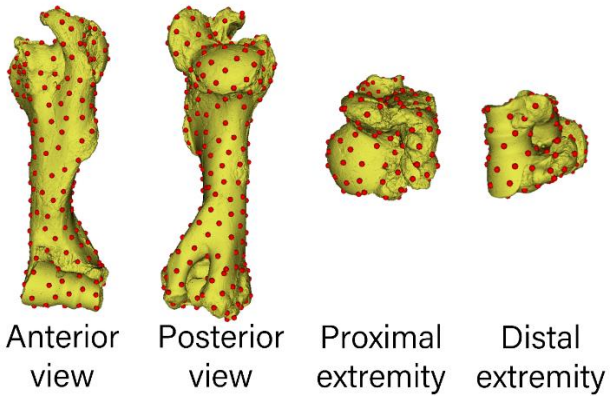

Figure 3. Projection of 285 landmarks of the humerus bone.

Shape Analysis: The semi-landmark coordinates were analyzed using Generalized Procrustes Analysis (GPA) (6, 31). GPA aligns the shapes by eliminating differences in position, orientation, and scale, thereby standardizing them for comparative analysis. This process involves translating, rotating, and scaling the landmark configurations to a common reference, enabling the assessment of true shape differences without the influence of confounding factors.

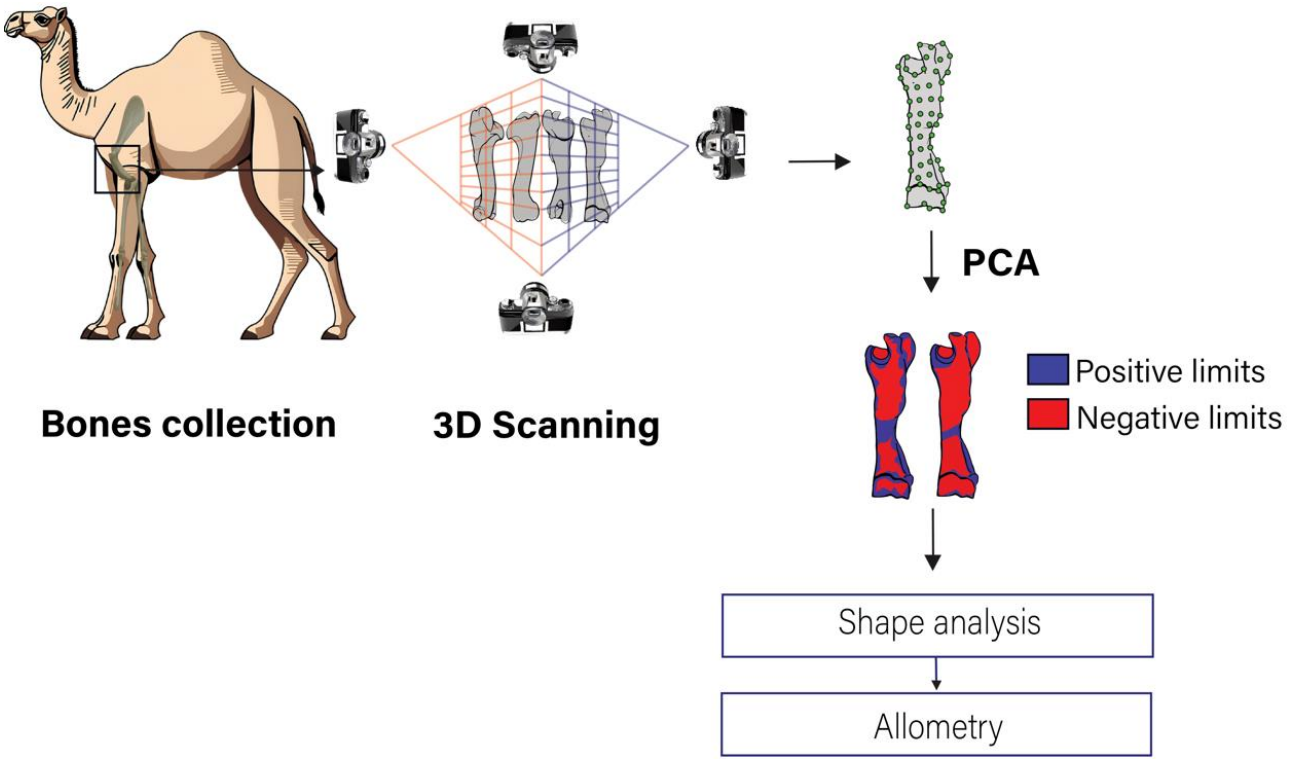

Figure 4. Workflow illustration of the methodology from bones sampling to data analysis and results extraction.

Principal Component Analysis: Principal Component Analysis (PCA) was performed on the aligned landmark coordinates to identify the primary axes of shape variation among the humeri. PCA reduces the dimensionality of the data by transforming it into principal components, which are orthogonal linear combinations of the original variables that capture the maximum variance in the dataset (12, 13). The principal components were subsequently analyzed to determine the extent of morphological variation and to identify shape differences between male and female humeri. Additionally, 3D changes were obtained from Slicer (version 5.4.0), and Procrustes distances were calculated for all samples.

Allometric Analysis: To investigate the relationship between shape and size, an allometric analysis was performed using centroid size as a proxy for humerus size. Centroid size is a measure of the geometric size of an object, is calculated as the square root of the sum of squared distances of each landmark from the centroid (5, 17). The shape variables (principal component scores) were regressed against the centroid size using R Studio (version 4.3.2) statistics programs, enabling the evaluation of how size influences shape variation. Multivariate regression analysis was used to investigate whether allometry existed in the dromedary humerus and to assess the statistical significance of the results (9). The overall workflow is presented in (Figure 4).

Statistical Analysis: To assess the statistical significance of shape and size differences between male and female humeri, Procrustes ANOVA was conducted to evaluate the variation in shape associated with group membership (sex) by partitioning the total shape variation into components attributable to sex (13, 17). The centroid size differences between sexes were analyzed using one-way ANOVA to determine whether size variation was statistically significant. One-way ANOVA was performed using R Studio (version 4.3.2), with a significance threshold set at $P < 0.05$.

Results

The humerus of the dromedary camel exhibits a robust and elongated structure, reflecting its adaptation to weight-bearing and locomotion. The proximal end is distinguished by a well-developed humeral head, which articulates with the scapula to form the shoulder joint. The greater and lesser tubercles are prominent, serving as attachment sites for key shoulder muscles. Additionally, The deltoid tuberosity, located along the cranial aspect of the shaft, is notably pronounced, indicating strong muscular attachment, particularly for the deltoid and brachialis muscles.

The humeral shaft displays a gentle curvature, contributing to biomechanical efficiency in load distribution during movement. The distal end is

characterized by a distinct trochlea and capitulum, which articulate with the radius and ulna to facilitate forelimb flexion and extension. Furthermore, medial and lateral epicondyles are well-defined (Figure 5).

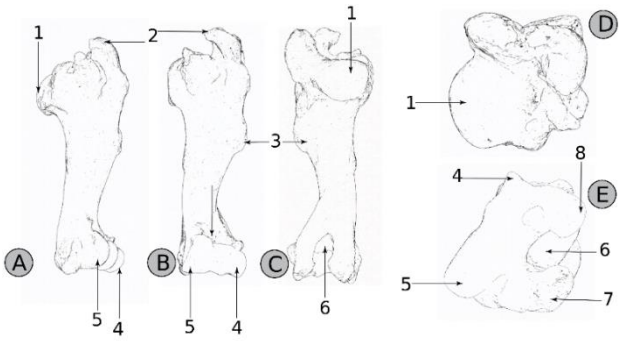

Figure 5. Left humerus of the camel (a) lateral view, (b) cranial view, (c) caudal view, (d) proximal view, (e) distal view; 1: The head, 2: Major tuberosity, 3: Deltoid tuberosity, 4: Capitulum, 5:Trochlea, 6: Olecranon fossa, 7: Lateral epicondyle, 8: Medial epicondyle.

Principal Component Analysis (PCA): The application of PCA to the humerus bones identified two primary components, PC1 and PC2, which account for substantial portions of the shape variance within the dataset. Specifically, PC1 explains 19.6% of the total variance, while PC2 accounts for 11.9%. The PCA scatterplot visually illustrates the distribution of the samples across these principal components, effectively highlighting the morphological diversity among the specimens (Figure 6).

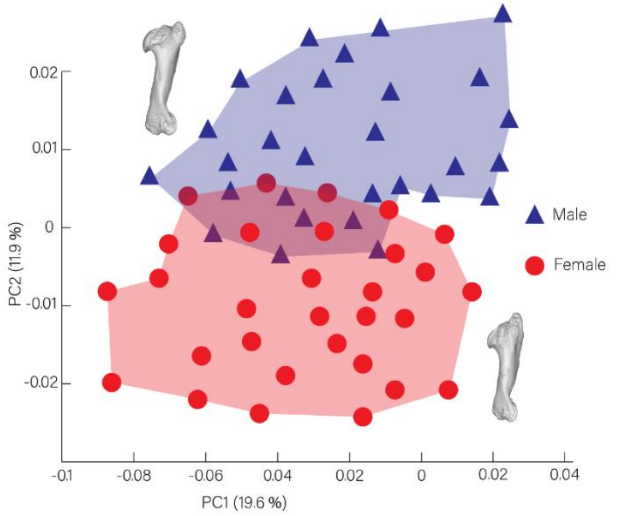

Figure 6. Principal component analysis (PCA) scatterplot of 3D geometric morphometrics of male and female humeri in dromedaries, including convex hulls delineating each sex (males in blue triangles, females in red circles).

Positive and Negative Values of PC1: The results of the principal component analysis (PCA) revealed significant morphological variations in the humeri of dromedary camels along the first principal component (PC1), highlighting pronounced sexual dimorphism. The lateral view demonstrated that the positive limits (blue) of PC1 were associated with a more robust humeral head, major tuberosity, and deltoid tuberosity, whereas the negative limits (red) corresponded to less prominent structures. The cranial view showed a more bulbous and extended head, along with more pronounced capitulum and trochlea in the blue regions, compared to flatter structures in the red regions. Similarly, in the caudal view, the olecranon fossa appeared deeper and more defined in blue, while shallower in red, with both lateral and medial epicondyles exhibiting similar size variations. The proximal view reinforced these observations, with larger and more robust head and major tuberosity in the blue regions. Finally, the distal view highlighted more prominent capitulum, trochlea, and epicondyles in the blue regions, contrasting with flatter and less distinct structures in the red regions, male camels exhibited more robust and structurally prominent humeri (blue regions), whereas female camels displayed more slender and delicate humeri (red regions), particularly in the head, major tuberosity, deltoid tuberosity, capitulum, trochlea, olecranon fossa, and epicondyles (Figure 7).

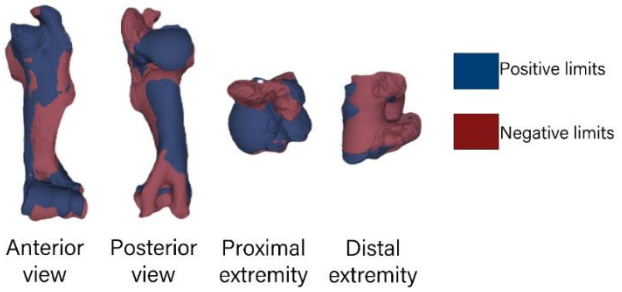

Figure 7. Distribution of principal component 1 (PC 1) values with negative (red) and positivie (blue) limits.

Positive and Negative Values of PC2: The results of the second principal component (PC2) analysis revealed significant morphological variations in the humeri of dromedary camels, indicating distinct shape differences. The lateral view demonstrated that the positive limits (blue) of PC2 were associated with a more expanded and pronounced humeral head, major tuberosity, and deltoid tuberosity, whereas the negative limits (red) corresponded to more compact and less prominent structures. The cranial view showed a more bulbous and expanded head, along with more pronounced capitulum and trochlea in the blue regions, compared to flatter structures in the red

regions. From the caudal view, the olecranon fossa appeared deeper and more defined in blue, while shallower in red, with both lateral and medial epicondyles exhibiting similar size variations. The proximal view reinforced these observations, with a larger and more robust humeral head and major tuberosity in the blue regions. Finally, the distal view highlighted more prominent capitulum, trochlea, and epicondyles in the blue regions, contrasting with flatter and less distinct structures in the red regions. Overall, the positive side of PC2 (blue regions) was associated with more expanded, pronounced, and robust humeral structures, whereas the negative side (red regions) corresponded to more compact, less pronounced, and delicate structures, particularly in the head, major tuberosity, deltoid tuberosity, capitulum, trochlea, olecranon fossa, and epicondyles (Figure 8).

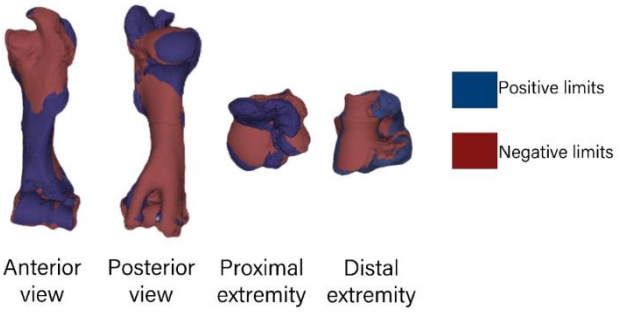

Figure 8. Distribution of principal component 2 (PC2) values with negative (red) and positive (blue) limits.

PCA Scatterplot Analysis: The Principal Component Analysis (PCA) scatterplot visually represents the shape variation within the humerus bones of the camel sample set, comprising twenty nine males and thirty females. This analysis elucidates the primary axes of morphological variation, with PC1 accounting for 19.6% of the total variance and PC2 capturing an additional 11.9% (Figure 6).

The PCA scatterplot, accompanied by convex hulls to encapsulate the variation within each group, demonstrates a notable separation of specimens along the PC1 axis. This separation suggests that PC1 is the predominant factor influencing shape variation in the humerus bones, likely capturing the major morphological differences between male and female specimens. The distinct clustering along this axis indicates significant sexual dimorphism in the shape of the camel humerus.

In contrast, there is some degree of overlap observed along the PC2 axis. This overlap implies that while PC2 captures shape variation, it represents secondary morphological differences that are less pronounced than those captured by PC1. The additional variation encapsulated by PC2 may be attributed to factors such as age-related changes, individual variation within sexes, or environmental influences on bone morphology.

The convex hulls drawn around the male and female groups highlight the dispersion and extent of morphological variation within each sex. These convex hulls serve as a visual boundary, illustrating the range of shape variations present in the sample set. The overlap between the convex hulls of males and females along PC2 further supports the presence of shared morphological traits between the sexes, despite the overall sexual dimorphism captured by PC1 (Figure 6).

Clusters of specimens with similar morphological traits are evident within the scatterplot. These clusters may correspond to subpopulations or distinct age groups within the sample set. Analyzing these clusters in greater detail could provide insights into the functional implications of the observed shape variations. For instance, variations in the robustness or gracility of the humerus bones could be related to differences in locomotor behavior or biomechanical loading patterns.

Interpretation of Morphological Variations: The observed morphological variations in the humerus bones provide valuable insights into the functional adaptations of camels. The humerus with positive PC1 values, characterized by its increased width and robustness, likely reflects adaptations for handling greater loads and accommodating substantial muscular attachments. This is particularly relevant for camels living in harsh desert environments, where such adaptations are crucial for effective locomotion and survival.

In contrast, the narrower and more gracile humerus associated with negative PC1 values may signify adaptations for less strenuous locomotion or represent different age or sex groups within the camel population. This variation highlights the diverse functional demands placed on the humerus depending on the camel's role and environment.

The variations captured by PC2 provide additional insights into the functional adaptations of the humerus. The elongated and slender humerus associated with positive PC2 values may suggest adaptations aimed at enhancing speed and agility, which could be advantageous in certain environmental or behavioral contexts. Conversely, the shorter and more robust humerus linked to negative PC2 values likely indicates adaptations for strength and endurance, potentially reflecting different functional or environmental pressures.

Procrustes ANOVA and Centroid Size Analysis: The results of the Procrustes ANOVA indicated no statistically significant shape differences between male and female camel humeri. Sex explained only 0.06% of the total shape variation ($R^2 = 0.0006$, $P = 0.97$), suggesting that humeral

morphology is highly conserved between sexes. The observed variation appears to be primarily attributable to individual differences rather than sex-specific traits. These findings imply that sexual dimorphism in humeral shape is either negligible or undetectable given the current dataset and sample size.

Similarly, centroid size analysis also revealed no significant differences between male and female camel humeri. ANOVA results showed a P-value of 0.827 (F = 0.049), indicating that size variation between sexes is not statistically significant. Similar to shape, most of the size variation appears to be driven by individual differences rather than sex. These results suggest that sexual dimorphism in humeral size is minimal or undetectable with the available data.

Allometric Effects on Humeral Shape: Allometric analysis demonstrated a strong and statistically significant relationship between centroid size and humeral shape ($p < 0.001$). Size accounted for 71.7% of the total shape variation ($R^2 = 0.717$), highlighting its major role in determining humeral morphology. The F-statistic (F = 119.11) and Z-score (Z = 7.27) further reinforce the strength of this relationship. While size is a key factor in shaping humeral morphology, a remaining 28.3% of shape variation is unexplained, likely due to individual differences or other non-size-related influences.

By integrating the findings from PCA with an understanding of camel biomechanics and ecology, the study contributes valuable knowledge on the morphological diversity of camel humerus bones. This understanding not only elucidates the functional adaptations of camels but also establishes a framework for exploring the adaptation processes that have shaped their skeletal morphology. The integration of PCA results allows for a comprehensive analysis of shape variations and how these variations relate to the biomechanical demands placed on camels in their natural habitats. This holistic approach sheds light on the complex interplay between form and function in camel skeletal structures. Moreover, the study underscores the importance of considering both major and minor sources of shape variation to gain deeper insights into the evolutionary processes influencing camel morphology. By doing so, it enhances our understanding of how camels have adapted to their environments over time, offering valuable perspectives for further research in the field.

Discussion and Conclusion

This study presents a geometric morphometric analysis of camel humerus bones, utilizing Principal Component Analysis (PCA) to elucidate shape variations within the sample set. The PCA results reveal significant insights into the morphological diversity and functional adaptations of the camel humerus, enhancing our understanding of The PCA identified two principal components, PC1 and PC2, which collectively capture a substantial portion of the shape variance in the camel humeri. PC1, accounting for 19.6% of the variance, represents a primary axis of morphological variation, while PC2, explaining 11.9% of the variance, captures additional, less pronounced differences. This finding aligns with previous studies that have demonstrated the effectiveness of PCA in capturing major and minor shape variations in skeletal elements (2, 18, 31).

The positive values of PC1, characterized by a wider and more robust humerus with pronounced tuberosities and curvature, reflect adaptations associated with increased muscularity and load-bearing capacities. Such traits are consistent with the findings of (18), who observed similar morphological adaptations in the humeri of large terrestrial mammals. These characteristics are likely advantageous for camels, which are subject to significant biomechanical stresses in their arid habitats. A robust humeral structure may facilitate greater muscle attachment areas, thereby enhancing locomotor efficiency and load distribution (29).

In contrast, negative PC1 values correspond to a narrower and more gracile humerus, with smaller tuberosities and less pronounced curvature. This morphology suggests adaptations for reduced muscular demands or or may indicate a different demographic within the population. Similar variations have been observed in other species, where gracile bone structures are often associated with less demanding locomotor and functional requirements (22). These findings suggest that camels with such traits may be younger or possess different functional adaptations compared to their more robust counterparts.

PC2, which captures 11.9% of the variance, provides additional insights into the functional adaptations of the camel humerus. Positive PC2 values are associated with a more elongated and slender shaft, smaller tuberosities, and less pronounced curvature. This morphology could be indicative of adaptations for speed and agility, supporting findings from similar studies on limb bone morphology (20). The elongated, slender humerus may be advantageous for camels engaged in activities requiring rapid, agile movements, although such adaptations might be less critical in their primary desert habitat.

Conversely, negative PC2 values correspond to a humerus with a shorter, more robust shaft, larger tuberosities, and a more pronounced curvature. This morphology suggests adaptations for strength and endurance, likely reflecting different functional requirements. The robust humerus with larger tuberosities may be indicative of greater muscular attachments, enhancing the camel's capacity for sustained physical

exertion (32). Such adaptations are consistent with observations in other large mammals, where robust bone structures are often linked to enhanced strength and load-bearing capabilities (20).

The PCA scatterplot demonstrates a clear separation of specimens along the PC1 axis, with some overlap observed along the PC2 axis. This separation indicates that PC1 predominantly captures the primary source of shape variation in the camel humerus, while PC2 reflects additional, less dominant variations. The clustering of specimens with similar morphological traits may represent different subpopulations or age groups within the sample set. This observation is in line with previous research that has utilized PCA to identify distinct morphological clusters within animal populations (16, 19).

The presence of morphological clusters suggests that the camel population exhibits a range of adaptations corresponding to different ecological or functional contexts. These clusters could represent variations in age, sex, or environmental pressures, providing valuable insights into the functional dynamics of camel humeral morphology. Similar clustering patterns have been observed in other studies examining skeletal diversity and its relation to ecological factors (5, 30).

The observed morphological variations in the camel humerus offer valuable insights into the functional adaptations of camels. The robust humerus associated with positive PC1 values likely reflects adaptations for enhanced load-bearing and muscular attachments, crucial for coping with the biomechanical stresses of desert environments. These findings are consistent with the notion that skeletal adaptations in large mammals often correlate with their functional and environmental demands (27).

Conversely, the gracile humerus associated with negative PC1 values may indicate adaptations for less strenuous locomotion or or reflect different demographic characteristics. Such variations highlight the plasticity of camel skeletal morphology in response to varying functional and environmental pressures (21).

PC2 variations further elucidate the functional adaptations of the camel humerus. The elongated and slender humerus associated with positive PC2 values may reflect adaptations for speed and agility, whereas a robust humerus with larger tuberosities linked to negative PC2 values likely indicates adaptations for strength and endurance. These findings contribute to a broader understanding of how skeletal morphology is shaped by adaptation and functional demands (23).

The geometric morphometric analysis of camel humerus bones, facilitated by PCA, provides a comprehensive understanding of shape variations and their functional implications. The results underscore the importance of considering both major and minor sources of shape variation in elucidating the adaptive significance of skeletal morphology. Future research could further explore the relationship between humeral morphology and other ecological or physiological factors, offering deeper insights into the adaptation dynamics of camel anatomy (31).

The influence of environmental and genetic factors on camel humerus morphology cannot be understated. Recent studies have highlighted how environmental pressures, such as arid conditions and terrain variability, shape skeletal adaptations in camels (3). Genetic factors also play a crucial role, with specific genetic markers being associated with skeletal robustness and morphological traits (1, 14). By integrating these aspects, future research can provide a more holistic view of the determinants of camel humerus morphology, linking phenotypic variations to underlying genetic and environmental contexts.

The geometric morphometric analysis of camel humerus bones, facilitated by PCA, provides a comprehensive understanding of shape variations and their functional implications. The results underscore the importance of considering both major and minor sources of shape variation in elucidating the adaptive significance of skeletal morphology. Future research could further explore the relationship between humeral morphology and other ecological or physiological factors, offering deeper insights into the adaptation dynamics of camel anatomy (31).

One limitation of this study is the relatively small sample size, which, while sufficient for identifying general trends in humeral morphology, may not fully capture the complete range of variation within dromedary camels from the El Oued region. The inclusion of only 59 specimens from a single population restricts the ability to conduct more detailed subgroup analyses, such as age-related variations or potential regional differences. Additionally, the dataset may not be fully representative of the broader dromedary population, as factors such as genetic diversity, environmental influences, and biomechanical demands could contribute to variations in humeral morphology that were not captured in this study. Future research with a larger sample size, incorporating additional geographic locations and age groups, would enhance the robustness of statistical analyses and provide a more comprehensive understanding of sexual dimorphism and allometric patterns in camel humeri. Expanding the dataset would also allow for the application of more advanced morphometric techniques, improving the resolution of shape differences and further elucidating the functional and evolutionary significance of humeral morphology in dromedary camels.

This study provides significant insights into the three-dimensional morphological variation and sexual

dimorphism in the humerus of dromedary camels (*Camelus dromedarius*) from the El Oued region. Using advanced geometric morphometric techniques, the research reveals substantial sexual dimorphism, with male camels displaying more robust and thicker humeri compared to the more slender and delicate humeri of female camels. The allometric analysis highlights notable size-related shape changes, particularly in the deltoid tuberosity and distal epiphysis regions. These findings underscore the functional adaptations and biomechanical demands placed on camelid skeletal structures, demonstrating the utility of geometric morphometrics in skeletal morphology studies. This research sets a new standard for future studies by integrating high-resolution 3D scanning with sophisticated morphometric analyses, contributing to a deeper understanding of the anatomical and functional diversity in dromedary camels.

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Ethical Statement

This study was conducted in accordance with the ethical standards for animal research. All procedures involving the handling and sampling of dromedary camels (*Camelus dromedarius*) were approved by the Committee of Ethics of the University of Souk Ahras in Algeria (Approval no: 2021/08704-180).

Conflict of Interest

The authors declared that there is no conflict of interest.

Data Availability Statement

The data supporting this study's findings are available from the corresponding author upon reasonable request.

Animal Welfare

The authors confirm that they have adhered to ARRIVE Guidelines to protect animals used for scientific purposes.

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The effects of the demographic characteristics of pet owners on their animal ownership and care behaviors

Ahmet Cihat TUNÇ[1,a], Durmuş Fatih BAŞER[1,b], Sercan Hüseyin BAYENDUR[1,c], Abuzer ACAR[1,d,✉]

[1]Afyon Kocatepe University, Faculty of Veterinary Medicine, Department of Internal Medicine, Afyonkarahisar, Türkiye.

[a]ORCID: 0000-0002-6296-6762; [b]ORCID: 0000-0003-4272-9011; [c]ORCID: 0000-0003-4246-8181; [d]ORCID: 0000-0002-4235-2763

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✉Corresponding author
abuzeracar@hotmail.com

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ABSTRACT

In this study, the answers were obtained and evaluated from the questionnaires that were applied to the owners of cats and dogs in at least one province from each region, covering all geographical regions throughout Türkiye. To do this, the original questions for this survey were prepared to evaluate the information about the marital status, gender, age range, education level, place of residence (province, district, town, etc.), economic income level, animal species, and animal care/ownership knowledge level of cat and dog owners. In the study, a face-to-face questionnaire was applied to a total of 1000 participants in the cities of Hatay, Mersin, Elazığ, Erzurum, Kars, Van, Afyonkarahisar, Aydın, Balıkesir, İzmir, Uşak, Gaziantep, Şanlıurfa, Ankara, Kırıkkale, Konya, Çorum, Bursa, İstanbul, Tekirdağ. A total of 962 (96.2%) participants completed the survey in its entirety, while 38 participants were excluded from the evaluation due to incomplete responses. According to the evaluation results of the data obtained; it has been revealed that different variables such as gender, income level, education level, and age of animal owners are highly effective on variables such as the specie, care and ownership knowledge level, and responsibility. As a result, the awareness level of individuals who own cats and dogs throughout Türkiye was measured under the leadership of different variables, and a very comprehensive study was put forward.

Introduction

Pet ownership is the most common form of interaction between humans and animals. Many studies have been published on the demographic characteristics of owning a pet and its effects on human health in the United States of America (USA), United Kingdom (UK), Canada, Australia, and various European countries. In these studies, the effects of demographic characteristics of pet owners on pet ownership and care behaviors were examined in detail. In these reports, the effects of demographic factors such as age, gender, income level, and education level of pet owners on their animal ownership and care behaviors were meticulously evaluated (8, 25, 26). However, it was revealed that there were significant differences in ownership and care behaviors among pet owners in different age groups when the age factor was studied (1). For example, it turned out that young adults own more pets and spend more time with them. On the other hand, it has been determined that older individuals generally provide better care due to being more experienced and patient (10). When the effects on gender were examined in the same studies, it was seen that the animals owned by women were more than the animals owned by men. Income level was also determined as another important factor affecting the ownership and care behaviours of pet owners. It has been revealed that pet owners with higher incomes pay more attention to the care of their animals because they have more resources and financial opportunities (8, 10, 11, 16). Studies have shown that the level of education can also affect the ownership and care behaviour of pet owners. It has been observed that individuals with higher education levels are generally more conscious and knowledgeable, have a better understanding of the needs of pets, and provide

appropriate care (20, 24). In studies on mental health, it has been concluded that having a pet can increase resistance to mental disorders by providing friendship, reducing loneliness, increasing socialization and giving meaning (12). Despite these positive findings, studies on examining sociodemographic factors suggest that individuals with pets are generally in an advantageous socioeconomic position compared to individuals who do not have pets, thus weakening the positive relationships between pet ownership and human health (2).

Türkiye has a unique diversity in pet ownership due to its cultural, economic, and social dynamics. In the literature, studies on pet ownership and care behaviours in Türkiye are limited and generally focused on Western cities (6, 22). This research aims to fill the knowledge gap in this area with data and innovative data specific to Türkiye. This study aims to explore the factors influencing pet ownership in Türkiye, specifically focusing on the demographic characteristics that drive the tendency to own cats and dogs.

Materials and Methods

Survey Population: The survey population materials of this study were collected in 20 provinces (Hatay, Mersin, Elazığ, Erzurum, Kars, Van, Afyonkarahisar, Aydın, Balıkesir, İzmir, Uşak, Gaziantep, Şanlıurfa, Ankara, Kırıkkale, Konya, Çorum, Bursa, Istanbul, and Tekirdağ), covering all geographical regions of Türkiye and including at least one province from each region. The cities were selected from the seven different geographic regions according to the existence of the University Veterinary Hospital or having high potential veterinary clinics. All the answers were obtained from the survey questions applied to cat and dog owners. The number of questionnaires was determined according to the estimated number of pet owners in each city of Türkiye used in the study. It was evaluated that 1000 participants represent a wide demographic spectrum. A face-to-face survey was applied to 1000 participants in total. The questionnaires of participants who answered all survey questions were included in the study, while those who left any questions unanswered were excluded.

Survey Questions: Survey questions were asked to the animal owners to evaluate the information about the marital status, gender, age, education level, place of residence (province, district, town, etc.), economic income level, species of the owned animal, and the level of knowledge about animal care/ownership of the cat and dog owners. Survey was composed of multiple-choice and open-ended questions. While designing the survey questions about income levels, questions were prepared on US dollar basis to make the evaluation of purchasing power and future years more objective. While preparing the survey questions included in the study, five different scientists who are experts in their fields were asked about their opinions, and after some additions and deletions, the validity of the questions in scale of measuring the demographics of animal owners and their ownership and care was decided. Likert-type questions aimed to evaluate the participants' attitudes towards pet ownership and care behaviors by measuring the extent to which they agree with certain statements. The Cronbach's Alpha value for the validity of the Likert-type questions in the scale was obtained as 0.72, and the data was analyzed by accepting that the scale was reliable. Previous studies have reported that the reliability level of the survey increases as Cronbach's Alpha value approaches 1 (5).

Statistical Analysis: Statistical comparisons of data were performed using the SPSS® software program (SPSS 22.0, Chicago, IL, USA). An independent Chi-Square (X^2) test was used to analyze variables, and the results were presented in the relevant tables. The significance level in the analysis was taken as $P<0.05$.

Results

In the research, a direct survey method was utilized involving 1,000 participants. Out of these, 962 individuals (96.2%) completed and returned all survey questions, while 38 participants were omitted from the analysis due to incomplete responses. The information on the demographics of the personal, environmental, educational, and income levels of the participants is given in Table 1 and Table 2.

The relationship between gender and the type of pet owned was tested and the results showed a statistically significant association ($P<0.05$), as indicated in Table 3. According to the results, it has been revealed that women mostly prefer to own cats, whereas men mostly prefer to own dogs. However, the ownership of both species is primarily seen in males.

Whether there is a relationship between the education level of the animal owners and the type of pet animals fed was tested with the X^2 test, and it was seen that there was a statistically significant relationship ($P<0.05$), as can be seen in Table 3. Accordingly, it was observed that with higher education level, the rate of cat ownership increased, and the rate of dog ownership decreased (Table 2). According to the results of the relationship between the education level of the animal owners and the care knowledge; It has been observed that as the education level of animal owner increases, the level of knowledge about care also increases. Accordingly, it has been revealed that animal owners with master's/doctorate degrees are more knowledgeable about care and ownership ($P<0.05$, Table 4).

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Table 1. Demographic information of the participants.

Variables	Group	Number of people	%
Gender	Female	418	43.6
	Male	540	56.4
Marital Status	Single	531	55.4
	Married	384	40.1
	Divorced	43	4.5
Age	<18	51	5.3
	18-30	492	51.4
	31-50	310	32.4
	>50	105	11
Living Place	Metropolis	503	52.5
	City	244	25.5
	County / District / Village	211	22
Type of Home	Flat	541	56.5
	Detached house	272	28.4
	Buildings	145	15.1
Household Members	1	142	14.8
	2	189	19.7
	3	249	26
	4	225	23.5
	≥5	153	16

Table 2. Demographic information about the education level and income of the participants.

Variables	Group	Number of people	%
Education	Primary School	49	5.1
	Secondary School	65	6.8
	High School	236	24.6
	Bachelor's degree	495	51.7
	Masters/PhD	113	11.8
Income (Monthly)	<410 $	133	13.9
	410-819 $	298	31.1
	820-1,294 $	221	23.1
	1,235-1,649 $	139	14.5
	>1,650 $	167	17.4

Table 3. The relationship between the gender and educational level of individuals in pet species.

Variables	Cat	Dog	Both species	P value
Female	61.7%	28.5%	9.8%	0.001
Male	37.4%	50.6%	12.0%	
Primary school	32.7%	57.1%	10.2%	0.002
Secondary school	35.4%	60.0%	4.6%	
High school	45.8%	44.9%	9.3%	
Bachelor's degree	51.1%	36.8%	12.1%	
Masters/PhD	53.1%	32.7%	14.2%	

Table 4. The relationship between the educational level and age of individuals in care-related knowledge.

Variables	High	Low
Primary school	59.2%	40.8%
Secondary school	66.2%	33.8%
High school	72.9%	27.1%
Bachelor's degree	79.4%	20.6%
Masters/PhD	83.2%	16.8%
<18	56.9%	43.1%
18 – 30	82.7%	17.3%
31 - 49	71.9%	28.1%
>50	68.6%	31.4%

Table 5. The relationship between the age and income levels of the owners and species of pet.

Variables	Cat	Dog	Both species	P value
<18	64.7%	31.4%	3.9%	
18 – 30	51.2%	38.2%	10.6%	0.013
31 - 49	43.2%	45.5%	11.3%	
>50	39.0%	44.8%	16.2%	
<410 $	51.1%	40.6%	8.3%	
410 – 819 $	42.6%	48.7%	8.7%	
820 – 1,234 $	53.8%	32.6%	13.6%	0.034
1,235–1,649 $	46.8%	39.6%	13.7%	
>1,650 $	48.5%	39.5%	12.0%	

Table 6. The relationship between the income level of animal owners and the compelling responsibility of owning an animal.

Variables	Yes	Sometimes	No	P value
<410 $	16.5%	47.4%	36.1%	
410 – 819 $	13.1%	55.4%	31.5%	
820 – 1,234 $	9.5%	56.1%	34.4%	0.104
1,235 – 1,649 $	12.9%	59.0%	28.1%	
>1,650 $	10.2%	47.3%	42.5%	

It was found that there was a statistically significant difference in the evaluation of the relationship between the age of the owners and the specie of animal they own($P<0.05$). Accordingly, it has been revealed that owners prefer to keep dogs over cats as their age increases (Table 5). Our results showed an increase in ownership knowledge in relation to increased education level and age. In this context, it was observed that the age group with the highest level of knowledge about the care and ownership of animals was in the 18-31 age group, while the <18 age group had the lowest level of knowledge on this subject (Table 4).

It was found that there is a statistically significant difference between the income level of the animal owners and the choice of animal species ($P=0.034$). According to the findings, it was revealed that the rate of cat ownership was highest in the group with the lowest income level; and as the income level increased, the rate of owning both cats and dogs increased (Table 5). In contrast, there was no statistically significant difference between the income level of animal owners and the difficulty of owning an animal ($P>0.05$). Also, as the income level increases, it is seen that the responsibility of owning animals decreases partially (Table 6).

In this study, the relationship between the gender of the animal owners and the knowledge of the specie characteristics of the animals, the level of care and nutrition of the animals they keep, the relationship between the gender of the animal owners and the level of knowledge about the bathing of the animals, the

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relationship between the gender of the animal owners and the compulsion of having an animal, and the education level of the animal owners. The relationship between the animal owners' age and the breed characteristics of the animals, the level of knowledge about the bathing of the animals, and the compulsion of the responsibility of owning the animals were insignificant (P>0.05). In addition, the relationship between the marital status of the animal owners and the knowledge of the animals' breed characteristics, the animal owners' income level, and the relationship between care and ownership knowledge level were examined. No statistically significant difference was found between these parameters (P>0.05).

Discussion and Conclusion

In the present study, it was concluded that the age, gender, and income parameters are affected directly in pet ownership and care in Türkiye. Murray et al. (18) found that there is a significant interaction between the presence of children under the age of 18 in a household in the United Kingdom and having a dog. In the same study, it was shown that there is a positive interaction between the tendency to own cats and households with children and dogs. In our study, similar to the study of Murray et al. (18), it is seen that there is generally a tendency to own cats in households with individuals under the age of 18 in Türkiye. However, the tendency to have both cats and dogs in households with individuals under the age of 18 is quite low compared to other age groups. Having an animal in a home with kids contributes positively to children's emotional, social, cognitive, and behavioural development (7, 8, 21). With the increase in the visibility of news with similar studies in parallel with the development of scientific communication day by day, makes us think that the tendency to own animals in households with children, and therefore individuals under the age of 18 increases. It has been observed that individuals between the ages of 18-30 have a higher level of knowledge about the care and ownership needs of animals compared to other age groups. While it was observed that the level of knowledge about animal ownership and care was the lowest in individuals under 18, it was determined that this level gradually decreased again in those over 30. Among the questionnaires who participated in our study, it was concluded that the level of responsibility and knowledge of those under the age of 18 was lower and that the older age group would be lower in terms of accessing information, using current communication tools, and information sources compared to those between the ages of 18-30. It is thought that the high level of knowledge of questionnaires between the ages of 18-30 is due to a high sense of responsibility and the use of technological opportunities. Therefore, it is more advantageous to access research and animal ownership information. The result of the present study showed that 18-30 age group generally tends to own cats. Among the possible reasons for this situation may be that the time allocated for pets has decreased due to the intense work and social life in the 18-30 age group, and that taking care of cats might be seen easier than dog care. In this study, it was observed that individuals aged 50 and over tended to have dogs, and it has been observed similarly with Friedmann et al. (10) that the tendency to own animals increases as getting older, in which the adopted animals are seen as a companion.

Considering the tendency to own animals based on gender, it is reported that this tendency is higher in females than males, but there is no gender difference in terms of preferred species (17). On the other hand, in our study, it was observed that women tended to have cats and men tended to have dogs, and a significant result was obtained that gender could play a role in animal species preference. In contrast to this study, our results showed that gender is essential in choosing a type of animal. Accordingly, women tend to prefer cats more, while men tend to prefer dogs more. It's known that cats are more independent and self-sufficient than dogs (9). It was thought that women tend to prefer cats because of their fewer care requirements than dogs. Also, the reasons why women prefer cats may be related to cultural, social, or practical matters. However, the relationship between owner gender and pet animal species needs to be researched in more detail.

Saunders et al. (23) reported that the tendency to keep cats is high in singles, especially in women, and the tendency to have dogs in married individuals, and they associated this with the size of the house and the working hours of the house members. In our study, although there was no clear preference for a breed in married individuals, it was observed that the tendency to breed cats was higher in single individuals, as previously mentioned, regarding the size of the house and working hours.

Martins et al. (14) showed that there was a significant relationship in the high tendency to own a dog with a higher household income, compared to a lower household income. In our study, however, there was no significant relationship between income level and preferred animal species. Our findings differ from those of Martins et al. (14), possibly due to cultural or regional differences in pet ownership behaviors in Türkiye compared to other countries. However, proportionally, it was observed that the tendency to keep both cats and dogs increased with higher income level. Pets in socioeconomically disadvantaged households have very limited access to quality food, preventive and curative health services (3, 4, 13, 19). When the burden of responsibility of owning an animal is examined with these findings, although there is no significant difference between the income levels, the fact that the difficulty is proportionally higher in the lower

income group suggests that the main difficulty is the health and quality nutritional requirements of animals in connection with the increasing cost of living.

In previous studies, it was not reported that there is a definite relationship between education level and tendency to own a pet. In some studies, it has been concluded that individuals with a lower education level have a higher tendency to own pets. In a report published in the Netherlands, it was concluded that the level of education, when age and gender were evaluated together, had a significant effect on the tendency to own animals (24). Our research identified a positive relationship between educational attainment and cat ownership, while a negative relationship was observed concerning the inclination to own a dog. According to Schollen's (24) report, while individuals with a lower education level were less interested in the pet animals they lived with, compared to individuals with a higher education level, it was observed that as the education level increased in Türkiye, the level of knowledge about care and nutritional requirements about pet animals also increased. As the level of education increases, people's living spaces are mostly city centers and apartments. As a natural result of this situation, it is thought that cats, which are easy to care for and have fewer needs, are preferred. As seen in our study, it was determined that people with primary and secondary school education levels prefer dog-owning more. In this case, it was thought that people with this level of education prefer dog ownership as living space and condition that will meet the needs of dogs because they live in rural areas.

Michel et al. (15) reported that pet owners' tendency to feed their animals is influenced by various factors such as the nutritional value of the food content, health and safety of the food, concerns about the pet food industry, and sources of information on animal dietary management. However, the report also argues that the reasons why commercial foods are not preferred in nutrition management are the distrust of the pet food industry and the pleasure of preparing food for pets. In our study, the rate of not using any commercial food for feeding the pets was determined as 13.2%, the rate of use of non-commercial foods together with commercial food was 31.2%, and it was concluded that home meals were preferred as non-commercial food. It can be argued that the reasons why commercial food is not preferred alone in the nutrition of pets in Türkiye are the widespread acceptance in Turkish society that healthy food is home-cooked food and the diversification of the food eaten by the animal, together with economic concerns. This study uniquely contributes to understanding pet ownership trends in Türkiye, a region with distinct cultural and economic dynamics.

This research indicates that pet owners exhibit a high frequency of veterinary visits within a three-month parasite control or only when a health problem is encountered. The fact that the rate of routine vaccination and follow-up of antiparasitic applications is 86.8% suggests that the anti-vaccination in pet animals in Türkiye is not at a level to cause concern now. One of the reasons that lead animal owners not to interrupt regular antiparasitic application is the concern about human health since these factors can also affect themselves.

In this study, demographic characteristics of individuals who own cats and dogs in Türkiye were examined. As a result, it was concluded that the factors that significantly affect the tendency to have a pet are age, gender, and education level. The level of knowledge about the care and dietary needs of cats and dogs living together was associated with age and education level.

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Ethical Statement

The study was reviewed and approved by the Animal Experiments Local Ethic Committee of Afyon Kocatepe University, Afyonkarahisar, Türkiye (Decision number: 49533702/114**).**

Conflict of Interest

The authors declared that there is no conflict of interest.

Author Contributions

ACT, DFB, SHB and AA conceived and planned the experiments. ACT, DFB, SHB and AA carried out the experiments. ACT, DFB, SHB and AA contributed to survey preparation. ACT, DFB, SHB and AA contributed to the interpretation of the results. AK took the lead in writing the manuscript. All authors provided critical feedback and helped shape the research, analysis and manuscript.

Data Availability Statement

The data supporting this study's findings are available from the corresponding author upon reasonable request.

