Ankara Üniversitesi Veteriner Fakültesi Dergisi

Veterinary Journal of Ankara University

ISSN 1300-0861 • E-ISSN 1308-2817 Volume 71 • Number 4 • Year 2024

Ankara Univ Vet Fak Derg - vetjournal.ankara.edu.tr - Open Acsess

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Ankara Üniversitesi Veteriner Fakültesi Dergisi
Volume: 71 • *Number*: 4 • *Year*: 2024
Veterinary Journal of Ankara University
Quarterly Scientific Journal
ISSN 1300-0861 E-ISSN 1308-2817

Publisher
On behalf of Ankara University, Faculty of Veterinary Medicine
Prof. Dr. Ender YARSAN
Dean

Publisher
Address
Ankara University, Faculty of Veterinary Medicine
Publication Subcommittee
06070 Ankara, Türkiye
Tel: 90 312 317 03 15, **Fax:** 90 312 316 44 72
E-mail: vfdergi@veterinary.ankara.edu.tr
URL: http://vetjournal.ankara.edu.tr
Publication Type: Peer- reviewed and published quarterly online by DergiPark Akademik

This journal is covered by **SCI-EXP** and **JCR** of Thomson Reuters®, **Cabells Journalytics, International Scientific Indexing, CAB Abstracts, Academindex, ABCD Index, Global Health, CAB Direct, Database Subsets; Scopus** and **TR Dizin** database systems.

Web Address
http://vetjournal.ankara.edu.tr

Ankara Üniversitesi Basımevi / *Ankara University Press*
İncitaşı Sokak No:10 06510 Beşevler / ANKARA
Tel: 0 (312) 213 66 55
Basım Tarihi: 23/09/2024

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EDITORIAL

Dear Esteemed Readers,

We are pleased and proud to present the fourth and final issue of our journal for the year 2024.

This issue features a total of 15 articles, including 12 research articles, 2 case reports, and 1 review article.

We extend our sincere gratitude and appreciation to all authors who have chosen our journal as a platform to disseminate their valuable research to the scientific community.

We hope that this final issue of the year will make a significant contribution to the advancement of science.

With our best regards...

Dr. Levent ALTINTAŞ
Editor in Chief
Ankara Üniversitesi Veteriner Fakültesi Dergisi

Exploring the dynamics of Human-Pet attachment: An In-Depth analysis of socio-demographic factors and relationships

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ARTICLE INFO

Article History
Received : 27.09.2023
Accepted : 20.02.2024
DOI: 10.33988/auvfd.1366652

Keywords
Attachment
Companion animal
Human-animal bond
Pet caretakers

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How to cite this article: Özen D, Bıçakcı NM, Salgırlı Demirbaş Y (2024): Exploring the dynamics of Human-Pet attachment: An In-Depth analysis of socio-demographic factors and relationships. Ankara Univ Vet Fak Derg, 71 (4), 385-393. DOI: 10.33988/auvfd.1366652.

ABSTRACT

This study examined the factors influencing pet attachment by investigating attachment dimensions and exploring the relationship between demographic factors and pet attachment. The study utilized the Pet Attachment Questionnaire (PAQ) to assess the level of attachment between pet owners and their animals. A demographic questionnaire was also administered to gather socio-cultural, economic, and health-related data from pet caretakers. Confirmatory factor analysis was applied to confirm the scale factor structure. Hypothesis testing procedures were used to reveal the relationship between the demographic characteristics of the participants and the attachment relationships. The study involved 304 volunteers who visited the animal hospital at Ankara University, Faculty of Veterinary Medicine, Türkiye. The findings revealed significant impacts of various factors on attachment dimensions, including age, household income, participant and household member anxieties and traumas, number of pets owned, pet health, and previous pet ownership. These results contribute to our understanding of the complex dynamics that shape the attachment between humans and animals. Further research is needed to understand the underlying mechanisms and potential interactions among these factors, advancing our knowledge of human-pet attachment.

Introduction

Researchers have paid a great deal of attention to the bond between pet owners and their animals, recognizing it as a significant and dynamic relationship (23, 29, 41). Research has indicated that humans and animals benefit from strong emotional bonds (14, 26). For example, owners with higher levels of attachment to their pets had improved mental health outcomes, such as reduced loneliness, depression, and anxiety (7). Dogs with secure attachments to their owners also exhibited fewer behavioral problems and improved overall welfare (38). These findings emphasize the mutual benefits of emotional support and companionship in the relationship between pet and owner. A key component of this relationship is the centrality of the bond formed between a pet and its caregiver. Bowlby (6) introduced the theory of pet attachment, which concerns the emotional bond and affection that arises between humans and their animal companions. It is a prime example of the deep emotional connections that can exist between species (15). Comprehending the nature and dynamics of this attachment is essential for the well-being of companion animals and humans alike.

Domestication has played a crucial role in the development of pet-owner attachment. Certain animal species, including domestic dogs and cats, have developed a unique ability to form profound emotional connections with humans (10, 29). Numeruous studies have investigated the factors that contribute to the formation and strength of the pet-owner relationship, categorized as follows: the characteristics of the owner, the characteristics of the pet, and the dynamics of their

interaction (16, 21, 27, 28). Individual owner characteristics may influence the extent of pet attachment, according to research. Individuals with an idealist personality type had higher attachment scores than those with other personality types, according to one study. One study reported that people with an idealistic personality type had higher attachment scores than people with other personality types (3). According to de Albuquerque et al. (9) a significant correlation was found between greater attachment to pets and neuroticism. Those with greater empathy tend to form stronger attachments to their companions (8). Likewise, previous favorable experiences with pets may increase the likelihood of developing stronger attachments to current pets (4). The species and age of the animal can affect the intensity of the attachment. For example, research indicates that dog owners tend to have higher attachment levels than cat owners (22). Moreover, due to their perceived vulnerability and dependence, puppies and kittens are more likely to inspire stronger attachments (2). Lastly, the dynamics of the owner-pet interaction contribute to the development of attachment. Since positive interactions, such as play, hygiene, and engaging in shared physical activities, have been found to increase attachment levels (17, 19), the quality of care may influence the strength of the bond.

Various tools and measures have been developed to assess the degree of pet-owner attachment, building upon evidence-based theories of human interpersonal relationships. These tools have been modified in various ways to provide the most appropriate explanations of the physiological and psychosocial effects on the well-being of both humans and animals (1, 25, 41). The majority of these instruments focus on the attachment between pets and their caretakers. Intimacy, commitment, emotional involvement, conflict, and other aspects of human-animal relationships vary significantly, just as they do between humans. These variations reflect the internal functioning patterns associated with expectations, emotions, and pet-related behavior. To better define this relationship and assess pet attachment orientations, Zilcha-Mano et al. (40) developed the self-report Pet Attachment Questionnaire (PAQ). This scale was developed based on the Experiences in Close Relationships form (13), which is one of the most widely attachment patterns between care takers and their pets through a series of questions pertaining to sentiments of closeness, dependence, and the overall quality of the relationship. This instrument has proven useful for comprehending the dynamics of pet-owner attachment and has provided researchers with standardized measures for assessing attachment levels across various populations.

While the emotional bond between humans and their pets, known as pet attachment, has been recognized as a significant and evolving relationship, previous studies have not comprehensively explored the human-related factors that may influence attachment types of caretakers towards their pets. Understanding the nature and dynamics of this attachment is crucial for comprehending the well-being of both companion animals and humans. This study aims to explore various factors, including socio-demographics and the experiences of pet caretakers, contributing to the pet-caretaker attachment type.

Materials and Methods

Sample Size Considerations and Participants: Prior to the study, a power analysis conducted. To detect the difference between the Cronbach alpha coefficient under the null hypothesis of 0.86 and the coefficient alpha under the alternative hypothesis of 0.89 using a two-sided F-test with a significance level of 0.05, a sample of 275 subject would be enough to achieve 80% power (5, 11).

In the current study, there were 304 individuals who visited the animal hospital at Ankara University, Faculty of Veterinary Medicine between March and June 2023, ranging in age from 18 to 65, and who live with at least one pet. There were 223 (73.4%) female participants and 81 (26.6%) male participants.

This study has obtained the necessary permission from the Ankara University Ethics Committee (dated 24.11.2022, decision number 20). The participants were given an "informed consent" form at the beginning of the study, in which they were assured about information and confidentiality about the research, and their consent was obtained.

Data Collection Tools

Demographic Information Form: The socio-demographic variables of the participants and the information including 26 questions about owning a pet were evaluated.

Pet Attachment Questionnaire (PAQ): The Pet Attachment Questionnaire, which consists of 26 items in total and examines the attachment relationships of the participants to the pets was used. Each item in the scale is scored on a Likert scale between 1 (strongly disagree) and 7 (strongly agree). The original scale exhibited a 2-factor structure as anxious and avoidant attachment style, and Cronbach's alpha values were found to be 0.86 and 0.89, respectively. The Turkish adaptation study of the scale was carried out by Şahin and Kahya (35), and the Cronbach alpha values of the study adapted into Turkish were found to be 0.86 and 0.79.

Statistical Analysis: Frequency (n) and percentage (%) were used for categorical data and median (minimum-maximum) was used for numerical data in describing the demographic characteristics of the participants. The chi-square test was used by considering the distribution of expected cells in the comparison of the frequencies of

categorical variables between groups. Prior to examining the differences in scale scores for each variable between the groups, the data were analysed using the Shapiro-Wilk test for conformity to normal distribution and the Levene test for homogeneity of variances. For the comparison among two groups, the Student t-test was used for variables that met the assumptions while the Mann Whitney U test was used for those that did not. For comparing more than two groups, a one-way analysis of variance (ANOVA) was used for variables that met the assumptions of the parametric test, and Kruskal-Wallis tests were used for variables that did not meet the assumptions. Cronbach's alpha coefficient was used to determine the internal consistency of the scale. In order to assess the factor structure of the scale, explanatory factor analysis was carried out using principal axis factoring and varimax rotation, in line with the original study of the scale. Kaiser-Meyer-Olkin (KMO) measure was used to determine sampling adequacy. Bartlett's test of sphericity was used to test null hypothesis that the correlation matrix is an identity matrix. Confirmatory factor analyses were used to confirm the scale factor structure. The $P<0.05$ criterion was used in all statistical evaluations. Stata 18 was used in the analysis of the data.

Results

Participants' pets had an average age of 5.23 ± 3.36 years (median: 4). In addition, the average duration of pet ownership of the participants was 11.01 ± 5.55 years (median: 8). A summary of the socio-demographic, economic, and history/experience with pets of caretakers is presented in Table 1.

To evaluate the factor structure of PAQ, explanatory factor analysis was conducted with basic axis factoring (Principle axis extraction method) and varimax rotation, consistent with the original study of the scale. Since item 1 in the Pet Attachment Scale is a reversed item, reverse coding was performed before factor analysis. The results showed that the data set obtained using PAQ was suitable for explanatory factor analysis (KMO = 0.815; Barlett Test $\chi2$ (325) = 2327, $P<0.001$). The eigenvalues, scree plot, and item distribution of the factors were taken into consideration while adhering to the original study of the scale and its adaption to Turkish, and the two-factor structure in its original form was preferred (35, 40). These two factors were theoretically named as anxious (PAQ-anxiety) and avoidant (PAQ-avoidant) attachment to pets, as indicated in the attachment literature. In the study, it was found that these two factors together accounted for 35.63% of the variation. 18.81% of the variation was explained by factor 1, which corresponds to the avoidance dimension, and 16.82% by factor 2, which corresponds to the anxiety dimension. The factors' eigenvalues were 4.63 and 4.37, respectively. The Cronbach alpha values of the study were found to be 0.796 and 0.813 for avoidance and anxiety dimensions, respectively (Table 2).

The confirmatory factor analysis, which was conducted to confirm the factor structure of PAQ by taking into account the original scale structure, adaptation study, and existing explanatory factor analysis findings, demonstrated acceptable fit between the model and the data. The fit of the model was assessed using indices such as RMSEA (0.058), CFI (0.911), TLI (0.902) and SRMR (0.076), which indicate the usability of the model as well as the verification of the factor structure of the scale (Table 3). The factor structure of the scale and the standardised values were presented in Figure 1. Results showed that all factor loadings were significant at the $P<0.001$ level. PAQ6 was the primary contributor to anxiety, whereas PAQ21 was the biggest contributor to Avoidance. We found the covariance between anxiety and avoidance to be insignificant (cov (Anxiety, Avoidant) =0.025; z=0.31, P=0.754) (Figure 1).

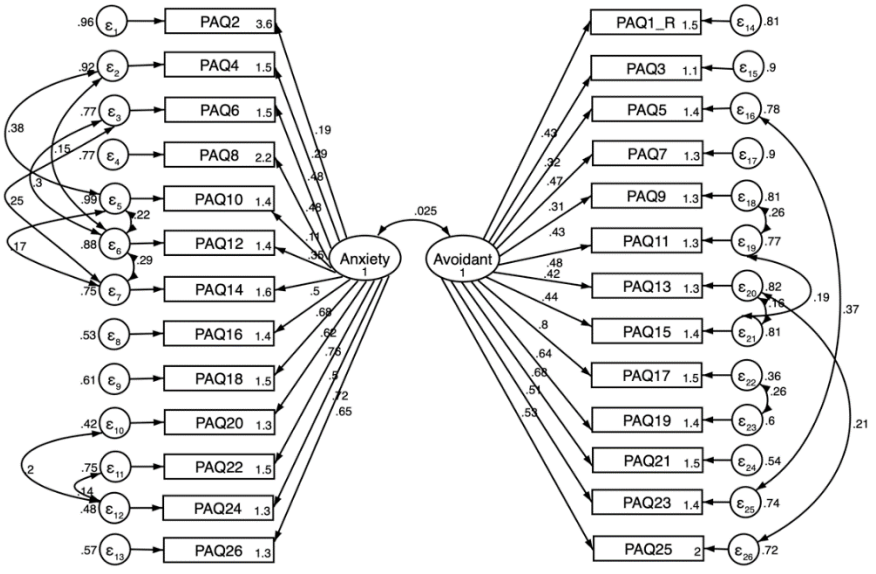

Figure 1. Standardized coefficients of the model for the two-factor structure of the Pet Attachment Questionnaire.

Table 1. Information on the socio-demographic and economic status of the patients.

Variables	Category	n (%n)
Sex	Female	223 (73.4%)
	Male	81 (26.6%)
Age (year)	<=20	10 (3.3%)
	21-40	184 (60.5%)
	41-60	91 (29.9%)
	>60	19 (6.3%)
Marital Status	Single	177 (58.6%)
	Married	103 (34.1%)
	Devorced	22 (7.3%)
Educational level	Secondary Ed.	37 (12.3%)
	Undergraduate	204 (67.5%)
	Postgraduate	61 (20.2%)
Working status	Yes	166 (55.1%)
	No	135 (44.9%)
Household income level	0 - 500 US$	52 (17.2%)
	501 - 1000 US$	145 (48%)
	>1000 US$	105 (34.8%)
Do you live with your family	Yes	172 (56.8%)
	No	131 (43.2%)
Has anyone in your home ever experienced trauma or a fear of animals?	Yes	41 (13.5%)
	No	263 (86.5%)
Is there a previous history of pet ownership in your family?	Yes	236 (77.6%)
	No	68 (22.4%)
Do you have a history of an animal-related allergy disease?	Yes	50 (16.4%)
	No	254 (83.6%)
Do you have a fear of animals or a traumatic history?	Yes	49 (16.1%)
	No	255 (83.9%)
Do you have children?	Yes	103 (33.9%)
	No	201 (66.1%)
What type of pet are you looking after?	Cat	221 (72.7%)
	Dog	52 (17.1%)
	Other	8 (2.6%)
	Cat and Dog	23 (7.6%)
How did you get your pet?	Shelter	20 (6.6%)
	Adopting a stray animal	165 (54.5%)
	Petshop	26 (8.6%)
	Familiar environment	92 (30.4%)
Did you pay a fee to adopt your animal?	Yes	43 (14.3%)
	No	258 (85.7%)
Before the animal you are currently caring for, did you have another pet?	Yes	230 (75.7%)
	No	74 (24.3%)
Have you ever had more than one pet at once?	Yes	197 (64.8%)
	No	107 (35.2%)
Are the animals you own the same species?	Yes	111 (49.6%)
	No	113 (50.4%)
Does your pet suffer from a physical condition or ongoing illness?	Yes	82 (27%)
	No	222 (73%)
Have you ever experienced losing a pet?	Yes	209 (69.2%)
	No	93 (30.8%)
Have you given up on your pet before?	Yes	35 (11.5%)
	No	269 (88.5%)

Table 2. Results of the explanatory factor analysis of PAQ items.

PAQ items	On-factor loadings
1. Being close to my pet is pleasant for me (reverse-scored)	0.432 (1)
2. I'm often worried about what I'll do if something bad happens to my pet	0.451 (2)
3. I prefer not to be too close to my pet	0.349 (1)
4. Sometimes I feel that I force my pet to show more commitment and desire to be close to me	0.401 (2)
5. I prefer to keep some distance from my pet	0.596 (1)
6. If I can't get my pet to show interest in me, I get upset or angry	0.598 (2)
7. Often my pet is a nuisance to me	0.375 (1)
8. Signs of affection from my pet bolster my self-worth	0.516 (2)
9. I feel distant from my pet	0.519 (1)
10. I often feel that my pet doesn't allow me to get as close as I would like	0.311 (2)
11. I'm not very attached to my pet	0.593 (1)
12. I get angry when my pet doesn't want to be close to me as much as I would like it to	0.489 (2)
13. If necessary, I would be able to give away my pet without any difficulties	0.482 (1)
14. I get frustrated when my pet is not around as much as I would like it to be	0.622 (2)
15. I have no problem parting with my pet for a long duration	0.586 (1)
16. I need shows of affection from my pet to feel there is someone who accepts me as I am	0.685 (2)
17. I get uncomfortable when my pet wants to be close to me	0.721 (1)
18. I feel frustrated if my pet doesn't seem to be available for me when I need it	0.679 (2)
19. I get nervous when my pet gets too close to me	0.645 (1)
20. Without acts of affection from my pet I feel worthless	0.767 (2)
21. I want to get close to my pet, but I keep pulling away	0.647 (1)
22. I am worried about being left alone without my pet	0.524 (2)
23. I try to avoid getting too close to my pet	0.564 (1)
24. I need expressions of love from my pet to feel valuable	0.736 (2)
25. When I'm away from my pet for a long period of time, I hardly think about it	0.596 (1)
26. I need a lot of reassurance from my pet that it loves me	0.684 (2)
Cronbach alpha	0.796 (1) 0.813 (2)
Percentage of explained variance in item scores (%)	18.81 (1) 16.82 (2)

(1): Avoidance, (2): Anxiety

Table 3. Goodness-of-fit criterias for the created model.

Goodness of fit criteria	Close approximate fit	Acceptable range	Achieved goodness of fitness
$\chi 2$	$0 \leq \chi 2 \leq 2sd$	$2sd \leq \chi 2 \leq 3sd$	499.278 (sd=247)
P value	$0.05 < P \leq 1.00$	$0.01 \leq P \leq 0.05$	<0.001
RMSEA	$0 \leq RMSEA \leq 0.05$	$0.05 \leq RMSEA \leq 0.08$	0.058 (PCLOSE=0.036)
CFI	$0.97 \leq CFI \leq 1.00$	$0.95 \leq CFI \leq 0.97$	0.911
TLI	$0.95 \leq TLI \leq 1.00$	$0.90 \leq TLI \leq 0.95$	0.902
SRMR	$0 \leq SRMR \leq 0.04$	$0.5 \leq SRMR \leq 0.10$	0.076

RMSEA: Root Mean Square Error of Approximation; TLI: Tucker-Lewis Index; CFI: Comparative Fit Index; SRMR: Standardized Root Mean Residual.

Table 4. The relationship between the demographic information of the animal owners and the PAQ.

		PAQ-Anxiety Median (Min - Max)	P	PAQ-Avoidance Median (Min - Max)	P
Sex	Female	37 (15 - 81)	**0.254**	22 (16 - 54)	**<0.001**
	Male	36 (13 - 66)		26 (18 - 49)	
Age (year)	<=20	39.5 (24 - 81) **a**	**0.047**	22 (19 - 30)**a**	**0.031**
	21-40	38 (16 - 80) **ab**		23 (19 - 49)**a**	
	41-60	33.5 (13 - 73) **b**		25 (16 - 54)**b**	
	>60	36 (25 - 59) **ab**		21 (18 - 40)**ab**	
Marital Status	Single	37 (16 - 81)	**0.526**	23 (19 - 49)	**0.244**
	Married	38 (13 - 79)		25 (16 - 54)	
	Devorced	31 (16 - 73)		22 (19 - 41)	
Educational level	Secondary Ed.	36 (16 - 71)	**0.701**	25.5 (16 - 54)**a**	**0.039**
	Undergraduate	37 (13 - 81)		22 (18 - 50)**b**	
	Postgraduate	37.5 (15 - 73)		23.5 (19 – 45)**b**	
Working status	Yes	36 (13 - 79)	**0.096**	23 (19 - 49)	**0.763**
	No	37 (18 - 81)		23 (16 - 54)	
Household income level	0 - 500 US$	34 (16 - 73)**ab**	**0.019**	24.5 (19 - 49)	**0.523**
	501 - 1000 US$	38 (19 - 81)**a**		22.5 (16 - 54)	
	>1000 US$	34 (13 - 71)**b**		23 (19 - 40)	
Do you live with your family	Yes	36.5 (13 - 80)	**0.644**	24 (16 - 54)	**0.238**
	No	37 (16 - 81)		23 (18 - 49)	
Has anyone in your home ever experienced trauma or a fear of animals?	Yes	44 (19 - 81)	**0.002**	25 (19 - 54)	**0.081**
	No	36 (13 - 80)		23 (16 - 50)	
Is there a previous history of pet ownership in your family?	Yes	36 (15 - 81)	**0.205**	23 (19 - 54)	**0.455**
	No	40 (13 - 74)		24 (16 - 49)	
Do you have a history of an animal-related allergy disease?	Yes	34 (16 - 81)	**0.64**	22.5 (19 - 49)	**0.715**
	No	37 (13 - 79)		24 (16 - 54)	
Do you have a fear of animals or a traumatic history?	Yes	45 (19 - 81)	**<0.001**	24 (19 - 54)	**0.43**
	No	36 (13 - 79)		23 (16 - 50)	
Do you have children?	Yes	35 (13 - 73)	**0.157**	25 (16 - 54)	**0.005**
	No	38 (16 - 81)		23 (19 - 49)	
What type of pet are you looking after?	Cat	37 (13 - 80)	**0.632**	23 (16 - 54)	**0.134**
	Dog	35 (16 - 79)		22 (19 - 42)	
	Other	45.5 (19 - 81)		26 (24 - 45)	
	Cat and Dog	37 (18 - 73)		23.5 (19 - 42)	
How did you get your pet?	Shelter	40 (15 - 73)	**0.899**	25 (19 - 34)	**0.18**
	Adopting a stray animal	36 (13 - 80)		22 (16 - 54)	
	Petshop	37 (18 - 81)		24 (18 - 42)	
	Familiar environment	37.5 (19 - 66)		25 (19 - 49)	
Did you pay a fee to adopt your animal?	Yes	38 (18 - 81)	**0.609**	24 (18 - 49)	**0.706**
	No	36.5 (13 - 80)		23 (16 - 54)	
Before the animal you are currently caring for, did you have another pet?	Yes	35.5 (15 - 81)	**0.014**	24 (16 - 54)	**0.674**
	No	41.5 (13 - 74)		23 (18 - 49)	
Have you ever had more than one pet at once?	Yes	35 (13 - 81)	**0.021**	23 (16 - 54)	**0.142**
	No	40.5 (18 - 80)		25 (18 - 49)	
Are the animals you own the same species?	Yes	34.5 (13 - 68)	**0.142**	23 (19 - 50)	**0.277**
	No	37 (15 - 81)		23 (19 - 54)	
Does your pet suffer from a physical condition or ongoing illness?	Yes	36 (16 - 79)	**0.938**	22 (19 - 54)	**0.675**
	No	37 (13 - 81)		24 (16 - 49)	
Have you ever experienced losing a pet?	Yes	36 (13 - 80)	**0.168**	23.5 (16 - 54)	**0.522**
	No	38 (19 - 81)		23 (19 - 50)	
Have you given up on your pet before?	Yes	47 (20 - 71)	**0.018**	23 (19 - 38)	**0.257**
	No	36 (13 - 81)		23 (16 - 54)	

a,b,c: Different letters in the same column for each variable show statistically significant difference (P<0.05).

In Table 4, the comparison of the variables of interest based on the participant's demographics and PAQ sub-factor scores is presented.

Considering the anxiety scores of the participants in the study, which express the anxious attachment style to their pets, "age (P=0.047)", "household income level (P=0.019)", "fear of animals in the household or in oneself (P<0.01)", "being ownership of a previous pet (P=0.014), "having more than one animal (P=0.021)", and "giving up on a pet before (P=0.018) had all statistically significant effect. Once these factors are analyzed further in detail, it is evident that anxiety affects those who are very young and very old. Anxiety levels were significantly higher in middle-class individuals, those who fear animals or have undergone trauma, people who have owned pets in the past, people who have multiple dogs, and people who have previously had to give up their pets (Table 4).

Gender (P=0.011), age (P=0.031), education level (P=0.039), and having a child (P=0.005) were all shown to be statistically significant when examining the avoidance scores of the study participants, which express the avoidant attachment to their pets. Considering the significant factors related to avoidance; men compared to women; middle and upper age group (>41 years) compared to young people (<40); those who have undergraduate and graduate education compared to secondary education, and those who have children have statistically significantly higher avoidance scores than those who do not (Table 4).

Discussion and Conclusion

The human-animal attachment, specifically regarding companion animals, has gained considerable attention in recent years (2, 18, 24, 31). Understanding the factors that contribute to pet attachment is crucial for comprehending the reasons, effects, and nature of this bond, as noted by Johnson et al. (18). This study aimed to investigate the influence of various factors on anxious and avoidant attachment styles towards pets, contributing to the understanding of human-pet attachment. To assess pet attachment, the study utilized the Pet Attachment Questionnaire (PAQ), a valid and reliable tool for measuring attachment levels and attributions of meaning to pets, particularly regarding anxiety and avoidance dimensions (20).

Consistent with previous research on the correlation between pet attachment level and age of care takers, age was found to be a significant factor influencing attachment types (3). Both younger and older individuals displayed higher levels of anxiety in their attachment to pets in comparison to other age groups (Table 4). This finding also aligns with existing literature suggesting that older individuals tend to have a higher level of attachment to pets, as pets compensate for the absence of human companionship and contribute to a reduction in negative moods (2, 36, 39).

Regarding attachment anxiety and avoidance, gender was found to have a significant effect only on the avoidance component. Men were more likely than women to exhibit an avoidant attachment to their dogs (Table 4). Previous studies have also shown differences in attitudes toward animals between females and males (3, 33, 34, 37). Although females generally exhibit more favorable attitudes toward animals, no significant gender effect was observed on attachment levels. Thus, it can be concluded that different attachment styles can influence one's attitude toward companion animals, even though this attitude does not directly affect the level of attachment.

Participants with higher levels of education, particularly those who had completed undergraduate and graduate education, demonstrated greater degrees of avoidance (Table 4). This finding may be attributed to individuals with higher education levels having more demanding work schedules or a preference for personal space and independence, both of which contribute to a more avoidant attachment style towards their pets (20). Contrary to previous findings (32, 36, 39), marital status did not statistically impact attachment anxiety and avoidance components. However, individuals without children exhibited a higher level of avoidant attachment compared to those who had children. One possible explanation is that individuals without children have better management of their time and energy, along with reduced caring responsibilities, allowing for a more avoidant attachment style toward pets.

The study revealed that prior pet ownership significantly impacted attachment anxiety, possibly due to the experience of losing a pet in the past (Table 4). Attachment anxiety and stronger attachment were positively correlated with more severe grief in pet caregivers, which may further influence their attachment style with a new pet (12). Furthermore, household income level emerged as a significant factor influencing attachment type. While previous studies by Johnson et al. (18) suggested that lower income was associated with stronger attachment, this research indicates that individuals with a middle-income level exhibit higher levels of attachment anxiety. Individuals with pre-existing irrational fears or phobias of animals also displayed higher levels of anxiety in their pet attachment, suggesting that addressing these issues is crucial for developing a healthy relationship between individuals and their dogs. Additionally, individuals who had previously given up a pet exhibited higher anxiety levels, possibly indicating a continued sense of connection insecurity (40). Despite caretakers with multiple pets generally having stronger attachments to their animals (3), this study found that

owning more than one animal was associated with higher levels of anxiety. This suggests that the obligations and demands associated with caring for multiple animals contribute to increased anxiety levels among owners.

An intriguing finding of this study is the connection between the presence of a child and increased levels of avoidance in the attachment to pets, despite the common perception of many people considering their companion animals as their children, as previously discussed by Sife (30). This phenomenon suggests that the attachment style can be influenced by the presence of a child in the family. The responsibilities and demands of parenting may redirect attention and resources away from the pet, leading to the development of an avoidant attachment pattern between the individual and their pet.

In conclusion, this study highlights the significant impact of various factors, including age, household income level, fear of animals, prior pet ownership, owning multiple animals, gender, education level, and having a child, on anxious and avoidant attachment styles toward pets. Understanding the elements that influence human-pet attachment can aid in improving animal care practices and environments. By revealing these influential factors, this research enhances our understanding of the complex dynamics that shape human-pet relationships. Furthermore, the study's observations regarding the impact of different demographic factors on attachment can assist pet owners in making informed decisions about the care of their pets. By informing and influencing the dynamics of human-animal interaction, these findings possess the capability to enhance the general quality of life for pets. Additionally, the findings have the potential to improve positive outcomes associated with strong, healthy relationships between humans and animals. Further investigation into the underlying mechanisms and potential interactions among these factors is crucial for deepening our comprehension of the intricate nature of attachment to pets.

Acknowledgements

The authors would like to thank all volunteers who patiently responded the survey. Also the authors wish to thank to Ankara University Veterinary Faculty Administration and Animal Hospital Chief Physician for their support and convenience.

Financial Support

This research received no grant from any funding agency/sector.

Conflict of Interest

The authors declared that there is no conflict of interest.

Author Contributions

DO designed the study. NMB contributed to data collection. DO analysed the data. YSD contributed to the interpretation of the results. DO and YSD lead in writing and the critical review of 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.

Ethical Statement

This study was carried out after the animal experiment was approved by Ankara University Local Ethics Committee (Decision number: 20, Date: 24.11.2022). All respondents read and accepted the consent form before responding to the survey.

Animal Welfare

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

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Exploring the neuroprotective effects of black garlic ethanol extract on acrylamide-induced brain damage through apoptotic and neurodegenerative pathways

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ARTICLE INFO

Article History
Received : 01.11.2023
Accepted : 08.03.2024
DOI: 10.33988/auvfd.1384531

Keywords
Acrylamide
Apoptosis
Black Garlic
Neurodegeneration
Neurotoxicity

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How to cite this article: Gezer A, Karadağ Sarı E, Gelen V, Eliş Yıldız S, Özkaraca M, Bedir G, Çalık F, Eliş İ (2024): Exploring the neuroprotective effects of black garlic ethanol extract on acrylamide-induced brain damage through apoptotic and neurodegenerative pathways. Ankara Univ Vet Fak Derg, 71 (4), 395-406. DOI: 10.33988/auvfd.1384531.

ABSTRACT

This research focused on exploring the therapeutic impact of black garlic ethanol extract (BGE) on the brain tissue of rats exposed to acrylamide (ACR). Twenty-four female rats were divided into four groups. Rats in the control group were given 1 ml of saline by oral gavage for 14 days. The BG group received 5 mg/200 g of BGE extract on a daily basis. The ACR group was administered 40 mg/kg of ACR daily. Rats in the BGE+ACR group received both 5 mg/200 g of BG extract and 40 mg/kg of ACR daily. Brain tissue samples were collected at the study's conclusion for histopathological, immunohistochemical, and biochemical analyses. Hematoxylin-eosin staining was performed to examine the general structure of the brain tissue. Erk1/2, p-ERK1/2, and c-fos were analyzed immunohistochemically; Bcl-2, Caspase-3, ATF6, CREB, and NfkB-p65 protein levels were analyzed by Western blotting; and MDA, SOD, CAT, GSH, TNF-α, IL-1β, and IL-6 activities and levels were analyzed using ELISA kits. It was determined that ACR application raised the levels of Erk1/2, p-ERK1/2, c-Fos, NfkB-p65, caspase-3, MDA, IL-6, IL-1-β, and TNF-α, and BGE supplementation decreased this increase. ACR exposure caused a decrease in Bcl-2, ATF6, CREB, CAT, GSH, and SOD expressions, and BGE supplementation prevented or increased this decrease. Based on the findings obtained, it can be said that the ethanol extract of black garlic has antioxidative and anti-inflammatory effects, prevents cell damage, and has positive effects on apoptosis in rat brain tissue.

Introduction

Acrylamide (ACR), which is widely exposed in daily life through environmental pollution and nutrition, is a crystalline, vinylic small molecule, a synthetic substance with high water solubility, and no specific color and odor. It was classified as a probable carcinogenic compound for humans by the International Agency for Research on Cancer in 1994 (15). As a result of various studies, it has been discovered that some heat-treated foods such as bread, coffee, and French fries contain high amounts of Acrylamide. The main way Acrylamide is formed in foods is the Maillard reaction between reducing sugars and the amino acid asparagine during heat treatments such as frying, baking, and roasting at temperatures above 120°C (6). Many health problems, including immunotoxic, hepatotoxic, neurotoxic, genotoxic, carcinogenic, and

teratogenic effects, can develop because of acrylamide toxicity (14). It has been observed that plant-based compounds with antioxidant, anti-inflammatory, and antiapoptotic effects are used to minimize the harmful effects of ACR or to treat the damage, and garlic extract has strong anti-inflammatory, antioxidant, and antiapoptotic effects (20).

Fresh garlic contains large amounts of sulfur compounds such as alliin, allicin and S-allyl cysteine (SAC) (3). In recent years we have witnessed the implementation of methods like heat treatment, drying, and fermentation to eliminate the unpleasant odor and taste associated with garlic (38). Thanks to these methods, irritating properties are eliminated, and the garlic is given a more delicious form. The product called "black garlic" was obtained by exposing the garlic heads to high temperatures (60°C and above), maintaining 85-90% relative humidity for 30-40 days, and then softening and darkening the garlic heads. During fermentation, the enzyme alliinase is broken down, thereby alliin cannot be converted into allicin (8). As a result of this event, the specific odor of garlic decreases. Alliin is converted into SAC and odorless components are formed. While the amount of SAC increases in the formation of black garlic, alliin, allicin, and polysaccharides decrease. In this way, the bitterness and pungent odor of garlic is reduced (5).

Erk1/2 plays significant roles in cell proliferation, differentiation, and survival, signaling from the cell surface to the nucleus and detecting cellular stress. It exerts crucial effects on inflammation, and there is communication between cell receptors and different nuclear transcription factors (48). p-ERK1/2 is a member of the MAPK family and contains an ERK protein within this family (32). c-fos is a protooncogene that is a member of the IEG (Immediate Early Gene) group, and the increase of its accumulation depends on the effect of various stimuli (24). Bcl-2 is one of the most important regulators of the intrinsic apoptosis pathway (45). Caspase-3, located at the end of caspase cascades activated by apoptotic pathways acts as a primary protein for apoptosis (30). ATF6 is an endoplasmic reticulum transmembrane glycoprotein, transcription factor, and ER stress sensor. It detects protein misfolding in the endoplasmic reticulum and triggers the Unfolded protein response (UPR) to maintain homeostasis (36). CREB, a general transcription factor, acts as an inhibitor for the cell survival mechanism, thus leading the cell to programmed death (44). NfkB-p65 is an essential transcription factor and translocates from the cytosol to the nucleus upon activation and controls the expression of numerous genes related to cell cycle, growth, viability, specialization, movement, bonding, and inflammation (9). MDA, a widely recognized result of lipid peroxidation, elevates in response to heightened levels of free radicals. Hence, the concentration of MDA serves as an indicator of oxidative stress. Within the category of antioxidants, SOD, GSH, and CAT are substances that impede the formation of free radicals (4). TNF-α is a transmembrane protein and a crucial proinflammatory cytokine produced by various cells. Dysregulation of TNF-α has been linked to such several pathological conditions as autoimmune disease, atherosclerosis, cancer, Alzheimer's disease, infection, and inflammatory bowel disease. TNF-α also has various functions in the regulation of different developmental and immunological processes, such as inflammation, differentiation, lipid metabolism, and apoptosis (28). IL-1-β is a cytokine that has a potent proinflammatory effect when inducing inflammation. IL-1β damages neuronal synapses during inflammation and causes neurodegeneration (40). IL-6 is a proinflammatory cytokine that triggers inflammation; the uncontrolled, heightened, and continuous production of IL-6 has adverse effects on both acute systemic inflammatory response syndrome and chronic immune-mediated disorders (11).

After being consumed through food products, acrylamide can be rapidly absorbed from the gastrointestinal system and transferred to all body tissues (such as the liver, thymus, brain, heart, and kidneys) through the bloodstream. Therefore, this experimental study aims to determine the effects of BGE on oxidative stress, inflammation, and apoptosis in ameliorating the adverse effects of ACR on brain tissue.

Material and Methods

Collection and Extract Preparation of BGE: The black garlic used in the study was obtained from Edovital (Kastamonu, Türkiye). The peeled black garlic cloves were cut into small pieces and dried on blotting papers in a dark area with air circulation and out of the sun. The dried black garlic parts were ground with the help of a grinder. The soxhlet device cartridge was washed with extraction solvent by taking 50 g of the ground garlic samples and placing them in a 500 ml soxhlet extractor. Ethanol was used as an extraction solvent. 650 ml of ethanol solvent was added to the boiling flask. The solvent was subjected to extraction (using 10-15 siphons) for roughly 10 hours until the solvent became transparent. At the end of the 10^{th} hour, the liquid extracts obtained were filtered through blue band filter paper and the particles were removed. The filtrated extract sample underwent evaporation using a rotary evaporator at temperatures between 35-45ºC. The garlic extract left in the flask was placed inside a desiccator for 12 hours. After removing all traces of solvent from the garlic extract, it was weighed with a precision of 0.1 mg, transferred to an extraction container, and stored at +4ºC for subsequent analysis (43).

DOI: 10.33988/auvfd.1384531

Animal and Experimental Groups: The study utilized rats sourced from the Kafkas University Medical Experimental Research and Application Center, where the experimental phase of the study was also conducted. The research adhered to the principles outlined in the Declaration of Helsinki. A total of 24 female Wistar albino rats were utilized in the study; these rats were in the estrous phase, aged 3-4 months, and weighed between 250-350 grams. It was calculated with the G*Power program that at least 24 rats for 4 groups (at least 6 rats for each group) should be included in the study at 80% power and 95% confidence level for the case where there is an effect size (f = 0.45) compared to the reference study (27). The groups were randomly formed. They were fed *ad libitum* with tap water in an environment with 12 hours of darkness and 12 hours of lighting and kept in standard cages. Control group were given 1 ml of saline by oral gavage for 14 days. Rats in the BGE group were administered 5mg/200g black garlic extract dissolved in saline by oral gavage for 14 days (26). The rats in the ACR group were administered 40 mg/kg acrylamide dissolved in saline i.p. once a day for 14 days (39). Rats in the BGE+ACR group were administered 5 mg/200 g black garlic extract by oral gavage and 40 mg/kg Acrylamide i.p. as a daily dose for 14 days.

Following the conclusion of the experiment, rats were deeply sedated using sevoflurane (Sevorane®, Abbott Lab. Istanbul, Turkey), cervical dislocation was then performed, and brain tissue samples were collected. Some of the obtained brain tissues were stored at -80^{0}C for ELISA and Western blot examinations. Brain tissue specimens were immersed in a 10% buffered neutral formalin solution, and following standard histological processes, they were embedded in paraffin wax.

Histopathological Examination: The 5µm sections taken on polylysine slides were stained with hematoxylin-eosin (H&E) and semiquantitatively evaluated as absent (-), mild (+), moderate (++), and severe (+++) in terms of pyknotic and degenerative changes seen in neurons in the cortex parts (22).

Immunohistochemical examination: The 5µm sections taken on polylysine slides were passed through xylol and alcohol series, washed with PBS, and then kept in 3% H_2O_2 for 10 min to inactivate endogenous peroxidase. To release the antigen in the tissues, they were treated with antigen retrieval solution at 500 watts for 2x5 min. The tissues were then washed with PBS and incubated with Erk1/2 (Santa Cruz, Catalog No. sc-514302), p-ERK1/2 (Affbiotech, Catalog No. AF1015), and c-fos (Santa Cruz, Catalog No. sc-166940) primary antibodies at a dilution ratio of 1/150 at +4ºC overnight. As secondary; The Large Volume Detection System employed anti-polyvalent, HRP (Thermofischer, Catalog no: TP-125-HL) as per the manufacturer's instructions. DAB (3,3′-diaminobenzidine) served as the chromogen. Subsequently, following counterstaining with Mayer's hematoxylin, the slides were sealed with entellan and inspected using a light microscope. In the examination, immunopositivity was evaluated semiquantitatively as absent (-), mild (+), moderate (++), severe (++++), and very severe (++++) (21).

Western Blot Analysis: The brain tissue samples were weighted and crushed in nitrogen gas, treated with radioimmunoprecipitation (RIPA buffer, Ecotech Bio, Turkey) supplemented with protease and phosphatase inhibitors, and homogenized using a tissue lyser device (Qiagen, USA) at 30 Hz for 30 sec to determine the relative protein expressions of Bcl-2, Caspase-3, ATF6, CREB, and NfkB-p65. A protein assay kit was used to quantify brain tissue's total protein (Pierce BCA, Thermo Sci., USA). 25 ug of protein were then put into the PVDF membrane after being separated by 10% SDS-PAGE. First, at room temperature, 5% bovine serum albumin was used to block the membranes for 90 minutes. Then, the membranes were incubated at 4°C overnight with the appropriate primary antibodies NfkB-p65 (AF5006, Affinity Biotech), ATF6 (DF6009, Affinity Biotech), CREB antibody (AF6188, Affinity Biotech), Caspase-3 (sc-56053, Santa Cruz), Bcl-2 (sc-7382, Santa Cruz), and Beta-actin (sc-47778, Santa Cruz). After primary antibody incubation, the PVDF membranes were washed with TBST and then incubated for an additional 90 minutes at room temperature with the second antibody (Santa Cruz, sc-2004/sc-2005) coupled to horseradish peroxidase. Afterward, the protein bands were recorded using the Western ECL substrate, an enhanced chemiluminescence reagent (Thermo, 3405), visualized and analyzed by Image Lab™ Software (Bio-Rad, Hercules, CA, USA).

Biochemical Examination: Brain tissue homogenates required for oxidative stress and inflammation biomarkers analyses were obtained as described in our previous study. Malondialdehyde (MDA), glutathione (GSH) levels, superoxide dismutase (SOD), and catalase (CAT) activity were determined in brain tissue. Moreover, the concentrations of interleukin-6 (IL-6), interleukin-1β (IL-1β), and tumor necrosis factor-alpha (TNF-α) were evaluated. Oxidative stress parameters and cytokine levels in brain tissue supernatants obtained from rats were quantified using a rat ELISA kit following the provided guidelines from the manufacturer. The assessments were conducted utilizing an ELISA Plate Reader (Bio-Tek, Winooski, VT, USA) following the standard instructions provided by the manufacturer, and the absorbance was recorded at 450 nm.

Statistical Analysis: Statistical analysis was performed using the SPSS (version 25.0; IBM SPSS Inc, Chicago, IL, USA) package program. The normality of data was determined with the Kolmogorov-Smirnov test. Descriptive statistical analyses (mean±standard deviation) were used. One-way ANOVA test and post-hoc Tukey test were performed to compare groups. P values less than 0.05 at the 95% confidence interval were considered statistically significant.

Results

Histopathological Results: Histopathologic evaluation revealed statistically significant differences between the groups (Table 1, P<0.05).