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Skull morphology of shepherd dogs in Poland

Edyta PASICKA[1,a], Maciej JANECZEK[1,b], Ozan GÜNDEMİR[2,c,✉]

[1] Wrocław University of Environmental and Life Sciences, Faculty of Veterinary Medicine, Department of Biostructure and Animal Physiology, Wrocław, Poland; [2] İstanbul University-Cerrahpaşa, Faculty of Veterinary Medicine, Department of Anatomy, İstanbul, Türkiye

[a]ORCID: 0000-0002-9852-400X; [b]ORCID: 0000-0003-4357-2271; [c]ORCID: 0000-0002-3637-8166

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✉**Corresponding author**
ozan.gundemir@iuc.edu.tr

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ABSTRACT

This study aims to assess the skull morphological features of shepherd dog breeds raised in Poland, with an emphasis on native breeds such as the Tatra Sheepdog and Polish Lowland Sheepdog, by utilizing a detailed dataset to analyze and compare the structural traits of their skulls. To achieve this, a total of 32 dog skulls were modeled in 3D, and geometric morphometric analysis was performed to reveal skull shape variations. Among the shepherd samples used, the Polish Lowland Sheepdog exhibited the smallest average skull size. The Tatra Shepherd Dog displayed a skull size similar to that of other sheepdog breeds, although it was larger than that of the Polish Lowland Sheepdog. The results indicate that the Tatra Shepherd Dog possesses a more robust and elongated skull structure compared to the Polish Lowland Sheepdog. Both of these Polish shepherd breeds share similar skull morphology with other shepherd breeds, with the notable exception of collies. Collie breeds exhibit a markedly dolichocephalic skull morphology that sets them apart from the other samples in this study. The analysis revealed that neither Procrustes distance nor shape variation from PC1 had a statistically significant effect on skull size. To enhance our understanding of Poland's shepherd dog diversity, future studies should focus on expanding the dataset to include additional native Polish breeds and exploring a broader range of morphological features beyond the skull.

Introduction

Livestock guardian dogs are specialized breeds used to safeguard livestock from predators and deter potential threats such as thieves (13, 25). In recent years, the protection of livestock grazing in mountainous and foothill regions from predators such as wolves, bears, and lynxes has become increasingly important, not only in Poland but across Europe (13). Unlike herding dogs, which are bred to “manage” and direct herds, livestock guardian dogs have been selectively bred to “protect” herds from external dangers (19). For many years, as a result of these breeding conditions, shepherd dogs have generally been characterized by their large body size and strong musculature; these traits enable them to withstand harsh climatic conditions and sustain prolonged physical activity. This common functional necessity may also indicate shared morphological traits among these breeds. However, significant structural differences can still be found among shepherd dogs, and these differences may provide insight into how they have morphologically adapted to specific environmental conditions and intended uses (6, 9). In this context, skull morphology serves as an anatomical reference for understanding functional differences among dog breeds. The shape and structure of the skull can influence various biological factors, such as chewing mechanics, visual perception, or brain size. Furthermore, it can be suggested that shepherd dogs raised in different geographical regions may have developed distinct morphological adaptations in response to ecological factors such as local climate and predator pressure.

Understanding the morphology of livestock guardian dogs can help uncover how their physical traits contribute to their protective roles and resilience in challenging environments, while also providing insights into their morphological adaptations. Additionally, studies conducted with different livestock guardian dog breeds allow for the identification of anatomical variations in

dogs with similar functional characteristics. These studies, which focus on detailed analyses of skull and skeletal morphology, can provide reference data on species-specific adaptations, such as their ability to withstand predators and endure harsh climatic conditions.

The skull, as the most critical structure of the axial skeleton, protects the brain and houses essential sensory organs such as the eyes and inner ears, playing a vital role in animal biology (4, 12, 15). The skull's anatomical design, with distinct regions like the neurocranium and viscerocranium, demonstrates its adaptation to various functions such as housing sensory structures, supporting feeding mechanisms, and providing muscle attachment sites for mastication and head movement. Its structure is a key element in determining breed, age, and sexual dimorphism, as well as being fundamental in veterinary anatomy for taxonomy and species identification (5, 12, 16, 22). Moreover, studying skull morphology provides practical applications in veterinary medicine, including guiding skull nerve anesthesia and supporting forensic investigations (11, 24). In carnivora, skull shapes and sizes show remarkable variation, shaped largely by ecological roles and dietary needs. This study evaluates the skull morphological characteristics of Polish shepherd dog breeds to characterize their breed-specific skull features and enhance understanding of their skull variation.

In recent years, geometric morphometrics has become increasingly prominent in veterinary anatomical studies, particularly in the field of taxonomy (2, 10, 23). The skull, as one of the most informative skeletal structures, was extensively analyzed using these techniques to identify species-specific traits and classify animals more accurately. Three-dimensional geometric morphometrics, in particular, advanced the precision of such studies, enabling the detailed examination of skull morphology (1). This method proved invaluable for exploring factors such as allometric changes, sexual dimorphism, and phylogenetic relationships (14, 15, 18, 20). By incorporating 3D models, researchers were able to capture subtle morphological differences, making this approach a crucial tool in both taxonomic classification and broader veterinary anatomical research.

The primary objective of this study is to evaluate the skull morphology of shepherd dog breeds raised in Poland, with a particular focus on native breeds (Tatra Sheepdog and Polish Lowland Sheepdog), using a comprehensive dataset to identify and compare the distinct morphological traits of their skulls. Understanding the morphological variations among these Polish breeds is essential for several reasons: it contributes to the preservation and conservation of Poland's rich canine heritage, while also enhancing our knowledge of breed-specific traits that may be associated with their historical adaptations and roles. By conducting a thorough analysis of skull morphology, this study aims to provide a valuable reference for future morphological research on these two breeds, ultimately aiding in their preservation and informing breeding practices. The findings will serve not only to enrich the scientific community's understanding of Polish dog breeds but also to foster greater appreciation for their unique characteristics and contributions to Poland's cultural identity.

Materials and Methods

Animals and Modeling: 26 skulls (2 Tatra Shepherd and 3 Polish Lowland Sheepdogs) from the bone collection at the Archaeozoology Laboratory and Museum of Standards, Department of Biostructure and Animal Physiology were used in this study. Most of the samples were sheepdogs, but there were also examples such as Cane Corso and Husky. Additionally, 6 Illyrian shepherd skull samples were taken from the study of Jashari (12). The breeds of each skull were known, and all samples belonged to adult dogs (Table 1). Skulls with extreme brachycephalic or dolichocephalic features were excluded, except for those of shepherd dogs (such as collies), which were the main focus of the study.

Table 1. Distribution of skull samples by breed

Breeds	Number	Breeds	Number
German Shepherd	5	Bracco Italiano	1
Bucovina Shepherd	1	Cane Corso	1
Shar Pei	1	Collie	6
Illyrian Shepherd	6	Siberian Husky	1
Labrador Retriever	1	Siberian Mastiff	1
Mioritic Shepherd	2	Moloss	1
Polish Lowland Sheepdog	3	Tatra Shepherd Dog	2
			Total: 32

The skulls were modeled in 3D using the Shining 3D EinScan Pro 2X scanner. To minimize errors during scanning, the rotary table, an accessory of the scanner, was used (manual scanning was not conducted). The scanning accuracy was set to 0.04 mm. After scanning, the 3D models were merged using EXScan Pro software (version 4.0.0.4) and saved in "ply" format.

Landmarking: A total of 19 landmarks were used in the study, all classified as type 1 and corresponding to specific

anatomical regions (Figure 1). The landmarks used in this study were selected from anatomical landmarks employed in previous studies (9). All landmarking procedures were performed manually by the same researcher to ensure consistency. Slicer software (version 5.2.2) was used for the landmarking operations (21).

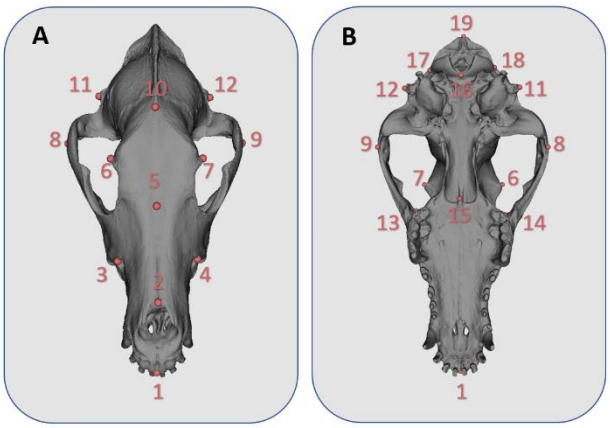

Figure 1. Landmarks

Geometric Morphometrics: The centroid size (CS) for each dog skull was calculated to represent the overall size of each specimen, providing a standardized measure for comparing the target breeds in the study—Tatra Shepherd and Polish Lowland Sheepdog. Additionally, procrustes distances were recorded within each dog to capture within-group variation and evaluate morphological diversity among individuals of the same breed.

To explore the skull shape differences across these breeds, Principal Component Analysis (PCA) was applied (3). PCA is a powerful method for simplifying complex datasets by transforming the original correlated variables (landmark coordinates) into a set of orthogonal, uncorrelated components known as principal components (PCs). The first two principal components, PC1 and PC2, accounted for the largest proportions of total variation in skull shape, and therefore, were the primary focus for identifying and interpreting significant shape differences. Components explaining less than 10% of the variation were excluded from further analysis to streamline results and focus on the most informative shape changes.

To visualize these results, scatter plots were generated to illustrate the distribution of individual specimens along PC1 and PC2 axes, highlighting shape patterns specific to each breed. Additionally, the study examined potential associations between skull size and shape by assessing the effect of size (as captured by centroid size) on Procrustes distance and shape variation along PC1. A multivariate regression analysis was performed to quantitatively assess the relationship between size and shape, aiming to determine whether size influences skull morphology in these breeds.

Results

In evaluating the skull morphology of Tatra Shepherd and Polish Lowland Sheepdog breeds, other herding and shepherd dog breeds in the sample pool, particularly the German Shepherd, Illyrian Shepherd, and Collie, were also included for comparison. This approach allowed for a more comprehensive analysis of breed-specific morphological traits by similarities and differences in skull structure that may relate to each breed's functional roles and environmental adaptations. Among the samples used, the Polish Lowland Sheepdog had the smallest average skull size (Figure 2). Although the other breeds were close in skull size, the Illyrian Shepherd had the largest skull on average, along with the greatest size variation. The Tatra Shepherd, meanwhile, displayed a skull size similar to the other sheepdog breeds, though larger than that of the Polish Lowland Sheepdog.

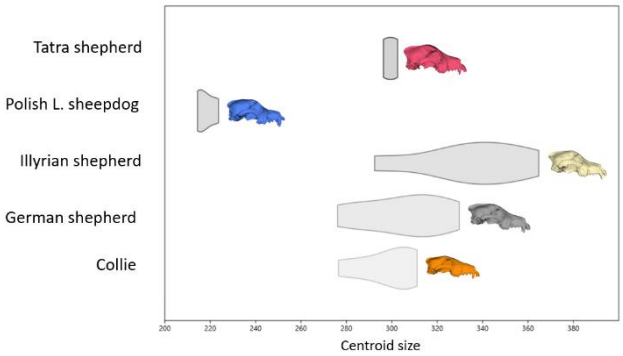

Figure 2. Results of centroid size for dog breeds in the study.

As a result of the shape analysis, it was observed that PC1 explained 37.2% of the total variation in skull morphology, while PC2 accounted for 25.1%. The prominent shape variation along PC1 represented a shift in skull morphology from mesocephalic to dolichocephalic characteristics. Specifically, in specimens with positive PC1 values, the facial structure was thinner and more elongated, creating a distinctly narrow and extended facial profile (Figure 3). In this positive PC1 range, the hard palate exhibited a longer, slender shape, whereas, in specimens with negative PC1 values, the palate was comparatively wider and more robust. The occipital region remained relatively consistent across PC1, with no significant shape differences detected in this area.

In PC2, notable morphological differences were observed, particularly in the nasal-frontal junction and the contour of the orbital region (Figure 3). At negative PC2 values, the junction between the nasal and frontal bones showed a more pronounced curvature, giving this region a rounded appearance. Conversely, in specimens with positive PC2 values, this section appeared straighter and more aligned. Furthermore, the shape of the lacrimal rim

within the orbit differed, with a straight configuration in positive PC2 values, while specimens with negative PC2 values displayed a more oval-shaped lacrimal rim, contributing to a distinctive orbital contour.

The distribution of samples along PC1 and PC2 is illustrated in Figure 4, highlighting distinct trends among the different shepherd breeds. Polish Lowland Shepherd samples tended to cluster within the negative range of PC1 values, indicating a broader, more robust skull morphology, while their distribution along PC2 was more centered, suggesting average variation in this dimension. Tatra Shepherd samples, on the other hand, displayed a central position along PC1, reflecting a balanced skull morphology, but were more positively distributed on PC2, implying subtle shape differences that distinguish them from the other shepherd breeds.

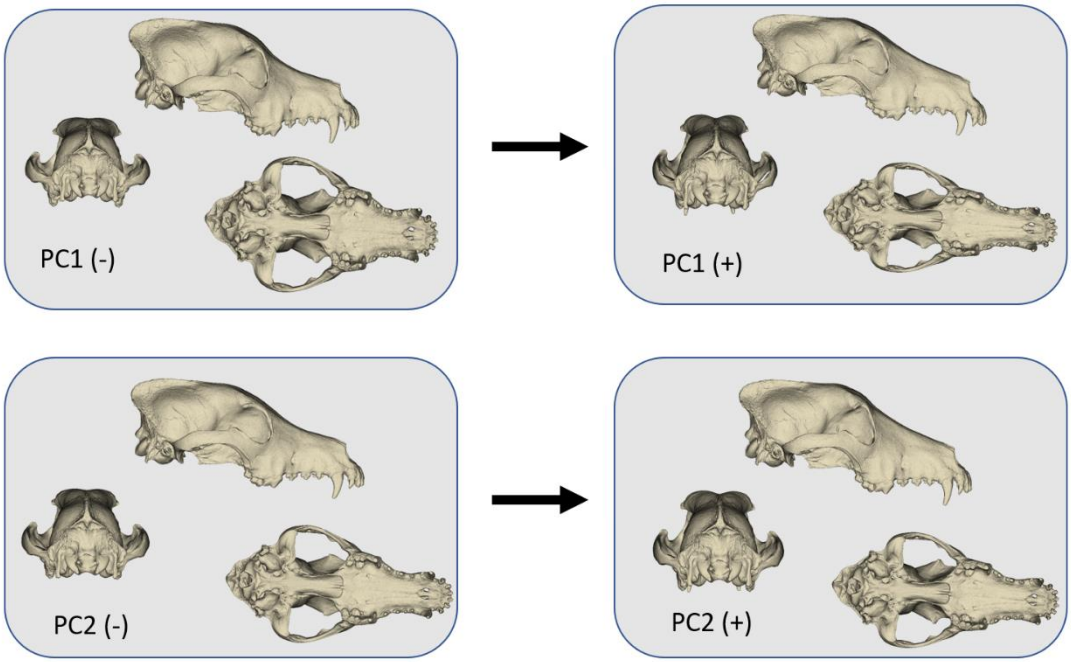

Figure 3. Models describing skull shape changes between the minimum and maximum values of PC1 and PC2.

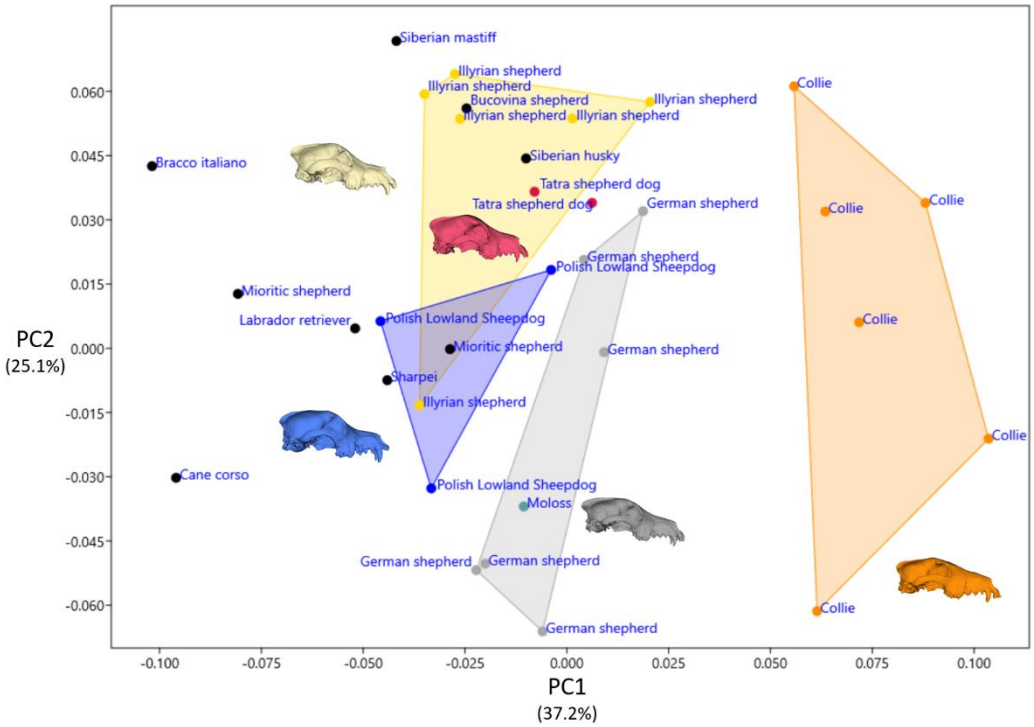

Figure 4. Principal component analysis plot of skull morphology illustrating variation along PC1 and PC2 axes. Convex hulls are shown, delineating the area occupied by each breed of shepherd dog, highlighting breed-specific clustering and overlap in skull form.

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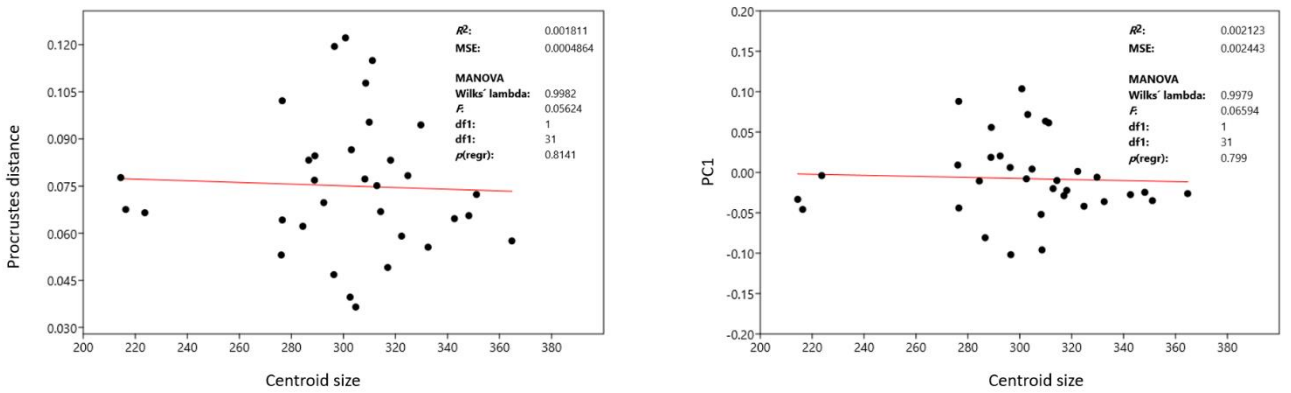

Figure 5. The effect of Procrustes distance and PC1 on skull size.

Collie samples were unique in that they occupied the positive boundary of PC1 exclusively, suggesting that the elongated, narrower skull features associated with positive PC1 values are characteristic of this breed. This distribution suggests that shape variation along PC1 may be largely influenced by the distinct skull morphology of Collies, who consistently exhibit a more dolichocephalic form. In contrast, the remaining shepherd breeds clustered closer together, showing more mesocephalic characteristics, with only slight variation along PC1.

In terms of PC2, no significant differences were observed among most shepherd breeds, reinforcing the notion that skull shape changes along this axis may represent individual variations rather than breed-level morphological trends. However, Tatra Shepherds did display a marginally more positive PC2 value in comparison to Polish Lowland Shepherds, suggesting minor distinctions in the nasal-frontal or orbital regions that set them apart. This finding implies that while shape variations along PC2 are subtle and may be individually based, certain breed-specific trends can still be observed, particularly between Tatra and Polish Lowland Shepherds.

Figure 5 illustrates the relationship between Procrustes distance, PC1, and skull size. Analysis showed that neither Procrustes distance nor PC1 had a statistically significant effect on skull size. This suggests that variations in skull shape captured by PC1, as well as the within-group variation measured by Procrustes distance, do not correlate strongly with changes in overall skull size across the samples.

Discussion and Conclusion

The findings of this study revealed the skull characteristics of the Tatra Shepherd Dog and the Polish Lowland Sheepdog by analyzing their skull morphology alongside other breeds from the same region, revealing species-specific skull features. The results indicate that the Tatra Shepherd Dog exhibits a longer skull structure compared to the Polish Lowland Sheepdog. Despite these differences, both Polish Shepherd Dog breeds share similar skull morphology with other shepherd breeds, excluding Collies. The most distinctive feature of Collies, which sets them apart from the other samples in this study, is their pronounced dolichocephalic skull morphology.

The dolichocephalic structure of the Collie contributed significantly to the primary axis of shape variation, highlighting its distinctive skull morphology compared to other breeds. In contrast, the remaining breeds displayed more mesocephalic characteristics, aligning with the general morphology observed in many shepherd dogs. Despite these differences, the results of the principal component analysis revealed different shape variations between the breeds, suggesting that skull morphology is influenced by factors beyond basic cephalic indices. Interestingly, even within the shepherd dog group, which shares close functional roles, notable morphological differences were observed. This variation could be attributed to breed-specific adaptations shaped by historical roles, environmental conditions, or selective breeding practices. For instance, shepherd dogs used in different terrains or climatic conditions may have developed subtle structural differences to better suit their environments. Additionally, the morphological distinctions observed might reflect genetic diversity within the group, further emphasizing the complex interplay between functionality, adaptation, and skull structure. These findings underscore the importance of detailed morphological analyses to uncover nuances that may not be immediately apparent from functional similarities alone. They also suggest that while shepherd dogs may share common tasks, their skull morphology is shaped by a combination of ecological, genetic, and selective pressures, resulting in a diversity of forms even within this functional group. Future studies could expand on these results by incorporating additional breeds and investigating the relationship between skull morphology, environmental adaptation, and genetic lineage.

In studies conducted on dogs, the primary skull variations generally showed similar results. The findings that revealed the most significant shape variation (PC1)

demonstrated that skulls are divided into brachycephalic, mesocephalic, and dolichocephalic types (6, 9, 17). However, the variations following the primary shape variation presented more specific shape differences depending on the sample groups used by the researchers. For instance, in a study on livestock guardian dogs, the main shape component described a gradient between brachycephalic and dolichocephalic skulls, while the second shape component was characterized by braincase shape features (9). In another study, the primary component variation showed a range from short, wide, and round skulls to those with a more elongated shape. However, the second shape variation was associated with flat muzzles in the dogs' skulls (6). In this study on Polish Shepherd Dogs, the primary shape variation was consistent with similar variations reported in previous studies. However, unlike other studies, the second shape variation captured morphological differences, particularly in the nasal-frontal junction and the contour of the orbital region. The classification of carnivora skulls into brachycephalic, mesocephalic, and dolichocephalic types as the primary shape variation is now well-supported by numerous studies in the literature. However, secondary shape variations in these studies often capture subtle but significant nuances. These features may provide detailed and essential information about skull morphological characteristics. For instance, in this study, while the primary shape component did not capture the morphological distinction between Tatra and Polish Lowland Shepherds, the secondary shape variation revealed that these two breeds exhibit distinct morphological patterns. This underscores the importance of examining secondary components to uncover fine-scale morphological differences within and between breeds.

The primary axis of variation (PC1) distinguished breeds with a longer and narrower facial profile from those with a broader and more robust skull morphology. This shape gradient aligns with the functional differences observed among various working and herding breeds. Dogs with higher PC1 values, such as Collies, exhibited a more dolichocephalic skull, a characteristic often associated with enhanced visual perception and agility—traits beneficial for fast-paced herding tasks. Conversely, breeds with lower PC1 values, such as Polish Lowland Sheepdogs, displayed a more compact and mesocephalic skull, which may contribute to stronger bite force and greater resistance to physical strain, advantageous for livestock protection and endurance in harsh environments. PC2 primarily captured shape differences in the nasal-frontal junction and orbital contour, indicating potential variations in olfactory capability and visual field adaptation. Breeds positioned at the negative end of PC2, such as the Illyrian Shepherd and Tatra Shepherd Dog, which are found in higher altitudes, exhibited a more pronounced nasal-frontal curve, which may suggest an adaptation for enhanced olfactory sensitivity—a crucial trait for detecting predators in mountainous terrains. Additionally, the variation in orbital shape may reflect differences in peripheral vision and depth perception, further influencing their ability to navigate and respond to environmental cues. The potential morphological differences observed in dogs living at higher altitudes highlight the need for studies with larger and more homogenous sample sizes to validate these findings. Expanding the dataset would allow for a more robust statistical analysis, helping to determine whether these traits represent adaptive modifications to environmental pressures or merely individual variation. Given the functional significance of skull morphology in sensory perception and survival strategies, such studies could serve as an important reference for future research. Further investigations integrating biomechanical modeling and ecological factors may provide deeper insights into the environmental adaptations of shepherd dog breeds.

Linear skull studies, which measure the distance between two points, provide valuable but limited information about skull dimensions. However, these measurements can be influenced by the curvature between the points or the shape of intervening anatomical structures, potentially affecting the accuracy of the results. In contrast, geometric morphometrics methods allow for a more detailed and holistic analysis of size and shape by incorporating multiple reference points and capturing the spatial relationships between them. In veterinary anatomy, this approach has proven especially useful for analyzing complex structures. For example, horn dimensions in ruminants and even the size of turtle carapaces have been calculated using geometric morphometrics (7, 8). These methods surpass traditional linear measurements by providing a comprehensive representation of the shape and size, enabling researchers to account for curvature, asymmetry, and other morphological nuances. In this study, 19 reference points were used to compare skull sizes. Additionally, these points were selected to ensure repeatability, making them suitable for use in future studies as well.

One limitation of this study was the small sample size for native Polish dog breeds, which included only 2 Tatra Shepherd Dogs and 3 Polish Lowland Sheepdogs. Due to the limited number of samples within groups, the study focused primarily on examining variation and allometric characteristics rather than conducting a detailed analysis of shape differences between groups. To perform more robust statistical analyses and achieve a deeper understanding of intergroup morphological differences, larger sample sizes would be necessary. Increasing the number of specimens in future research would enable the application of advanced statistical tools, allowing for a

more comprehensive evaluation of skull morphology and a better assessment of the factors contributing to shape variation. Expanding sample sizes across all groups would also help capture the full spectrum of morphological diversity, providing a stronger basis for comparing native Polish breeds with other shepherd dogs.

In veterinary practice, understanding breed-specific skull morphology can assist in procedures such as anesthesia administration, surgical planning, and diagnosing cranial deformities that may be more common in certain breeds. For instance, the differences in orbital and nasal structure observed in Polish Lowland Sheepdogs and Tatra Shepherd Dogs could influence approaches to ophthalmic and respiratory treatments in these breeds. Additionally, skull morphology plays a crucial role in forensic and archaeological applications, where geometric morphometric techniques can aid in identifying breed origins from skeletal remains. Given that many shepherd dogs are working breeds with historical significance, their skeletal characteristics could provide valuable insights into past breeding practices and population genetics.

To further enhance our understanding of Poland's canine diversity, future studies should aim to expand the dataset to include additional local Polish breeds and investigate a broader range of morphological traits. Exploring the functional implications of skull morphology on behavior, health, and performance could provide a more comprehensive perspective on how these traits influence breed abilities and their interactions with the environment. Moreover, conducting longitudinal studies to examine how these morphological traits adapt over time in response to changing environmental pressures and breeding practices will be essential.

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Ethical Statement

This study does not present any ethical concerns.

Conflict of Interest

The authors declared that there is no conflict of interest.

Author Contributions

E.P conceived and planned the experiments. O.G. carried out the experiments. M.J. contributed to the interpretation of the results. E.P. and O.G. took the lead in writing the manuscript. All authors provided critical feedback and helped shape the research, analysis and manuscript.

Data Availability Statement

The data supporting this study's findings are available from the corresponding author upon reasonable request.

Animal Welfare

The authors confirm that they have adhered to ARRIVE Guidelines to protect animals used for scientific purposes.

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Treatment of acetabular fractures in cats and dogs with locking veterinary acetabular plates

Merve BAKICI[1,a,✉], Barış KÜRÜM[1,b]

[1]Kırıkkale University, Faculty of Veterinary Medicine, Department of Surgery, Kırıkkale, Türkiye

[a]ORCID: 0000-0001-8833-3499; [b]ORCID: 0000-0002-5559-7815

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✉Corresponding author
merve.blsy@gmail.com

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ABSTRACT

This study aimed to assess the intraoperative application and postoperative outcomes of locking acetabular plates in the management of acetabular fractures in dogs and cats. The study analyzed intraoperative and postoperative data from feline and canine patients diagnosed with acetabular fractures. Variables evaluated included fracture location, degree of displacement, severity of lameness, coexisting orthopedic conditions, neurologic deficits, interval between injury and surgery, quality of fracture reduction, and postoperative complications. A total of 19 acetabular fractures were repaired: 9 fractures in 8 dogs and 10 fractures in 10 cats. The acetabular plate was utilized in 16/19 cases. In 3/19 cases of acetabular fractures, a reconstruction plate was utilized due to the inability to apply an acetabular plate for various reasons. However, these cases were included in the study. As a result of the study, it was observed that acetabular plates provide successful results, especially in fractures located in the central region; however, in fractures located in the caudal regions, reduction may be difficult due to the limited bone stock where the plate can be placed. In cases involving concurrent fractures of the ilium or ischium fracture, more easly shaped implants, such as reconstruction plates, were found to be more advantageous. These findings underscore the importance of tailoring implant selection to the specific anatomical and clinical characteristics of each fracture to optimize surgical outcomes.

Introduction

Acetabular fractures represent a challenging clinical entity for surgeons due to the complexity of the surgical approach, difficulties in achieving precise anatomical reduction, and the risk of causing permanent injury to adjacent vital structures, such as the sciatic nerve (*nervus ischiadicus*) and the colon. These fractures are relatively uncommon in veterinary practice. Reported incidence rates in dogs range between 3% and 7.5% (7, 24, 27), while in cats, they account for approximately 4.5% to 5.2% of fractures (19, 26).

The management of intra-articular fractures, such as acetabular fractures, necessitates the preservation of joint integrity to maintain optimal joint functionality. Post-traumatic osteoarthritis, a common complication following such fractures, is attributed to two primary factors: (1) damage to joint structures, particularly hyaline cartilage, caused by the high-energy forces transmitted during the initial trauma, even in the absence of a fracture; and (2) joint incongruity resulting from incomplete or suboptimal anatomical reduction (13). The treatment objectives for acetabular fractures include achieving precise anatomical reduction, ensuring rigid and stable fixation, and establishing interfragmentary compression at the earliest opportunity to restore joint congruity and minimize the risk of degenerative joint disease. These principles are critical for optimizing functional outcomes and mitigating long-term complications (2, 4, 11, 24, 33, 35, 38).

Many methods are used for the fixation of acetabular fractures, including dynamic compression plates (17), veterinary acetabular plates (3), string of pearl (16), reconstruction plates (13), plate luting (2), and screw wires with or without polymethylmethacrylate (4, 23).

Although MIPO (10) and external fixator use (15) have been reported in recent years, it is still unclear whether these methods provide additional benefits. Preformed locking acetabular plates reduces the need for plate contouring, which is one of the challenging aspects of acetabular fracture surgery, while also providing the biomechanical advantages of locking plates (1).

The aim of this study is to present the information obtained regarding the intraoperative application and postoperative outcomes of acetabular fractures treated with locking veterinary acetabular plates.

Materials and Methods

The study population consisted of cats and dogs presented to the Surgery Clinic of the Kırıkkale University Faculty of Veterinary Medicine Research and Practice Hospital between 2020 and 2024, which were diagnosed with acetabular fractures. Signalment data for all patients were collected, including species, breed and age. Comprehensive clinical evaluations were performed to assess the degree of lameness, co-existing orthopedic conditions, and neurological deficits. Radiographic assessments were conducted to determine the fracture localization and degree of displacement. Data from these assessments were systematically recorded. The success of fracture reduction achieved during surgical intervention was evaluated using immediate postoperative radiographs. Postoperative outcomes included monitoring the time to initial limb usage and final functional recovery of the affected extremity.

The acetabular fractures were categorized based on a modified classification system initially described by Butterworth et al. (1994). This classification included the following categories: cranial, central, and caudal. As the number of cases increased, two additional classifications were introduced: "craniocentral" (fractures involving the physis between the cranial and central regions) and "centrocaudal" (fractures involving the physis between the central and caudal regions).

The degree of acetabular fracture displacement was classified into three grades (8). However, certain cases in this study presented with free fracture fragments displaced into the pelvic canal. To account for these instances, a fourth category, "Grade 4: Severe comminuted fractures with significant displacement," was added to the classification.

Lameness was assessed using a composite scoring system derived from multiple grading scales (9, 12, 30). The severity of lameness was classified into six grades: grade 0: Normal gait, grade 1: Mild lameness (noted with the trained eye), grade 2: Moderate lameness (typically with distinct “head bob”), grade 3: Severe, weight-bearing lameness with ground contact only by the toe, grade 4: Non-weight-bearing lameness, characterized by ambulation on three limbs, grade 5: Unable or unwilling to rise.

Neurological deficits were systematically evaluated in the preoperative period and monitored postoperatively to assess improvement, based on established grading criteria (32). According to this classification system, it was divided into 6 groups as grade 0 (normal neurological function), grade 1 (presence of pain without associated neurological deficits, grade 2 (paresis, with or without pain, characterized by varying degrees of motor and proprioceptive impairment), grade 3 (plegia, defined as a complete loss of voluntary movement in the affected limbs and/or tail), grade 4 (plegia accompanied by loss of voluntary urinary control), grade 5 (plegia with concurrent loss of voluntary urinary function and absence of conscious perception of noxious stimuli (deep pain perception) in the affected limbs and/or tail).

Radiographic evaluation of fragment reduction was performed immediately post-surgery and categorized according to a predefined classification system (14): grade 1 (anatomic reduction), grade 2 (good reduction and functional outcome), grade 3 (malaligned and possibly requires revision) and grade 4 (unable to achieve adequate reduction).

Postoperative complications were categorized following the criteria defined by Cook et al. (2010) into three groups: minor (requiring minimal intervention), major (requiring additional surgery or medical treatment) and catastrophic (unacceptable function).

Functional outcomes of the affected limb were assessed using the criteria defined by Troger et al. (2008): grade 1 (return to complete normal function), grade 2 (very mild/intermittent lameness after prolonged exercise), grade 3 (frequent mild/moderate weight bearing lameness), grade 4 (permanent moderate severe lameness). The evaluation was based on the latest follow-up visit, ensuring a standardized and comprehensive assessment of extremity function.

Preoperative Management: The operation was planned after laterolateral and frog leg radiographs taken in the preoperative period. The anesthesia protocol was performed with 0.2 mg/kg butorphanol, 0.02 mg/kg medetomidine hydrochloride and 2 mg/kg ketamine hydrochloride. The patient was intubated after propofol administration intravenously (1.5 mg/kg), and anesthesia was maintained with isoflurane (1-3%).

For intraoperative analgesia, butorphanol (0.2 mg/kg/h), ketamine (0.1 mg/kg/h) and lidocaine (3 mg/kg/h) (only in dogs) were administered as a constant rate infusion. Cefazolin (25 mg/kg) was added 20 minutes before the incision and additional doses were administered at one-hour intervals until the operation was completed.

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Surgical Technique: The patients were placed lateral recumbency on the surgery table with the affected acetabulum uppermost. Great trochanter osteotomy (Gorman method) or gluteal muscle tenotomy approach of the acetabulum was used (21). At the end of the operation, orthogonal radiographs were taken to evaluate the implant position and fracture reduction. In this study, locking acetabular plates and locking reconstruction plates made of grade 5 medical titanium (TiAI4V6) were used (Figure 1).

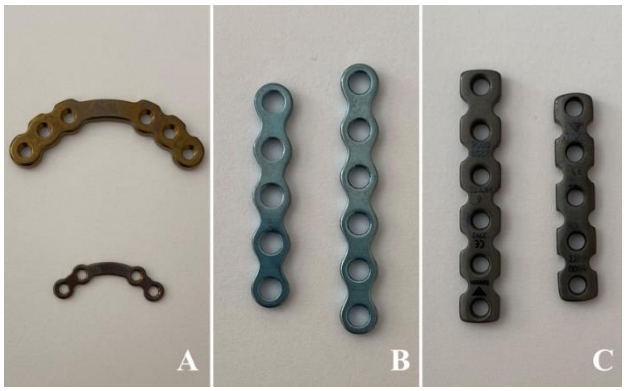

Figure 1. Locking acetabular plate for large dog and cats/small breed dog (A), 1.5 mm reconstruction plates with different number of holes for cat/small breed dog (B) and 3.5 mm reconstruction plates for large breed dog (C).

Postoperative Management: Meloxicam (0.2 mg/kg) was given for its anti-inflammatory and analgesic properties, while amoxicillin-clavulanic acid (12.5 mg/kg) was administered to provide broad-spectrum antimicrobial coverage. Strict cage confinement was advised for the first 10 days to limit patient movement and promote stable healing. No external support, such as bandages or splints, was applied to the operated limb to avoid unnecessary immobilization of surrounding structures. Long-term follow-up evaluations were conducted via clinical examinations and radiographic imaging. During follow-up, extremity functions were evaluated by physical examination, and radiographic images were taken to evaluate the presence/absence of the fracture line, callus formation, and fixation quality.

Results

As the majority of cases involved stray animals, precise age determination was not feasible. Therefore, cases were classified as either mature or immature based on the status of the epiphyseal plates, specifically whether they were open or closed. Out of the 18 animals diagnosed with acetabular fractures (10 cats and 8 dogs), five were stray animals for which the precise time of trauma could not be determined. For the remaining 13 animals with known histories, the mean interval from injury to surgical intervention was 3.7 days, ranging from a minimum of 1 to a maximum of 10 days. Weight and breed distributions are given in detail in tables 1 and 2.

Acetabular fractures demonstrated distinct patterns of localization between species. In dogs, fractures were most frequently observed in the central region (62%) of the acetabulum, whereas in cats, they predominantly occurred in the cranial (40%) region. Bilateral acetabular fractures were identified in 12.5% (1/8) of the canine cases, whereas all feline cases exhibited unilateral fractures.

Neurological evaluations in the preoperative period revealed proprioceptive neuropathy in two of the eight canine cases. Both cases experienced immediate resolution of neurological deficits following surgical intervention. Postoperatively, the average time to initiate weight-bearing on the affected limb in seven dogs was recorded as two days. Data for case 11 could not be obtained for postoperative follow-up, as the owner transferred the animal to another veterinary facility shortly after surgery.

Preoperative neurological evaluations revealed that seven of the ten cats with acetabular fractures exhibited normal proprioceptive reflexes, while three cats presented with proprioceptive neuropathy. Postoperatively, the average time to regain weight-bearing ability on the affected limb in cats with intact proprioceptive reflexes (excluding case 13) was one day. In case 13, however, weight-bearing was delayed to five days post-surgery. Among the three cats with proprioceptive neuropathy, two (excluding case 18) resumed limb use approximately one month postoperatively. Case 18 was euthanized on the 10th postoperative day due to a systemic infection, preventing sufficient time for neurological recovery. It is presumed that the neurological deficits in this patient remained unresolved at the time of euthanasia.

The plate, which was contoured during the intraoperative period, was first fixed to the caudal fragment. This approach was adopted for two primary reasons. Firstly, the caudal region of the acetabulum provides limited bone stock; therefore, securing the plate to the cranial fragment first could result in difficulty identifying adequate bone stock for screw placement in the caudal segment. Secondly, the cranial fragment exhibited greater inherent stability, which facilitated precise repositioning and fixation of the caudal fragment. This strategic order of fixation ensured optimal stability and alignment of the fractured acetabular components.

In animals receiving acetabular plates, a full set of screws was placed in some cases (four screws in cats and eight screws in dogs). However, in others, certain screw holes were left empty, particularly in cranial and/or caudal regions where bone stock was insufficient due to the plate's contouring. Especially in fractures in the cranial or caudal region, since the plate could not be placed symmetrically, it was observed that the most cranial screw hole and the most caudal screw hole in fractures in the caudal region remained empty (Figure 2).

Table 1. Preoperative period – Dog.

Case	Breed	Bone growth	Weight (kg)	Sex	Location	Side affected	Degree of displacement	Severity of lameness on presentation	Grade of neurological deficit	Concomitant injury
1	German Shepherd	Immature	20	F	Centrocaudal	R	1	4	3	Ischial fracture (C) Pubis fracture (B) Hip dysplasia
2	Mixed	Mature	32	M	Central (comminuted)	R	4	5	4	Coxofemoral luxation (C) Ischial fracture (B)
7	Mixed	Mature	35	M	Central	R	3	4	1	Ilial fracture (I) Sacroiliac luxation (C)
8	Mixed	Immature	22	M	Centrocaudal	L	2	5	2	Ilial fracture (I)
11	Anatolian Shepherd	Immature	22	F	Centrocaudal (comminuted)	R	2	5	1	Pubis fracture (I) Radial paralysis (L)
14	Mixed	Immature	24	F	Central	L	2	3	1	Femoral fracture (C)
16	Mixed	Immature	11	F	Central	L	3	2	1	Ilial fracture (I)
20	Chihuahua	Mature	3.2	F	Central (L) Craniocentral (R)	B	2 (L) 3 (R)	4	1	-

F: Female, M: Male, C: Contraletral, B:Bilateral, I: Ipsilateral, L:Left, R: Right.

Table 2. Preoperative period – Cat.

Case	Breed	Bone growth	Weight (kg)	Sex	Location	Side affected	Degree of displacement	Severity of lameness on presentation	Grade of neurological deficit	Concomitant injury
3	Tabby	Immature	1.5	F	Cranial	R	2	3	1	Coxofemoral luxation (C) Pelvic symphysis separation
4	Tabby	Immature	2.6	F	Cranial	R	3	3	1	-
9	Tabby	Immature	2.5	M	Cranial	L	1	4	1	-
10	Tabby	Immature	3.1	F	Craniocentral	L	2	4	1	Ilial fracture (I)
12	Tabby	Immature	2.8	M	Craniocentral	L	3	3	1	-
13	Tabby	Immature	2.4	F	Cranial	L	3	3	1	-
15	Tabby	Mature	3.4	F	Caudal	L	2	5	1	Sacroiliac luxation (B)
17	Tabby	Mature	3.5	M	Centrocaudal	L	1	4	3	-
18	Tabby	Immature	3.4	M	Caudal	L	2	4	3	Tibial fracture (C) Sacroiliac luxation (C) Urinary bladder herniation Pelvic symphysis separation
21	Tabby	Immature	2.1	M	Central	R	Grade 1	Grade 4	Grade 3	Sacroiliac luxation (C)

F: Female, M: Male, C: Contralatreal, B: Bilateral, I: Ipsilateral, L: Left, R: Right.

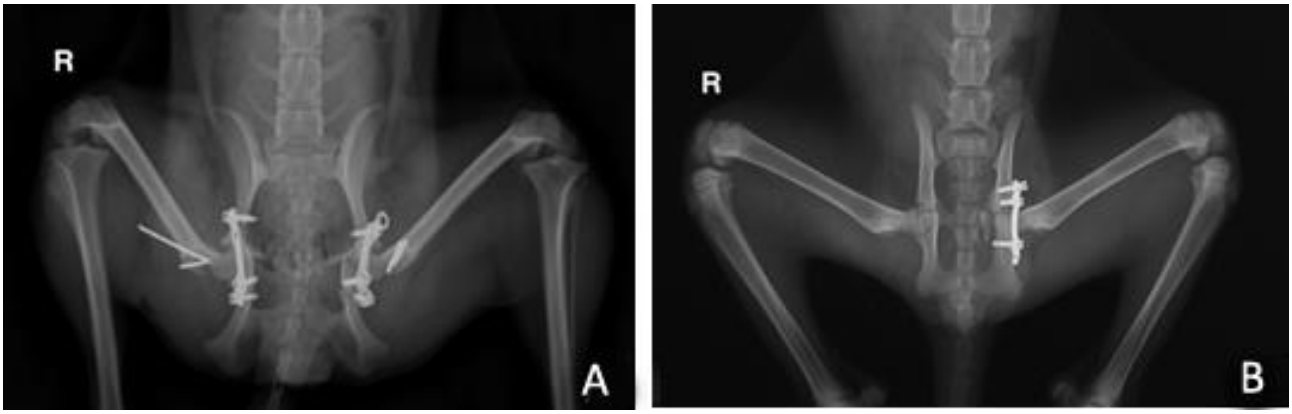

Figure 2. Radiographic images taken immediately after surgery. There is an empty screw hole in the cranial section of case 20 (A) and another in the caudal section of case 12 (B).

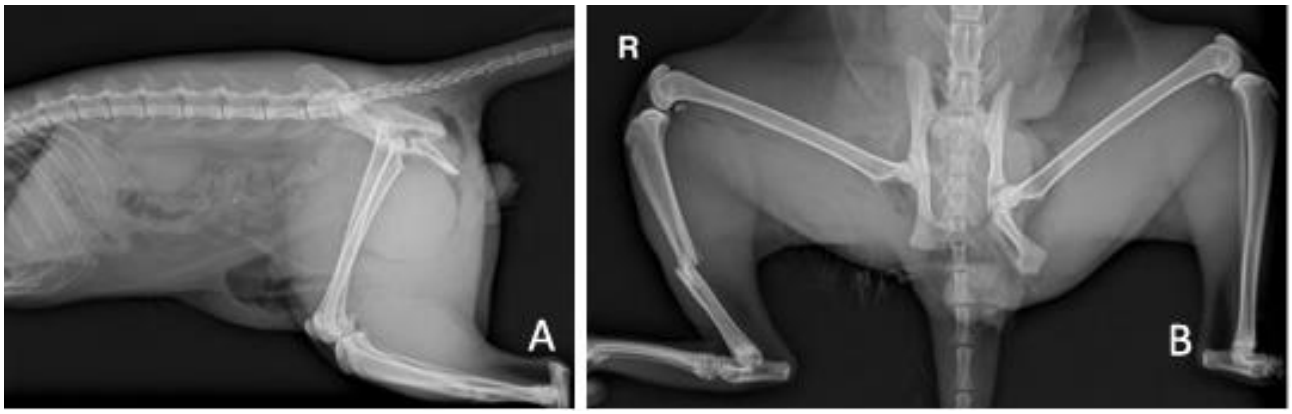

Figure 3. Laterolateral and frog-legged radiographic image of case 18 with acetabular fracture and concomitant righ tibial fracture and right sacroiliac luxation in the contralateral extremity and urinary bladder herniation into the pelvic canal.

In one cat (case 13), a second screw could not be placed in the caudal segment of the plate due to plate breakage during intraoperative contouring. Despite the presence of only a single screw in the caudal portion of the plate, no implant-related complications were observed during the postoperative period.

Major complications were observed in only one case (1/18). In case 7, both the cranial and caudal screw positions were incomplete due to insufficient bone stock. By the postoperative seventh day, the two screws in the caudal segment had broken, resulting in displacement of the caudal acetabular fragment into the pelvic canal. Therefore, a second revision surgery was performed.

Concomitant orthopedic injuries were encountered in 5 out of 10 cats and all dogs except the case 20. In case 18, in addition to the orthopedic concomitant injury, herniation of the urinary bladder into the pelvic canal was encountered (Figure 3).

A surgical approach was applied to cats with acetabular fractures with great trochanteric osteotomy (7/10) or gluteal muscle tenotomy (3/10). In all dogs, the surgical approach involved great trochanteric osteotomy. It is established that gluteal muscle tenotomy does not provide adequate exposure in large animals (21). Consequently, gluteal muscle tenotomy was not performed in any of the dogs included in this study. In cats, the decision to perform the procedure was left to the surgeon's preference. In patients who underwent a surgical approach with great trochanteric osteotomy, the osteotomized bone was fixed with Kirschner wire and/or screw. No tension band was utilized for any of the fixation performed with Kirschner wires. No complications such as pin/screw loosening or non-union were encountered in any of the patients who underwent greater trochanter osteotomy.

Long-term follow-up (>1 year) was possible for only three of the ten cats treated for acetabular fractures. Of the remaining seven, three died to presumed viral infections, while the other four were released back to the streets, preventing further follow-up. Similarly, none of the eight dogs treated for acetabular fractures could be followed for the long term due to similar circumstances. Preoperative, postoperative and long-term radiographs of a case with acetabular plate are shown in Figure 4. Detailed preoperative data for all patients are provided in tables 1 and 2, while postoperative outcomes are presented in tables 3 and 4.

Table 3. Postoperative period – Dog.

Case	Implant	Reduction grade	Weight bearing on the affected limb (postoperative-day)	Complication	Neurological condition	Follow-up (day)	Injury-to-surgery (day)	Limb function (Final follow-up)
1	AP (6 screw)	1	2nd	-	Normal	12	2	1
2	AP (1 screw missing-cranial)	3	3th	-	Normal	30	10	1
7	AP (2 screws missing-cranial/caudal)	2	1st	Minor (discharge) Major (The screw head broke on the 6th postoperative day)	Decreased proprioception 6th postoperative day (temporary)	30	Unknown	3
8	RP	3	4th	-	Normal	10	4	4
11	AP (6 screw)	2	Could not be followed up	Could not be followed up	Could not be followed up	Could not be followed up	Unknown	Could not be followed up
14	AP (2 screws missing-cranial/caudal)	4	2nd	-	Normal	5	5	3
16	AP (6 screw)	1	1st	-	Normal	10	4	1
20	AP (1 screw missing-cranial)	1 (both)	1st	-	Normal	10	2	1

AP: Acetabular plate, RP: Reconstruction plate.

Table 4. Postoperative period – Cat.

Case	Implant	Reduction grade	Weight bearing on the affected limb (postoperative-day)	Complication	Neurological condition	Follow-up (day)	Injury-to-surgery (day)	Limb function (Final follow-up)
3	AP (1 screw missing-cranial)	2	1st	-	Normal	12	Unknown	1
4	AP (4 screw)	2	1st	-	Normal	14	Unknown	2
9	AP (4 screw)	2	1st	-	Normal	>1 year	2	1
10	RP	1	1st	Lameness occurred on the 18th postoperative day (temporary)	Normal	75	Unknown	1
12	AP (1 screw missing-cranial)	1	1st	-	Normal	11	4	1
13	AP (1 screw missing-caudal)	2	5th	-	Normal	11	3	3
15	RP	2	1st	-		15	4	1
17	AP (4 screw)	1	35th	-	Normal (5th week)	>1 year	1	1
18	AP (4 screw)	1	Euthanasia	-	Delay in proprioception	10	2	4
21	AP (4 screw)	1	30th	-	Normal (4th week)	>1 year	6	1

AP: Acetabular plate, RP: reconstruction plate.

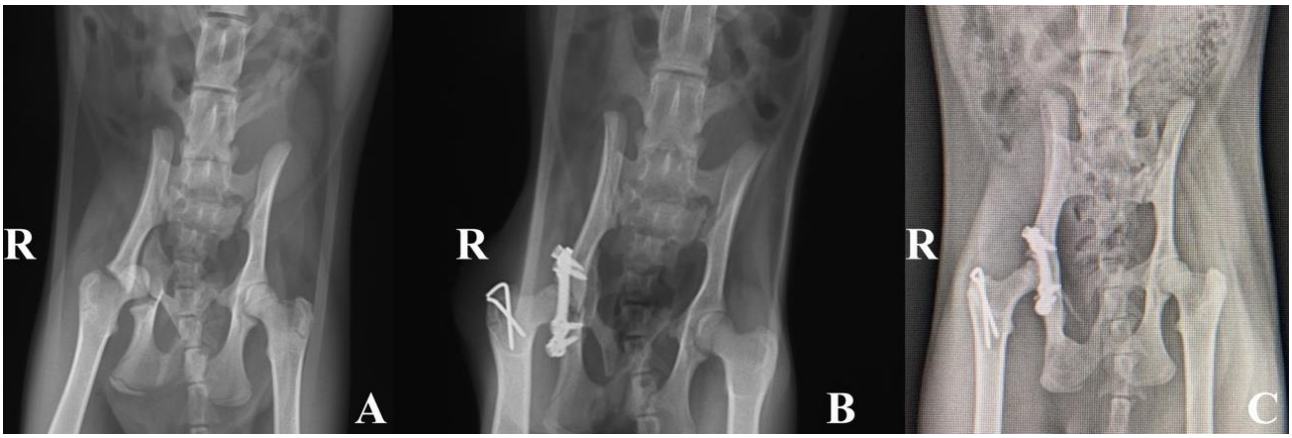

Figure 4. Preoperative (A), postoperative (B) and long-term (11 months) radiographic images (C) of case 21.

Discussion and Conclusion

In a biomechanical analysis conducted by Prieur et al. (1980), it was observed that the primary load transmitted to the hip joint in running dogs occurs predominantly in the horizontal plane and is directed forward. Based on this finding, some authors have argued that fractures of the caudal acetabulum occur in regions subjected to minimal biomechanical stress and, consequently, this region can be treated conservatively (8, 13, 20, 28). However, subsequent research has challenged this perspective. A study focusing on the canine acetabulum (29) demonstrated that the cranial and caudal thirds of the acetabulum endure loads approximately 7.9 and 13.1 times greater, respectively, than the central region. Similarly, Beck et al. (2005) reported in a feline acetabulum study that the central and caudal regions bear significantly higher mechanical loads than the cranial region. Despite these biomechanical insights, clinical fracture patterns present an intriguing contrast. In our study, 50% of acetabular fractures in dogs were located in the central third, a region identified as being subject to lower mechanical stress in prior studies (29). In feline cases, the highest proportion of fractures was observed in the cranial third, an area noted for bearing the least load according to Beck et al. (2005). These findings suggest that factors beyond mechanical loading, such as species-specific anatomical differences, bone morphology, and potential variations in trauma mechanisms, may play a critical role in fracture distribution patterns in both dogs and cats.

It was reported that most animals with acetabular fractures had a high rate (46/49) of concomitant orthopedic injuries, including pelvic fractures, and these were mostly seen in small breeds and young animals (36). In this study, concurrent orthopedic injuries were seen in 87% of dogs (7/8) and 50% of cats (5/10). Additionally, a study reported that non-orthopedic injuries, such as urinary system trauma and neurological complications, were present in approximately 59–72% of patients with pelvic fractures (22). In one of the cases included in our study (case 18), the urinary bladder herniated into the pelvic canal, and this hernia was repaired at the same time as the acetabular fracture. Cases with concomitant orthopedic injuries were managed either medically or surgically. In all cases requiring surgical intervention, the additional orthopedic injuries were addressed concomitantly with acetabular fracture repair. In 10 of the 12 cases, no complications were observed aside from prolonged operative time. Of the remaining two cases, one developed septicemia secondary to multiple trauma and was euthanized, while the other experienced postoperative screw breakage, necessitating revision surgery.

In a previous study, it was stated that there was 23% peripheral nerve dysfunction in patients with pelvic fractures in the preoperative period (25). In this study, the rates of peripheral nerve dysfunction were determined as 30% in cats and 20% in dogs. Among the feline cases in our study, two of the three cats diagnosed with preoperative sciatic neuropraxia exhibited functional recovery of the affected limb within an average of four weeks following surgical intervention. Long-term evaluations revealed that these cats regained excellent extremity functionality. Similarly, two canine patients presented with preoperative sciatic neuropraxia, which resolved completely postoperatively. The resolution of neuropraxia in both species is likely attributable to the decompression of the sciatic nerve, achieved through anatomical reduction and stabilization of the acetabular fractures. These findings underscore the critical role of timely surgical intervention in mitigating secondary neural compression and promoting functional recovery in patients with pelvic fractures complicated by sciatic nerve involvement.

There is a possibility of iatrogenic neurotrauma during surgery in acetabular fractures. It was reported that 5 out of 16 dogs without preoperative neuropraxia

developed postoperative neuropraxia, but the extremity regained full function at the end of 6 weeks (13). In this study, in one case (case no 13), the patient was normal in the preoperative period, but a loss of proprioceptive reflexes associated with sciatic neuropraxia was observed in the first five postoperative days, but this problem resolved after the fifth day.

In studies examining acetabular fractures, conflicting findings have been reported regarding the relationship between the degree of fragment displacement and the success of fracture reduction. Some studies suggest that a higher degree of displacement negatively impacts fracture reduction outcomes (17), whereas others have found no significant association (36). In our study, we observed no significant correlation between the degree of fragment displacement and the achieved fracture reduction. However, it was observed that in case 2, which involved an acetabular fracture with a displacement classified as "grade 4," the degree of reduction was assessed as "grade 3." This finding suggests a potential positive correlation between the severity of displacement and the extent of postoperative reduction. However, in this particular case, it remains uncertain whether the suboptimal reduction was primarily attributable to the severity of displacement itself or to the presence of fibrous tissue at the fracture site, which may have impeded reduction, given that the interval between trauma and surgical intervention was 10 days.

A statistically significant correlation was observed between the duration of trauma prior to surgical intervention and the degree of fracture reduction achieved. However, due to incomplete trauma time data for two dogs and a prolonged trauma-to-operation interval of 10 days in one case, these three subjects were excluded from the final analysis. Consequently, calculations were performed on the remaining five cases. Based on the revised dataset, the mean trauma-to-surgery interval for these five dogs was determined to be 3.2 days. The average degree of fracture reduction achieved was classified as 2nd degree. In feline cases, due to the lack of precise trauma timing in three individuals, only seven cases were considered for analysis. Among these, the mean interval from trauma to surgical intervention was calculated as 3.1 days, while the mean reduction degree was determined to be 1.4. Although the average time from trauma to surgical intervention was comparable between species, at approximately three days, the degree of reduction differed, with cats demonstrating an average reduction grade of 1.4 compared to 2 in dogs. This disparity may be attributed to anatomical differences between the species, with the smaller size and more delicate skeletal structure of cats potentially facilitating precise anatomical reduction with minimal force during surgical manipulation. Further studies with larger sample sizes are necessary to establish a more definitive relationship between animal size and reduction degree outcomes.

In the two canine cases presenting with preoperative neurological dysfunction, the interval between trauma and surgical intervention was 2 days in one case and 10 days in the other. Despite this variation, both dogs regained functional limb use within an average of 2 days postoperatively. Similarly, among the two feline cases with preoperative neurological deficits (excluding case 18), the time from trauma to surgery was 1 day in one case and 6 days in the other. The mean time to regain functional limb use in cats was 1 month. No significant correlation was identified between the duration from trauma to surgical intervention and the recovery time of neurological function. However, given the limited sample size, these findings should be interpreted with caution.

The use of appropriate plates for fixation in acetabular fractures is widely regarded as the optimal approach for achieving precise anatomical reduction and providing rigid stabilization. However, several challenges associated with this technique have been documented. In certain fractures, implant size may be constrained by anatomical limitations, particularly in cases involving the caudal acetabular fragment, where limited bone stock can restrict optimal screw placement. Additionally, in bilateral acetabular fractures, early postoperative loading of the affected extremity can lead to complications such as screw loosening or breakage (3, 13, 18, 31). Although screw loosening is uncommon in locking plate systems, previous reports suggest that angulation of locking screws may compromise the locking mechanism, potentially leading to screw loosening (34). In the present study, particularly in feline cases, some locking screws had to be inserted at an angle due to insufficient caudal acetabular bone stock. Despite this deviation from the optimal screw trajectory, no instances of screw loosening were observed in any of the cases (except case 7). A notable example from our study involved case 7, which presented with an ipsilateral ilium fracture and contralateral sacroiliac dislocation in addition to the acetabular fracture. On the postoperative sixth day, screw heads in the caudal fragment failed due to the biomechanical stresses imposed. Corrective surgery was performed due to the displacement of the caudal fragment into the pelvic canal and the intra-articular nature of the fracture.

In some cases, the failure to achieve optimal reduction following plate fixation can primarily be attributed to inadequate contouring of the plate to conform precisely to the anatomical shape of the acetabulum. Although C-type acetabular plates and reconstruction plates were utilized in this study, and locking mechanisms were employed to enhance stability, achieving perfect contouring proved challenging. Despite efforts to shape

the plates intraoperatively, anatomical mismatches occasionally persisted. Implant failure was observed in case 13 due to over-shaping of the acetabular plate used. Therefore, one screw was placed incompletely in the caudal acetabulum. No complications were observed in the postoperative period. This outcome may be attributed to the use of locking plates, which are known to maintain a degree of mechanical stability even with a reduced number of screws, though this is suboptimal (33). Although stainless steel is known to be softer than titanium and exhibits greater plastic deformation before fracturing, a direct comparative analysis of these materials in the context of acetabular plate application would provide more definitive insights (6).

The findings and clinical insights gained from this study indicate that achieving optimal reduction of fractures in the central acetabular region using "C" plates is associated with highly favorable clinical outcomes. For fractures located in the cranial acetabular region, effective reduction and fixation can be achieved with reconstruction plates extending towards the corpus ossis ilium. In contrast, fractures in the caudal acetabulum present greater challenges for reduction. This difficulty arises from the limited bone stock available for secure screw placement in the caudal fragment, compounded by the ventral curvature and termination of the acetabular arch. Despite these challenges, reconstruction plates remain a viable option for fixation in this region, similar to their use in cranial fractures. Based on our surgical experience with 19 acetabular fractures, it is evident that these injuries result in significant lameness. However, with precise anatomical reduction and stabilization, excellent clinical outcomes can be achieved. Consequently, primary surgical repair should be prioritized over conservative management or salvage procedures such as excision arthroplasty. While excision arthroplasty may be considered as a secondary option, it should be reserved for cases where primary repair is unfeasible, given the potential for orthopedic complications to develop in the contralateral limb over time. These findings reinforce the importance of prioritizing anatomical reduction and stabilization to optimize functional recovery and long-term patient outcomes (24, 38).

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Ethical Statement

This study was carried out after the animal experiment was approved by Kırıkkale University Local Ethics Committee (Decision number: 99-257526).

Conflict of Interest

The authors declared that there is no conflict of interest.

Author Contributions

MB and BK conceived the idea and planned the manuscript. MB, and BK contributed to sample preparation. MB and BK have made significant scientific support and also contributed to the interpretation of the results. Both authors provided significant contributions by giving feedback and help shape the manuscript.

Data Availability Statement

The data supporting this study's findings are available from the corresponding author upon reasonable request.

Animal Welfare

The authors confirm that they have adhered to ARRIVE Guidelines to protect animals used for scientific purposes.

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Nanofiber encapsulation of probiotic cultures via electrospinning: fabrication and quality compliance with ISO/IEC 17043 and ISO 22117 standards

Ahmet KOLUMAN[1,a], Çiğdem AKDUMAN[2,b], Mahmed Sari NJJAR[1,c], Meltem DELİMANLAR[1,d,✉], Ulviye ADAMCI[3,e], Mehmet Kıvanç ALAY[3,f], Mustafa SOYLU[3,g]

[1]Pamukkale University, Faculty of Technology, Department of Biomedical Engineering, Denizli, Türkiye; [2]Pamukkale University, Denizli Vocational School of Technical Sciences, Department of Textile Technology, Denizli, Türkiye; [3]VERITAS Textile, Denizli, Türkiye

[a]ORCID: 0000-0001-5308-8884; [b]ORCID: 0000-0002-6379-6697; [c]ORCID: 0000-0003-2494-1086; [d]ORCID: 0000-0003-4152-7805; [e]ORCID: 0000-0001-6311-5284; [f]ORCID: 0009-0005-8033-9905; [g]ORCID: 0009-0005-5701-263X.

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✉Corresponding author
delimanlarmeltem@gmail.com

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ABSTRACT

Probiotics offer numerous health benefits, including inhibiting pathogenic growth, supporting intestinal microbiota, and synthesizing essential biomolecules. However, their viability during storage remains a challenge due to sensitivity to environmental conditions. This study investigates the encapsulation of *Lactobacillus rhamnosus* and *Lactobacillus acidophilus* in polyvinyl alcohol (PVA) nanofibers via electrospinning to enhance stability and viability. Near-optimized electrospinning parameters, including solution concentration, voltage, and collector distance, were used to produce nanofibers, which were characterized using Field Emission Scanning Electron Microscopy (FESEM). The results showed non-uniform fiber diameter distributions, with 16 kV producing thicker fibers with an average diameter of 479.11 nm. Homogeneity assessment confirmed uniform probiotic distribution within the nanofibers, with a coefficient of variation of 5.3%. Storage stability tests at 4°C over 15 days were conducted following ISO/IEC 17043 and ISO 22117 standards. The findings demonstrated that encapsulation effectively preserved *L. rhamnosus* viability in 16LR/PVA nanofibers, whereas *L. acidophilus* exhibited reduced viability at both 10 kV and 16 kV.

Introduction

The term "probiotic" originates from Greek, meaning 'for life'. Probiotics, tasked with functions such as preventing the development of pathogenic species, maintaining intestinal flora, improving bowel movements, facilitating mineral absorption, and synthesizing vitamins and antimicrobial substances, positively impact human health when consumed in sufficient quantities (10). Due to the well-known effects of probiotics, there has been an increased demand for both probiotic medications and probiotic foods in recent times. According to a report by the Food and Agriculture Organization, probiotics are defined as living microorganisms that positively influence the health of their host when consumed in sufficient quantities (20). The majority of probiotics belong to the *Lactobacillus* and *Bifidobacterium* genera. However, besides these genera, some cocci, non-lactic acid bacteria, yeasts (*Saccharomyces cerevisiae*, *Saccharomyces boulardii*), and certain other species (EcN, *Sporolactobacillus* spp.) have been observed to exhibit probiotic properties (26). The primary mechanisms that contribute to the positive impact of probiotics on health include the production of inhibitory metabolites such as organic acids, hydrogen peroxide, and bacteriocins, as well as their ability to colonize adhesive regions in the intestines, contribute to pathogen inhibition through

nutrient competition, suppress toxin production, and lower intestinal pH (34). In tissue engineering, various studies have investigated the potential use of probiotics, reporting benefits such as wound healing, protection against ultraviolet radiation, and enhanced innate immunity through topical or systemic applications (10, 31). Wound healing involves processes such as homeostasis, inflammation, proliferation, and tissue remodeling. In the initial stage of homeostasis, platelets are activated, and growth factors, cytokines, and substances present in platelets are released (49). These molecules activate mechanisms such as chemotaxis, cell proliferation, angiogenesis, extracellular matrix accumulation, and tissue remodeling (8). Probiotics are suggested to be effective in wound healing by influencing these mechanisms (49).

For probiotics to be effective, they must remain in sufficient numbers throughout their shelf life and survive harsh environmental conditions to reach the target area. However, since these microorganisms are highly sensitive to their surroundings, they need greater resistance to stay alive. Their ability to maintain metabolic activity is essential for supporting host health. However, many of these microorganisms are sensitive to various environmental factors, including the presence of oxygen, acidity, process temperature, storage temperature, and product processing conditions, all of which can constrain their viability (45). Given that these microorganisms are affected by both process and storage conditions as well as environmental factors, the initial inoculum becomes crucial for realizing the benefits of these microorganisms on the host. Considering the sensitivity of probiotics to environmental conditions and processing parameters during the production of probiotic products, various encapsulation methods have been developed to enable these microorganisms to maintain their metabolic activities and viability for longer durations (49).

In recent years, encapsulation has proven to be a promising method for preserving bacterial cells (15). Various studies have reported different techniques for probiotic encapsulation, such as extrusion, emulsification, and spray drying (4, 16). However, most of these methods involve the use of high temperatures or organic substances that can cause significant cell death in probiotic cells (19). The electrospinning method can be used as an alternative and suitable encapsulation technique for delicate foods and bioactive compounds, thanks to its ability not to damage active agents (22).

The electrospinning method is a simple nanofiber production technique that allows encapsulation both in capsule and fiber forms at the submicron and nanometer scales. It consists of a high-voltage power source, a collector, a syringe pump, and a needle used as a nozzle (37). The electrospinning system has advantages over both traditional encapsulation methods and other nanofiber processes (36), including temperature adaptability, the generation of products with a large surface area, a high surface-to-volume ratio, the use of a wide range of polymers and solutions with various properties, simplicity, cost-effectiveness, and the ability to scale up for industrial production (42). Additionally, the electrospinning method allows the production of materials with desired characteristics and dimensions (micro, submicron, nano) by adjusting polymer properties, system parameters, and environmental conditions (9). To preserve the viability of probiotics, the materials used in the electrospinning method are limited to those that can be successfully drawn in either a water medium or a solution with mild acidity, such as an acetic acid solution. The encapsulation of probiotics is achieved by using a combination of synthetic polymers or synthetic/biopolymers. Many biocompatible synthetic polymers can be directly drawn into ultrafine fibrous matrices. Among these, PVA, polyethylene oxide (PEO), and polyvinylpyrrolidone (PVP) polymers are commonly used. PVA is a hydrophilic polymer with a semi-crystalline structure that is known as Generally Recognized as Safe (GRAS), showing no toxic properties and possessing high thermal and chemical stability (25). Due to its high biocompatibility and cost-effectiveness, PVA is commonly utilized in the electrospinning system (33). Its water-soluble nature facilitates the easy recovery of cells, making it suitable for the encapsulation of probiotics using the electrospinning method (24).

In a study by Amna et al. (3), it was found that *Lactobacillus gasseri* encapsulated within PVA nanofibers remained viable *in vitro* for several months. In another study by Han et al. (27), *E. coli* cells were encapsulated with PEO, glycerol, and dextran using the electrospinning method. The resulting encapsulated fibers were observed to maintain the viability of cells at room temperature for a longer duration compared to free cells. *Lactobacillus paragasseri* K7 was paired with sodium alginate (NaAlg) and PEO polymers, employing a structured electrospinning method with a high electric field and a smooth nozzle (46).

In another study, Mojaveri et al. (38) produced chitosan/PVA nanofibers loaded with inulin-carrying *B. lactis* BB-12 as a probiotic. When compared to pure PVA nanofibers, the hybrid chitosan/PVA nanofibers were noted to provide better protection through intermolecular hydrogen bonding between chitosan and PVA molecules for encapsulated bacteria.

These studies demonstrate that the electrospinning method can be utilized as a tool for producing polymeric fibers containing probiotic bacteria. By using different polymers and electrospinning methods for each bacterial species, the resulting fibers were aimed to have distinct properties (21).

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Ensuring the shelf life and viability of probiotics is crucial for harnessing their health benefits effectively. Probiotics, which are live microorganisms, are delicate and can easily degrade if not stored properly. Factors like temperature, humidity, packaging, and the specific strains of probiotics all play pivotal roles in determining their longevity and effectiveness (48). Refrigeration is often recommended, especially for strains like *Lactobacillus* and *Bifidobacterium*, as they tend to thrive at lower temperatures and can lose potency at room temperature (39). Moreover, the type of packaging used also matters; vacuum-sealed or blister-packed probiotics tend to have longer shelf lives due to reduced exposure to moisture and oxygen. Encapsulation methods, such as electrospinning, provide an additional layer of protection during processing and storage, shielding probiotics from environmental stressors. It's worth noting that the shelf life of probiotics can vary widely, ranging from 1 to 4 years, depending on factors like formulation, storage conditions, and strain specificity (12). By adhering to recommended storage guidelines and choosing high-quality probiotic supplements, consumers can ensure that they receive the maximum health benefits from these beneficial microorganisms (50).

ISO/IEC 17043 (29) is an international standard that outlines the general requirements for the competence of proficiency testing (PT) providers. PT is a critical component of laboratory quality assurance. It is a way of evaluating the performance of laboratories by comparing their results with the results of other laboratories using the same method (29). PT is used to evaluate laboratory proficiency, confirm measurement precision, and pinpoint areas in need of development (5). ISO 22117 (28) expands on the principles of ISO/IEC 17043, concentrating specifically on microbiological testing in food products (28). ISO 22117 covers the preparation and distribution of microbiological samples, emphasizes safety measures, and incorporates specialized statistical methods to ensure the accurate detection and quantification of microorganisms (28).

This study aimed to utilize the electrospinning process for encapsulating *L. acidophilus* and *L. rhamnosus* while examining their response to the process. Validation experiments were conducted in accordance with ISO/IEC 17043 and ISO 22117 standards, specifically focusing on stabilization and homogenization. Production conditions were investigated for *L. rhamnosus and L. acidophilus*, followed by an examination of their viability after encapsulation. Within this scope, the focus is on preserving the viability of probiotics, achieving homogeneous distribution within nanofibers, and investigating the parameters of the directed electrospinning process.

Materials and Methods

Polyvinyl alcohol (PVA) (125,000 MW, 99% hydrolyzed) used in the production of nanofibers was purchased from Sigma-Aldrich Co. (St. Louis, U.S.A.). Distilled water was employed to prepare PVA solutions. For probiotic preparation, *L. rhamnosus* ATCC 7469 and *L. acidophilus* ATCC 4356, Trypticase Soy Broth (TSB) (Oxoid, England), and De Man–Rogosa–Sharpe (MRS) agar (Merck, Darmstadt, Germany) were used.

Preparation of Probiotics: *L. acidophilus* and *L. rhamnosus* strains were used as reference cultures. The strains were transferred into TSB and incubated at 37ºC for 18 hours. After incubation, the cultures were streaked onto MRS agar. Colonies were examined by further incubation at 30ºC for 24 hours to assess colony morphology. The choice of 30°C for further incubation is ideal for lactic acid bacteria, as it closely matches their natural habitat (e.g., fermentation processes). This temperature can enhance the growth and activity of these specific organisms compared to higher temperatures. The relevant ISO standard for incubation at 30 °C is ISO 4833-1 (30). This standard specifies a horizontal method for the enumeration of microorganisms that can grow and form colonies in a solid medium after aerobic incubation at 30 °C. It applies to various products intended for human consumption, animal feed, and environmental samples related to food production and handling. The incubation period specified in this standard is typically 72 hours under aerobic conditions at 30 °C. Subsequently, a single colony was picked, retransferred into TSB, and the enrichment process was repeated. A 5 mL aliquot was taken from the obtained enrichment, centrifuged at 3000 rpm to discard the supernatant, and this step was repeated until a bacterial pellet formed at the bottom. The resulting pellets were diluted with physiological saline solution (0.9% NaCl) until reaching a 0.5 McFarland turbidity. This process rendered the obtained probiotics suitable for the electrospinning process.

Preparation of Electrospinning Solutions: A 15% (w/w) PVA solution was prepared by dissolving 1.5 g of PVA in 8.5 g of distilled water with gentle stirring using a magnetic stirrer at 100°C until fully dissolved, followed by cooling to room temperature under sterile conditions. PVA solutions have been successfully prepared using similar procedures (2, 41). To each PVA solution, 5 mL of *L. rhamnosus* suspension was added, resulting in an *L. rhamnosus*/PVA solution with a final PVA concentration of 10% (w/w). The concentrations used in this study were based on those described by Nagy et al. (40), who applied similar concentrations in their work. The solution was stirred for one hour under the same sterile conditions. Sterility was assessed by measuring total bacterial counts

and conducting swab sampling in the production area, where no bacterial growth was detected. The same procedure was repeated for *L. acidophilus*.

Fabrication of Probiotic Nanofibers: Based on previous studies (23, 33, 40, 43, 47, 50), the parameters for the electrospinning process were set as follows: a distance of 15 cm between the collector and the needle, applied voltages ranging between 10 and 16 kV, and solution feeding to the system at a rate of 0.8 ml/h using a syringe pump. The experiments were performed at room temperature (25°C) in a fume hood. The electrospinning was carried out using a Nanoliz electrospinning device (Nanoliz, Ankara, Türkiye). Throughout the electrospinning process, nanofibers produced through the application of high voltage to the solutions were gathered on a rotating cylindrical collector covered with sterile aluminum foil. The nanofibers obtained at 10 kV were labeled as 10LA/PVA and 10LR/PVA, while those obtained at 16 kV were labeled as 16LA/PVA and 16LR/PVA. The prepared samples are summarized in Table 1. The samples obtained were stored in a sterile box for further analysis.

Field Emission Scanning Electron Microscopy (FESEM): The morphology of the samples was investigated using a FESEM. Each sample was coated with a thin layer of gold-palladium to provide conductivity (Quorum Q150R). All FESEM images were captured using a SUPRA 40VP microscope (Carl Zeiss, Germany). The magnification used for imaging was 5000X and 10000X. The average fiber diameters of the nanofibers were calculated from these images using the ImageJ program.

Validation and Verification of Probiotic Distribution and Storage Stability:

Homogeneity Assessment of Probiotic Distribution: A homogenization process was conducted to ensure uniform distribution of probiotics within the nanofiber matrices. Following electrospinning, the collected nanofibers were manually separated into smaller, randomized sections and thoroughly mixed to form a composite batch. Representative samples were randomly selected from this batch for further analysis. Homogeneity was assessed by analyzing ten randomly chosen sub-samples from different regions (central, peripheral, and intermediate) of the batch. Probiotic viability within these sub-samples was determined using the plating procedure under standardized conditions to minimize variability. The bacterial counts (CFU/g) were recorded, and the data were subjected to statistical analysis to evaluate distribution uniformity. The coefficient of variation (CV) was calculated using the following formula:

$$CV = \frac{\sigma}{\mu} \times 100 \quad (1)$$

where σ denotes the standard deviation of bacterial counts, whereas μ represents the mean bacterial count across all sub-samples.

Storage Stability and Viability Assessment: The viability of *L. acidophilus* and *L. rhamnosus* in the nanofiber matrices was evaluated under refrigerated storage conditions (4°C) over 15 days. Sampling was performed on days 0, 5, 11, and 15 to monitor bacterial stability. The validation of these experiments followed ISO/IEC 17043 (29) standards, with stabilization and homogenization procedures based on ISO 22117 (28) guidelines. Prior to validation, initial methodological procedures were implemented, referencing standard microbiological protocols to ensure reproducibility and reliability (13). To determine cell viability, a standardized plating procedure was conducted under controlled biosafety conditions within a biosafety cabinet. One gram of the nanofiber sample was accurately weighed and diluted in 9 milliliters of maximum recovery diluent (MRD), serving as the primary dilution for serial dilutions. The serial dilution factor (DF) was determined using the following formula:

$$\mathrm{DF} = \frac{V_{total}}{\mathrm{V_{sample}}} \quad (2)$$

V_{total} represents the total diluted volume, while V_{sample} denotes the initial sample volume. This dilution ensured that bacterial counts were within the quantifiable range. Precise aliquots (100 microliters) from each dilution were plated onto plate count agar using a Drigalski spatula to ensure uniform distribution. The plates were incubated in a controlled chamber at 30°C for 24 hours. If no microbial growth was observed within this

Table 1. Encapsulated probiotics and fabrication parameters.

Sample Coding	Probiotic	Voltage (kV)	Flow Rate (mL/h)	Distance (cm)
10LA/PVA	*L. acidophilus*	10	0.8	15
10LR/PVA	*L. rhamnosus*	10	0.8	15
16LA/PVA	*L. acidophilus*	16	0.8	15
16LR/PVA	*L. rhamnosus*	16	0.8	15

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period, an additional 12-hour incubation was performed to enhance detection. The bacterial count was determined using the formula:

$$\frac{CFU}{g} = \frac{N \times DF}{V_p} \quad (3)$$

Where N denotes the counted colony number, DF represents the dilution factor and V_p corresponds to the plated sample volume.

Results

FESEM Analysis Results: The morphology and diameters of electrospun fibers were investigated by field scanning electron microscopy. The diameters of 100 randomly selected fibers from the FESEM images of probiotic/PVA nanofibers were measured using ImageJ software. Subsequently, the average diameter of the fibers was calculated. This process was applied to all samples. The minimum, maximum, and average diameters of the measured nanofibers are presented in Table 2. The average fiber diameter of 10LA/PVA was 271.04 nm, while the average fiber diameters of 10LR/PVA and 16LR/PVA were 232.16 nm and 479.11 nm, respectively.

Figure 2 shows FESEM images and histogram profile of nanofiber samples. All samples displayed non-uniform diameter distributions. The histogram in Figure 2a illustrates a diameter distribution for 10LA/PVA, centered around 270 nm. For 10LR/PVA, a broader distribution was observed, with notable peaks at approximately 215 nm and 275 nm (Figure 2b). The fiber diameters of 16LR/PVA (Figure 2c) ranged from 228 nm to 800 nm, with the majority falling between 400 nm and 500 nm.

The morphology of *L. acidophilus* and *L. rhamnosus* and their arrangement in electrospun fibers were investigated by scanning electron microscopy. Figure 3 shows the formed fibers for the 16LR/PVA sample in different magnifications. The polymer fibers got thicker by encompassing rod-shaped single or interconnected bacteria.

Figure 4 shows the size of *L. acidophilus* and *L. rhamnosus* embedded in PVA nanofibers.

The FESEM images showed *that L. acidophilus* had a length of 2.35 µm and a width of 0.87 µm, while *L. rhamnosus* had a length ranging between 2.74 and 3.97 µm and a width ranging from 0.74 to 1.79 µm.

Validation and Verification:

***Homogeneity Assessment of Probiotic Distribution*:** Quantitative analysis indicated minimal variance in probiotic counts across the 16LR/PVA sub-samples, confirming a homogeneous distribution. CV for the bacterial counts was calculated at 5.3%, well within the acceptable threshold outlined in ISO/IEC 17043 (29) standards (<7%). Table 3 summarizes the probiotic counts for the tested sub-samples.

Table 3. Probiotic counts for the tested sub-samples

Sample ID	log 10 (CFU/g Mean)
Sample 1	6.494
Sample 2	6.489
Sample 3	6.491
Sample 4	6.497
Sample 5	6.490
Sample 6	6.493
Sample 7	6.496
Sample 8	6.487
Sample 9	6.491
Sample 10	6.493
Median	6.492
Standard Deviation	0.281

Storage Stability and Viability Assessment: The viability of *L. rhamnosus* in 16LR/PVA nanofibers produced at 16 kV was preserved from the 5th day onward. However, the electrospinning process at 10 kV had a negative impact on its viability. Similarly, the electrospinning process negatively affected the viability of *L. acidophilus* at both 10 kV and 16 kV. Figure 1 illustrates the stabilization of the 16LR/PVA nanofiber sample.

Table 2. Minimum, maximum, average diameters, and standard deviation of probiotic nanofibers.

Sample	Minimum Diameter (nm)	Maximum Diameter (nm)	Average Diameter (nm)	Standard Deviation
10LA/PVA	132.01	466.44	271.04	54.64
10LR/PVA	119.24	371.56	232.16	50.22
16LR/PVA	227.99	799.93	479.11	127.77

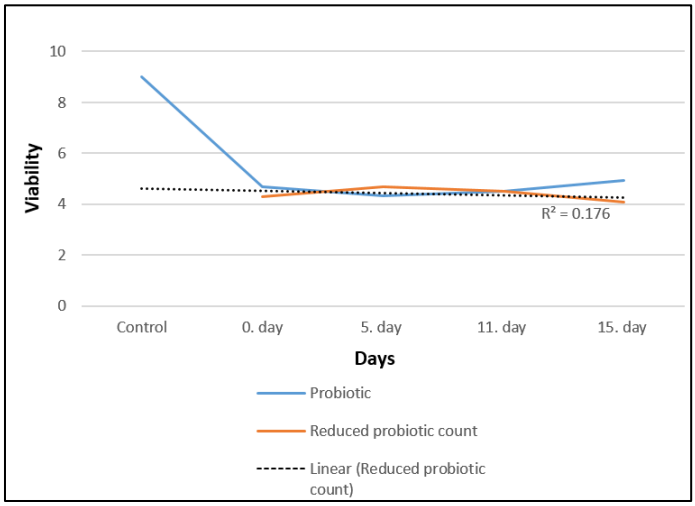

Figure 1. Stabilization of the 16LR/PVA nanofiber sample over 15 days.

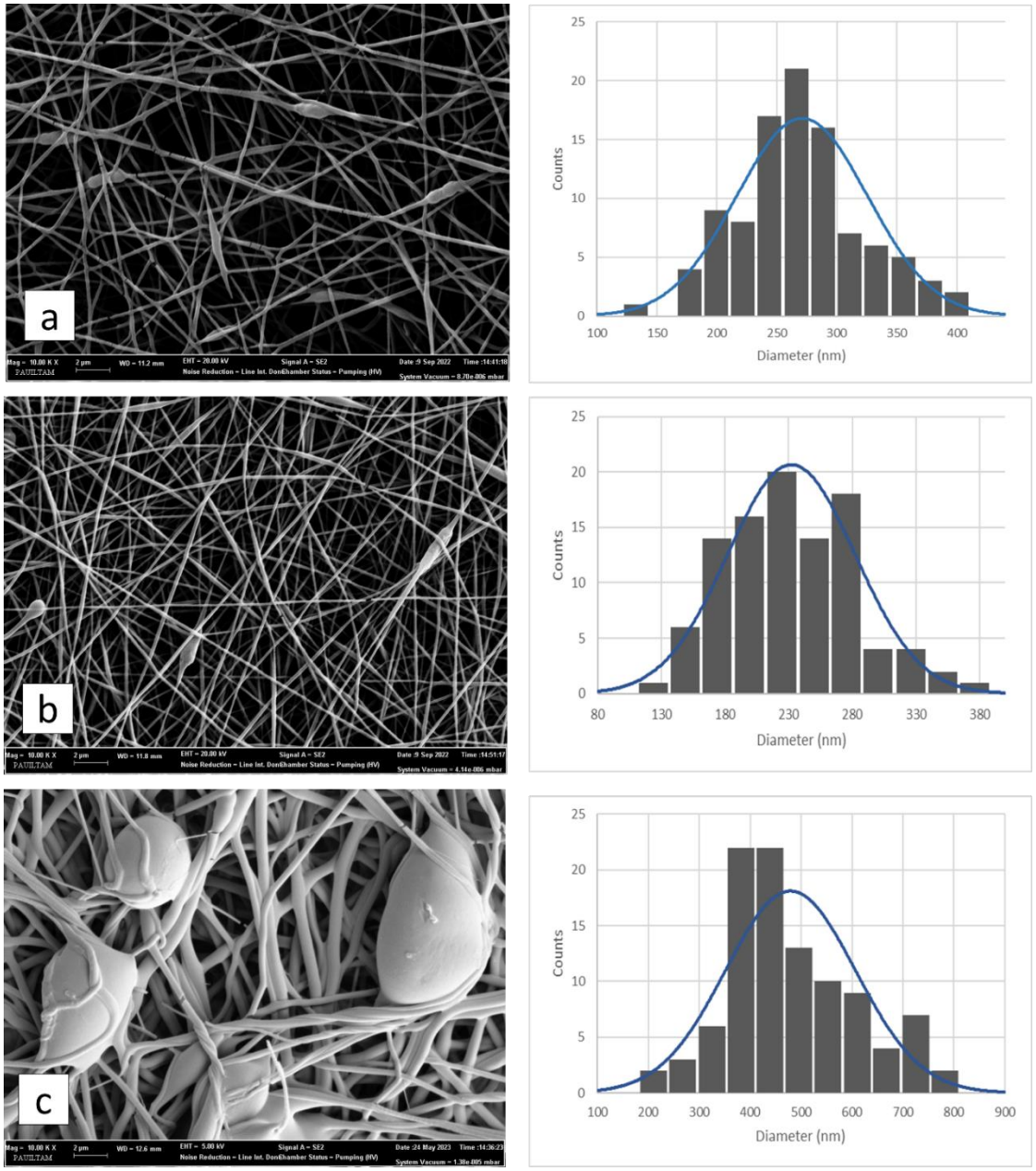

Figure 2. FESEM images 10000X magnification and histogram profile of nanofibers: a) 10LA/PVA, b) 10LR/PVA, c) 16LR/PVA.