The neurons in the cortex of the brain of rats in the control and BGE groups had a normal histologic appearance. While severe pyknotic and degenerative changes were observed in neurons in the ACR group, these changes were found to be moderate in the BGE+ACR group. Microscopically, the nuclei were dark and shrunken in pyknotic and degenerative neurons (Figure 1).

Immunohistochemical Results: Immunohistochemical staining for Erk1/2, p-ERK1/2, and c-Fos revealed statistically significant differences between the groups (Table 2), (P<0.05).

While Erk1/2 immunopositivity was very severe in the control and BGE group, it was mild in the ACR group. There was a correlation between p-ERK1/2 and c-Fos immunopositivity and Erk1/2 immunopositivity in the same direction. p-ERK1/2 and c-Fos immunopositivity were mild in the control and BGE groups, severe in the ACR group, and moderate in the BGE+ACR group (Figure 2). Immunohistochemical staining with Erk1/2, p-ERK1/2, and c-Fos showed neuron localization (Figure 2, 3, 4).

Table 1. Pyknotic and degenerative changes in neurons.

Groups	Cortex
Control	0.33 ± 0.21^{a}
BGE	0.33 ± 0.21^{a}
ACR	2.83 ± 0.16^{b}
BGE+ACR	1.66 ± 0.21^{c}

[a,b,c] The difference between groups in the same column (P<0.05).

Table 2. Comparison of the groups in terms of Erk1/2, p-ERK1/2 and c-Fos.

Groups	Erk1/2	p-ERK1/2	c-Fos
Control	1.16 ± 0.40^{a}	1.00 ± 0.00^{a}	0.83 ± 0.40^{a}
BGE	1.66 ± 0.51^{a}	1.16 ± 0.40^{a}	1.00 ± 0.00^{a}
ACR	3.83 ± 0.40^{b}	2.83 ± 0.40^{b}	2.83 ± 0.40^{b}
BGE+ACR	2.83 ± 0.40^{c}	1.83 ± 0.40^{c}	2.00 ± 0.00^{c}

[a,b,c] The difference between groups in the same column (P<0.05).

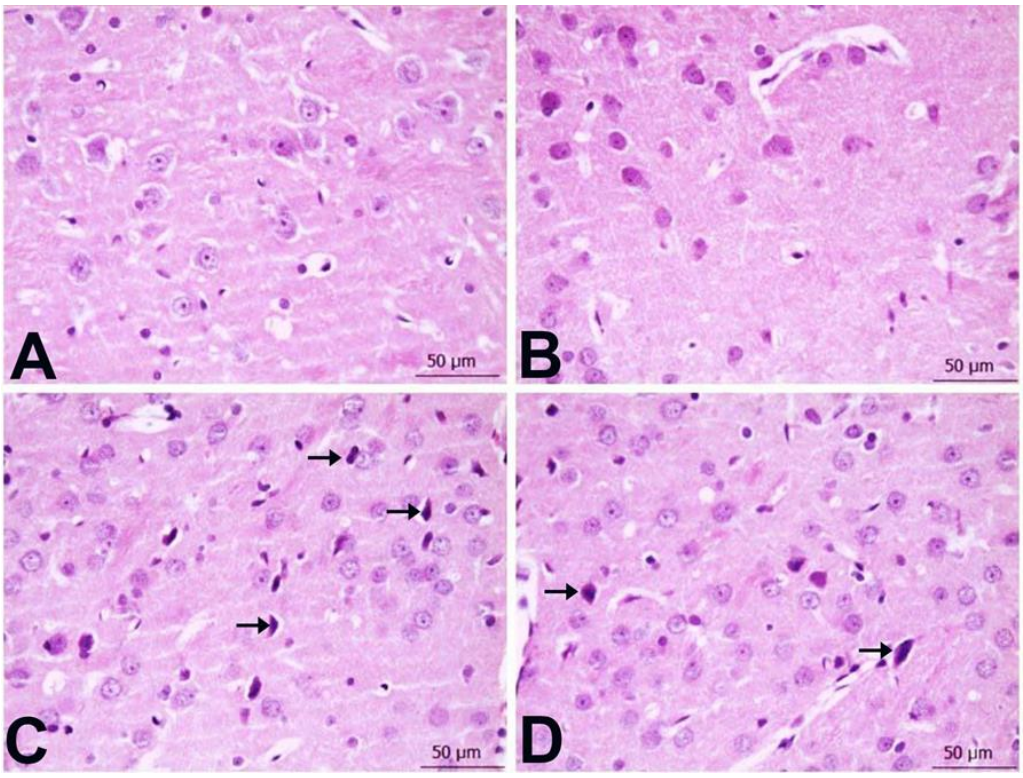

Figure 1. Histological changes in rat brain cortex.
A) Control group, B) BGE group; in normal histologic appearance, C) ACR group; severely pyknotic and degenerative neurons, D) BGE+ACR group; moderately pyknotic and degenerative neurons (arrows). Bar: 50 µm, H&E.

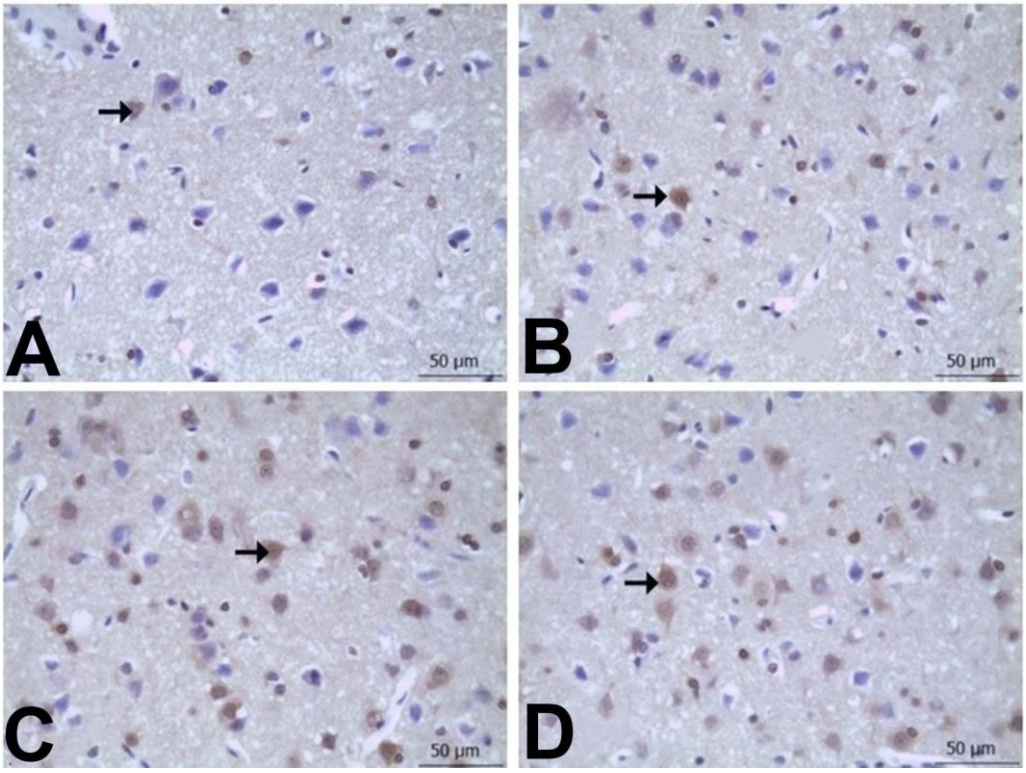

Figure 2. Erk1/2 immunohistochemistry.

A) Control group; mild level, B) BGE group; mild level, C) ACR group; very severe level, D) BGE+ACR group; moderate level Erk1/2 immunopositivity (arrows). Bar: 50 µm, IHC.

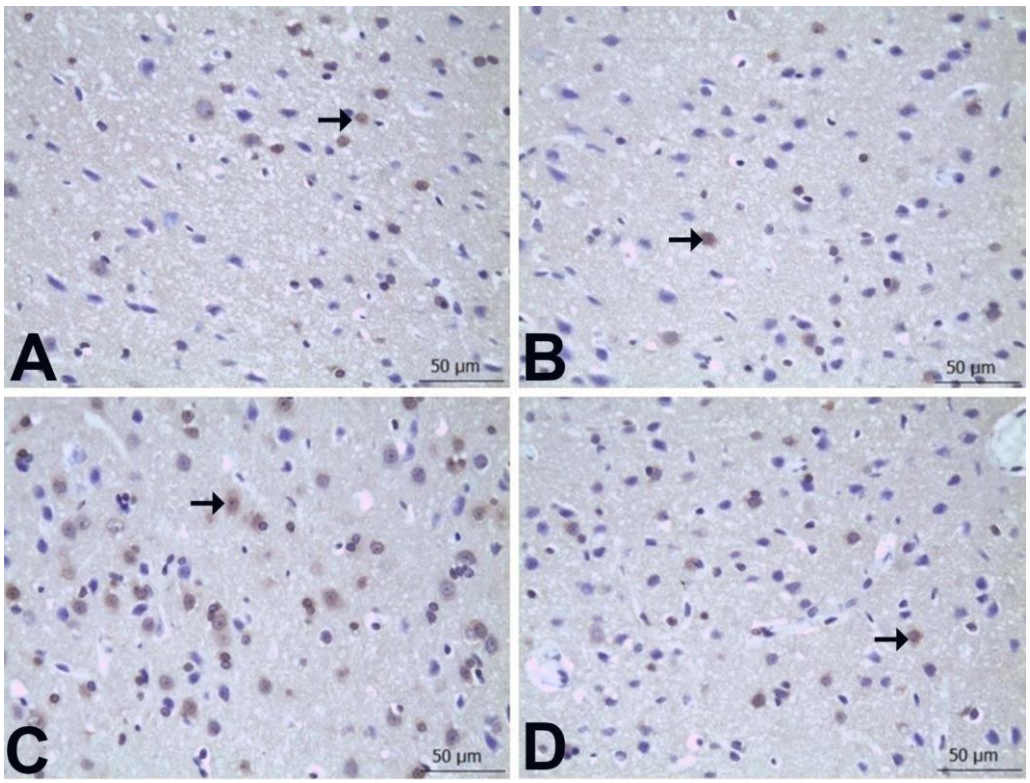

Figure 3. p-ERK1/2 immunohistochemistry.

A) Control group; mild level, B) BGE group; mild level, C) ACR group; severe level, D) BGE+ACR group; moderate level p-ERK1/2 immunopositivity (arrows). Bar: 50 µm, IHC.

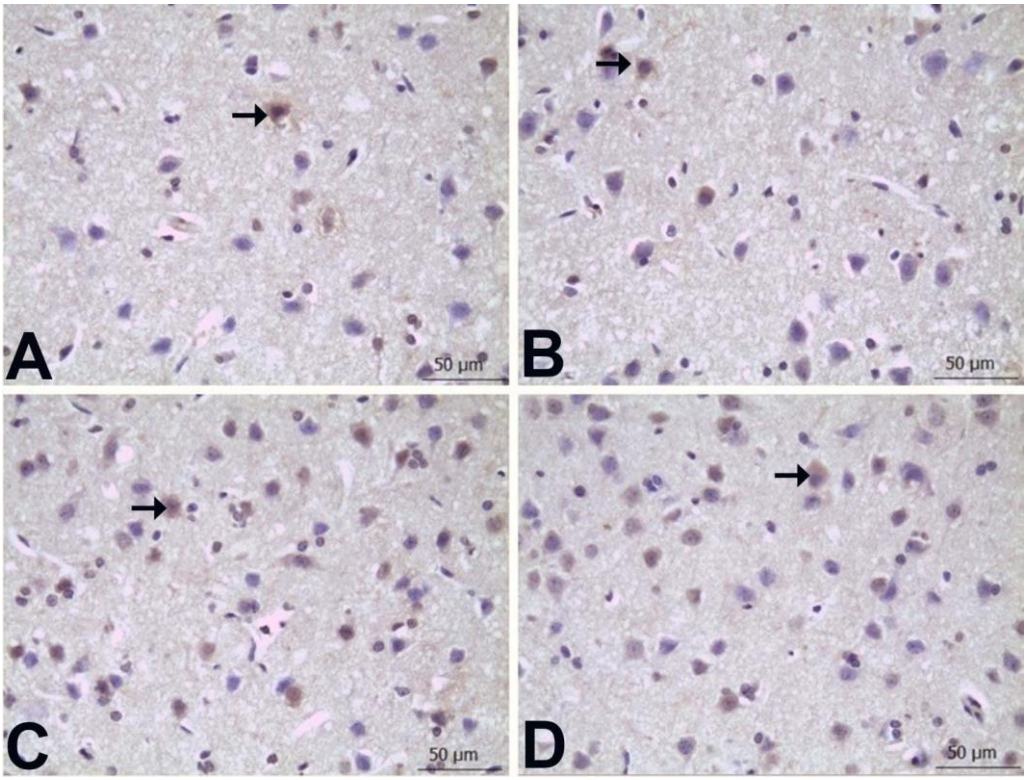

Figure 4. c-Fos immunohistochemistry.
A) Control group; mild level, B) BGE group; mild level, C) ACR group; severe level, D) BGE+ACR group; moderate level c-Fos immunopositivity (arrows). Bar: 50 µm, IHC.

Relative expressions of Bcl-2, Caspase-3, ATF6, CREB, and NfkB-p65 proteins: In the relative protein expression analysis, the CREB and Bcl-2 protein expression levels were found as the highest in the control group, while a dramatic decrease was detected in the ACR group. However, an increase was observed in the BGE+ACR group compared to the ACR group. In the analysis of Nf-kB, Caspase-3, and ATF6 protein expression levels, a significant increase was seen in the ACR group, while a relative decrease was achieved in the BGE+ACR groups. The lowest protein expression level was determined in the Control and BGE groups compared to other groups. Protein gel images and analysis graphics for all groups are presented in (Figure 5).

Biochemical Results

Effects of Black Garlic Ethanol Extract on ACR-Induced Brain Tissue Oxidative Stress: The effects of BGE administration on ACR-induced brain tissue oxidative stress are summarized in Figure 6. The MDA level was significantly higher in the ACR group than in the control group ($P<0.05$). BGE significantly ($P<0.05$) prevented ACR-induced lipid peroxidation. BGE administration alone did not cause any change in MDA level ($P>0.05$). SOD and CAT enzyme activities were significantly decreased in the ACR group compared to the control, BGE+ACR and BGE groups ($P<0.05$). In addition, GSH levels decreased in the ACR group compared to the BGE+ACR group, but this decrease was not statistically significant ($P>0.05$).

Effects of Black Garlic Ethanol Extract on ACR-Induced Inflammation in Brain Tissue: The effects of BGE administration on inflammation in ACR-induced brain tissue are summarized in (Figure 7). TNF-α, IL-1-β, and IL-6 levels were significantly increased in the ACR group compared to control, BGE, and BGE+ACR groups ($P<0.05$). BGE administration appears to prevent ACR-induced cytokine increase.

DOI: 10.33988/auvfd.1384531

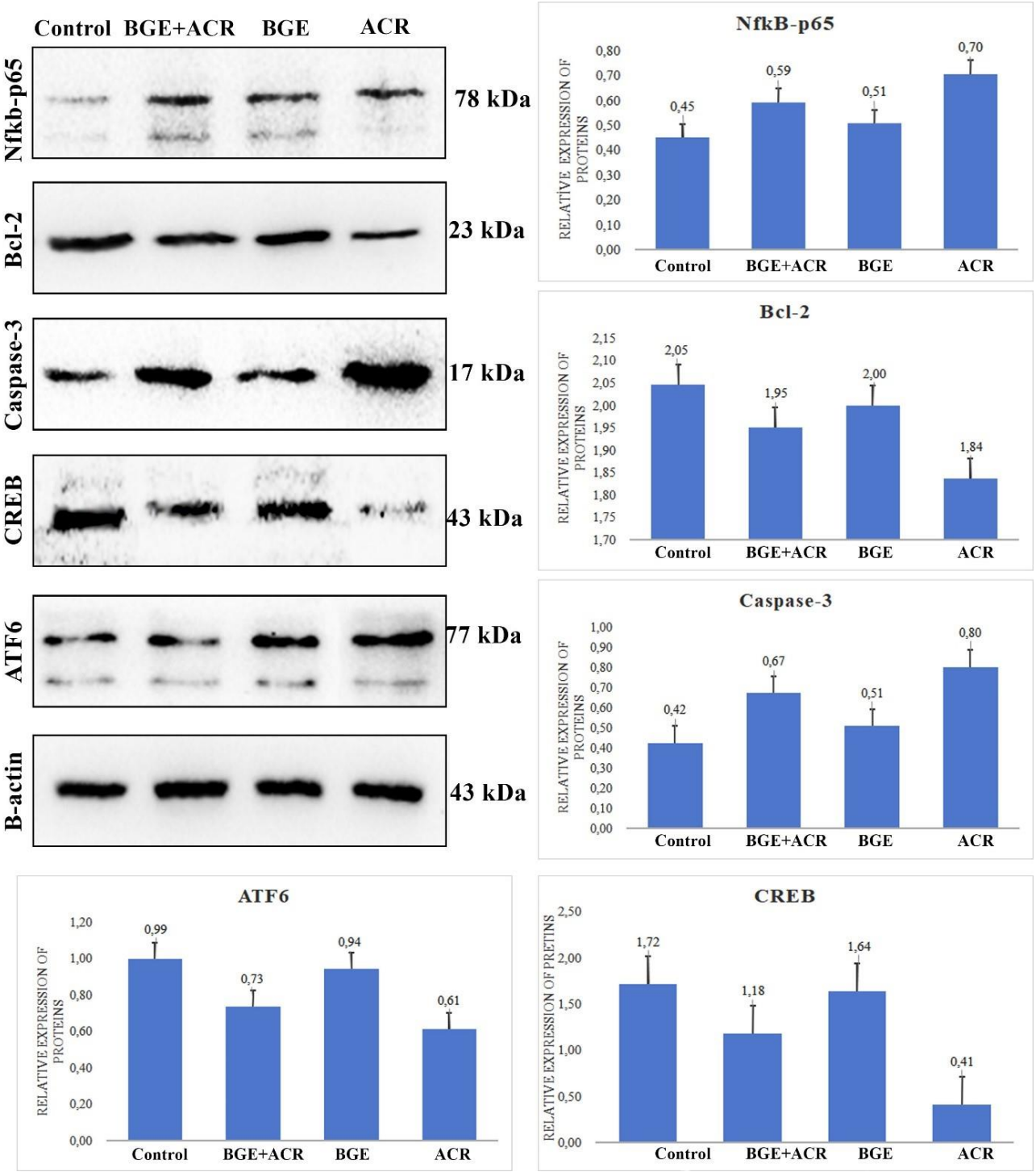

Figure 5. Relative expression of proteins for ATF6, Bcl-2, CREB, Caspase-3, NfkB-p65.

The values are given as mean ± SD (n=6) and analyzed by one-way ANOVA followed by the Tukey test. Distinct letters denote statistically significant variances, ($P<0.05$).

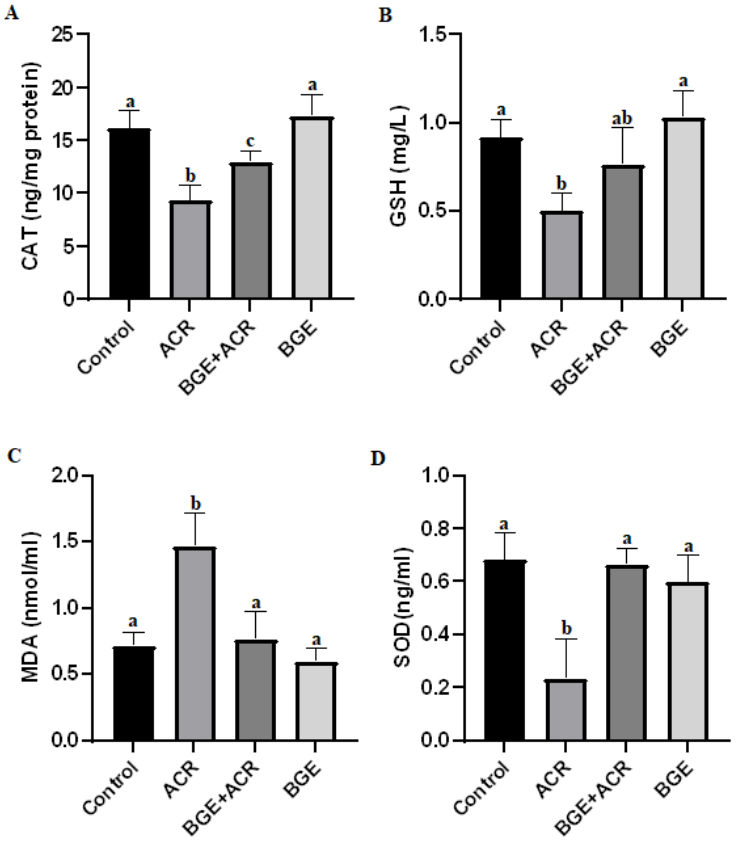

Figure 6. Effects of BGE against ACR-induced brain tissue oxidative stress in rats.

Values are expressed as mean ± SD. Different letters in columns (a-c) indicate statistical difference (P<0.05).

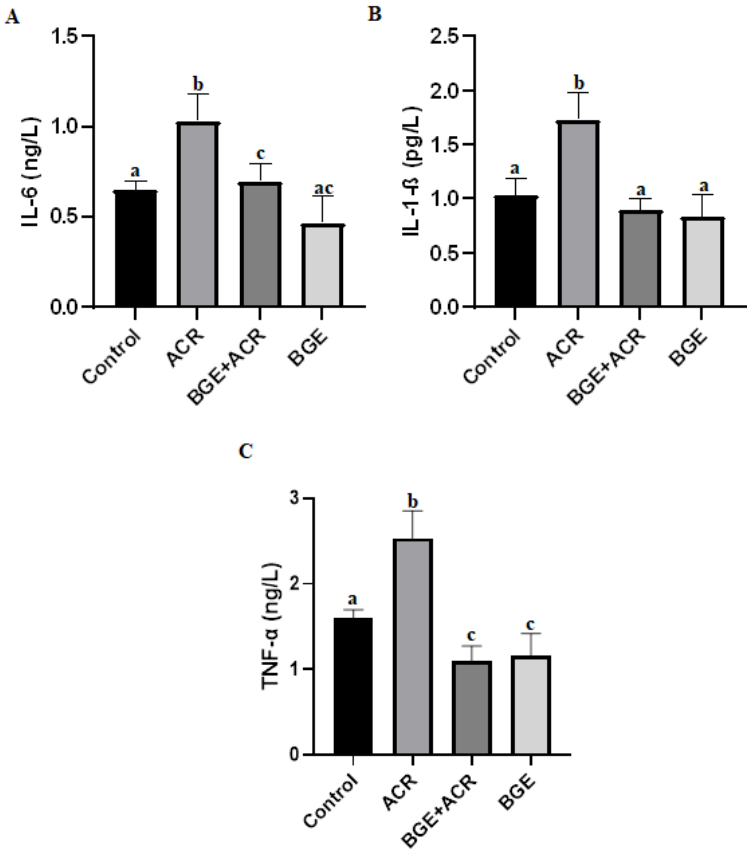

Figure7. Effects of BGE against ACR-induced brain tissue inflammation in rats.

Values are expressed as mean ± SD. Different letters in columns (a-c) indicate statistical difference (P<0.05).

Discussion and Conclusion

ACR, despite its toxic effects, finds application as an industrial chemical in various fields, including polymers, paper, cosmetics, fabrics, wastewater treatment, and the manufacture of laboratory gels. It is also formed during the high-temperature cooking of starchy foods (15). ACR exposure has been recorded through consumption of ACR-containing foods, drinking water contaminated with polyacrylamide coagulants, and occupational exposure through dermal contact or inhalation of industrial production dust (42). Adults typically have an average daily ACR intake of 1 µg/kg body weight/day. Exposure to ACR has been documented to induce diverse toxic effects in experimental animals (23). Exposure to ACR can notably trigger apoptosis and oxidative stress in pertinent tissues and cells and has been suggested to disrupt the intracellular oxidant and antioxidant balance system in cells (20). Various doses of ACR have been demonstrated to lead to neurotoxicity, reproductive toxicity, genotoxicity, and embryonic toxicity (47).

It has been documented that ACR results in severe damage to brain tissue, and the utilization of substances possessing antioxidant and anti-inflammatory properties are increasingly prevalent to mitigate or address this damage (46). In line with this information, in this study, we investigated the protective effects of BGE administration against ACR-induced neurotoxicity in rats.

It was determined that ACR caused abnormal histopathological findings in the cerebral cortex and meninges in rats. It was reported that there was significant occlusion in the submeningeal blood vessels and degeneration in the cerebral cortex (12). In our study, ACR application caused severe pyknotic-degenerative changes in neurons and BGE had a protective effect. Hermawati et al. (26) reported that BGE was protective against monosodium glutamate (MSG)-induced brain damage. The fact that BGE administration was effective against ACR-induced brain tissue damage in the histopathological findings we obtained in our study suggests that BGE may have a multifaceted protective effect when evaluated with the findings of Hermawati et al. (26). Because it has been reported that BGE may be protective against neurodegenerative diseases in the brain. For example, it has been reported that BGE application against oxidative stress caused by MSG in experimentally induced Alzheimer's disease is a powerful antioxidant that has a positive effect on memory (34) and helps to increase pyramidal neurons in hippocampus regions (7).

Immunohistochemically, the levels of Erk1/2, p-ERK1/2, and c-fos in the brain were examined. It was found that while these protein levels increased with ACR application, their levels decreased with BGE application. It is known that high ROS activates the MAPK pathway and thus Erk1/2 in response to glutamate-induced oxidative stress (32). Furthermore, Erk1/2 activation has been demonstrated in response to neurological injuries (35) and phosphorylated as p-ERK1/2 to provide neuroprotection. BGE significantly enhances Erk signaling suppressed by acrylamide in the hippocampus (13). The present study revealed that ACR administration increased Erk1/2, p-ERK1/2, and c-Fos expression in brain tissue and that BGE administration prevented this increase.

There is a close relationship between oxidative stress caused by ROS and the apoptosis mechanism. ROS has a significant role in stimulating the apoptotic mechanism in the cell (19). Apoptosis occurs through a mechanism induced by members of the Bcl-2 protein family, including Caspase-3 and Bcl-2 (10). In ACR-induced brain toxicity, increased Caspase-3 expression and decreased Bcl-2 expression were reported. BGE has been reported to suppress apoptosis by decreasing Caspase-3 expression and increasing Bcl-2 expression in various tissues (49). In this study, ACR significantly increased Caspase-3 expression and decreased Bcl-2 expression in brain tissue, while BGE decreased Caspase-3 expression and increased Bcl-2 expression. CREB serves as a significant nuclear transcription factor involved in the nervous system. CREB activation serves as a protective factor of brain tissue against damage (17, 31). It has been reported that ACR treatment decreased CREB expression in human neuroblastoma cell lines (29), while garlic extract increased CREB levels (13). The data we obtained in our study are consistent with this and show that while ACR application decreases CREB expression in brain tissue, BGE application prevents this decrease. It has been reported that garlic extract dropped the elevated NfkB-p65 level in Alzheimer's disease (37) and decreased ATF 6 level in this Alzheimer's disease increased with garlic extract (41).

In addition, the effect of BGE against inflammation induced by ACR was examined in the present study. Inflammation is associated with many chronic diseases, especially cancer, diabetes, cardiovascular, and neurological diseases. It has been emphasized that ACR induces inflammation by increasing proinflammatory cytokine levels and decreasing anti-inflammatory cytokine levels (1). Garlic has been found to reduce proinflammatory cytokines such as IL-1β, IL-6, and TNF-α (41). In addition, BGE was found to prevent/reduce inflammation experimentally (2). It was determined that neuronal inflammation induced by ACR increased proinflammatory cytokine levels and decreased anti-inflammatory cytokine levels. BGE prevented ACR-induced neuronal inflammation with an anti-inflammatory effect.

Studies in experimental animals have reported that ACR causes oxidative stress and cell damage (18, 33). Gur et al. found that SOD, CAT activity, and GSH levels decreased, and MDA levels increased in ACR-induced brain damage (25). It was reported that ACR application significantly decreased SOD and GSH activities and significantly increased MDA levels (16). BGE administration significantly reduced ACR-induced lipid peroxidation by decreasing MDA levels and increased SOD, GSH, and CAT expression levels (13). In our study, we determined that ACR administration stimulated oxidative stress in brain tissue, MDA level increased, GSH levels, SOD and CAT activities decreased as a result of induction of lipid peroxidation. BGE is an agent with a proven protective effect against various tissue toxicities caused by numerous agents on the brain (7, 26). We found that BGE administration significantly prevented ACR-induced oxidative stress in rats in our study. These findings were consistent with the literature.

The findings of the current study provide robust evidence for the anti-inflammatory properties of BGE, given its ability to suppress both NFKB and pro-inflammatory cytokines. We found that BGE attenuates oxidative damage after ACR exposure, resulting in reduced neuroinflammation and signaling expression, and neurological improvement. It provides an effective intervention strategy against neurotoxins and neurological diseases by providing anthocyanin-enriched foods. Our results suggest that BGE is a promising agent to eliminate ROS and thus may alleviate brain injuries mediated by oxidative stress. We think the findings will contribute to future research, but further studies are needed.

Financial Support

This research received no grant from any funding.

Conflict of Interest

The authors declare that all data were generated in-house and that no paper mill was used.

Author Contributions

AG, EKS, VG, SEY, MÖ, GB, FÇ and İE experiment design, experiment application, samples collection. AG, EKS, SEY, MÖ, GB and İE histopathological and immunohistochemical, investigation. VG and FÇ western blot analysis, determination antioxidant enzyme activities and of inflammation markers. All the authors contributed to the writing and editing, and they read and approved the manuscript.

Data Availability Statement

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

Ethical Statement

The approval was obtained by the Animal Experiments Local Ethics Committee of Kafkas University, Faculty of Veterinary Medicine, on the date of 03.10.2023 with the ethics committee number 2023/11 and decision no 108 for this study.

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 the preparation, characterisation, and release properties of Thymol-Containing Gelatin-Based Hydrogels for Varroosis control

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ARTICLE INFO

Article History
Received : 27.11.2023
Accepted : 25.02.2024
DOI: 10.33988/auvfd.1396538

Keywords
Controlled release system
Gelatine
Hydrogel
Thymol
Varroosis

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How to cite this article: Demir O, Pulat M, Bilgili A (2024): Evaluation of the preparation, characterisation, and release properties of Thymol-Containing Gelatin-Based Hydrogels for Varroosis control. Ankara Univ Vet Fak Derg, 71 (4), 407-416. DOI: 10.33988/auvfd.1396538.

ABSTRACT

The aim of this study was to investigate the swelling and degradation behavior of a controlled-release system using gelatin, thymol loading capacity, characterization, morphology, and thymol release level in relation to the recommended therapeutic dose for varroosis control. In this context, a series of hydrogels were first produced using a glutaraldehyde (GA) crosslinker and swelling tests were performed. Thymol loading was performed on the appropriate hydrogels, with swelling values between 269% and 431%. Thymol loading efficiency was determined to be between 20.07% and 29.80%. The chemical structures of the hydrogels with and without thymol loading were compared by Fourier transform infrared spectrometry (FT-IR) and it was determined that thymol was loaded into the structure. The morphological structures of the thymol-loaded and unloaded hydrogels were examined by scanning electron microscopy (SEM). It was observed that the non-thymol-loaded hydrogel had larger pores than the thymol-loaded hydrogel. A model release environment and measurement system were developed to predict the release type, level, and duration of the controlled release system in the hive air environment. In this model release environment, release tests were carried out for four weeks using different thymol application systems and the measurements were compared. As a result, it was found that the controlled thymol release system developed for the control of varroosis showed a more stable release compared to existing application systems.

Introduction

Varroosis is an external parasitic disease of bees characterized by the invasion of honey bee colonies by the *Varroa destructor* mite, which feeds on the haemolymph of adult and young bees and causes various damages to the colony (4). When measures are not taken against mites, colony productivity decreases, susceptibility to other diseases increases and colonies may die in more advanced stages. Many control methods have been developed against the mite, and chemical control methods are the most important of these. Thymol is a chemical used for varroosis control since the 1980s (1, 13, 35).

When *Varroa* mites inhale thymol, which sublimates and passes into the hive air, GABA-gated chloride channels are blocked. Thus, the acaricidal effect occurs as a result of excessive stimulation and convulsions in the central nervous system (2, 9, 10, 15). When thymol is applied to colonies, acaricidal activity varies in relation to environmental temperature and humidity (14). When thymol-containing drugs are applied to colonies at ambient temperatures lower than 15 °C, queen death may occur, and when applied at temperatures higher than 30 °C, agitation, flight, brood, and adult bee mortality may increase (3, 5, 7). Thymol is a chemical with a narrow therapeutic window that acts on *Varroa* mites and honey bees by the same mechanism. The topical LD_{50} of thymol is 56.1 µg for adult mites, 210.3 µg for adult worker bees and 150.7 µg for bee larvae (17). Thymol is effective

against the mite by inhalation at a concentration of 5–15 µg/L, but at an air concentration of 20 µg/L, it causes 90% mortality in bees (20).

Controlled release systems (CRS) are drug delivery systems that allow the active substance to be administered at the desired location, concentration, rate and duration. In CRS, the active substance is located in a drug reservoir coated with a film or in a matrix in which the drug is dispersed. From here, slower and more stable release processes are realized with different kinetics (11). CRS can provide benefits such as administering the dose within the therapeutic range, minimizing the amount of drug required, reducing side effects, and decreasing the frequency of administration (25, 27). In addition, CRS can increase the stability of the active substance against harmful effects such as temperature, oxidation, moisture, and microorganisms and can limit its high volatility (31, 8). On the other hand, CRS may have various disadvantages, such as toxicity or non-biocompatibility of the polymer used, unpredictable and poor correlation between in vitro release and in vivo release, unwanted degradation products, difficulty in dosage adjustment and cost (18).

Hydrogels are indispensable materials for CRS. Hydrogels are cross-linked, three-dimensional, hydrophilic polymeric structures that are insoluble in water and can absorb at least 20% or more of their dry mass. Cross-links between polymers can be shaped chemically (covalent or ionic) or physically (crystallinity) and they are insoluble in water thanks to these bonds (28). Hydrogels are widely used in biomedical fields such as contact lenses, artificial heart, muscle, and skin materials due to their biocompatibility (28), as well as in the pharmaceutical industry as support materials for controlled drug release systems and enzyme immobilizations (29).

Gelatine is a natural polymer used in pharmaceutical and biomedical fields such as drug coating, microencapsulation, and hydrogel production. In order for the materials to be produced from its aqueous solutions to be used for a long period of time, gelatine must be subjected to cross-linking, which increases both its thermal and mechanical stability. The most widely used chemical crosslinker for this purpose is glutaraldehyde (GA). The swelling and failure behavior of hydrogels can be controlled by changing the crosslinker and polymer ratios (30).

The aim of this study was to develop and characterize controlled thymol-releasing hydrogel systems for use in varroosis control. To this end, hydrogel systems were developed using gelatin polymers, GA crosslinkers, and thymol as the active substances. Swelling and thymol loading tests were carried out on the produced hydrogels. In addition, their chemical structures were examined by Fourier transform infrared spectrometry (FT-IR) and their morphological structures were examined by scanning electron microscopy (SEM). Finally, release tests of a thymol-loaded hydrogel were carried out in a model release environment for four weeks. It was planned to select the appropriate hydrogel for varroosis control studies in honey bee colonies.

Materials and Methods

Materials: Bovine gelatin (Food grade, Alfasol), GA (Technical grade, 25%, Merck), sunflower oil (Food grade, Yudum), thymol (Technical grade, 99% purity, Sigma-Aldrich), and distilled water were used for hydrogel production. For chromatographic analyses, thymol reference standard (99.6% purity, Dr. Ehrenstorfer), acetonitrile (HPLC grade, Sigma-Aldrich), n-methyl-2-pyrrolidone (NMP) (Technical grade, BASF), and distilled water were used.

Preparation of Hydrogels: 8% and 10% gelatin solutions were prepared using distilled water. The solutions were kept in an ultrasonic bath at 50 °C for one hour to completely dissolve the gelatin. Gelatin solutions and GA solutions were added into Falcon tubes at different ratios, as shown in Table 1. The tubes were mixed at 1000 rpm for 2 minutes and kept at room temperature for 24 hours. The hydrogels were removed from the tubes and cut into 0.5-cm-thick discs with a scalpel. The discs were washed three times successively with distilled water to remove gelatin and GA that did not enter the crosslinking reaction. The hydrogel discs were placed in an oven (IN 260, Memmert) set at 30 °C. The discs were weighed daily with a precision balance (AK160, Mettler Toledo, Ohio, USA), and the drying process was terminated when a constant weight was reached. The discs were stored in moisture- and light-proof bags until they were used in further studies.

Table 1. Gelatin Hydrogel Preparation Conditions.

Hydrogel Code	Gelatin (%)	GA (%12.5 mL)	GA/Gelatin Ratio
G-1	8	0.2	6.25×10^{-3}
G-2	8	0.4	12.50×10^{-2}
G-3	8	0.8	25.00×10^{-2}
G-4	8	1.6	50.00×10^{-2}
G-5	8	2.4	75.00×10^{-2}
G-6	8	3.2	1.00
G-7	8	4.0	1.25
G-8	10	0.2	5.00×10^{-2}
G-9	10	0.4	10.00×10^{-2}
G-10	10	0.8	20.00×10^{-2}
G-11	10	1.6	40.00×10^{-2}
G-12	10	2.4	60.00×10^{-2}
G-13	10	3.2	80.00×10^{-2}
G-14	10	4.0	1.00

DOI: 10.33988/auvfd.1396538

Swelling Test: The dried hydrogel discs were placed in sample containers containing 100 mL distilled water and placed in an oven (IN 260, Memmert) set at 30 °C. The discs were removed from the water at regular intervals and dried superficially with filter paper. The weighed discs were returned to the water and placed in the oven. Weighing was continued until a constant weight was reached for each disc. The test was carried out on three samples. The mean percentage swelling values (S%) were calculated by Equation 1 (29) (md: dry weight, mw: wet weight).

$$S\ (\%)= \frac{mw-md}{md} \times 100 \quad (1)$$

Thymol Loading and Loading Efficiency Determination: Thymol-loaded samples were prepared by using G-3 and G-10 hydrogels, which were prepared with gelatin solutions at different concentrations and exhibited the most ideal properties in terms of swelling and structural stability. Although thymol's solubility in water is very low, its solubility in vegetable oils is high (6). Accordingly, thymol was added to the gelatine solutions in two different ways before the cross-linking step, following the procedure, described in the section on the preparation of hydrogels. In the first group (T), pure thymol in solid form was added at weights of 0.5 g, 1 g, and 1.5 g. In the second group (OT), 1 mL, 2 mL, and 3 mL of 50% thymol-sunflower oil solutions were added to the tubes, respectively. Then, thymol-loaded hydrogels were produced by adding GA crosslinker. Table 2 presents the prepared samples and their codes.

Table 2. Preparation Conditions of Thymol Loaded Gelatin Hydrogel.

Hydrogel Code	Gelatin (%)	GA (%12,5, mL)	Thymol (g)	Thymol/Sunflower Oil Solution (mL)
(G-3)-T1	8	0.8	0.5	-
(G-3)-T2	8	0.8	1.0	-
(G-3)-T3	8	0.8	1.5	-
(G-3)-OT1	8	0.8	-	1.0
(G-3)-OT2	8	0.8	-	2.0
(G-3)-OT3	8	0.8	-	3.0
(G-10)-T1	10	0.8	0.5	-
(G-10)-T2	10	0.8	1.0	-
(G-10)-T3	10	0.8	1.5	-
(G-10)-OT1	10	0.8	-	1.0
(G-10)-OT2	10	0.8	-	2.0
(G-10)-OT3	10	0.8	-	3.0

The hydrogel discs were extracted with acetonitrile to determine thymol loading efficiencies. Three samples were prepared for each hydrogel series and the thymol content was measured using HPLC-DAD (Thermo Scientific, Dionex Ultimate 3000). Analytical separation was achieved with a C18 (250 mm x 4.60 mm, 5 µ ACE) column. Acetonitrile and water were used as the mobile phase in a 75/25 ratio at a flow rate of 1 mL/min. Samples were injected into the system in a volume of 20 µL. The detection of thymol peaks was performed in 5 minutes and at 278 nm. The drug loading efficiency of the hydrogel series was calculated by Equation 2 (38).

$$\text{Loading Efficiency}\ (\%)= \frac{\text{Total Thymol Mass in Hydrogel}}{\text{Total Hydrogel Mass}} \times 100 \quad (2)$$

FT-IR Analysis: Fourier Transform Infrared Spectroscopy (FT-IR) analyses (Spectrum Two™, Perkin Elmer) were performed to determine whether cross-linking between gelatin and GA was present and to prove the presence of thymol loaded in the hydrogel structure. For this purpose, direct spectra of dry samples of pure gelatin, G-10 hydrogel, and thymol-loaded (G-10)-OT1 hydrogel were taken. The scans were carried out in the wavelength range 4000-450 cm1 with a MIR TGS detector at 4 cm^{-1} resolution and 0.2 cm^{-1} scan rate conditions and the spectra were compared.

Scanning Electron Microscopy (SEM) Analysis: For the determination of hydrogel morphology, surface topography, and microstructure, 1 cm-thick discs were formed from thymol-loaded hydrogel ((G-10)-OT1) and non-thymol-loaded hydrogel ((G-10)-O1) with the help of a scalpel. The hydrogel discs were allowed to swell in distilled water at room temperature for 48 hours. The discs removed from the water were kept in the refrigerator at -20 °C overnight and frozen. The frozen discs were placed in a lyophilization device (FreeZone1, Labconco, Canada) and dried at -40 °C for 10 days (22). From the dried discs, smaller sized samples were prepared with a razor blade. The samples were coated with a 3.0 nm (±0.5 nm) thick layer of gold (Au) in a coating device (Cressington Sputter Coater 108 Auto, Watford, UK). The coated samples were imaged by SEM (VEGA3 SBU-EasyProbe, TESCAN, Czech Republic) under 15.0 kV (19).

Release Study: Release studies of thymol-loaded (G-10)-OT1 hydrogel were performed in order to examine the kinetics of thymol release under laboratory conditions and to evaluate the suitability in terms of the therapeutic window. A model release environment shown in Figure 1 was created with variables such as temperature, humidity, and hive ventilation that are effective on the pharmacological and toxicological processes of thymol in

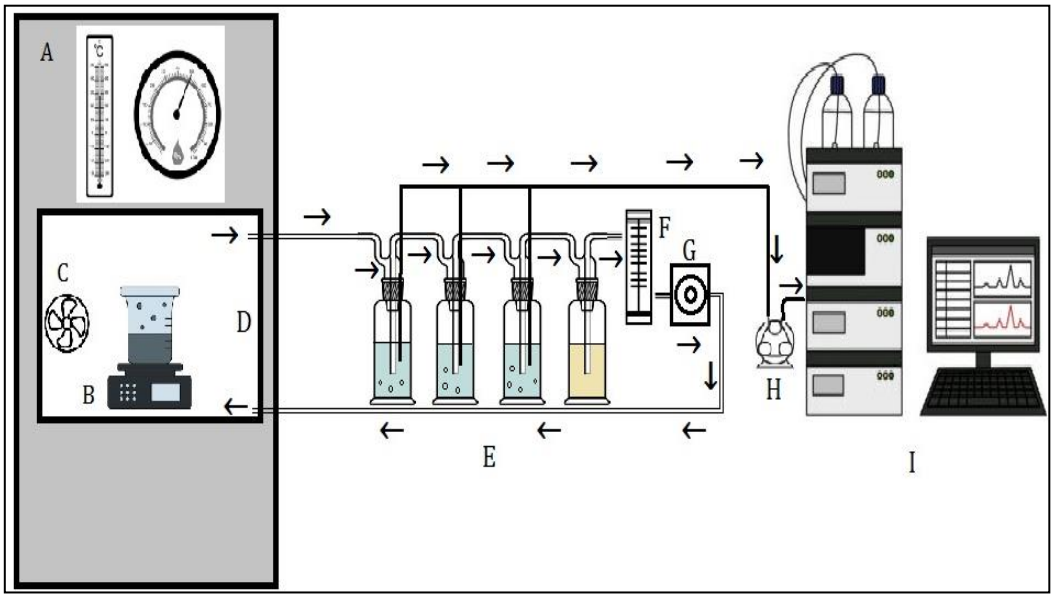

Figure 1. Release System: A-acclimatisation cabinet, B- balance, C-fan, D-hive, E-gas washing bottles, F-flow meter, G-air vacuum pump, H-peristaltic pump, I-HPLC DAD system.

colonies in nature (12, 14, 16, 26, 33, 34, 36). In this setup, bee, honey, and beeswax compartments and variables that may affect the release level, such as fluid dynamics, were ignored. For the release test, three experimental groups containing equal weights of thymol were formed. A reference drug containing thymol approved for use in the control of varroosis, pure thymol, and hydrogel ((G-10)-OT1) samples containing 12.5 g of thymol were used for four weeks.