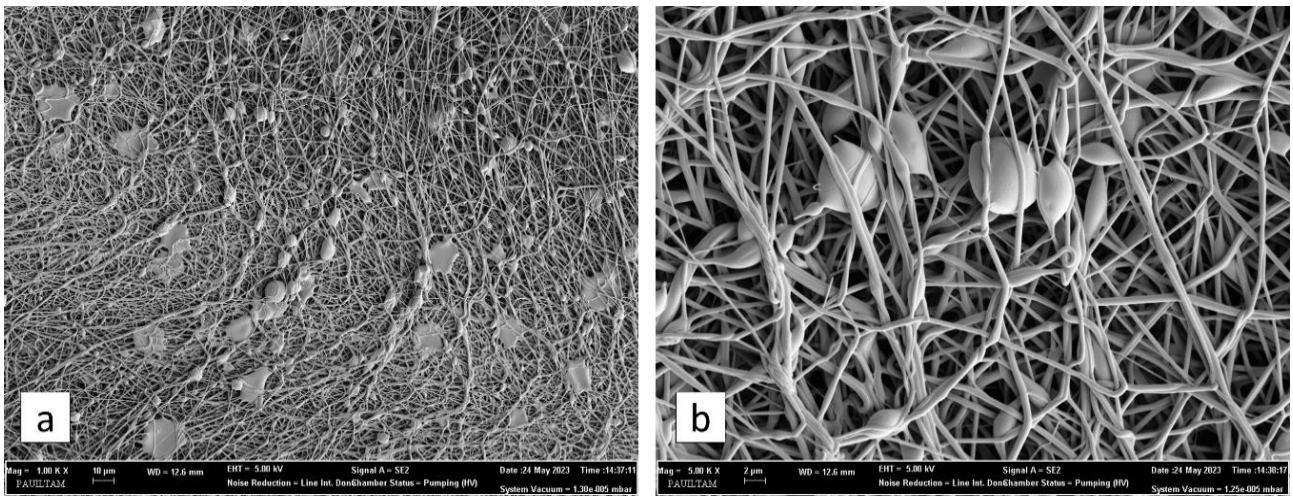

Figure 3. FESEM images of 16LR/PVA nanofibers at different magnifications. a) 1000X b) 5000X

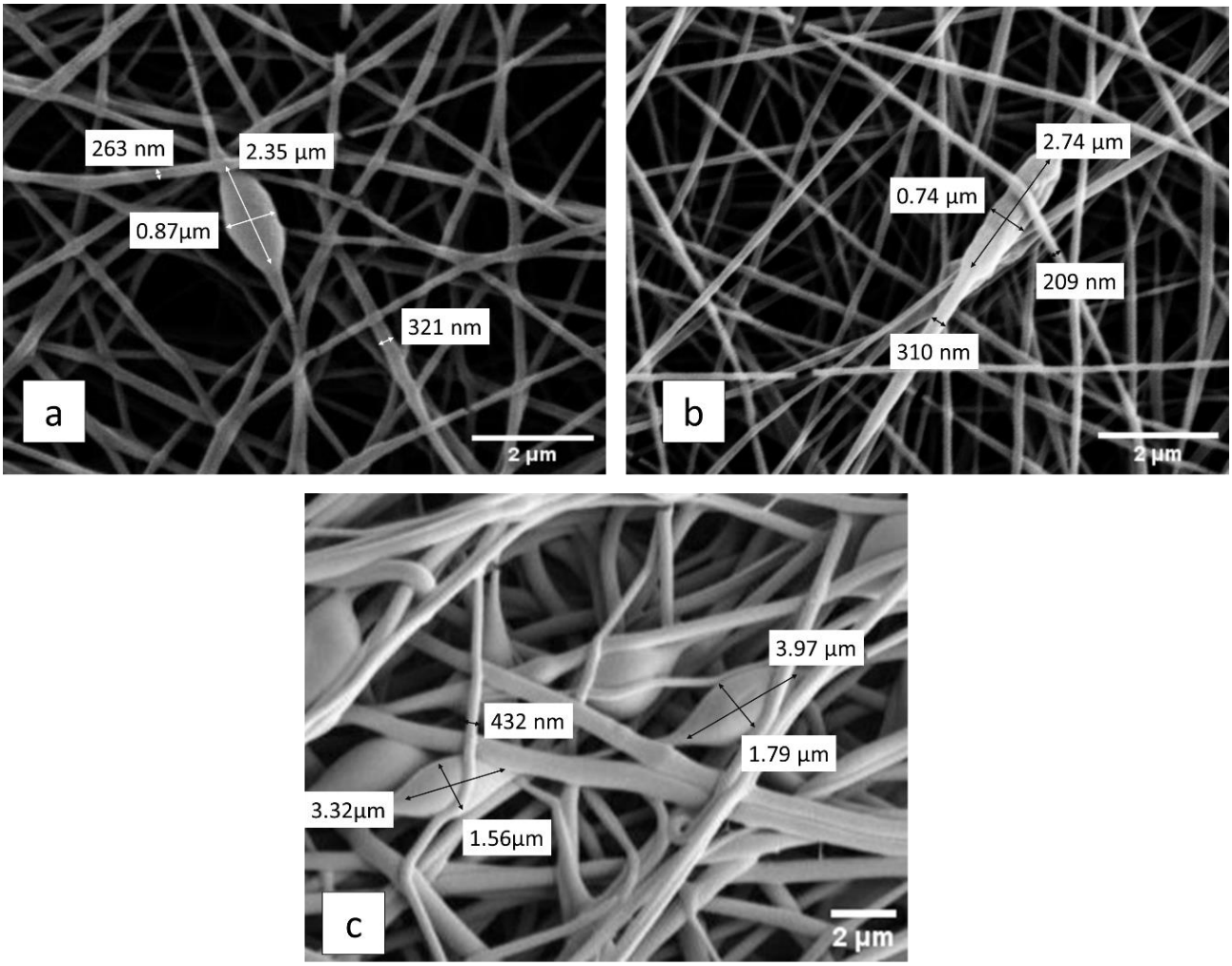

Figure 4. FESEM image of *L. acidophilus* and *L. rhamnosus* embedded in PVA nanofibers with thickness data: a) 10LA/PVA, b) 10LR/PVA, c) 16LR/PVA.

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Discussion and Conclusion

Considering the literature reviews, PVA polymer is generally recognized as safe (GRAS), showing non-toxic properties, high thermal and chemical stability, and is a semi-crystalline, hydrophilic polymer (14). Due to its high biocompatibility and low cost, it is frequently used in electrospinning systems. Therefore, PVA polymer was preferred for the production of nanofibers containing probiotics in this study.

Previous research (17, 37, 51) indicates that developing practical applications for electrospun nanofibers necessitates a complete understanding of the electrospinning parameters, since the structural morphology and diameter of the electrospun nanofiber will have an impact on the finished product. There are numerous parameters that can potentially influence the electrospinning process. Therefore, examining all of them within a single study is almost impossible. However, certain parameters can be kept constant during experiments. To conduct the experiments under controlled environmental conditions, ambient factors such as temperature and humidity are maintained consistently (51). The electrospinning was carried out in a fume hood. The continuous ventilation provided constant humidity during the process owing to the same inlet air. The relative humidity was around 55%, and the temperature was 25°C.

At first the electrospinning process of polymers was optimized without bacteria, and the collected nanofibers were investigated by FESEM. Probiotics containing polymer solutions were prepared at the determined optimal concentration. Koski et al. (33) observed that low solution concentrations resulted in the electrospinning of beaded fibers. Therefore, it was hypothesized that a concentration range of 8% to 12% would promote the formation of stable, bead-free fibers with circular cross-sections. According to Rwei and Huang (43), a 10% solution concentration is in the range that yields an acceptable electrospinning process. Similar results have been reported in the literature (1, 40). Thus, for this study, the polymer concentration was set as 10% (w/w).

Applied voltage exerts two significant effects on fiber diameter. Firstly, increasing the voltage enhances the electric field strength, leading to a greater electrostatic stretching force that accelerates the jet within the electric field, thereby promoting the formation of thinner fibers. Secondly, since charge transport in the electrospinning process is solely conducted by the polymer flow (50), an increase in voltage would result in more surface charges on the jet. This, in turn, increases the mass flow rate from the needle tip to the collector, causing the solution to be drawn more rapidly from the needle tip and potentially increasing the fiber diameter. The interplay of these two effects determines the final fiber diameter. Consequently, increasing the applied voltage may decrease, increase (7, 35), or have no effect (6, 32) on the fiber diameter.

The histogram (Figure 2a) indicated a non-uniform diameter distribution centered at approximately 270 nm for sample 10LA/PVA. As can be seen in Table 2, the average fiber diameter of 10LA/PVA was found to be 271.04 nm. 10LR/PVA displayed a broader range of data, with notably higher frequencies observed around 215 nm and 275 nm compared to other measurements (Figure 2b). The diameter distribution of 16LR/PVA was non-uniform (Figure 2c), with fiber diameters ranging from 228 nm to 800 nm. The majority of the nanofibers had diameters between 400 nm and 500 nm. As can be seen in Table 2, the average fiber diameters of 10LR/PVA and 16LR/PVA were found to be 232.16 and 479.11 nm, respectively. Even though there is no clear correlation, it may be concluded that the diameter of the fibers increased with the increase of the voltage. The increase in the diameter of the *L. rhamnosus* samples could be explained with the model proposed by Ziabari et al. (51). The change in fiber diameter as a function of voltage is dramatically influenced by spinning distance. At a short distance, the electric field is a high and dominant factor. Whereas, at long distances where the electric field is low, the effect of the mass of the solution would be a determining factor according to which fiber diameter increased with applied voltage.

The use of 5-20 cm for spinning distance was reported in the literature (22). Short distances are suitable for highly evaporative solvents, whereas it results in wet coagulated fibers for nonvolatile solvents due to insufficient evaporation time. Since water was used as a solvent for PVA in this study, short spinning distances were not expected to be favorable for dry fiber formation. Afterwards, this was proved by experimental observations, and 15 cm was considered as the effective spinning distance. This can also be supported by the results of Nagy et al. (40).

When investigating probiotic encapsulation, it is critical to take into account the complex impact of electrospinning parameters. To be more precise, the voltage and concentration have a substantial impact on the nanofibers' properties (17, 50). Nonetheless, there is remarkable variation in the impact of voltage on probiotic encapsulation amongst research investigations (18). According to some research findings, voltage may not always affect probiotics' encapsulation efficiency even while it has a discernible effect on the characteristics of nanofibers (31). This complex association calls for a closer look at the complex interactions between the particular behavior of the probiotics throughout the encapsulation process and the electrospinning parameters. The necessity of high voltage in electrospinning for nanofiber production juxtaposes concerns regarding its

potential deleterious effects on probiotics. Škrlec et al. (47) explored the impact of voltage on the viability of *L. plantarum* cells, revealing optimal viability at 15 kV (0.81 log reduction compared to theoretical loading). However, viability decreased with increased voltage (2.03 log reduction at 20 kV) or decreased voltage (1.30 log reduction at 10 kV). Additionally, electrospinning at 10 kV exhibited lower efficiency than at 15 kV or 20 kV, resulting in reduced fiber production rates. Conversely, Feng et al. (23) demonstrated that elevating the applied voltage from 10 to 16 kV did not significantly alter the viability of *L. plantarum* cells, with loaded cells maintaining high viability levels even under heightened voltage conditions.

In this study, *L. rhamnosus* maintained its viability at 16 kV, and a decreased viability was observed at 10 kV. The electrospinning process had a negative impact on *L. acidophilus* samples both at 10 and 16 kV. Additionally, electrospinning at 10 kV was less efficient than that at 16 kV and resulted in lower nanofiber production per unit time, similar to Škrlec et al.'s results (47). The primary concern for medical applications lies in the biological activity of probiotics within the nanofibers, which was assessed after their dissolution. The impact of the electrospinning process on bacterial viability was investigated over a 15-day period at 4°C. As can be seen in Figure 1, the probiotic viability of 16LR/PVA nanofibers obtained at 16 kV was preserved from day 5 onwards. The viability of *L. acidophilus* and *L. rhamnosus* was reduced from day 1 after the electrospinning process was carried out. The homogeneity test results confirm that the probiotic nanofiber batches produced via the described electrospinning process demonstrate a consistent distribution of probiotics. This ensures the reliability of further analyses and verifies compliance with ISO/IEC 17043 (28) standards.

The morphology of probiotics and their arrangement in electrospun fibers was also investigated by scanning electron microscopy. As can be seen in Figure 3, FESEM showed that the polymer fibers got thicker by encompassing rod-shaped single or interconnected bacteria. The polymer coating was formed around bacteria as a result of the electrospinning process. Similar findings have been reported by Nagy et al. (40) and Ceylan et al. (11), who observed an increase in fiber diameter associated with probiotic incorporation. The probiotics aligned along the nanofibers, consistent with the observations of Salalha et al. (44). Their findings revealed that bacteria, which were initially scattered randomly in a polymer solution, tend to position themselves within the Taylor cone during electrospinning, largely following the streamlines. This alignment remains consistent throughout the jet creation process, eventually becoming entrenched in the formed nanofibers.

Observations from the FESEM images indicate that *L. rhamnosus* in sample 16LR/PVA tends to aggregate, forming small clusters. The sizes of bacteria can be seen in Figure 4, which is similar to that of their original form (typical size of *L. acidophilus*: width ~ 0.6 - 0.9 µm, length: ~ 1.5-6.0 µm; typical size of *L. rhamnosus*: width ~ 0.8 - 1.0 µm, length: ~ 2.0 - 4.0 µm). The FESEM images showed that *L. acidophilus* had a length of 2.35 µm and a width of 0.87 µm (Figure 4a). While *L. rhamnosus* had a length ranging between 2.74 and 3.97 µm and a width ranging from 0.74 to 1.79 µm (Figures 4b, 4c).

In conclusion, this study explored the potential of electrospinning for encapsulating probiotic microorganisms, namely *L. rhamnosus* and *L. acidophilus*. The findings revealed a differential effect on probiotic viability. Electrospinning negatively impacted the viability of *L. acidophilus*, but *L. rhamnosus* encapsulated within 16LR/PVA nanofibers exhibited sustained viability from day 5 onwards. These results demonstrate the potential of electrospinning as a method for preserving the viability of certain probiotic strains. This approach holds promise for industrial-scale production of probiotics with enhanced stability and efficacy.

However, limitations were identified in the efficiency of electrospinning *L. acidophilus*. Further research is warranted to optimize the homogenization and encapsulation process parameters specifically for this probiotic strain. Additionally, broader investigations are needed to optimize parameters for encapsulating a wider range of probiotic species and maintaining their viability for extended periods. Future studies could explore the impact of different factors on the success of this technique:

Microorganism Selection*:* The selection of probiotic strains for encapsulation is crucial. This study focused on *L. rhamnosus* and *L. acidophilus*, but exploring strains with varying morphologies, stress tolerance, and surface properties could yield valuable insights. Strains exhibiting greater inherent robustness during electrospinning would be ideal candidates.

Polymer Selection: Polyvinyl alcohol (PVA) was used in this study, but other polymers with tailored properties for probiotic encapsulation should be investigated. Biocompatible and biodegradable polymers with controllable degradation rates could be explored.

Process Optimization: Optimizing homogenization and electrospinning parameters is critical. Future studies could explore variables such as needle size, flow rate, and the distance between the collector and the needle to improve encapsulation efficiency and minimize stress on the probiotics.

Storage Conditions: The long-term viability of encapsulated probiotics is directly affected by storage conditions. Future studies should investigate the impact of temperature, light exposure, and humidity on the encapsulated probiotics' viability and functionality.

By addressing these limitations and expanding the research scope, electrospinning can be established as a robust and versatile technique for the development of novel and effective probiotic delivery systems. Exploring the factors mentioned above can lead to the development of optimized protocols for encapsulating a diverse range of probiotic strains with enhanced viability and functionality. This holds the potential to revolutionize the production and delivery of probiotics for various applications, including functional foods, dietary supplements, and even targeted drug delivery systems.

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Ethical Statement

Ethics committee approval is not required for this study.

Conflicts of Interest

The authors declared that there is no conflict of interest.

Author Contributions

All authors have contributed equally to all aspects of this study, including the conception, methodology, data analysis, and manuscript preparation.

Data Availability Statement

The authors confirm that the data supporting the findings of this study are available in the article.

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Aqueous parsley (*Petroselinum crispum*) extract ameliorated methotrexate-induced brain and small intestine damage in rats

Ercan DURSUN[1,a], Sümeyye YILMAZ KARAOĞLU[2,b], Güzin Göksun SİVAS[1,c], Elif TUFAN[1,d], Özlem SACAN[3,e], Refiye YANARDAĞ[3,f], Göksel ŞENER[4,g], Tugba TUNALI AKBAY[5,h,✉]

[1]Marmara University, Institute of Health Sciences, Department of Biochemistry, Istanbul, Türkiye; [2]Fenerbahçe University, Vocational School of Health Services, Istanbul, Türkiye; [3]Istanbul University Cerrahpaşa, Faculty of Engineering, Department of Chemistry, Istanbul, Türkiye; [4]Fenerbahçe University, Faculty of Pharmacy, Department of Pharmacology, Istanbul, Türkiye; [5]Marmara University, Faculty of Dentistry, Department of Basic Medical Sciences, Istanbul, Türkiye

[a]ORCID: 0000-0001-6025-9565; [b]ORCID: 0000-0001-5529-7380; [c]ORCID: 0000-0001-7347-490X; [d]ORCID: 0000-0003-0684-3693; [e]ORCID:0000-0001-6503-4613; [f]ORCID: 0000-0003-4185-4363; [g]ORCID: 0000-0001-7444-6193; [h]ORCID: 0000-0002-2091-9298

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✉Corresponding author
ttunali@marmara.edu.tr

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ABSTRACT

Methotrexate (MTX) is a widely used antiarthritic and chemotherapeutic agent known to cause damage to various tissues. This study investigated the potential protective effects of parsley extract against MTX-induced brain and intestinal tissue damage. Sprague-Dawley rats were divided into control, control + parsley, MTX, and MTX + parsley. MTX (20 mg/kg, i.p.) was administered to the MTX and MTX + parsley groups. The control + parsley, and MTX + parsley groups were administered 2 g/kg parsley extract by oral gavage for five consecutive days. After the fifth day, brain and small intestinal tissues were taken. Total protein, nitric oxide, lipid peroxidation, glutathione levels, tissue factor, superoxide dismutase, and glutathione S-transferase activities were determined in these tissues. The protein profiles of the tissues were evaluated using SDS polyacrylamide gel electrophoresis. Parsley administration caused a decrease in lipid peroxidation levels in both tissues of the MTX group. On the other hand, glutathione level, glutathione-S-transferase, and superoxide dismutase activities were found to be increased. On the other hand, parsley decreased the nitric oxide level which was increased in the intestinal tissues of the MTX group. There was no significant change in brain nitric oxide level and tissue factor activity between groups. MTX and parsley administration altered protein expression, leading to the appearance or disappearance of specific bands in intestinal and brain tissues. In conclusion, parsley alleviated MTX-induced damage in brain and intestinal tissues by reducing lipid peroxidation and modulating antioxidant defenses.

Introduction

Methotrexate is a cytotoxic agent widely used in the treatment of cancer and autoimmune diseases such as rheumatoid arthritis and psoriasis. The mechanism of action of methotrexate (MTX) is based on the inhibition of dihydrofolate reductase and other related enzymes involved in purine and thymidine synthesis and ultimately inhibition of DNA, RNA, and protein synthesis. The cessation of these biosynthesis prevents biochemical processes such as vital ATP molecule synthesis and cell division (2, 3). However, as with other chemotherapeutic agents, its side effects affect the quality of life of patients and cause limitations in the use of the drug.

Studies have shown that MTX treatment can cause cognitive impairments such as decreased memory and learning by causing neuroinflammation and oxidative stress in rats (36, 40, 46). MTX-induced intestinal irritation sends signals to the brain via the gut-brain axis, activating microglia and increasing cytokines, which eventually leads to neuroinflammation (19, 37).

It has been reported that the cause of MTX-induced damage in the brain and intestine, as in many tissues, is due to oxidative stress. Although the mechanism of neurotoxicity is not clear, it is stated that the apoptosis pathway stimulated by the disruption of folate metabolism caused by MTX, disorders in myelin synthesis, and oxidative stress may be involved (30). It has been shown that reactive oxygen species have some roles in the formation of this damage. MTX causes a decrease in nicotinamide adenine dinucleotide phosphate (NADPH) within the cell. Since NADPH is a cofactor of glutathione reductase, which is involved in glutathione (GSH) homeostasis, this causes a decrease in GSH levels (18, 21). In addition, the reactive species formed disrupt the structures of molecules such as proteins, lipids, and nucleic acids, causing DNA damage and lipid peroxidation. Since the cells cannot adequately defend against this oxidative damage, MTX-induced tissue damage occurs (21). Similarly, studies have shown that MTX-induced oxidative stress also plays a role in intestinal toxicity (29, 32, 35). One of the critical pathways implicated in MTX-induced toxicity is the activation of tissue factor (TF), a transmembrane glycoprotein and a key initiator of the extrinsic coagulation cascade. Although TF is primarily known for its role in thrombosis, emerging evidence suggests that it also plays a significant role in inflammation, cell injury, and tissue damage. Elevated TF expression has been associated with the pathophysiology of several diseases, including cancer, cardiovascular disorders, and organ-specific damage (8). Studies indicate that TF activation leads to inflammatory responses, endothelial dysfunction, and increased vascular permeability, contributing to tissue injury (9).

Parsley (*Petroselinum crispum*), a green plant that is a member of the Umbelliferae family, has antioxidant, antidiabetic, anti-inflammatory, and antiapoptotic properties (11). Phytochemical analyses have shown that it contains many molecules with biological properties, such as flavonoids, coumarins, carotenoids, apiol, myristicin, phthalides, sesquiterpenes, monoterpenes and ascorbic acid (44). Among these molecules, flavonoids, carotenoids, tocopherols, essential oils, and ascorbic acid, preventing free radical formation, exhibit antioxidant properties, while the essential oils in their content play a role in suppressing autoimmune and chronic inflammatory disorders and allergies (24). Just as there is a close relationship between changes in the intestinal microbiota and various neurological disorders, there is also a close relationship between changes in the intestinal microbiota and chemobrain (22, 47). MTX administration in rats alters the link between the small intestine and the brain, principally by inducing intestinal inflammation, disrupting gut microbiota, and resulting in neuroinflammation and cognitive impairment (5, 37). Understanding these mechanisms indicates the role of the gut-brain axis in mediating MTX side effects and proposes possible treatment methods to maintain gut and brain health. Therefore, this study aims to evaluate the effect of aqueous parsley extract on the rat intestinal and brain oxidant-antioxidant balance disrupted by MTX administration. The selected parameters, including the specific biochemical markers assessed—such as total protein, nitric oxide, lipid peroxidation, glutathione levels, and the activities of tissue factor, superoxide dismutase, and glutathione S-transferase—were used to evaluate the extent of tissue injury and the protective effects of parsley. By analyzing these parameters, the study provides valuable insights into the mechanisms through which parsley mitigates MTX-induced oxidative stress and tissue injury.

Materials and Methods

Preparation of Aqueous Parsley Extract: The aqueous parsley extract was prepared at the Istanbul University Cerrahpaşa Faculty of Engineering, Department of Biochemistry.

The leaves of the parsley plant purchased from a local greengrocer were thoroughly washed and dried at room temperature for 3 days and stored in cellophane bags. Dried parsley leaves (100 g) were extracted with 1000 ml of distilled water and boiled for 30 minutes. The extract was filtered, and the solvent in the obtained extract was evaporated to dryness under reduced pressure in a rotary evaporator (12). The obtained powdered parsley extract was stored at -20 °C.

Experimental Animal Model: This experimental study was conducted with the permission of the Marmara University Animal Experiments Local Ethics Committee dated 11.01.2022 and protocol numbered 02.2022mar A number of thirty-two female and male Sprague Dawley rats of three months old weighing 200-300 g were used in the study. The rats were kept in conventional cages with a condition of a 12-hour light-dark cycle and a room temperature of 22 ± 2 °C. The rats were fed standard pellet feed and drinking water ad libitum throughout the experimental period.

Rats were divided into control, control+parsley, methotrexate (MTX), and MTX+parsley groups. Each group had 8 rats. In the control group, to imitate methotrexate administration, saline was given to rats intraperitoneally. To imitate the oral gavage method, 0.5 ml of drinking water was given by oral gavage for 5 days. In the control+parsley group, rats were given 2 g/kg parsley extract dissolved in 0.5 ml of water by the oral gavage method for 5 days after a single dose of intraperitoneal saline administration. MTX was dissolved in physiological saline and administered to the animals as

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a single dose of 20 mg/kg (intraperitoneally) in the MTX group (4). 0.5 ml of drinking water was also given by oral gavage for 5 days. In the MTX+parsley group, a single dose of 20 mg/kg MTX was administered intraperitoneally, and 2 g/kg parsley extract was also administered to the rats by oral gavage for 5 days.

Rats in all groups were decapitated, and their brain and small intestine tissues were taken at the end of the fifth day. 10% tissue homogenates were prepared using physiologic saline solution for biochemical analysis.

Determination of Lipid Peroxidation: Malondialdehyde (MDA) is one of the end products formed through the decomposition of lipid peroxidation products. MDA level was measured by using the method of Ledwozvy et al. (23). In this method, the sample undergoes a reaction with TBA under acidic conditions, resulting in the formation of an MDA-TBA complex. The intensity of the pink color formed is proportional to the MDA concentration and can be quantified by measuring absorbance at around 532 nm. Brain and small intestine MDA levels were expressed as nmol MDA/g tissue.

Determination of Glutathione Level, Superoxide Dismutase, and Glutathione-S-Transferase Activity: Brain and small intestine glutathione (GSH) levels were determined by the modified Beutler's method (6). In this method, after the addition of a precipitation solution to the homogenate, the sample is centrifuged. The supernatant was treated with Na_2HPO_4 and Ellman's reagent. Absorbance was measured at 412 nm. GSH levels were expressed as mg GSH/g tissue.

Brain and small intestine superoxide dismutase (SOD) activities were determined by the method of Mylroie et al. (28). The method involves measuring the absorbance of potassium phosphate buffer, riboflavin, o-dianisidine, and tissue extract. The mixture was illuminated with fluorescent light and absorbance was measured at 460 nm. SOD activities were expressed as U/g tissue.

Determination of GST activity is based on the principle of measuring the absorbance at 340 nm of the product formed by the conjugation of glutathione (GSH) and 1-chloro-2,4-dinitro-benzene (CDNB) by spectrophotometric method (16).

Determination of Total Protein, Nitric Oxide Levels, and Tissue Factor Activity: Brain and small intestine total protein levels were determined by the method of Lowry et al. (45). This method involves the reaction of protein with a copper ion in an alkaline solution, followed by the addition of a Folin-Ciocalteu reagent. The resulting color change is proportional to the protein concentration. The total protein levels were expressed as mg protein/g tissue.

The method of Miranda et al (27). was used for the detection of brain and small intestine NO levels. The method involves measuring the conversion of NO to its stable metabolite, nitrite, which can be quantified. The process typically includes the addition of reagents that react with nitrite, producing a color change, and the absorbance is measured at 540 nm. NO levels were expressed as µmol NO/g tissue.

Brain Tissue factor (TF) activity was determined by using the modified Quick's one-stage method (20). This method involves measuring the time it takes for blood clotting to occur in the presence of a tissue extract. This method assesses the ability of TF to initiate the coagulation cascade by adding a plasma sample to the tissue extract and measuring the clotting time. The clotting time is inversely related to TF activity, with shorter clotting times indicating higher TF activity.

Brain and Small Intestine Protein Electrophoresis: An electrophoretic examination of brain and small intestine proteins was carried out by using Laemmli SDS–polyacrylamide gel electrophoresis (SDS-PAGE) (17). SDS–PAGE was performed by using the BIO-RAD Mini Protean Precast II Dual Slab Gel Apparatus (BIO-RAD, USA). Mini PAGE gels (any kD precast polyacrylamide gel, 8.6 × 6.7 cm [W × L], Catalog Number: 4569033, BIO-RAD, USA) were used for protein electrophoresis. High-resolution photographs of the gels were taken using a Canon EOS 700D camera with an 18–55 lens to evaluate protein bands after electrophoresis, and the images were exported as JPEG files. Densitometric graphs of protein bands were plotted using ImageJ software (33).

Statistical Analysis: GraphPad Prism 8.0 (GraphPad Software, San Diego, CA, USA) was used for the statistical analysis of the data. The normality of the data was checked using the Shapiro-Wilk test before applying parametric tests. All results were presented as mean and standard error of mean (SEM). One-way analysis of variance (ANOVA) (post-hoc Tukey test) was used for comparison between groups. $P<0.05$ was considered statistically significant. Statistical power analysis was performed on the small intestine and brain NO levels using Faul et al.'s (13) method.

Results

Small Intestine Results: Significant decreases were found in the total protein and glutathione levels, glutathione-S-transferase, and superoxide dismutase activities of the methotrexate group compared to the C and C+Parsley groups. Parsley administration to the MTX group significantly increased these intestinal parameters (Figure 1). Malondialdehyde and nitric oxide levels significantly increased in the MTX group compared to the C and

C+Parsley groups. Parsley administration to the MTX group significantly decreased malondialdehyde and nitric oxide levels. Parsley administration to the control group also significantly decreased malondialdehyde and nitric oxide levels and significantly increased glutathione levels, glutathione-S-transferase, and superoxide dismutase activities and did not change total protein levels. The power analysis for the small intestine NO levels was conducted with a specific effect size, sample size (n = 32 per group), and a significance level of α = 0.05. The resulting power value of 0.95 indicates that there is a 95% probability of correctly rejecting the null hypothesis, assuming a true effect exists. This high power ensures that the study was well-equipped to detect significant differences in NO levels.

Brain Results: Significant decreases were found in the brain glutathione level, glutathione-S-transferase, and superoxide dismutase activities of the methotrexate group compared to the C and C+Parsley groups. Parsley administration to the MTX group significantly increased these parameters in brain tissue (Figure 2). Brain malondialdehyde levels significantly increased in the MTX group compared to the C and C+Parsley groups. Parsley administration to the MTX group did not change the MDA level. No significant differences were detected between all the groups in nitric oxide levels and tissue factor activities of the brain tissue. Parsley administration to the control group significantly increased glutathione level and superoxide dismutase activity and did not change the other parameters. The power analysis for the brain NO levels was conducted with a specific effect size, sample size (n = 32 per group), and a significance level of α = 0.05. The resulting power value of 0.95 indicates that there is a 95% probability of correctly rejecting the null hypothesis, assuming a true effect exists.

Electrophoretic Evaluation of Small Intestine and Brain: According to the electrophoresis results of intestinal tissue, protein bands with molecular weights of approximately 80, 90, and 100 kDa were not found in the C and C+Parsley groups but were seen in the MTX and MTX+Parsley groups. It was determined that the 66 kDa protein decreased with the administration of MTX and that the parsley administration to the MTX group caused this band to decrease even more. The 36 and 45 kDa proteins were present in the C, C+Parsley, and MTX groups but disappeared in the MTX+Parsley group (Figure 3).

According to the electrophoretic evaluation of brain tissue, proteins weighing 24, 36, and 45 kDa disappeared in the parsley-administered MTX group, while they remained unchanged in the other groups (Figure 4).

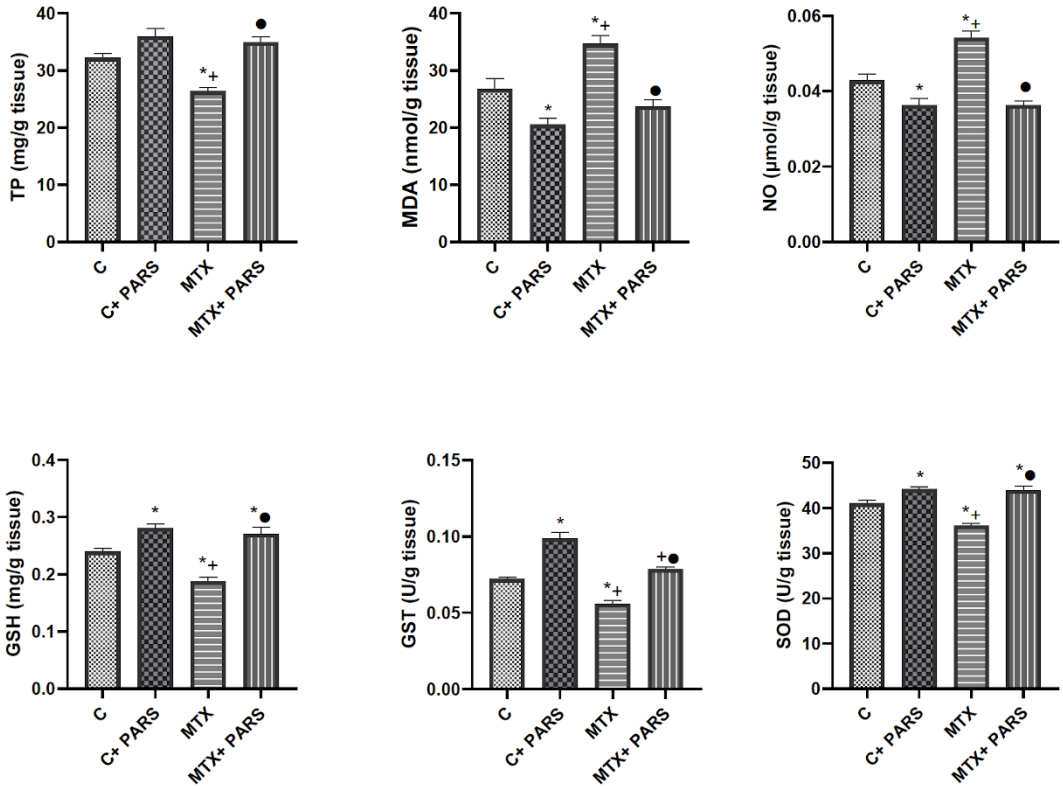

Figure 1. Biochemical results of small intestine tissue

C: Control, **C+Parsley**: Control+parsley, **MTX**: Methotrexate, **MTX+Parsley**: Methotrexate+ parsley group, results were presented as Mean ± Standard Deviation, n=8 in each group. (*) P<0.05 is significant according to the C group, (+) P<0.05 is significant according to C+ Parsley, (●) P<0.05 is significant according to the MTX group.

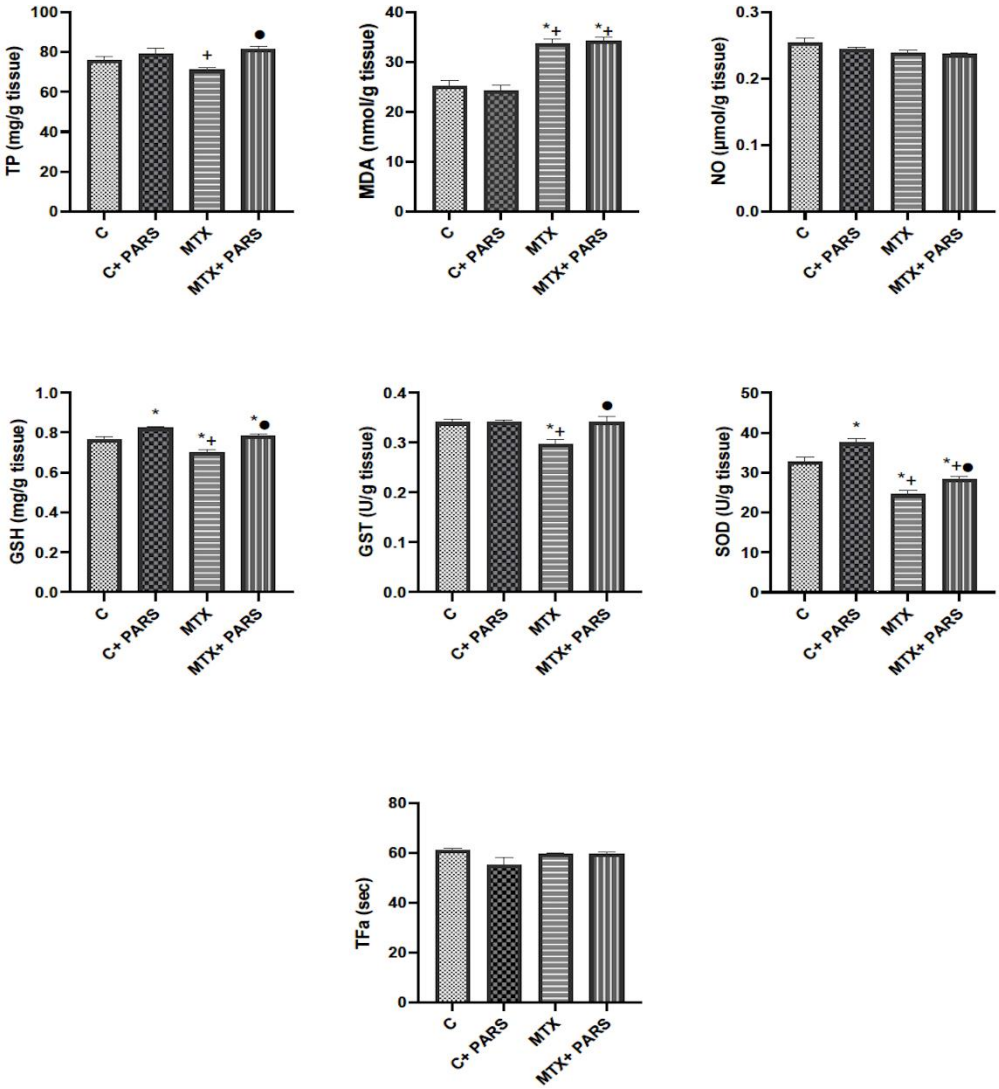

Figure 2. Biochemical results of brain tissue.

C: Control, **C+Parsley**: Control+parsley, **MTX**: Methotrexate, **MTX+Parsley**: Methotrexate+ parsley group, results were presented as Mean ± Standard Deviation, n=8 in each group. (*) P<0.05 is significant according to the C group, (+) P<0.05 is significant according to C+ Parsley, (●) P<0.05 is significant according to the MTX group.

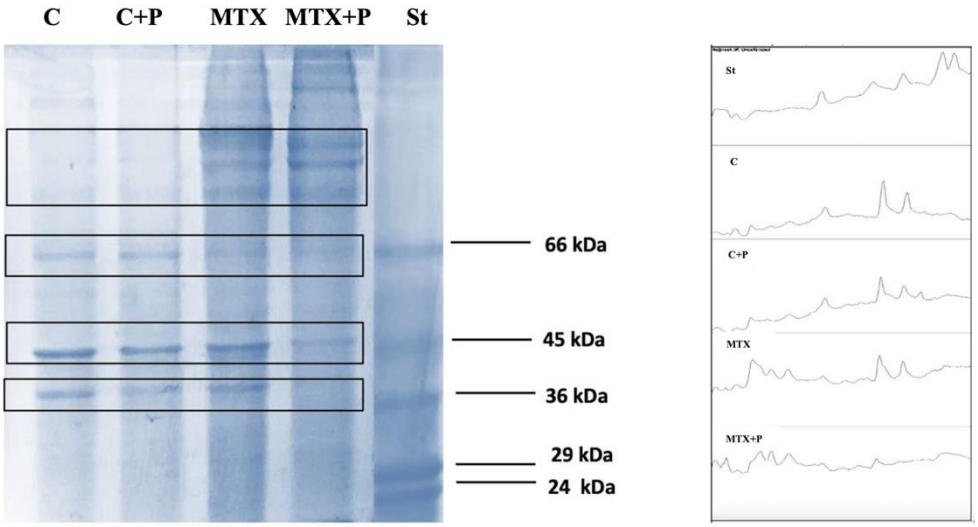

Figure 3. Electrophoretic evaluation of small intestine tissue proteins**.**

C: Control, **C+P**: Control+Parsley, **MTX**: Methotrexate, **MTX+P**: Methotrexate+Parsley group, **St:** Standard protein marker.

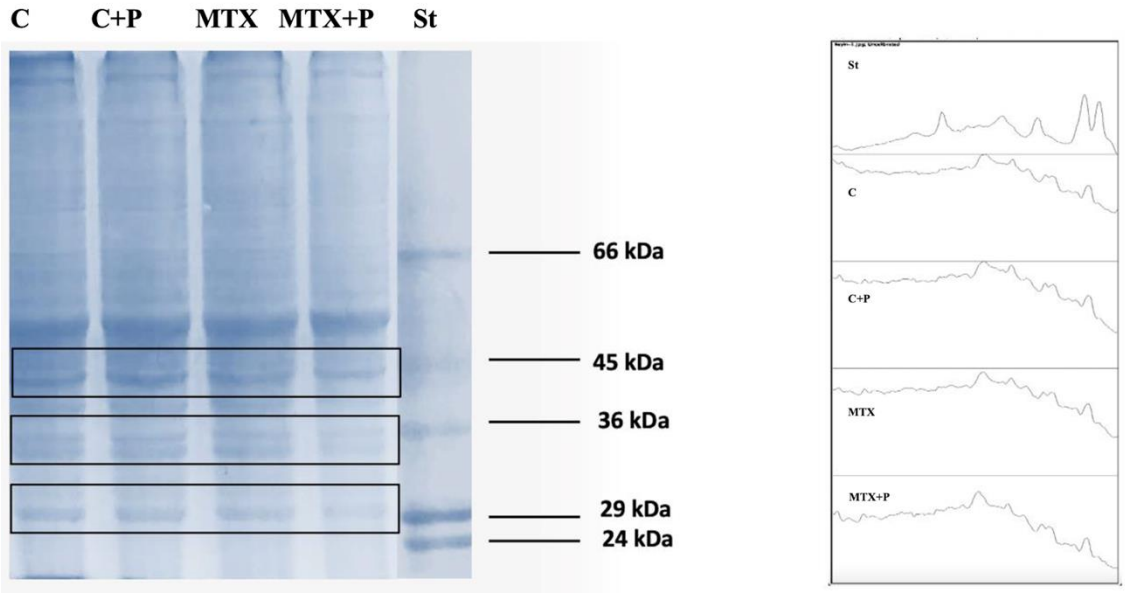

Figure 4. Electrophoretic evaluation of brain tissue proteins.
C: Control, **C+P**: Control+Parsley, **MTX**: Methotrexate, **MTX+P**: Methotrexate+Parsley group, **St:** Standard protein marker.

Discussion and Conclusion

This study provides new insights into the biochemical and physiological effects of methotrexate (MTX) administration on brain and small intestine tissues. A significant increase was observed in MDA levels, accompanied by a decrease in glutathione levels, superoxide dismutase activity, and glutathione-S-transferase activity, indicating oxidative stress and impaired antioxidant defense mechanisms. Yılmaz et al. (48) have reported that a single dose of intraperitoneal MTX (20 mg/kg) administration induced brain damage by increasing MDA and decreasing antioxidant parameters (GSH, GST, and SOD). They also found that MTX administration caused an increase in the NO levels of intestinal tissue but did not cause any significant change in the brain. Rtibi et al. (32) have found an increase in MDA levels and a decrease in antioxidant parameters (SOD, CAT, and GPx) of intestinal tissue of rats administered orally 100 mg/kg MTX. Similarly, El-Baghdadi et al. (10)observed increased NO and MDA levels and decreased GSH levels in the small intestine tissue. It was also revealed that MTX administration can increase the mRNA expression and synthesis of NOS enzymes (34).

In this study, in line with the literature, MTX administration increased intestinal and brain MDA levels and decreased GSH levels and SOD and GST activities. In addition, MTX administration increased NO levels in the small intestine but did not change NO levels in the brain. Tissue factor activity is another parameter examined in this study. Tissue factor is a coagulation protein involved in the extrinsic pathway of the coagulation mechanism (14). In this study, TF activity could not be detected in the intestinal tissue. In brain tissue, MTX administration did not change TF activity in all groups.

Many studies have examined the effects of various antioxidant supplements and substances, such as whey proteins, naringenin, bromelain, L-carnitine, melatonin, apricots, and beta-carotene, on MTX-induced oxidative stress-induced damage (15, 25, 38, 39, 41, 42). In this study, the effects of parsley, which has antioxidant properties thanks to molecules such as apiol, myristicin, apiin, luteolin, and beta-carotene in its content, against intestinal and brain damage caused by experimental MTX were investigated.

Parsley has antidiabetic, antibacterial, antioxidant, anticoagulant, and immune system-strengthening properties due to many phytochemicals in its structure (24, 31). Ertaş et al. (11) stated that parsley prevents lipid peroxidation in liver damage caused by MTX by increasing GSH levels. In the study conducted by Maodaa et al. (26), it was determined that parsley reduces lipid peroxidation and increases GSH levels and glutathione peroxidase enzyme activity in oxidative damage caused by cadmium in the brain. In another study conducted on the brain, it was stated that parsley extract reduced MDA levels and increased the activities of antioxidant enzymes SOD and glutathione peroxidase in brain damage caused by D-galactose (43). It has also been found that parsley has a gastroprotective effect in a gastric ulcer model created in intestinal tissue by pyloric ligation (1). In this study, when parsley extract was given to rats treated with MTX, a decrease in MDA levels was detected in the small intestine and brain tissues. In addition, parsley extract

administration to the MTX group caused a significant decrease in intestinal NO levels but did not change brain NO levels and TF activity.

Boukhettala et al (7) . stated that MTX treatment altered the intestinal mucosa and protein metabolism by decreasing protein synthesis and increasing proteolysis mediated by the lysosomal pathway. Methotrexate can cause villous atrophy. Therefore, morphological changes such as decreased villus length and increased crypt depth can be observed. In this study, when the protein profile of intestinal and brain tissues was examined with SDS-PAGE, a decrease in some protein bands and an increase in some protein bands were detected in both tissues in the MTX and parsley-treated MTX groups. In the small intestine tissue, protein bands with molecular weights of approximately 80, 90, and 100 kDa were not found in the C and C+Parsley groups but were seen in the MTX and MTX+Parsley groups. It was also determined that the 60-65 kDa protein band decreased with the administration of MTX and that the parsley administration to the MTX group caused this band to decrease even more. Two protein bands (36 kDa and 45 kDa) of the C, C+Parsley, and MTX groups also disappeared in the MTX+Parsley group (Figure 1). The exact causes and consequences of changes in proteins can be examined in detail and determine the context of changes in their expressions. According to the electrophoretic evaluation of brain tissue, proteins weighing 24, 36, and 45 kDa disappeared in the parsley-administered MTX group, while they remained unchanged in the other groups (Figure 2).