A plastic hive with Langstroth hive dimensions was placed in an air-conditioned cabinet (Weiss WK 111–180). A fan, data logger, and balance equipment were installed in the hive. Samples were placed on the balance sheet. The hive was isolated in such a way that air inlets and outlets were provided only by pipes. The fan was operated at 400 rpm (37). The temperature of the chamber was periodically increased by 5 °C from 10 °C to 35 °C with software (Simpati 4.50) and then decreased again until the temperature reached 10 °C, creating a 24-hour temperature cycle. Hive air passed through pipes (A-60-G, Tygon) was vacuumed by an air pump (Aco 9601, Hailea) at an average speed of 0.42 L/min, adjusted by a flowmeter (LZT 4 T). The vacuumed air was sequentially passed through three gas washing bottles containing 500 mL volumes of a 10% NMP solution. The air was then returned to the hive through a gas scrubber bottle containing silica gel to trap excess moisture. The humidity level inside the hive was monitored in terms of 40%–60% relative humidity limits. The total amount of thymol retained in the solutions sent from the gas washing bottles to the HPLC loop system by capillary tubes and peristaltic pumps was measured daily by the HPLC-DAD device. At the end of four weeks, thymol adsorbed on the hive wall was collected according to a method given in the literature (32). In addition, the weight changes of the samples were measured daily with a balance.

Results

Swelling Test Results: Among the hydrogels synthesized with different ratios of gelatin and GA composition, it was aimed to determine at least two hydrogels that provided the highest level of swelling while maintaining their robustness as a result of swelling tests. As shown in Figure 2 and Figure 3, it was determined that the %S initially increased with time and then remained constant at the end of 48 h. The average %S values were determined as 431% (± 42.90) for the most swelling hydrogel G-1 and 269% (± 17.61) for the least swelling hydrogel G-14. It was determined that all hydrogels completed the 48-hour swelling test without disintegration and with an average swelling of 349% (± 53.94). Two hydrogels (G-3 and G-10) with the highest level of swelling, robust structure, and homogenous appearance were selected to be tested in thymol loading studies.

Thymol Loading Results: The thymol loading efficiency results for the synthesized hydrogels were compared as shown in Figure 4. The average thymol loading efficiency was calculated to be 25.26% (± 3.05). In general, it was observed that in hydrogels loaded with pure solid thymol, the thymol was oriented towards the outer surface of the hydrogel. Thymol loaded as dissolved in sunflower oil was found to be homogeneously distributed in the hydrogel matrix structure. The hydrogel coded (G-10)OT1, which exhibited a robust structure and homogeneous appearance as well as above-average loading efficiency, was selected for use in release trials.

DOI: 10.33988/auvfd.1396538

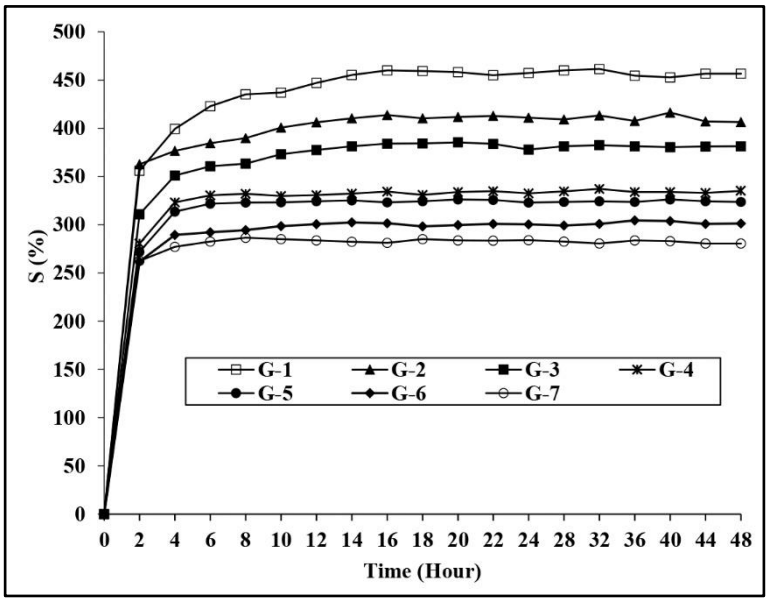

Figure 2. Swelling-Time Graph of Hydrogels (8%).

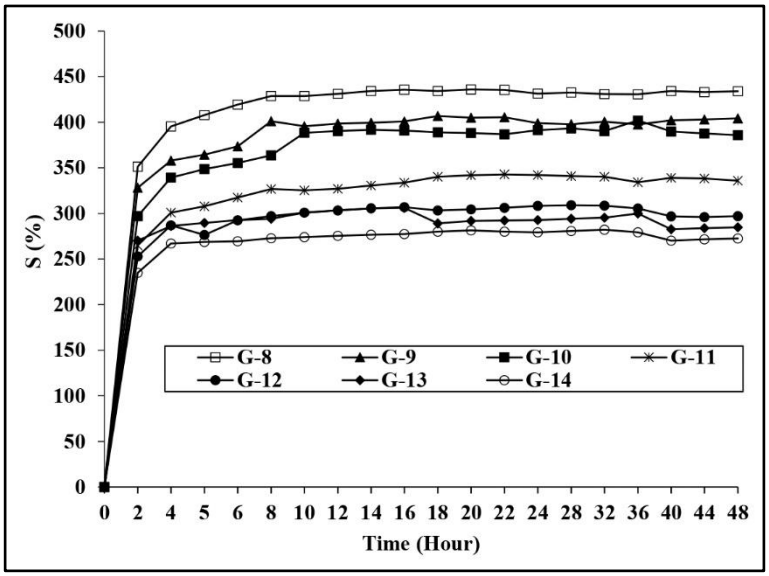

Figure 3. Swelling-Time Graph of Hydrogels (10%).

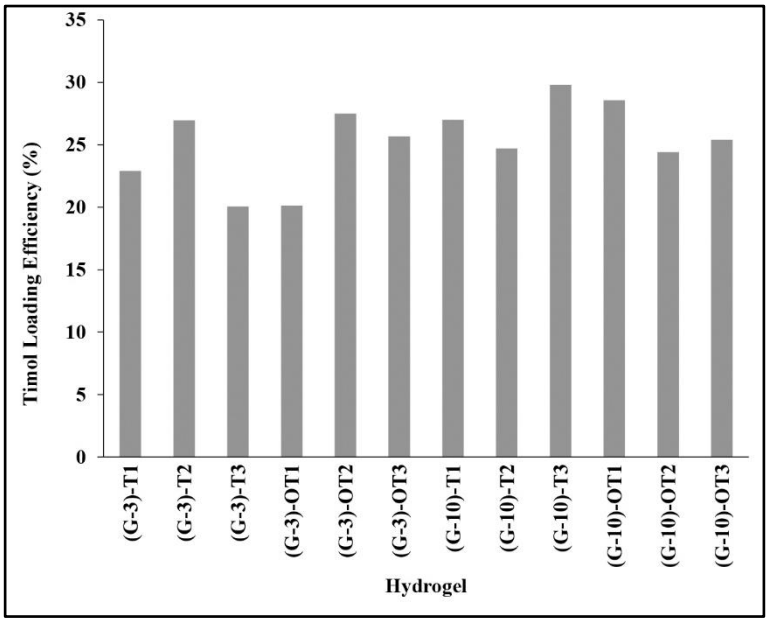

Figure 4. Timol Loading Efficiency Graph of Hydrogels.

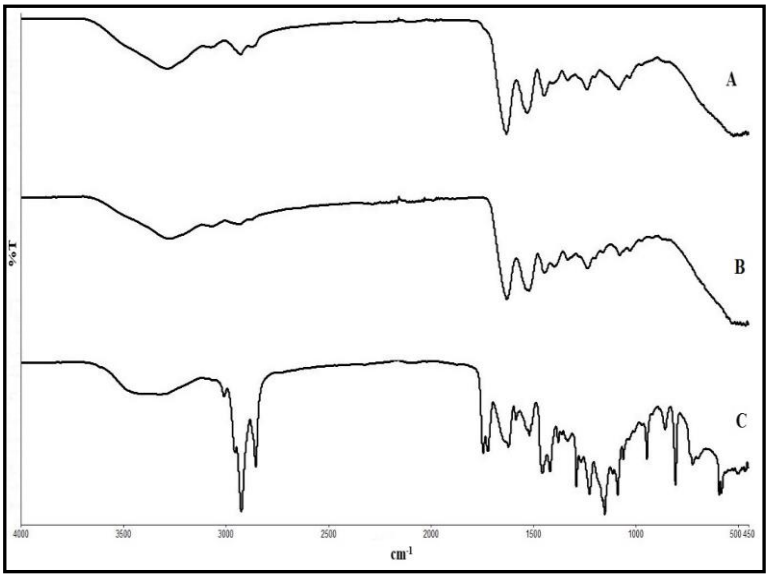

Figure 5. FT-IR Spectra of gelatin (A), Hydrogel (G-10) (B), Thymol Loaded Hydrogel (G-10)OT1 (C).

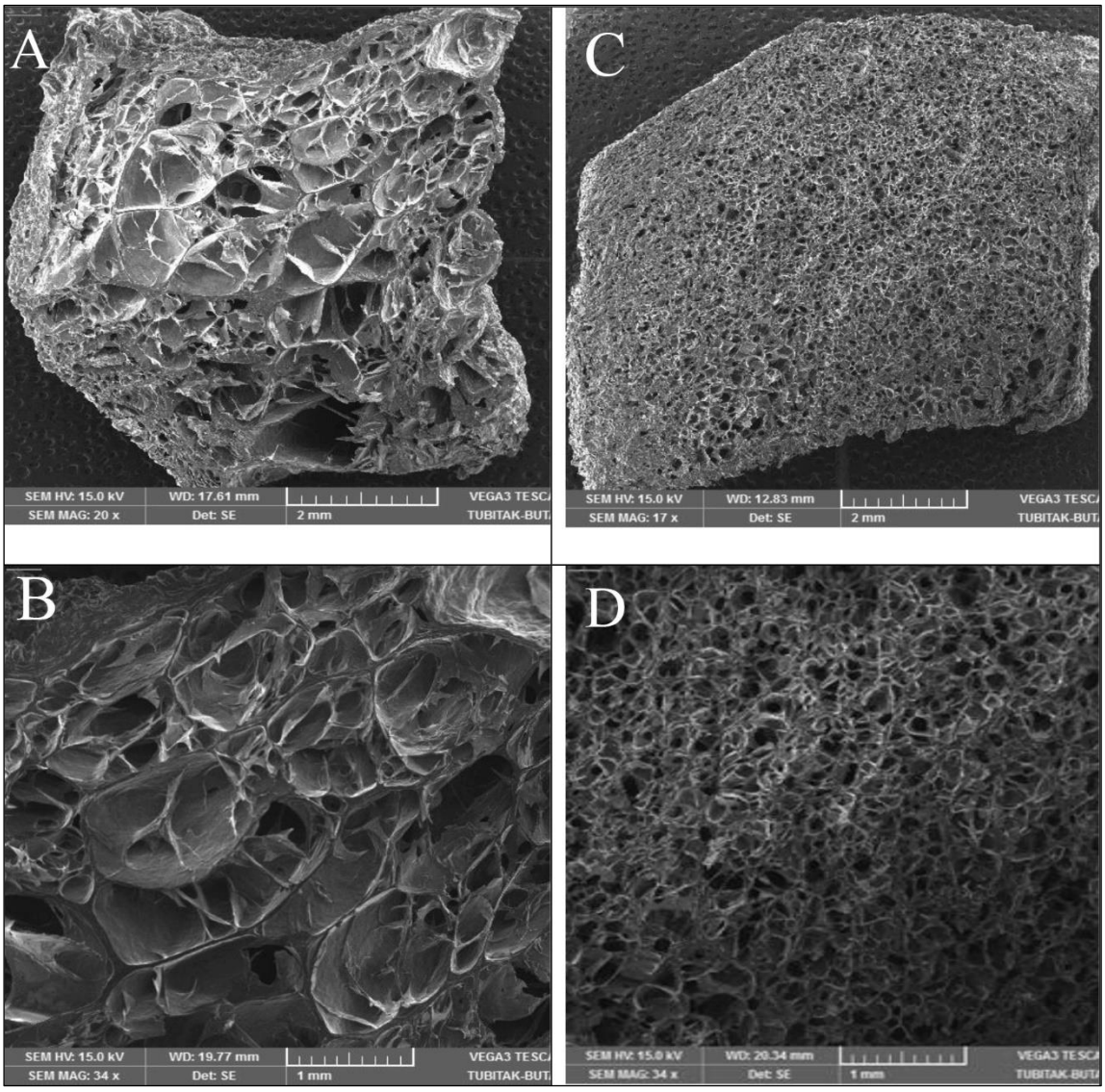

Figure 6. SEM Images of Non-Thymol Loaded Hydrogel (G-10)O1 (A- 2 mm, B-1 mm) and Thymol Loaded Hydrogel (G-10)OT1 (C- 2 mm, D-1 mm).

DOI: 10.33988/auvfd.1396538

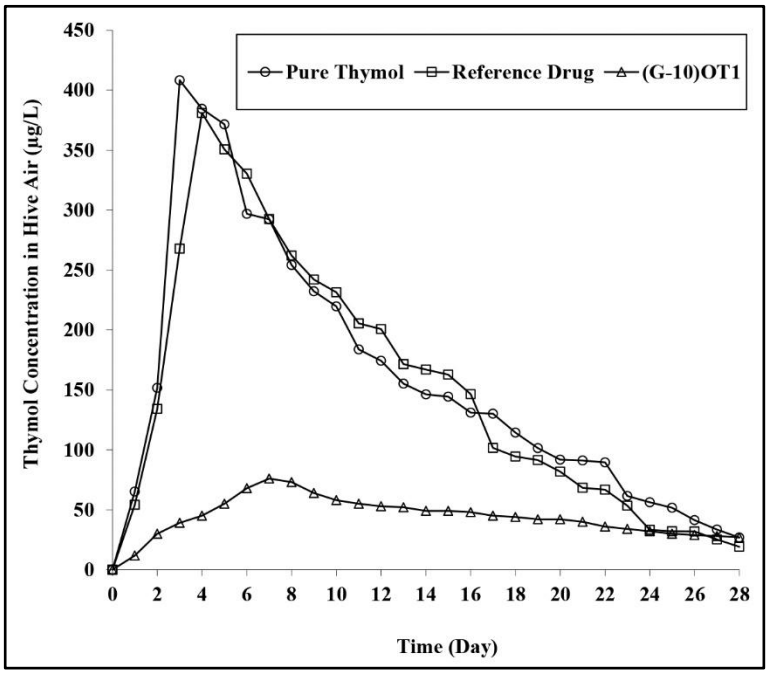

Figure 7. Time Graph of Thymol Concentration in Hive Air.

FT-IR Results: The spectra of pure gelatin, G-10 hydrogel, and (G-10)-OT1 hydrogel were compared as shown in Figure 5. The spectra of gelatin and G-10 hydrogel are generally similar to each other. After the crosslinking reaction, the narrowing of the amine (N-H) peak at 3283 cm^{-1} of gelatin and the decrease in the primary amine content of gelatin in the hydrogel (G-10) structure were observed. In the spectrum of (G-10)-OT1 hydrogel, characteristic C-H bond peaks of thymol were observed at 2924 cm^{-1} and 2854 cm^{-1}.

Scanning Electron Microscopy Results: When evaluated in terms of macroscopic findings, it was observed that the hydrogels obtained after freezing at -20 °C and the subsequent lyophilization process were suitable for disintegration. As shown in Figure 6, when compared to microscopic findings, there are significant differences between the hydrogels in terms of pore size and internal structure appearance. It was determined that there were pores in the matrix structure with an average diameter of 280 µm (±17.74) for the non-thymol-loaded (G-10)O1 hydrogel and 78.39 µm (±11.63) for the thymol-loaded (G-10)OT1 hydrogel.

Results of Release Study: At the end of the release studies, it was determined by gravimetric measurements that 5.28 g of pure thymol, 5.38 g of reference drug and 1.87 g of hydrogel ((G-10)OT1) occurred due to the sublimation of thymol. The amounts of thymol released from pure thymol, the reference drug, and thymol-loaded gelatin hydrogel (G-10)OT1 for four weeks and measured daily were converted to determine the amount of thymol in 1 L of in-hive air, and the in-hive air thymol concentration time plot is shown in Figure 7. The in-hive air thymol concentrations were calculated as the lowest 26 µg/L, 19 µg/L, 27 µg/L, the highest 408 µg/L, 381 µg/L, 76 µg/L, and the mean 160.72 µg/L (± 109.30), 153.53 µg/L (± 107.88), and 44.81 µg/L (± 14.95) for pure thymol, reference drug, and hydrogel, respectively. The release continued continuously for four weeks in all three groups.

Discussion and Conclusion

According to the results of the swelling tests, it was determined that increasing the crosslinker ratio in the hydrogel led to lower swelling values. In this respect, results consistent with published studies were obtained (29, 30).

As a result of the cross-linking reaction between gelatin and GA, the aldimine bond (CH=N) is formed between the amino groups of gelatin and the aldehyde groups of GA, and the primary amine content of gelatin is reduced. In addition, in the comparison of the samples, only in the spectrum of (G-10)OT1 hydrogel, characteristic C-H bond peaks of thymol were observed at 2924 cm^{-1} and 2854 cm^{-1}. As a result, the FT-IR findings in terms of cross-linking with GA in the hydrogel structure and the presence of thymol in the matrix structure are consistent with the literature (24, 39).

Using the hydrogel technique with gelatin, a natural polymer, a thymol-loadable CRS was formed with an average yield of 25.26% (± 3.05). The pores in the hydrogel matrix structure were measured at 280 µm

(±17.74) for (G-10)O1 hydrogel and 78.39 µm (±11.63) for thymol (G-10)OT1 hydrogel. In terms of these properties, it is evaluated that the results are compatible with the previous studies on controlled release of thymol (8, 23, 25). When evaluated in terms of macroscopic findings, it is in accordance with the literature that the hydrogels obtained after freezing at -20 °C and subsequent lyophilization exhibit a structure suitable for disintegration (22). This may be thought to be due to thymol, which is the only known difference between the two hydrogel compositions. Probably, thymol in the structure creates a difference in the heat transfer rate in the freezing process and therefore larger ice crystals are formed in the non-thymol structure during freezing. Larger ice crystals also push the gelatin chains more and cause larger pore sizes (19, 22).

As a result of gravimetric measurements, pure thymol and the reference drug, which initially contained equal amounts of thymol, released thymol at similar weights. Similarly, it is noteworthy that the lowest and highest concentrations reached in the in-hive air thymol concentration-time graphs were very similar for pure thymol and the reference drug. This indicates that the reference drug formulation was designed for rapid release of thymol. The observation of thymol concentrations above the therapeutic window in all three release trial groups is probably due to the unpredictable and poor correlation between in vitro and in vivo test results (21). However, disadvantages such as these may be experienced in the development stages of CRS (18). This situation may be caused by factors that cannot be established in vitro but may be important in the pharmacokinetics of thymol. Hydrogel release was lower than that of pure thymol and the reference drug in both gravimetric measurements and in vitro air thymol concentration-time graph evaluation. In addition, the closeness of the average, highest, and lowest thymol concentration values obtained with hydrogel release indicates that a more stable release is exhibited compared to the other groups. It is evaluated that the controlled thymol release system developed in terms of a slow, continuous, and constant rate of thymol release, more efficient use of the active substance, and minimizing environmental damage is compatible with the literature (25, 27).

In conclusion, the controlled thymol release system developed and tested in this study for the control of varroosis infestation was found to exhibit more stable release compared to existing application systems. The controlled release system has promising potential advantages in terms of the effective use of highly volatile active substances with a narrow therapeutic window, such as thymol, in the control of varroosis and the protection of colony health.

Acknowledgements

This work is derived from the first author's PhD thesis entitled "Development of a Controlled Thymol Release System for Varroosis Struggle and Investigation of its Effectiveness" (PhD Thesis, Ankara University, Ankara, Türkiye, 2022).

Financial Support

This research was funded by the Republic of Türkiye Ministry of Agriculture and Forestry, General Directorate of Agricultural Research and Policies. TAGEM/HSGYAD/A/20/20/20/A5/P6/1943. (Project Start-End date: 01.01.2020-01.01.2022).

Conflict of Interest

The authors declared that there is no conflict of interest.

Author Contributions

First, second and third author conceived and planned the experiments. First and second author carried out the experiments and contributed to the interpretation of the results. 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.

Ethical Statement

This study was carried out after the animal experiment was approved by Pendik Veterinary Control Institute Local Ethics Committee (Decision number: 202-17/2018).

Animal Welfare

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

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Phylogenetic characterization of Cochroaches (Insecta: Blattaria) in Türkiye and determination of their vector potential for medically important parasites

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ARTICLE INFO

Article History
Received : 31.10.2023
Accepted : 22.02.2024
DOI: 10.33988/auvfd.1300194

Keywords
Cockroach
DNA barcoding
ITS-2
mt-COI and mt-COII
Vector

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How to cite this article: Cevahir F, Düzlü Ö, Atelge M, Yıldırım A (2024): Phylogenetic characterization of Cochroaches (Insecta: Blattaria) in Türkiye and determination of their vector potential for medically important parasites. Ankara Univ Vet Fak Derg, 71 (4), 417-426. DOI: 10.33988/auvfd.1300194.

ABSTRACT

This study was performed to investigate the phylogenetic characters of the cockroaches in the Kayseri region for mitochondrial cytochrome c oxidase subunit I (mt-COI), mt-COII, and internal transcribed spacer-2 (ITS-2) gene regions. It was also aimed to determine their mechanical transmission of medically important parasites. PCR-restriction fragment length polymorphism (RFLP) was performed by using mt-COI, mt-COII, and ITS-2 DNA gene regions to identify cockroach species (n=220) collected from different regions. Differentiation of cockroach species was based on RFLP models using two restriction enzymes: AvaI and EciI. For phylogenetic analysis, mt-COI, mt-COII, and ITS-2 DNA barcode regions were amplified with standard primers. The obtained amplicons were purified and sequenced using the PCR primers. According to PCR-RFLP, the cockroach species were identified as *Blattella germanica* (n=105), *Blatta orientalis* (n=86), and *Periplaneta americana* (n=29). A total of 13 haplotypes were detected and maximum likelihood (ML) analyses revealed that the sequences of all three species showed a monophyletic structure for the three gene regions. The cockroaches were examined for the presence of parasites. It was found that of the 58 parasitic forms identified, 46 (79.3%) belonged to helminth species and 12 (20.7%) to protozoan species. The results showed that *B. germanica* (58.6%) had the highest prevalence, followed by *Bl. orientalis* (32.8%) and *P. americana* (8.6%). The results of the study not only contribute to the molecular epidemiology of cockroaches but also confirm their important role as mechanical vectors of protozoan and helminth parasites.

Introduction

Cockroaches are one of the most important pests found in apartments, houses, restaurants, hospitals, and health care facilities. Especially German cockroaches show an exploitative effect in poor living conditions. Cockroaches feed on garbage, rotting food, and even the feces of other insects. They are important vectors because they carry pathogens to meals, dishes, kitchen surfaces and other areas around the house. They can cause food poisoning in humans by leaving pathogens such as fungi, viruses, and bacteria on the food (34).

Moreover, they cause allergic reactions in many people and can trigger asthma. 95% of cases of food poisoning are caused by humans consuming cockroach saliva, feces, and the nutrients left by their eggs. They mechanically transmit parasites, bacteria, and viruses by crawling on feces and other organic materials to obtain food. In this respect, they are of medical and economic importance (49).

It has been found that until today, molecular-based studies on cockroaches and their vector potentials are limited in the world, and they are not yet available in Türkiye. In this context, it is aimed to determine the molecular characters of cockroaches and to reveal the phylogenetic structures between cockroach populations in our study and in the world. It is also aimed to determine

the level of genetic differences and the current situation of the mechanical vectoring of the samples determined on the basis of species in terms of parasitic infections. The study yielded data, indicating the first molecular information on cockroaches in Türkiye. In addition, the results provided important scientific data on the zoonotic risks of the mechanical vectoring potential of cockroaches, which are widespread in the study area.

Materials and Methods

Sample Collection and DNA Extraction: A total of 220 adults and nymphs belonging to *Blattella germanica*, *Bl. orientalis*, and *P. americana* species were trapped from different locations such as hospitals, food companies and houses in Kayseri region of Türkiye. Cockroaches were individually placed in plastic containers, inactivated at -20°C, and then identified using morphological keys (22, 42). Genomic DNA extraction from the legs of cockroaches was performed using the AxyPrep Multisource Genomic DNA Miniprep Kit (Axygen Biosciences, USA). Total DNA was eluted to the elution tube and stored at -20 °C until use.

PCR-RFLP: Before the RFLP analyses, nested PCR protocol (43) was used to amplify the gene regions for small subunit ribosomal RNA (SSU rRNA). The first PCR step employed IMS-GR1-SSUF1 (5'-TAARGTGAAA CCGCGAATG-3') and IMS-GR1-SSUR1 (5'-ACCTTGTTACGACTTTTAC-3') primers to amplify the relevant gene region and produce 1.793 bp (43). The internal primers IMS-GR1-SSU-F2-(5'-ACCGC GAATGGCTCATTAAATC-3') and IMSGR1-SSU-R2-(5'-TACGACTTTTACTTCCT C-3') were utilized in the second step to amplify the 1.775 bp segment of the corresponding gene region (43). A 50 µl PCR reaction was comprised of 25 pl of HotStarTaq Master Mix (QIAGEN) and 25 pl of a solution containing 200 nM of each primer, 1.5 mM of additional $MgCl_2$, and template DNA (50 ng) that was diluted in PCR-grade water (43). The reactions were performed for 35 cycles, each consisting of 94°C for 45 s, 50°C for 45 s, and 72°C for 60 s, in a thermocycler, with an initial hot start at 94°C for 15 min and a final extension at 72°C for 10 min. For the second round of PCR, a 1.775 bp fragment was amplified from 2.5 ml of primary PCR reaction. The PCR conditions for this round were the same as in the primary PCR, except for a higher annealing temperature of 55°C. The resulting PCR products were analyzed using agarose gel electrophoresis and visualized following ethidium bromide staining.

For the RFLP analysis, AvaI and EciI (New England Biolabs, Beverly, MA) restriction enzymes were selected by the manufacturer. 15 µL of the PCR products were digested in a 25 µL reaction mixture containing the enzymes and 2.5 µL of the appropriate restriction buffer at 37°C by overnight according to the manufacturer's instructions. The digested products were fractionated on a 1.5% agarose gel and visualized by ethidium bromide staining under ultraviolet light.

Nucleotide Sequencing and Phylogenetic Analysis: For the phylogenetic analysis of gDNAs obtained from individual cockroach samples, the mt-COI, mt-COII, and ITS2 gene regions were amplified by PCR using the primers C1J1718MF (5'-GGAGGATTTGGAAATT GATTAGT-3') and C1N2191BR (5'-CAGGTAAAATTA AAATATAAACTTCDGG-3') (17); COIIF (5'-AGAGCWTCACCTATTATAGAAC-3') and COIIR (5'-GTARWACRTCTGCTGCTGTTAC-3') (38); ITS2F (5'-CGATGAA GAACGCAGCAAA-3') and ITS2R (5'-TCCTCCGCTTATTGATATGC-3') (13), respectively. Recombinant plasmid DNAs containing Mt-COI, mt-COII and ITS2 target gene regions were bidirectionally sequenced using pJET1.2 forward and reverse primers. After careful analysis of the chromatograms of the plasmids whose bidirectional DNA sequence was determined, the final sequences of the isolates were obtained by determining the inserted target gene region in the vector nucleotide sequence and by performing pairwise alignments of the forward and reverse sequences using Geneious software (27). DnaSP 5.10.01 software (32) was used to determine DNA polymorphism and haplotype structure in the isolates characterized in the study. Intra- and inter-specific genetic differences were performed in MEGA 7 software (46) by using the Kimura two-parameter (K2P) distance model (28, 36). Bayesian (BA) inference and maximum likelihood (ML) analyses were used to determine the phylogenetic structures of cockroach species. jModelTest v.0.1.1 (40) was used to determine the most appropriate substitution model for sequence evolution in BA and ML analyses, and the models with the lowest AIC (Akaike Information, Criterion, Correction) value were used to construct the phylogenetic trees. BA and ML analyses were performed with the Geneious R10 software (27), using the MrBayes (25) and PhyML (21) plug-ins, respectively. A bootstrap test with 1000 replicates was used to determine the reliability of the trees generated by the ML analysis.

Investigation of Parasitic Forms of Medical Importance in Cockroaches: Cockroaches were transferred to appropriate sterile vials and 0.9% sterile physiological saline was added to them. They were then subjected to mechanical agitation in the Tissue Lyser LT (Qiagen) device for 2 minutes. After that, the obtained suspension was divided into two separate microcentrifuge tubes of 1 ml each. The first dividing tube was centrifuged at 2,000 rpm for 5 minutes, and after removing the supernatant, the sediment stained with %1 Lugol's iodine was examined under a light microscope for parasitic forms (10).

DOI: 10.33988/auvfd.1300194

Results

Identification of Cockroach Species: In the study, adult and nymph cockroaches were classified through morphological analysis as 128 (58.2%) *B. germanica*, 71 (32.3%) *Bl. orientalis* and 21 (9.5%) *P. americana*. The study identified that out of the 220 cockroaches examined, 105 (47.7%) (71 adults, 34 nymphs) were classified as *B. germanica*, 86 (39.1%) (54 adults, 32 nymphs) as *Bl. orientalis*, and 29 (13.2%) (21 adults, 8 nymphs) as *P. americana* species using PCR-RFLP results.

SSU rRNA Nested PCR and RFLP Analysis Results: Table 1 presents the band profiles of cockroach samples obtained through individual gDNA extraction via nested PCR and subsequent analysis of the partial SSU rRNA gene region using RFLP techniques. The results indicate that the *AvaI* and *EciI* enzymes consistently cleave these band profiles in all samples.

Table 1. Some band profiles determined after RFLP with *AvaI* and *EciI* restriction enzymes in positive samples.

Type of cockroach	*AvaI* (bp)	*EciI* (bp)
Blattella germanica	124, 865	1052
Blatta orientalis	613, 831	650, 1018
Periplaneta americana	834	1021

The band profile images obtained from the analysis of positive isolates using *AvaI* and *EciI* restriction enzymes and RFLP analysis, in a 1.5% agarose gel, are displayed in Figure 1 and Figure 2, respectively.

Phylogenetic Analysis Results: A total of 13 haplotypes, five, three and five, respectively, were determined for the mt-COI, mt-COII and ITS-2 gene regions of the related species. The mean haplotype diversities were 0.962±0.017, 0.842±0.047, and 0.810±0.080, respectively. The intraspecific nucleotide differences of *B. germanica*, *Bl. orientalis*, and *P. americana* species in the data sets were 1.0±0.2%, 0.4±0.1%, 2.4±0.5% for mt-COI, 0.1±0.1%, 0.1±0% for mt-COII, 0.3±0.1%, and 0.7%±0.3% and 0.8±0.3% for ITS-2, respectively. The interspecific nucleotide differences are for mt-COI, mt-COII and ITS-2: *B. germanica* and *Bl. orientalis* 22.7±2.5%, 26.0±2.9%, 45.7±6.1%; between *B. germanica* and *P. americana* 24.6±2.5%, 29.5±3.3%, 43.6%±5.8%, and between *Bl. orientalis* and *P. americana* 14.5±1.8%, 15.7%±2.1%, and 17.9±2.7%. According to ML analysis, the sequences of all three species showed monophyletic structure for three gene regions (Figure 3-5). The phylogenetic analysis of the isolates of all three-cockroach species showed similarity rates of 98.6-100% were determined with similar isolates in the world, although it varied depending on the species and gene region.

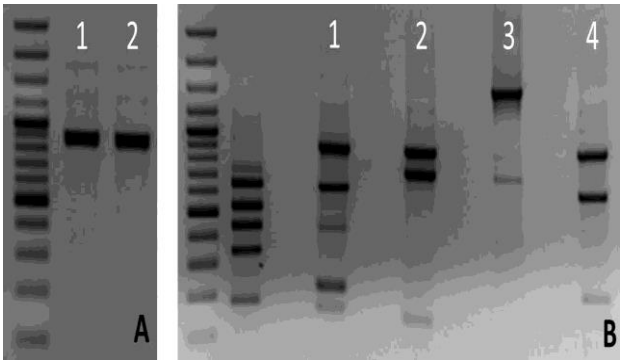

Figure 1. The gel electrophoresis of the band profiles obtained by RFLP analysis with AvaI restriction enzyme of the products obtained after the amplification of the partial SSU rRNA gene region in some cockroach isolates. Marker (100bp).
(A) 1-2: *P. americana*; (B) 1,4: *Bl. orientalis*; 2, 3: *B. Germanica*.

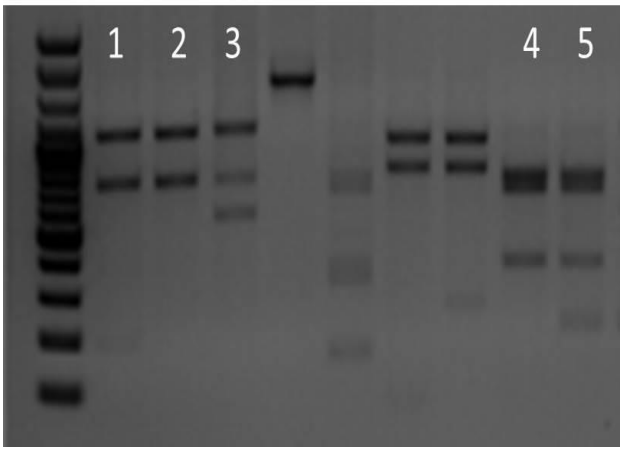

Figure 2. The gel electrophoresis of the band profiles obtained by RFLP analysis with Ecil restriction enzyme of the products obtained after the amplification of the partial SSU rRNA gene region in some cockroach isolates. Marker (100bp).
1-3: *B. germanica*; 4: *P. americana*; B. 5: *Bl. Orientalis.*

Table 2. Protozoan and helminth numbers detected in cockroach species.

Type of cockroach	Protozoa n (%)	Helminths n (%)	Total n (%)
Blattella germanica	26 (56.5)	8 (66.7)	34 (58.6)
Blatta orientalis	16 (34.8)	3 (25.0)	19 (32.8)
Periplaneta americana	4 (8.7)	1 (8.3)	5 (8.6)
Total	46 (100.0)	12 (100.0)	58 (100.0)

Parasitic Forms Detected in Cockroaches: It was found that 47 (21.4%) of the 220 cockroaches examined were infective with at least one parasitic form, and some cockroach samples were found to be infective with several parasitic forms (Table 2). Table 2 shows that *B. germanica* (58.6%) was the most parasitized cockroach. This was

followed by *Bl. orientalis* (32.8%) and *P. americana* (8.6%). It was determined that 46 (79.3%) of the total 58 parasitic forms identified belonged to protozoan species and 12 (20.7%) to helminth species. *Toxocara* spp. (4 eggs, 8.5%), Trichostrongylid type eggs (3 eggs, 6.4%), *Trichuris* spp. (3 eggs, 6.4%), *Ascaris lumbricoides* (2 eggs, 4.3%); *Blastocystis* sp. (12 vacuolar form, 25.5%), isosporoid type oocyst (10 oocyst, 21.3%), *Eimeria* spp. (7 oocysts, 14.9%), *Cryptosporidium* spp. (17 oocysts, 36.2%) were identified among protozoa (Figure 6-7).

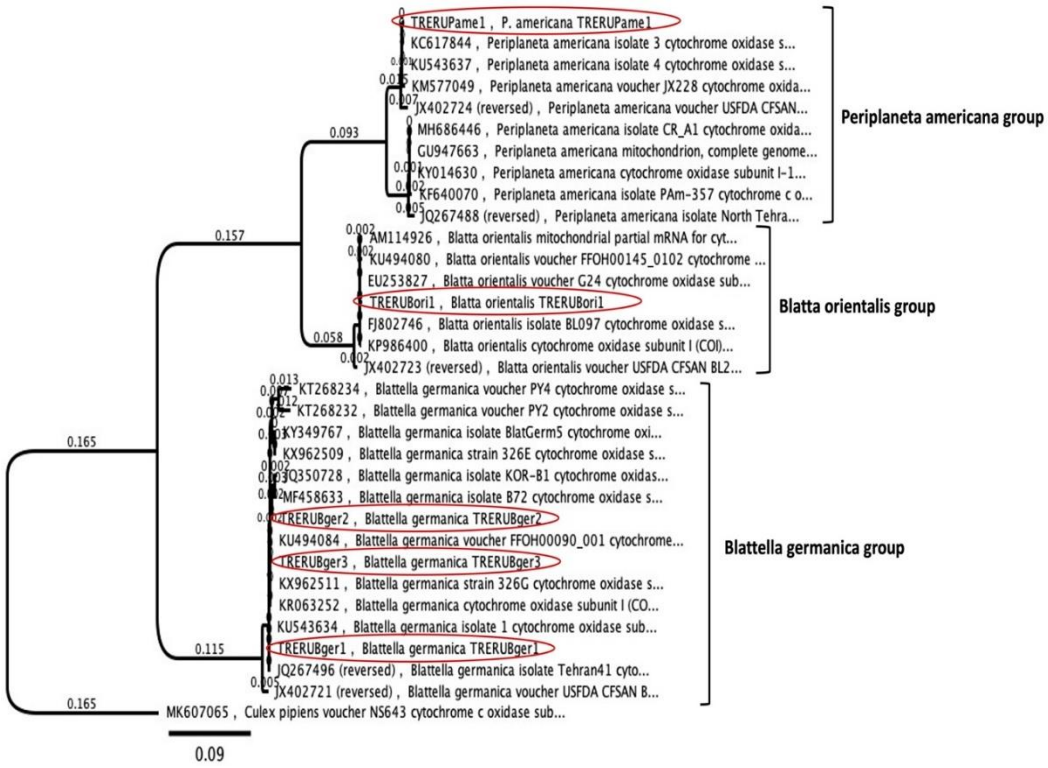

Figure 3. Phylogenetic relationships of cockroach isolates isolated from Kayseri region and other cockroach isolates registered in GenBank according to mt-COI gene region.

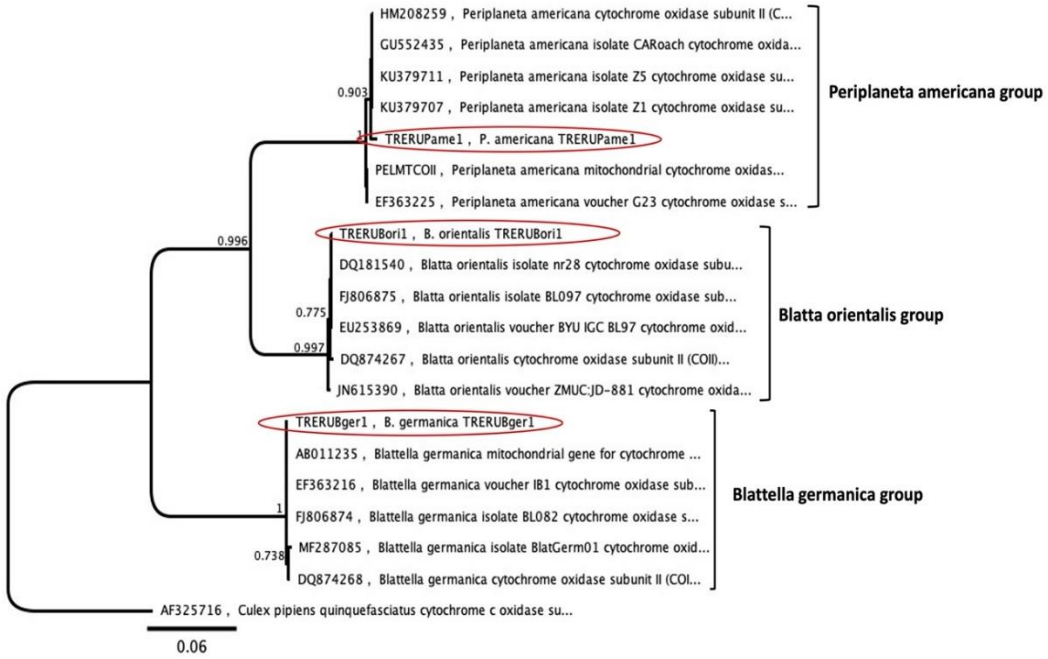

Figure 4. Phylogenetic relationships of cockroach isolates isolated from Kayseri region and other cockroach isolates registered in GenBank according to mt-COII gene region.

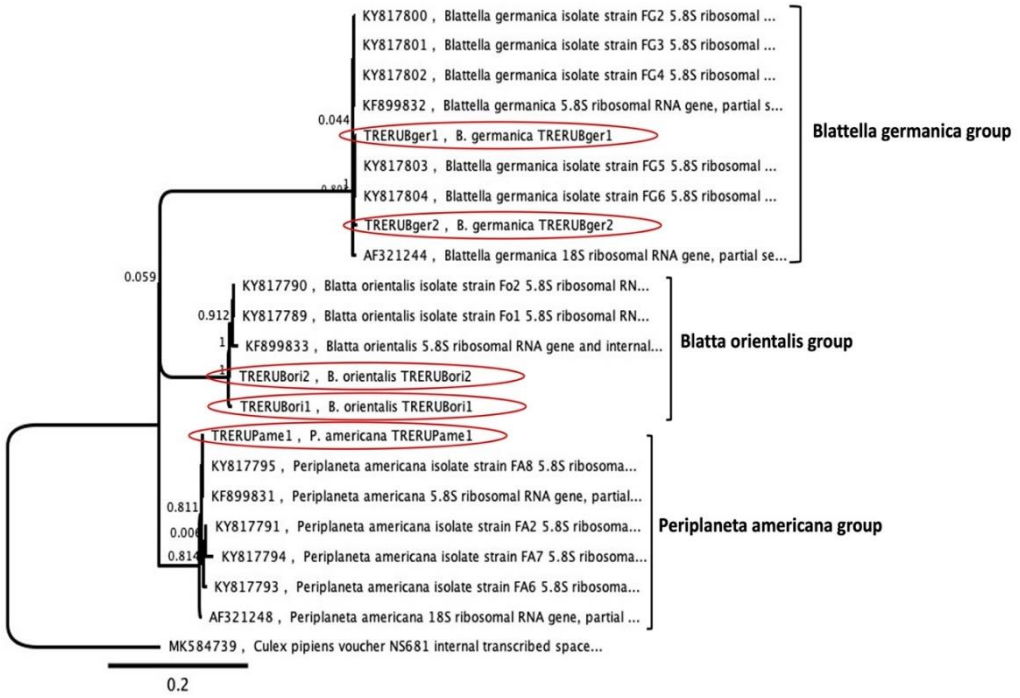

Figure 5. Phylogenetic relationships of cockroach isolates isolated from Kayseri region and other cockroach isolates registered in GenBank according to ITS-2 gene region.

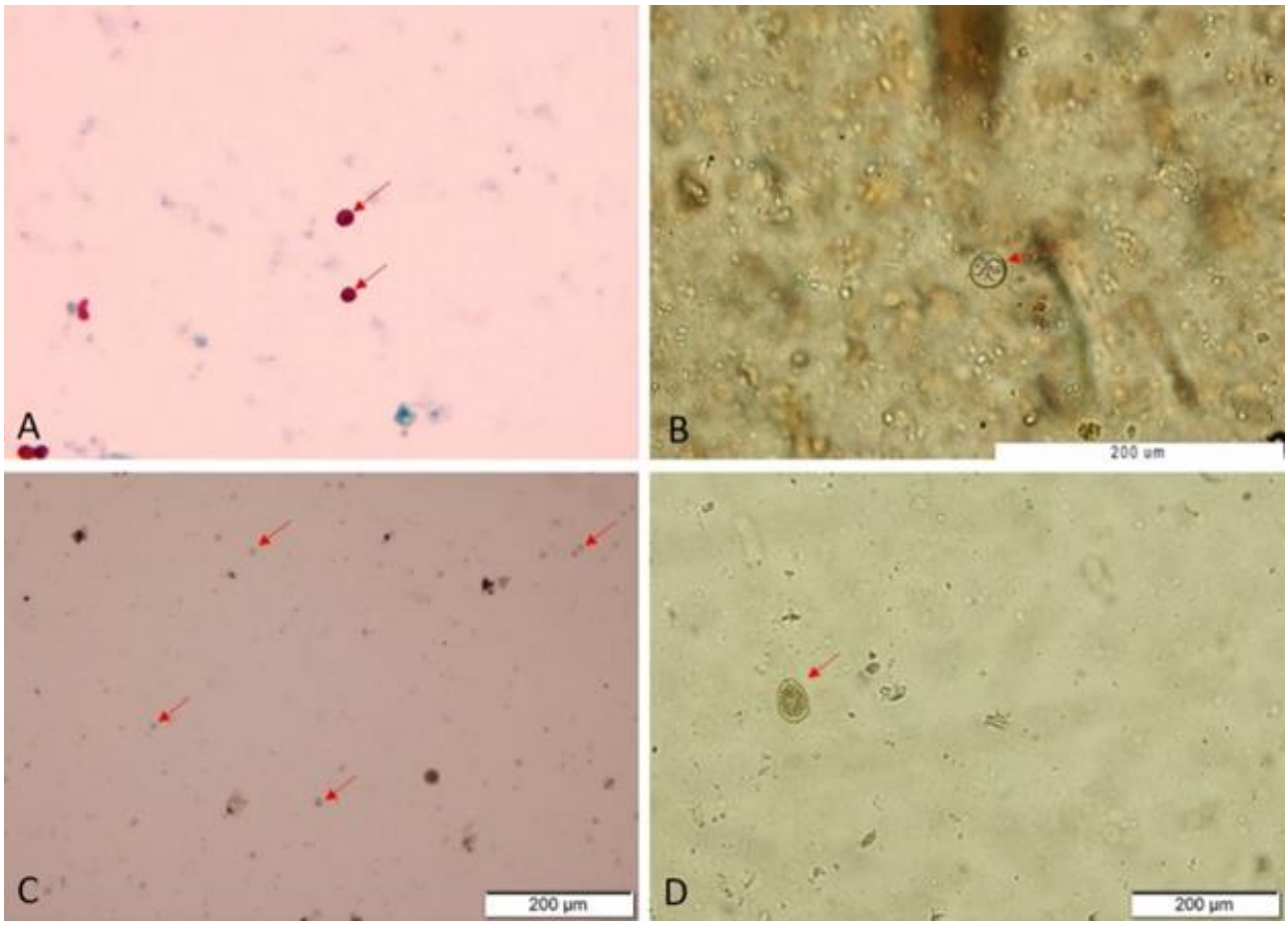

Figure 6. Protozoan species detected in cockroaches.

A. *Cryptosporidium* spp. oocysts, B. Sporulated isosporoid type oocyst, C. *Blastocystis* sp. vacuolar forms, D. Unsporulated *Eimeria* spp. oocyst.

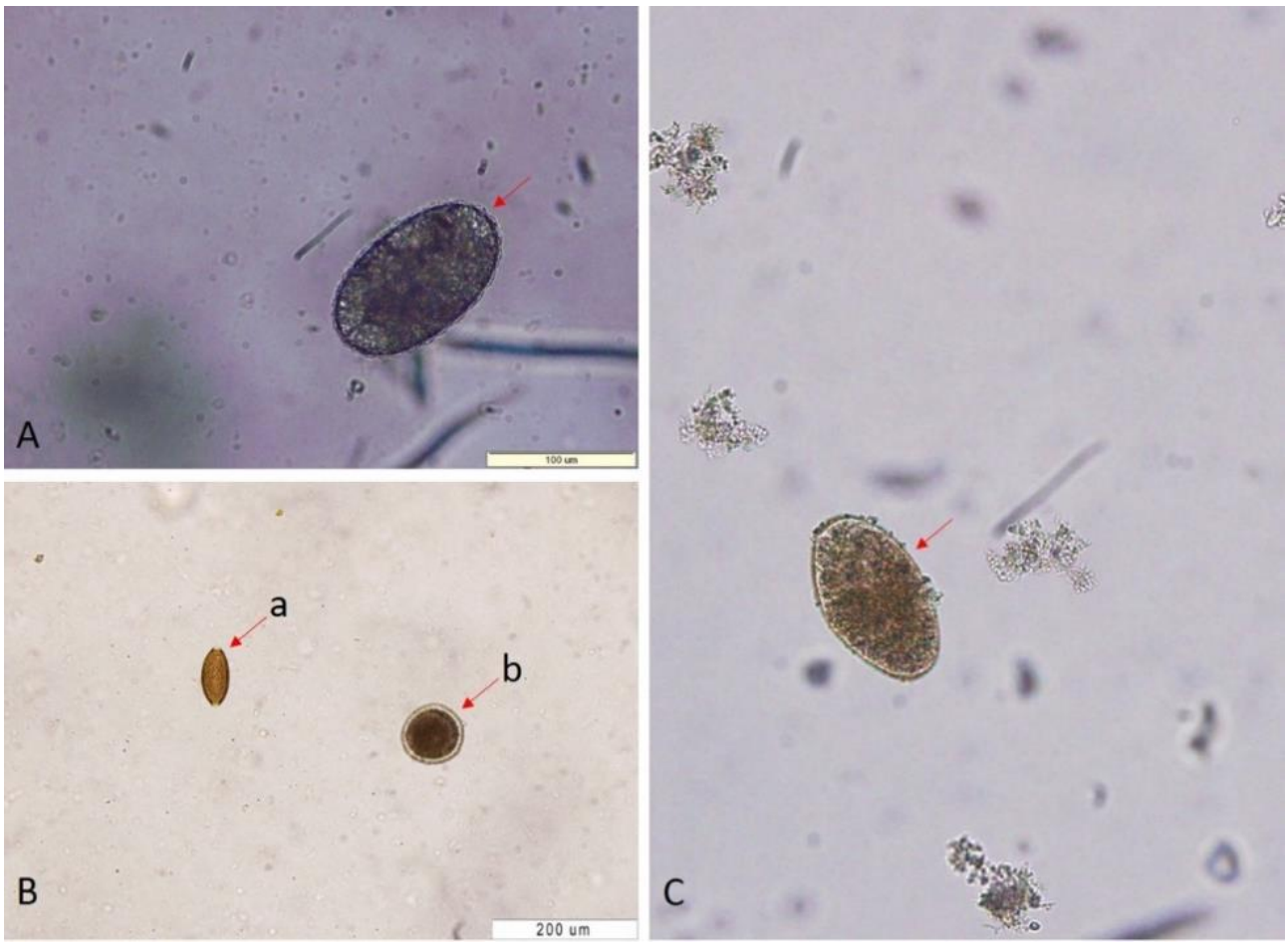

Figure 7. Helminth species detected in cockroaches.
A. Infertile *Ascaris lumbricoides* egg, B. a. *Trichuris* sp. egg, b. *Toxocara* sp. egg, C. *Trichostrongylid* type egg.

Discussion and Conclusion

In Türkiye, the existence of *Bl. orientalis*, *P. americana*, *P. australasiae* belonging to Blattidae family and *B. germanica* species belonging to Blattellidae family have been reported. In comparison with other studies conducted in Türkiye (30, 37), *B. germanica* was found to be the most dominant species in our current study in line with these studies. However, the prevalence of *B. orientalis* and *P. americana* in our study was quite high compared to other studies. Two other studies (30, 37) collected cockroach samples from houses and hospitals. However, in our current study, samples were obtained from hospitals and houses, as well as from food establishments. While *Bl. orientalis* and *P. americana* species tend to live in wet and humid areas due to their high moisture requirements, *B. germanica* is mostly adapted to living in areas such as kitchens, basements, and hospitals (1). Therefore, it is hypothesised that the variance in prevalence rates could be attributed to the fact that the cockroaches grouped by habitat originate from varying environments and regions.

Furthermore, it is worth mentioning that both studies relied solely on morphological criteria for the identification of cockroaches. According to the literature (6, 12, 16, 48, 51), it has been reported that it is very difficult to determine the species based on the morphological characteristics of adult individuals and young nymphs, especially in cockroaches. The diagnosis of all cockroaches (146 adults, 74 nymphs) sampled in our study was confirmed by RFLP and sequence analysis. In our study, we confirmed this situation through the varying rates of molecular and morphological prevalence we obtained.

It is usually very difficult to distinguish between adult and nymphal stages of cockroaches. Close-knit species often have very similar morphological characteristics. Cockroaches vary greatly in their developmental stages. Especially externally, differences in morphological criteria such as spination, setation and coloration make it very difficult to distinguish between species (6, 12, 48). Therefore, to overcome this situation, simple, accurate and easily applicable methods that can distinguish all developmental stages of cockroaches are needed (48, 51).

In this context, diagnostic methods based on DNA barcoding have been developed in recent years to determine the species of cockroaches and other insects

with higher accuracy. To date, the number of studies using the mt-COI or ITS gene region to differentiate cockroach species is quite limited worldwide. Knebelsberger and Miller (29) used COI sequences to distinguish three conspecific morphotypes of *Phyllodromica iberica*, and to identify phylogenetic relationships among species in the subaptera-group. Evangelista et al. (11) used the COI gene region to confirm the existence of *P. japonica*, a new invasive cockroach species they found in New York. Similarly, Yue et al. (49) used a DNA barcoding system to determine that both macropterous and brachypterous females and males of *Hebardina concinna* belonged to this species. Evangelista et al. (12) used both morphological and genetic barcode information to reveal the species richness of the Blattodea family. Che et al. (5) determined the phylogenetic affinities of cockroaches of the family Ectobiidae collected in China by amplification of the COI gene region. Hashemi-Aghdam et al. (24) amplified the mt-COI gene regions of *B. germanica*, *Bl. orientalis*, *P. americana*, *Shelfordella lateralis* and *Supella longipalpa* species for DNA barcoding of cockroaches and developed the PCR-RFLP method for rapid identification of these species in their study in Iran. Similarly, Sulaiman et al. (43), developed the PCR-RFLP technique based on the SSU rRNA gene region for differentiation of *B. germanica*, *Bl. orientalis*, *P. americana* and *S. longipalpa* species. The same researchers (44) performed DNA barcoding of these four species according to the mt-COI gene region in 2016. Cheng et al. (7) extracted the complete mitochondrial genomes of the cockroach species *Gromphadorhina portentosa*, *Panchlora nivea*, *Blaptica dubia* in the family Blaberidae and *S. lateralis* in the family Blattidae. Mukha et al. (35) reported that the 28S rDNA gene region, together with the ITS-1 and ITS-2 gene regions, can be used to differentiate cockroaches in the *Blattella* and *Periplaneta* lineages. Similarly, Everaerts et al. (13) have reported that complex species of *Cryptocercus punctulatus* in the family Cryptocercidae utilized the 16S, mt-COII and ITS-2 gene regions for DNA barcoding. Park et al. (38) performed DNA barcoding of the mt-COII, 16S and 18S rRNA gene regions of *Cryptocercus* cockroach species native to North Asia. Farmani et al. (14) used the ITS-2 gene region for DNA barcoding of seven cockroach species (*P. americana*, *S. lateralis*, *Bl. orientalis*, *B. germanica*, *S. longipalpa*, *Polyphaga aegyptiaca*, *P. saussurei*) after morphological examination in Iran. They pointed out the importance of confirming the species by molecular techniques.

All studies on cockroaches in Türkiye have been carried out according to morphological criteria and no study has been revealed the molecular characters of cockroaches. In this sense, our present study has the feature of the first qualification in which the molecular characters of cockroaches in the *B. germanica*, *Bl. orientalis* and *P. americana* strains in Türkiye were revealed and their phylogenetic affinities with similar isolates in the world were determined. In our study, mt-COI, mt-COII and ITS-2 gene regions were used for DNA barcoding of three cockroach species in parallel with studies in the world. As a result of the study, it was confirmed that all three genes can be used as molecular markers to differentiate *B. germanica*, *Bl. orientalis* and *P. americana* species.

Cockroaches are mechanical vectors of many pathogens that infect both humans and animals by carrying them from one place to another with their bodies. In studies on this subject, it has been reported that cockroaches have reached dimensions that threaten human health by infecting humans with saprophytic and pathogenic microorganisms with this role (1, 4). Besides, many areas such as especially kitchens, rooms, basements of houses, sewer systems, manholes, storerooms, patient rooms, examination rooms, study rooms, warehouses, kitchens, laundries, warehouses, meeting rooms, canteens, tea stoves, toilets, and bathrooms in hospitals. They create ideal environments for insects to breed. The abundance of cockroach species captured in hospital environments via varied trapping methods suggests that hospitals represent vital zones for control purposes since these species serve as vectors of pathogenic microorganisms (2, 8, 18-20, 41). In addition to the many bacterial pathogens that cockroaches carry as mechanical vectors, one of the most important issues is the parasitic pathogens that they carry. Protozoa such as *Toxoplasma gondii*, *Blastocystis hominis*, *Cryptosporidium* spp., *Balantidium coli*, *Entamoeba histolytica*, *Giardia intestinalis*, and infective forms of some helminth species such as *Ascaris lumbricoides*, *Enterobius vermicularis*, *Ancylostoma duodenale*, *Necator americanus*, *Hymenolepis diminuta*, *Trichuris trichuria*, *Gongylonema pulchrum* have been detected in cockroaches. Although the possibility of cockroaches being biological vectors for these species is worth considering, recent reports suggest that this may be linked to cockroaches' feeding habits (1, 15, 31, 33, 39, 40, 47). A study by Hamu et al. (23) reported that *A. lumbricoides*, *T. trichiura*, *Taenia* spp., *Strongyloides* spp., *E. histolytica/dispar/moshkovski*, *G. duodenalis* and *B. coli* parasites were detected on the external surface of approximately 11% of 2,010 *B. germanica* cockroaches. El-Sherbini and El-Sherbini (9) found parasitic pathogens such as *E. histolytica*, *C. parvum*, *Cyclospora cayetenensis*, *Isospora belli*, *B. coli*, *A. lumbricoides*, *A. duodenale*, *E. vermicularis*, *T. trichura*, and *S. stercoralis* on the external surfaces of various species of cockroaches collected from toilets, kitchens, and bedrooms in their study in Egypt. Chamavit et al. (3) found parasitic forms on the external surfaces of about 54% of a total of 920

cockroaches collected from 18 public supermarkets in Thailand and reported that 56% of them were protozoans [*Cyclospora* spp. (1.3%), *Endolimax nana* (1.3%), *B. hominis* (1.2%), *I. belli* (9.6%), *E. histolytica/dispar* (4.6%), *Cryptosporidium* spp. (28.1%), *Chilomastix mesnilli* (0.3%), *E. coli* (4.0%), *B. coli* (5.8%), *Iodamoeba butschlii* (0.1%)], 1.5% pathogenic helminth species [*S. stercoralis* (0.8%), *A. lumbricoides* (0.3%), *T. trichiura* (0.3%), *Taenia* spp. (0.1%)] and 42.5% of them were non-pathogenic helminth species. Similarly, Jarujareet et al. (26) reported in their study on *P. americana* cockroaches that these insects carry sporulated *E. tenella* oocysts on their external surfaces. In the first study in Türkiye to detect parasitic infections in cockroaches (37), it was reported that 48% of 138 cockroaches collected in the Van region and diagnosed as *B. germanica* were infected with parasitic forms. In the same study (19), about 97% of the parasitic forms detected were protozoa and the rest were helminth species [*Toxocara* sp. (3%), *A. lumbricoides* (3%), *Trichostrongylus* sp. (1.5%), *T. trichiura* (1.5%), *E. nana* (7.6%), *B. hominis* (41%), *E. histolytica/E. dispar* (16.7%), unsporulated coccidial oocysts (7.6%), *C. mesnilli* (4.5%), *E. coli* (35%), *Giardia* spp. (13.6%), *I. butschlii* (7.6%)] were detected. In our current study, 47 (21.4%) of the 220 cockroaches we collected were found to be infective with at least one parasitic form, and some cockroach samples were found to be infective with several parasitic forms. In our study, *B. germanica* (58.6%) was found to be the most parasitized cockroach, followed by *Bl. orientalis* (32.8%) and *P. americana* (8.6%). It was found that 79.3% of the identified parasites belonged to protozoan species [*Blastocystis* sp. (25.5%), isosporoid type oocysts (21.3%), *Eimeria* spp. (14.9%), *Cryptosporidium* spp. (36.2%)] and 20.7% to helminth species [*Toxocara* spp. (8.5%), *Trichostrongylid* type eggs (6.4%), *Trichuris* spp. (6.4%), *A. lumbricoides* (4.3%)]. It has been concluded that the obtained results are similar to the results of studies on parasitic cockroaches both in the world and in Türkiye in terms of parasite species and prevalence rates.

In conclusion, this study presents the first molecular characterization of the mt-COI, mt-COII, and ITS-2 gene regions of cockroaches in Türkiye. Furthermore, the study establishes the phylogenetic relationship of these isolates with similar ones from around the world. It was concluded that the obtained results could contribute to the limited knowledge of the molecular epidemiology of cockroaches. Moreover, cockroaches were found to play a role as mechanical vectors of parasite-related diseases. In this regard, further studies should be conducted, such as the prevalence and status of cockroach-related parasitic diseases affecting health risks in different habitats and their appropriate control measures in our environment.

Acknowledgements

This study was derived from the the first author's doctoral dissertation.

Financial Support

This study was supported by the Erciyes University Scientific Research Projects Unit with project number TDK-2017-7760.

Conflict of Interest

The authors declared that there is no conflict of interest.

Author Contributions

Consept: FC, OD, AY, Design: FC, OD, AY, Sample Collection: FC, Processing: FC, MA, Analysis and Interpretation: FC, OD, AY, Literature Search: FC, OD, AY, Writing: FC, OD.

Data Availability Statement

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

Ethical Statement

This study does not present any ethical concerns.

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Phylogenetic characterization of two common sandflies, *Phlebotomus major* and *P. kandelakii,* in Inebolu District of the West Black Sea Region, Türkiye based on mitochondrial gene sequence analysis

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ARTICLE INFO

Article History
Received : 07.10.2023
Accepted : 25.02.2024
DOI: 10.33988/auvfd.1372324

Keywords
COI
Cytb
Haplotype
Sand fly
West Black Sea Region

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How to cite this article: Karademir GK, Atelge M, Yetişmiş K, Yetişmiş G, Uslu S, Çiloğlu A, Önder Z, Özbel Y, Pekmezci GZ, Yıldırım A, Düzlü Ö, Toz S, Pekmezci D, İnci A (2024): Phylogenetic characterization of two common sandflies, *Phlebotomus major* and *P. kandelakii,* in Inebolu District of the West Black Sea Region, Türkiye based on mitochondrial gene sequence analysis. Ankara Univ Vet Fak Derg, 71 (4), 427-436. DOI: 10.33988/auvfd.1372324.

ABSTRACT

Phlebotomus major and *P. kandelakii* are members of the *Larroussius* subgenus, which includes important vector sand fly species. Most members of the subgenus *Larroussius* have the ability to transmit *Leishmania infantum*, the causative agent of visceral leishmaniasis. Here, we investigated the genetic diversity within each species collected from the West Black Sea Region of Türkiye using mitochondrial DNA markers, specifically cytochrome oxidase I (COI) and cytochrome b gene sequences (Cytb). A total of 1889 sand fly specimens were collected from the study area in June 2021 and August 2022; 1596 (84.49%) were identified as *P. major* sensu lato, and 253 (13.40%) were identified as *P. kandelakii*. Nine and four haplotypes of *P. major* were determined in the study area based on COI and Cytb sequences, respectively. Analysis of the phylogenetic datasets generated from our isolates and published isolates in GenBank revealed high haplotype diversities within *P. major* (COI = 0.933, Cytb = 0.714). For *P. kandelakii*, we detected four and three haplotypes within the COI and Cytb sequences, and the haplotype diversities were also high in the datasets, including our isolates and published isolates in GenBank (COI = 0.978, Cytb = 1.000). Pairwise mean genetic distances calculated from the COI and Cytb datasets were 0.4% and 1.4% for *P. major* and 1.0% and 0.2% for *P. kandelakii*, respectively, suggesting the absence of cryptic species. Phylogenetic analyses revealed three and two major clusters of the *Larroussius* subgenus in the COI and Cytb datasets, respectively. Our study contributes to molecular information for *P. major* and *P. kandelakii* distributed in Türkiye and provides valuable insights into the phylogenetic relationships among species within the subgenus *Larroussius.*

Introduction

The subfamily Phlebotominae of the order Diptera includes hematophagous insects that play a role in the transmission of various pathogens such as protozoa (*Leishmania* spp.), viruses (Toscana virus), and bacteria (*Bartonella* sp.) (10, 35). It has also been reported that they have a potential role in the transmission of nematodes in the Onchocercidae family (6). Their most important role

in human and animal health is the transmission of pathogens that cause diseases such as cutaneous (CL) and visceral leishmaniasis (VL), which affect millions of people each year (4, 34). Species identification of sand flies has therefore been undertaken in endemic areas since the discovery of their association with these diseases. Understanding sand fly ecology and host-parasite interactions is crucial for predicting future outbreaks and controlling existing ones (5). Furthermore, it is essential to establish the taxonomy and systematics of Phlebotominae in all areas of research aimed at controlling leishmaniasis (5).

Sand flies play a crucial role in the transmission of *Leishmania* parasites in the Mediterranean Basin. Approximately 20 species of sand fly are involved in this transmission, with the subgenus *Larroussius* containing the most significant vectors of *L. infantum* (14, 36, 45). One of the most important and widespread species of *Larroussius* is *P. major*, a member of the “Major Group." This group consists of morphologically similar species with mixed taxonomic status, geographic distribution, and vector potentials (21, 28). *Phlebotomus major* was the first species identified within the "major group" and was previously considered to be the only species in this complex (1). A thorough analysis of the morphological features of specimens from different biogeographical regions (2, 25, 30, 31, 41, 42) showed that *P. major* is a complex of species. Currently, six species with morphologically similar and largely allopatric names (*P. major, P. neglectus, P. notus, P. syriacus, P. wenyoni*, and *P. wui*) have been recorded within the *P. major* group (3, 13). However, the status of the species within this taxon is still unclear. *Phlebotomus kandelakii*, which is also common in several regions of Türkiye, has also been reported as one of the most common vectors of *L. infantum* in the north-east and north-west of Iran and Georgia (14, 33, 36, 45). However, data on the genetic diversity of this species is scarce, with only about 26 COI or Cytb sequences in GenBank (access date: November 25, 2023), mainly from Türkiye and Azerbaijan.

Mitochondrial genes have been widely used for Phlebotomine sandfly systematics (13) due to their slow evolutionary rate, which is interesting for population studies; they are haploids and easily amplified, in addition to their low recombination. The COI and Cytb genes have commonly been utilized for DNA barcoding and phylogenetic characterization of several sand fly species. They are appropriate markers for analyzing the genetic structure and phylogeny due to their high mutation rate (7, 13).

This study aimed to highlight the genetic diversity of *P. major* and *P. kandelakii*, the dominant sand fly species of the subgenus *Larroussius* in the study area, by analyzing the COI and Cytb gene regions and comparing them with the published haplotypes from other countries available in GenBank.

Materials and Methods

Origin of the specimens of P. major and P. kandelakii: The specimens of *P. major* and *P. kandelakii* were collected in June 2021 and August 2022 from the populations distributed in the Inebolu district of Kastamonu province in the West Black Sea Region of Türkiye. Inebolu is located approximately 25 km from the 42 north parallel and 34 east meridian, which run through the north of Anatolia (Figure 1.). Inebolu generally has a

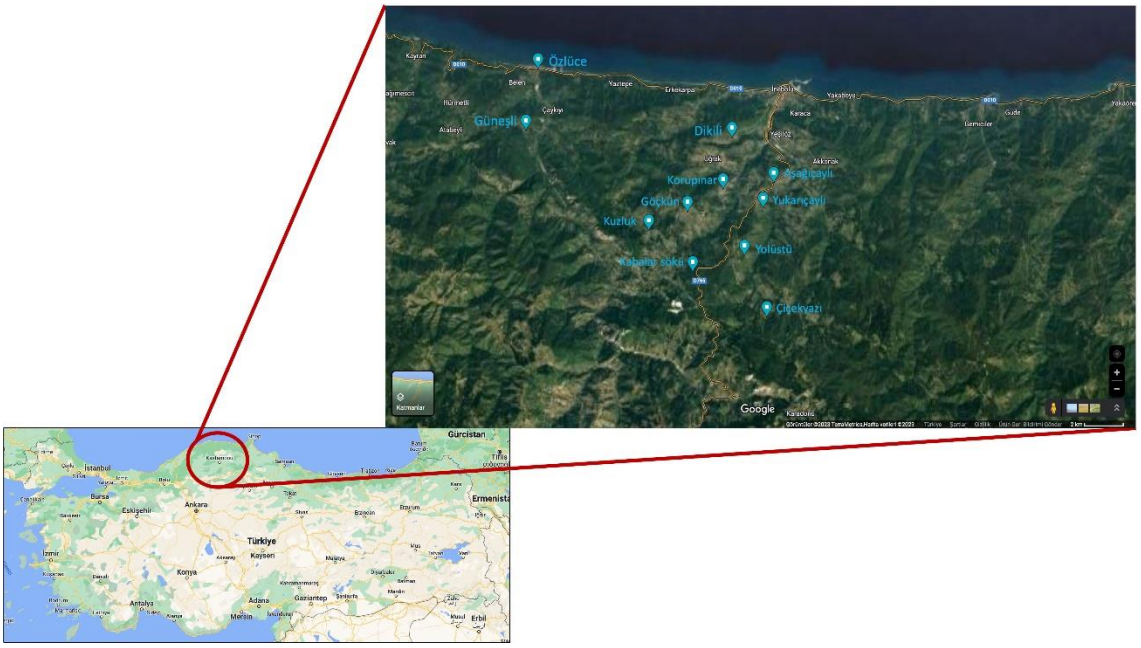

Figure 1. The map shows the Inebolu district of the West Black Sea Region, Türkiye. The blue pins indicate the collection sites of sand flies (Google maps was used to indicate the collection localities).

climate typical of the Black Sea Region, with the fog that occurs in the spring. Winters: mild and rainy; summer months: hot but not dry. It has a warm and mild climate with high relative humidity levels in all seasons, with the highest rainfall occurring between December and March. The average annual temperature of the district is 13.1 degrees, and the average annual rainfall is around 1000 mm. Inebolu is in a strategic position due to its geographical location and is the closest port to Anatolia (23).

For the collection of sand flies, CDC light traps (John W. Hock, USA) were placed in or near animal pens and in courtyards adjacent to houses in 11 locations in Inebolu district (Figure 1). They were set at each site before sunset, when sand flies are active for breeding and feeding, and collected before sunrise. Based on my personal observations, several animals, mostly cattle and also chickens and dogs, that might serve as hosts for the blood of sandflies were found in most of the collection sites.

Captured sand fly specimens were collected from the traps using a manual aspirator and preserved in tubes containing absolute ethanol. The specimens were then transported to the laboratory in a cold chain using ice boxes. Microscopic identification of each specimen was performed by head and genital morphology using an Olympus BX51 light microscope (Olympus, Tokyo, Japan) equipped with an Olympus DP70 (Olympus, Tokyo, Japan) digital camera and imaging software cellSens Standard v.1.13 (Olympus, Tokyo, Japan) (2, 24, 28, 29, 39, 40). After identification, the body parts of the specimens were stored at -20 ºC for subsequent analyses.

Genomic DNA isolation and polymerase chain reactions (PCR): We included 5 specimens of *P. major* from each of the 11 collection sites, resulting in a total sample size of 55 for phylogenetic analyses. While the sample size of *P. kandelakii* included a total of 25 specimens due to the detection of low numbers in some collections, Each sand fly sample included in the survey was crushed to a fine powder using liquid nitrogen in pre-cooled microcentrifuge tubes with sterile pestles prior to DNA extraction. The PureLink™ Genomic DNA Mini Kit (Thermo Scientific, Waltham, MA, USA) was used to extract the total genomic DNA (gDNA) according to the manufacturer's protocol, with a final elution volume of 35 µl. The extracted gDNA was then quantified using a Qubit 3.0 fluorometer quantitation instrument (Thermo Scientific, Waltham, MA, USA) and stored at −20 °C for downstream applications.

For phylogenetic characterization, we targeted the COI and Cytb genes of the sand fly species. The partial 709 bp segment of the COI and 788 bp of the Cytb gene regions were amplified by PCR using LCO1490 (5'-GGTCAACAAATCATAAAGATATTGG-3') and HCO2198 (5'-TAAACTTCAGGGTGACCAAAAAATCA-3') (19), and CB1-SE (5′TATGTACTACCCTGAGGACAAATATC3′) and CB-R06 (5′TATCTAATGGTTTCAAAACAATTGC 3′) (37) primers, respectively, according to the described protocols (18, 35). Electrophoresed gels were analyzed using the Fusion FX Gel Documentation System (Vilber Lourmat, France).

Sequence and phylogenetic analysis: The amplified fragments were sequenced bidirectionally with the PCR primers on the Sanger sequencing platform using the automated DNA sequencer ABI 3730XL (Macrogen Corporation, Korea). Paired nucleotide sequences of COI and Cytb from sand flies were processed and aligned using Geneious Prime 2022.1.1 software (http://www.geneious.com) in order to obtain a single consensus sequence. The obtained sequences were deposited in GenBank with the accession numbers OR511616-OR511628 for COI and OR520133-OR520139 for Cytb.

Using the BLASTn algorithm, the final sequences were searched in the GenBank database to compare fragments and generate the datasets for phylogenetic analyses of the related sand flies. The COI and Cytb datasets consisted of a total of 52 and 31 sequences, respectively. All sequences were aligned using MAFFT (22) through the plugin available in Geneious Prime. The parameters for MAFFT were set up as follows in Geneious Plugin as recommended (22); algorithm: “Auto”, scoring matrix: “200PAM/k=2”, gap open penalty: “1.53”, offset value: “0.123”. MEGA version 11 (49) was used to calculate the intra- and inter-specific genetic differences based on the Kimura-2-Parameter (K2P) distance model (25), as well as the ts/tv bias (R) in each codon. Haplotype number (K) and diversity (H), nucleotide composition, AT bias, and genetic diversity (π) indices were calculated using DnaSP v.5.1 (30). Neutrality tests, including Tajima’s D (48) and Fu’s F (16), were performed in DnaSP v.5.1.

Both maximum likelihood (ML) and Bayesian inference (BI) were used for phylogenetic analyses of sand fly isolates in the COI and Cytb datasets. The most appropriate DNA-substitution models for ML (GTR+G+I) and BI (GTR+G+I) phylogenetic analyses of the COI and Cytb datasets were selected based on Akaike’s information criterion (AIC) (42) and Schwarz's Bayesian information criterion (BIC) (46) algorithms, respectively, using jModeltest v.0.1.1 (42). Analyses were performed using PhyML (17) and MrBayes version 3.2.6 (20), respectively, through the plugins available with Geneious Prime. A bootstrap analysis was conducted in ML using 1000 replicates. For posterior probability calculations in BI, two Markov Chain Monte Carlo simulations were run

simultaneously for 10 million generations, with sampling every 200 generations. After discarding 25% of the initial trees in each run as burn-in, a majority consensus tree was constructed.

Results

Morphological identification of sand fly specimens: Based on the morphological identification results, the majority of the specimens 1596 (84.49%) and 253 (13.40%) out of the 1889 captured specimens were identified as *P. major* and *P. kandelakii*, respectively, in the study area. The detailed information about the collections and identifications is given in Supplementary Table S1. The morphological structures of the two species are shown in Figure 2. The remaining 40 specimens (%2.11) were morphologically identified as *P. papatasi*, *P. tobbi*, *P. sergenti*, *P. halepensis*, *P. alexandri*, and *Sergentomyia dentata* with 1, 3, 18, 15, 2, and 1 specimens, respectively.

COI and Cytb sequence analyses and divergence of sand flies: We successfully recovered the barcode sequences of the 658 bp region of the COI and the 711 bp region of the Cytb genes from the 55 specimens of *P. major* and 25 specimens of *P. kandelakii*. The absence of insertions, deletions, or stop codons in the COI and Cytb indicates that all sequences were functional mitochondrial products. Of the nine and four COI haplotypes detected in the study for *P. major* and *P. kandelakii*, respectively, seven from *P. major* and three from *P. kandelakii* were new to the respective sand fly species according to the blast searches in the GenBank and BOLD Systems databases. The remaining two *P. major* and one *P. kandelakii* haplotypes were previously reported from the Black Sea Region of Türkiye (Figure 3). COI-BSPmaj1 was the most common haplotype of *P. major*, comprising 21 out of 55 isolates. This was followed by the haplotypes COI-BSPmaj2 to COI-BSPmaj9, with 1 to 14 isolates within each haplotype (Figure 3). For the COI sequences of *P. kandelakii*, COI-BSPkan1 was the dominant known haplotype, with a total of 10 isolates. The new haplotypes COI-BSPkan2, COI-BSPkan3, and COI-BSPkan4 included seven, four, and four isolates, respectively (Figure 3). We identified three haplotypes (one known and two new) of *P. major* and three haplotypes (all new) of *P. kandelakii* based on the Cytb sequence analyses (Figure 3). The best-known haplotype of *P. major* is Cytb-BSPmaj1, representing a total of 37 isolates. The new haplotypes Cytb-BSPmaj2 and Cytb-BSPmaj3 included 10 and 8 isolates, respectively. Cytb-BSPkan1 was the dominant haplotype of *P. kandelakii*, represented by 20 isolates and the remaining two haplotypes, Cytb-BSPkan2 and Cytb-BSPkan3, were presented by three and two isolates, respectively (Figure 3).

The fragments of both COI and Cytb base compositions in the assembled datasets containing 51 and 31 sequences, respectively, showed significant variation with a total of 39.4% and 37.7% polymorphic sites among the species of the subgenus *Larroussius* of the genus *Phlebotomus*. The AT and GC composition of the entire datasets ranged from 68.4 to 64.4% and 31.6–35.6% for COI and 71.5 to 74.6% and 25.4–28.5% for Cytb, respectively, with an AT bias. The transition/transversion bias (R) was higher in the 2nd (19.59) codon position, followed by the 1st (4.39) and 3rd (1.64) codon positions for the COI dataset. While the R was higher at the 2nd (5.87) and 3rd (4.94) codon positions compared to the 1st (1.29) position for the Cytb dataset, The genetic diversity indices and the results of the neutrality tests for the COI and Cytb datasets are shown in Table 1. The overall haplotype and nucleotide diversities were 0.99 and 0.16 for COI and 0.99 and 0.16 for Cytb, respectively, among the *Larroussius* species, with low levels of haplotype and nucleotide diversity within each species. Tajima's D (48) and Fu's F (16) were significant ($P < 0.05$) only for *P. major* taxa.

The pairwise genetic distance matrix of the COI and Cytb gene regions among the *Larroussius* species is given in the supplementary tables (S2 and S3). The mean intraspecific genetic distance for the COI sequences of *P. major* was determined to be 0.4%, and our sequences showed 98.9% to 100.0% identity with isolates reported from the Middle Black Sea Region of Türkiye (GenBank accessions: OQ826546, ON093827, ON093829, and MN086538). The *P. major* haplotypes also showed 99.1% to 99.8% similarity with the *P. neglectus* isolates from the southern region of Türkiye (GenBank accession: OL352136) and Leros, Greece (GenBank accession: OL352154). The analyses of the COI sequences of *P. kandelakii* in the dataset showed a mean intraspecific genetic distance of 1.0%, and our haplotypes were found to be 96.7% to 100.0% identical to the haplotypes reported from the West Black Sea Region of Türkiye (GenBank accessions: ON093832, ON093833, ON093835, MN086479, MN086487, and MN086490).

Cytb sequence analyses of *P. major* revealed a mean intraspecific genetic distance of 1.4% in the dataset and our sequences showed 99.7% to 100.0% identity with haplotypes reported from several sites in the West Black Sea Region of Türkiye (GenBank accessions: ON097122, ON097127). The identified Cytb haplotypes were also 95.5% to 95.6% identical to the Iranian specimen (GenBank accession: GQ169334). Analysis of the *P. kandelakii* Cytb dataset revealed a low level of intraspecific genetic distance (0.2%) between the haplotypes identified in the study and published haplotypes from the West Black Sea Region of Türkiye (GenBank accessions: OQ846925, ON097120).

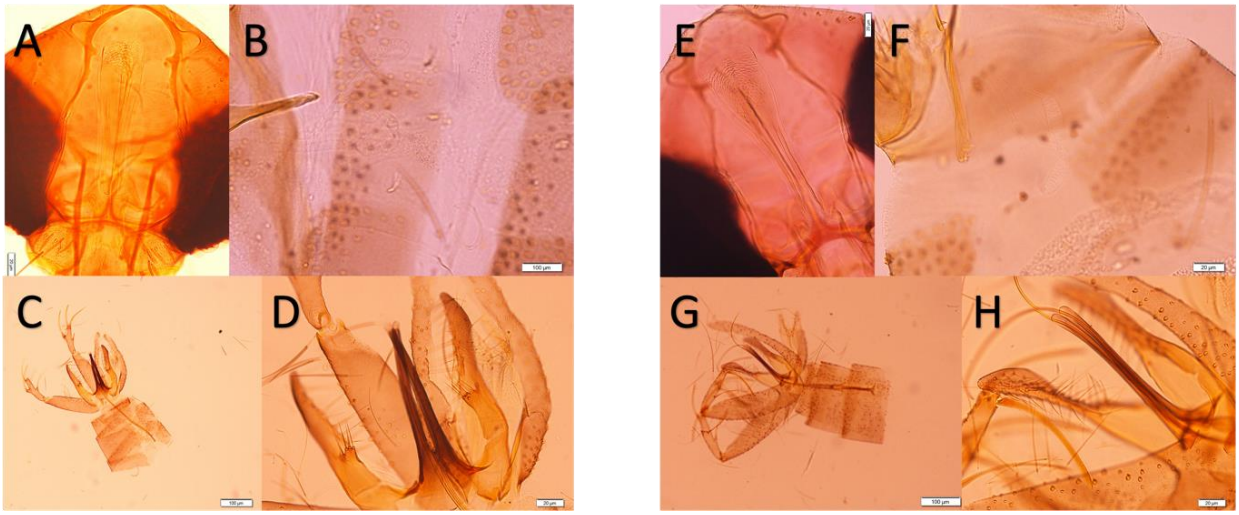

Figure 2. Morphological characteristics of *Phlebotomus kandelakii and P. major*. *P. kandelakii female.*
(A-B): pharynx and cibarium (A), spermathecal body (B); P. kandelakii male genitalia (C-D); P. major female (E-F): pharynx and cibarium (E), spermathecal body (F); *P. major* male genitalia (G-H).

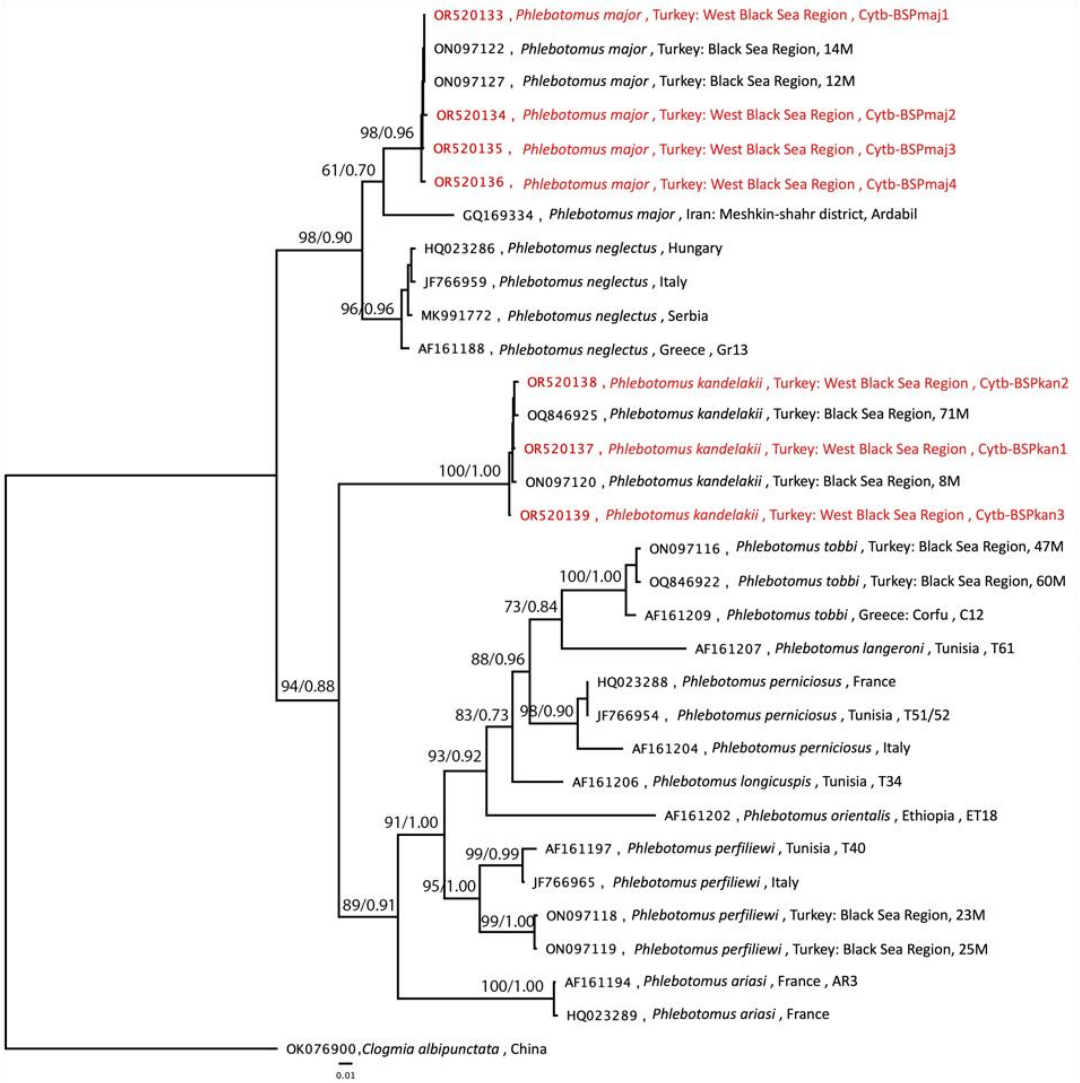

Figure 3. COI phylogenetic tree of the species of *Larroussius* subgenus including the haplotypes of *P. major* and *P. kandelakii* identified in the study (in red).

Numbers at the nodes represent the bootstrap values (1000 replicates) and posterior probabilities, respectively. The sequences were given as GenBank accession number, country and isolate name if available. *Clogmia albipunctata* sequence was used as the outgroup. The scale bar represents 0.01% divergence.