In conclusion, it was determined that parsley has a protective effect against MTX-induced oxidative damage in small intestine and brain tissues. It has the potential to improve gut-brain axis communication altered by MTX administration. Further studies are needed to investigate whether the effects of parsley are dose-dependent, as this could provide valuable insights into its therapeutic potential and provide more clarity on how it interacts with methotrexate.

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Ethical Statement

This study was carried out after the animal experiment was approved by Marmara University, Animal Experiments Local Ethics Committee (Decision Number: 02.2022mar)

Conflict of Interest

The authors declared that there is no conflict of interest.

Author Contributions

ED was responsible for organizing and conducting the biochemical experiments. SYK, GGS, and ET performed biochemical analyses. ÖS, RY, and GŞ conceived and designed the experiments. TTA supervised the study, contributed to manuscript writing, and interpreted the results. All authors provided critical feedback and contributed to shaping the research, analysis, and manuscript.

Data Availability Statement

The data supporting this study's findings are available from the corresponding author upon reasonable request.

Animal Welfare

The authors confirm that they have adhered to ARRIVE Guidelines to protect animals used for scientific purposes.

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Principal component and discriminant function analysis of cranium and mandible in domestic buffalo (*Bos bubalis*)

Semine DALGA[1,a,✉], Kadir ASLAN[1,b]

[1]Kafkas University, Faculty of Veterinary Medicine, Department of Anatomy, Kars, Türkiye

[a]ORCID:0000-0001-7227-2513; [b]ORCID:0000-0002-7617-0175

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✉Corresponding author
seminedalga@kafkas.edu.tr

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ABSTRACT

The purpose of this study was to use a geometric morphometric approach to ascertain the gender-related differences in the morphology of the domestic buffalo's skull and mandible. The skulls yielded a total of 20 main components. The first principal component (PC1) alone was responsible for 37.066% of the variation among these principal components. The first principal component (PC1) alone was responsible for 26.242% of the total variation among the lateral principal components. PC1 showed a medial extension of the posterior portion of the ectorbital, while PC2 showed a lateral extension. In PC2 and PC3, the right facial tuber displayed a cranial and linear expansion, respectively. In PC1, the left facial tuber was directed caudally, and in PC2, it was directed cranially. The anterior border of the first premolar had a caudo-ventral extension in PC1 and a cranio-dorsal extension in PC2 and PC3, according to lateral studies. In PC1 and PC2, the anterior side of the ectorbital displayed a caudo-dorsal extension, but in PC3, it displayed a dorsal extension. The anterior margin of the first premolar displayed a caudal extension in the extension evaluation of the three principal component analyses with the highest values in the mandibles, where the data are completely integrated with one another. PC1 showed a caudo-dorsal extension, PC2 showed a cranio-dorsal extension, and PC3 showed a dorsal extension of the landmark at the level of the incisura vasorum facialum. It is anticipated that this research will add to the body of knowledge about a particular breed, zoo archaeology, or be used as an animal model in relevant health professions.

Introduction

The buffalo, which has an important economic activity in the world as a dairy, meat, and draught animal, is raised in Southeast Asia, South America, North Africa, all Mediterranean countries except France, Balkan countries, some Central European countries, and Australia (39). The European bison (*Bison bonasus*), the largest mammal in Europe, is still a protected species, although its number has increased with breeding programs that put its species under protection after World War I (18). Water buffalo is a species that can cause confusion, especially in North America and Asia, about which animal is being referred to by the English term water buffalo. The word water buffalo in Turkish is thought to come from Manda, a geographical region in India. The domestication of the buffalo, whose domestication process dates back 5000 years (39). Buffalo breeding in the world has not lost its importance from past to present, and has even increased in both numbers and production over times (2), is now widely cultivated in 38 countries around the world (30). Buffaloes are a family of double-hoofed ruminant cattle. The first buffalo belongs to the Bubalus family. There are two types of Bubalus groups, and they are classified as Asian buffaloes (bubalina) and African buffaloes (synserina) (3).

Knowing the morphology of the skull is crucial to comprehending the systematics and phylogeny of the skull species under study (18). One sex-determining characteristic of male and female bovids is their horns, horn protrusions, and length, which have a robust structure (20, 21, 27, 40). The brain, hearing and balancing organs, respiratory and olfactory routes, and other vital tissues are all protected by the skull's mostly comprised bones (8, 26).

Markland (29) stated that in relation to nutritional factors, the facial tuber (tuber faciale) on the maxilla bone is related to the molar tooth articulation and the articulation role of the incisors in this region, as well as the changes in these regions and nutrition (29). The differences observed laterally in the points representing the rostral edge of the incisive bone and nasomaxillar fissura, which represent the mouth and nose regions, are thought to be related to diet and climate-altitude variations (28). Other points where the shape change is evident include the ventral margin of the jugular process, the midpoint of the margo supraorbitalis, and the external lacrimal fossa (5).

The majority of earlier research on large ruminants was based on traditional morphometric techniques, which included a variety of skull measurement instruments and reference data. Regarding species and sex relationships, these data are quite important (8). Skull morphology was described, and sex differences were attempted to be explained by researchers who performed a linear analysis and comparison of the skulls of domestic cattle and water buffalo (32). The study on the Indian Mithun's skull is the largest ruminant study currently accessible (7). In a similar vein, Ko-brýnczuk (27) used linear measures to analyze European bison and identify sex differences in skulls. Apart from cattle, numerous other animal species have been the subject of sex analysis research using the linear approach (16, 19, 22, 23).

The method of geometric morphometry, which easily reveals the shape differences between the skull and mandible, has become an important part of anthropology and archaeology studies in recently. It is possible both to evaluate discoveries and to re-evaluate old discoveries. Since the traditional morphometry method does not reveal all the information about the shape, this deficiency led to the birth of the geometric morphometry method. This new method reveals the whole geometric shape taken from the coordinates formed by anatomical points that are found in the same way in all samples. In this way, it reveals more information than the classical morphometry method (18, 36, 42). In recent years, there have been many studies on different species and different bones to determine the differences between the sexes of animals by the geometric morphometry method (1, 14). There have also been geometric morphometric studies on three-dimensional bone materials (5, 6, 15, 25, 33). There are also studies using the geometric morphometric method on materials obtained from archaeological excavations (35).

In this study, geometric morphometric approaches are used to assess the mandible and skull of farmed buffaloes based on the sex factor. We believe that the information gathered from the study will greatly advance real-world uses in buffalo management and breeding. In order to guide future research on buffaloes from microclimate regions and other parts of the world, as well as to support zooarchaeological studies, it is believed that the morphological characteristics of these unique animals, whose breeding dates back 5000 years, can be used for humanity in every aspect of life.

Materials and Methods

Sampling: Skulls obtained from local producers and slaughterhouses in Iğdır province were used in the study. 14 animals (7 female/7 male) of both sexes were used. All female and male subjects were 2 years old with an average body weight of 123-166 kg and 142-179 kg, respectively. Skulls weighed between 14 and 20 kg.

Preparation of Samples: They were separated from the skin and muscles. Then boiled and macerated. After bleaching and drying, mandibles were photographed from the left lateral direction and skulls from the dorsal and lateral directions. The recorded photographs were transferred to the computer in JPEG format and then converted to a Tps file with the TpsUtil (Version 1.79) program. Homologous landmarks were marked on the photographs with the TpsDig2 (Version 2.31) program (37). The Cartesian coordinates of the landmarks were determined in this way. A homologous landmark verification test was performed with the TpsSmall (Version 1.34) program (34, 36). Since there may be differences in size, direction, and position in the photographs obtained in the study, superimposition was applied (38).

Analysis: Principal Component Analysis (PCA) was performed on the new coordinates obtained as a result of superimposition. Thus, covariance analysis between factors was used to determine the degree of separation of specimens by sex (43). In addition, the MorphoJ program was used to determine the landmark level and direction of the shape difference (24).

Results

14 buffalo skulls, 7 female and 7 male, were analyzed laterally and dorsally for this study. Ten pointing procedures were used to investigate the dorsal and lateral directions in the geometric morphometry method. Twenty major components in all were found. The first principal component (PC1) alone was responsible for 37.066% of the variation among these principal components. Of the overall variation, 28.65% was explicated by the second main component (PC2) alone, and 12.65% was explained by the third principal component (PC3) alone. The first principal component (PC1) alone account for 26.242% of the total variation among the lateral principal components. Of the overall variation, 24.143% can be explicated by the

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second principal component (PC2) alone, and 18.097% can be explained by the third main component (PC3) alone. The mandibles photographed from the left lateral direction yielded a total of 13 major components of variation. Of them, PC1 alone was responsible for 38.947% of the variation. The third principal component was responsible for 12.586%, whereas the second principal component (PC2) was in charge of 22.886%. More than half of the variation was explained by PC1, PC2, and PC3 in both materials, according to the principal component analysis. Significant percentages of variance were also found in each of the two materials after analysis. Table 1 contains the reference principal component analysis data for both materials. The plots show that PC1, PC2, and PC3 are accountable for over half of the variation. Significant percentages of variation are also present in each of the examined materials separately.

Figures 1, 2, and 3 display the shape variation for principal components 1, 2, and 3 derived from dorsal and lateral skulls and left lateral mandibles. Additionally, Figures 4, 5, and 6 display graphic representations of the variance distributions. The average form for each analysis is shown by dots. The extensions represent the positive bounds for PC1, PC2, and PC3. The posterior section of the occipital bone extends caudally in PC1 and PC2, particularly in PC2, as can be seen in the dorsal analysis when taking into account the significant deviations. In PC1 and PC2, the posterior region of the ectorbitale was found to exhibit a medial and lateral expansion, respectively. PC2 displays a cranial expansion of the right facial tuber, while PC3 has a linear extension. It was discovered that the left facial tuber had a cranial extension in PC2 and a caudal extension in PC1. It was observed that the septal process extended cranially in the direction of the skull's cranial aspect.

The anterior edge of the first premolar had a caudo-ventral expansion in PC1 and a cranio-dorsal extension in PC2 and PC3, according to the lateral studies. When seen from the lateral face, the root section of the cornual process (landmark number 5) had a ventral extension in PC3 and a linear cranial extension in PC1 and PC2. In PC1 and PC2, the anterior portion of the ectorbitale displayed a caudo-dorsal extension, but in PC3, it displayed a dorsal extension. In PC1 and PC2, the frontal tuber displayed a medially directed extension, whereas, in PC3, it displayed a caudo-ventrally directed extension. In PC1, the anterior border of the os incisivum seems to reach the skull's tip.

In the skulls of buffaloes obtained from the province with microclimate characteristics, stronger and larger anatomical formations are expected in male individuals in direct proportion to the purpose of use. Especially in females used for milk production, the horn size has strikingly attracted attention. It was observed that these formations remained weaker in male individuals where competition is common due to the environment and the labor force they use.

In both PC1 and PC3, the infradental gap appears to extend anteriorly from the cranial apex in the mandibles, where the data are completely integrated. According to the extension evaluation of the three principal component analyses with the highest values, the anterior border of the first premolar exhibits a caudal expansion. In PC1, the posterior border of the coronoid process extends ventrally, but in PC2 and PC3, it extends caudally. In every component analysis, the caudal gonion was revealed to have a cranio-dorsal extension. PC1 showed a caudo-dorsal extension, PC2 showed a cranio-dorsal extension, and PC3 showed a dorsal extension of the landmark at the level of Incisura vasorum facialum. The extension study of the projective landmark on the ventral margin of the mental foramen indicates that PC1 has a ventral extension, while PC2 and PC3 have a caudal extension. In PC2, the anterior border of the first premolar tooth also extends cranially.

Table 1. Component analysis obtained from the cranium and mandible

PC No	Eigenvalues (Dorsal)	Variance (%)	Eigenvalues (Lateral)	Variance (%)	Eigenvalues (Mandible)	Variance (%)
PC1	0.00112949	37.066	0.00034026	26.242	0.00138678	38.947
PC2	0.00087327	28.657	0.00031303	24.143	0.00081489	22.886
PC3	0.00038550	12.651	0.00023464	18.097	0.00044815	12.586
PC4	0.00033131	10.872	0.00015805	12.190	0.00040680	11.425
PC5	0.00011805	3.874	0.00008274	6.381	0.00022611	6.350
PC6	0.00009635	3.162	0.00005893	4.545	0.00010374	2.913
PC7	0.00007088	2.326	0.00004464	3.443	0.00008174	2.296
PC8	0.00002706	0.888	0.00003467	2.674	0.00003564	1.001
PC9	0.00000940	0.309	0.00002322	1.791	0.00002467	0.693
PC10	0.00000594	0.195	0.00000642	0.495	0.00001574	0.442

PC: Principal component

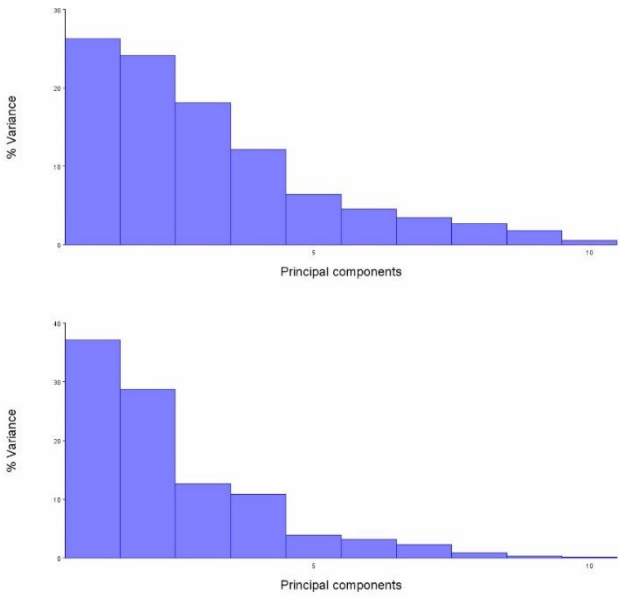


Figure 1. Variation distribution graph of principal component analysis (Dorsal (top), Lateral (bottom)).

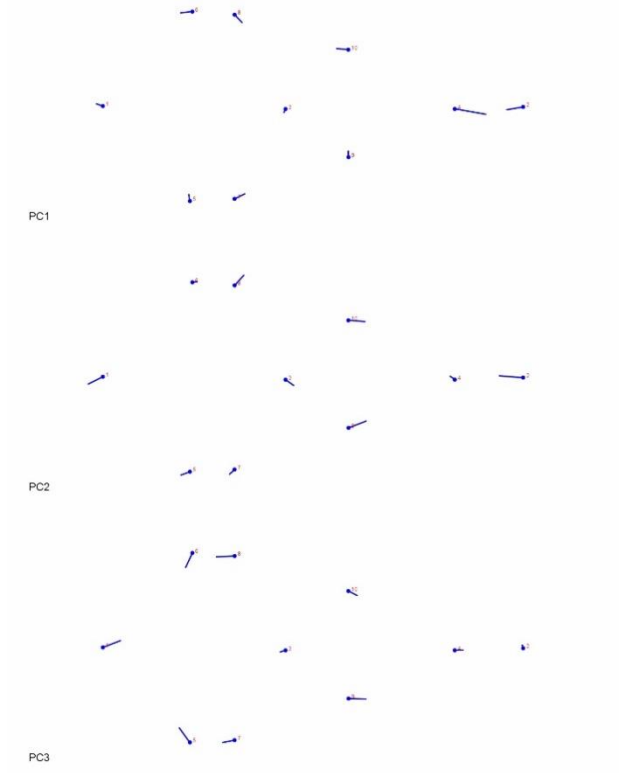


Figure 2. Shape variation of principal components 1, 2, and 3; 1: Posterior boundary of the occipital bone, 2: Tip of the incisive bone, 3: Fronto-nasal suture, 4: Tip of the nasal process, 5-6: Roots of the right and left cornual process, 7-8: Posterior edge of the ecto-orbital, 9-10: Right and left facial tuber (Dorsal Analysis). PC: Principal component

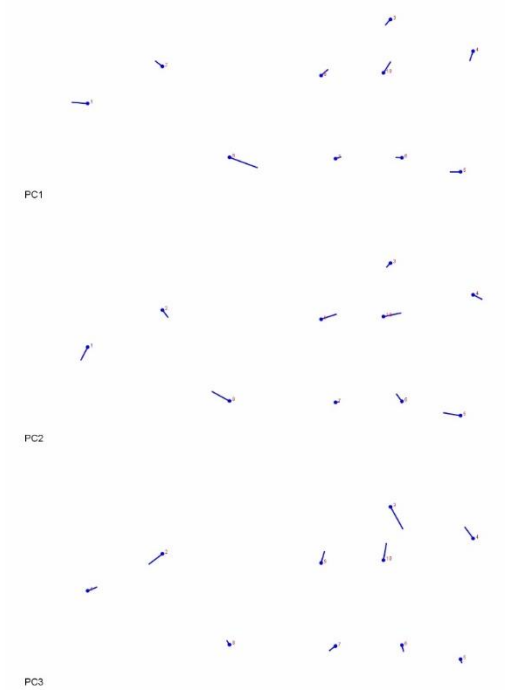


Figure 3. Shape variation of principal components 1, 2, and 3 in the lateral view; 1: Tip of the incisive bone, 2: Tip of the nasal process of the nasal boner 3: The frontal tuber of the frontal bone, 4-5: Roots of the condylar process, 6: Lateral edge of the muscular process, 7: Posterior edge of the last molar tooth, 8: Anterior edge of the first premolar tooth, 9: Anterior edge of the ectorbital, 10: Posterior edge of the ectorbital. PC: Principal component

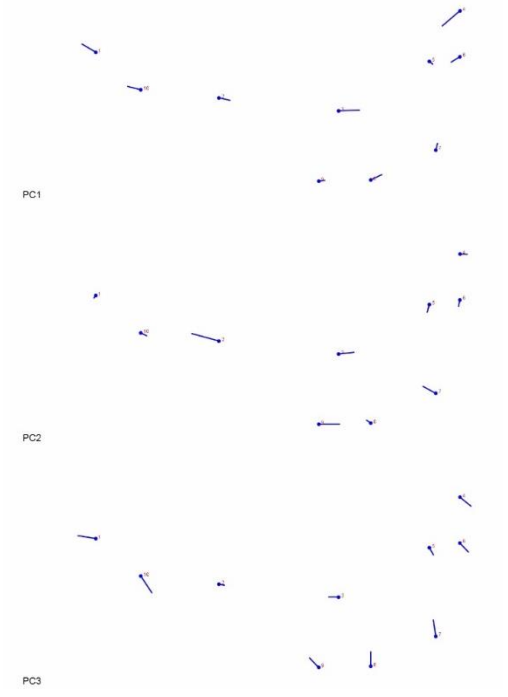


Figure 4. Shape variation of PC1, PC2, PC3; 1: Infradental space, 2: Anterior edge of the first premolar tooth, 3: Posterior edge of the last molar tooth, 4: Posterior edge of the coronoid process, 5: The ıncisura of mandible, 6: Posterior edge of the condylar process, 7: Caudal gonion, 8: Incisura vasorum facialum, 9: Ventral gonion, 10: Distance from the ventral edge of the mental foramen (Mandible). PC: Principal component

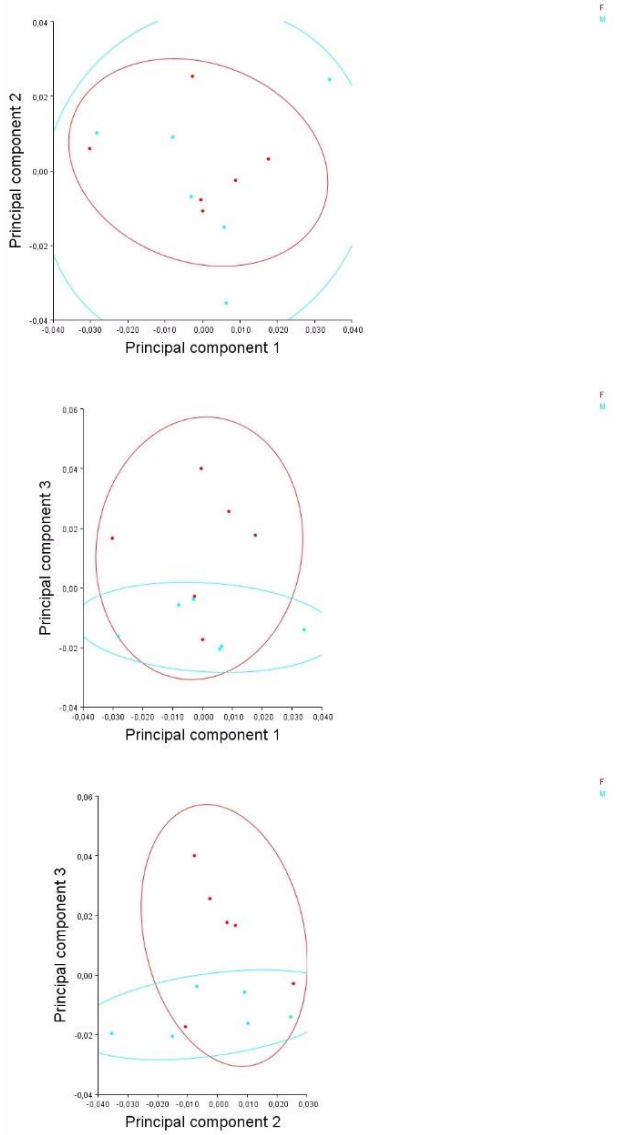


Figure 5. Variation distribution of principal components. Red points: Female, Green points: Male (Dorsal).

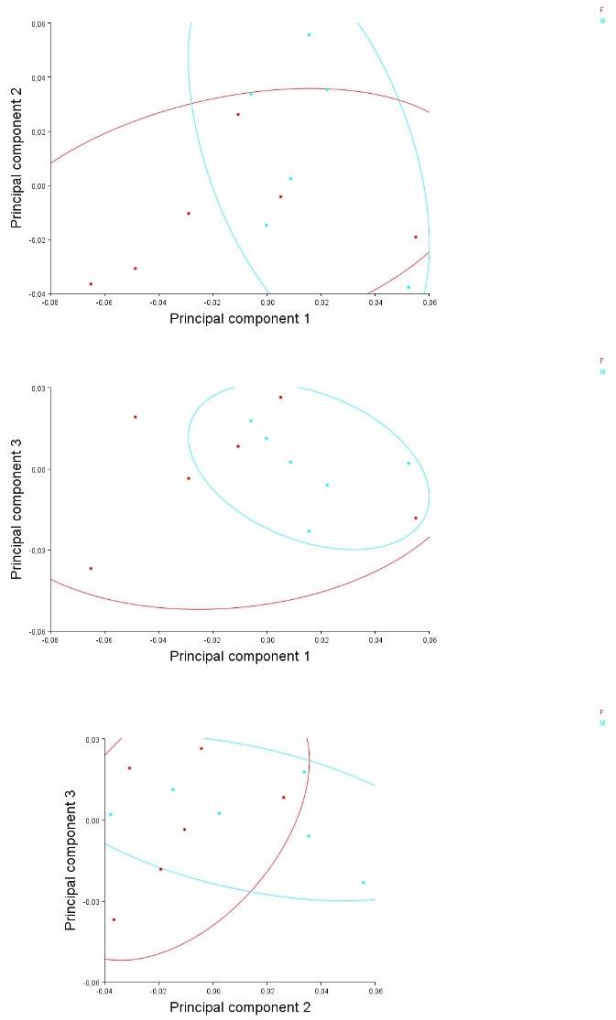


Figure 6. Variation distribution of principal components. Red points: Female, Green points: Male (Lateral).

Figures 7–11 display the primary components derived from the materials' principal component analysis. More than half of the variance was explained by the first three components (PC1, PC2, and PC3) for all materials. Despite having a large overall variance across all three tests, there was no statistically significant separation between the materials' shapes.

The study employed a discriminant function analysis (DFA) to evaluate sex differences objectively. The buffalo skull and mandible did not exhibit any statistically significant differences between the sexes, according to DFA. Nonetheless, the mandible and skull are both different shapes. Figure 12 shows the gender disparities in the discriminant function analysis graphically with shape modifications. The complete separation of both genders in terms of shape is also seen in the graph.

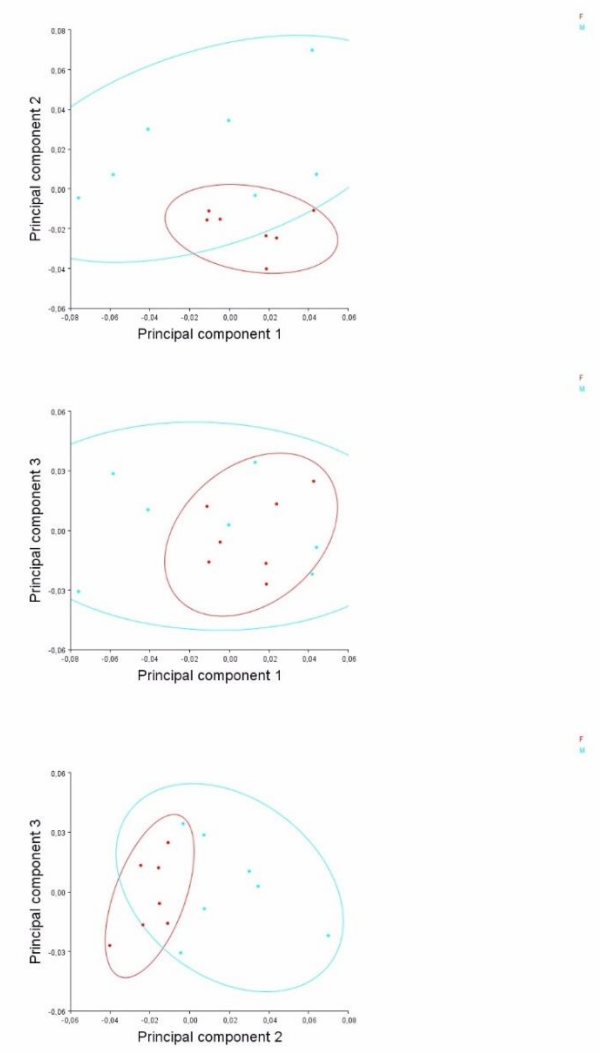
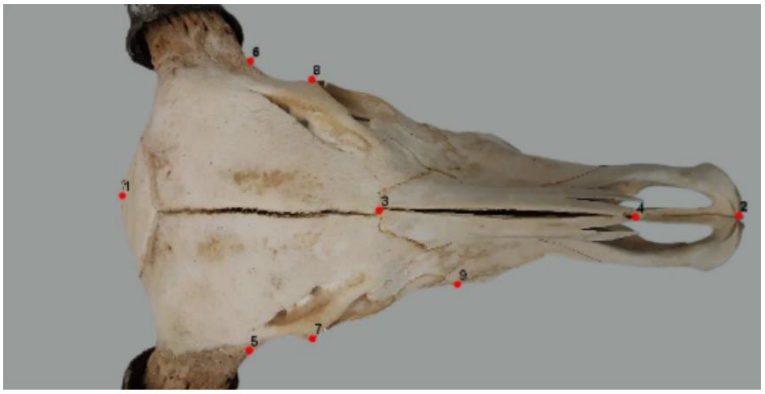


Figure 7. Variation distribution of principal components. Red points: Female, Green points: Male (Mandible).



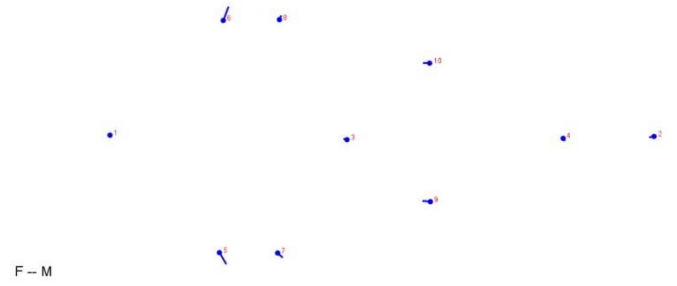


Figure 8. Principal component variation. 1: Posterior boundary of the occipital bone, 2: Apex of the incisive bone, 3: Fronto-nasal suture, 4: Apex of the nasal process, 5-6: Roots of the right and left cornual process, 7-8: Posterior edge of the ectorbitale, 9-10: Right and left sides of the facial tuber (Dorsal Analysis).

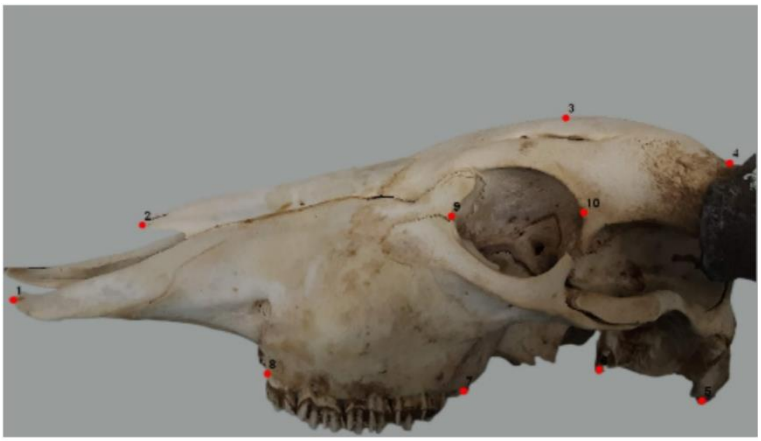


Figure 9. Principal component variation. 1: Apex of the incisive bone, 2: Apex of the nasal process of the nasal bone, 3: Frontal tuber of the frontal bone, 4-5: Root of the condylar process, 6: Lateral edge of the muscular process, 7: Posterior edge of the last molar tooth, 8: Anterior edge of the first premolar tooth, 9: Anterior edge of the ectorbitale, 10: Posterior edge of the ectorbitale.

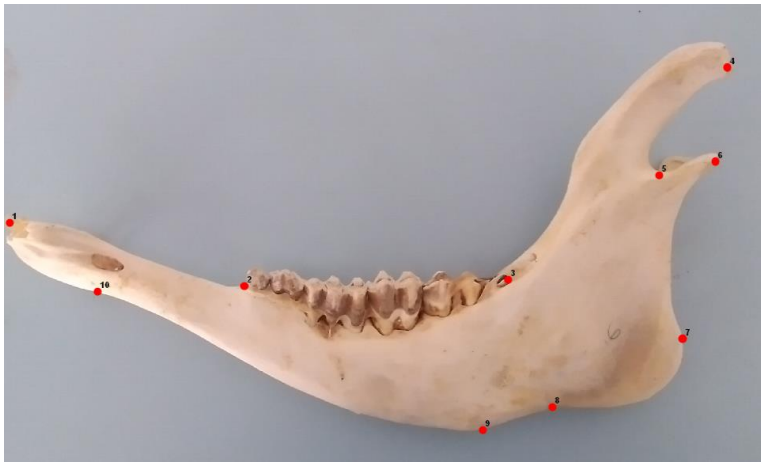


Figure 10. Principal component variation. 1: Infradental space, 2: Anterior edge of the first premolar tooth, 3: Posterior edge of the last molar tooth, 4: Posterior edge of the coronoid process, 5: Incisura mandibulae, 6: Posterior edge of the condylar process, 7: Caudal gonion, 8: Incisura vasorum facialum, 9: Ventral gonion, 10: Distance from the ventral edge of the mental foramen.

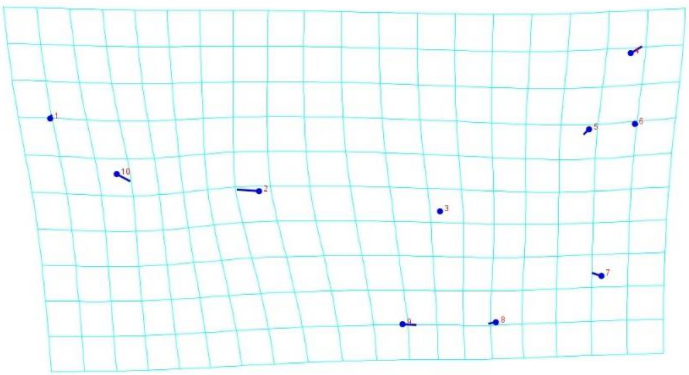


Figure 11. Discriminant fonction analyses of mandible; 1: Infradental space, 2: Anterior edge of the first premolar tooth, 3: Posterior edge of the last molar tooth, 4: Posterior edge of the coronoid process, 5: Incisura mandibulae, 6: Posterior edge of the condylar process, 7: Caudal gonion, 8: Incisura vasorum facilium, 9: Ventral gonion, 10: Distance from the ventral edge of the mental foramen. F: Female, M: Male

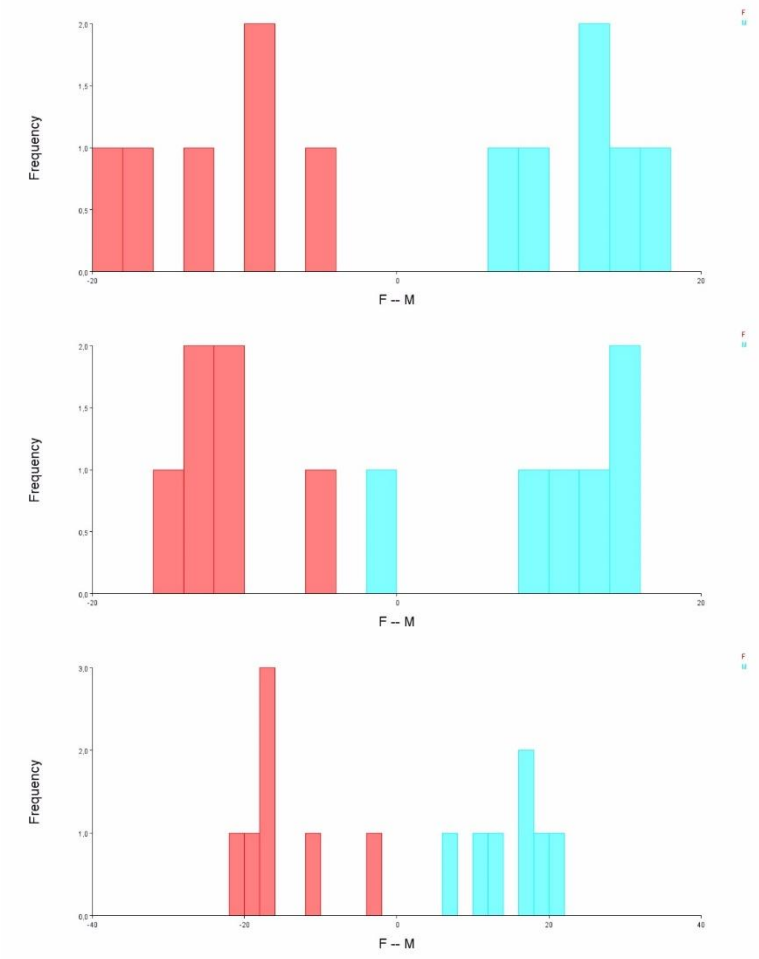


Figure 12. Sex distribution graph in Discriminant Function Analysis. Red: Female (F), Green: Male (M) (Top - Dorsal / Middle - Lateral / Bottom - Mandible).

Discussion and Conclusion

Male and female buffalo skulls from Eastern Anatolia were employed in this investigation. Dorsal and lateral markings were followed by a geometric morphometric examination of the materials. The geometric morphometric approach was used to conduct discriminant function analysis and principal component analysis. Among the variations derived from principal component analysis, the first three analyses were taken into account. The materials that did not exhibit a statistically complete separation varied in shape, even though the overall variance was considerable across all three tests. Similarly, sex differences were objectively evaluated using the discriminant function analysis (DFA). Gender differences in buffalo skulls and mandibles were not statistically significant ($P>0.05$), according to DFA. That being said, the mandibles and skulls differed in shape. There may not have been a statistically significant difference due to the small number of materials.

The skull and lower jaw bones of animals provide a great deal of morphological information for individuals and different races within the same family. Thanks to this morphological information, the effects of the environment in which living things are located on their morphological structures can be interpreted, and today's technologies allow the construction of human, animal-based surgical models to evaluate objects obtained from remains (25, 34, 42). Studies are conducted to comprehend how genetic variation, sexual selection, and environmental factors affect skull shape (16). According to previous reports, these morphological data can show relationships between living beings, especially when obtained using geometric methods (4, 10, 35, 43, 44). Although it has been widely used in recent years, classical morphological methods that have been used since ancient times are still used (31).

According to a study on male Holstein and Simmental cattle, PC1 accounted for 60.30% of the overall variation, whereas PC2 explained 12.67%. Additionally, it was highlighted that the Holstein breed's skull length was greater than that of the Simmental breed (8). According to a study by Gündemir and Szara (16) on the skulls of 57 European bison (*Bison bonasus*), males had larger heads and horns than females. Additionally, it was mentioned that the frontal, nuchal, and maxillary regions showed morphological modifications (18).

It is easy to find studies on small ruminant skulls and mandibles that fall under the category of geometric morphometry in the literature. Apart from ruminant skull studies, where dimorphism is most evident, some researchers have also studied the mandible and reported their findings (15). Numerous research studies have been conducted on the metapodium (41), mandible (12, 15, 22), and skull (10, 11, 13) in sheep. The precise anatomical distinctions of the species were assessed using a variety of methodologies in terms of species and sex, just like in our investigation, and analyses were conducted over the determined durations in each of these studies. The geometric morphometry method has been used by researchers studying sexual dimorphism in several animals, including turtles (24), in addition to ruminants, to uncover the structural differences among related species. It must be because the materials belonging to small ruminants are easily obtained that they are frequently used by researchers in terms of understanding and interpretation. For this reason, the findings we obtained regarding our study materials, buffaloes, have always been compared with the existing literature on small ruminants.

When the mandibles of Honamlı and Hair goats were subjected to geometric morphometric analysis, the researchers found that there was a pronounced gender difference between the two species. They claimed that male goats were considerably more grouped than females in terms of race. In our investigation, there was a difference in shape even though the lower jaw was not fully sexually differentiated (11). Studies on Awassi sheep revealed that when utilizing the geometric morphometry approach for analysis, the initial PCA accounted for 24.92% of the total form difference (12). Furthermore, it was claimed that there was no discernible gender difference in the mandibles. In a similar vein, other researchers discovered that 30.409% of the Morkaraman sheep mandible's overall shape variance could be described by the first main component (9). However, it was shown that the first main component alone accounted for 38.947% of the overall shape variance in the studies taken from buffalo mandibles.

Using principal component analysis, researchers who attempted to assess mammalian morphology from a paleoecological standpoint reported that PC1 accounted for 45.59% of the variation in mandible morphophysiology (41). They added that dietary practices have an impact on the mandible's morphology. The researchers who studied Anatolian wild sheep (42) reported that there is a noticeable difference in the subjaws at the level of LM9 parameters, and this difference is related to environmental factors, feeding practices, and domestication adaptations. It was noted that the LM9 value in Awassi sheep varied significantly (12). Additionally, it was mentioned that although there were variations in LM2, LM8, and LM10 levels, they were minimal. The ventral gonion, identified as a marker, was observed to increase caudally at PC2 as a consequence of the form study of buffalo mandibles. The first two fundamental analyses in 2D image inspections were found to account for 45.59% and 14.70% of the shape variance, respectively, in the study looking at the association between cattle's mandible and nutrition (41). There was no gender dimorphism in the principal component analysis of buffalo mandibles, as reported in Anatolian wild sheep (42), Awassi sheep (12), and Morkaraman sheep (9), when we compared the study to other studies in the literature. However, changes in shape were noted when we examined the data using discriminant function analysis. Dorsal sexual dimorphism was not entirely isolated in our study. Once more, 37.066% of the form differences were explained by PCA-1 alone. By comparing two unique cattle breeds, the researchers found that there was a noticeable difference between the breeds based on principal component 2's measurements of the occipital bone's height and the frontal bone's width (8).

In a geometric morphometric analysis of skull bones from various breeds, the researchers found that PCA-1 accounted for 42.268% of the form variance in males and 50.628% in females. When they examined the dorsal side of the skulls of animals of various breeds and sexes, they discovered that sexual dimorphism was breed-specific. According to the same researchers, who dorsally analyzed the skulls of Honamlı, Kilis, Saanen, and Kıl goats, there was a notable clustering in female Honamlı goats and a limited amount of dorsal separation between the skulls. They reported that among males, there was a clear grouping among those from the Saanen and Honamlı families (43). According to a study on Balkan sheep, the first two primary components can account for 61.18% of the overall shape variation in the dorsal direction. This demonstrates the form differences between Bardhoka and Ivesi sheep (17). In both dorsal and ventral directions, the researchers examined the skulls of female Akkaraman and Anatolian wild sheep (42). Dorsal analyses revealed that both principal components accounted for 70.03% of the overall shape difference. The PCA-1 value for both breeds was determined to be 65.93% based on the ventral analysis. These studies all provide evidence for both intraspecific and interspecific variances. According to the same researchers, in PCA graphs created from the dorsal and basal sides, the skull bones of Anatolian wild sheep were clustered to the right of the “y” coordinate, whereas those of Akkaraman sheep were clustered to the left. The dorsal and left lateral sides of the Awassi sheep's skulls were examined by researchers, who found that PCA analysis explained 37.719% and 44.238% of the overall form variance for each side, respectively (13).

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In principal component analysis, PC1 is typically given more attention than the other three analyses. It was shown that PC2 accounted for 12.67% of the overall variation in Simmental and Holstein breeds for PC2 study. The second main component analyses revealed that Simmental cattle had a positive mean for metrics like the largest breadth of the skull, the length of the occipital bone, and the width of the frontal bone, but Holstein cattle had a negative mean for these same parameters. This difference was believed to be a reflection of the morphological traits unique to the species (8). This figure was reported to be 14.53% for the lateral area and 27.84% for the dorsal area in Awassi sheep in another study that focused on PC2 analysis (12). Although our study did not include any racial differences, PC2 accounted for 28.65% of the overall gender difference.

It is believed that the morphological analyses and morphometric findings of the native bison skull and mandible, as well as the identification and determination of osteological materials obtained from archaeological excavations, the development of three-dimensional models, and the application of these morphological analyses on animal and human models will greatly advance the research to be conducted in this field. We believe that the findings obtained from this study will also be valuable in terms of biogeographic, phylogenetic, and system studies in terms of their primary widespread impact. Principal Component Analysis was also used to evaluate the shape changes between males and females and to analyze the principal component variation values between males and females based on race. The primary component of the study, discriminant function analyses, was also used to evaluate sex determination. We think that there is no statistical difference due to the number of skulls used in the study. However, it was clearly demonstrated that the sexes are completely separate in terms of shape.

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Ethical Statement

This study was approved by the Kafkas University Animal Experiments Local Ethics Committee (Approval no: 2022/027).

Conflict of Interest

The authors declare no conflicts of interest.

Author Contributions

SD designed the study. SD, and KA performed the morphometric analysis. SD carried out the statistical analysis. SD and KA performed the imaging all section. The manuscript was written by SD and KA. All authors approved the final version.

Data Availability Statement

The data supporting this study's findings are available from the corresponding author upon reasonable request.

Animal Welfare

The authors confirm that they have adhered to ARRIVE Guidelines to protect animals used for scientific purposes.

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Evaluation of urine samples of diabetic rats treated with metformin and different natural product combinations

Yeliz KAYA KARTAL [1,a,✉], Tevhide SEL[1,b]

[1] Ankara University, Faculty of Veterinary Medicine, Department of Biochemistry, Ankara, Türkiye

[a]ORCID: 0000-0002-3661-5504; [b]ORCID: 0000-0002-9753-779X

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✉Corresponding author
yelizkaya06@gmail.com
ylzkaya@ankara.edu.tr

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ABSTRACT

Diabetes is highly prevalent worldwide, and urine analyses with dipstick methods are important tools to monitor glucosuria and nephron status in diabetic animals. The aim of this study is to follow glucosuria, ketonuria, and proteinuria changes in hypoglycemic drug use in diabetes and drug and natural product combinations. Male wistar albino rats were used in the study and type 2 diabetes was induced with streptozotocin (65 mg/kg, i.p.) and nicotinamide (110 mg/kg, i.p.). The drug and natural products were administered orally for a period of 84 days (healthy control, diabetic, diabetic+metformin, diabetic+metformin+cherry laurel, diabetic+metformin+rutin and diabetic+metformin+alpha lipoic acid groups), and the urine samples were collected at the end of the experiment. The urinalysis (glucose, ketone, and protein) was done with a dipstick. The results were scored between 0 and 3, and Kruskal-Wallis analysis was applied. There was a significant difference between the untreated diabetic group (DM) and the remaining groups in glucose, but ketone and protein analysis did not show any statistically significant differences. The results showed that drug and drug+natural product combination reduced urinary glucose excretion in diabetes. In conclusion, the use of metformin and/or natural product combinations decreased the glucose output in urine. With an easy and cheap monitoring method such as a dipstick, the metabolic state can be revealed. And the effect of drug and natural product combinations can be monitored.