Table 1. Summary of genetic diversity indices and results of neutrality tests (Tajima's D and Fu's F) in the mitochondrial COI and Cytb datasets of the species of *Larroussius* subgenus.

Species	n	k	K	h (±SD)	Mean π	Tajima's*D*	Fu's *F*
COI gene							
P. major	15	33	11	0.933 ± 0.054	0.00848	-2.04*	-2.83*
P. kandelakii	10	26	9	0.978 ± 0.054	0.00969	-1.46	-1.96
P. ariasi	1	1	N	N	N	N	N
P. chadlii	1	1	N	N	N	N	N
P. guggisbergi	2	2	5	1.000 ± 0.500	0.0076	N	N
P. keshishiani	1	1	N	N	N	N	N
P. longicuspis	3	1	N	N	N	N	N
P. neglectus	5	2	20	0.400 ± 0.237	0.01216	-1.32	-1.23
P. orientalis	3	3	3	1.000 ± 0.272	0.00304	N	N
P. perfiliewi	3	2	25	0.667 ± 0.314	0.02533	N	N
P. perniciosus	3	3	11	1.000 ± 0.272	0.01114	N	N
P. smirnovi	1	1	N	N	N	N	N
P. syriacus	1	1	N	N	N	N	N
P. tobbi	3	3	19	1.000 ± 0.272	0.01925	N	N
Overall	52	172	31	0.994 ± 0.022	0.09330	0.04	0.07
Cytb gene							
P. major	7	4	31	0.714 ± 0.181	0.01308	-1.65*	-1.88*
P. kandelakii	5	5	4	1.000 ± 0.126	0.00225	-1.09	-1.11
P. ariasi	2	2	1	1.000 ± 0.500	0.00141	N	N
P. longicuspis	1	1	N	N	N	N	N
P. neglectus	4	4	8	1.000 ± 0.177	0.00587	-0.45	-0.44
P. orientalis	1	1	N	N	N	N	N
P. perfiliewi	4	4	32	1.000 ± 0.177	0.02981	1.46	1.70
P. perniciosus	3	3	16	0.667 ± 0.314	0.01502	N	N
P. tobbi	3	3	8	1.000 ± 0.272	0.00751	N	N
P. langeroni	1	1	N	N	N	N	N
Overall	31	27	206	0.985±-0.015	0.11652	0.54	0.76

n: number of sequences; k: number of variable sites; K: number of haplotypes; *h*: haplotype diversity; π: nucleotide diversity; N: value could not be calculated due to insufficient data ∗significant at the 0.05 level.

Phylogenetic analysis: Figures 3 and 4 show the consensus trees generated by maximum likelihood (ML) analyses with the corresponding nucleotide substitution models for the COI and Cytb datasets, which included the alignment of 52 and 31 nucleotide sequences, respectively. A similar topology for both gene regions was produced by the tree based on Bayesian inference (BI). Therefore, posterior probabilities were presented with bootstrap values on the ML trees. We used COI and Cytb sequences of a non-hematophagous insect, *Clogmia albipunctata*, within the same family (Psychodidae: Diptera) as an outgroup taxon to generate the dendrogram for phylogenetic analyses.

The COI phylogenetic tree revealed the presence of three major clusters within the subgenus *Larroussius*, supported by a bootstrap value of 83%-99% and a posterior probability of 0.88-1.00. The first cluster included *P. major*, *P. neglectus*, *P. syriacus*, and *P. keshishiani*. The phylogenetic resolution of this cluster was also supported by bootstrap values of 88%-93% and posterior probabilities of 0.88-1.00, except for the sister relationship between the clade containing the *P. neglectus* from Serbia (GenBank accession: KY848830) and the clade containing all *P. major* and *P. neglectus* haplotypes, which had a relatively moderate bootstrap (76%) and posterior probability (0.84) support. The second cluster was divided into two sub-clusters. The first sub-cluster included only *P. kandelakii* haplotypes from the Black Sea Region of Türkiye. The phylogenetic resolution in this sub-cluster was strongly supported, with a bootstrap value of 100.0% and posterior probabilities of 1.00. The second sub-cluster contained the monophyletic taxa consisting of

P. orientalis, *P. smirnovi*, *P. tobbi*, *P. longicuspis*, *P. perniciosus*, *P. perfiliewi*, and *P. guggisbergi*. The phylogenetic resolution of this sub-cluster was also supported by bootstrap values of 94%-100% and posterior probabilities of 1.00. The third major cluster was an outer taxon of the first and second major clusters and included the *Larroussius* species *P. ariasi* and *P. chadlii*. The phylogenetic relationships in this cluster had a high bootstrap value of 99% and a posterior probability of 1.00 (Figure 3).

Phylogenetic analyses of the Cytb dataset revealed two major clusters within the subgenus *Larroussius*, supported by bootstrap values of 89%-100% and posterior probabilities of 0.89-1.00. The first cluster was subdivided into two sub-clusters. The first sub-cluster was comprised of the *P. major* haplotypes and the second one included the *P. neglectus* haplotypes from several countries. The monophyletic resolution of both sub-clusters was well supported by bootstrap values of 94%-98% and posterior probabilities of 0.96–1.00, except for the sister relationship of the *P. major* Iran specimen (GenBank accessions: GQ169334), which exhibited a moderate bootstrap (61%) and posterior probability (0.70). The second cluster is also divided into two monophyletic sub-clusters and supported by bootstrap values of 89%-100% and posterior probabilities of 0.91-1.00. The first sub-cluster included *P. kandelakii* haplotypes and the second included *P. tobbi*, *P. langeroni*, *P. perniciosus*, *P. longicuspis*, *P. orientalis*, *P. perfiliewi*, and *P. ariasi*. *P. ariasi* was placed as an outer taxon within the second sub-cluster (Figure 4).

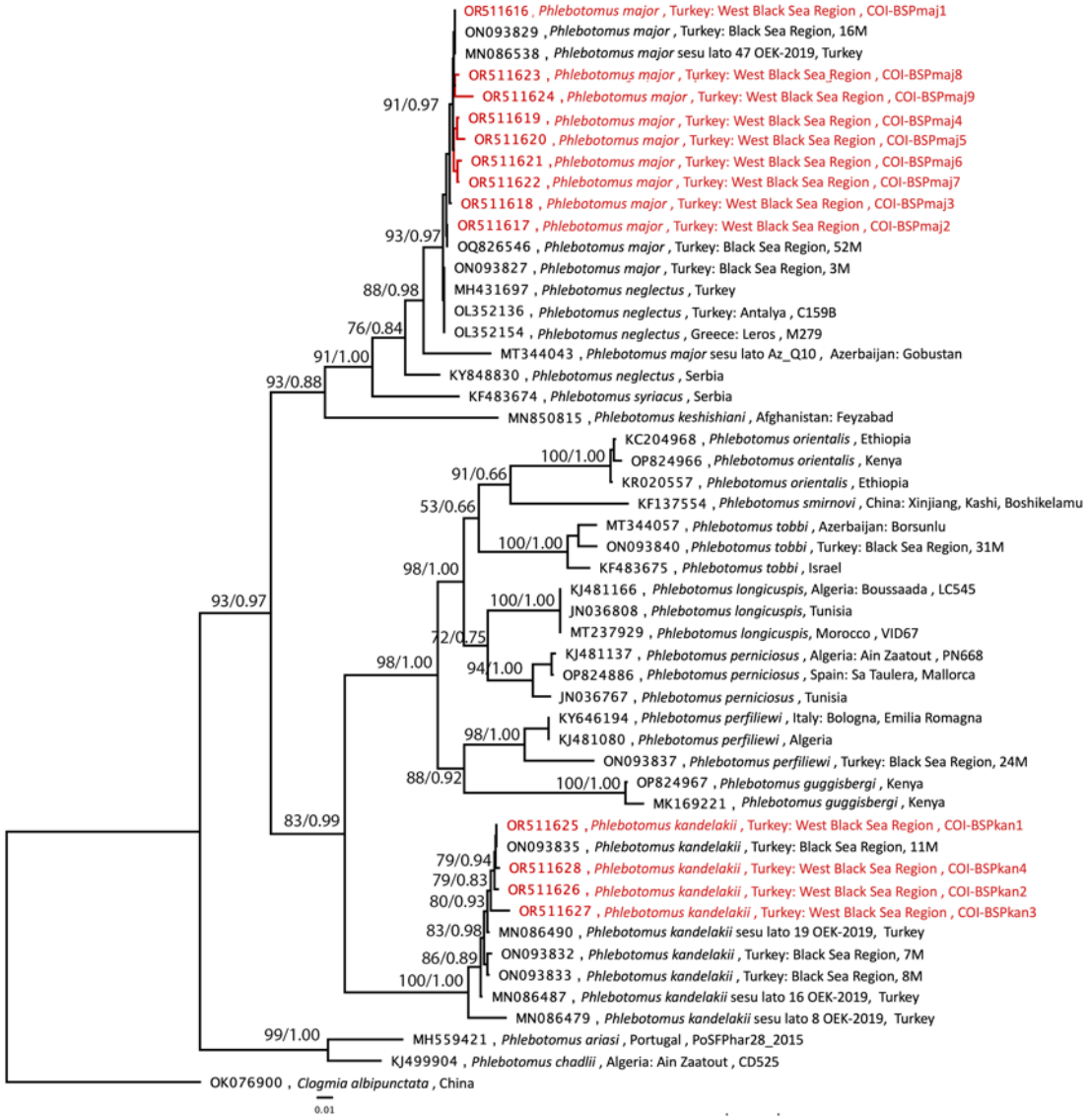

Figure 4. Cytb phylogenetic tree of the species of Larroussius subgenus including the haplotypes of *P. major* and *P. kandelakii* identified in the study (in red).

Numbers at the nodes represent the bootstrap values (1000 replicates) and posterior probabilities, respectively. The sequences were given as GenBank accession number, country and isolate name if available. *Clogmia albipunctata* sequence was used as the outgroup. The scale bar represents 0.01% divergence.

Discussion and Conclusion

The members of *Phlebotomus major* s.l. are one of the most prevalent sand fly species in almost all geographical regions of Türkiye (21, 38). The members of this complex are known as competent vectors of *L. infantum* and also have an overlapping distribution with the endemic area of VL around the world (21, 28, 33, 45). The major group of *Larroussius* has had a complex taxonomy since its first description by Annandale (1910) and currently six species are recognized within this taxon, including *P. major*, *P. neglectus*, *P. syriacus*, *P. wui*, *P. notus*, and *P. wenyoni* (3, 13). The females of *P. major* s.l. are mainly distinguished from other *Larroussius* species mainly by the shape of their pharyngeal armatures and this typical morphological character was observed in all *P. major* s.l. specimens in our study. The male morphological characters, including the aedeagus, palpal formulae, length of the style to coxite, and pharyngeal armature, are the only known characters used for the identification of the corresponding six species of major complex (3, 29). The morphological analyses of the male specimens of *P. major* s.l. in our study revealed the same characteristics of the aedeagus, the length of the style to coxite, and the pharyngeal armature as those of the species *P. major*. Nevertheless, the taxonomic situation of this complex is not yet resolved and the status of the described species as valid or conspecific within taxa is still unclear.

Both mitochondrial COI and Cytb gene fragments are considered to be valid molecular markers for distinguishing several sand fly species, such as *P. chinensis*, *P. stantoni*, *P. papatasi*, *P. sergenti*, *P. ariasi* and *P. tobbi* (7, 9, 46). DNA barcoding using these mitochondrial markers has been widely used to characterize and identify sand flies (11, 15, 18, 26, 27, 31, 41). The barcoding gap between the species in most phlebotomine taxa was considered to contain sufficient sequence diversity for species delimitation (7, 8, 31, 34). In the current study, both mitochondrial markers were evaluated for their ability to discriminate between the species of the subgenus *Larroussius* using the characterized sequences of *P. major* and *P. kandelakii* and the sequences of related taxa within the subgenus. Considering the major complex, a low level of intraspecific genetic distance (0.2% to 0.9%) was found between *P. major* COI sequences. However, some of the *P. neglectus* sequences from different parts of Türkiye and Greece (OL352154, OL352136, and MH431697; Figure 3) showed interspecific genetic differences <0.9, with the *P. major* sequences indicating an overlap with the intraspecific difference. On the other hand, the Serbian isolate of *P. neglectus* (KY848830; Figure 3) showed 3.2% to 4.0% intraspecific genetic differences with *P. major*. The only available COI sequence of *P. syriacus* from Israel (KF483674; Figure 3) also showed a 5.9% to 7.2% difference with *P. major* sequences. All these data suggest that the COI sequence can be used as a DNA barcode to distinguish *P. syriacus* from *P. major* and *P. neglectus*. It appears that the overlap between the COI sequences does not allow *P. major* and *P. neglectus* to be distinguished. However, the overlapping sequences of *P. neglectus* from Türkiye and Greece could also be related to misidentification. In fact, the Serbian isolate of *P. neglectus* has a genetic difference of more than 3.0% from *P. major*, which serves as a suitable barcode gap between the species. Further data based on the combination of morphological and molecular analyses is needed to clarify the efficiency of COI-based barcoding within this complex. With regard to the Cytb sequence analyses, it appears that *P. neglectus* differs from *P. major* with 5.2% to 8.0% genetic distance and the phylogenetic tree clustered the isolates into monophyletic clades (Figure 4). Even though the *P. major* haplotypes from Türkiye were close to each other with an overall identity of 99.8%, the Iranian *P. major* isolate showed a mean genetic difference of 4.5% from the Turkish haplotypes and formed a separate clade in a phylogenetic tree (Figure 4). There is also a lack of information on the Cytb sequence characterization of other members of the major complex in GenBank. While the data obtained in our study provides initial evidence for the usefulness of Cytb-based barcoding to distinguish the members of the major complex, the lack of sequence information from other species within the taxa limits the comprehensive evaluation of Cytb barcoding.

The second widespread sand fly species in the study area is *P. kandelakii*, which is also capable of transmitting *L. infantum* (14, 33, 45). Data on this species of COI and Cytb sequence diversity were limited, with only a few sequences mainly from different regions of Türkiye. The phylogenetic analyses of the COI and Cytb datasets clearly indicated a monophyletic taxon for this species with an overall identity of 99.0 for COI and 99.7% for Cytb among the haplotypes of *P. kandelakii*. Both COI and Cytb trees clustered the *P. kandelakii* haplotypes in a separate clade closer to the cluster comprised of *P. orientalis*, *P. smirnovi*, *P. tobbi*, *P. longicuspis*, *P. perniciosus*, *P. perfiliewi*, and *P. guggisbergi* rather than the cluster of major complex species.

Although we determined several haplotypes of *P. major* and *P. kandelakii* based on COI and Cytb sequences, the overall haplotype and nucleotide diversities were low for both species. Different statistical tests have been utilized to test the selective neutrality and population growth of several organisms (44). We used two frequently used tests to analyze population growth that have variable power: Tajima's D-test and Fu's F-test (43). The outputs indicated negative values for both *P. major* and *P. kandelakii* with P-values < 0.05. This result might indicate an excess of recently derived haplotypes of both species and an excess of low-frequency polymorphism in the

populations, which was also observed in other sand fly populations in different regions, such as *P. sergenti* (12).

In conclusion, our results contribute to the current knowledge of the species' genetic diversity in the subgenus *Larroussius* of sand flies. We also provide further data on the utility and usefulness of COI and Cytb barcoding for delimiting species within this subgenus, focusing on the two widespread species, *P. major* and *P. kandelakii*. Further investigations with large-scale samplings from different regions using both morphological and molecular approaches are proposed to clarify the genetic diversity and taxonomic status of the members of the major complex.

Acknowledgments

Gupse Kübra Karademir and Sadullah Uslu were supported by the Council of Higher Education (YOK) 100/2000 PhD scholarship. Thanks to YOK for 100/2000 PhD scholarship.

Financial Support

This research was supported by the Erciyes University Research Fund (grant number TCD-2021-11185).

Conflict of Interest

The authors declared that there is no conflict of interest.

Author Contributions

GKK, GZP, MA, DP, KY, GY, and SU planned and carried out the field samplings. GKK, MA, KY, YO, ST, ZO, and AI contributed to the preparation of slides and morphological identifications. GKK, OD, AY, MA, and GY carried out the DNA extraction, PCR, and sequencing. GKK, AY, AC, and GZP contributed to the molecular and phylogenetic analyses. GKK, YO, and AI provided a conception of research, methodology, and supervision. All authors provided critical feedback and helped shape the research, analysis, and manuscript.

Data Availability Statement

Accession numbers given by NCBI for *P. major* and *P. kandelakii* isolates identified in this study (https://www.ncbi.nlm.nih.gov/) are OR511616-28 and OR520133-9.

Ethical Statement

This study does not present any ethical concerns.

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The principal component of body height linear type traits and its relationship level to milk yields as Holstein cattle selection criterion

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ARTICLE INFO

Article History
Received : 16.02.2023
Accepted : 10.01.2024
DOI: 10.33988/auvfd.1251983

Keywords
Body measurement
Correlation
Dairy cows
Height dimension

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How to cite this article: Prabowo S, Garip M (2024): The principal component of body height linear type traits and its relationship level to milk yields as Holstein cattle selection criterion. Ankara Univ Vet Fak Derg, 71 (4), 437-444. DOI: 10.33988/auvfd.1251983.

ABSTRACT

The essential body height characteristics associated with milk yields must be carefully identified. In particular, this study sought to identify the most relevant body height dimension trait as a selection criterion for the milk yield increase program. The test animals for the study were 121 heads of Holstein cows, and seven characteristics of body height were recorded for each cow separately. Principal component analysis (PCA), correlation, and regression were used to analyze the data. As an analytical tool, the R program 4.2.1 with RStudio was employed. The primary elements discovered in PCA's output were the wither height (WTH), back height (BCH), rump height (RMH), thurl height (TLH), tail-head height (THH), and pins height (PNH). Afterward, the correlation and regression analysis findings showed that the rear udder height (RUH) had the highest priority in correlating with milk yields, followed by the thurl height (TLH). In conclusion, it is proposed that the RUH be utilized for the cow selection scheme while the TLH is used for the calf and heifer selection programs.

Introduction

Generally, taller cows produce more milk than shorter cows (42). Meanwhile, dairy cattle have various linear traits related to body heights, such as wither height (43), back height (31), rump height (43), tail-root height (40), pin bone height (19), thurl height (53), and rear udder height (45). Therefore, this is considered a large number of parameters to be executed in the selection program for dairy cattle.

It is challenging to investigate this matter since dairy cattle have a significant share of the dimensional variable of body height. It necessitates excessive time, energy, and a research budget to identify dairy cattle's body height's most essential linear traits. The principal component analysis (PCA) method can address those problems. An article explained that principal component analysis could reduce the number of characteristics that should be assessed for milk production and composition and make an essential contribution to data quality by explaining the characteristics of Holstein cows (1).

The canvas of this topic could be more varied nowadays. Indeed, the protruding body height of cows concerning milk yield is still labeled equivocally and unconfidently. Such exploration only revealed the significant contribution of height regions of Rhodope Shorthorn cows to growth performance; meanwhile, milk capacity needs to be improved (30). Another study merely analyzed the relation of the height dimension to daily milk production (37). Briefly, the prime body height to milking potency information is inadequate; thus, the selection program lacks impracticality and is uneconomical, particularly for small-scale farms. Consequently, this theme should be delved into shortly.

Additional analyses of Pearson's correlation and regression analysis are used to capture the level of association between body height traits and milk yields, like a study done by American investigators on the relationship between body size and milk supply potency to recognize the supreme height frame structure (24). As a result, dairy cattle's superior body height and linear features could be firmly established over time. The current study proposes a substantial body height feature as a selection criterion based on the degree of correlation with milk yield to address this issue.

Materials and Methods

Data compilation: In terms of execution, this exploration was completed using 121 Friesian Holstein cows on a commercial dairy farm, namely UD. Saputra Jaya, East Java, Indonesia. Also, the sample age range was 2–6 years old, and the cows were entirely in the lactation period. Then, entire body height variables were measured using a cattle stick gauge with a centimeter (cm) scale so that the data was of the interval data type. Body height variables and their detailed descriptions are displayed in Table 1.

Statistical Analysis: Regarding data analysis, R application type 4.2.1 with RStudio was applied to perform principal component analysis (PCA), correlation, and regression analysis alternately. Then, the mathematical model of the PCA is as follows:

$$\mathrm{PC}_i = \beta_{i1}\mathrm{X}_1 + \beta_{i2}\mathrm{X}_2 + \beta_{i1}\mathrm{X}_1 + \beta_{i3}\mathrm{X}_3 + \cdots + \beta_{im}\mathrm{X}_m \quad (38)$$

with β_i: coefficients$i^{\text{-th}}$;X_m: variable $m^{\text{-th}}$.

Meanwhile, the formulas of correlation (a) and regression (b) are reflected as

$$r^2 = \frac{[\sum_{i=1}^{n}(x_i - \bar{x})(y_i - \bar{y})]^2}{\sum_{i=1}^{n}(x_i - \bar{x})^2 \sum_{i=1}^{n}(y_i - \bar{y})^2} \quad \text{(a) (46)}$$

$$Y = \alpha + \beta x + \mathcal{E} \quad \text{(b) (15)}$$

which$\bar{x} = \frac{\sum_{i=1}^{n} x_i}{n}$ and $\bar{y} = \frac{\sum_{i=1}^{n} y_i}{n}$ are the means of a sample. Then,$\alpha$is the independent term, $\beta$is the slope of the straight line, and $\mathcal{E}$is a mark of the perturbation element.

Continuing with the test interval method of milk yield ($\mathrm{MY_{tim}}$) mathematical model is as follows:

$$\mathrm{MY_{tim}} = I_0 M_1 + I_1\left(\frac{(M_1 + M_2)}{2}\right) + I_2\left(\frac{(M_2 + M_3)}{2}\right) + I_{n-1}\left(\frac{(M_{n-1} + M_n)}{2}\right) + I_n M_n$$

The $\mathrm{MY_{tim}}$ is the total milk yield estimation, and the M_1, M_2, and M_n are the 24-hour milk yield. Then, theI_1, I_2, and I_{n-1} are the days between two milking days. Henceforth, theI_0 is the interval between the start of lactation and the milking day's first recording. Meanwhile, the I_n is the interval between the last recording of the milking day and the dry time (21). Then, the milk yieldsare standardized at 305 days ($\mathrm{MY_{s305}}$), and the milk yield's mature equivalents ($\mathrm{MY_{me}}$) (28)are also determined.

Results

Table 2 shows the probe results of Holstein's body height linear traits as a descriptive statistic. WTH, BCH, RMH, TLH, THH, PNH, and RUH range scores were generally broad. The PNH trait had the most extensive data range, whereas the RUH trait had the narrowest data range. In the meantime, Table 3 emerged with the outright data of Kaiser Meyer Olkin Measures Sampling Adequacy (KMO-MSA) and Bartlett's test of sphericity. All body height features scored higher than 0.5 except for the RUH trait. Even though RUH's KMO-MSA score was only 0.12 individually, the average overall score was still above 0.5. Furthermore, Bartlett's test p-value was less than 0.01.

Table 1. Description and symbols of dairy cattle body heights

Body height	Symbols	Description	References
Withers height	WTH	Measured as the vertical distance from the top of the wither *spine* to the floor	(11)
Back height	BCH	Measured as the vertical distance from the top of the back *spine* last rib to the ground	(11)
Rump height	RMH	Measured from the anterior edge of the *sacrum* between the hips vertically when a cow was standing	(11)
Thurl height	TLH	Measured as the height at the greater *trochanter* to the floor	(35)
Tail-head height	THH	Measured from the anterior edge of the *caudal* (*sacrococcygeal* region) vertically to the floor base	(20)
Pins height	PNH	Measured as the height of pin or *tuber ischium* to the concrete	(11)
Rear udder height	RUH	Downmost point of the vulva to the uppermost point of the ligament suspensory rear-view	(10)

Table 2. Descriptive Statistics of dairy cattle body heights and milk yields

Body height	Min	1st quartile	Median	Mean		3rd quartile	Max
				Statistic	St. error		
WTH (cm)	120.1	128.6	132.2	133.0	0.57	136.6	152.6
BCH (cm)	117.6	128.9	132.4	133.2	0.58	137.3	151.4
RMH (cm)	121.7	128.7	132.1	132.8	0.57	136.2	151.4
TLH (cm)	90.6	104.2	108.7	108.4	0.63	112.7	125.4
THH (cm)	122.1	128.9	132.6	133.3	0.54	136.2	152.3
PNH (cm)	109.4	117.4	121.9	122.5	0.64	126.9	146.5
RUH (cm)	7.20	14.60	17.80	17.84	0.39	20.80	27.6
MY_{tim} (kg)	1789	2314	2538	2556	29.96	2729	3673
MY_{s305} (kg)	1985	2263	2448	2482	27.17	2646	3357
MY_{me} (kg)	2105	2551	2764	2809	33.77	3043	3853

WTH: wither height; BCH: back height; RMH: rump height; TLH: thurl height; THH: tail head height; PNH: pins height; RUH: rear udder height; MY_{tim}: milk lactation total of test day; MY_{s305}: the whole milk lactation 305 days; MY_{me}: the overall milk lactation matured evenly.

Table 3. KMO-MSA and Bartlett's test of dairy cattle body height

Test type						Score	
Kaiser-Meyer-Olkin factor adequacy (Overall MSA):						0.83	
MSA for each item:	WTH	BCH	RMH	TLH	THH	PNH	RUH
	0.88	0.84	0.79	0.93	0.79	0.81	0.12
Bartlett's test of sphericity	Chi-squared:		1122.9				
	df:		21				
	p-value:		0.001				

WTH: withers height; BCH: back height; RMH: rump height; TLH: thurl height; THH: tail head height; PNH: pins height; RUH: rear udder height.

Table 4. Eigenvector of the dairy cattle body heights principal component

Traits	PC_1	PC_2	PC_3	PC_4	PC_5	PC_6	PC_7
WTH	0.4138	-0.3283	0.0632	-0.0153	-0.6485	0.5074	0.1967
BCH	0.4275	-0.2979	-0.0198	-0.0949	-0.2521	-0.7220	-0.3663
RMH	0.4193	-0.2852	-0.0367	-0.0844	0.5048	-0.1421	0.6778
TLH	0.3843	0.6091	-0.5295	-0.4393	-0.0514	0.0721	-0.0113
THH	0.4038	-0.1789	0.0439	0.0791	0.5045	0.4288	-0.5987
PNH	0.3992	0.5504	0.4425	0.5650	-0.0496	-0.1071	0.0933
RUH	-0.0094	-0.1270	-0.7185	0.6821	-0.0377	-0.0212	0.0221

WTH: wither height; BCH: back height; RMH: rump height; TLH: thurl height; THH: tail head height; PNH: pins height; RUH: rear udder height; PC_{1-7}: the principal component number one to seventh.

An important next-level consideration in the principal component analysis is the eigenvector or loading factor and Eigen value output. Tables 4, 5, and 6 give rise to the eigenvector, loading factor, and Eigen value from this investigation, respectively. However, loading factor analysis can be used for simplicity and more comprehensive dimension reduction. Table 5 details the loading factor for a primary component in this investigation. The further issue is that the early principal component (PC_1) had an explained capability of 75.4% by the total proportion of variance, as illustrated in Table 6. Furthermore, PC_2 to PC_7 only explained capabilities that begin at 8.92% and gradually decrease. THE LINEAR EQUATION OF DAIRY CATTLE BODY HEIGHT IS GIVEN because PC_1 is the only combination with a percentage of variance explained above 10%.

$$PC_1 = 0.414\ log\ (x_1) + 0.427\ log\ (x_2) + 0.419\ log\ (x_3) + 0.384\ log\ (x_4) + 0.404\ log\ (x_5) + 0.399\ log\ (x_6)$$

with PC_1: principal component 1;x_1: wither height (WTH); x_2: back height (BCH); x_3: rump height (RMH); x_4: thurl height (TLH); x_5: tail-head height (THH); x_6: pins height (PNH) in that order.

The correlation coefficient among the body-height linear traits is given in Table 7. This table revealed that the correlation among body height linear traits was almost entirely positive; additionally, it had a relatively high association among variables. Only RUH negatively correlates with the other body height features, making it clear that RUH stands out. It also ran in parallel with the PCA output. The RMH and THH traits had the highest correlation coefficient; RUH and THH had the lowest. When body height traits were linked to milk yields, the most significant association was delivered by RUH, followed by TLH.

The regression coefficient for Table 8's linear model to predict the milk yield test interval using body height features was given as follows:

$$MYT_{1st} = 2962.059 - 22.783(x_7)$$
$$MYT_{2nd} = 2269.664 - 22.717(x_7) + 6.382(x_4)$$

While the following model is consistent with estimating milk yield at 305 days:

$$MYS_{1st} = 2884.602 - 22.565(x_7)$$
$$MYS_{2nd} = 1824.636 - 22.463(x_7) + 9.770(x_4)$$

eventually, calculating the milk yield of mature equivalents will apply to this equation

$$MYM_{1st} = 3182.516 - 20.922(x_7)$$
$$MYM_{2nd} = 1855.119 - 20.794(x_7) + 12.235(x_4)$$

MYT_{1st} Is the first formula to estimatethe milk yield test interval; MYT_{2nd} is the second formula to predict the milk yield test interval. Then, the MYS_{1st} is the first formula to compute the milk yield standardized at 305-d; theMYS_{2nd} is the second formula to calculatethe milk yield standardized at 305-d. Henceforth, theMYM_{1st} is the first formula to assess themilk yield of the mature equivalent; and theMYM_{2nd} is the second formula to evaluate the milk yield of the mature equivalent. Meanwhile, thex_4 isTLH,and thex_7 is RUH.

Again, the RUH trait is indicated as having a prominent capacity to predict milk yields according to the regression analysis stepwise method; besides, the TLH trait was also detected as an essential character in the dairy cattle body height. Hence, these two traits should be observed meticulously due to the evidence and analysis output directed at them.

Table 5. Loading factor of the dairy cattle body heights principal component

Traits	PC_1	PC_2	PC_3	PC_4	PC_5	PC_6	PC_7
WTH	0.414	0.328			0.649	0.507	0.197
BCH	0.427	0.298			0.252	-0.722	-0.366
RMH	0.419	0.285			-0.505	-0.142	0.678
TLH	0.384	-0.609	-0.529	0.439			
THH	0.404	0.179			-0.505	0.429	-0.599
PNH	0.399	-0.505	0.442	-0.565		-0.107	
RUH		0.127	-0.718	-0.682			

WTH: wither height; BCH: back height; RMH: rump height; TLH: thurl height; THH: tail head height; PNH: pins height; RUH: rear udder height; PC_{1-7}: the principal component number one to seventh.

Table 6. Eigenvalue of the dairy cattle body heights principal component

Level	PC_1	PC_2	PC_3	PC_4	PC_5	PC_6	PC_7
Standard deviation	14.221	4.892	4.232	4.107	1.687	1.376	0.844
Portion of Variance	0.7545	0.0892	0.0730	0.0629	0.0106	0.0071	0.0027
Cumulative Portion	0.7545	0.8437	0.9167	0.9797	0.9903	0.9973	1.0000

PC_{1-7} is the principal component number one to seventh.

Table 7. Phenotypic correlation between dairy cattle body heights and milk yields

Corr.	WTH	BCH	RMH	TLH	THH	PNH	RUH	MY_{tim}	MY_{s305}	MY_{me}
WTH	1.00									
BCH	0.95**	1.00								
RMH	0.94**	0.96**	1.00							
TLH	0.63**	0.68**	0.68**	1.00						
THH	0.93**	0.93**	0.97**	0.68**	1.00					
PNH	0.68**	0.67**	0.67**	0.63**	0.75**	1.00				
RUH	-0.30	-0.03	-0.02	-0.01	-0.00	-0.07	1.00			
MY_{tim}	0.09	0.11	0.07	0.14	0.08	0.11	-0.30**	1.00		
MY_{s305}	0.20*	0.23*	0.19*	0.23*	0.18*	0.14	-0.33**	0.90**	1.00	
MY_{me}	0.15	0.20*	0.16	0.23**	0.17	0.15	-0.24**	0.73**	0.85**	1.00

WTH: wither height; BCH: back height; RMH: rump height; TLH: thurl height; THH: tail head height; PNH: pins height; RUH: rear udder height; MY_{tim}: milk lactation total of test day; MY_{s305}: the whole milk lactation 305 days; MY_{me}: the overall milk lactation matured evenly.
** Significantly correlated at the 0.01 degree (2-tailed).
* Significantly correlated at the 0.05 degree (2-tailed).

Table 8. Regression coefficient of body height of dairy cattle related to milk yield.

Model		Milk yield-test day (MY_{tim})		Milk yield-standardized 305d (MY_{s305})		Milk yield-mature equivalent (MY_{me})	
		β	Adj. R sq.	β	Adj. R sq.	β	Adj. R sq.
1	Intercept	2962.059	0.081**	2884.602	0.098**	3182.516	0.051**
	RUH	-22.783		-22.565		-20.922	
2	Intercept	2269.664		1824.636		1855.119	
	RUH	-22.717	0.091	-22.463	0.142*	-20.794	0.096**
	TLH	6.382		9.770		12.235	

**P-value < 0.01.
* P-value < 0.05.

Discussion and Conclusion

A comparative study with another relevant investigation was published: mature Holstein cows have a wither height (WTH) of 124–158 cm (11, 17, 23, 31, 32, 42, 48), a back height (BCH) of 116–160 cm (11, 17, 31), a rump height (RMH) of 125–162 cm (11, 17, 31), and a pins height (PNH) of 119–153 cm (9, 11, 31). Meanwhile, the range score of thurl height (TLH) is 122–130 cm (4), tail-head height (THH) is 113–121 cm (27), and rear udder height (RUH) is 21.95 cm on average (10). The range of cow's body highness data previously exposed is compiled under the minimum and maximum highness points from the manifold of cited references.

Based on the literature on the body height of dairy cattle mentioned before, this investigation is in the tolerable normal range, even though some linear traits are in the outer boundary area. The outlying data gap of the present watchfulness could be clouted by the availability of cow research samples on the farm being very from small to oversized frames of a cow. Thus, the range of data variance is broader than the references. Another factor is caused by the distinctiveness of the cattle breeds used in the present study, the excerpted quotation, and limited sources such as appeal articles on several body height properties. Wither height (WTH) correlates relatively significantly with live weight, carcass weight, and meat yield (39, 51). A positive correlation between those linear traits caused by the height of the withers correlated positively with feed intake (FI), body weight (BW), average daily gain (ADG), feed conversion ratio (FCR), and residual feed intake (RFI). However, it has a negative genetic correlation with residual gain (RG) (13). In parallel, growth in wither height is 0.120±0.002 cm/kg LW/day for Friesian-Holstein breed cows, mainly (18). However, the swiftest gains in live weight, wither relative height development, and feed cost efficiency for those traits of the calf occurred throughout the time before reaching six months of age (23). In addition, body height is considered for calculating the cubicle width, headspace, lunging space, cubicle partition, top-bottom rail, and separation wall (16). Another study in linear terms discovered that the result of PCA in the diverse body of linear traits with cubic dimensions revealed 51.4% of the total variability, with the first and second factors

accounting for 40.2% and 11.1% of the total variability, respectively. The WTH is also classified as a significant trait in another breed of cattle by PCA (47, 50). Nonetheless, when interconnected, milk capacity is characterized as trivial (2, 33). Propitiously, this merit has a high heritability capacity (55).

Back or loin height (BCH) insignificantly and weakly correlates to trunk or relative body length (37). However, it relates to live weight significantly in Holstein and Jersey breeds (31). Quite similar to WTH, the BCH also has the profitable nature of a moderate to high heritability score (5, 29). Afterward, this feature is also highly correlated to the other highness structure of the body in the dairy cattle breed (34). Nonetheless, the evidence about this feature linked to milk yield still needs to be densely detected.

Rump or hip height (RMH) has vigorously replied to routine milk delivery volume (41). The RMH is recommended as a selection criterion in Holstein and their crossbreed at calf to heifer period (8). Another researcher also suggested that the RMH trait be applied as a sorting factor for the selection (43).The underlying logic is a very high score of heritability on this property (6, 7, 12, 54). In addition, the RMH relative to WTH could be applied as an overgrowth indicator (26).

Thurl height (TLH) could be used as a criterion to cull a cow for disposal of inabilities or disease, and the higher the TLH, the lower the probability of being culled in Holstein breeds (53). However, criticism of the thurl is more frequently emphasized on the trochanter placement or position between the hip and pin bone (3), and it has a 0.22 heritability score (36).Reasonably, a greater taper corner on the thurl “V” shape will possess a lower thurl height than the obtuse one, even though it has a tantamount highness of body.

Tail head height (THH) has a 0.25 adjusted heritability score (44). This nature is commonly connected to the parturition course, but the opposing viewpoint says the calving difficulty is not associated with tail-head or tail-root height (20). Therefore, this trait of milk delivery should be discussed more. Comparable to fettle, the height of the pin (PNH) is seldom chewed over as well. Nevertheless, an article stated that the PNH is poor, negatively correlates with the milk supply, and is insignificant (37). Vice versa, this property links to body weight significantly in Holstein and Jersey cattle breeds (31).

A study shows that the rear udder height (RUH) is significantly related to milk yields (52), but the heritability is low to moderate (25). Selection on the RUH combined with the other udder traits is given significant distinction on Holstein's predicted transmitting ability for type (PTAT) score (14). Moreover, the total milk volumes, total lactation number, and total day in milk (DIM) are leveraged simultaneously by this characteristic (22). Unquestionably, the RUH is a crucial trait for milk yield characteristics because it is a section of the udder's properties. As a note, the golden expansion period of the udder occurs in the heifer stages and is saliently influenced by environmental factors (49).

Briefly, the present outturn of the PCA, confronted with the magnitude of works of literature in the pertaining field, is signified by an imitation issuance. It is bestowed on the WTH, BCH, RMH, TLH, THH, and PNH as crucial factors in dairy cattle and eliminates the RUH. Contrarily, the correlation and regression analyses are inclined toward the RUH as the most pertinent linear trait to milk yield, followed by the TLH. Later, the WTH, BCH, RMH, TLH, THH, and PNH are outwardly closer to the growth performance characteristics than the milk capacity. Encapsulates this investigation given the confirmation that the milk yield improvement program could prioritize TLH for calves and heifers because these stages are the golden growth curve period and the RUH does not spring up yet. Meanwhile, the lactation cows should be focused on the RUH owing to the critical period of udder growth in the heifer phase. By virtue, the lactation cows have already passed through that precarious moment, and the udder structure has been steadfastly positioned and settled by now.

Acknowledgments

The writers would like to thank all the Zootechnic Veterinary Department of Selçuk University staff and UD. Saputra Jaya for their valuable contributions to the study.

Financial Support

This research received no grant from any funding agency or sector.

Conflict of Interest

The writers stated there are no conflicts of interest. This investigation is a section concisely of Sigid PRABOWO Ph.D. thesis.

Author Contributions

SP carried out the experiments comprised planned, carried out the simulations, contributed to sample preparation, and wrote the manuscript. MG contributed to the interpretation and analysis of the manuscript.

Data Availability Statement

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

Ethical Statement

This study was carried out after the animal experiment was approved by Airlangga University Local Ethics Committee (Decision number: 3.KE.137.12.2021).

Animal Welfare

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

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DOI: 10.33988/auvfd.1251983

Micellized conjugated linoleic acid as an immune modifier feed additive for suckling calves

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ARTICLE INFO

Article History
Received : 02.11.2023
Accepted : 08.03.2024
DOI: 10.33988/auvfd.1383903

Keywords
Calf
Conjugated linoleic acid
Emulsion
Immune response
Healthy milk

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How to cite this article: Khalili B, Abdi-Benemar H, Seifdavati J, Zamanloo MR (2024): Micellized conjugated linoleic acid as an immune modifier feed additive for suckling calves. Ankara Univ Vet Fak Derg, 71 (4), 445-452. DOI: 10.33988/auvfd. 1383903.

ABSTRACT

This study attempted to assess the effects of micellized conjugated linoleic acid (CLA) as a feed additive for suckling calves on their growth performance and blood metabolic, oxidative, and immune parameters. Forty-eight Holstein calves were divided among four experimental groups (12 calves/treatment), including 1) calves with no CLA supplementation (CON), 2) calves supplemented with 1 gr CLA/d as micellized form by dissolving 5 mL/d of a CLA-contained emulsion in milk (CLA1), 3) calves supplemented with 2 gr CLA/d as micellized form by dissolving 10 mL/d of a CLA-contained emulsion in milk (CLA2), and 4) calves supplemented with 3 gr CLA/d as micellized form by dissolving 15 mL/d of a CLA-contained emulsion in milk (CLA3). Calves in the CON group received 10 mL of the emulsion medium with no CLA. Feeding micellized CLA via whole milk resulted in a linear increase in blood glucose concentration on day 40 ($P=0.04$) and total protein ($P<0.01$), albumin ($P<0.01$), and triglyceride ($P=0.02$) concentrations on day 20 of the experiment. The inclusion of micellized CLA resulted in a linear decrease ($P<0.05$) in blood malondialdehyde concentration at both periods but had no effect on blood total antioxidant status. On day 20, tumor necrosis factor-alpha level in the blood of suckling calves exhibited a quadratic effect with micellized CLA inclusion; however, interleukin-6 concentration was not affected. The use of 3 g per day of micellized CLA, via daily milk has the potential to reduce inflammation in young calves during the pre-weaning period.

Introduction

Calf rearing is very challenging and the highest mortality in dairy cattle occurs from birth to the weaning period. Neonatal calves are born with no active immunity and rely only on passive immunity that comes from colostrum to protect them from environmental pathogens. Due to their higher susceptibility to both disease and death, improving the health and nutrition of dairy calves are important factors for maintaining viable and sustainable dairy farms. A better understanding of the immune system of calves will result in lower antibiotic use and the production of high-quality replacement heifers for the dairy herd (2, 25).

Conjugated linoleic acids (CLA), a class of LA isomers characterized by a conjugated double bond, are found mainly in milk and meat from ruminant animals due to their rumen microbial action on the LA. Cis-9, trans-11 CLA and trans 10, cis-12 CLA are the most important isomers, among CLA isomers, with some functional effects on human and animal health including body fat and serum cholesterol reduction, antioxidant activity, anticarcinogenic and immune-enhancing effects (6, 29). CLA feed supplements have been studied extensively in dairy cattle nutrition due to their effects on depressing milk fat, enhancing milk yield, and alleviating negative energy balance in early lactating dairy cows (6,

reducing monocyte apoptosis (3). Maternal CLA supplementation has been reported to influence metabolic changes and factors related to neonatal insulin response and had some effects on the growth of intestinal mucosa in calves (29).

Emulsions are the mixture of two or more liquids that are naturally immiscible together and mix with each other by the process called emulsification. Emulsion systems have been used widely to deliver lipophilic bioactive compounds in food, cosmetic, and pharmaceutical products (22). Oil-in-water emulsions are the best medium for incorporating and delivering lipophilic nutraceuticals in milk and dairy products for domestic animals and humans (14). CLA and other lipophilic bioactive compounds cannot be administrated directly to suckling calves via milk due to their insolubility in milk. Naturally, milk is an oil-in-water emulsion and therefore, the use of emulsion systems to deliver lipophilic bioactive compounds via mixing in milk may be the best administration route.

There are efforts on micellizing CLA for use in human nutrition (31, 35, 19). However, there is no report focusing on feeding CLA in a micellized form to suckling calves. The present study aimed to evaluate an emulsion-based delivery system for administering CLA as a bioactive lipophilic compound via milk for suckling calves. This study was an attempt to assess the effects of micellized conjugated linoleic acid as a feed additive for suckling calves on their growth performance, and blood metabolic, oxidative and immune parameters.