Introduction

Diabetes mellitus is the most common cause of chronic kidney diseases in the world, and urine analysis is an important laboratory finding in kidney diseases. Urine tests are crucial to get information about renal dysfunction and metabolic disorders. The vital roles of kidneys are filtration and the output of waste products by urine. That's why urine tests can give the metabolic state of the body (25). In normal patients, glucosuria is an undetectable result because kidneys reabsorb all the glucose in a routine metabolic phase (45). If hyperglycemia exceeds the renal threshold, glucosuria can be detected. The most common cause of glucosuria, as it is known, is diabetes. However, in some animals, like cats and birds, severe stress can also be the reason for glucosuria (33). Experimental studies with rats exhibited that severe pain could cause glucosuria in healthy rodents (21). In glucosuria, sometimes the problem could be due to congenital or acquired proximal tubular diseases. In these situations, it is named as normoglycemic glucosuria (41). Congenital diseases could be Fanconi's syndrome, primary renal glucosuria, and congenital renal dysfunction, and acquired diseases could be acute renal failure, toxicosis (heavy metals, nephrotoxic drugs), and chronic renal failure (10). It is stated that enrofloxacin and cephalexin use in dogs could cause false positive glucosuria (35).

Proteinuria has several reasons depending on the type of protein or amino acid. Mostly, a minute amount of protein can be seen in urine, which is named as trace and is a physiological condition. Since test strips often measure albumin, a significant increase in albumin suggests proteinuria when using dipsticks for chemical

analysis. The reasons for proteinuria can be classified as prerenal, glomerular, tubular, hemorrhagic, or inflammatory and protein-losing nephropathy, and renal failure (15). As previously stated, trace protein in urine is physiological and typically corresponds to +1 in dipstick evaluations. However, if the urine is alkaline or a concentrated urine sample, additional methods are required to define proteinuria because the dipstick result may be an artifact; otherwise, it is a pathological finding (37). In a study, nephropathy was induced with doxorubicin in rats, and the urine ketone, glucose, and protein output was analyzed. According to the study, there was no change in ketonuria, but glucosuria and proteinuria gave a statistical difference between the control and nephropathy-induced rats (8). In diabetes, a drop in glycemia gives rise to a decline in HbA1c levels, and every 0.9% decrease in HbA1c lowers the risk of diabetic nephropathy by about 30% (1, 13).

Ketonuria is an important diagnostic parameter for diabetes, but it is not the only reason. Ketone bodies are related to the rise of gluconeogenesis, but if lipid catabolism is increased, ketone bodies will arise in the blood too. Mostly, increased gluconeogenesis is seen in diabetes because there is an impaired ability to use carbohydrates. The other reasons for increased gluconeogenesis could be starvation and fasting (lack of carbohydrates) or loss of carbohydrates (mostly seen in renal or digestive problems). In pregnancy, ketonuria can occur as a physiological condition. The renal threshold to clear the ketone bodies from blood is low, so even a low increase in plasma ketone will result in ketonuria. Since dipsticks often employ the nitroprusside (Rothera) test method, test strips fail to detect ß-hydroxybutyric acid. This situation can sometimes lead to false negative results. For example, extreme dehydration is a reason for hypoxia, and this can increase ß-hydroxybutyric acid levels, which will be undetectable with the dipstick urinalysis method (6, 34, 43, 46).

In physiological conditions, the control mechanism of renal excretion is glomerular filtration, which is a passive transport; tubular resorption, which can be active or passive; and tubular secretion, which is active. In the glomerular filtration process, solutes, depending on their molecular size and electrical charge, can pass the glomerular filtration barrier. When the molecular size is less than 2.5 nm, all solutes can easily pass the barrier, while the size is between 2.5 and 3.4 nm, some of the solutes can pass the barrier, but when the size of the molecules is higher than 3.4 nm, none of the solutes can pass the barrier. It is also hard for the molecules to pass the barrier if they bear a negative charge. Albumin is not anticipated to be present in urine samples, particularly in felines, equines, or bovines, because albumin possesses a negative charge and has a molecular size of 3.5 nm.

Glucose can pass through the filtration barrier, but all of the filtered glucose is reabsorbed in the proximal tubules passively. Smaller proteins and amino acids are filtered too, but these molecules are reabsorbed in the proximal tubules. The transport of glucose is done by carrier proteins. In hyperglycemia, mostly the carrier proteins carry the glucose until there are no more carrier proteins, and at least hyperglycemic glucosuria is formed (12, 15, 40, 41).

With advances in research on the pathophysiology of diabetic kidney diseases, some new treatments targeting kidney inflammation and oxidative stress have gradually entered clinical practice. In fact, some drugs that are useful in mitigating the progression of diabetic kidney diseases have anti-inflammatory properties, such as metformin. Metformin remains the first-line treatment for type 2 diabetes. Natural products with antioxidant effects are effective molecules in preventing oxidative stress and inflammation. The lack of literature on urine analysis findings in diabetic rats gave us the opportunity to evaluate urine results from a different perspective. The goal of this study was to observe how metformin and antioxidant combinations affect urine protein, glucose, and ketone levels in rats with type 2 diabetes, as well as the development of long-term issues like diabetic nephropathy.

Materials and Methods

Chemicals: Streptozotocin (STZ; Sigma cat. no.: S0130-1G), nicotinamide (NA; Sigma cat. no.: 72340-100G), and alpha lipoic acid (ALA; Sigma cat. no.: 62320-25G-F) were supplied by Sigma Aldrich. Rutin flavonoid (R; Alfa Easer cat. no.: A13570.22) was bought from Thermo Fisher Scientific, and metformin (Met; Novartis Glukofen® 1000 mg) was bought from the pharmacy. Dimethylsulfoxide (DMSO) was purchased from Honeywell fluka (cat. no.: 41640-1L), and phosphate buffer solution (PBS) was obtained from Merck (cat. no.: P4417-100TAB). Cherry laurel fruit was obtained from a market in Istanbul. The fruits were grown in the northwest region of the Black Sea, in Zonguldak and Bartin provinces.

Extraction of L. officinalis: The extraction of *L. officinalis* was done according to the method of Agcam and Akyildiz (2). The methanolic extraction solution (methanol: HCl (0.1 N); 85:15, v/v) was prepared with reference to Bronnum Hansen et al. (9). After the cherry laurel was extracted, methanol was evaporated in a vacuum oven and dissolved in DMSO not to exceed 1% of the total solution and diluted in PBS.

Animals and Urine Collection: Eight-week-old 250-350 g weighed male Wistar albino rats were used. It is

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demonstrated that the estrogen hormone in females has a protective effect on beta cell damage (27). This is the rationale behind the choice of male rats for the purpose of this study.

Twelve hours of light and 12 hours of darkness on a regular basis were provided. Water and food were given *ad libitum*. The room temperature was set to 25-27℃. Five rats were in a cage, and each group contained 10 animals. Rats were grouped randomly.

The induction of type 2 diabetes was done with STZ (65 mg/kg) and NAD (110 mg/kg) injection (28). As laboratory animals, 8-week-old Wistar albino male rats were chosen, and the rats were grouped into 6 groups, with 10 rats in each group. Before the induction of diabetes, rats were grouped randomly because their weights were close to each other. In Table 1, the groups and doses of given products can be seen.

To assess diabetes, an OGT test has been performed (24). After diabetes developed, metformin and natural products were given for 84 days (12 weeks). Some of the animals could not complete the study. Urine samples of the rats were collected before the sacrificial process. During the weighing process, the rats handled spontaneously urinated into the weighing cup, and the urine was collected from the weighing cup with the help of a syringe. Samples could not be taken from some rats because their bladders were empty. The urine volume that rats urinate into the weighing cup was not enough to do all the parameters listed on the dipstick. The volume was about 0.2 cc/animal. The weight and glucose measurements after ending the protocol are given in the PhD thesis of Kaya Kartal (24).

Urinalysis: Samples were measured semi-quantitatively with a dipstick (microcult, REF: 116010). Only glucose, protein, and ketone were measured, and the results were numbered from 0 to 3 according to the color on the dipstick. The working principle of glucose measurement in dipstick is based on the enzymatic reaction. First, glucose is oxidized and then forms gluconic acid and H_2O_2 in the presence of glucose oxidase. Ketone body detection in urine samples with the dipstick method uses the nitroprusside test (Rothera test) principle, but in this evaluation, β-hydroxybutyric acid cannot be detected. To measure proteinuria, the reaction is based on the phenomenon known as the protein error of pH indicators, where an indicator that is highly buffered will change color in the presence of proteins (anions) as the indicator releases hydrogen ions to the proteins (17).

Statistical Analysis: The SPSS 21.00 package program was used to determine the statistical differences. For semi-quantitatively measured results, the Kruskal Wallis test was applied. Only glucose gave a statistically significant difference between groups, and $P<0.05$ means statistical significance (13). The results are given as median (Q1-Q3).

Results

There was a statistical significance between DM and all other groups ($P<0.05$). Especially in the control group and the DM+Met+ALA group, no glucose has been found in the urine of a rat. The results are given in Table 2. In ketonuria, no statistical significance was seen ($P>0.05$). The results of urine ketones are 0.0 (0.0-1.0) in control, 1.0 (0.5-1.0) in DM, 2.0 (1.0-2.0) in DM+Met, 1.0 (0.75-1.0) in DM+Met+CL, 1.0 (1.0-2.0) in DM+Met+R, and 1.0 (1.0-2.0) in the DM+Met+ALA group. In urine protein measurement, there was no statistical significance between groups ($P>0.05$). The results of proteinuria were too close in all groups, and proteinuria was observed in all rats.

Table 1. Name of the groups and doses of drugs and natural products and the induction of diabetes.

Groups	n	Flavonoid dose	Metformin dose (30)	STZ+NAD (27)
Control	10	-	-	-
DM	10	-	-	65 mg/kg STZ+110 mg/kg NAD
DM+Met	10	-	500 mg/kg	65 mg/kg STZ+110 mg/kg NAD
DM+Met+CL	10	100 mg/mL	500 mg/kg	65 mg/kg STZ+110 mg/kg NAD
DM+Met+R	10	60 mg/kg (16)	500 mg/kg	65 mg/kg STZ+110 mg/kg NAD
DM+Met+ALA	10	100 mg/kg (21)	500 mg/kg	65 mg/kg STZ+110 mg/kg NAD

DM: Diabetic, DM+Met: Diabetic+Metformin, DM+Met+CL: Diabetic+Metformin+Cherry Laurel, DM+Met+R: Diabetic+Metformin+Rutin, DM+Met+ALA: Diabetic+Metformin+α Lipoic Acid. STZ+NAD: Streptozotocin+Nicotinamide.

Table 2. Results of urine glucose, ketone, and protein measurement (median (Q1-Q3)).

Parameter	Control	DM	DM+Met	DM+Met+CL	DM+Met+R	DM+Met+ALA	P
Glucose	0.0 (0.0-0.0)[a]	2.0 (2.0-2.0)[b]	0.0 (0.0-0.0)[a]	0.0 (0.0-0.0)[a]	0.0 (0.0-0.0)[a]	0.0 (0.0-0.0)[a]	<0.05
Ketone	0.0 (0.0-1.0)	1.0 (0.5-1.0)	2.0 (1.0-2.0)	1.0 (0.75-1.0)	1.0 (1.0-2.0)	1.0 (1.0-2.0)	>0.05
Protein	2.0 (1.5-2.0)	3.0 (2.0-3.0)	3.0 (1.0-3.0)	3.0 (1.75-3.0)	3.0 (2.25-3.0)	2.0 (2.0-3.0)	>0.05
N	5	5	5	10	8	7	

DM: Diabetic, DM+Met: Diabetic+Metformin, DM+Met+CL: Diabetic+Metformin+ Cherry Laurel, DM+Met+R: Diabetic+Metformin+ Rutin, DM+Met+ALA: Diabetic+Metformin+ α Lipoic Acid· **[a-b]: The difference between the means with different letters in the same row is significant (P<0.05).**

Discussion and Conclusion

Hard (19) researched the urine protein content of humans and rats. According to the review, the protein output of rat urine is much higher than that of human urine. Protein output by urine differs between sexes too because studies showed that male rats' urine protein content is approximately 10-fold higher than female rats' urine protein content. Male rats mostly excrete the urine protein as α2μ-globulin because its secretion is controlled by androgens (44). There is a decrease in urine α2μ-globulin output by age, but the decrease is compensated by an increase in albumin output. So, in young mature rats, the proteinuria is defined as physiological proteinuria, while the latter is defined as pathological albuminuria in later phases (32).

When handled for restraint, most rodents urinate and defecate spontaneously, so collecting urine by placing the rodent in a plastic bag can be a urine collection method. Diabetic rodents urinate 10 times more than normal rodents, which makes it easier to collect the urine samples with the mentioned collection method (30).

In normal conditions, glucose is not detected in urine, but according to studies, stress, pain, and fright can cause glucose output in urine. Glucosuria and ketonuria are mostly present in diabetes, but in some rodents with dental and gastrointestinal (cecum) problems, ketonuria can occur (21). In a study in rats fed with a low-carbohydrate, high-fat diet, conducted by Bielohuby et al. (7), the ketonuria measurements with urine dipsticks (two different companies) and laboratory methods (GC/MS) were compared. According to the study, wet chemistry methods are more reliable than urine dipsticks for ketonuria (7). The output of ketone, even in healthy rats, in the current study could be due to the false positive result of the dipstick. There were no significant differences in ketonuria between the groups, and this reveals that dipstick ketone measurement is not reliable. On the other hand, glucose monitoring via urine dipsticks can be beneficial in diabetes.

Masrika et al. (29) studied male Wistar rats fed with a high-protein, low-carbohydrate, and low-fat diet and evaluated urine samples with a dipstick. The ketone and protein outputs in standard diet-fed rats were significantly higher, even if the rats were healthy. Glucose output was not detected in two of the diets. According to these findings in the current study, ketonuria and proteinuria in healthy rats could be regarded as usual, which could explain why there was no statistically significant difference between the healthy and diabetic groups.

Hoffman et al. (20) compared the urine collection and stress markers in rats housed in normal cages with hydrophobic sand and metabolic cages and did not find any significant difference in urinalysis between the collection methods. Urinalysis was done with a dipstick. According to their results, glucose was not detected in any of the healthy rats, but ketone and protein were detected in small amounts. Another study compared wire-bottom and solid-bottom cages, and according to the urinalysis, which was measured with a dipstick, none of the rats' urine samples contained glucose, but approximately all of the healthy rats had ketonuria, and there was no statistically significant difference between the housing types (39).

According to the studies, it can be said that the collection method and the cages in which the rats are housed did not influence the results of urinalysis. But especially according to the study of Bielohuby et al. (7), ketone measurement can differ when done with a dipstick.

Studies on healthy rats and diet, housing, and urine collection methods and the effects on urinalysis were discussed extensively, but the results of nontreated diabetic rats and those treated with drugs, natural products, and their combinations still needed to be evaluated. The lowest blood sugar levels in treated diabetic rats were found in the DM+Met+ALA and DM+Met+CL groups, which are consistent with urinalysis results (24). So, the analysis of glucosuria and blood glucose is an essential diagnostic parameter in diabetes. Studies showed that metformin in combination therapies

is more useful than using metformin alone in type 2 diabetes (5).

The use of lipoic acid alone is a known antidiabetic agent because of the positive effect on insulin resistance and glucose tolerance. Lipoic acid improves hepatic insulin sensitivity (36), so administration of lipoic acid with metformin shows that its antihyperglycemic effect is improved. Magnesium content of natural products is crucial for regulating glucose impairment because magnesium enhances insulin action and has an insulin-like impact on the metabolism of glucose (38). Cherry laurel has a high magnesium content (11), and the mechanism of action against type 2 diabetes could be due to its mineral content, which still needs further investigation. So, according to the current study, the combination of cherry laurel and metformin has positive effects on blood sugar and glucosuria levels. In a study conducted by Sun et al. (42), the effects of rutin on hyperglycemic rats were studied, and rutin reduced blood sugar and lipids and had positive effects on damaged islet cells and antioxidant activity. Based on the current study, it can be said that rutin has positive effects on decreasing the urine glucose output too if used in combination with metformin.

In addition to the classical complications of proteinuria, such as hypoalbuminemia, edema, and acidosis, there is increasing evidence in laboratory animals and humans that proteinuria can cause glomerular and tubulointerstitial damage and lead to progressive nephron loss (16).

In a study, it was found that the microalbuminuria in diabetic patients shows pseudoesterase activity, while the overt albuminuria group did not show this activity. To measure the activity, urine proteins were first isolated, and pseudoesterase activity was measured with electrophoretic measurements (26). This is a good biomarker for understanding renal failure in diabetic patients, but the dipstick method is an easier, less time-consuming, and low-cost method when electrophoresis is not readily accessible.

In a study, the dipstick method for urinalysis in glucosuria was checked in dogs and cats. The dipstick readings were done with visual observation and with an automated approach, and the results showed that visual observation is more sensitive than the automated approach. Glucosuria measurement with a dipstick can lead to false negatives, so if glycemia occurs but no glucosuria is observed, other approaches for glucosuria should be carried out for verification. Dipstick methods for glucosuria in cats were more useful than in dogs (3). Another study with cats used four different dipstick models for glucosuria and found that not all dipstick models are useful to detect glucosuria, but for cats with diabetes, to control the glucosuria, a dipstick with high analytical sensitivity is still useful (47).

A study with diabetic male mice revealed the importance of circadian rhythm for hyperglycemia. In this study, the blood glucose levels between the diabetic and diabetic+treated groups were close, but in urinary glucose, the glucosuria of diabetic mice was higher than that of the diabetic treated group, but there was no statistical difference, and it was concluded that the change in circadian rhythm can lead to a worse effect on diabetes (4).

In the DM+Met+R group, in one rat, the urine glucose level was scored as +2; this is due to the blood glucose level (583 mg/dL). The other animals in this group did not show a high blood glucose level like this. At the beginning of the experiments, there was no statistical difference between groups according to the weights, but after the experiment, the DM+Met+R group showed a significant difference between the other groups (24). There may have been a decrease due to the debilitating effect of rutin flavonoid. The studies showed that the rutin flavonoid decreases the food intake in rats (22).

In conclusion, metformin and metformin + natural product combinations in glucosuria measurement show statistically significant differences between nontreated DM groups. There was still a glucose output in some animals, but the results were closer to those of the control group. However, using metformin alone and using it with different natural products did not produce any statistically significant differences. Urinalysis with dipsticks is mostly used in various housing, urine sample collection methods, and different diets. Urine collection from rats using the spontaneous collection method and dipstick analysis is easy and cheap. So, it can be used in some metabolic disorders to see the metabolic state of animals. The hypoglycemic effects of metformin and antioxidant administration in type 2 diabetes are critical for adjusting treatment strategies, preserving renal function, and improving prognosis. When we look at the additive effects of natural products on the hypoglycemic action of metformin, it is understood that it has no modifying effect on metformin use. Proteinuria measurement is not preferable for revealing diabetic nephropathy in rats due to the results we found. Diseases are mostly induced in laboratory animals like rats, and the results are interpreted for humans or other animals (dogs, cats). In veterinary medicine, dipsticks for urinalysis are commonly used, and according to this study, glucose monitoring from urine could be a choice for diabetic animals since it causes less stress. But for ketonuria and proteinuria, using the dipstick needs more study in the veterinary field to be sure of following diabetic nephropathy and ketoacidosis.

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Ethical Statement

This study was carried out after the animal experiment was approved by Ankara University Local Ethics Committee of Animal Experiments (Decision number: 2021-5-29).

Conflict of Interest

The authors declared that there is no conflict of interest.

Author Contributions

Y.K.K. and T.S. conceived and designed the study, performed the sample collection, conducted the analyses, wrote drafted the manuscript.

Data Availability Statement

The data supporting this study's findings are available from the corresponding author upon reasonable request.

Animal Welfare

The authors confirm that they have adhered to ARRIVE Guidelines to protect animals used for scientific purposes.

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Beekeeping practice-related factors that impact nosemosis prevalence in honey bees in the Republic of Tatarstan, Russia

Nikolai Dmitrievich SHAMAEV[1,2,3,a,✉], Eduard Arkadievich SHURALEV[1,2,4,b], Oleg Vladimirovich NIKITIN[5,c], Malik Nilovich MUKMINOV[1,2,d]

[1]Russian Medical Academy of Continuous Professional Education, Central Research Laboratory, Kazan, Republic of Tatarstan, Russia; [2]Kazan Federal University, Institute of Environmental Sciences, Department of Applied Ecology, Kazan, Republic of Tatarstan, Russia; [3]Kazan State Medical University, Kazan, Republic of Tatarstan, Russia; [4]Kazan State Agrarian University, Kazan, Republic of Tatarstan, Russia; [5]Ekoaudit LLC, Kazan, Republic of Tatarstan, Russia.

[a]ORCID: 0000-0002-0575-3760; [b]ORCID: 0000-0003-0650-3090; [c]ORCID: 0000-0002-6753-0597; [d]ORCID: 0000-0002-5996-0271

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✉**Corresponding author**
nikolay1157@gmail.com

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ABSTRACT

To ensure pollination services for agriculture and implement effective management strategies to protect honey bee populations, it is necessary to understand the prevalence of pathogens and pests and the factors that impact their occurrence. The aim of this study is to investigate potential links of nosemosis prevalence in the Republic of Tatarstan, Russia. Multivariate logistic regression was used to evaluate the following factors as potential risk factors for *Nosema apis* and *N. ceranae* PCR positivity: district, wintering type, honey bee breed, hive material, varroosis, ascosferosis or nosemosis observed in the previous year, colony strength, feeding in winter, and amitraz, fluvalinate, or thymol usage. Our results show that only the variable counting for thymol usage fits the data well, where the actual observed prevalence of *N. ceranae* infection is significantly higher in honey bee populations that use thymol compared to those that do not. Honey bee populations with thymol usage in the current study with decreased, but not eliminated, *N. ceranae* infection, possibly faced preventive, uncontrolled, and excessive use of miticide in beekeeping practice.

Introduction

Honey bees are useful for managing the environment and are crucial pollinators of commercially significant crops. Biotic and abiotic factors (diseases, pesticide use, land use, and climate change) affect insect development and the quantity and quality of honey bee-related products (9, 14, 18). In recent years, there has been increased interest in the effects of *Nosema* species on honey bee colonies (the original parasites of Asian and Western honey bees are *N. ceranae* and *N. apis*, respectively) (37, 39). Sharing habitats, contaminated food sources, trophallaxis, asymptomatic and tolerant honey bees in hives, and the commerce in honey bees and their products are all factors that contribute to the spread of *Nosema* species. The primary way that foraging insects become contaminated with *Nosema* species is through environmental spores. Particularly in areas with long, harsh winters, a high incidence of infection with both *Nosema* species against variations in temperature, relative humidity, and brood rearing in mid-winter may be connected to the health of honey bees (39). The number of managed honey bee colonies has been steadily declining over time in geoclimatic regions with long, cold winters, including Russia. Various phytotherapeutics, organic acids, essential oils, polysaccharides, and metabolites are examples of organic control techniques that reduce the size of the *Nosema* parasite population; they are accessible in many countries, pose little threat to consumer safety by contaminating bee products, and are environmentally benign (14). However, as it is typical for the apiary to have

a few pathogens and diverse rearing methods (9), the lack of multifactor effect data of treatment on *Nosema* spp. in the bee operations is a disadvantage. In Russia, very few investigations on honey bee nosemosis prevalence have been carried out (28, 35, 37, 39, 40). Beekeepers can report the illness status, but this passive surveillance of honey bee pathogens must be verified because it mostly depends on their observations. Additionally, identifying the presence of a pathogen and treatment strategies that may aid in identifying colonies that are more likely to carry a pathogen is pertinent to targeted sampling in the context of pathogen monitoring. The aim of this study is to investigate potential links of nosemosis prevalence in the Republic of Tatarstan, Russia.

Materials and Methods

Sample Collection and Sample Size Estimation: Honey bee sampling was performed in the Republic of Tatarstan, Russia, in spring 2024 in the private-sector apiaries (Figure 1, Table 1, Table 2). The sample collection procedure was described by Shamaev et al (37). There are overall 43 districts in the Republic of Tatarstan. Districts are just administrative borders that have no relation to host-pathogen interaction (38). In this study 13 districts were selected, which is a proportion of the entire honey bee population in the Republic of Tatarstan. Among 13 districts, 11 were chosen for sampling because nosemosis-infected honeybees were reported there: with a high rate of infection (Almetyevsky, Aznakaevsky, Buinsky, Elabuzhsky, Laishevsky, Menzelinsky, Muslyumovsky, and Sabinsky) – 8 districts; with either no infection cases or a single positive sample (Apastovsky, Verkhneuslonsky, and Zelenodolsky) – 3 districts (39). Additionally, we included 2 districts that were not surveyed previously (Kamsko-Ustinsky and Vysokogorsky) – they border the above-mentioned districts and have different honey bee breeds. According to the latest data from the Ministry of Agriculture and Food of the Republic of Tatarstan (27), the number of apiaries in the selected districts for 2022 is 496 in Aznakaevsky district, 181 in Almetyevsky district, 395 in Apastovsky district, 225 in Buinsky district, 286 in Verkhneuslonsky district, 220 in Vysokogorsky district, 128 in Elabuzhsky district, 155 in Zelenodolsky district, 213 in Kamsko-Ustinsky district, 178 in Laishevsky district, 136 in Menzelinsky district, 354 in Muslyumovsky district, and 381 in Sabinsky district. The minimum number of apiaries to be sampled was determined through the following formula (1, 48):

$$n = \frac{Nt^2p(1-p)}{\mathrm{d}^2(N-1) + t^2p(1-p)}$$

In the formula, N is 3384, which is the total number of apiaries in all 13 districts, i.e., in the selected proportion of the entire population. According to Aguila and Gonzalez-Ramırez, the formula is acceptable to calculate a proportion (1). The prevalence value P was considered as 0.059, since the average value of nosemosis prevalence in Russia is 5.9% (28, 39), while d2 is 0.0025 (a minimum error of 5% was chosen), which is the margin of error in the sample, and t2 is 3.8416 (for 95% CI). According to this formula, the minimum number of apiaries to be selected in all 13 districts, i.e., in the selected proportion of the entire population, was determined as 21. We used this information as the border of a minimum number of apiaries. Overall, 26 apiaries were studied, which is 2 apiaries per district.

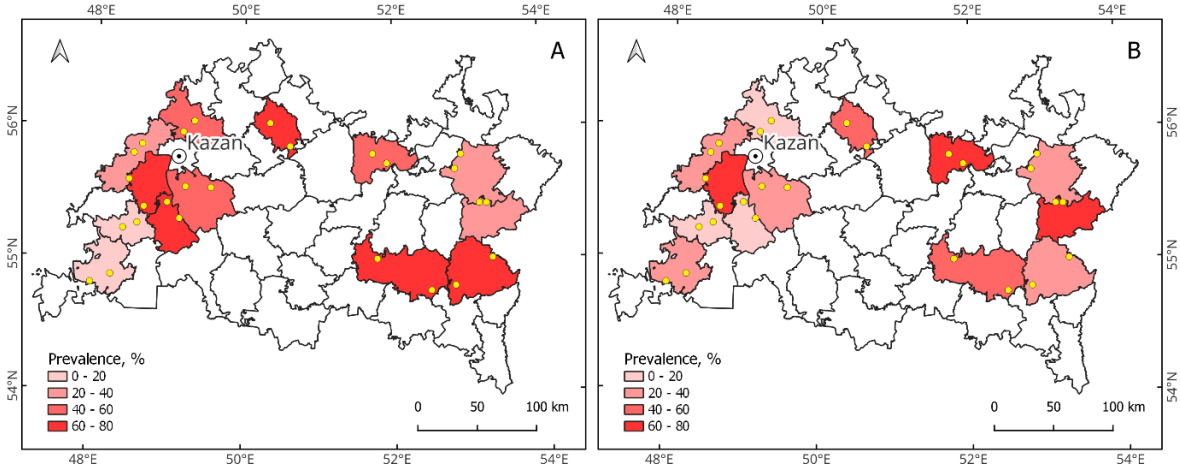

Figure 1. Cartographic data visualization on *Nosema* spp. prevalence in the private-sector apiaries in the districts of Republic of Tatarstan, Russia. (A) *N. apis*. (B) *N. ceranae*. Spatial referencing of sampling sites and data visualization was carried out using a global positioning system (GPS) and the free and open-source geographic information system QGIS 3.28 (https://qgis.org). Geodetic coordinates were projected into planar rectangular coordinates in the Universal Transverse Mercator projection on the WGS-84 ellipsoid (Universal Transverse Mercator (UTM), zone 39N, EPSG:32639). The coordinates on the maps are presented as geodetic coordinates (WGS-84, degrees north latitude and east longitude). To visualize thematic objects (administrative boundaries, regional capital), a set of vector data layers called NextGIS (https://data.nextgis.com) was used. Data license: ODbL. Prevalence data was reflected in the form of a background cartogram (choropleth map) for five equivalent classes (0-20, 20-40, 40-60, 60-80, and 80-100%).

Table 1. *N. apis* prevalence in private sector apiaries

Category		Examined	Positive	Negative	Prevalence (%)	95% CI
District	Almetyevsky	41	32	9	78.04	61.96-88.88
	Apastovsky	79	15	64	18.98	11.35-29.69
	Aznakaevsky	48	36	12	75	60.1-85.89
	Buinsky	107	21	86	19.62	12.82-28.66
	Elabuzhsky	46	21	25	45.65	31.17-60.84
	Kamsko-Ustinsky	63	41	22	65.07	51.94-76.36
	Laishevsky	76	33	43	43.42	32.25-55.25
	Menzelinsky	40	9	31	22.5	11.4-38.85
	Muslyumovsky	46	18	28	39.13	25.45-54.6
	Sabinsky	45	30	15	66.66	50.94-79.56
	Verkhneuslonsky	52	32	20	61.53	47.01-74.36
	Vysokogorsky	37	17	20	45.94	29.85-62.86
	Zelenodolsky	71	21	50	29.57	19.63-41.75
Wintering type	Winter shelter	363	139	224	38.29	33.3-43.53
	Insulated hives	388	187	201	48.19	43.13-53.28
Subspecies	*A. m. carnica*	350	162	188	46.28	41-51.66
	A. m. carpatica	102	43	59	42.15	32.57-52.34
	A. m. mellifera	94	39	55	41.49	31.56-52.12
	A. m. caucasica	42	24	18	57.14	41.07-71.92
	Not identified	163	58	105	35.58	28.35-43.5
Hive material	Wood	317	114	203	35.96	30.72-41.54
	Polystyrene	434	212	222	48.84	44.06-53.65
Varroosis reported previously	No	290	115	175	39.65	34.02-45.55
	Yes	461	211	250	45.77	41.16-50.44
Ascoferosis reported previously	No	596	143	453	24	20.65-27.66
	Yes	155	24	131	15.48	10.36-22.36
Nosemosis reported previously	No	332	105	227	31.62	26.71-36.96
	Yes	419	221	198	52.74	47.84-57.59
Colony strength	≥ 6 frames	406	143	263	35.22	30.61-40.11
	< 6 frames	345	183	162	53.04	47.62-58.38
Feeding in winter	Sugar-honey	104	69	35	66.34	56.33-75.13
	Sugar	540	211	329	39.07	34.95-43.34
	None	107	46	61	43	33.57-52.91
Amitraz used	No	609	296	313	48.6	44.57-52.65
	Yes	142	30	112	21.12	14.91-28.93
Fluvalinate used	No	645	270	375	41.86	38.03-45.78
	Yes	106	56	50	52.83	42.93-62.51
Thymol used	No	428	151	277	35.28	30.79-40.03
	Yes	323	175	148	54.17	48.57-59.68
Infected with *N. ceranae*	No	467	159	308	34.04	29.79-38.56
	Yes	284	167	117	58.8	52.82-64.54
Total		751	326	425	43.4	39.84-47.04

Table 2. *N. ceranae* prevalence in private sector apiaries

Category		Examined	Positive	Negative	Prevalence (%)	95% CI
District	Almetyevsky	41	24	17	58.53	42.19-73.29
	Apastovsky	79	10	69	12.65	6.56-22.49
	Aznakaevsky	48	19	29	39.58	26.11-54.70
	Buinsky	107	25	82	23.36	15.95-32.72
	Elabuzhsky	46	33	13	71.73	56.31-83.54
	Kamsko-Ustinsky	63	9	54	14.28	7.13-25.89
	Laishevsky	76	24	52	31.57	21.66-43.37
	Menzelinsky	40	10	30	25	13.24-41.52
	Muslyumovsky	46	33	13	71.73	56.31-83.54
	Sabinsky	45	27	18	60	44.37-73.93
	Verkhneuslonsky	52	40	12	76.92	62.82-87.01
	Vysokogorsky	37	3	34	8.1	2.11-23.02
	Zelenodolsky	71	16	55	22.53	13.8-34.28
Wintering type	Winter shelter	363	142	221	39.11	34.1-44.36
	Insulated hives	388	142	246	36.59	31.83-41.63
Subspecies	*A. m. carnica*	350	135	215	38.57	33.48-43.91
	A. m. carpatica	102	48	54	47.05	37.18-57.15
	A. m. mellifera	94	34	60	36.17	26.69-46.78
	A. m. caucasica	42	19	23	45.23	30.16-61.16
	Not identified	163	37	126	22.7	16.67-30.04
Hive material	Wood	317	105	212	33.12	28.02-38.64
	Polystyrene	434	179	255	41.24	36.59-46.04
Varroosis reported previously	No	290	102	188	35.17	29.73-41.00
	Yes	461	182	279	39.47	35.01-44.12
Ascoferosis reported previously	No	596	257	339	43.12	39.11-47.21
	Yes	155	27	128	17.41	11.98-24.51
Nosemosis reported previously	No	332	117	215	35.24	30.15-40.67
	Yes	419	167	252	39.85	35.16-44.73
Colony strength	≥ 6 frames	406	112	294	27.58	23.34-32.25
	< 6 frames	345	172	173	49.85	44.46-55.24
Feeding in winter	Sugar-honey	104	73	31	50.69	42.27-59.07
	Sugar	540	190	350	35.18	31.18-39.39
	None	107	21	86	19.62	12.82-28.66
Amitraz used	No	609	249	360	40.88	36.97-44.91
	Yes	142	35	107	24.64	17.97-32.71
Fluvalinate used	No	645	203	442	31.47	27.93-35.23
	Yes	106	81	25	76.41	67.00-83.88
Thymol used	No	428	118	310	27.57	23.44-32.11
	Yes	323	166	157	51.39	45.80-56.94
Infected with *N. apis*	No	425	117	308	27.53	23.38-32.08
	Yes	326	167	159	51.22	45.66-65.75
Total		751	284	467	36.35	32.92-39.92

Table 3. List of districts, used for the sample collection and the PCR-RFLP results

District	Number of colonies	Number of samples succeed in PCR-RFLP	Breed based on the PCR-RFLP
Almetyevsky	1	27/27	*A. m. caucasica*
	1	11/14	*A. m. caucasica*
		3/14	NA
Apastovsky	1	41/41	*A. m. carnica*
	1	3/38	*A. m. carnica*
		35/38	NA
Aznakaevsky	1	21/21	*A. m. carnica*
	1	14/27	*A. m. carpatica*
		13/27	NA
Buinsky	1	27/59	*A. m. carpatica*
		32/59	NA
	1	48/48	*A. m. carnica*
Elabuzhsky	1	31/31	*A. m. carnica*
	1	15/15	*A. m. carnica*
Kamsko-Ustinsky	1	22/22	*A. m. carnica*
	1	41/41	*A. m. carpatica*
Laishevsky	1	15/34	*A. m. mellifera*
		19/34	NA
	1	18/42	*A. m. mellifera*
		24/42	NA
Menzelinsky	1	15/22	*A. m. caucasica*
		7/22	NA
	1	7/18	*A. m. carnica*
		11/18	NA
Muslyumovsky	1	26/26	*A. m. carnica*
	1	20/20	*A. m. carpatica*
Sabinsky	1	38/38	*A. m. carnica*
	1	7/7	*A. m. carnica*
Verkhneuslonsky	1	23/23	*A. m. carnica*
	1	29/29	*A. m. mellifera*
Vysokogorsky	1	32/37	*A. m. mellifera*
	1	4/5	*A. m. mellifera*
		1/5	NA
Zelenodolsky	1	60/60	*A. m. carnica*
	1	11/11	*A. m. carnica*

NA: samples with no result obtained.

We used the following approach for the minimum sample size estimation in the selected proportion of the entire population (13 districts). According to the existing rules/guidelines of sample size, there is a ratio of 20-to-1 (10), where a study with 1 item (question) requires 20 samples. We had 20 main questions about the relationship between nosemosis prevalence and: 1. Usage of winter shelter as a wintering type; 2. Usage of insulated hives as a wintering type; 3. Usage of wood as a hive material; 4. Usage of wood as a hive material; 5. Honey bee breed *A. m. mellifera*; 6. Honey bee breed A. *m. carnica*; 7. Honey bee breed A. *m. carpatica*; 8. Honey bee breed *A. m. caucasica*; 9. Data from beekeeper regarding varroosis in the previous year; 10. Data from beekeeper regarding ascoferosis in the previous year; 11. Data from beekeeper regarding nosemosis in the previous year; 12. Usage of sugar-honey in feeding in winter; 13. Usage of sugar in feeding in winter; 14. No feeding in winter; 15. Colony strength ≥ 6 frames; 16. Colony strength < 6 frames; 17. Amitraz usage; 18. Fluvalinate usage; 19. Thymol usage; 20. Another *Nosema* species occurrence in study. In total, a minimum sample size should be 400 (20x20) in the selected proportion of the entire population (13 districts). We used this information as the border of a minimum sample size. Overall, 751 honey bees were studied (the number of honey bees collected in each apiary is shown in the Table 3). A survey of beekeepers was conducted to assess the beekeeping practices used in the apiary,

evaluate the use of pharmaceutical products or other additives, and consider recorded cases of honey bee diseases in the colonies annually.

DNA Extraction and PCR: Only worker bees were studied. Prior to DNA extraction, each individual honey bee was washed in ethanol and sterile water and then ground in 1 ml of newly added sterile water. DNA extraction was performed for each individual honeybee. DNA was extracted using the AmpliPrime kit (NextBio, USA) following the previously established protocol (42). Duplex PCR was performed to amplify the 321 bp and 218 bp fragments corresponding to the 16S ribosomal gene of *N. apis* and *N. ceranae*, respectively. PCR-RFLP was performed to amplify the cytochrome oxidase 1 gene region and evaluate the honey bee breed among the samples. PCR procedures with corresponding primer sequences are detailed in the previous report (34, 37).

Statistical Analysis: All analyses were performed using R Statistical Software (version 4.3.0) (43). Multivariate logistic regression was used to evaluate the different factors as potential risk factors for PCR positivity with *Nosema* species. Quantitative data were replaced with 0 or 1 dummy variables. Honey bee breed variables were replaced by 0, 1, 2, and 3 for *A. m. mellifera*, *A. m. carnica*, *A. m. carpatica*, and *A. m. caucasica*, respectively. Feeding in winter was replaced by 0, 1, and 2 for honey, sugar-honey, and sugar, respectively. Multicollinearity among the explanatory variables was assessed using Spearman's rank correlation coefficient. None of the Spearman's coefficients were greater than 0.6 (Figure 2). To find the best-fitting model, a backward selection procedure was used. Predictive performance analysis, model fitting, and computation of the standard errors for the predicted probabilities, as well as the list of software packages used in this study, were described previously (38). P value from the CI of estimated *N. ceranae* prevalence was calculated using a method reported previously (2). The P-values less than 0.05 were considered statistically significant.

Results

Among 751 worker honey bees from 26 colonies in 13 districts, 326/751 (43.4%, 95% confidence interval; CI: [39.84–47.04]) and 284/751 (36.35%, 95% confidence interval; CI: [32.92–39.92]) of the honey bee samples showed *N. apis* and *N. ceranae* positivity, respectively (Figure 1, Table 1, Table 2).

Hive conditions were not counted as factors for nosemosis because the beekeepers do regular inspections every 2 weeks to monitor colonies' health and progress, look for symptoms associated with established pests and diseases of honey bee colonies. Average temperature in the hive was 77-82.4 F and relative humidity 55-70%, which did not exceed the values in other regions with similar climates (25, 47). We found that 38.29% (139/363) and 48.19% (187/388) of the honey bees managed in the winter shelter and insulated hives were *N. apis* positive, and 39.11% (142/363) and 36.59% (142/388) were *N. ceranae* positive, respectively. 35.96% (114/317) and 48.84% (212/434) of the honey bees managed in the wooden and polystyrene hives were positive for *N. apis*,

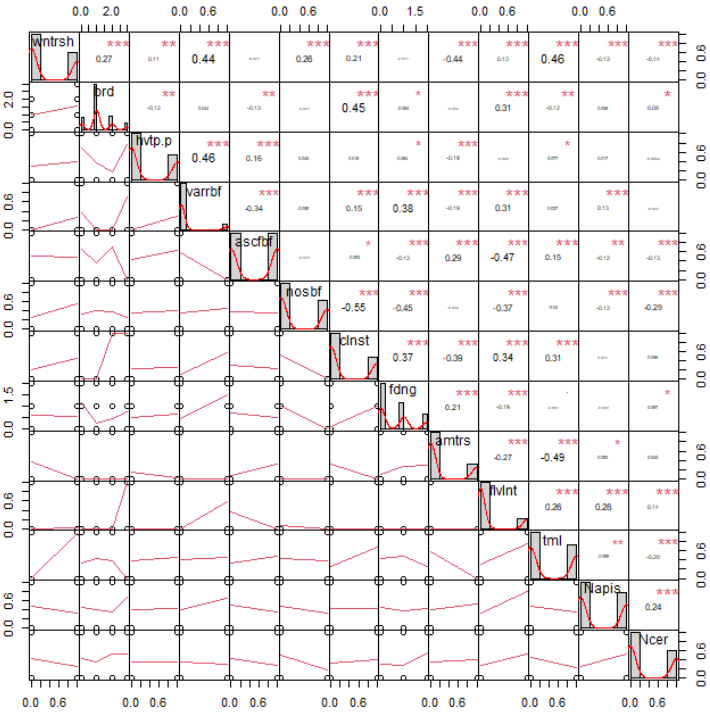

Figure 2. Multicollinearity of the explanatory variables using Spearman's coefficient. None of the Spearman's coefficients were greater than 0.6. Variable designations: Wntrsh - Wintering type, brd - Subspecies, hvtp.p - Hive material, varrbf - Varroosis reported previously, ascbf - Ascoferosis reported previously, nosbf - Nosemosis reported previously, clnst - Colony strength, fdng - Feeding in winter, amtrs - Amitraz used, flvnt - Fluvalinate used, tml - Thymol used, Napis - Infected with *N. apis*, Ncer - Infected with *N. ceranae*.

and 33.13% (105/317) and 41.24% (179/434) were positive for *N. ceranae*, respectively. *N. apis* and *N. ceranae* positivity among honey bee colonies with other pathogens reported previously were as follows: 45.77% (211/461) and 39.47% (182/461) for varroosis, 15.48% (24/155) and 17.41% (27/155) for ascosferosis, and 52.74% (221/419) and 39.85% (167/419) for nosemosis.

According to the PCR-RFLP results, four distinct subspecies were identified, although some samples exhibited multiple bands or yielded negative results. Those samples were abbreviated as "NA" and were included in the statistical analysis (Table 3). *N. apis* and *N. ceranae* positivity among honey bee subspecies were 46.28% (162/350) and 38.57% (135/350) for *A. m. carnica*, 42.15% (43/102) and 47.05% (48/102) for *A. m. carpatica*, 41.49% (39/94) and 36.17% (34/94) for *A. m. mellifera*, 57.14% (24/42) and 45.23% (19/42) for *A. m. caucasica*, and 35.58% (58/163) and 22.7% (37/163) for those honey bee samples that were not identified. *Nosema* species positivity among honey bee colonies with a colony strength of 7 frames or more and less than 7 frames were as follows: 35.22% (143/406) and 53.04% (183/345) for *N. apis*, and 27.58% (112/406) and 49.85% (172/345) for *N. ceranae*. *N. apis* and *N. ceranae* positivity among honey bee colonies with different feeding in winter was as follows: 66.34% (69/104) and 50.69% (73/104) for sugar-honey syrup, 39.07% (211/540) and 50.69% (190/540) for sugar syrup, and 43% (46/107) and 19.62% (21/107) for no feeding.

There are various registered names for thymol, fluvalinate, and amitraz available for purchase for beekeepers in Russia, and all the products have the same quantities of active ingredient that should be applied in the hive. According to information obtained from 26 beekeepers in spring, all of them used the exact portion of product according to the product instructions for use. For thymol, it was fed to honey bees together with syrup (3 g of thymol powder diluted in 25 L of 50% syrup, and 100 mL was added in the hive feeder for each frame). Such treatment was applied 4 times during 1 month with an equal interval between treatments. For fluvalinate, all the beekeepers used 2 strips per 8-12 frame hive. 1 strip was used with a lesser number of frames. Each strip contains 80 mg of fluvalinate. Because there was no difference in quantity and feeding period, thymol, fluvalinate, and amitraz were included in the statistical analysis as “used” or “not used”. *N. apis* and *N. ceranae* positivity among honey bee colonies treated with synthetic or organic chemicals and compounds was as follows: 21.12% (30/142) and 24.64% (35/142) for amitraz, 52.83% (56/106) and 76.46% (81/106) for fluvalinate, and 54.17% (175/323) and 51.39% (166/323) for thymol usage.

Multivariate logistic regression analysis was performed to separately validate risk factors for *Nosema* spp. infection. Using a backward selection procedure, three models were generated. A best-fitted model 1 to estimate the risk factors for *N. apis* infection included the following factors: *N. ceranae* infection, previously observed nosemosis, colony strength, amitraz usage, feeding in winter, and previously observed varroosis. A best-fitted model 2 to estimate the risk factors for *N. ceranae* infection included the following factors: fluvalinate usage, *N. apis* infection, feeding in winter, thymol usage, previously reported ascosferosis, colony strength, hive material, and previously observed nosemosis. A best-fitted model 3 to estimate the risk factors for infection with both *Nosema* species included the following factors: thymol usage, previously reported nosemosis, feeding in winter, and fluvalinate usage. A plot of the modified Akaike information criterion (AICc) of several models showed that model 3 minimizes AICc, and is therefore chosen as the best model out of this set (Figure 3). To assess the estimates of the actual prevalence of the honey bee population obtained from the model 3 and evaluate its goodness of fit, we plotted the model-based estimates of prevalence against the raw prevalence from the population (Figure 4). Among variables, only the variable counting for thymol usage fits the data well, where the actual observed prevalence of *N. ceranae* infection was also significantly higher ($P < 0.05$) in honey bee populations with thymol usage than in the populations without it.

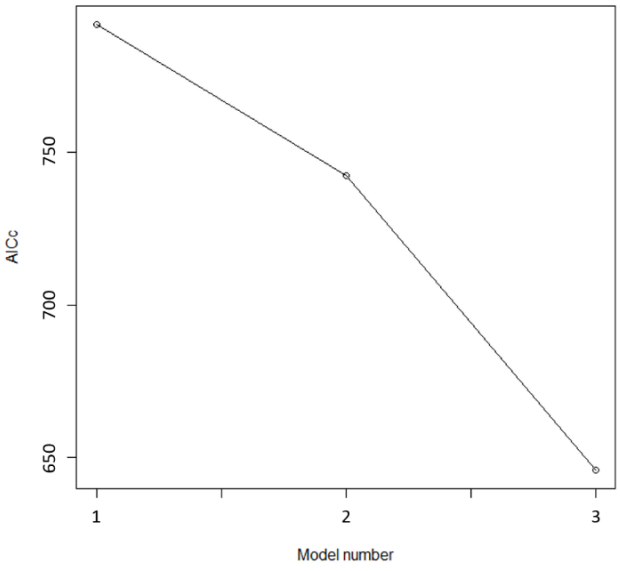

Figure 3. A plot of AICc of several models, where model 3 minimizes AICc, and is therefore chosen as the best model out of this set

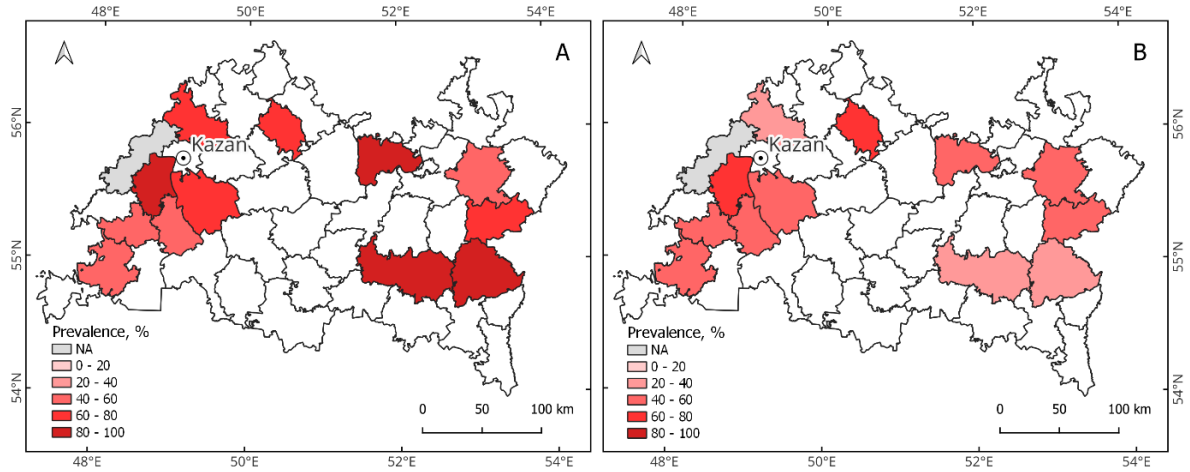

Figure 4. Map of estimated *Nosema* species prevalence in the honey bees where thymol was either used or not used. Estimated prevalences among the honey bees where (A) thymol was used and (B) was not used. The observed prevalence and 95% CIs are shown from the fitted model. Values for wintering type, honey bee breed, hive material, colony strength, feeding in winter, amitraz or fluvalinate usage, reported varroosis, ascosferosis or nosemosis were set to zero in the model.

Discussion and Conclusion

Variety of pests and pathogens, including microsporidians *N. apis* and *N. ceranae* are responsible for mass bee colony losses in Russia (28, 33, 36, 39). In this study, we surveyed the prevalence of nosemosis and the factors that impact its occurrence among 13 districts in Tatarstan, Russian Federation. Wintering type (winter shelter or insulated hive) was chosen because the wintering technique had an impact on honey bee survival; colonies that spent the winter indoors had lower mortality rates when infected with *Nosema* species and a quicker spring population build-up than colonies that spent the winter outdoors (32). Hive material reflects the internal conditions within a hive, too. According to a survey, keeping bees in wooden hives preserves ideal temperature conditions in the brood-rearing zone, which benefits queen egg production, worker bee flight activity indicators, and colony strength. As opposed to wooden hives, polyurethane foam hives are difficult to sterilize, have no vapor permeability, and water is not absorbed; instead, it flows down and stays on the bottom (46). The prevalence of nosemosis infection is naturally found at a high infection rate in *A. mellifera* populations (8), but it remains unknown within subspecies present at the same study area. Such variables as varroosis, ascosferosis, and nosemosis (observed in the apiary in the previous year or not) were included in the analysis, as the findings of studies conducted on bee colonies in different regions of Russia (Arkhangelsk, Belgorod, Voronezh, Kirov, Leningrad, Moscow, Orenburg, Penza, Tomsk, Tula, and Tyumen regions; Altai, Krasnodar, Perm, and Stavropol krai; Republics of Mari El, Tatarstan, and Udmurtia) indicate that varroosis-nosemosis and varroosis-nosemosis-ascosphaerosis are included in the list of the most prevalent infection-invasions of bees (12). Feeding in winter was included in the analysis because different winter feed types may be associated with any significant differences in nosemosis prevalence (5). Nosemosis has been associated with its negative impact on colony strength and productivity in several studies (30). Since robust colonies consistently produce more broods, there is a direct correlation between colony strength and brood raising. More worker bees can make more honey and feed and care for more broods. According to Bhusal and Thapa (2006), honey output from less than six frames is much lower than that from six, eight, and ten frame types (3). We counted frames in order to gauge the strength of the honey bee colonies under examination (≥ 6 frames or < 6 frames). Other variables included in the analysis were amitraz, fluvalinate, or thymol usage. For example, beekeepers can leave strips soaked in amitraz in the hive for longer than necessary (49). It is reported that in the honey bee family, nosemosis and exposure to the commonly used in-hive acaricide amitraz are common stressors that both result in higher mortality rates than bees exposed separately, with no difference in the development of parasites (22). It is also a typical practice in Russia, where higher dosages of amitraz, thymol, and fluvalinate active ingredients resulted in higher fatality rates or decreased reproductive performance in colonies (7, 24). The rate at which pests and pathogens are eliminated from the colony allows beekeepers to calculate its appropriate dosage. However, according to data from beekeepers in this study, they used the exact dosage according to the product instructions. Using logistical regression analysis, we found that the honey bee populations with thymol usage significantly impacted *N. ceranae* prevalence but not wintering type, honey bee breed, hive material, colony strength, feeding in winter, amitraz, fluvalinate, or thymol

usage, and varroosis, ascosferosis or nosemosis observed in the previous year. This result is reinforced by the fact that *N. ceranae* prevalence among the honey bees treated with thymol was significantly higher statistically than that without thymol. Honey bee populations in the current study may have faced preventative, uncontrolled, and excessive thymol treatment in beekeeping practices. *Thymus vulgaris* is the natural source of thymol (3-hydroxy-p-cymene), which is an essential oil constituent utilized for decades in *Varroa* control due to its anti-parasitic properties (19). Different studies in which honey bees fed on thymol report that it may be able to control *N. ceranae* to varying extents (*N. ceranae* spore load reduction or no effect) (4). Thymol itself may cause certain disorders that affect bee survival, lowering oxidative capacity, and downregulating some immune-related gene expressions in *Nosema*-free bees, but in *Nosema*-infected bees, some studies show increasing levels of immune-related genes and values of oxidative stress parameters in addition to decreasing *Nosema* spore loads (16). Other studies also show reduced survival in the honey bees and genotoxic effects of thymol (17). To understand the potential detrimental effects on brood growth after thymol treatment, its usage should be further examined in the honey bees exposed to both common stressors (varroosis and nosemosis).

The overall *N. apis* and *N. ceranae* prevalence in honey bees was 43.4% and 36.35%, respectively. There is sufficient information regarding the prevalence of *N. apis* and *N. ceranae* in the Republic of Tatarstan, Russia, from other researchers: 5.9% prevalence of nosemosis on the regional level, including the Republic of Tatarstan and one N. *apis*-infected honey bee reported in the Republic of Tatarstan (28, 39, 47). Comparing our results collected from the same apiary in Laishevsky district in February between 2023 and 2024, infection prevalences became 2.6 (16.66% vs. 43.42%) and 7-fold times higher (4.44% vs. 31.57%) for *N. apis* and *N. ceranae*, respectively (35, 39, 40). Interestingly, co-infection with both species decreased 2.6-fold times (38.88% vs. 14.47%). Also, in the same study, we found a moderate differentiation in the genetic structure of *N. apis* (na1.1 haplotype) and *N. ceranae* subpopulations (nc1.4, nc7.1, nc13.3, nc17.1, nc20.3, nc35.1, and nc1.1, nc4.1, nc4.4, nc5.1, nc6.2, nc11.1, nc24.1, and nc29.1 haplotypes) (39). In another study in the same apiary in 2024, we observed the negative effect of high infection loads on *N. apis* spore size by the depletion of resources needed for spore production (37). With an increase in spore load, more atypical *N. apis* spores were observed (including the data from honey bees co-infected with both *Nosema* species). *N. apis* in the current study was found to be more prevalent in honey bees than *N. ceranae*. However, the drastic increase of *N. ceranae* prevalence from 2023 to 2024, the presence of *N. ceranae* multiple haplotypes, and atypical *N. apis* spores as a result of resource depletion altogether can be related to the higher adaptability of *N. ceranae*, which seems that the situation in the Republic of Tatarstan reflects broader global trends in Europe, where *N. ceranae* became increasingly dominant compared to *N. apis*.

The intraspecific taxonomic affiliation of honey bee colonies determines their susceptibility to nosemosis; in temperate and northern latitudes, colonies of bees belonging to the subspecies *A. m. ligustica*, *A. m. caucasica*, and *A. m. carnica* are more likely to be infected with *Nosema* species than colonies of *A. m. mellifera* (44). Unlike the reported data, in our study, *N. apis* prevalence among *A. m. carnica*, *A. m. carpatica*, *A. m. mellifera* and *A. m. caucasica* was in the range 41.49-57.14%. *N. ceranae* prevalence among *A. m. carnica*, *A. m. carpatica*, *A. m. mellifera* and *A. m. caucasica* was in the range 36.17-47.05%. Among domestic *A. mellifera* honeybee subspecies, there are some differences in resistance to nosemosis, which are assumed from the expression of immune genes, mortality rates, events of hybridization or the prevalence of pathogens (6, 20). Kharitonov found that severity of *N. apis* and *N. ceranae* infection in *A. m. mellifera* was significantly lower than in *A. m. caucasica* (20). Petukhov et al. observed that *A. m. caucasica* and *A. m. carpathica* tend to be affected by *N. apis* in a more intensive manner than *A. m. mellifera* (31). Kaskinova et al. found that *A. m. mellifera* and *A. m. carnica* were equally infected with *N. apis*, but *A. m. mellifera* were 3-fold times more infected with *N. ceranae* than *A. m. carnica* (26). Tozkar found that the highest responses from immune genes against *N. ceranae* were in *A. m. carnica*, compared to *A. m. caucasica* (45). Prevalence of either *N. apis* or *N. ceranae* was not significantly different between subspecies in the current study (Figure 5). However, *N. ceranae* prevalence in not identified subspecies was

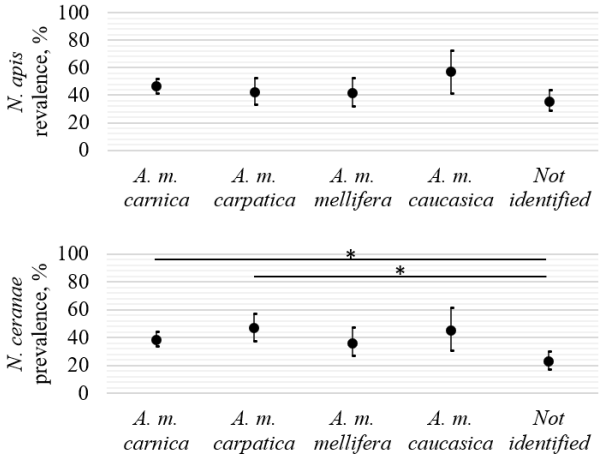

Figure 5. Difference among *N. apis* and *N. ceranae* prevalence among *A. mellifera* subspecies. Asterisk show the statistical significance with P-values less than 0.05.

significantly different from *A. m. carnica* and *A. m. carpathica* subspecies, but not *A. m. mellifera* and *A. m. caucasica*. It can be explained that when different honey bee subspecies form hybrids, new genotypes are formed and genetic imbalance arises, which leads to changes in resistance against diseases. It is not clear whether *N. ceranae*-resistant *A. mellifera* hybrids derived from *A. m. mellifera* with *A. m. caucasica* in the current study. We assume that it is unlikely because our own morphological observations of honey bees from the Tatarstan Republic revealed a positive correlation between the *A. m. mellifera* / *A. m. caucasica* hybrid and a high *Nosema* spp. spore load (40). Also, Ostroverkhova et al. reported that *N. apis* and *N. ceranae* presence was increased in naturally resistant Central Russian *A. m. mellifera* after hybridization with honey bee subspecies from southern regions (29). At last, Fontbonne et al. found that pure *A. m. carnica* and *A. m. carpathica* mortality for *N. ceranae* was up to 50%, while for *A. m. carnica* /*A. m. carpathica* / *A. m. mellifera* hybrids and for *A. m. caucasica* /*A. m. carnica* /*A. m. carpathica* hybrids it was up to 100% (13). To prove the hypothesis about genetic imbalance and know the degree to which hybridization alters resistance, further experiments with whole genome sequencing are necessary.

N. apis and *N. ceranae* can lead to Colony Collapse Disorder (CCD), a dangerous disease of the honey bee *A. mellifera* that causes the sudden death of the entire colony (11). However, in the apiaries from Spain, Switzerland, France, and Germany, almost all colonies vulnerable to CCD were infected with *N. apis* and *N. ceranae*, while in apiaries from Russia and Serbia, Bosnia and Herzegovina, and Montenegro, none of the colonies infected with *N. apis* and *N. ceranae* were susceptible to CCD (23). Although nosemosis may play a role in CCD development, other factors, including viral infection and/or honey bee intoxication from sublethal pesticide dosages and/or heavy metals, must coexist for CCD to be effective (15, 21, 23).

At last, for some explanatory variables related to beekeeping practices, it is likely that some of the colonies cannot be considered independent if they belonged to the same beekeeping operation or apiary location. For example, beekeepers might either apply thymol or not apply thymol in their apiaries. This means that if one colony belonging to the beekeeper was treated with thymol, the second colony also must have received thymol, even if the beekeeper said that only a particular colony was treated. The two colonies within the apiary may not be independent with respect to thymol treatment. However, even if the relationship between two colonies within the apiary may increase the chance of a type I error, the multicollinearity among the explanatory variables using Spearman's rank correlation coefficient didn't show values greater than 0.6. For better accuracy, we recommend using more districts, but not more than one colony per apiary, to study the beekeeping practices. In conclusion, the study indicates that honey bee populations exposed to higher levels of thymol are more likely to experience *N. ceranae* infection, possibly due to the uncontrolled use of miticides in beekeeping practices.

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Ethical Statement

Not applicable.

Conflict of Interest

The authors declared that there is no conflict of interest.

Author Contributions

NDS conceived the idea; MNM performed the field work; NDS, EAS, OVN and MNM carried out the laboratory experiments and data analysis. NDS and MNM wrote the manuscript with help from EAS.

Data Availability Statement

The data supporting this study's findings are available from the corresponding author upon reasonable request.

Animal Welfare

Not applicable.

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Combined use of essential oils with organic acids in modifying performance, intestinal health, caecal microflora, and selected blood and bone parameters in broilers

İlyas ONBAŞILAR[1,2,a,✉], Sakine YALÇIN[3,b], Handan ESER[4,c], Muhammad Shazaib RAMAY[5,d], Suzan YALÇIN[6,e], Bülent ÖZSOY[7,f], Fatma Kübra ERBAY ELİBOL[8,g], Süleyman TABAN[9,h], Selma Tuna KOÇOĞLU[10,ı], Emrah TORLAK[11,j]

[1]Hacettepe University, Transgenic Animal Technologies Research and Application Center, Ankara, Türkiye; [2]Hacettepe University, Health Science Institute, Ankara, Türkiye; [3]Animal Nutrition Science Association, Ankara, Türkiye; [4]Bolu Abant İzzet Baysal University, Faculty of Agriculture, Department of Poultry Breeding, Bolu, Türkiye; [5]Ankara University, Faculty of Veterinary Medicine, Department of Animal Nutrition and Nutritional Diseases, Ankara, Türkiye; [6] Selçuk University, Faculty of Veterinary Medicine, Department of Food Hygiene and Technology, Konya, Türkiye; [7]Aydın Adnan Menderes University, Faculty of Veterinary Medicine, Department of Animal Nutrition and Nutritional Diseases, Aydın, Türkiye; [8]TOBB Economics and Technology University, Faculty of Engineering, Department of Biomedical Engineering, Ankara, Türkiye. [9]Ankara University, Faculty of Agriculture, Department of Soil Science and Plant Nutrition, Ankara, Türkiye; [10]Bolu Abant İzzet Baysal University, Seben İzzet Baysal Vocational School, Department of Plant and Animal Production, Bolu, Türkiye; [11] Necmettin Erbakan University, Faculty of Science, Department of Molecular Biology and Genetics, Konya, Türkiye.

[a]ORCID: 0000-0002-1464-4654; [b]ORCID: 0000-0001-8640-2729; [c]ORCID: 0000-0002-7617-6059; [d]ORCID: 0000-0002-7061-0483; [e]ORCID: 0000-0002-3937-6705; [f]ORCID: 0000-0003-0045-3790; [g]ORCID: 0000-0002-4117-1098; [h]ORCID: 0000-0002-7997-9412; [ı]ORCID: 0000-0003-4794-2655; [j]ORCID: 0000-0003-4636-7791

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✉Corresponding author
ilyas@hacettepe.edu.tr

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ABSTRACT

Essential oils (EOs) and organic acids (OAs) are promising feed additives with crucial roles in promoting animal health and performance. This study aimed to assess the combined effects of phytobiotics with organic acids (EOAs) in broiler diets for 39 days. A total of daily 300 male chicks were assigned to three groups, each containing 100 chicks with 5 replicates. The basal diet was supplemented with 0, 0.1, and 0.2% EOAs, respectively. EOAs supplementation did not significantly affect performance values. The villus height/crypt depth ratio in the jejunum and villus dimensions in the ileum showed improvement with EOAs. Dietary EOAs supplementation led to a reduction in the count of *Enterobacteriaceae* and an increase in *Lactobacillus* in the caecum. Serum IgA and IgG levels increased with EOAs. Ultimate load, yield load, and the levels of ash, calcium, phosphorus, zinc, and manganese in tibia and femur were higher at high levels of EOAs than in the other groups. In conclusion, the study suggests that 0.1% EOAs usage in diets could be a viable option for enhancing intestinal health, immunity, and bone mineralization as an alternative growth promoter, especially in commercial broiler production.

Introduction

Feed additives such as phytobiotics (Ps) and organic acids (OAs), have gained increasing recognition as natural growth promoters in poultry diets. Ps can be defined as natural compounds derived from plants, which encompass whole plants, plant components, extracts, or essential oils (EOs). The positive impacts of Ps can be attributed to various factors such as the stimulation of feed

consumption, improved nutrient digestion and absorption, modulation of gut microbiota, decreased colonization of harmful pathogens in the gut, and reinforcement of the birds' immune status. They also exhibit antibacterial, antiviral, antioxidant, and anti-inflammatory properties. In addition to Ps, OAs have found widespread use in poultry nutrition due to having vital roles in lowering pH, decreasing the survival of pathogens, enhancing the activity of digestive enzymes, improving intestinal morphology, and consequently fostering a healthier gut microflora. This, in turn, contributes to enhanced performance and increased profitability in poultry production (7, 13, 19, 37).

Researchers (1, 5, 30, 40, 43, 49, 50) have studied the usage of the combined use of OAs with EOs (EOAs) in broilers. The synergistic effects of EOs and OAs can be attributed to the improved efficiency of digestive enzymes in acidic conditions (43). Yang et al. (49) indicated that the addition of the blends of sorbic acid, fumaric acid, and thymol during the grower phase increased efficiency, possibly by improving intestinal morphology and increasing digestive enzyme activities of broiler chickens.

Leg weakness, lameness and various bone abnormalities linked to metabolic disorders continue to pose challenges in rapidly growing meat-type chickens, resulting in significant production losses and adverse effects on the welfare of birds (26, 42). Modern broiler lines often exhibit poor bone calcification and high porosity, which can increase the susceptibility to bone damage (44). Nutrition plays a crucial role in the development of these bone disorders, and optimizing dietary factors may help reduce the severity of leg lesions in broilers. Liu et al. (29) reported that supplementation with a mixture of essential oils and organic acids (citric acid, sorbic acid, thymol, and vanillin) had no significant effect on leg bone growth or bone length in broilers. Notably, there is a lack of published research specifically examining the detailed effects of dietary EOAs on broilers, particularly concerning bone parameters. We hypothesize that the combined use of essential oils and organic acids in broiler diets will enhance growth performance, improve intestinal health, and positively modulate caecal microflora. Additionally, this supplementation is expected to influence selected blood parameters and support bone development, potentially mitigating bone disorders associated with rapid growth in broilers. Building on this hypothesis, the objective of this study is to evaluate the effects of dietary supplementation with a combination of essential oils and organic acids on growth performance, caecal fermentation, intestinal health, and selected blood and bone parameters in broilers.

Materials and Methods

***Experimental Design and Diets*:** A total of 300 daily Ross 308 male broiler chicks were divided into three groups, each consisting of 100 chicks. Within each group, there were five replicates, each containing 20 chicks. The chicks were housed in pens (2m x 1m) with wood shavings as litter. The experimental period lasted for 39 days, during which the broilers were fed with different diets as follows: starter diets from day 0 to 13, grower 1 diets from day 14 to 24, grower 2 diets from day 25 to 36, and finisher diets from day 37 to 39. Basal diets were formulated according to the commercial management guide (6) and supplemented with EOAs. The EOAs included thyme oil, orange oil, garlic oil, sorbic acid, acetic acid, malic acid, lactic acid, citric acid, tri-sodium citrate, tartaric acid, salicylic acid, ascorbic acid (Nafoil A Plus, Biotem Ltd Company, İstanbul, Türkiye) at three levels: 0% (EOA0), 0.1% (EOA1), and 0.2% (EOA2). The ingredients and chemical composition of the basal diets are presented in Table 1. The diets were provided in mash form, and feed and water were available *ad libitum* throughout the experiment. Room temperature was 32±2°C during the first week and gradually reduced to an average of 24 to 26°C, which was maintained until slaughter age.

***Traits Measured*:** The nutrient composition of the diets was determined using the methods described in AOAC (4), and metabolisable energy values were calculated using the equation of Carpenter and Clegg as reported by Yalçın et al. (45). The volatile oil profile of the EOAs was determined by the GC-MS (Agilent:6890 MS:5973, New Jersey, USA) with an HP-5 MS column (30 meters).

Individual bird weights were recorded at the beginning of the experiment and on the 13th, 24th, 36th, and 39th days to calculate live weight gains. Daily monitoring of the birds was conducted, and feed intake was measured and expressed in g per bird per period. The feed conversion ratio (FCR) was determined as kg feed consumed per kg weight gain. Percentage of livability {(number of broilers at the end of the study x 100)/number of chicks at the beginning} and European Production Efficiency Factor {EPEF, % = [(livability, % x live weight, kg x 100)/(age, day x FCR, kg feed/kg gain)]} values were calculated (24).

At the end of the experiment (day 39), 10 broilers from each group were weighed, and slaughtered by severing the jugular vein, and their hot carcass weights and carcass yields were determined. The absolute and proportional weights of abdominal fat, liver, gizzard, heart, bursa Fabricius, and spleen were recorded. Duodenal, jejunal, and ileal samples were collected after slaughtering to evaluate morphological changes as reported by Onbaşılar et al. (32). Samples were stained with Mallory's trichrome, and sections were analysed under a light microscope (Olympus BX-40). Measurements were done using Cellsens CS-ST-V1.8 (Standard) software program. For measurement 10 well-oriented crypt-villus units were selected for each intestinal cross-section. Villus height (VH)

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Table 1. The ingredients and chemical composition of the basal diets (as-fed basis)

Items (%)	Broiler starter (0-13 d)	Broiler grower-1 (14-24 d)	Broiler grower-2 (25-36 d)	Broiler finisher (37-39 d)
Ingredients (%)				
Corn	36.55	33.79	35.54	37.71
Soyabean meal, 46% CP	18.83	11.32	4.65	10.47
Fullfat soya	18.00	18.00	19.00	12.50
Wheat	13.50	13.00	14.50	15.50
Sunflower seed meal, 34% CP	4.00	7.00	8.00	5.00
Red dog	2.50	4.50	5.00	5.00
Rice	0.00	3.00	3.00	3.00
Meat and bone meal	3.00	1.75	2.14	1.01
Poultry rendering meal	0.00	3.50	4.00	5.00
Soyabean oil	1.13	1.88	2.19	2.78
Limestone	0.80	0.81	0.74	0.79
Lysine sulphate	0.40	0.41	0.39	0.39
Methionine	0.35	0.29	0.23	0.23
Monocalcium phosphate	0.30	0.20	0.10	0.10
Salt	0.18	0.19	0.18	0.18
Treonine	0.13	0.08	0.06	0.06
Vitamin premix[1]	0.10	0.10	0.10	0.10
Mineral premix[2]	0.10	0.10	0.10	0.10
Choline chloride	0.08	0.08	0.08	0.08
Anticoccidial[3]	0.05	0.00	0.00	0.00
Chemical composition (Analysed values)				
ME[4] (kcal/kg)	3030	3124	3160	3223
Crude protein (%)	23.27	22.16	20.99	20.12
Calcium (%)	0.98	0.93	0.90	0.87
Total phosphorus (%)	0.68	0.66	0.63	0.63

[1]: Supplied per kg: 11 000 000 IU vitamin A, 3 500 000 IU vitamin D3, 100 g vitamin E, 3 g vitamin K3, 3 g vitamin B1, 6 g vitamin B2, 15 g calcium D-pantothenate, 1 g vitamin B6, 20 mg vitamin B12, 35 g niacin, 1.5 g folic acid, 200 mg D-biotin
[2]: Supplied per kg: 30 g Cu, 120 g Mn, 110 g Zn, 2 g I, 300 mg Se, 50 g Fe
[3]: Salinomycin
[4]: Metabolisable energy content of diets was estimated as stated in Yalçın et al (45)

was measured from the tip of the villi to the villus crypt junction, and crypt depth (CD) was defined as the depth of the invagination between adjacent villi. The ratio of villus height to crypt depth (VH/CD) was calculated.

Blood samples were collected from vena brachialis under the wing from 10 broilers from each group (two from each replicate) at day 39 and centrifuged at 3220 x g for 5 min for collection serum. Levels of triglyceride, total cholesterol, aspartate aminotransferase (AST), alkaline phosphatase (ALP), and alanine aminotransferase (ALT) were determined using an autoanalyzer (Olympus AU400) using their accompanying commercial kits. ELISA kits were used to determine the level of Immunoglobulins (Ig) G (BT-E0019Ch, Bioassay Technology Laboratory, Shanghai, China) and IgA (ab157691, Abcam, Shanghai, China) in blood serum according to the instructions.

Caecal digesta was collected immediately after slaughtering for the determination of total aerobic bacteria (17), *Enterobacteriaceae* (23), and *Lactobacillus* spp. (22). Data were expressed as $\log_{10}$ colony-forming units/g caecal digesta.

The right tibia and femur of broilers were removed, cleaned from all tissue, and then weighed. The lengths of the bones at the distance between the distal and proximal ends and the outer diameter at the narrowest point of the bone shaft were measured by a digital caliper. The breaking strength was determined by 3-point bending with Material Testing Machines (Instron 5944 testing frame, Instron, USA) using Instron Plus software and a standard 2 kN load cell. Distances of 50 mm were established between the two fixed points supporting the bone. The weight load was applied to the midpoint of the shaft under a crosshead speed of 5 mm/min until failure. The Load-Displacement curve was obtained by using the load and displacement values recorded throughout the test. By using the curve obtained, values of stiffness, yield point, elongation at yield point, and maximum load were obtained. The stiffness value was obtained by calculating

the slope of the load-displacement graph. For yield point and yield point elongation values, the load and displacement values at the point that the linear region ends and bone deformation begins were determined. The ultimate load was determined as the maximum load point resisted by the bone sample (33, 38). Bones were prepared for mineral analysis as explained by Yalçın et al. (47). Calcium, phosphorus, magnesium, zinc, and manganese concentrations were determined (9) using ICP-OES (Perkin Elmer OptimaTM DV 2100 Model, Dual View, Perkin Elmer Life and Analytical Sciences).

***Statistical Analysis*:** Data distribution was checked for normality using the Kolmogorov-Smirnov test. The effects of different levels of the EOAs were analysed using One-way ANOVA with five replicates for each dietary treatment. The significance of mean differences between groups was tested by Tukey. Linear effects were determined using polynomial contrasts. Statistical significance was considered at $P \leq 0.05$ (12).

Results

The volatile oil profile of EOAs is detailed in Table 2. It is noteworthy that the volatile oil was particularly rich in carvacrol (40.23%) and linalool (17.59%). The effects of EOAs on various performance parameters are presented in Table 3. Body weight, weight gain, feed intake, FCR, and EPEF values remained largely unaffected by the inclusion of EOAs. However, it is important to note that as the levels of EOAs increased, there was a significant improvement in livability ($P < 0.001$). There were no significant effects of EOAs on carcass yield and the relative weights of various organs (Table 4). Nonetheless, a linear increase ($P = 0.039$) in carcass yield was observed with increasing levels of EOAs.

Table 2. Volatile oil profile of the mixture of essential oils and organic acids (% of volatile oil)

Components	% of volatile oil
Carvacrol	40.23
Linalool	17.59
Diallyl disulphide	10.40
Limonene	10.25
Thymol	7.52
Diallyl trisulphide	5.32
Allyl methyl trisulphide	3.91
p-Cymene	2.41
Allyl methyl disulphide	2.37

Table 3. Effects of combined use of essential oils with organic acids on performance

Parameters	Mixture of essential oils and organic acids (%)			P-value	
	0	0.1	0.2	Combined	Linear
			Body weight (g)		
d 0	43.05±0.52	42.90±0.35	42.95±0.49	0.973	0.880
d 39	2929.20±12.13	2921.72±17.72	2927.82±18.36	0.979	0.972
			Body weight gain (g/bird)		
d 0-13	399.95±5.63	388.44±5.92	388.85±6.29	0.334	0.212
d 14-24	923.12±10.74	881.43±14.99	903.31±12.75	0.116	0.068
d 25-36	1187.30±17.48	1207.90±26.83	1224.71±18.08	0.481	0.237
d 37-39	375.79±10.82	401.06±22.85	368.01±12.29	0.354	0.168
d 0-39	2886.15±12.64	2878.82±20.13	2884.87±19.78	0.953	0.765
			Feed intake (g/bird)		
d 0-13	440.55±12.52	413.53±13.12	422.35±12.27	0.338	0.329
d 14-24	1214.01±16.60	1169.11±13.65	1174.03±10.44	0.078	0.063
d 25-36	2002.76±25.86	2043.21±27.22	2031.52±16.89	0.594	0.485
d 37-39	751.78±11.26	759.87±13.79	754.15±14.68	0.908	0.902
d 0-39	4409.11±46.65	4385.71±56.47	4382.05±31.06	0.915	0.703
			Feed conversion ratio (kg feed intake/kg weight gain)		
d 0-13	1.10±0.03	1.06±0.02	1.09±0.04	0.714	0.767
d 14-24	1.32±0.02	1.33±0.03	1.30±0.01	0.502	0.511
d 25-36	1.69±0.02	1.69±0.03	1.66±0.03	0.637	0.484
d 37-39	2.01±0.06	1.90±0.05	2.06±0.07	0.210	0.548
d 0-39	1.53±0.02	1.52±0.01	1.52±0.01	0.927	0.703
Livability, %	94.64±0.25^{b}	99.00±1.00^{a}	100.00±0.00^{a}	<0.001	<0.001
EPEF, %	465.55±6.75	486.93±4.95	494.49±7.54	0.081	0.033

n: 5, EPEF: European production efficiency factor

^{a,b}: Means within a row with different superscripts differ significantly at $P < 0.05$.

Table 4. Effects of combined use of essential oils with organic acids on carcass yield (%) and relative organ weights (%)

Parameters (%)	Mixture of essential oils and organic acids (%)			P-value	
	0	0.1	0.2	Combined	Linear
Carcass yield	74.52±0.44	74.87±0.31	75.51±0.17	0.108	0.039
Liver	1.93±0.07	2.01±0.05	2.00±0.06	0.552	0.375
Heart	0.54±0.03	0.52±0.02	0.53±0.02	0.823	0.781
Spleen	0.10±0.01	0.11±0.01	0.11±0.01	0.365	0.176
Bursa Fabricius	0.06±0.01	0.07±0.01	0.08±0.01	0.320	0.135
Gizzard	0.99±0.07	1.00±0.06	0.98±0.05	0.965	0.897
Abdominal fat	1.10±0.07	1.08±0.05	1.10±0.06	0.981	0.977

n:10

Table 5. Effects of combined use of essential oils with organic acids on intestinal histomorphology of broilers

Parameters	Mixture of essential oils and organic acids (%)			P-value	
	0	0.1	0.2	Combined	Linear
		Duodenum			
Villus height (µm)	1881.01±48.90	1855.95±39.42	1855.31±38.30	0.931	0.742
Crypth depth (µm)	131.31±5.00	145.69±7.89	150.88±7.17	0.128	0.052
Villus width (µm)	224.11±6.99	201.47±7.00	220.80±7.85	0.127	0.778
Villus height/crypt depth	14.54±0.71	13.14±0.86	12.59±0.71	0.234	0.101
		Jejunum			
Villus height (µm)	1169.42±70.81	1192.38±68.13	1280.13±71.89	0.510	0.275
Crypth depth (µm)	137.44±7.33	115.08±6.62	116.03±8.64	0.079	0.056
Villus width (µm)	275.61±15.44	290.29±20.45	269.26±15.02	0.677	0.796
Villus height/crypt depth	8.65±0.56^{b}	10.60±0.71^{ab}	11.53±0.93^{a}	0.033	0.011
		Ileum			
Villus height (µm)	821.30±18.83^{b}	952.93±21.56^{a}	899.10±23.06^{b}	0.004	0.038
Crypth depth (µm)	91.51±4.37	102.48±3.56	100.50±4.78	0.173	0.148
Villus width (µm)	163.94±7.72^{b}	212.25±10.11^{a}	209.96±9.94^{a}	0.018	0.015
Villus height/crypt depth	9.14±0.44	9.34±0.23	9.19±0.63	0.953	0.931

n:10
^{a,b}: Means within a row with different superscripts differ significantly at $P < 0.05$.

The influence of EOAs on intestinal morphology is summarized in Table 5. VH/CD ratio in the jejunum, villus height, and villus width in the ileum exhibited improvements with the addition of EOAs ($P < 0.05$). In contrast, the values of villus height, crypt depth, villus width, and the VH/CD ratio in the duodenum remained unaffected by the addition of EOAs.

Table 6 provides data on the microbial populations in the caecum and blood serum parameters. No significant differences were observed in the total aerobic bacteria in the caecum among the groups. However, the inclusion of EOAs in the diets led to an increase in *Lactobacillus* spp. count ($P = 0.048$) and a decrease in *Enterobacteriaceae* count ($P = 0.009$) in the caecum. Blood serum levels of triglycerides, total cholesterol, and the activities of ALT, ALP, and AST were not significantly affected by the inclusion of EOAs. Notably, there were significant increases in serum IgA levels ($P < 0.001$) and IgG levels ($P = 0.005$) with increasing levels of EOAs in the diets.

Table 7 presents data on the tibia and femur characteristics. Wet weight, length and diameter, and yield load displacement values of the tibia and femur were not significantly influenced by the use of EOAs. However, it was observed that ultimate load ($P = 0.002$), yield load ($P = 0.001$), and stiffness ($P = 0.001$) values were significantly higher with the inclusion of 0.2% EOAs in the diets compared to the other groups in the tibia. In the femur, the ultimate load and yield load values were significantly increased with both levels of inclusion in the diets compared to the control group ($P < 0.001$). Moreover, linear increases ($P < 0.05$) were observed in ash content and the levels of calcium, phosphorus, zinc, and manganese in both tibia and femur bones due to the inclusion of EOAs. Magnesium levels in bones, however, were not affected by the inclusion of EOAs.

Table 6. Effects of combined use of essential oils with organic acids on caecal microorganisms and some blood biochemical indices in broilers

	Mixture of essential oils and organic acids (%)			P-value	
	0	0.1	0.2	Combined	Linear
Caecal microorganisms (log_{10}cfu/g)					
Total aerobic bacteria	6.83±0.09	6.82±0.08	6.83±0.09	0.992	0.955
Lactobacillus	6.32±0.15^{b}	6.75±0.15^{a}	6.81±0.14^{a}	0.048	0.023
Enterobacteriaceae	6.47±0.09^{a}	6.03±0.14^{b}	5.98±0.10^{b}	0.009	0.005
Blood serum biochemical indices					
Total cholesterol (mg/dl)	215.40±10.43	203.10±6.82	193.80±8.61	0.234	0.092
Triglyceride (g/l)	10.80±0.84	9.50±0.83	9.40±0.78	0.414	0.236
ALT (U/l)	18.90±1.19	18.40±1.30	18.80±0.77	0.945	0.950
ALP (U/l)	258.40±17.91	270.00±16.83	259.30±16.45	0.868	0.971
AST (U/l)	121.20±9.16	129.10±8.50	123.30±9.75	0.820	0.872
IgA (mg/dl)	56.00±2.10^{b}	70.30±3.19^{a}	73.10±2.90^{a}	<0.001	<0.001
IgG (mg/dl)	135.30±7.20^{b}	160.40±5.53^{a}	164.90±5.90^{a}	0.005	0.002

n:10
^{a,b}: Means within a row with different superscripts differ significantly at $P < 0.05$.

Table 7. Effects of combined use of essential oils with organic acids on bone parameters in broilers

	Mixture of essential oils and organic acids (%)			P-value	
	0	0.1	0.2	Combined	Linear
Tibia					
Wet weight (g)	15.23±0.37	14.50±0.34	14.81±0.39	0.377	0.429
Length (mm)	99.40±0.97	98.26±0.81	97.41±0.51	0.217	0.084
Diameter (mm)	9.17±0.16	9.17±0.14	9.45±0.29	0.553	0.354
Ultimate load (N)	307.91±14.54^{b}	332.68±12.60^{b}	387.15±15.82^{a}	0.002	0.001
Yield load (N)	261.15±15.84^{b}	294.64±12.83^{b}	343.27±12.33^{a}	0.001	<0.001
Yield load displacement (mm)	2.09±0.14	2.32±0.13	2.37±0.12	0.269	0.135
Stiffness (N/mm)	124.80±5.40^{b}	125.27±5.45^{b}	157.79±7.48^{a}	0.001	0.001
Ash (%DM)	48.29±0.22^{b}	49.12±0.23^{a}	49.45±0.24^{a}	0.005	0.002
Calcium (g/kg DM)	215.10±3.29	220.63±3.21	225.07±2.53	0.084	0.028
Phosphorus (g/kg DM)	106.63±1.04^{c}	110.20±1.18^{b}	113.83±0.99^{a}	<0.001	<0.001
Magnesium (g/kg DM)	4.94±0.08	5.07±0.07	5.05±0.10	0.505	0.375
Zinc (mg/kg DM)	153.91±4.89^{b}	175.81±4.86^{a}	189.44±4.99^{a}	<0.001	<0.001
Manganese (mg/kg DM)	4.36±0.11^{c}	5.06±0.13^{b}	5.76±0.15^{a}	<0.001	<0.001
Femur					
Wet weight (g)	11.39±0.23	11.14±0.24	11.50±0.31	0.619	0.764
Length (mm)	73.98±0.71	72.75±0.25	72.55±0.87	0.277	0.140
Diameter (mm)	10.00±0.15	9.88±0.10	9.71±0.22	0.458	0.218
Ultimate load (N)	255.52±9.04^{b}	296.63±5.99^{a}	320.83±8.18^{a}	<0.001	<0.001
Yield load (N)	231.69±9.04^{b}	264.21±7.42^{a}	293.63±8.77^{a}	<0.001	<0.001
Yield load displacement (mm)	2.83±0.17	2.97±0.08	2.99±0.07	0.567	0.324
Stiffness (N/mm)	81.79±3.67^{b}	89.65±2.25^{ab}	97.02±3.51^{a}	0.009	0.002
Ash (%DM)	47.12±0.28^{b}	48.22±0.25^{a}	48.25±0.27^{a}	0.008	0.006
Calcium (g/kg DM)	210.85±2.12^{b}	215.28±2.31^{ab}	220.22±1.99^{a}	0.017	0.005
Phosphorus (g/kg DM)	104.43±1.36^{b}	108.00±1.36^{ab}	111.63±1.30^{a}	0.003	0.001
Magnesium (g/kg DM)	4.82±0.05	4.87±0.06	4.84±0.09	0.878	0.917
Zinc (mg/kg DM)	160.46±4.49^{c}	181.66±3.46^{b}	195.29±3.52^{a}	<0.001	<0.001
Manganese (mg/kg DM)	4.16±0.12^{c}	4.71±0.15^{b}	5.51±0.13^{a}	<0.001	<0.001

n:10, DM: dry matter ^{a,b,c}: Means within a row with different superscripts differ significantly at $P < 0.05$.