Materials and Methods

Emulsion preparation and evaluation: Emulsion preparation was based on the method described by Asghari et al. (2). In brief, 25 g of lecithin (Ehsan supplying group, Ardabil, Iran) was mixed with 200 mL of CLA oil (CLA oil 80%, 39.9% cis-9, trans-10 CLA, 39.4% cis-10, trans 12; CLA Suzhou Vit-ajoy Bio-Tech CO., Ltd. China) on a heater (55 ºC) equipped with a magnetic mixer until full mixing to make the lipid portion. To make the aqueous portion, 2 g of gum Arabic (Sigma-Aldrich, CAS Number, 9000-01-5) and 20 g of whey protein (Ehsan supplying group, Ardabil, Iran) were dissolved in 500 mL distilled water until full mixing on a laboratory heater (40 ºC). Then, the lipid portion was added slowly to the aqueous portion while mixing with a magnetic stirrer. After full mixing, distilled water was added to the emulsion to have a 1000 mL final volume. To avoid microbial contamination, sodium benzoate (0.1% w/w, 98.5% extra pure, Dr. Mojallali Industrial Chemical Complex Co.) and potassium sorbate (0.1% w/w, 99%, Mobtakeran Chemistry, Tehran, Iran) was added to the mixture. Butylated hydroxyl toluene (0.2% w/w, extra pure, supplied by CCIRAN Co.) was used as an antioxidant agent to protect the CLA fatty acids from oxidation. Thereafter, the emulsion was further mixed with a blender at 8000 rpm for 10 minutes and homogenized with a high-speed lab homogenizer (IKA T25, Germany) at 20000 rpm for 10 minutes. The final product, containing 200 g of CLA per L of the emulsion, was stored in a 1 L dark polyethylene bottle. A CLA-free emulsion was made and used for the control treatment.

After 3 weeks of storage at room temperature, the dynamic light scattering technique (DLS) was used to measure the droplet size of the micellized CLA (Nanopartica SZ-100V2 Series, HORIBA Scientific Instrument, Kyoto, Japan) and electrophoretic light scattering technique was applied to assess the surface charge density (Zeta potential) of the prepared emulsion droplets in triplicates. Atomic force microscopy (Core AFM Nano surf, Liestal, Switzerland) was used to take an image of the emulsion droplets (Figure 1). The samples were diluted to 1:1000 with distilled water before the measurements.

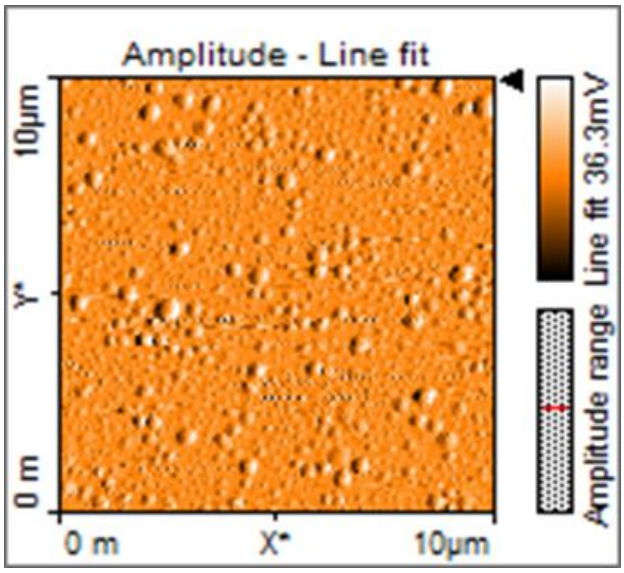

Figure 1. Atomic force microscopy image of the prepared emulsion sample.

Experimental design and animal management: 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.1402.019) 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. Forty-eight male Holstein calves (6-day old; initial body weight of 39.43 ± 4.0 kg) were selected from farm-2 calf rearing station of (Parsabad, Ardabil, Iran) and assigned randomly to four groups (calves/treatment) in a completely randomized design. The experimental groups were: 1) the control

group with no CLA supple-mentation (CON), 2) calves supplemented with 1 g of micellized CLA/d by dissolving 5 mL/d of the prepared emulsion in milk (CLA1), 3) calves supplemented with 2 g of micellized CLA/d by dissolving 10 mL/d of the prepared emulsion in milk (CLA2) and 4) calves supplemented with 3 g of micellized CLA/d by dissolving 15 mL/d of the prepared emulsion in milk (CLA3). Calves in the CON group received 10 mL of the CLA-free emulsion. The calculated amount of the emulsions was mixed with morning milk meal immediately before milk feeding for the first 42 days of the experiment and weaning was done on d 56.

Calves received 4 kg Colostrum after birth for 3 days and thereafter, a step-rise and step-down milk feeding program was applied for feeding calves. Briefly, 4 kg of whole milk was fed daily for the first 2 weeks, 6 kg from the third to sixth weeks, 4 kg for the seventh week in two equal meals at 8:00 and 18:00 and 2 kg of milk only for a morning meal for the eighth week to the weaning at day 56 (56 days). Milk feeding was performed by using plastic buckets and free access to a starter diet and water from d 7 was allowed. Ground chopped alfalfa hay (10%, w/w) was mixed with the starter diet from d 20. Calves were reared in individual pens (1 × 2.5 m) that were cleaned and re-bedded daily with dry straw. The ingredients and chemical composition of the starter feed, alfalfa hay, and milk (dry matter basis) are shown in Table 1.

Table 1. Ingredients and chemical composition of the starter feed, alfalfa hay and milk (dry matter basis).

	Starter feed	Alfalfa hay	Milk
Ingredients, g/kg			
Maize grain, grounded	350	-	-
Barley grain, grounded	220	-	-
Wheat bran	40	-	-
Soybean meal	360	-	-
Salt	5	-	-
Limestone	10	-	-
Vit and Min premix[1]	10	-	-
Dicalcium phosphate	5	-	-
Chemical composition, g/kg			
Dry matter	910	878	122
Crude protein	185	142	30.1
Ether extract	275	25.5	33.5
Neutral detergent fiber	166	561	-
Acid detergent fiber	81	380	-
Calcium	6.2	15	-
Phosphorus	5.2	4	-

[1] 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/kg; Fe, 100 mg; Zn, 16500 mg/kg; Mn, 9000 mg; I, 120 mg/kg; Co, 90 mg/kg; Se, 90 mg/kg.

Sampling, data collection, and chemical analysis: Growth performance and body weight gain were done by serial weighing of all calves on d 14, 28, and 56 (weaning day) of the experiment. Daily solid feed intake was measured by recording daily feed offered and feed refused. The feed conversion ratio (FCR) was calculated by dividing daily feed intake by weight gain without considering the consumption of milk. On d 20 and 40, two blood samples were taken 3-4 hours after the morning meal from the jugular vein to obtain plasma and serum samples. Plasma and serum samples were taken by 15 min centrifugation at 3500 × g at 4°C and frozen at -20°C. On analysis day, plasma samples were thawed at room temperature and analyzed for blood metabolites such as glucose, triglycerides, cholesterol, total protein, albumin, and blood urea nitrogen (BUN) by using Pars Azmoon commercial kits (Pars Azmoon Co., Tehran, Iran). Ranbut assay was used for determining beta-hydroxybutyric acid (BHBA) concentration in serum samples (Ranbut assay, Randox Laboratories, Crumlin, UK). Liver enzymes including aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were measured in serum samples by using Pars Azmoon commercial kits. Total antioxidant status (TAS) and malondialdehyde (MDA) concentrations were determined in serum samples by Randox assay (Randox Laboratories, Crumlin, UK) and the method described by Moore and Robert (1998), respectively. Bovine tumor necrosis factor-alpha (TNF-α) and bovine interleukin-6 ELISA kits (Shanghai Crystal Day Biotech Co., LTD. Shanghai, China) were used for determining blood TNF-α and IL-6 concentrations, respectively.

Chemical analysis of starter and alfalfa hay was done to determine the contents of dry matter, crude protein, ether extract by methods AOAC (1), neutral detergent fiber (NDF), and acid detergent fiber (ADF) (30) and calcium and phosphorous contents (AA-670, Shimadzu, and Tokyo, Japan). Chemical analysis of milk samples was performed by Delta dairy analyzer (CombiScope FTIR 600/300 Hp - Dairy Analyser, Delta Instruments, Drachten, and Netherland).

Statistical Analysis: Performance data (daily gain and feed intake) were analyzed as repeated measurements by using the Mixed Procedure of SAS (13) as a completely randomized design. CLA feeding effect as the treatment effect, time effect, and CLA and time interaction effect were considered in the statistical model as fixed effects and individual effects of calves in treatments as random effects. Based on smaller Schwarz Bayesian criteria, the UN covariance structure was applied for the repeated measures analysis (13). The interaction between CLA feeding and time was not significant and therefore was excluded from the model. The effect of time was significant for all performance data ($P<0.01$). The orthogonal

polynomial CONTRAST statement of SAS (13) was used for linear and quadratic contrasts among experimental groups. Statistical analysis of test-day data including blood parameters was performed by GLM procedure of SAS with the effect of CLA feeding as a fixed effect. The covariate effect of the body weight of calves at the onset of the experiment was included in the statistical model. Significant effects were declared at $P \leq 0.05$ and $0.05 < P < 0.10$ was discussed as the significance trend.

Results

Emulsion characteristics: Emulsion characteristics measured three weeks after preparation are shown in Table 2. Based on dynamic light scattering (DLS) data, the micellized CLA had 423 nm mean particle size and 100 percent stability after two months of storage at room temperature. Consistent with high stability index, the micellized CLA had a high negative zeta potential values (-69.5 mV mean zeta potential).

Table 2. The CLA contained emulsions characteristics.

	Droplet size (nm)[a]	Z-average (nm)	Zeta potential (mV)	Stability (%)
Mean ± SD	423 ± 303	1109 ± 676	-69.5 ± 3.5	100[b]

[a] Measured by dynamic light scattering technique.
[b] measured as the percentage of creaming layer formed after two months.

Calves' growth performance: The final body weight of the claves was not affected by feeding the micellized CLA (Table 3). Supplementation with micellized CLA did not affect the average daily gain of calves on d 1−14. However, orthogonal contrast between the CON group and calves that received the micellized CLA showed a tendency for increased average daily gain on 14-28 of the experiment (P=0.09). The total average daily gain of calves was not influenced by increasing doses of the micellized CLA. Feeding different levels of the micellized CLA did not affect the daily feed intake of calves. However, a tendency for improved feed conversion ratio (FCR) was noted for calves supplemented with the micellized CLA compared with CON calves (P=0.07).

Blood metabolites: Feeding micellized CLA via milk increased the blood concentration of glucose on d 40 of the experiment (L; P=0.04) and CLA-fed calves also had higher blood glucose levels compared to the CON calves (P=0.03, Table 4). Blood concentrations of cholesterol, urea, and beta-hydroxybutyric acid were not affected by feeding micellized CLA. Total protein (P=0.001), albumin (P=0.002), and triglyceride (P=0.021) concentrations increased linearly on d 20 with increasing doses of the micellized CLA, however, after 40 days, their blood metabolite concentrations were similar. Blood levels of AST and ALT were not affected by CLA supplementation.

Blood oxidative and immune markers: The blood total antioxidant status (TAS) of the calves was not affected by feeding the micellized CLA in milk. However, CLA supplementation reduced blood malondialdehyde concentration (MDA) in d 20 (L; P=0.01) and d 40 (L; P=0.03) of the experiment. On d 20, tumor necrosis factor-alpha (TNF-α) level in the blood of suckling calves decreased (L; P=0.001) with micellized CLA inclusion, however, interleukin-6 (IL-6) concentration was not affected (Table 5).

Table 3. Effects of feeding micellized CLA on feed intake and growth performance of suckling calves.

	CON	CLA1	CLA2	CLA3	SEM	P-value		
						O	L	Q
Initial weight (kg)	39.4	39.3	39.5	39.3	1.62	0.982	0.986	0.969
Final weight (kg)	87.1	90.3	89.1	91.6	2.14	0.211	0.216	0.885
Feed intake (g/d)								
1-14 days	168.5	175.6	142.1	192.2	15.76	0.935	0.597	0.183
14-28 days	454.8	496.1	494.6	419.8	44.83	0.330	0.599	0.357
Total	881.7	835.8	818.4	856.0	26.4	0.420	0.773	0.238
ADG (g/d)								
1-14 days	401.7	471.2	435.7	480.3	37.21	0.176	0.247	0.744
14-28 days	539.3	548.2	503.5	575.0	51.36	0.090	0.122	0.715
Total	734.6	784.6	762.5	804.8	35.02	0.234	0.241	0.914
Total FCR[2]	1.18	1.09	1.08	1.07	0.046	0.068	0.138	0.370

CON = control group with no CLA supplementation, CLA1= calves received 1 g CLA/d; CLA2 = calves received 2 g CLA/d; CLA3= calves received 3 g CLA/d. ADG = average daily gain. Total FCR = total feed conversion ratio calculated as total feed intake divided by total ADG. O = orthogonal contrast between the control group and CLA receiving groups, L = linear effect of feeding emulsified CLA, Q = quadratic effect of feeding emulsified CLA. SEM = standard error of the mean.

Table 4. Effects of feeding micellized CLA on blood metabolites of suckling calves.

	CON	CLA1	CLA2	CLA3	SEM	P-value		
						O	L	Q
Glucose (mg/dl)								
d 20	117.0	99.3	114.4	110.8	9.25	0.416	0.939	0.448
d 40	57.2	68.1	82.3	73.4	6.77	0.035	0.048	0.155
Cholesterol (mg/dl)								
d 20	95.1	93.8	101.4	111.9	7.73	0.412	0.601	0.911
d 40	104.6	103.9	85.0	105.6	10.29	0.591	0.733	0.308
Triglyceride (mg/dl)								
d 20	14.6	15.5	18.9	19.8	1.714	0.095	0.021	1.000
d 40	22.0	27.0	22.7	28.9	3.179	0.263	0.260	0.856
BUN (mg/dl)								
d 20	36.6	36.5	42.1	42.0	3.72	0.412	0.203	1.000
d 40	10.6	17.2	21.6	12.1	2.95	0.073	0.509	0.011
Albumin (g/dl)								
d 20	3.4	3.6	4.3	4.2	0.20	0.011	0.002	0.498
d 40	3.6	3.6	3.7	3.5	0.08	0.777	0.772	0.773
Total protein(g/dl)								
d 20	6.5	6.7	7.1	7.4	0.14	0.001	0.001	0.760
d 40	7.2	7.0	6.9	6.9	0.21	0.221	0.224	0.666
BHBA (mg/dl)								
d 20	0.14	0.11	0.11	0.12	0.02	0.135	0.273	0.338
d 40	0.30	0.27	0.39	0.27	0.04	0.849	0.904	0.262
AST (U/ml)								
d 20	23.0	23.0	20.5	24.3	3.21	0.911	0.931	0.562
d 40	64.0	58.5	63.3	63.4	2.93	0.501	0.824	0.343
ALT (U/ml)								
d 20	26.1	25.5	28.1	25.4	3.63	0.961	0.982	0.772
d 40	20.7	21.6	20.8	20.3	0.90	0.879	0.579	0.420

CON = control group with no CLA supplementation, CLA1= calves received 1 g CLA/d; CLA2 = calves received 2 g CLA/d; CLA3= calves received 3 g CLA/d. BHBA = beta-hydroxybutyric acid, BUN = Blood urea nitrogen, AST = Aspartate aminotransferase, ALT = Alanine aminotransferase. O = orthogonal contrast between the control group and CLA receiving groups, L = linear effect of feeding emulsified CLA, Q = quadratic effect of feeding emulsified CLA. SEM = standard error of the mean.

Table 5. Effects of feeding micellized CLA on blood oxidative and immune parameters of suckling calves.

	CON	CLA1	CLA2	CLA3	SEM	P-value		
						O	L	Q
TAS (mmol/l)								
d 20	0.55	0.60	0.59	0.66	0.06	0.358	0.249	0.873
d 40	0.56	0.55	0.54	0.52	0.03	0.543	0.280	0.713
MDA (mmol/l)								
d 20	2.29	2.38	1.49	1.30	0.16	0.006	0.001	0.396
d 40	2.83	3.03	2.09	2.26	0.26	0.23	0.035	0.963
TNF-α (ng/l)								
d 20	183.5	90.8	139.4	116.6	5.80	0.001	0.001	0.001
IL-6 (ng/l)								
d 20	31.8	38.9	35.3	38.5	3.02	0.115	0.242	0.512

CON = control group with no CLA supplementation, CLA1= calves received 1 g CLA/d; CLA2 = calves received 2 g CLA/d; CLA3= calves received 3 g CLA/d. MDA = Malondialdehyde, TAS = Total antioxidant status, TNF-α = Tumor necrosis factor-alpha, IL-6 = Interleukin-6. O = orthogonal contrast between the control group and CLA receiving groups, L = linear effect of feeding emulsified CLA, Q = quadratic effect of feeding emulsified CLA. SEM = standard error of the mean.

Discussion and Conclusion

The incorporation of lipid-based nutrients and bioactive components in aqueous-based foods and drugs is faced with some challenges due to their hydrophobic nature and water insolubility. The application of emulsion-based delivery systems is the best route to overcome the problem of administering lipophilic bioactive compounds such as conjugated linoleic acid and other functional fatty acids, herbal essential oils, fat-soluble vitamins, etc (2, 22). Due to increased digestion, absorption, stability, and bioavailability, emulsion-based delivery systems are used not only for delivering lipophilic bioactive compounds but also for delivering water-soluble micronutrients such as vitamin C (9).

Milk is an oil-in-water emulsion with its fat globules dispersed in an aqueous medium along with other nutrients such as protein, carbohydrates, vitamins, and minerals (2). In the present study, CLA, as a lipidic bioactive molecule, was incorporated in an oil-in-water emulsion to deliver via milk to suckling calves. Milk fat micelles (globules) had a wide range of diameter from 100 to 10000 nm with an average diameter of around 4000 nm (34). The micellized CLA droplet size was similar to milk fat micelles and their incorporation into calves' daily milk intake had no digestive problems during the experiment period. In addition, the droplets of the micellized CLA had a large negative surface charge (-69.5 mV) that shows excellent stability. Zeta-potential values of greater than ±60 mV are classified as having excellent stability and emulsions with values greater than ±30 mV are classified as having good stability (12).

CLA isomers, including cis-9, trans-11 and cis-10, trans-12, have been studied extensively due to their potential effects on immune system modulation, disease prevention, lipid and glucose metabolism, and body composition alteration in animals (6, 23, 24). In this study, feeding CLA isomers as the micellized form to suckling calves did not improve daily weight gain which resulted in better FCR values for supplemented calves compared to CON ones. This observation was consistent with their higher blood glucose, albumin, and total protein concentrations and lower tumor necrosis factor-α (TNF-α) levels. The highest and lowest FCR values were recorded for calves in the CON and CLA3 groups, respectively. Increased blood glucose level by feeding micellized CLA observed in the present study is consistent with previous results reported by Moloney et al. (17) and Risérus et al. (21). The effect of CLA on blood glucose was attributed to reduced insulin sensitivity (12). Cantwell et al. (4) reported that incubating hepatocytes with mixed CLA isomers resulted in increased protein synthesis and this report is consistent with our results on the positive effects of CLA supplementation on blood protein parameters with higher albumin and total protein concentrations after 20 days of feeding the micellized CLA. Previous studies in animals and humans have reported CLA effects on energy, lipid, and glucose metabolism (7, 8, 11, 33). Increased triglycerides (TG) concentration in the blood of CLA-received claves could have been caused by reduced insulin sensitivity (17), increased lipolysis (7), and induction of adipocyte apoptosis (8). Increased TG level with micellized CLA supplementation, observed in the present study, was in agreement with some earlier works (7, 33).

In farm animals, stimulated immune function coincides with decreased production performance due to inflammatory functions and conversely, increased growth rate and milk production suppresses the immune system (5, 20). Inflammatory cytokines are produced when the immune system is stimulated to increase the proliferation of immune cells to attack pathogens. However, these cytokines have some extra catabolic effects on body tissues and can result in a decrease in feed intake and the breakdown of skeletal muscle (32). CLA has been recognized as an anti-inflammatory compound that can decrease the production of pro-inflammatory cytokines and increase the production of anti-inflammatory cytokines (27). Miller et al. (16) reported that feeding mice with CLA prevented anorexia and weight loss induced by bacterial lipopolysaccharide injection. Therefore, decreased blood TNF-α level by CLA supplementation, in this study, is consistent with other reports (16, 27) and can be attributed to the suppressive effect of the micellized CLA fed to the calves. IL-6 is a pro-inflammatory cytokine that causes the induction of the hepatic acute phase protein response and stimulation of B- and T-cell responses (15). TNF-α, produced by white blood cells, especially macrophages, is one of the most important pro-inflammatory cytokines and is involved in vasodilatation and edema formation, and contributes to oxidative stress in sites of inflammation (36). Therefore, a reduction in TNF-α production may be associated with higher cell integrity and lower tissue peroxidation. This matter may explain the lower malondialdehyde (MDA) concentration in the blood of calves that received the emulsified CLA supplement. MDA is a good marker for the tissue oxidative stress produced from the peroxidation of polyunsaturated fatty acids. The hypothesis that the effects of CLA could be mediated by preventing the production of some pro-inflammatory cytokines, particularly TNF-α and the activation of peroxisome proliferator-activated (PPAR) receptors have been proposed in some previous studies (10, 23, 28).

This study was an attempt to use an emulsion-based delivery system to make CLA soluble in an aqueous medium like milk and to design a CLA feed additive for suckling calves. The application of this method introduces a new administration route for feeding lipophilic nutrients like CLA for suckling calves. The micellized CLA fed to

the calves was effective in causing metabolic changes such as enhancing blood glucose and protein and decreasing blood TNF-α levels. Based on the current results, micellized CLA can be introduced as an anti-inflammation feed additive for suckling calves, especially for recovery periods after infectious diseases. The results suggest that 3 g per day of the micellized CLA can be used for suckling calves via daily milk to maximize its positive effects.

Acknowledgements

Authors are grateful to the University of Mohaghegh Ardabili for their help.

Financial Support

This research received no grant from any funding agency/sector.

Conflict of Interest

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

Author Contributions

BK, HAB, JS designed the experiment, carried out the research and laboratory analysis. The re-search was conducted under the supervision of HAB, JS, and MRZ. The final version of the manuscript was submitted by agreement of all authors.

Data Availability Statement

All data and materials are available.

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.1402.019) and an cooperation contract was signed between University of Mohaghegh Ardabili and Moghan Agro-Industrial and Animal Husbandry company for the participation of the animals.

Animal Welfare

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

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The effect of dairy farm size on the economic structure and feed consumption: A case study of the Aegean Region

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ARTICLE INFO

Article History
Received : 05.07.2023
Accepted : 20.02.2024
DOI: 10.33988/auvfd.1332777

Keywords
Aegean Region
Dairy farming
Economic structure
Feed consumption

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How to cite this article: Güler D, Saner G (2024): The effect of dairy farm size on the economic structure and feed consumption: A case study of the Aegean Region. Ankara Univ Vet Fak Derg, 71 (4), 453-461. DOI: 10.33988/auvfd.1332777.

ABSTRACT

Dairy farming has an important role in agriculture and livestock sector in Türkiye. Cow's milk production constitutes 92.11% of the total milk production in Türkiye. The purpose of this study is to demonstrate the effect of scale size on the economic structure and feed consumption in dairy farms. The research data were obtained from face-to-face surveys conducted with 147 farmers in the Aegean Region. In the study, the farms were divided into four groups based on the number of cows: 5-15 cows, 16-25 cows, 26-40 cows, and 41 cows and above. The total variable cost per livestock unit in the 4th group farms was 26.07% less than the farms in the 1st group. The total production cost per livestock unit in the largest farm group is 25.81% less than that of the smallest farm group. The research findings indicate that large-scale farms take advantage of economies of scale, resulting in lower cost per livestock unit. Additionally, it was observed that as farm size increases, the feed conversion ratio also increases. As farms grow larger, they often have access to economies of scale, better management practices, and improved infrastructure.

Introduction

Dairy farming has an important role in agriculture and livestock sector in Türkiye. The total amount of milk produced was 23.20 million tons in 2021 and cow's milk made up 92.11% (21.37 million tons) of the total milk production in Türkiye. 2.86% of the global cow's milk production, which was 746.06 million tons in 2021, was realized in Türkiye (12). Cow's milk production value in 2020 was 46.82 billion Turkish Liras (TL) (6.68 billion US Dollars) in Türkiye. This value constitutes 8.51% of total agricultural production value in Türkiye. In addition, cow's milk production value made up 43.11% of the total animal product value (36).

İzmir and Manisa, where the study was conducted, are located in the Ege Region at level-1 sub-region according to the Statistical Regions Classification. In 2019, the Aegean Region of Türkiye produced 3.75 million tons of cow's milk, which accounted for 18.05% of the total production of 20.78 million tons. Within the Aegean Region, 37.81% (1.42 million tons) of cow's milk was produced in İzmir and Manisa (36).

To determine feed efficiency has significant importance in the economic analysis of dairy farming. Feed efficiency (FE) is an essential parameter used to evaluate the productivity of livestock, especially in the context of milk production. It provides valuable insights into how effectively animals convert the feed they consume, specifically the dry matter, into milk or other animal products. Feed efficiency is crucial for dairy farming because it directly impacts the profitability and sustainability of their operations. Feed efficiency is typically measured as the amount of milk produced per unit of dry matter intake. This parameter allows comparing the revenue generated from the milk sale based on the amount of dry matter ingested by the animal (1, 16).

There are numerous studies available that assess the economic structure of dairy farms in Türkiye (6, 7, 9, 13, 14, 18, 20, 22, 25, 27, 28, 37). In some of these studies,

along with the economic structure, the feed conversion ratio has also been presented.

The primary objective of this study is to examine the economic structure of dairy farms based on the farm size. Besides that, the study presents feed efficiency and feed conversion ratio according to the farm size scale.

In line with these objectives, the following hypotheses were tested in the research: (i) the demographic characteristics of the farmers differ according to the farm size; (ii) the existence of family and paid labor differs according to the farm size; (iii) the existence of property, rented, and common land differs according to the farm size; (iv) milk yield, lactation period, and annual milk yield per cow differs according to the farm size; and (v) the variable costs per livestock units differs according to the farm size.

Materials and Methods

The main material of this study consists of data obtained from face-to-face surveys conducted with dairy cattle farmers in İzmir and Manisa in Türkiye. İzmir and Manisa are located in the Aegean Region, which ranks first in cow's milk production among the 12 regions designated by Turkish Statistical Institute (TURKSTAT). Approximately 6.83% of the cow's milk produced in Türkiye was produced in İzmir and Manisa in 2019 (36).

The number of registered farmers (3175) in İzmir and Manisa Cattle Breeders' Association has been considered as the main population, and the sample size has been calculated. Accordingly, the number of farmers to be interviewed is calculated as 143, with a 95% confidence interval and an 8% margin of error. The sample size has been increased by 4 to reach 147. When calculating the sample size, the formula for proportional sample size has been utilized (23).

$$n = \frac{Np(1-p)}{(N-1)\sigma_{px}^2 + p(1-p)}$$

In the formula, n represents the sample size; N is the total number of dairy farmers, and p indicates the proportion of farmers that will be included in the sample. σ_{px}^2 represents the variance of the proportion. In order to achieve the maximum sample size in the calculation, "P" has been set as 0.50, and (1-p) is also taken as 0.50.

The surveys were conducted with dairy farmers in the research area in 2018. The survey data were analyzed using SPSS statistical software. The data were initially evaluated using basic statistical methods such as percentages and means, and the results were presented in tables. Differences between farm groups were tested statistically. The Mann-Whitney U test was applied to compare two groups for continuous variables that did not follow a normal distribution and had heterogeneous variances. The Kruskall-Wallis test was used to compare more than two groups for the same type of variables.

The farms were divided into four groups based on the number of cows: 5-15 cows, 16-25 cows, 26-40 cows, and 41 cows and above. In order to enable comparisons between the groups, it was important to have a minimum of 30 farms in each group and to ensure that the percentage distribution of farms was relatively similar for homogenous distribution. There were 32 farms in the 1st group, 46 farms in the 2nd group, 36 farms in the 3rd group, and 33 farms in the 4th group.

In the study, in order to evaluate the animal populations in a homogeneous manner, the animal populations in the farms were converted into livestock units (LU). The conversion coefficients for livestock units were taken into account as 1.00 for cow, 1.40 for bull, 0.70 for heifer, 0.50 for calf and 0.20 for calf less than six months old (11).

The male labor unit (MLU) was taken into account in determining the current labor force in farms. Accordingly, the calculation is as follows: 0.50 for males and females in the 7-14 age group, 1.00 for males and 0.75 for females in the 15-49 age group, and 0.75 for males and 0.50 for females who are 50 years and older (26).

Feed efficiency is a parameter frequently used in animal husbandry and is also called dairy efficiency. FE is calculated by dividing the amount of milk expressed by the amount of dry matter consumed (19).

$$FE = \frac{Daily\ milk\ yield\ of\ cow\ (kg)}{Daily\ dry\ matter\ consumption\ (kg)}$$

Feed conversion ratio (FCR) is the gross production value (GPV) obtained per value of feed consumed. FCR was calculated by the following formula in this study (28):

$$FCR = \frac{Gross\ Production\ Value}{Value\ of\ Feed\ Consumed} x\ 100$$

To calculate the gross profit margin, total variable expenses have been subtracted from the gross production value, and the result has been divided by the gross profit.

Results

Information about the Farms: The average age of the farmers is 47.12 years, the duration of education is 7.29 years, and the number of family members is determined to be 3.61 individuals. Furthermore, the experience duration in crop production is 25.53 years, while the experience in dairy farming is found to be 22.75 years.

Although there is no difference in terms of age, number of individuals in the family, plant production experience and dairy farming experience by the size of the farm, it has been determined that there is a difference

between the groups in terms of education level (P<0.05). The period of education of the farmers in large-scale farms is longer than the farmers in small-scale farms (Table 1).

The existence of family labor force in the farms has been calculated in terms of male labor units (MLU), and the average is determined to be 2.73 MLU. As for paid labor, there are 0.37 MLU employed in the farms.

It has been determined that the existence of paid labor force (P<0.01) and total labor force (P<0.01) differs by size of farm size. As the size of the farm increases, the existence of paid labor force and total labor force also increase (Table 2).

The total land of the farms is an average of 76.80 decares. Within the total land, the property land is 51.88 decares, the rented land is 24.51 decares, and the common land is 0.41 decares. The average number of parcels per farm is 4.79.

According to farm size, there is a statistical difference between the farms in terms of property (P<0.01), rented (P<0.01), and total land (P<0.01), and the number of parcels (P<0.01). It has been determined that as the size of the farm increases, the number of parcels of land increases as well as the property, rented and total land (Table 3).

Table 1. Demographic characteristics of farmers by farm size (head).

Characteristics	1. Group (32) 5-15 Head		2. Group (46) 16-25 Head		3. Group (36) 26-40 Head		4. Group (33) 41≥ Head	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Age (year)	46.94	9.43	47.37	11.38	46.50	12.16	47.64	10.07
Education level (year)*	6.13	2.12	7.02	2.84	7.33	2.70	8.73	4.13
Number of individuals in the family	3.37	1.01	3.70	1.13	3.50	1.08	3.85	1.15
Plant production experience (year)	22.53	12.52	26.72	12.57	27.25	12.15	24.91	11.60
Dairy farming experience (year)	21.59	10.20	23.65	13.08	23.17	12.63	22.15	11.04

* P<0.05.

Table 2. The existence of labor force by farm size (Male labor unit).

Labor Force	1. Group (32) 5-15 Head		2. Group (46) 16-25 Head		3. Group (36) 26-40 Head		4. Group (33) 41≥ Head	
	Person	%	Person	%	Person	%	Person	%
Family labor force	2.75	100.00	2.74	97.51	2.65	93.97	2.80	67.31
Paid labor force **	-	-	0.07	2.49	0.17	6.03	1.36	32.69
Total labor force**	2.75	100.00	2.81	100.00	2.82	100.00	4.16	100.00

** P<0.01.

Table 3. The existence of land by farm size (decares).

Land Ownership	1. Group (32) 5-15 Head			2. Group (46) 16-25 Head			3. Group (36) 26-40 Head			4. Group (33) 41≥ Head		
	Mean	SD	%	Mean	SD	%	Mean	SD	%	Mean	SD	%
Property**	14.87	16.69	61.73	45.04	65.07	72.75	40.56	41.90	62.69	109.67	275.82	67.74
Rented **	9.22	12.40	38.27	16.72	32.51	27.01	23.22	38.60	35.89	51.61	51.62	31.88
Common	-	-	-	0.15	1.03	0.24	0.93	5.50	1.42	0.61	3.48	0.38
Total **	24.09	21.45	100.00	61.91	81.33	100.00	64.71	51.47	100.00	161.89	306.13	100.00
Number of parcels **	3.42			4.19			5.83			5.82		

** P<0.01.

Table 4. Feed conversion ratio by farm size (head).

Incentive Status	Variables	1. Group (32) 5-15 Head	2. Group (46) 16-25 Head	3. Group (36) 26-40 Head	4. Group (33) 41≥ Head
Without Incentive	GPV	159188	277194	427752	1098972
	Value of Feed Consumed	108277	180254	266130	652892
	FCR	147.02	153.78	160.73	168.32
With Incentive	GPV	162021	281736	435409	1120168
	Value of Feed Consumed	108277	180254	266130	652892
	FCR	149.64	156.30	163.61	171.57

Table 5. Milk yield (kg), lactation period (day) and annual milk yield per cow (kg/year) by farm size.

Variables	1. Group (32) 5-15 Head		2. Group (46) 16-25 Head		3. Group (36) 26-40 Head		4. Group (33) 41≥ Head	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Milk Yield	22.06	4.30	23.33	4.11	23.11	2.48	23.79	4.39
Lactation Period	290.68	6.23	292.25	7.37	291.60	3.98	288.50	16.36
Annual Milk Yield	6406.25	1215.45	6817.08	1211.40	6738.85	723.98	6856.02	1301.31

Feed Efficiency and Feed Conversion Ratio: In cows with 150-225 days of milking, feed efficiency is considered normal between 1.4-1.6. However, FE varies depending on many factors and can take values between 1.2-2.0 (16, 19). From the first group to the fourth group, FE was 1.50, 1.54, 1.61 and 1.69, respectively. In addition, FE was calculated as 1.61 across the farms.

Feed conversion ratio was calculated as 162.09 across the farms. It was determined that FCR with incentive calculated by adding milk incentive pay and calf support, increased to 167.09. As the size of the farm increases, the feed conversion ratio also increases. Accordingly, from the first group to the fourth group, FCR was 147.02, 153.78, 160.73 and 168.32, respectively (Table 4).

Production Cost: The daily average milk yield per herd size is 23.10 kg, the lactation period is approximately 290.91 days, and the annual milk yield per cow is 6717.23 kg. There is no statistically significant difference in milk yield, lactation period, and annual milk yield per cow among the groups based on farm sizes (Table 5).

According to the research results, the share of feed cost in variable costs is 87.95%. As the size of the farm increases, the share of concentrate feed in total feed cost increases, but the concentrate feed cost per livestock units decreases. It was determined that there was a significant difference at the level of 1% between the farm groups in terms of feed, veterinary, pharmaceutical and vaccine, artificial insemination, water-electricity, fuel, temporary labor, cleaning materials and salt costs. There was a significant difference at the level of 5% between the farm groups in terms of repair and maintenance costs. Such that, as the size of the farm increases, the cost per livestock units decreases. In order to compare the variable cost between the farm groups, an index was prepared by equating the total variable cost per 1 LU to 100 units in the 1st group. The variable cost of the other groups were evaluated by taking the variable cost of 1st group as a reference. According to the index, while the total variable cost is 100.00 units in the 1st group which includes the small farms, it is 91.10 units in the 2nd group farms, 81.39 units in the 3rd group farms, and 73.93 units in the 4th group farms. The total variable costs per LU in the 4th group farms is 26.07% less than the farms in the 1st group (Table 6). In addition, total variable costs constitute 71.30% of the total production costs.

When the fixed costs of the farms are examined by their sizes, there is a statistical difference between the farm groups in terms of fixed cost components. Among the examined farms, the total fixed costs of the farms in the 4th group are 418.15% higher than those in the 1st group. The most significant factor causing this difference is the depreciation value in the 4th group farms. In terms of the proportion of depreciation in total fixed costs based on farm sizes, it is 55.99% in the 1st group, 54.77% in the 2nd group, 55.88% in the 3rd group, and 52.38% in the 4th group (Table 7).

In the index prepared by assigning a value of 100 units to the first group, the total production cost is found to be 155.50 units in the 2nd group, 214.33 units in the 3rd group, and 513.78 units in the 4th group. Therefore, the total production cost per livestock unit in the largest farm

group is 25.81% less than that of the smallest farm group (Table 8).

The gross profit margins by the size of the farms increase from the first group to the fourth group, with percentages of 21%, 29%, 34%, and 41%, respectively. This indicates that larger farms tend to have higher gross profit margins compared to smaller farms. As the scale of the farm increases, the total variable cost per livestock unit tend to decrease. This situation has enabled larger farms to achieve a higher gross profit margin, despite the lower gross production value per livestock unit in these farms (Table 9).

Table 6. The variable costs by farm size.

Variable Costs	1. Group (32) 5-15 Head			2. Group (46) 16-25 Head			3. Group (36) 26-40 Head			4. Group (33) 41≥ Head		
	Cost (TL)	%	Cost per LU (TL)	Cost (TL)	%	Cost per LU (TL)	Cost (TL)	%	Cost per LU (TL)	Cost (TL)	%	Cost per LU (TL)
Feed**	103964	82.29	5617	171686	87.00	5364	245395	87.36	4853	574670	88.85	4483
Concentrate**	65291	62.80	3527	94531	55.06	2953	130773	53.29	2586	301774	52.51	2354
Roughage**	38674	37.20	2089	77155	44.94	2410	114622	46.71	2267	272896	47.49	2129
Veterinary, Pharmaceutical, Vaccine**	5208	4.12	281	6100	3.09	191	8699	3.10	172	21438	3.31	167
Artificial Insemination**	3479	2.75	188	4418	2.24	191	6647	2.37	131	13082	2.02	102
Water-Electricity**	2377	1.88	128	3416	1.73	107	5323	1.89	105	13554	2.10	106
Fuel**	3535	2.80	191	3518	1.78	110	5178	1.84	102	11433	1.77	89
Temporary Labor**	2592	2.05	140	2828	1.43	88	3615	1.29	71	4811	0.74	38
Repair, Maintenance*	1749	1.38	95	1926	0.98	60	2409	0.86	48	3075	0.48	24
Cleaning Materials**	1461	1.16	79	1469	0.74	46	1633	0.58	32	2431	0.38	19
Bedding	1454	1.15	79	1495	0.76	47	1465	0.52	29	1565	0.24	12
Salt**	512	0.41	28	491	0.25	15	553	0.20	11	756	0.12	6
Total Variable Cost	126334	100.00	6825	197348	100.00	6218	280916	100.00	5555	646815	100.00	5046
Index		-	100.00	-	-	91.10	-	-	81.39	-	-	73.93

* P<0.05 ** P<0.01.

Table 7. The fixed costs by farm size.

Fixed costs	1. Group (32) 5-15 Head		2. Group (46) 16-25 Head		3. Group (36) 26-40 Head		4. Group (33) 41≥ Head	
	Cost (TL)	%	Cost (TL)	%	Cost (TL)	%	Cost (TL)	%
Depreciation**	29085	55.99	43754	54.77	56541	55.88	140986	52.38
Building Depreciation*	9814	33.74	11093	25.35	10213	18.06	26103	18.51
Equipment Depreciation**	7836	26.94	12689	29.00	15455	27.33	36699	26.03
Cow Depreciation**	11435	39.32	19972	45.65	30874	54.60	78183	55.45
Land Rent**	7245	13.95	13115	16.42	14851	14.68	39405	14.64
Interest on Debt **	5973	11.50	9875	12.36	11662	11.52	34128	12.68
General Administrative Expenses**	3790	7.30	5920	7.41	8427	8.33	19404	7.21
Permanent Labor Costs**	-	-	1330	1.67	3967	3.92	28873	10.73
Family Labor Wage	5775	11.12	5764	7.22	5556	5.49	5870	2.18
Cooperative-Association Dues**	74	0.14	123	0.15	181	0.18	476	0.18
Total Fixed Costs	51943	100.00	79881	100.00	101185	100.00	269142	100.00
Index	100.00		153.79		194.80		518.15	

* P<0.05 ** P<0.01.

Table 8. Total production costs by farm size (TL).

Costs	1. Group (32) 5-15 Head		2. Group (46) 16-25 Head		3. Group (36) 26-40 Head		4. Group (33) 41≥ Head	
	Per Farm	Per LU	Per Farm	Per LU	Per Farm	Per LU	Per Farm	Per LU
Total Variable Costs (1)	126334	6825	197348	6165	280916	5555	646815	5046
Total Fixed Costs (2)	51943	2806	79881	2496	101185	2001	269142	2100
Total Production Costs(1+2)	178277	9631	277229	8661	382101	7556	915957	7146
Index	100.00	100.00	155.50	89.92	214.33	78.45	513.78	74.19

Table 9. Gross production value, total variable costs and gross profit by farm size (TL)*.

Variables		1. Group (32) 5-15 Head		2. Group (46) 16-25 Head		3. Group (36) 26-40 Head		4. Group (33) 41≥ Head	
		Per Farm	Per LU	Per Farm	Per LU	Per Farm	Per LU	Per Farm	Per LU
Revenues	**Milk Income**	111243	6010	194834	6087	301879	5970	763035	5953
	Increase in Inventory Value	15760	851	23840	745	31440	622	89080	695
	Calf Income	23740	1283	43915	1372	71360	1411	188375	1470
	Manure Income	8445	456	14605	456	23073	456	58482	456
Without Incentive	**Gross Production Value (1)**	159188	8600	277194	8660	427752	8459	1098972	8574
	Total Variable Costs (2)	126334	6825	197348	6165	280916	5555	646815	5046
	GROSS PROFIT (1-2)	32854	1775	79846	2494	146836	2904	452157	3528
	GROSS PROFIT MARGIN (1-2)/(1)	0.21	0.21	0.29	0.29	0.34	0.34	0.41	0.41
With Incentive	**Gross Production Value (1)**	162021	8753	281736	8801	435409	8610	1120168	8739
	Total Variable Costs (2)	126334	6825	197348	6165	280916	5555	646815	5046
	GROSS PROFIT (1-2)	35687	1928	84388	2636	154493	3055	473353	3693
	GROSS PROFIT MARGIN (1-2)/(1)	0.22	0.22	0.30	0.30	0.35	0.35	0.42	0.42

* During the research period (2018), 1 dollar is approximately 4.40 Turkish Lira.

Discussion and Conclusion

The research results indicate that total variable costs constitute 71.30% of the total production costs. In the study conducted by Semerci (31), the share of total variable costs was determined to be 64.26%. According to the research results, the share of feed cost in variable costs is 87.95%. When previous studies are examined, this rate is found to be 71.30% (4), 87.50% (5), 81.68% (8), 86.30% (20), 72.82% (24), 74.80% (33), and 81.60% (34).

Feed conversion ratio was calculated as 162.09 in this research. In previous researches in Türkiye, it was calculated as 273.17 (6), 194.00 (9), 207.43 (14), 226.00 (17), and 195.72 (31). In addition, feed efficiency was calculated as 1.61 in this research. In another study conducted in İzmir (2), the feed efficiency was calculated as 1.60.

The most produced forage crop among feed crops is corn silage. Additionally, sorghum production is widespread for use as artificial pasture on farms. Corn silage is cultivated in 56.83% of the total forage crop planting area, while sorghum is grown in 16.66% of it. Furthermore, barley, oats, alfalfa, peas, and triticale are produced for use as feed for animals. Except for a small amount of wheat grown on the farms, grain feed production is not widespread. It can be said that this situation increases the farms' concentrated feed expenses. Indeed, a study conducted by Demir et al. (10) in Kars found that the proportion of feed expenses in the total costs was quite low (25%). This was attributed to the intensive use of pasture and forage areas for animal feeding in the region. In a study conducted by Santos et al. (29) in Brazil, which examined three family farms, it was determined that the share of feed expenses in the total costs was 63.09%. The high share of feed expenses was explained by the direct relationship of feed costs with milk production, and the fact that cows were allowed to feed freely without being dependent on milk yield.