Discussion and Conclusion

Numerous studies have investigated the effects of OAs and EOs when used as feed additives in poultry diets, and these additives have shown positive effects on various aspects of poultry production (1, 25, 40, 41). However, in the present study, no statistically significant differences were observed in body weight, weight gain, feed intake, and FCR among groups that received different levels of EOAs. A noteworthy finding was the significant increase in livability percentages in the groups that received EOAs at 0.1% and 0.2% levels compared to the control group (EOA0), with ($P < 0.001$). This suggests that the inclusion of EOAs in the diets improved livability, although it may not have been at a sufficient level to affect body weight gain and feed efficiency. Similar findings were reported by Kaya and Tuncer (28) and Fascina et al. (16), where growth performance and FCR were not affected by EOAs supplementation. Liu et al. (30) revealed that there were no significant differences in feed intake, weight gain, and FCR among the three groups during the first stage (days 0-21) using the protected EOAs, however, reduced feed intake and improved FCR at 22-42 days of age. Basmacıoğlu-Malayoğlu et al. (8) indicated that EOAs (formic acid, propionic acid, oregano, clove, cumin) did not affect feed intake but had positive effects on body weight gain and feed efficiency in broilers. In another study (21) body weight and weight gain were not affected, while FCR was significantly worsened when the diet was supplemented with a combination of plant extracts and organic acid salts compared to a control diet.

Consistent with our findings, Fascina et al. (16) indicated that EOAs (lactic acid, benzoic acid, formic acid, acetic acid, citric acid, citrus extract, turmeric extracts, grape seed extract + Chinese cinnamon essential oil, fenugreek seeds, Chile Boldo leaves) supplementation did not affect productive efficiency index. EOAs (benzoic acid, thymol, eugenol, piperine) supplementation (5) and different Ps (thymol and cinnamaldehyde, cumin, mint, cloves, and anise or thymol) inclusion (24) significantly increased EPEF. The variability in these findings may be attributed to differences in the chemical composition and dosages of EOs, OAs, and EOAs, as well as differences in diet formulation, diet composition, poultry breed, age, health status, and environmental conditions (8, 18, 34).

In the present experiment, the effects of EOAs on carcass yield showed no significant differences among groups. However, a significant linear increment ($P = 0.039$) in carcass yield was observed with increasing levels of EOAs. The relative weight percentages of abdominal fat, liver, heart, spleen, bursa Fabricius, and gizzard were not significantly affected by the inclusion of EOAs. These results are in line with previous studies (8, 15, 28) where carcass yield and relative organ weight percentages were not significantly affected by EOAs supplementation. Similarly, Dong et al. (14) reported that the percentages of liver, bursa of Fabricius, and abdominal fat were not affected but spleen index value was increased with the usage of EOA. The immune system, in conjunction with lymphoid tissue and immune cells, is largely composed of immune organs. These immune organs, including the thymus, spleen, and bursa Fabricius, are typically responsible for the generation, proliferation, differentiation, and maturation of immune cells (10). In line with the findings of the present study, Liu et al. (30) similarly reported that the usage of protected EOAs did not yield significant differences in the immune organ indexes, specifically the spleen and bursa indexes.

In the present study, the improved VH/CD ratio in the jejunum and enhanced VH in the ileum with the addition of EOAs suggest an improvement in the digestion and absorption efficiency of the diet. The increased counts of *Lactobacillus* spp. ($P = 0.048$) and decreased counts of *Enterobacteriaceae* ($P = 0.009$) in the caecum indicate that the inclusion of EOAs positively affected the intestinal microbiota. These changes in intestinal morphology and microbial populations may have contributed to better digestibility and a reduction in pathogenic coliforms.

Yang et al. (49) found that EOA supplementation during the finisher period increased VH and muscular layer thickness in the duodenum, as well as improved VH and the VH/CD ratio in the jejunum. Similarly, Basmacıoğlu-Malayoğlu et al. (8) reported a significant increase in VH in both the jejunum and ileum following EOA supplementation. Pham et al. (34) also demonstrated that adding different levels of EOAs to broiler diets significantly reduced CD and improved the VH/CD ratio, particularly in the context of a necrotic enteritis challenge. Additionally, Liu et al (29) noted that EOAs enhanced intestinal VH due to an increase in goblet cell content. However, Liu et al. (30) reported that dietary EOA supplementation had no effect on duodenal and ileal morphology at 21 and 42 days, as well as jejunal morphology at 21 days, but significantly increased VH and CD in the jejunum at 42 days of age. In contrast to the present study, Dong et al. (14) observed that EOA supplementation had no influence on small intestinal morphology.

Consistent with the present study, Yang et al. (48) demonstrated that diets supplemented with EOAs enhanced the regenerative capacity of epithelial cells, leading to improved intestinal absorptive capacity, primarily due to a reduction in *E.coli* populations in the ileal contents at 42 d of age. Furthermore, EOAs were found to be effective in protecting the intestinal mucosa, which plays a critical role in safeguarding animals against microbial infections, as noted by Stefanello et al. (40). Giannenas et al (18) reported that a combination of benzoic acid and essential oils improved growth performance, reduced the pH levels in caecal contents, increased lactic acid bacteria populations, and decreased coliform bacteria in the caecum of turkey poults. Several

authors (2, 11, 30) have also suggested that supplementation of EOs, OAs, or EOAs can increase the proportion of *Lactobacillus* spp. in chickens. Similarly, Dong et al. (14) reported that EOA supplementation significantly decreased *E. coli* counts while increasing *Lactobacillus* in excreta. The antibacterial effects of organic acids and essential oils were shown to be synergistic. Essential oils, due to their high hydrophobicity, increase bacterial membrane permeability, allowing more organic acids in their undissociated form to penetrate the bacterial cytoplasm. This process ultimately leads to the death of pH-sensitive bacteria such as *E.coli* (14). In contrast, Pham et al. (34) reported that dietary EOAs did not affect *E.coli* and *Lactobacillus* counts in the caecum.

The antibacterial effects of EOAs against pathogenic bacteria have been explained by various researchers (8, 27) through three main hypotheses: a) the membrane-damaging effect of EOs may render bacteria more susceptible to acidic environments, b) EOs exhibit increased hydrophobicity or antilisterial activity at low pH, making them more soluble in the lipids of bacterial cell membranes, and c) OAs appear to be particularly effective in the feed, crop, and gizzard, while EOs seem to work more efficiently in the lower segments of the intestinal tract.

Another significant finding in the present study is the increase in serum IgA ($P < 0.001$) and IgG ($P=0.005$) levels with higher levels of EOAs in the diet. This suggests that EOAs may have immunomodulatory effects. Both IgA and IgG play crucial roles in the immune system, with IgG being responsible for neutralizing pathogens, toxins, and viruses (39), while IgA serves as a reliable serum biomarker for assessing intestinal inflammation (35). Dong et al. (14) reported that EOA supplementation increased IgA and IgM levels, contributing to enhanced immune status. The rise in serum IgA may be linked to the potential stimulation of B and T lymphocytes (14). Additionally, supplementation with a high level of mixed OAs (including formic acid, ammonium formate, propionic acid, acetic acid, lactic acid, malic acid, citric acid) increased serum IgA levels but had no effect on IgG in broilers aged 42 days of age (31).

In the current study blood serum levels of total cholesterol, triglycerides, and the enzyme activities of ALT, ALP, and AST were not affected by the inclusion of EOAs in the diet. The activities of ALT, AST, and ALP in the blood serve as indicators of liver integrity (3). The findings suggest that EOAs did not have any detrimental effect on liver function. Similarly, Iqbal et al. (20) reported that serum cholesterol levels in the EOA-supplemented group (containing oregano, rosemary, cinnamon, chili pepper extract, and sodium diformate) were comparable to those in the negative control group. Kaya and Tuncer (28) also found that serum triglyceride and cholesterol levels remained unchanged with EOA supplementation. However, Liu et al. (29) observed a significant reduction in triglyceride concentration, while cholesterol levels were unaffected by EOA inclusion. Dong et al (14) reported that EOA treatment significantly decreased the serum total cholesterol levels but had no effect on serum triglyceride levels. Ajibaiye et al. (3) found that different levels of EOAs supplementation had no impact on serum ALT and ALP levels but led to a reduction in serum AST levels at certain supplementation levels. Additionally, Yalçın et al. (46) found that serum cholesterol concentration increased with different levels of lactic acid supplementation in quail fattening, while triglyceride levels remained unchanged.

Ultimate load, yield load, and stiffness values were found to be significantly higher at the usage of 0.2% EOA in the diets than those of other groups in the tibia. The ultimate load and yield load values in femur were significantly increased when EOA was used at both levels in diets compared to the control group. With increasing doses of EOAs usage, linear increases were reported in levels of ash, calcium, phosphorus, zinc, and manganese in the tibia and femur bones. In a study conducted by Sevim and Çufadar (36), it was observed that the tibia Ca, Mg, and P contents were higher in the group that was fed the diet supplemented with thyme essential oil as compared to the control group due to the stimulating effect of thyme essential oil on osteoblast proliferation, and thereby potentially influencing tibia mineral concentrations positively. However, it is worth noting that despite these effects on mineral content, thyme essential oil did not have a significant effect on tibia-breaking strength in broilers (36). In another study by Ruff et al. (35), tibia-breaking strength and total ash content were increased significantly with supplementation of EOs (*Lippia origanoides*, *Rosmarinus officinalis*) or EOs+betaine compared to the control group in chickens exposed to cyclic heat stress. Liu et al. (29) reported that EOA supplementation had no effect on length of tibia and femur. The enhancement of bone characteristics and mineralization could be attributed to the specific composition of the EOAs and their interactions with the broilers' metabolism.

In the evaluation of the results, the dietary inclusion of EOAs could positively affect in various aspects of poultry production such as livability, intestinal health, immunity, and bone mineralization. Further research is needed to optimize the use of EOAs and to better understand the underlying mechanisms responsible for the observed effects. Nevertheless, these findings indicate the potential of EOAs as a viable alternative to antibiotic growth promoters in the poultry industry, supporting intestinal health, immunity, and bone mineralization.

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Ethical Statement

All procedures involving animal care, handling and sampling were approved by the Animal Experiments Local Ethics Committee of the Ankara University, Veterinary Faculty, Ankara, Türkiye (2020-2-14).

Conflict of Interest

The authors declare that there are no conflicts of interest associated with this work.

Author Contributions

The study design and the trial were carried out by İO, HE and SY. All authors performed the analysis. The manuscript was written by İO and SY. All authors approved the final version.

Data Availability Statement

The data supporting this study's findings are available from the corresponding author upon reasonable request.

Animal Welfare

The authors confirm that they have adhered to ARRIVE Guidelines to protect animals used for scientific purposes.

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Effect of nanomicelles of *Thymus vulgaris*, *Carum copticum*, *Mentha longifolia*, and *Lavandula angustifolia* essential oils on the performance and health status of suckling calves

Mojtaba ALIPOUR AINUDDIN[1,a], Jamal SEIFDAVATI[1,b,✉], Hossein ABDI BENEMAR[1,c], Reza SEYEDSHARIFI[1,d]

[1]University of Mohaghegh Ardabili, Faculty of Agriculture and Natural Resources, Department of Animal Sciences, Ardabil, Iran.

[a]ORCID: 0009-0003-8162-2855; [b]ORCID:0000-0001-6794-4450; [c]ORCID:0000-0001-5318-4585; [d]ORCID:0000-0003-4593-2058

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✉Corresponding author
jseifdavati@uma.ac.ir

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ABSTRACT

This study investigated the effects of nanomicelles of four essential oils on functional and antioxidant parameters of 48 male Holstein newborn calves. The calves were assigned to six groups and monitored over a 46-day period. The groups included one control group and five treatment groups, each receiving 400 mg of a specific nanomicellized essential oil (NEO) – *Thymus vulgaris* (TNEO), *Carum copticum* (CNEO), *Mentha longifolia* (MNEO), *Lavandula angustifolia* (LNEO) – or a blend of all four (BNEO), administered daily via 8 ml of milk emulsion. No significant differences in average daily weight gain (ADG) were observed among the groups during days 0-14 and 14-32. However, during days 32-46, calves supplemented with TNEO and LNEO showed significantly higher ADG (P=0.004). Calves receiving LNEO exhibited significantly higher blood concentrations of total protein (P=0.022) and albumin (P=0.046) compared to both the control and other treatment groups. Alanine aminotransferase (ALT) levels were lower in the TNEO, LNEO, MNEO, and BNEO groups compared to the control (P=0.012). Blood glutathione peroxidase (GPx) (P=0.009) and superoxide dismutase (SOD) (P=0.001) activities were elevated in the TNEO and BNEO groups. Malondialdehyde (MDA) concentrations were reduced in all NEO-supplemented groups, with the lowest levels observed in the TNEO group (P=0.009). Although the control group exhibited the lowest total antioxidant capacity (TAC), no significant differences were detected among the NEO-treated groups. Overall, these findings suggest that nanomicellized essential oils of *Lavandula angustifolia* and *Thymus vulgaris* confer the most notable benefits to suckling calves, enhancing weight gain, feed consumption, blood parameters, and oxidative stress markers.

Introduction

Although antibiotics are effective in enhancing animal performance and health, their prolonged use raises concern due to the potential development of antibiotic-resistant microorganisms, which can lead to economic losses and treatment challenges. These resistant strains can act as reservoirs for resistance genes, posing a serious risk to public health (28). As a result, there is increasing interest in identifying alternative strategies that provide similar benefits to antibiotics but with fewer side effects and a lower risk of resistance development (39).

Essential oils (EOs) have emerged as promising alternatives due to their well-documented antioxidant, antimicrobial, and anti-inflammatory properties (7). *Thymus vulgaris*, from the Lamiaceae family, is a widely recognized aromatic herb known for its medicinal value, with its EO containing active compounds such as thymol, p-cymene, carvacrol, and γ-terpinene (24). *Lavandula angustifolia*, a perennial evergreen species, produces EO rich in linalool, linalool acetate, lavandolol, and γ-terpineol, exhibiting various biological and therapeutic activities (34). *Carum copticum*, belonging to the

Apiaceae family and the Trachyspermum genus (13), is another medicinal plant whose EO contains thymol, carvacrol, γ-terpinene, and p-cymene as major constituents (14). Similarly, *Mentha longifolia*, a perennial herb from the Lamiaceae family, is valued for its pharmacological properties, with its EO predominantly comprising carovene, limonene, iso-dihydro-carovene, and caryophyllene (9,11).

Despite their beneficial properties, EOs present several challenges, including volatility, hydrophobicity, and susceptibility to degradation by light, oxygen, and heat (21). To overcome these limitations, nanoemulsion-based encapsulation has been developed as a modern strategy to enhance the stability, bioavailability, and solubility of EOs (4, 16). Therefore, the present study aimed to determine the effects of nanomicelles containing essential oils of *Thymus vulgaris*, *Carum copticum*, *Mentha longifolia*, and *Lavandula angustifolia* on growth performance, blood parameters, and antioxidant status in suckling Holstein calves.

Materials and Methods

Preparation and Emulsification of Essential Oils: To create an oil-in-water emulsion, the lipid phase was composed of essential oils (Barij Essence Kashan, Iran), with lecithin serving as the emulsifier. Whey protein (Ehsan Confectionery, Ardabil, Iran) and gum Arabic (Sigma-Aldrich, CAS Number 9000-01-5) were incorporated as stabilizers. The EO blend was prepared by mixing equal proportions of EOs from four medicinal herbs. Nanomicellized essential oil (NEO) formulations were produced according to the method described by Asghari et al. (3). Briefly, the lipid phase was prepared by mixing 25 g of lecithin with 50 g of each essential oil. The mixture was stirred using a magnetic stirrer at 60°C for 10 minutes. In parallel, the aqueous phase was prepared by dissolving 2 g of gum Arabic and 10 g of whey protein in distilled water. This solution was homogenized at 3000 rpm for 15 minutes using a laboratory homogenizer. The lipid phase was then slowly added dropwise to the aqueous phase under gentle stirring. Distilled water was subsequently added to bring the total volume to 1000 ml. The final emulsions were stored in opaque plastic containers, with each milliliter containing 50 mg of essential oil. A control emulsion, identical in composition but lacking essential oils, was also prepared.

Characterization of Nanoemulsions: The droplet size distribution and surface charge density (zeta potential) of the nanoemulsions were analyzed using dynamic light scattering and electrophoretic light scattering techniques, respectively (nanoPartica SZ-100V2 Series, HORIBA Scientific Instrument, Kyoto, Japan). Prior to analysis, the emulsions were diluted 1:100 with distilled water. Measurements were performed in triplicate using three independently prepared samples.

Determination of Antioxidant Activity: The antioxidant activity of the nanoemulsions was assessed using the DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging method (37). A 0.1 mmol DPPH solution was prepared in 99% methanol (Mayer, Mexico). For each test, 1 ml of the DPPH solution was added to 3 ml of 99% methanol in a test tube, followed by the addition of 50 µl of nanoemulsion. The mixture was incubated in the dark at room temperature for 30 minutes. Absorbance was measured at 517 nm using a UV-visible spectrophotometer.

The percentage of free radical scavenging activity was calculated using the following equation:

$$\text{Scavenging activity (\%)} = [(A_0-A_1)/A_0]\times 100$$

Where A_0 is the absorbance of the control (1 ml DPPH + 3.05 ml methanol) and A_1 is the absorbance of the sample. All measurements were performed in triplicate, and results are expressed as mean values.

Emulsion Characteristics: The physical stability of the emulsions was evaluated after 21 days under ambient conditions, and the results are listed in Table 1. Droplet size ranged from 151.9 to 213.9 nm, with a Z-average particle size between 153.8 and 398.4 nm. The zeta potential values varied from -27.0 to -48.6 mV. Results of the DPPH assay showed that nanoemulsions containing essential oils from *Carum copticum* and *Thymus vulgaris* exhibited greater free radical scavenging activity compared to those prepared with *Mentha longifolia* and *Lavandula angustifolia*.

Table 1. Emulsion characteristics and antioxidant activity of nanomicellized essential oils[a]

Traits	TNEO	CNEO	MNEO	LNEO	BNEO
Droplet size (nm)[b]	198.1	188.2	152.4	213.9	151.9
Z-average (nm)	174.8	197.2	398.4	184.7	153.8
Zeta potential (mV)	-27.0	-31.4	-48.6	-33.2	-48.5
Electrophoretic Mobility (cm2/Vs)×10^{-4}	-2.1	-2.4	-3.8	-2.6	-3.7
DPPH radical scavenging activity (%)	74.54	81.48	20.14	14.58	72.11

TNEO= *Thymus vulgaris* nanomicellized essential oil; CNEO= *Carum copticum* nanomicellized essential oil; MNEO= *Mentha longifolia* nanomicellized essential oil; LNEO= *Lavandula angustifolia* nanomicellized essential oil; BNEO= Blend of nanomicellized essential oils. [a]Samples: Dilution 1:100. [b]Measured by dynamic light scattering technique.

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Table 2. The chemical composition of the starter diet, alfalfa hay and milk (dry matter basis).

Item, g/kg	Starter[a]	Alfalfa hay	Milk
Dry matter	912	886	125
Crude protein	194	147	34.20
Ether extract	29.10	23.10	37.10
Neutral detergent fiber	150	561	-
Acid detergent fiber	77	375	-
Calcium	7.00	15.10	-
Phosphorus	5.60	2.90	-

[a] Starter diet contained 410 kg ground corn, 130 kg ground barley, 50 kg wheat bran, 380 kg soybean meal, 5 kg salt, 10 kg calcium carbonate, 5 kg dicalcium phosphate and 10 kg vitamin and mineral premix (Vitamin Premix provided per kg of diet: vit A, 200000 IU; vit D, 300000 IU; vit E, 10000 IU; vit K, 2 mg; Butylated hydroxytoluene 1000 mg/kg. Mineral premix provided per kg of diet: Cu, 3300 mg; Fe, 100 mg; Zn, 16500 mg; Mn, 9000 mg; I, 120 mg; Co, 90 mg; Se, 90 mg.

Experimental Design and Animal Management: A total of 48 male Holstein calves (average body weight: 43.02 ± 3.60 kg; average age: 12.08 ± 4.37 days) were randomly selected from the dairy herd of Moghan Agriculture and Animal Husbandry dairy herd (Parsabad, Ardabil, Iran). The calves were assigned to six experimental groups using a completely randomized design and monitored over a 46-day period. The groups included a control group and five treatment groups receiving 400 mg/day of nanomicellized essential oils: TNEO, CNEO, MNEO, LNEO, or a blend of all four nanomicellized essential oils (BNEO), administered via 8 ml of milk emulsion.

From day 7 of age until weaning on day 46, the specified emulsion dose was added to the calves' morning milk feeding. During the first three days after birth, each calf received 4 kg of colostrum per day via teat bucket. In the subsequent two weeks, they were fed 4 kg of milk daily, divided into two meals at 8:00 and 18:00. From weeks 3 to 5, milk allowance increased to 6 kg per day. In week 6, the amount was reduced to 4 kg per day (split into two feedings), and in week 7, calves received 2 kg of milk only in the morning, and weaning occurred on day 46. Milk was provided in buckets.

From day 7 onward, calves had ad libitum access to a calf starter (Table 1) and fresh water. From day 20, chopped alfalfa was introduced at 10% of the starter feed. Calves were individually housed in 1 × 2.5 m stalls. Bedding was inspected daily, and wet bedding was replaced with clean, dry straw.

Sampling and Analysis: Body weights of the calves were recorded on the first day of the experiment and then on days 14, 32, and 46, prior to the morning meal. The daily feed intake was calculated by subtracting feed refusals from the amount offered the previous day. The chemical composition of milk, starter feed, alfalfa hay, and milk is presented in Table 2. The starter diet and alfalfa hay were analyzed for dry matter (DM: AOAC (2) method 930.15), crude protein (CP: method 984.13, Kjeldahl N×6.25), ether extract (method 920.39), and ash (method 924.05). The fiber insoluble in neutral detergent (NDF) and fiber insoluble in acidic detergent (ADF) were measured according to Van Soest et al. (38). NDF analysis was conducted without the use of heat-stable alpha-amylase but in the presence of sodium sulfite. Calcium and phosphorus concentrations in the starter and hay were measured using atomic absorption spectrophotometry (AA-670, Shimadzu, Tokyo, Japan). Milk composition (DM, CP, and fat) was determined using a milk analyzer (CombiScope FTIR 600/300 Hp - Dairy Analyser, Delta Instruments, Drachten, Netherlands).

Fecal Scoring and Health Monitoring: Fecal consistency was scored daily using the 5-point scale described by Khan et al. (17): score 1: hard and tubular feces; score 2: soft to loose; score 3: loose to watery; score 4: watery with mucus and traces of blood; and score 5: watery with mucus and visible blood. Calf health was assessed daily following the criteria of Lowe et al. (22), including visual evaluation of appearance, nasal and eye secretions, eye depression, and rectal temperature.

Microbiological Analysis: In the third and sixth weeks of the experiment, feces were collected rectally from each calf to assess *Escherichia coli*. Fecal samples were cultured on eosin methylene blue agar for coliform enumeration. Plates were incubated inverted at 37°C for 48 hours. Colonies were counted using a digital colony counter, and results were expressed as $\log_{10}$ colony-forming units per gram of dry matter ($\log_{10}$CFU/g DM).

Blood Sampling and Biochemical Analysis: On day 20, approximately 3 ml of blood was collected from the jugular vein 4 hours post-feeding in the morning. Blood was drawn into tubes with and without sodium heparin (Pars Azmoon Co., Tehran, Iran). Blood samples were centrifuged at 3000 rpm for 15 minutes at 4 °C and were kept at -20°C and thawed at room temperature before analysis. Serum biochemical parameters, including glucose, cholesterol, triglycerides, total protein, albumin,

aspartate aminotransferase (AST), and alanine aminotransferase (ALT), were measured using commercial kits (Pars Azmoon Co., Tehran, Iran). After measuring total hemoglobin, blood samples were centrifuged at 4000 rpm for 10 minutes. The buffy coat and plasma layers were gently separated, and the erythrocytes were washed three times with 0.9% saline solution. Hemolysates were then prepared and used for the analysis of GPx and SOD activities using Ransel and Ransod kits, respectively (Randox Laboratories, Crumlin, UK).

Hematological and Oxidative Status Analysis: Hematological parameters, including red blood cell (RBC) count, hemoglobin concentration, hematocrit (HCT), white blood cell (WBC) count, neutrophils, lymphocytes, monocytes, and eosinophils, were measured using an automatic cell counter (Sysmex XT-2000iV analyzer, Japan) following the manufacturer's protocol with bovine-specific settings.

Serum total antioxidant status (TAS) was assessed using a commercial kit (Randox Laboratories, Crumlin, UK). Serum MDA levels were determined according to the method described by Moore and Robert (26) as an indicator of lipid peroxidation.

Statistical Analysis: Data on average daily gain and feed intake were analyzed using a repeated-measures ANOVA in a completely randomized design. Calf body weight at biweekly intervals was considered a repeated measure using the MIXED procedure of SAS (35). The model included treatment, time, and their interaction as fixed effects, with individual calf as a random effect. Non-significant interactions were removed from the final model. Time had a significant effect on all performance parameters ($P < 0.01$).

Blood samples were analyzed using a completely randomized design with the General Linear Model (GLM) procedure in SAS, considering the different levels of emulsified essential oils as the treatment effect. Initial body weight was included as a covariate in all models. Differences were considered statistically significant at $P \leq 0.05$, and trends toward significance were noted when $P < 0.10$.

Results

As shown in Table 3, supplementation of milk with NEOs did not significantly influence the final body weight of calves. There were no significant differences in ADG among treatment groups during days 0–14 and 14–32. However, during the 32–46 day period, calves receiving TNEO and LNEO exhibited significantly higher ADG compared to the other groups ($P = 0.004$). Additionally, the total ADG over the experimental period was significantly higher in calves supplemented with LNEO, TNEO, and BNEO than in the control group ($P = 0.018$), with the highest value observed in the LNEO group.

Table 3. Effects of different nanomicellized essential oils on feed intake and growth performance of suckling calves.

Traits	CON	TNEO	CNEO	MNEO	LNEO	BNEO	SEM	P-value
Initial weight (kg)	43.9	43.7	43.7	43.7	43.8	43.7	1.78	1.000
Final weight (kg)	65.9	70.7	69.2	67.3	71.8	69.9	2.03	0.374
ADG (g/d)								
0-14 days	337.2	390.5	433.3	378.6	402.9	394.0	0.03	0.394
14-32 days	512.5	587.5	565.0	532.5	638.0	615.8	0.04	0.243
32-46 days	^{b}612.5	^{a}808.3	^{b}677.8	^{b}643.1	^{a}805.0	^{b}698.6	0.03	0.004
Total	^{c}523.4	^{ab}640.9	^{abc}607.1	^{bc}563.5	^{a}668.1	^{ab}624.2	0.03	0.018
Average daily feed intake (g/d)								
0-14 days	146.6	144.3	169.0	154.3	167.5	176.9	0.02	0.780
14-32 days	377.9	379.5	392.7	362.9	416.6	435.5	0.04	0.816
32-46 days	^{ab}944.0	^{ab}927.9	^{b}813.8	^{ab}910.9	^{a}1039.4	^{ab}953.1	0.07	0.032
Total	489.5	483.9	458.5	476.0	541.2	521.8	0.03	0.595
FCR								
0-14 days	0.44	0.39	0.40	0.41	0.42	0.49	0.06	0.883
14-32 days	0.74	0.65	0.72	0.69	0.69	0.69	0.07	0.961
32-46 days	^{b}1.54	^{a}1.16	^{a}1.22	^{ab}1.43	^{ab}1.30	^{ab}1.34	0.08	0.038
Total	^{b}0.94	^{a}0.76	^{a}0.76	^{ab}0.84	^{ab}0.82	^{ab}0.83	0.04	0.083

CON = control diet with no additive; TNEO= *Thymus vulgaris* nanomicellized essential oil; CNEO= *Carum copticum* nanomicellized essential oil; MNEO= *Mentha longifolia* nanomicellized essential oil; LNEO= *Lavandula angustifolia* nanomicellized essential oil; BNEO= Blend of nanomicellized essential oils. ADG = average daily gain; FCR = feed conversion ratio calculated as feed/gain without considering the consumed milk. SEM = standard error of the mean. $^{a-e}$Values within a row with different superscripts differ significantly at $P < 0.05$

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Table 4. Effects of different nanomicellized essential oils on blood metabolites and enzymes.

Traits	CON	TNEO	CNEO	MNEO	LNEO	BNEO	SEM	P-value
Glucose, mg/dl	116.00	113.17	119.17	111.83	126.83	116.17	9.24	0.992
Cholesterol, mg/dl	114.00	107.67	138.83	115.00	124.83	124.17	10.86	0.453
Triglyceride, mg/dl	35.33	29.33	28.17	34.67	35.50	31.17	3.37	0.154
Total protein, g/dl	6.25^{b}	6.47^{b}	6.47^{b}	6.30^{b}	7.10^{a}	6.67^{ab}	0.19	0.022
Albumin, g/dl	2.95^{b}	2.92^{b}	3.00^{ab}	2.90^{b}	3.22^{a}	2.88^{b}	0.08	0.046
AST, U/ml	53.83	50.50	50.00	58.50	39.50	52.83	6.54	0.722
ALT, U/ml	13.17^{b}	10.17^{a}	11.83^{ab}	10.33^{a}	9.83^{a}	10.50^{a}	0.65	0.012

CON = control diet with no additive; TNEO= *Thymus vulgaris* nanomicellized essential oil; CNEO= *Carum copticum* nanomicellized essential oil; MNEO= *Mentha longifolia* nanomicellized essential oil; LNEO= *Lavandula angustifolia* nanomicellized essential oil; BNEO= Blend of nanomicellized essential oils. AST = Aspartate aminotransferase, ALT = Alanine aminotransferase. SEM = standard error of the mean. $^{a-e}$Values within a row with different superscripts differ significantly at $P < 0.05$

Table 5. Effects of different nanomicellized essential oils on blood oxidative parameters and antioxidant status.

Traits	CON	TNEO	CNEO	MNEO	LNEO	BNEO	SEM	P-value
GPx, U/g Hb*	65.00^{b}	79.10^{a}	69.85^{ab}	72.28^{ab}	73.92^{ab}	76.05^{a}	3.11	0.009
SOD, U/g Hb	1067.78^{b}	1339.25^{a}	1324.58^{a}	1292.40^{a}	1274.87^{a}	1305.13^{a}	55.29	0.001
MDA, mmol/l	2.50^{b}	0.97^{a}	1.75^{ab}	2.03^{b}	1.65^{ab}	1.65^{ab}	0.32	0.009
TAC, mmol/l	0.38^{b}	0.47^{a}	0.49^{a}	0.55^{a}	0.48^{a}	0.48^{a}	0.03	0.002

CON= control diet with no additive; TNEO= *Thymus vulgaris* nanomicellized essential oil; CNEO= *Carum copticum* nanomicellized essential oil; MNEO= *Mentha longifolia* nanomicellized essential oil; LNEO= *Lavandula angustifolia* nanomicellized essential oil; BNEO= Blend of nanomicellized essential oils. GPx= Glutathione peroxidase, SOD= Superoxide dismutase, MDA= Malondialdehyde, TAC= Total antioxidant capacity. *Hb= Hemoglobin. SEM = standard error of the mean. $^{a-e}$Values within a row with different superscripts differ significantly at $P < 0.05$

Feed intake did not differ significantly among groups during days 0–14 and 14–32. However, from days 32 to 46, feed intake was significantly increased in calves supplemented with NEOs, with the LNEO group showing the highest consumption ($P = 0.032$). Furthermore, supplementation with NEOs significantly improved the feed conversion ratio (FCR) during both the 32–46 day period and the overall trial period. Specifically, calves in the CNEO and TNEO groups had lower FCRs compared to control calves ($P = 0.038$).

As presented in Table 4, blood concentrations of glucose, cholesterol, and triglycerides were not significantly affected by NEO supplementation. However, calves receiving LNEO exhibited significantly higher total protein levels compared to those in the control, TNEO, CNEO, and MNEO groups ($P = 0.022$). Similarly, LNEO supplementation led to a significant increase in blood albumin levels ($P = 0.046$). While NEO supplementation did not significantly alter AST levels, ALT concentrations were significantly lower in calves receiving TNEO, LNEO, MNEO, and BNEO compared to the control group ($P = 0.012$).

Dietary supplementation with NEOs significantly enhanced the activities of glutathione peroxidase (GPx) and superoxide dismutase (SOD) enzymes (Table 5). GPx activity was significantly higher in the TNEO and BNEO groups compared to the control group ($P = 0.009$), and all NEO-supplemented groups showed significantly higher SOD activity than the control ($P = 0.001$). Moreover, NEO supplementation reduced serum MDA concentrations, with the lowest MDA level recorded in the TNEO group ($P = 0.009$). Total antioxidant capacity (TAC) of the blood was also significantly improved by NEO supplementation ($P = 0.002$); control calves had the lowest TAC values, while no significant differences were observed among the NEO-treated groups.

There were no significant differences among experimental groups in terms of red blood cell (RBC) count, white blood cell (WBC) count, or hemoglobin (Hgb) concentration (Table 6). Similarly, differential counts of immune cells—including neutrophils, lymphocytes, monocytes, eosinophils, and basophils—were not significantly affected by NEO supplementation.

Calves' general appearance, nasal secretions, rectal temperature, and fecal *Escherichia coli* counts were not significantly influenced by dietary NEOs. However, fecal consistency scores were significantly affected by NEO supplementation, with treated calves displaying higher fecal consistency scores compared to the control group ($P=0.032$).

Table 6. Effects of different nanomicellized essential oils on hematological parameters.

Traits	CON	TNEO	CNEO	MNEO	LNEO	BNEO	SEM	P-value
RBC (10^6/ul)	6.96	7.03	7.58	7.17	7.59	7.58	0.63	0.953
Hgb (g/dl)	7.55	7.28	7.22	8.22	7.83	8.05	0.75	0.912
WBC (10^3/ul)	7.02	8.22	7.51	8.94	7.77	7.13	1.00	0.757
Neutrophil (%)	45.54	37.66	39.42	43.61	39.21	38.65	4.83	0.829
Lymphocyte (%)	52.29	60.22	58.41	54.66	58.72	59.16	4.86	0.845
Monocyte (%)	0.93	1.21	1.19	0.93	1.08	1.35	0.31	0.921
Eosinophil (%)	0.91	0.70	0.73	0.59	0.61	0.70	0.21	0.907
Basophils (%)	0.31	0.22	0.24	0.22	0.39	0.15	0.12	0.762

CON = control diet with no additive; TNEO= *Thymus vulgaris* nanomicellized essential oil; CNEO= *Carum copticum* nanomicellized essential oil; MNEO= *Mentha longifolia* nanomicellized essential oil; LNEO= *Lavandula angustifolia* nanomicellized essential oil; BNEO= Blend of nanomicellized essential oils. RBC = Red Blood Cell, Hgb= Hemoglobin, Hct= Hematocrit, WBC= White blood cell. SEM = standard error of the mean.

Table 7. Effects of different nanomicellized essential oils on health indicators of suckling calves.

Traits	CON	TNEO	CNEO	MNEO	LNEO	BNEO	SEM	P-value
Appearance	0.00	0.07	0.03	0.10	0.03	0.07	0.042	0.258
Eye discharge	1.01	0.90	0.70	0.63	0.77	1.07	0.227	0.518
Nasal discharge	0.00	0.03	0.10	0.03	0.00	0.03	0.043	0.287
Rectal temperature (°C)	38.95	38.90	39.02	38.97	38.83	38.80	0.078	0.606
Fecal consistency	2.73^{a}	1.92^{ab}	1.76^{b}	1.73^{b}	1.54^{b}	1.94^{ab}	0.120	0.032
Feces microorganisms, log10 CFU/g dry matter								
Escherichia coli count	6.48	6.40	6.26	5.84	6.40	6.53	0.258	0.776

CON = control diet with no additive; TNEO= *Thymus vulgaris* nanomicellized essential oil; CNEO= *Carum copticum* nanomicellized essential oil; MNEO= *Mentha longifolia* nanomicellized essential oil; LNEO= *Lavandula angustifolia* nanomicellized essential oil; BNEO= Blend of nanomicellized essential oils. SEM = standard error of the mean. $^{a-e}$Values within a row with different superscripts differ significantly at $P < 0.05$

Discussion and Conclusion

Essential oils, as secondary metabolites derived from medicinal plants, have attracted considerable interest in animal nutrition due to their broad-spectrum biological activities, including antibacterial, antifungal, anticoccidial, and antioxidant effects (40). Nevertheless, their practical use in livestock production remains limited due to their lipophilic nature and low water solubility, particularly in liquid feed systems such as milk (5). Nanoemulsion technology offers a promising approach to overcome these limitations by enhancing EO solubility, stability, and bioavailability. It also protects active compounds from degradation and increases cellular uptake, thereby improving antimicrobial and antioxidant efficacy (10).

In this study, EO-based nanoemulsions were successfully formulated with droplet sizes ranging from 151.9 to 213.9 nm, which fall within the accepted nanometric scale (20–200 nm) (15). The zeta potential values of these emulsions ranged from –0.27 to –48.6 mV. Emulsions with absolute zeta potential values greater than 30 mV are typically considered physically stable due to electrostatic repulsion between particles (8). These physical properties suggest that the formulated NEOs were suitably stable for oral administration in milk.

Essential oils (EOs) derived from medicinal plants are complex mixtures, primarily composed of terpenoids along with smaller amounts of non-terpenoid compounds. These constituents are well recognized for their antibacterial, anti-inflammatory, and antioxidant activities. Although the antioxidant potential of EOs has been widely documented, their anti-inflammatory and antihypertensive effects are often attributed to these antioxidant mechanisms (6). However, conventional chemical assays that assess redox activity—such as the DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging method—may not accurately represent the true antioxidant capacity of EOs in biological systems. The DPPH assay relies on the neutralization of a stable free radical and is frequently used to evaluate the *in vitro* antioxidant potential of plant extracts, including EOs (37). In the present study, *Lavandula angustifolia* and *Mentha longifolia* EOs demonstrated relatively low DPPH scavenging activity. The relatively low antioxidant activity observed for *Lavandula angustifolia* and *Mentha longifolia* essential oils in the present study aligns with the findings of Chen et al. (6), who reported that the DPPH radical scavenging capacity of essential oils is largely influenced by their chemical composition. Specifically, essential oils rich in thymol and eugenol exhibit strong

antioxidant activity in DPPH assays, whereas those dominated by linalool and menthol—such as *L. angustifolia* and *M. longifolia*—demonstrate comparatively weaker radical scavenging potential.

The present study demonstrated improved ADG in calves supplemented with NEOs, consistent with previous findings (3, 33). In particular, supplementation with *Lavandula angustifolia* and *Mentha longifolia* NEOs resulted in significantly higher ADG compared to other treatment groups. These findings are in agreement with Pawar et al. (30), who reported increased weight gain in buffalo calves fed *Carum copticum* seed oil. Similarly, Asghari et al. (3) showed that emulsified blends of essential oils derived from *Thymus vulgaris*, *L. angustifolia*, *Salvia officinalis*, and *Capparis spinosa* improved growth performance in suckling dairy calves. Moreover, Pawar et al. (30) observed enhanced dry matter intake in calves receiving *C. copticum* EO, supporting the potential of EO supplementation in improving feed efficiency and weight gain.

In line with the performance data, NEO supplementation was associated with reductions in blood levels of hepatic enzymes, particularly ALT and, to a lesser extent, AST. Elevated levels of AST and ALT are widely recognized biomarkers of hepatic stress or damage (23, 31). While AST levels were not significantly affected by treatment, ALT concentrations were significantly lower in NEO-supplemented calves than in controls. Given that ALT activity is more specific to hepatocellular damage due to its higher abundance in liver cells compared to AST, it serves as a more sensitive indicator of liver health (25). The reduction in liver enzyme levels in NEO-fed calves is consistent with the hepatoprotective effects of essential oils, which are largely attributed to their antioxidant activity (32, 36). These findings are further supported by previous *in vivo* and *ex vivo* studies demonstrating the protective effects of *L. angustifolia* EO on hepatic cells in both rodent and human models (18, 36). The superior growth performance observed in the LNEO group may thus reflect improved liver function and reduced oxidative stress.

Although NEO supplementation did not significantly alter blood concentrations of glucose, cholesterol, or triglycerides, a notable increase in total protein and albumin levels was observed in the LNEO group. The liver is the primary site for the synthesis of albumin and other serum proteins, and elevated levels of these biomarkers are indicative of enhanced liver function and metabolic health. Therefore, the increased blood protein concentrations in calves receiving LNEO may further support the notion of improved hepatic integrity and overall physiological status in this group.

SOD and GPx are key enzymatic antioxidants that play a central role in cellular defense against oxidative stress by scavenging reactive oxygen species (ROS) and neutralizing free radicals (20). Oxidative stress arises when the equilibrium between pro-oxidant and antioxidant systems is disturbed, leading to the accumulation of oxidative damage markers such as lipid peroxides in tissues (19). In the present study, calves supplemented with NEOs exhibited elevated blood levels of SOD and GPx, suggesting an enhancement of systemic antioxidant capacity and a reduction in oxidative stress. Among the treatment groups, the highest SOD and GPx activities were observed in calves receiving nanoemulsions of TNEO and CNEO. These increases were consistent with lower MDA concentrations and higher TAC in blood, as corroborated by the DPPH radical scavenging assay. The findings reflect the potent antioxidant properties of *T. vulgaris* and *C. copticum* essential oils, particularly in their nanoemulsified forms, which enhance their bioavailability and efficacy. These observations align with previous results reported by Asghari et al. (3), who demonstrated that supplementation with emulsified essential oils in suckling calves reduced lipid peroxidation (as indicated by decreased MDA levels) and elevated systemic antioxidant capacity (increased TAC). In addition to their direct antioxidant effects as exogenous radical scavengers (1), essential oils have also been shown to modulate the expression of genes involved in xenobiotic metabolism and endogenous antioxidant defense systems (27), further contributing to their protective role against oxidative stress at the molecular level.

The gastrointestinal tract of neonatal animals is colonized by *Escherichia coli* (E. coli) shortly after birth through environmental exposure. These bacteria constitute a significant component of the commensal intestinal microbiota throughout the animal's life. Although many *E. coli* strains are of low pathogenicity, they can act as opportunistic pathogens, causing extraintestinal infections such as wound infections, pneumonia, meningitis, and septicemia under certain conditions (29). In the present study, the absence of significant changes in fecal *E. coli* counts following NEO supplementation was consistent with the lack of significant differences in clinical health parameters, including general appearance, nasal secretions, and rectal temperature. This suggests that while NEOs may not drastically alter gut microbial load, they contribute positively to health status through their antioxidative, antimicrobial, and anti-inflammatory properties (7). Furthermore, the enhanced ADG observed in NEO-supplemented calves may be attributed to several mechanisms, including stimulation of antioxidant and anti-inflammatory pathways (12), preservation of liver integrity, modulation of metabolism (29), and potentially increased fecal shedding of *E. coli* as reported in previous

studies (3). Collectively, these findings support the potential of essential oils, particularly in nanoemulsified form, as viable alternatives to conventional antibiotics in livestock production.

In conclusion, this study evaluated the effects of essential oils (EOs) from four medicinal plants formulated into a water-soluble nanomicellized form and administered via milk to suckling calves. Supplementation with nanoemulsified essential oils from *Lavandula angustifolia*, *Mentha longifolia*, and a four-plant blend significantly improved ADG, with the highest growth performance observed in the *L. angustifolia* NEO (LNEO) group. LNEO supplementation also led to increased serum levels of total protein and albumin, alongside a reduction in liver enzyme concentrations (ALT and AST), suggesting enhanced liver function. Additionally, all NEO treatments—most notably those containing *Thymus vulgaris* EO—enhanced antioxidant defense, as evidenced by elevated blood levels of GPx, SOD, and TAC, along with decreased MDA concentrations. These findings suggest that nanomicellized essential oils derived from *Lavandula angustifolia* and *Mentha longifolia* represent promising dietary supplements for suckling calves, exerting beneficial effects on daily weight gain, feed intake, blood metabolic profiles, and oxidative stress biomarkers.

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Ethical Statement

Protocols applied for this experiment were approved by the Animal Ethics Committee of the University of Mohaghegh Ardabili (Ardabil, Iran) (Approval Number: IR.UMA.REC.1402.099) and a cooperation contract was signed between the University of Mohaghegh Ardabili and Moghan Agro-Industrial and Animal Husbandry Company for the participation of the animals.

Conflict of Interest

The authors declare that when conducting their search, there were no business or financial relationships that may be interpreted as constituting a conflict of interest.

Author Contributions

MAA, HAB, JS designed the experiment, carried out the research and laboratory analysis. HAB, JS supervision the student, RS, MAA, HAB, JS did the data analysis, wrote the manuscript, and revised the manuscript. All authors reviewed and agreed on the final manuscript.

Data Availability Statement

The data supporting this study's findings are available from the corresponding author upon reasonable request.

Animal Welfare

The authors confirm that they have adhered to ARRIVE Guidelines to protect animals used for scientific purposes.

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