Despite corn silage being the most produced forage crop, it is also the most purchased feed. Following corn silage, the most purchased roughage feeds, in order, are hay and alfalfa. Among concentrated feeds, dairy feed is

the most commonly purchased feed. Following dairy feed, there are beef feed, corn flakes, and calf growing feed, in that order. When examining feed consumption by type of roughage in the farms, it was found that 9.32 kg of roughage and 5.08 kg of concentrated feed were consumed daily. Among roughage feeds, corn silage has the highest consumption share (73.50%), while among concentrated feeds, dairy feed is the most consumed (65.75%). Considering this situation is important for reducing the feed costs of dairy farming when planning support for livestock farming.

The daily milk yield per herd average is 23.10 kg, the lactation period is approximately 290.91 days, and the annual milk yield per cow is 6717.23 kg in this research. The studies conducted in Türkiye, milk yield has been found to vary across different provinces. In the research conducted by Talim et al. (34) in Balıkesir, İzmir and Manisa, the average daily milk yield per cow was 19.84 kg and the annual milk yield was 6090 kg. In the research conducted by Tandoğan (35) in Afyonkarahisar, it was determined that the milk yield was 5187 lt/year. According to the size of the farms, the milk yield in small (1-15 head), medium (16-35 head), and large (36 and above head) farms was 5159 lt, 5155 lt, and 5313 lt, respectively. In the research conducted in the Thrace region (20), milk yield was 21.6 lt per day and 6093.6 lt per year. In this research, according to the size of the farm, the daily milk yield was 18.6 lt (5204.2 lt/year) in small (1-5 heads) farms, 20.0 lt (5611.8 lt/year) in medium (6-9 head) farms and 26.3 lt (7685.7 lt/year) in large (10 and above head) farms. In the research conducted by Sarıözkan et al. (30) in Kayseri, the daily milk yield was determined to be 22.7 liters per cow, and the lactation milk yield was found to be 6925 liters per cow. In another research conducted in İzmir (22), the daily milk yield per cow was 21.40 kg and the milk yield obtained in a lactation period was 5711.9 kg. In the study conducted by Semerci (31) in Hatay, milk yield in a lactation period was determined to be 5.619 lt/year.

It is known that in addition to the animal's genetic characteristics, environmental factors also have an impact on milk yield (15). In the examined farms, almost all of the animals are of the Holstein breed. However, there is also a presence, albeit in small numbers, of hybrid and Simmental breeds. To increase milk yield, it is advisable to improve shelter conditions on the farms, especially by using cooling fans during the hot summer months when temperatures rise significantly and by promoting the use of feed mixers for homogeneous ration mixing. Additionally, feed mixers that save labor contribute to reducing labor costs. It was determined that feed mixers are present in 39.46% of the farms.

The calculations of feed efficiency and feed conversion ratio were used to assess the influence of scale size on feed consumption in this research. In particular, the inclusion of the gross production value in the formula of the feed conversion ratio leads to its correlation with the economic structure of the farm. Indeed, the research findings indicate that large-scale farms take advantage of economies of scale, resulting in lower cost per livestock unit. Additionally, it was observed that as farm size increases, the feed conversion ratio also increases. Moreover, as the size of the farm increases, the cost per livestock units decreases. The average total land ownership in the farms is 76.80 acres, of which 67.55% (51.88 acres) are owned lands. The average number of parcels is 4.79. It has been determined that in larger-scale farms with a higher number of cows, irrigated farmland and total land ownership are greater compared to smaller-scale farms. This situation provides an advantage to larger-scale farms in terms of being able to produce the feed they need on their farms and reduce farm expenses. The research findings are similar to the results of a study conducted in Balıkesir by Mat and Cevger (21). In their study, a clear distinction was noted in the profit and loss statuses of the farms based on their scales, showing a rise in profitability levels as farms transitioned from small to large scale. Additionally, in the study conducted by Akbay et al. (3), which covers the seven geographical regions of Türkiye, it has been determined that with the increase in farm size, the farm land, forage crop planting area, and the share of forage crop planting area within the farm land have increased. In the study conducted by Semerci and Çelik (32) in Hatay, dairy farms were evaluated in three size categories: small, medium, and large. Accordingly, the highest yield per cow, milk production value per cow, and the highest profit per liter were achieved in the large-scale dairy farms.

As farms grow larger, they often have access to economies of scale, better management practices, and improved infrastructure. These factors can contribute to increased efficiency in animal production, including better feed utilization.

It was determined that 42.86% of the farmers sell their milk to dairies, 27.89% to middlemen, and 23.81% to cooperatives. In practice, milk collectors (buyers) often require the farmers from whom they purchase milk to also buy feed from them. Farmers with strong capital can manage to meet this requirement, but those facing capital issues are often obliged to comply with this requirement. The potential of dairies and middlemen to provide capital support to farmers through cash advances is much higher compared to cooperatives, which influences the preference of farmers to choose dairies and middlemen for milk collection.

The results of the study indicate that larger-scale farms are more advantageous compared to smaller-scale

farms. At the same time, larger-scale farms have a lower need for borrowing/credit, and their participation rates in cooperatives are higher. Semerci and Çelik's (32) study has revealed that organized dairy farms sell their milk at a higher unit price and achieve higher profit per liter.

It has been determined that farms derive 80.19% of their total gross production value from dairy farming and fully specialize in this production branch. Specialization, although considered unfavorable in terms of capital risk distribution, is a positive aspect in terms of achieving efficiency. Farms can enhance profitability by supporting their specialization trend in dairy farming with technological innovations.

Acknowledgements

This study was derived from the PhD thesis of the first author.

Financial Support

This research received no grant from any funding agency/sector.

Conflict of Interest

The authors declared that there is no conflict of interest.

Author Contributions

DG and GS designed the research. DG conducted the surveys and obtained the data. 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.

Ethical Statement

This study does not present any ethical concerns.

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Investigation of quality characteristics of industrially produced halloumi cheese

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ARTICLE INFO

Article History
Received : 17.04.2023
Accepted : 24.10.2023
DOI: 10.33988/auvfd.1218926

Keywords
Chemical analysis
Food safety
Halloumi
Microbiology
Quality

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How to cite this article: Ulusoy BH, Yıldırım FK, Kaynarca DH, Berkan Ş, Kademi Hİ, Hecer C (2024): Investigation of quality characteristics of industrially produced halloumi cheese. Ankara Univ Vet Fak Derg, 71 (4), 463-470. DOI: 10.33988/auvfd.1218926.

ABSTRACT

Χαλλούμι (Halloumi)/Hellim is the traditional cheese of Cyprus and belongs to the whole Island with Turkish and Greek names. Especially with the spotlights more on the product nowadays. In the current study, cheese samples were collected as fresh and mature halloumi/hellim (sheep/goat, bovine, or both milk) and were analyzed to evaluate the physicochemical and microbiological status. As a physicochemical analysis, the potential of hydrogen (pH), titratable acidity (TA), salt-sodium chloride (NaCl), and dry-matter (DM) ratios were analyzed. For examining the quality and safety indicators; total mesophilic aerobic bacteria (TMAB), yeast and molds, lactic acid bacteria (LAB), coliform bacteria, coagulase-positive staphylococci, *Listeria monocytogenes*, *Salmonella* spp. analyzes were performed. This study will help to establish the quality profile of halloumi/hellim cheese and identify potential hazards and sources of contamination.

Introduction

Χαλλούμι (Halloumi)/ Hellim is the traditional cheese of Cyprus and belongs to the whole of the divided Island with Turkish and Greek names. Historical documents show that this cheese has been produced in Cyprus since 1554. Knowledge of the production process has been carried out from one generation to the next (1). Χαλλούμι (Halloumi)/Hellim cheese has a tight and elastic structure without holes and can be sliced easily. Χαλλούμι (Halloumi)/Hellim, which gives an elastic feeling in the mouth, does not melt like kashar cheese when fried, and due to this feature, it can be consumed by frying in a pan or on the grill. Therefore, Χαλλούμι (Halloumi)/Hellim cheese, which has a wide consumption area, can be consumed for breakfast, as well as fried or grated and added to omelets, pasta, pastries, wraps, pita bread, soups, and salads (11). The first references to halloumi were seen in 1554 when Florio Bustron referred to a cheese (calumi in Italian) made from the milk of the sheep and goats of Cyprus. Kiprianos Church of Cyprus in 1778, mentioned the taste of halloumi and that it was unique (31). Halloumi is an important part of the Cyprus dairy sector and even the economic resources of the island in general (1, 15).

The shelf-life and quality of Χαλλούμι (Halloumi)/ Hellim are reported to be affected by several factors such as the milk quality and the hygienic practices during processing (3). Although Χαλλούμι (Halloumi)/Hellim is by boiling the curds in whey, several studies have reported

the presence of contaminated microorganisms in the end product due to the low quality of raw milk (29) or due to the poor hygiene during the production process (7). On the other hand, thermoduric microorganisms may survive boiling and LAB has also been reported to be found in fresh and mature halloumi/hellim cheese (34). The microbiological load and profile in the end product may be originated from different sources (milk, starters, and contaminating microorganisms) and the growth of the microorganisms may be affected by factors such as the applications of raw milk and the maturating conditions.

The sensory qualities of halloumi/hellim may also be affected by *Lactobacillus cypricasei*, named special for halloumi/hellim. It provides a wide range of enzymatic activities in cheese and may influence the trait of the end product (9). Several research studies (4, 8, 20, 27, 35) have been performed on industrially and traditionally produced cheese varieties to promote sustained quality and safety standards for halloumi manufacturers as well as to define the unique attributes of the cheese to preserve it.

The main feature of halloumi/hellim cheese production technology is to be produced from raw milk in traditional scale production without using a starter culture. On the other hand; with the recommendations of Commission Implementing Regulation (EU) 2021/591 of 12 April 2021, the cheese should be produced by pasteurized milk. However, halloumi/hellim is made using varying proportions and types of milk, and according to PDO, the most crucial issue for the product is that it should be made with local sheep and goats (the Chios sheep and the Damascus goat) milk (1). Since no starter culture is used, the milk is coagulated by rennet at 33±1 °C and the clot is cut into 1 cm^3 pieces. In this case, after resting for 10 minutes, it is heated at 40 °C for 15 minutes, transferred to cheese vats, and applied pressure. It is then cut into dimensions of 8±1 cm x 10±1 cm x 4±1 cm. Then the whey is mixed and heated until it is brought to 80 °C. At this stage, albumin and globulin, which are serum proteins, coagulate and rise to the surface. This clot is carefully collected from the surface, pressed, and another local cheese type called "nor" is produced, which can be consumed fresh or dried (11).

It is known that different practices are followed in some process steps in the traditional production of halloumi cheese, which is common among the public of the Island. This situation causes differences in the standard features of the product. For this reason, The Regulation of PDO brought a unique and single standard for the whole island to provide exemplary quality production without changing its unique character and to maintain and increase its market share in the globalized competition. Depending on our scientific paper evaluation on halloumi/hellim quality investigation, there is not much paper regarding the situation on Northern Cyprus. That's why the current study aimed to characterize the some of the physicochemical and microbiological indices of halloumi/hellim produced in Northern Cyprus create a scientific data for the literature.

Materials and Methods

Samples collection: Totally, of 85 halloumi/hellim samples were collected from retail outlets of Nicosia. The samples were brought to the laboratory under a cold chain in their original vacuum packaging which are nearly 200-250 g in weight. The samples were collected under standard retail conditions and from the markets that have high capacity of halloumi/hellim retail.

Microbiological Analysis Of The Halloumi/Hellim Cheeses: 10 g of sample was homoginazated in 90 mL of Maximum Recovery Diluent (MRD). Then, serial dilutions were prepared and inoculated on sterile media. Total mesophile bacteria (TMAB) were enumerated on Plate Count Agar (PCA; LAB149) after incubation at 35 °C for 48h. Total coliform bacteria were enumerated on Violet-Red Bile Glucose Agar (VRBA; LAB031) at 35°C for 24h. Staphylococcal-micrococcal bacteria, Lactic Acid Bacteria (LAB), and mold/yeast were enumerated on Baird Parker Medium Agar (BPA; LAB085), MRS Agar, and Yeast Glucose Chloramphenicol Agar (YGC; LAB122), respectively. Thermophilic anaerobes, producing hydrogen sulphite were detected by Sulfite Polymyxin Sulfadiazine Agar (SPS; MERCK 110235). The media and incubation conditions are shown in Table 1. At the end of incubation, Petri dishes containing 30-300 colonies were counted and the result was reported as $\log_{10}$ cfu/g.

Physicochemical Analysis Of The Halloumi/Hellim Cheeses: After the packages of the halloumi/hellim cheeses were opened and used for microbiological analyses, the samples were prepared for physicochemical analyses. Hanna HI 98230 penetration pH meter (Hanna Instruments, Italy) calibrated with pH 4 and 7 buffers were used to determine the pH of the samples at 24°C. % lactic acid value was obtained by titration method. Humidity and dry matter (DM) analyses were performed by a humidity meter device (Shimadzu Corporation, MOC63u), and sodium chloride (NaCl) was analyzed by the Mohr method.

DOI: 10.33988/auvfd.1218926

Table 1. Media used incubation conditions, and references of methods.

Micro-organisms	Analytical reference method	Media name	Incubation conditions		
			Incubation temp.	Incubation period	O_2 requirement
Total Mezophile Aerobe Bacteria (TMAB)	ISO 4833	Plate Count Agar (LAB 149)	30°C ± 1 ºC	72 h ±3 h	Aerobic
Staphylococci *Staphylococcus aureus*	ISO 6888-1: 1999 + A1:2003	Baird Parker Medium Agar (LAB 085) + Egg Yolk Tellurite Emulsion (X 085)	35 º- 37 ºC	24 h ± 2 h	Aerobic
		Brain Heart Infusion Broth (LAB 049)	Confirmation for *Staphylococcus aureus*		
		Rabbit Plasma (X086)			
Salmonella spp.	ISO 6579:2002 + A1:2007	Buffered Peptone Water (LAB 204)	37 ºC ± 1 ºC	18 h ± 2 h	Aerobic
		Rappaport Vassiliadis Medium (R.V.S) single component (LAB 086)	41.5 ºC ± 1 ºC	24 h ± 3 h	
		X.L.D. Agar (LAB 032)	37 ºC ± 1 ºC	24 h ± 3 h	
		Triple Sugar Iron Agar (LAB 053)	Confirmation		
		Urea Broth Base (LAB 131)			
Coliform bacteria	ISO 4832:2006	Violet Red Bile Glucose Agar (LAB 031)	44 ºC (for *E.coli*) 30 ºC - 37 ºC	24 ± 2 h	Microaerophilic
		Brilliant Green Bile Broth (LAB051)	Confirmation		
Listeria monocytogenes	ISO 11290-1: 1996 + A1:2004	Half Fraser Broth Base (LAB 164)	30 ºC	24 h ± 2 h	Aerobic
		Fraser Broth Base (LAB 164)	37 ºC	24 h	
		Palcam Agar (LAB 148)	37 ºC	24 h ± 3 h	
		Tryptone Soya Yeast Extract Broth (LAB004)	Confirmation for *Listeria* spp.		
		Sheep Blood Agar (LAB028)	Confirmation for *L. monocytogenes*		
Lactic Acid Bacteria (LAB)	ISO 15214:1998	MRS Agar (LAB223)	30 ºC	2-3 days	Anaerobic
Yeast and mold	ISO 6611: 2004	Yeast Glucose Chloramphenicol Agar (LAB 122)	25 ºC	5 days	Aerobic
Thermophilic anaerob bacteria		Sulfite Polymyxin Sulfadiazine Agar	44 ºC	48 h	Anaerobic

Results

Results Of Physicochemical Analysis: The physicochemical analysis results of halloumi cheese are presented in Table 2. According to the physicochemical results, the minimum and maximum DM were 20-50 %, pH levels were between 4.94-6.87, salt ratios were 1-7; the titratable acidity was calculated as 0.07-0.28. With the results within the current study, it was observed that the pH of the samples is mostly in the range which stops undesired microorganism growth. The amount of salt in the analyzed halloumi cheeses was found to be min 1.00%, max 7%, and the mean value was 3% (Table 2). DM content of our samples was between 20-50% and at the mean of 40%.

Table 2. Physicochemical analysis results of halloumi cheese.

	N	Mean	Min-Max.
Dry matter (DM) (%)	85	40	20-50
pH	85	6.2	4.94-6.87
NaCl (%)	85	3	1-7
Lactic acid (LA) (%)	85	0.18	0.07-0.28

Results Of Microbiological Analysis: The microbiological analysis results of our study were presented in Table 3. Because the products are from different enterprises, very different results were obtained in TMAB and Staphylococci counts. The results were given as

Staphylococci, since all of the colonies counted on BPA agar had negative *S. aureus* confirmation test. *Listeria monocytogenes* and *Salmonella* spp. were not detected in any samples because of that, they were not included in Table 3. According to this study on the samples of halloumi/hellim cheese in the Cyprus market, *Salmonella* spp. and thermophilic anaerobe bacteria, producing hydrogen sulphite were not isolated in any of the samples. The TMAB was found to be min 3 $\log_{10}$ cfu/g, max 6.70 $\log_{10}$ cfu/g, and on average 4.54 $\log_{10}$ cfu/g in our study (Table 3). In this study, a max 2.41 $\log_{10}$ cfu/g coliform was obtained (Table 3). Considering the microbiological distribution in the samples, it was observed that only 3 (3.52%) samples were detected to contain 1.30-2.41 $\log_{10}$ cfu/g of coliform. One of the bacteria that is an indicator of hygienic quality and causes food poisoning is *E. coli*. This bacterium was not found in any of the analyzed halloumi cheeses. *Staphylococcus* spp. in halloumi cheeses was determined as a maximum of 6.70 $\log_{10}$ cfu/g and an average of 3.15 $\log_{10}$ cfu/g (Table 3). No coagulase-positive *Staphylococcus aureus* was detected in any of the samples. Yeast and molds generally affect the shelf life, quality, and flavor of foodstuffs. Yeast and molds were found to max 4.36 $\log_{10}$ cfu/g and an average of 2.50 $\log_{10}$ cfu/g in the examined halloumi cheese samples (Table 3). In the study, it was obtained a maximum LAB count of 6.70 log10 cfu/g and an average of 3 log10 cfu/g in halloumi cheeses (Table 3).

Table 3. Microbiological analysis results ($\log_{10}$ cfu/g).

	N	Mean	Min-Max.
TMAB	85	4.54	3-6.70
Staphylococci	85	3.15	1-6.70
Yeast/Mould	85	2.50	1-4.36
LAB	85	3	1-6.70
Coliform	85	1	1-2.41

Discussion and Conclusion

As reported in the scientific reports, the factors for controlling the growth of microorganisms in cheese include water activity, salt concentration, oxidation–reduction potential, pH, NO_3, temperature, and maybe the production of bacteriocins that are secreted by some of the microorganisms (19). The pH value is one of the important criteria that affect the growth of microorganisms and the shelf life of foods. Most of the bacteria require neutral pH for optimum growth. On the other hand, LAB, especially *Lactobacilli* spp., generally grows at a pH below 7, such as 4.0; most yeasts and molds can grow at a pH of 5–7 but can grow at pH values <3.0 (19). This can be accepted as a positive condition that the natural pH levels allow LAB to grow and unique properties are promoted by these LAB. In the results of various studies on halloumi cheese, the pH values were detected between 4.50–4.90 (33), and 4.79-6.12 (18) with an average of 5.38 (6), and an average of 5.97 (17).

The acidity value is also one of the important parameters in determining the chemical durability of the products. As presented in Table 2, LA% values were min 0.07, max 0.28 and mean value was 0.18. Atasever et al. (2) and Gün and Şimşek (18) determined the acidity values in halloumi as 0.53% and 1.68%, respectively. On the other hand, in the study performed by İncili et al. (21) 0.15% LA mean value was obtained which is similar to the results of our study. The main reason for the difference between the % lactic acid values is thought to be because of the microbial load of the raw milk processed into cheese, the different initial acidity, or different pressure applications.

The amount of salt added to foods affects the flavor, aroma, and also the shelf life of the products. The salt ratio is also a pivotal factor in microbiological growth. These values were found to be very low compared to the findings observed by Atasever et al. (2); Demirci and Arıcı (6); İncili et al. (21) with the results of 5.09%; 6.14% and 6.84% respectively. On the other hand, we obtained that the salt ratio diversity in commercial samples is high. This is because there is no uniformity in the production process. In the industrial process, the way the cheese is salted differs from traditional production. The cooked cheese blocks are not dry salted but left to cool in whey brine (12% NaCl) at 4ºC for ~18 h then sprinkled with dry, sterilized mint (24). As Guniee (16) mentioned the salt content of cheese varies according to the method of salting, the cheese type, cheese geometry, cheese size, etc. This affects not only its flavor but also most of its physicochemical characteristics. In addition, it is possible that the salt ratios can be different in the samples collected at different stages of their shelf lives. Within the study performed by Gün and Şimşek (18), a similar average salt ratio (4.76 ± 0.74%) was obtained. Similarly, the salt and pH values of halloumi samples were determined by Papademas and Robinson (26); Keles et al. (29); Milci et al. (33) show similarities with the results he determined. In the traditional process, the fresh cheese is dry salted and sprinkled with dry, crushed leaves of mint (*Mentha viridis*) and then kept in salted whey (12% NaCl). Storage in brine and the ratio of salt may change cheese properties such as the organic acids profile, volatile aroma compounds, and sensory features (23, 24). EU requirement for salt is maxed at 3% and 6% for fresh and mature halloumi/hellim respectively in a Single Document of the European Commission (CY-PDO-0005-01243-

17.7.2014). In this document two types of halloumi/hellim are described: fresh and mature (13).

DM of the cheese is strictly related to the DM of the raw milk that the cheese is produced from, and this also affects the yield of the cheese. Different types of milk used for halloumi/hellim production have different ratios of dry matter. This reflects the yield and the DM ratio in cheese samples The milk required to produce a kg of halloumi/hellim cheese was 5.44, 8.85, 11.30, and 6.70 kg for sheep, goat, cow, and mixed milk (sheep and goat), respectively (9). On the other hand, seasonal factors influence the DM of halloumi/hellim. As Esendağlı (12) concluded, DM ratios of halloumi/hellim samples that were produced in North Cyprus were lower in the months between April and October (min 48.8 % and max 50.14). The researcher obtained 54.1% of DM in December. The max DM that we obtained is 50% which is also below the criteria published in the Single Document of the European Commission (CY-PDO-0005-01243-17.7.2014). DM should be min 54% and 63% for fresh and matura halloumi/hellim, respectively (13). All our samples were produced with cow milk. This may be the reason for lower DM in samples. EU requires halloumi/hellim to be produced in sheep milk. Different results were concluded by researchers and those differences may depend on the origin of milk, the season of milking, the physiological and pathological condition of the animal, and the processing steps of halloumi/hellim. The obtained results were 55.02±1.78% on average as concluded by Gün and Şimşek (18), 58.89% as concluded by Atasever et al. (2) and 60.21% as concluded by Demirci and Arıcı, (6). İncili et al. (21) examined 30 halloumi samples in their study and determined that the dry matter ratio was min 37.21%, max 54.88%, and an average of 48.77±5.25 (21). In our study the avarage results are lower.

According to "Ready-to-eat foods able to support the growth of *L. monocytogenes*, other than those intended for infants and for special medical purposes" in Commission Regulation (EC) No 2073/2005 of 15 November 2005 (Chapter 1/1.2) on microbiological criteria for foodstuffs, *L. monocytogenes* should be absent as we obtained in our samples (5). On the other hand, the absence of other pathogens such as *Salmonella* spp. and sulfite producing anaerobes is a satisfactory result for these samples in terms of food safety and public health. The same result was reported by Özçil (30). The halloumi/hellim microbiological profile can be considered the fingerprint that creates the unique traits of the product Halloumi/ hellim is characterized by unsurpassed organoleptic characteristics and a rising contribution of the indigenous microflora in the milk (32). That's why, other than checking the pathogens in terms of food safety and the spoilage microorganisms, the presence of desired natural microorganisms is also indisputable. As Kamilari et al. (22) mentioned halloumi/hellim was a globally produced and consumed cheese up to date but the autochthonous microbial communities that affect its unique organoleptic properties and safety of the product, have not been fully evaluated yet. Moreover, these days when halloumi PDO registration is on the agenda, defining the microbial profile specific to this product will be a part of its regional uniqueness. Accordingly, Kamilari et al. (22) also mentioned that the microbiome may be used as an additional tool to define the typicity of Cyprus Halloumi/Hellim. Lawson et al. (28), characterized a novel, salt-tolerant species in halloumi/hellim cheese and named as *Lactobacillus cypricasei* sp. nov., which was later found to be a heterotypic synonym of *Lactobacillus acidipiscis* (22). *Lactobacillus cypricasei* is now mentioned and described in Regulation (EU) 2021/591 (12 April 2021) which is about PDO registration of the cheese (1). The microbiological profile of this cheese is affected by several factors, including; the heat process and curd cooking procedures, the microbiological load of rennet, as well as salt and Mentha Viridis (mint) leaves, microbial contamination from the production area environment, and the microbiological contamination from brined whey (22).

Usca and Erol (1998) reported 4.51 $\log_{10}$ cfu/g TMAB (38). Demirci and Arıcı (1989) and İncili et al. (2019) obtained TMAB counts of 8.60 and 6.39 $\log_{10}$ cfu/g, respectively (6, 21). As halloumi/hellim is produced without using a starter culture, TMAB may be used as a tool to have an idea of the microbiological quality and shelf life of the product. As Kamleh et al. (25) concluded in their study which is focused on the shelf life of halloumi/hellim, at the end of storage at 5°C, packaged halloumi cheese is expected to have TMAB of 3.8–4.0 $\log_{10}$ cfu/g.

Coliform bacteria are accepted as hygiene indicators and cause flavor, structure, and aroma disorders in cheeses. This group of bacteria can also cause technological errors during the ripening of cheeses. The number of coliform bacteria may vary depending on the technological processes applied, the type of cheese made, and whether the cheese is fresh or maturated. It is seen that the levels of coliform bacteria detected in the halloumi cheese samples examined were at levels (26% and 31.5%) lower than the levels found (6, 38). As İncili et al. (21) reported the mean number of coliforms was 2.29±0.73 $\log_{10}$ cfu/g.

Although halloumi cheese is a cheese whose curd is heat-processed due to its production technology, it is thought that the presence of *S. aureus* is probably due to the lack of personnel hygiene after heat treatment, the lack of hygiene in the use of tools and equipment that contacted with the cheese. It is known that some *Staphylococcus* strains play an essential role in foodborne poisoning. For this reason, especially *S. aureus* should not be present in

foods. *Staphylococcus* spp. in halloumi cheeses was determined as a maximum of 6.70 $\log_{10}$ cfu/g and an average of 3.15 $\log_{10}$ cfu/g (Table 3). Since Staphylococci are of human or animal origin, their myriad in foods indicates insufficient sanitation or heat treatment. İncili et al. (21) obtained the mean number for *S. aureus* as 2.81±1.54 $\log_{10}$ cfu/g. Özçil (30) reported that *S. aureus* was detected in two of them, but the researcher concluded the amounts found did not pose a public health hazard according to the Turkish Food Codex Microbiological Criteria.

It was observed that the results in this study were quite lower than the results of some researchers (2, 38). As İncili et al. (21) reported the mean numbers of yeast mold were found as 3.16±1.14 $\log_{10}$ cfu/g. This is probably due to the possible contamination from food handlers' hands during folding, the air of the production area especially where the fresh cheeses are kept together one day in brine, and the quality of the packaging materials. Brine itself can be the contamination source. As Ulusoy et al. (37) concluded in their study which focused on the contamination sources of halloumi/hellim throughout the production steps, brine may have a high microbiological load including yeast and mold. In another investigation, Atasever et al. (2), studied the effect of production technology on the vacuum-packed and brined halloumi on the microbiological quality of halloumi/hellim. They found that the coliform, total mesophilic aerobic bacteria, yeast, and mold counts were higher in vacuum-packaged products than in brine. Halloumi/hellim from different types of milk may include different species of yeast. While *Debaryomyces hansenii*, *Candida parapsilosis*, *C. boidinii*, *C. versatilis*, and *Pichia membranifaciet* were isolated from the matured halloumi cheese from sheep milk, *Cryptococcus albidus* and *Pichia membranifaciet* were isolated from halloumi cheese made from cow's milk (3). On the other hand, mold growth should also be considered in terms of the level of aflatoxins and other mycotoxins important for public health. Elkak et al. (10) reported that Aflatoxin M1 was detected in 21 samples in their analysis of 31 halloumi samples collected for sale in Lebanon and 8 of these samples exceeded the 250 ng/kg limit set by the European Union Commission.

LAB is effective on the flavor, structure, aroma, and shelf life of the products. It is a group of bacteria that can dominate in native microbial profiles and provide forming unique sensorial properties. *Lactobacilli* constitute the dominant microflora in halloumi as in cheeses such as Cheddar and Domiati, where starter culture is not used. It has been reported that lactic acid bacteria are a part of the microflora in the final product, although the milk is pasteurized in the production of halloumi and the curd is boiled during the production phase (3, 28, 33). As we previously mentioned *Lactobacillus cypricasei*, is one of the LAB that was obtained to be unique for halloumi and facultative anaerobe, homofermentative, and resistant to high salt concentrations and low pH values (28). *Enterococcus faecium*, which is one of the thermoduric microorganisms found in the natural flora of milk, can also be frequently isolated from halloumi/hellim. It has been reported that some Enterococcus strains are proteolytic and may contribute to the taste of halloumi made from sheep's milk (14).

With the results obtained by other researchers, the spore-forming bacteria, such as *Bacillus*, as well as thermophilic species, such as members of the genus *Lactobacillus* and *Enterococcus*, in addition to yeasts were identified in the cheese samples by culture-based techniques (3, 36). Kamilari et al. (22) used metagenomic analysis to characterize halloumi cheese's bacterial communities. According to the results of this study, the predominant bacteria were LAB genera, such as *Lactobacillus*, *Leuconostoc*, *Pediococcus*, *Weissella*, and *Marinilactibacillus*. Additionally, spore-forming bacteria, including the genus *Bacillus*, psychrophilic or psychrotolerant bacterial genera such as *Psychrobacter*, the halophilic genus *Halomonas*, as well as the genera *Pseudomonas*, *Staphylococcus*, *Acinetobacter*, *Macrococcus* and *Vibrio*, member of which may cause food spoilage were also commonly detected (22).

Halloumi is of great importance culturally and economically for the Turkish and Greek communities in Cyprus. Production by the registration standards prepared jointly by the two communities of Cyprus and submitted to the European Commission as a result of the application will cause halloumi to gain an important place in the world market. Both traditional small-scale enterprises and high-capacity industrial production enterprises must be subjected to inspections according to the standard set by the European Commission for registration to preserve the original character of the product. Hygienic, pathogen-free, and food-safe production is as important as quality and standard productivity.

As the conclusion of the microbiological and physicochemical analysis performed in this study, it was determined that the product did not pose a serious public health threat. However, whether small-scale or high-capacity facilities make production primarily in line with good hygiene practices (GHP) and good manufacturing practices (GMP), the product will be of higher microbiological quality.

Financial Support

This research has been supported by the content of the project no SAG-2017-01-037 by Near East University Scientific Research Projects Unit.

Conflict of Interest

The authors declared that there is no conflict of interest.

Author Contributions

BHU, FKY, and HDK conceived and planned the experiments. BHU and FKY carried out the experiments. BHU, ŞF and HIK planned and carried out the simulations. BHU, FKY, HDK and CH contributed to sample preparation. BHU, FKY, HDK, ŞF, HIK and CH contributed to the interpretation of the results. BHU and CH 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.

Ethical Statement

This study does not present any ethical concerns.

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Bee bread boosts probiotic Yoghurt: Unveiling the impact on physiochemical, microbiological, and sensory attributes

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ARTICLE INFO

Article History
Received : 13.07.2023
Accepted : 08.03.2024
DOI: 10.33988/auvfd.1326701

Keywords
Bee bread
Yoghurt
Probiotic

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How to cite this article: Keyvan N, Yurdakul Ö (2024): Bee bread boosts probiotic Yoghurt: Unveiling the impact on physiochemical, microbiological, and sensory attributes. Ankara Univ Vet Fak Derg, 71 (4), 471-480. DOI: 10.33988/auvfd.1326701.

ABSTRACT

This study aimed to investigate the effects of bee bread ratios of 0.5%, 1%, and 2%, respectively on some parameters in probiotic yoghurt production. The bee bread composition contained the elements B, Ca, Fe, K, Mg, Na, P, and Zn. The analysis of sugar composition revealed the presence of fructose, glucose, and sucrose. The organic acid and phenolic substance content were assessed. The following values were obtained: oxalic acid (1.26 mg/g), malic acid (7.79 mg/g), ascorbic acid (0.91 mg/g), citric acid (2.73 mg/g), *p*-coumaric acid (15.3 µg/g) and kaempferol (5.562.4 µg/g). The study determined the tocopherol content, specifically alpha (7.09 µg/g), beta (0.4 µg/g), gamma (0.77 µg/g), and delta (0.31 µg/g). A total of 55 distinct components were identified while analyzing the volatile and aroma profiles. This study found that the IC_{50} value of bee bread was 1.414 mg/mL. Bee bread did not affect physicochemical parameters such as pH, acidity, dry matter, ash, milk fat, and water holding capacity ($P>0.05$) but affected protein and syneresis ($P<0.05$). The addition of bee bread positively affected *Streptococcus thermophilus* and *Lactobacillus bulgaricus,* and *Lactobacillus acidophilus* LA-5 activity was preserved at around 10^7 kob/g during storage ($P<0.05$). Adding bee bread affected the color parameters L*, a*, and b* values ($P<0.05$). Consumers preferred the group to which 0.5% bee bread was offered following sensory analytical evaluation. The study has demonstrated that adding bee bread during yoghurt production can effectively maintain probiotic activity.

Introduction

Bee bread is primarily composed of pollen, honey, and secretions from the salivary glands of honey bees (43). Bees utilize nectar as their primary carbohydrate source, whereas pollen is a crucial source of proteins, lipids, vitamins, and minerals for bee bread production (42). The substance provides food for worker bees and developing larvae (27). Bee bread is considered a more easily digestible form of pollen because the bee's enzymes digest the pollen's outer shell during fermentation (18). The fermentation process carried out by lactic acid bacteria in the honey stomach of bees contributes to the transformation and preservation of the stored pollen, resulting in the formation of bee bread (39). Several research studies into the chemical composition of bee bread have revealed that it typically consists of water, protein, free amino acids, carbohydrates, fatty acids, minerals, vitamins, and numerous types of other bioactive compounds, including kaempferol, rutin, quercetin, luteolin, and rosmarinic acid (4, 5, 11, 23). Bee bread has many biological properties, including antioxidant, antibacterial, antifungal, antiviral, anti-inflammatory, and anticancer properties (5). Bee bread has been the subject of extensive research due to its unique nutritional qualities and possible benefits for health (31). Several studies have indicated that bee bread could enhance the immune system, promote digestion, and provide anti-inflammatory properties (30). Bee bread is considered a nutritional supplement due to its biological effects (34).

Probiotic yoghurt contains live microorganisms, known as probiotics, which confer health benefits on the host when consumed in adequate amounts (17). These

probiotics can improve the composition of the colonic microflora and exert health benefits independent of gastrointestinal colonization (22). The use of probiotic bacteria in yoghurt production has been explored to enhance its prophylactic properties (38). Probiotic yoghurt has also been found to inhibit pathogenic microorganisms such as *Staphylococcus aureus*, which may be attributed to the probiotic bacteria or the antibacterial substances they secrete (40). In addition to its cardiovascular and antimicrobial effects, probiotic yoghurt has been studied for its potential benefits in various health conditions. For example, daily probiotic yoghurt consumption has improved the albumin-to-creatinine ratio, estimated glomerular filtration rate, and metabolic parameters in patients with type 2 diabetes with nephropathy (16). Probiotic yoghurt has also been found to have potential anticarcinogenic effects, hypocholesterolemic effects, and the ability to alleviate lactose malabsorption and allergies (38). The study's objective was to investigate the effect of bee bread on physiochemical, microbiological, and sensory properties during the production of probiotic yoghurt.

Materials and Methods

The milk used for probiotic yoghurt production was obtained from the Official Milk Production Store of Burdur Mehmet Akif Ersoy University. Nu-trish LA5 (*Lactobacillus acidophilus* LA-5) and yoghurt culture YF-L903 (*Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus thermophilus*) were purchased from Christen Hansen Laboratories in Copenhagen, Denmark. The bee bread used for the experimental group studies was obtained from hives in Karaman Province, Türkiye (37°08'50.7"N 33°31'45.2" E). The chemicals used for the analyses were purchased from Sigma-Aldrich Co. (St. Louis, USA).

Study Design: *L. acidophilus* LA5 and bee bread were not included in the study's control groups, which were designated as group A. The only starters that were used were yoghurt starters. A combination of yoghurt starter cultures and *L. acidophilus* LA5 probiotic bacteria was included in the control test group's composition, which was Group B. A study strategy was created wherein the experimental groups, designated as C, D, and E, were assigned bee bread ratios of 0.5%, 1%, and 2% respectively. The purpose of these groups was to assess the physicochemical, microbiological, and sensory impacts of adding bee bread to yoghurts.

Characterization of Bee Bread Samples: The mineral composition of bee bread was analyzed by using inductively coupled plasma-optic emission spectroscopy (ICP-OES, Perkin Elmer OPTIMA 5300 DV, USA), with a focus on macroelements and microelements (33). High-performance liquid chromatography (HPLC, Shimadzu HPLC 10A VP, Shimadzu, Japan) was used to analyze samples for *p*-coumaric acid, quercetin, kaempferol, and free sugar using the method provided by Veberic et al. (44). Barros et al. (6) assessed the tocopherol content using HPLC, following the methodology previously described. In addition, the content of oxalic acid, one of the organic acid components of bee bread, was determined by HPLC (28). The volatile and aroma profile was determined by gas-chromatography–mass spectrometry (GC/MS) (Shimadzu GC-2010 Plus, Japan; Shimadzu GCMS-QP2010 SE (Detector)) solid-phase micro-extraction (SPME) (6). The 1,1-diphenyl-2-picrylhydrazyl (DPPH) method was used to evaluate the antioxidant activity of bee bread samples (32).

Production of Probiotic Yoghurt with Bee Bread: In the yoghurt production process, the milk was supplemented with 3% skimmed milk powder (Bagdat Baharat, Türkiye). After applying heat treatment at a temperature of 90°C for 10 minutes, the homogenized milk received a subsequent cooling process to reach a temperature of 43°C. Subsequently, the milk was divided into five equal portions by introducing 2% starter and probiotic cultures. The experimental groups were administered bee bread dissolved in water at concentrations of 0.5%, 1%, and 2%. Subsequently, 100 grams of polystyrene containers were filled and subjected to incubation at a temperature of 42°C for a duration of 3.5 hours. Following the incubation period, the yoghurt samples were subjected to a cooling process, reducing their temperature to 4°C. Subsequently, these samples were stored at this specific temperature for a duration of 28 days, as reported by Tamime and Robinson (41) and Ozcan et al. (36). The study was designed to include three replications, and data analyses were conducted at intervals of 0, 7, 14, 21, and 28 days.

Microbiological Analysis: The samples of yoghurt that were examined for *S. thermophilus* were cultivated using M-17 agar (Oxoid CM785) (9). The current study used MRS 5.4 Agar (De Man Ragosa Sharpe, Difco 288210) as the medium for the examination of *L. bulgaricus* (10). The method ISO 20128/IDF192 reported was used to detect probiotic *L. acidophilus* LA-5 (20).

Physicochemical Analysis: The physicochemical parameters, including pH, acidity, dry matter, ash, milk fat, and water holding capacity, were assessed for yoghurt products using the Official Methods of Analyses (2). The

Kjeldahl method was used to conduct a crude protein analysis of yoghurt samples (45). Syneresis was analyzed using the methodology described by Wu et al. (46). The color analysis was performed using a colorimeter (Konika Minolta, CR 400, Osaka, Japan). The analysis involved the assessment of the L* (lightness), a* (red/green), and b* (yellow/blueness) parameters according to the Hunter scale.

Sensory Analysis: A study was conducted to evaluate the sensory characteristics of yoghurt samples over a period of 28 days under cold storage conditions. The evaluation was carried out by a panel of 10 individuals who had received comprehensive training in dairy product assessment, following the methodology proposed by Canbulat and Özcan (8).

Statistical Analysis: The study was replicated three times, and triplicate measurements were conducted for each parameter on the 1st, 7th, 14th, 21st and 28th day of storage. The statistical analysis of the data was conducted using SPSS 25.0 software (SPSS Inc., USA). The physicochemical composition data, including pH, acidity, dry matter, ash, milk fat, syneresis, and water holding capacity, were assessed using the generalized linear mixed model (GLMM) procedure. Additionally, microbiological analysis and sensory evaluation were also conducted and included in the evaluation. In the statistical design, fixed effects were assigned to groups and storage duration, whereas a random effect was assigned to replications. The Tukey multiple comparison test was employed to assess significant disparities among the average means. Statistical significance was determined when the p-value was less than 0.05 for differences observed among mean values. The chemical composition data, including protein, fat, and ash concentrations, as well as color attributes, were subjected to examination using a one-way analysis of variance (ANOVA). The findings were presented as mean values accompanied by standard errors (SE) of the mean.

Results

Content Analysis of Bee Bread: The data presented in Table 1 were obtained by analyzing the macroelement and microelement composition of the bee bread sample. The evaluation involved examining the presence of various elements, including B, Ca, Cr, Fe, K, Mg, Mn, Mo, Na, P, and Zn. In this study, an investigation was conducted on the sugar content of bee bread, resulting in the determination of fructose (149.4 mg/g), glucose (92 mg/g), and sucrose (21.1 mg/g). The assessment of the organic acid content in bee bread revealed the presence of oxalic acid (1.26 mg/g), malic acid (7.79 mg/g), ascorbic acid (0.91 mg/g), and citric acid (2.73 mg/g). In the scope of this study, the evaluation of the phenolic compound content of bee bread sample revealed the presence of *p*-coumaric acid (15.3 µg/g) and kaempferol (5562.4 µg/g). The concentrations of tocopherols, including alpha (7.09 µg/g), beta (0.4 µg/g), gamma (0.77 µg/g), and delta (0.31 µg/g), was determined by the study. In this study's parameters, 55 different components were identified by analyzing volatile/aroma profiles using GC/MS SPME. The components that were detected in the highest proportions are as follows: acetic acid (42.89%), octane (6.64%), 6-Methyl-5-hepten-2-one (5.62%), 3,5,5-Trimethyl-2-cyclohexanone (3.92%), 9-Nonadecane (3.78%), dimethyl sulfide (3.53%), nonanal (2.71%), methyl acetate (2.10%), and penten-3-one (2%). Other components were detected in proportions below 2% (Table 2). In the current study, the antioxidant activity of bee bread was determined using DPPH, and the IC_{50} value of bee bread was found to be 1.414 mg/mL (Figure 1).

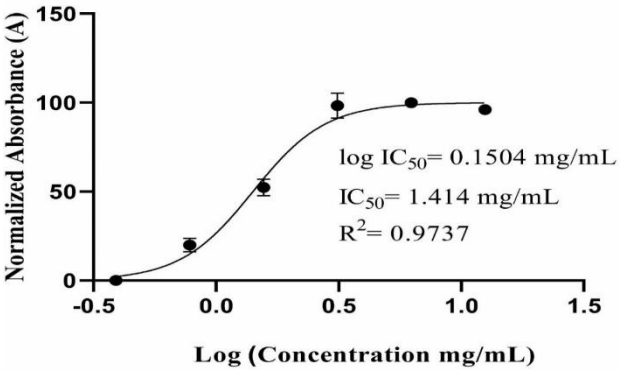

Figure 1. Bee bread IC_{50} value.

Table 1. The mineral content of bee bread.

Elements	Wavelenght (nm)	Content (mg/g)
B	249.677	0.013 ± 0.002
Ca	317.933	1.585 ± 0.162
Cr	267.716	< 0.005 ppm
Fe	238.204	0.111 ± 0.017
K	766.490	3.422 ± 0.043
Mg	285.213	0.564 ± 0.017
Mn	257.610	< 0.005 ppm
Mo	202.031	< 0.010 ppm
Na	589.592	0.056 ± 0.009
P	213.617	2.893 ± 0.076
Zn	206.200	0.013 ± 0.001

Table 2. Bee bread volatile compounds using GC/MS SPME.

Peak	R. Time	Name	Area	Area%
1	1.372	Ethyl alcohol	122132	0.68
2	1.443	Isopropenyl alcohol	264215	1.48
3	1.512	Dimethyl sulfide	632605	3.53
4	1.530	Methyl acetate	375229	2.10
5	1978	Acetic acid	7676538	42.89
6	2.217	2-Butenal	76342	0.73
7	2.250	3-Hydroxybutanal	46932	0.26
8	2.337	2-Pentanone	68819	0.38
9	2.554	Penten-3-one	358612	2.00
10	2.715	Heptanal	287448	1.61
11	2.815	2.5-Dimethylfuran	10899	0.06
12	3.428	Dimethyl disulfide	235749	1.32
13	3.665	(E)- 2-Pentenal	148120	0.83
14	3.818	3-Methyl-3-butenenitrile	119998	0.67
15	3.868	Toluene	101126	0.57
16	4.5627	Octane	1187763	6.64
17	5.531	Furfural 2-Furaldehyde	208555	1.17
18	6.6160	2-Hexenal	111234	0.62
19	6.611	o-Xylene	59561	0.33
20	6.660	p-Xylene	27292	0.15
21	7.322	Styrene	324620	1.81
22	7.668	Nonane	280952	1.57
23	7.731	Heptanal	121700	0.68
24	8.005	2-Acetylfuran	16516	0.09
25	8.030	Butyrolactone 2(3H)-Furanone. dihydro-	44587	0.25
26	8.107	2,6-Dimethylpyrazine	260670	1.46
27	8.549	Methyl caproate	40122	0.22
28	8.793	alpha- Pinene	95342	0.53
29	9.549	gamma- Valerolactone	21618	0.12
30	9.889	Benzaldehyde	41721	0.23
31	10.054	Dimethyl trisulfide	60981	0.34
32	10.284	Sabinene	25470	0.14
33	10.808	6-Methyl-5-hepten-2-one	1005430	5.62
34	11.285	3-Ethyl-1,4-hexadiene	78005	0.44
35	11.400	Decane	145345	0.81
36	11.512	Octanal	171343	0.96
37	11.656	cis- Ocimene	41193	0.23
38	11.865	(E,E)-2,4-Heptadienal	89802	0.50
39	12.276	Para Cymene	17543	0.10
40	12.466	Limonene	351807	1.97
41	13.050	Benzeneacetaldehyde	56476	0.32
42	13.206	beta-trans-Ocimene	21532	0.12
43	13.623	gamma- Terpinene	36112	0.20
44	14.155	(3E,5E)-3,5-Octadien-2-one	48730	0.27
45	14.954	2-Nonanone	13912	0.08
46	15.315	2,3,3-Trimethyloctane	160251	0.90
47	15.473	Nonanal	484166	2.71
48	16.047	3,5,5-Trimethyl-2-cyclohexenone	701488	3.92
49	16.964	2,6,6-Trimethyl-2-cyclohexene-1,4-dione	33128	0.19
50	17.920	3,5,5-Trimethyl-1,4-cyclohexanedione	17958	0.10
51	19.151	Dodecane	47638	0.27
52	19.354	Decanal	152864	0.85
53	21.877	2,3,6,7-Tetramethyl octane	31218	0.17
54	28.250	Tetradecane	61462	0.34
55	29.281	9-Nonadecene	677095	3.78

Table 3. The effect on protein values during storage in yoghurt experimental groups.

	Days				
Group	**1st day**	**7th day**	**14th day**	**21st day**	**28th day**
A	3.73 ± 0.01^{ze}	$3.81\pm0,04^{kd}$	$3.98\pm0,01^{yc}$	$4.23\pm0,02^{xa}$	4.11 ± 0.01^{xb}
B	3.74 ± 0.05^{zc}	3.91 ± 0.06^{zb}	3.92 ± 0.04^{yb}	4.18 ± 0.06^{xa}	4.28 ± 0.03^{xa}
C	3.86 ± 0.01^{yc}	4.00 ± 0.01^{yzbc}	3.89 ± 0.06^{yc}	4.14 ± 0.03^{xab}	4.18 ± 0.10^{xa}
D	3.83 ± 0.02^{yb}	4.04 ± 0.02^{yb}	3.49 ± 0.14^{zc}	4.14 ± 0.01^{xa}	4.17 ± 0.09^{xa}
E	4.02 ± 0.03^{xb}	4.31 ± 0.01^{xb}	4.31 ± 0.02^{xa}	4.27 ± 0.20^{xab}	4.15 ± 0.09^{xab}

a, b, c, d (→) Significant differences exist between the days indicated by different letters on the same line (P<0.05). x, y, z, k (↓) Significant differences exist between groups that are indicated by different letters in the same column (P<0.05). A: Control *L. acidophilus* LA5 (-), B: Control *L. acidophilus* LA5 (+), C: %0.5 bee bread, D: %1 bee bread, E: %2 bee bread.

Table 4. The effect on syneresis values during storage in yoghurt experimental groups.

	Days				
Group	**1st day**	**7th day**	**14th day**	**21st day**	**28th day**
A	14.47 ± 0.31^{ya}	$13.44\pm0,15^{yb}$	11.35 ± 0.50^{yd}	12.31 ± 0.02^{zc}	12.33 ± 0.31^{xc}
B	17.66 ± 0.07^{xa}	13.73 ± 0.19^{yb}	12.82 ± 0.56^{xc}	12.52 ± 0.38^{zc}	12.55 ± 0.60^{xc}
C	17.86 ± 0.01^{xa}	15.62 ± 0.32^{xb}	9.77 ± 0.35^{zkc}	9.97 ± 0.10^{yc}	8.79 ± 0.48^{yd}
D	17.62 ± 0.11^{xa}	15.60 ± 0.06^{xb}	8.80 ± 0.59^{kc}	9.26 ± 0.82^{yc}	8.87 ± 0.52^{yc}
E	17.76 ± 0.15^{xa}	15.19 ± 0.14^{xb}	10.38 ± 0.56^{yzc}	9.12 ± 0.01^{yd}	9.50 ± 0.41^{ycd}

a, b, c, d (→) Significant differences exist between the days indicated by different letters on the same line (P<0.05). x, y, z, k (↓) Significant differences exist between groups that are indicated by different letters in the same column (P<0.05). A: Control *L. acidophilus* LA5 (-), B: Control *L. acidophilus* LA5 (+), C: %0.5 bee bread, D: %1 bee bread, E: %2 bee bread.

Physicochemical Parameters: The physicochemical parameters, including pH, acidity, dry matter, ash, milk fat, and water holding capacity, were not significantly affected by adding bee bread (P>0.05). After analyzing the protein ratios of the yoghurt experiment groups, no significant difference was observed between the groups on the 21st and 28th days of the storage period (P>0.05). The group with the greatest protein ratio among all the groups was identified as E, with a statistical significance of (P<0.05). Protein levels in all groups increased over the last days of storage. (Table 3). As a result of examining the syneresis values of the various yoghurt experiment groups, it was found that the maximum syneresis occurred on the first day of storage (P<0.05). The evaluation indicated that there was a decrease in syneresis as the storage period increased, with a statistical significance of (P<0.05). The maximum syneresis value was observed on the initial day of study and in group C. The groups A and B demonstrated the highest syneresis value on the 21st and 28th days, while the groups containing bee bread showed a comparatively lower syneresis value (Table 4).

Microbiological Analysis: The addition of bee bread positively affected the growth of *S. thermophilus* and *L. bulgaricus*. Additionally, the activity of *L. acidophilus* LA-5 remained stable at approximately 10^7 kob/g throughout the storage period, with statistical significance (P<0.05). The results of the microbiological analysis are presented in Table 5.

Color Analysis: The L* values of the yoghurt experiment groups are presented in Table 6. During the initial analysis, it was observed that the L* value in group A was significantly greater than that in group E (P<0.05). There is an increase in the storage period towards the end compared to the initial days (P>0.05). The a* values of the yoghurt experimental groups are shown in Table 6. Group E had the greatest values up until the 14th day of storage, as shown by statistical analysis (P<0.05). During other analysis days, despite the apparently increased numbers, the statistical difference is not significant (P>0.05). Control groups A and B exhibited the lowest values on the 1th, 7th, and 14th days of storage, as assessed with statistical significance (P<0.05). While several groups exhibited statistical differences over different days, these variations could not be explained by the duration of storage. Upon analyzing the b* values of the different yoghurt experiment groups, it was noted that group E had the highest values (P<0.05). During the past two days of analysis, there was a significant difference in concentration between groups C and D (P<0.05). The control groups had significantly lower values compared to the other groups (P<0.05) (Table 6).

Table 5. *S. thermophilus*, *L. bularicus*, and *L. acidophilus* LA-5 bacteria growth values (log_{10} cfu/g) during storage in yoghurt experimental groups.

			***S. thermophilus* (log cfu/g)**		
Group	**1st day**	**7th day**	**14th day**	**21st day**	**28th day**
A	8.24 ± 0.24^{ya}	7.90 ± 0.09^{ya}	6.84 ± 0.25^{yb}	7.01 ± 0.07^{yb}	7.04 ± 0.02^{zb}
B	7.84 ± 0.19^{za}	8.03 ± 0.19^{ya}	7.99 ± 0.42^{xa}	7.48 ± 0.12^{xya}	7.77 ± 0.26^{ya}
C	8.87 ± 0.12^{xa}	8.15 ± 0.24^{xybc}	7.81 ± 0.42^{xbc}	7.74 ± 0.31^{xyc}	8.38 ± 0.06^{xab}
D	8.65 ± 0.17^{xa}	8.32 ± 0.40^{xya}	8.03 ± 0.16^{xab}	7.19 ± 0.99^{yb}	8.09 ± 0.05^{xyab}
E	8.82 ± 0.07^{xa}	8.64 ± 0.23^{xab}	8.39 ± 0.13^{xb}	8.41 ± 0.12^{xab}	8.45 ± 0.29^{xab}
			***L. bulgaricus* (log cfu/g)**		
Group	**1st day**	**7th day**	**14th day**	**21st day**	**28th day**
A	6.28 ± 0.05^{zc}	6.56 ± 0.09^{za}	6.94 ± 0.12^{zb}	6.39 ± 0.32^{zc}	5.58 ± 0.02^{kbc}
B	7.75 ± 0.14^{ya}	7.58 ± 0.15^{ya}	7.07 ± 0.21^{yb}	6.63 ± 0.33^{yb}	6.05 ± 0.03^{zc}
C	8.05 ± 0.22^{xya}	7.69 ± 0.31^{ya}	7.06 ± 0.06^{yb}	6.84 ± 0.33^{yb}	6.78 ± 0.19^{yb}
D	8.14 ± 0.19^{xa}	7.89 ± 0.14^{ya}	6.99 ± 0.16^{yb}	7.13 ± 0.04^{yb}	7.23 ± 0.23^{yb}
E	8.18 ± 0.10^{xab}	8.42 ± 0.13^{xa}	8.08 ± 0.09^{xab}	8.09 ± 0.04^{xab}	7.76 ± 0.40^{xb}
			***L. acidophilus* LA-5 (log cfu/g)**		
Group	**1st day**	**7th day**	**14th day**	**21st day**	**28th day**
A	-	-	-	-	-
B	7.88 ± 0.11^{za}	7.87 ± 0.10^{ya}	7.28 ± 0.04^{yb}	7.37 ± 0.08^{yb}	6.47 ± 0.08^{zc}
C	7.98 ± 0.05^{za}	7.74 ± 0.24^{ya}	7.24 ± 0.16^{yzb}	6.79 ± 0.02^{kc}	6.91 ± 0.02^{yc}
D	8.33 ± 0.15^{ya}	7.77 ± 0.18^{yb}	7.05 ± 0.08^{zc}	7.18 ± 0.08^{zc}	7.01 ± 0.07^{yc}
E	8.73 ± 0.15^{xa}	8.25 ± 0.13^{xb}	$7.75\pm0.11x^{c}$	7.67 ± 0.07^{xc}	7.70 ± 0.10^{xc}

a, b, c (→) Significant differences exist between the days indicated by different letters on the same line (P<0.05). x, y, z (↓) Significant differences exist between groups that are indicated by different letters in the same column (P<0.05). A: Control *L. acidophilus* LA5 (-), B: Control *L. acidophilus* LA5 (+), C: %0.5 bee bread, D: %1 bee bread, E: %2 bee bread.

Table 6. The effect of storage on L*, a*, b* color levels in different groups of yoghurt experiments.

			L* value		
Group	**1st day**	**7th day**	**14th day**	**21st day**	**28th day**
A	92.66 ± 2.39^{xa}	91.81 ± 3.08^{xa}	95.50 ± 0.37^{xa}	94.57 ± 0.53^{xya}	93.57 ± 0.51^{xa}
B	88.44 ± 1.95^{xyab}	87.33 ± 3.97^{xb}	87.28 ± 4.02^{yb}	95.51 ± 0.79^{xa}	94.09 ± 1.22^{xab}
C	86.45 ± 4.30^{xya}	84.85 ± 4.74^{xa}	86.64 ± 4.21^{ya}	91.24 ± 0.53^{xya}	89.10 ± 1.71^{ya}
D	88.44 ± 0.29^{xya}	86.92 ± 1.09^{xa}	89.99 ± 1.44^{xya}	87.12 ± 6.30^{ya}	91.03 ± 1.13^{xya}
E	84.49 ± 1.60^{ya}	83.69 ± 1.76^{xa}	86.51 ± 1.19^{ya}	$87.42\pm2,77^{xya}$	88.59 ± 1.59^{ya}
			a* value		
Group	**1st day**	**7th day**	**14th day**	**21st day**	**28th day**
A	1.60 ± 0.18^{za}	1.54 ± 0.45^{za}	1.60 ± 0.07^{za}	1.34 ± 0.11^{ya}	1.21 ± 0.17^{za}
B	0.93 ± 0.02^{zb}	1.35 ± 0.28^{zab}	1.24 ± 0.07^{zb}	1.37 ± 0.06^{yab}	1.77 ± 0.28^{zka}
C	2.45 ± 0.07^{ya}	2.97 ± 0.54^{ya}	3.16 ± 0.71^{ya}	2.45 ± 0.14^{ya}	2.67 ± 0.42^{yza}
D	3.09 ± 0.25^{ya}	2.97 ± 0.28^{ya}	3.45 ± 0.05^{ya}	3.58 ± 0.89^{xa}	3.80 ± 0.96^{xya}
E	4.98 ± 0.53^{xab}	4.49 ± 0.19^{xabc}	5.06 ± 0.10^{xa}	4.20 ± 0.15^{xc}	4.28 ± 0.23^{xbc}
			b* value		
Group	**1st day**	**7th day**	**14th day**	**21st day**	**28th day**
A	4.23 ± 0.51^{za}	3.53 ± 0.09^{ka}	4.37 ± 1.46^{za}	3.30 ± 0.38^{ka}	3.04 ± 0.13^{ka}
B	4.73 ± 0.59^{zb}	5.89 ± 0.11^{za}	3.89 ± 0.12^{zc}	2.24 ± 0.09^{ld}	2.88 ± 0.14^{kd}
C	9.61 ± 0.29^{yb}	13.40 ± 0.59^{ya}	9.76 ± 0.03^{yb}	8.01 ± 0.10^{zc}	9.42 ± 0.05^{zb}
D	11.50 ± 1.38^{yb}	13.42 ± 0.49^{ya}	9.96 ± 0.16^{ybc}	9.27 ± 0.01^{yc}	9.79 ± 0.14^{ybc}
E	18.58 ± 0.01^{xa}	18.27 ± 0.15^{xa}	16.51 ± 0.30^{xb}	13.86 ± 0.03^{xd}	15.76 ± 0.04^{xc}

a, b, c, d (→) Significant differences exist between the days indicated by different letters on the same line (P<0.05). x, y, z, k, l (↓) Significant differences exist between groups that are indicated by different letters in the same column (P<0.05). A: Control *L. acidophilus* LA5 (-), B: Control *L. acidophilus* LA5 (+), C: %0.5 bee bread, D: %1 bee bread, E: %2 bee bread.

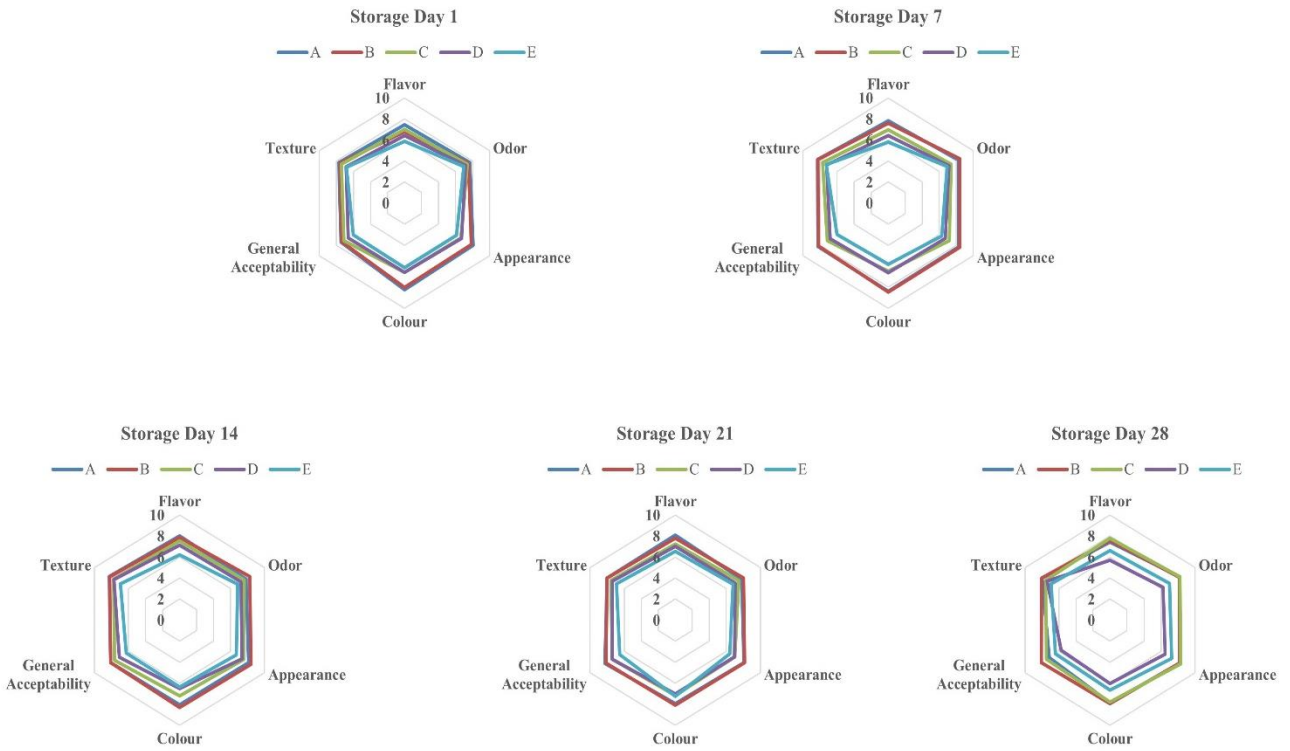

Figure 2. A graphical representation showing the sensory analysis values observed on the 1st, 7th, 14th, 21st, and 28th days of storage.

Sensory Analysis: Following the conclusion of a sensory analysis, consumers indicated that they favored the group that was provided with 0.5% bee bread. The results of the sensory evaluation are shown in Figure 2.

Discussion and Conclusion

Bee bread has been identified as a significant protein source (19.96/100g). Additionally, it is rich in total free sugar (18 grams per 100 grams), macroelements, microelements, polyunsaturated fatty acids, tocopherol, and natural antioxidants. Furthermore, it was ascertained that bee bread exhibited antioxidant properties and demonstrated efficacy against all examined bacteria and fungi (5). Similar to this study, Bakour et al. (4) identified fructose (118 mg/g) and glucose (57 mg/g) as primary components. In a study conducted in Romania, Dranca et al. (14) identified the presence of gluconic acid, formic acid, acetic acid, propionic acid, and butyric acid. Bakour et al. (4) identified the presence of oxalic acid in bee bread in another study. According to a recent investigation conducted by Bayram et al. (7), an analysis of phenolic components in pollen and bee bread demonstrated elevated levels of protocatechuic acid, 2,5-dihydroxybenzoic acid, and kaempferol in bee bread. The kaempferol content of the data obtained from this study shows similarities. In a survey conducted by Bakour et al. (4), it was observed that the α-tocopherol content measured 10.5 µg/g, while the δ-tocopherol content measured 0.40 µg/g, exceeding the levels observed in the present study. In a study conducted by Hryniewicka et al. (19), the researchers determined that the α-tocopherol content of bee bread was measured to be 80±30 µg/g. Differences in values could potentially be attributed to variations in botanical provenance. In the context of this research, it is important to determine the existence of tocopherol in bee bread. GC/MS SPME aroma profile study by Kaškonienė et al. (25) found 32 components in bee bread and honey. Dimethylsulfide, acetic acid, furfural, nonan, and 1-heptadekene are 20.0%, 13.4%, 9.8%, 10.4%, and 13.9%, respectively. According to the findings of Bakour et al. (4), the mineral content of bee bread in this study exhibited comparable values. Specifically, the mineral content of bee bread was found to be as follows: calcium (Ca) at 1.98 mg/g, iron (Fe) at 0.273 mg/g, potassium (K) at 3.38 mg/g, magnesium (Mg) at 0.61 mg/g, sodium (Na) at 0.142 mg/g, zinc (Zn) at 0.0331 mg/g, phosphorus (P) at 2.51 mg/g, and manganese (Mn) at 0.026 mg/g. In a previous study conducted by Andjelkovic et al. (1), the primary mineral identified as potassium (K), with phosphorus (P), calcium (Ca), and magnesium (Mg) following as secondary minerals. The primary origin of mineral substances within bee bread is from flower pollen, which is a significant mineral reservoir in both nectar and water. According to Andjelkovic et al. (1), geographical conditions can influence the mineral substance content. Ivanišová et al. (21) found that 15.78 mg TEAC/g was the highest antioxidant activity in bee bread samples collected from

five distinct localities within Ukraine. The IC_{50} value of bee bread was calculated to be 1.414 mg/mL in this study.

According to the findings of Khider et al. (26), adding 1% pollen to yoghurts resulted in a decrease in syneresis, an improvement in texture, and a pleasant aroma. The individual stated that the rheological characteristics and the presence of advantageous bacteria in fermented beverages were altered upon the addition of bee pollen. The study conducted by Yerlikaya (47) found no discernible adverse consequences associated with the incremental addition of pollen. Another study investigated the impact of different pollen rates (0%, 5%, 1%, 2.5%, and 3%) on the bio-functional properties of yoghurt produced from cow, sheep, and goat milk. Research findings have indicated that the inclusion of pollen in yoghurts increases their antioxidant capacity and total phenolic content. Furthermore, enhancements were observed in the sensory attributes of yoghurt, including taste, aroma, visual appeal, and texture. According to a study conducted by Karabagias et al. (24), it has been suggested that incorporating bee pollen into yoghurts could potentially serve as a cost-effective means of producing functional food products, thereby holding significant promise for future applications. A research study using bee bread as an additive determined that the pH level exhibited greater intensity than the control group.

The color attribute of foods is regarded as a significant factor in determining consumer acceptance (29). The observed disparity in L* values between groups A and E in this study may be attributed to the absence of bee bread in the first group. According to Ozcan et al. (35), there is a negative correlation between adding pollen to yoghurt and the L* value, indicating a decrease in the L* value as the amount of pollen increases. The higher b* and a* values observed in group E could potentially be attributed to the excess concentration of bee bread. The control groups in both color groups were found to have lower values compared to the other groups. The potential explanation for this could be the lack of bee bread within these groups. In their study, Ozcan et al. (35) conducted an evaluation that revealed that the inclusion of pollen resulted in a reduction of b* and a* values. The observed differences are believed to have originated from the related structural differences between bee bread and pollen. Insufficient research has been conducted on using bee bread to produce yoghurt.

This study targeted to investigate the potential impact of bee bread on probiotic bacteria during the storage period of yoghurt. Upon analysis of the acquired data, it was discovered that the experimental groups, which were supplemented with bee bread, exhibited a notable enhancement in the population of *L. acidophilus* LA-5. In an additional study, the impact of integrating pine honey into yoghurt at varying concentrations (2%, 4%, 6%) on the activity of *L. acidophilus* was assessed. The study findings revealed a notable reduction in the population of microorganisms during the preservation procedure, specifically a lower count of *L. acidophilus* compared to the mentioned study. The highest recorded count was determined to be 7.70 log cfu/g. It can be said that bee bread demonstrates a greater impact on probiotic activity compared to pine honey (12). This study suggests that the inclusion of bee bread generally resulted in higher probiotic activity in the respective groups. The maintenance of probiotic activity is estimated to be sustained at approximately 10^7 colony-forming units per gram (cfu/g). According to the findings of Demirci et al. (13), it is recommended that the concentration of probiotic bacteria should be no less than 10^7 colony-forming units per gram (cfu/g) in order to produce beneficial health effects. In their study, Panesar et al. (37) observed that the probiotic microorganisms *L. acidophilus* and *B. bifidum* maintained their continuity in probiotic yoghurt containing *Aloe vera* even after storage.

The data collected from the panelists during the sensory analysis conducted in this study generally exhibited values of 5 or higher. The panelists generally rated Group C (0.5%), one of the experimental groups that received bee bread supplementation, as more acceptable. Certain storage durations and sensory characteristics have been observed to produce a higher preference for groups containing added probiotic bacteria than those without. The observed improvements in sensory parameters can be attributed to the introduction of probiotic bacteria, as suggested by Atallah (3). According to a study conducted by El-Kholy et al. (15), yoghurts that incorporated nano-encapsulated pollen were found to have satisfactory sensory attributes. Insufficient sensory analysis data is available for producing yoghurt using bee bread. Hence, the significance of this study cannot be overstated.

In conclusion, the study findings indicate that bee bread possesses significant importance as a bee product due to its composition, which includes high levels of sugar content, organic acids, tocopherols, mineral substances, phenolic components, volatile components, and antioxidant substances. The presence of natural components in the composition of bee bread has been found to have advantageous impacts on human health. Bee bread, because of its high content of mineral and phenolic components, maintains the potential for the development of food products or food additives. Studies can be conducted to explore the innovative aspects of the antioxidant activity observed in bee bread. The study has demonstrated that adding bee bread during yoghurt production can effectively maintain probiotic activity. The inclusion of bee bread in yoghurts has been found to be positively perceived by consumers as a health-promoting

DOI: 10.33988/auvfd.1326701

product. This study presented significant data regarding the utilization of bee bread within the food industry.

Acknowledgements

This study was derived from the Ph.D. thesis of the first author.

Financial Support

This research has been supported within the context of project no: 0770-DR-21/2017K12-41003 by The Scientific Research Projects Committee of Burdur Mehmet Akif Ersoy University.

Conflict of Interest

The authors declared that there is no conflict of interest.

Author Contributions

The experiments were conceived and planned by NK and OY. NK and OY conducted the experiments. NK and OY conceived and executed the simulations. NK and OY contributed to the interpretation of the results. NK managed the composition of the manuscript. All authors provided constructive feedback and contributed to developing the research, analysis, and manuscript.

Data Availability Statement

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

Ethical Statement

This study does not present any ethical concerns.

Animal Welfare

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

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DOI: 10.33988/auvfd.1326701

Evaluation of colostrum quality and passive transfer immunity in terms of heat stress and disease incidence in Holstein cattle in Central Anatolia

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ARTICLE INFO

Article History
Received : 27.08.2023
Accepted : 08.03.2024
DOI: 10.33988/auvfd.1346189

Keywords
Colostrum Quality
Heat stress
Passive transfer failure
Passive transfer immunity
Temperature humidity index

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How to cite this article: Kara H, Güven M (2024): Evaluation of colostrum quality and passive transfer immunity in terms of heat stress and disease incidence in Holstein cattle in Central Anatolia. Ankara Univ Vet Fak Derg, 71 (4), 481-486. DOI: 10.33988/auvfd.1346189.

ABSTRACT

The effects of heat stress on colostrum quality, passive transfer failure, and disease incidence were investigated in a large population in order to prevent calf morality and yield losses in Holstein cows and calves. There was a statistically significant correlation between colostrum quality and the daily temperature humidity index, 7-day average temperature stress, and average temperature humidity indexes experienced by the cows during the dry period ($P<0.001$). It was observed that passive transfer failure occurred in 21% of the calves. There was a significant positive correlation ($P<0.05$) between the relative humidity and the temperature and humidity index values of the day of birth and the calf serum brix value. A negative and significant correlation was observed between Temperature-Humidity Index (THI) and serum brix value ($r = -10$, $P<0.01$). It was observed that the passive transfer success and diarrhea and pneumonia that were overcome in the first 365-day period showed a negative correlation ($P<0.01$). As a result, it has been observed that the heat stress experienced by cows and calves affects colostrum quality and passive transfer success, which also affects development and protection from diseases.

Introduction

Due to the special placental structures of cows, newborns are born with agammaglobulinemia (25). Calves need maternal Ig transferred with colostrum for the natural formation of the immune system in the neonatal period (22). In calves, macromolecules such as Ig taken with colostrum after birth are absorbed directly by intestinal epithelial cells without any change and go into circulation (10). Ideal colostrum feeding should be done by giving colostrum in sufficient quantity and quality as soon as possible from the moment the calf is born (3).

The most critical period is the neonatal period, as it is the period with the highest incidence of diseases and deaths for dairy cattle enterprises (20). Successful passive transfer immunity is required for economically sustainable herd management (1). For a successful passive immune transfer, the calf's serum IgG concentration is expected to rise above 10 g/L between 32 and 48 hours (8). Failure of passive transfer is not a disease, but it increases the likelihood of disease and adversely affects the development of the calf (25).

It is thought that heat stress may have potential effects on colostrum quality and passive transfer success (PTS) (12, 26). Heat stress occurs when body temperature exceeds the thermoneutral range due to inadequate temperature regulation. It is evaluated using this method, as it also evaluates the effect of increasing humidity on reducing heat loss (7). An alternative methodology for evaluating cooling requirements in cattle involves the utilization of the Temperature Humidity Index (THI). This composite metric, incorporating both ambient temperature and relative humidity, has demonstrated superior efficacy

compared to the sole consideration of temperature in gauging the environmental influences on lactating cattle (5).

The aim of this study is to investigate the effects of heat stress, which may affect maternal productivity and cause calf deaths, on colostrum quality, passive transfer success, and disease incidence in calves.

Materials and Methods

Animals: The animal material for this study was obtained from a professional dairy farm with a 2000 dairy cows' capacity in the Bala district of Ankara, located between 39° north latitude and 33° east latitude. The data we used in our study belongs to 1043 Holstein calves and their mothers born between June 2020 and July 2021. Stillbirth, abortion, animals that died in the first 48 hours, and animals whose animal health cards could not be accessed were excluded from the study. The cows were housed in a semi-open free-stall dairy barn, and the calves were housed in calf huts. Cows were fed a total mixed ration (soybean meal, canola meal, silage, straw, vitamin-minerals, molasses, and water). Feeding and vaccination programs during the dry period and postpartum are standardized. On the farm, newborn calves are fed the colostrum that the calf accepts to drink every 2 hours, starting within the first hour after birth. Calves that drink less than 2 liters of milk in the first feeding are fed with an oesophageal probe, and the minimum colostrum volume in the first feeding is completed to 2 liters.

Data Collection: The data included in the study were obtained from the witness samples recorded and stored under the newborn protocols of the farm. Date of birth, time of birth, colostrum quality, diarrhea, and cases of pneumonia in the first 365 days were recorded.

Colostrum quality was measured by trained delivery room personnel with an optic brix refractometer (ATC LYK SUR-1 Clinical Refractometer, China) with a range of 0 to 32% brix. When evaluating the results, the brix 22% value was accepted as equivalent to 50 g/L Ig density, which is considered the limit of good-quality colostrum, and below this value was classified as poor-quality colostrum (3). The blood serum taken from the calf between 32 and 48 hours after birth was removed and frozen at -20 °C. Cryo-serum samples were thawed in the laboratory and re-evaluated by a researcher using an optic brix refractometer (Index Instruments, Cambridge, UK) with a range of 0 to 32% Brix and recorded as "serum brix value." When evaluating the results, brix 8.4% was accepted as the cut-off value for PTS, and below this value was considered passive transfer failure (serum IgG < 10 g/L) (2). The birth score was evaluated according to the degree of intervention at birth. The unassisted birth of the cow was numbered with 1 point. Operation caesarean section was evaluated at 5 points (16).

The temperature-humidity index (THI) was calculated by taking the lowest and highest temperature and relative humidity data from the general directorate of meteorology. It was calculated using the formula THI [(Temperature, Humidity) = (9/5 x temperature + 32) – (11/20 – 11/20 x humidity) x (temperature – 26)] (19). The daily THI was calculated using the meteorological data on the day the cows gave birth. By using the calculated daily temperature and humidity indices, the average of the stress experienced by the cows during the dry period (2 months), the stress experienced during the 7 days before birth, and the 2-day stress level of the calf were calculated. The cut-off value was not used during statistical analysis to prevent data loss. However, THI was evaluated as 68–71 normal stress, 72–79 average stress, 80–89 severe stress, and 90–98 very severe stress (5).

Statistical Analysis: The data were analyzed with RStudio with the R 4.3 version and are presented as mean ± SD. Because the samples did not follow a normal distribution according to the Shapiro-Wilk test ($P<0.05$), non-parametric tests were applied for statistical analysis. We used Spearman correlation to investigate the relationship between variables. A two-sample Wilcoxon test (Mann-Whitney equivalent) was used for the comparison of groups.

Results

The average quality of the evaluated colostrum was calculated as brix 28.75%. 13% of the cows produced poor-quality colostrum (brix<22%). In 21% of the calves, serum brix values were below 8.4%, which is considered the cut-off value for failure of passive transfer (FPT) (Figure 1). The factors affecting colostrum quality and passive transfer success are reported in Table 1. In the first 365 days, diarrhea was observed in 13.71% and pneumonia in 24.35% of all calves. In calves with passive transfer failure, 16.81% diarrhea and 30.97% pneumonia were observed in the first 365 days.

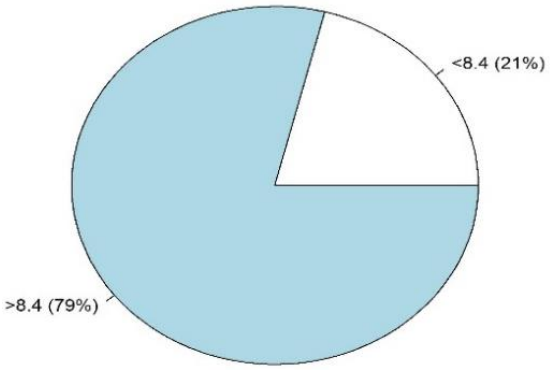

Figure 1. Serum Brix Value in Calves.

The minimum temperature during the study period was -5.4 °C, and the maximum was 36.1°C. Humidity varied between 10.5% and 95.8%. Heat stress was determined by using the temperature-humidity index formula according to the daily temperature and humidity findings to which the animals included in the study were exposed. THI ranged from 24.7 to 92.43 over the study period. According to the THI scale, 68.39% of the 1043 animals were at a normal stress level or below, 21.37% at a moderate stress level, 9.77% at a severe stress level, and 0.47% at a severe stress level. When the average THI was calculated during the dry period, 86.83% of the cows were under normal stress, 13.08% were under average stress, and 0.09% were under severe stress. There was a positive and statistically significant correlation between the temperature stress experienced by the cows during the dry period (P<0.001), last week (P<0.001), calving day (P<0.001), and colostrum quality. Relative humidity had no significant effect on colostrum quality (P>0.05) (Table 1).

The effects of factors such as diarrhea (Table 2, Fig. 2) and pneumonia (Table 3, Fig. 3) on colostrum quality and passive transfer success in pairwise comparison have been reported.

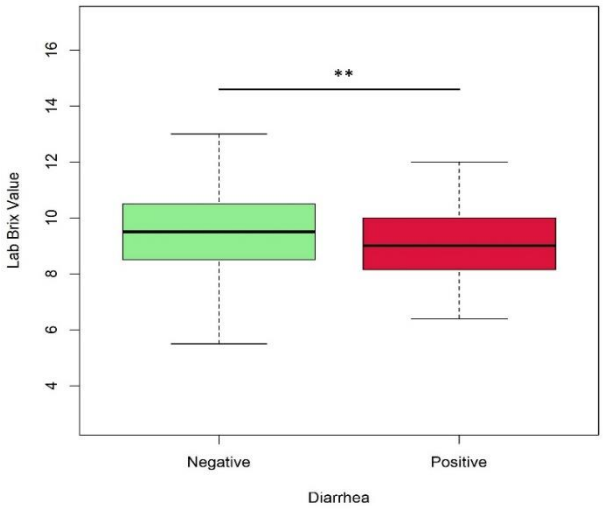

Figure 2. Relationship between diarrhea and PTS.
(* P<0.05, ** P<0.01, *** P<0.001, ns: not significant).

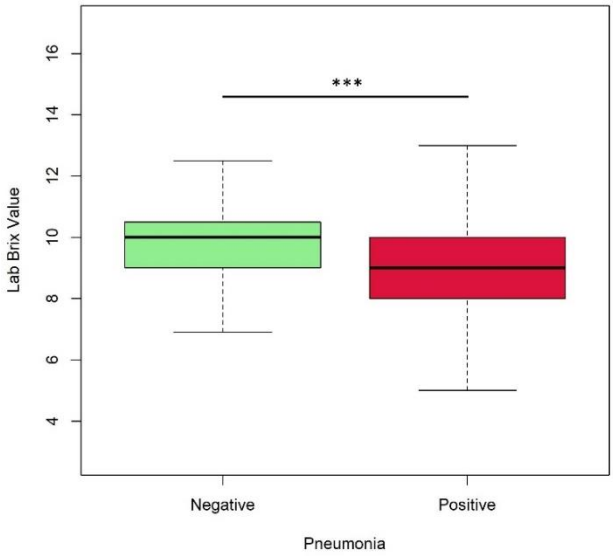

Figure 3. The relationship between pneumonia and PTS.
(* P<0.05, ** P<0.01, *** P<0.001, ns: not significant).

Table 1. Relationships between colostrum quality, PTS, and other factors.

	n	Mean	St. Dev.	Q1	Median	Q3	Corr. with Serum Brix value		Corr. with Colostrum Quality	
							r	P value	r	P value
Serum Brix Value	878	9.47	1.49	8.50	9.50	10.50	-	-	-0.02	0.54
Colostrum Qual.	789	28.75	3.34	27.00	30.00	31.00	-0.02	0.54	-	-
Daily THI	878	62.65	14.61	52.18	64.31	74.21	-0.10	<0.01	0.25	<0.001
Pre-natal THI (2-month)	878	57.38	10.64	48.43	53.43	68.72	-0.14	<0.001	0.36	<0.001
Pre-natal THI (7-day)	878	62.29	13.56	50.62	64.77	74.05	-0.15	<0.001	0.27	<0.001
Post-natal THI (2-day)	878	62.80	14.58	51.20	64.71	74.93	-0.10	<0.01	0.27	<0.001
Humidity	878	59.60	17.83	47.50	61.60	73.05	0.09	<0.01	-0.03	0.49

n: sample size, Mean: Arithmetic mean, St. Dev: Standard deviation, Q1: first quartile, Q3: third quartile, r: correlation coefficient.

Table 2. Relationship between diarrhea and serum Brix value.

	Min	Q1	Median	Mean	Q3	Max.	n	St. Dev.
Diarrhea	5.100	8.150	9.000	9.226	10.000	16.000	127	1.524
Healty	2.800	8.500	9.500	9.515	10.500	17.000	751	1.482

min: Minimum, Q1: First quartile, Q3: Third quartile, Max: Maximum, n: sample size, St. Dev.: Standard deviation.

Table 3. Relationship between pneumonia and serum Brix value.

	Min	1st Qu.	Median	Mean	3rd Qu.	Max.	n	sd
Pneumonia	3.200	8.000	9.000	9.094	10.000	16.000	232	1.615
Healty	2.800	9.000	10.000	9.609	10.500	17.000	646	1.420

min: Minimum, Q1: First quartile, Q3: Third quartile, Max: Maximum, n: sample size, St. Dev.: Standard deviation.

Discussion and Conclusion

In our study, a significant, positive correlation was detected between the heat stress exposed to cows and colostrum quality (Table 1). There is no significant effect of relative humidity on colostrum quality ($P > 0.05$). There are doubts about the effect of heat stress on colostrum quality. Zentrich et al. (28) reported a negative correlation between colostrum quality and heat stress in a study of 2500 Holstein Friesian cows. Nardone et al. (18) reported in a study that they investigated the effect of temperature on colostrum quality and found that cows that are exposed to heat stress have lower colostrum quality. Consistent with our study, Gulliksen et al. (11) reported that colostrum increased during the period when seasonal temperatures increased. Additionally, Nardone et al. (18) reported that the amount of colostrum decreased in cows exposed to high temperatures. It is thought that the effect of heat stress on colostrum quality can be explained by the decrease in the amount of colostrum due to heat stress (18), the decrease in the amount of colostrum causing an increase in IgG concentration, as in low milk yielding cows (14), and as heat stress increases, the amount of IgG passing into the colostrum increases by increasing the permeability in the blood vessels due to the effect of vasodilation (21).

Heat stress, which we determined with the average THI values of the dry period, 7 days before birth and 2 days after birth, was compared with calf serum brix values, from which we obtained information about the passive transfer success of calves. A negative correlation was determined between prenatal 2-month THI ($P<0.001$), prenatal 7-day THI ($P<0.001$), postnatal 2-day THI ($P<0.01$), and calf serum brix value (Table 1). Tao et al. (23) reported a significant decrease in serum IgG ($P=0.03$), total protein ratio ($P<0.01$), and absorption efficiency ($P<0.01$) in calves born to cooled and high temperature-exposed cows (24). In a similar study by Laporta et al. (15), it was reported that the IgG ratio and absorption efficiency measured at 24 hours were significantly ($P<0.05$) lower in calves of cows exposed to high temperatures before birth. This is thought to be caused by impairments in intestinal surface area and absorption rather than colostrum quality (6). There was a positive correlation between the heat stress to which the calf was exposed on the day of birth and the serum brix value obtained at 32–48 h, and it was statistically significant.

No statistically significant result was observed between colostrum quality and serum brix value. Colostrum quality is important for PTS, but for successful passive transfer, colostrum delivery rate and colostrum quantity are as important as quality (9). It has been reported that the second colostrum administration within the first 12 hours in newborns leads to an increase in serum Ig levels at 24-48 hours (17). Jester et al. (13) reported serum IgG levels of 38.6 mg/mL and 45.6 mg/mL, respectively, in a study in which 4 L of colostrum was administered to calves immediately after birth as a whole and divided into two applications. Similarly, in this study, calves received colostrum as much as they needed immediately after birth and were fed every 2 hours. Therefore, although colostrum quality and serum protein concentrations were not significant, this is thought to be the reason for the high PTS of the herd.

When the incidence of diarrhea and pneumonia in PTS was evaluated, it was observed that there was a positive correlation and statistically significant relationship between serum brix value and diarrhea ($P<0.01$) and pneumonia ($P<0.001$). Consistent with these results, Caffarena et al. (4) reported that serum IgG and serum protein concentrations were lower and statistically significant in groups with diarrhea. Again, in accordance with our results, Windeyer et al. (27) found passive transfer failure in 32% of 2874 calves. It was reported that the probability of pneumonia before 5 weeks was 13% in those without PTF, while this rate increased to 18% in

those with PTF. Successful passive transfer immunity was found to be effective in protecting against diarrhea and pneumonia in the early stages of life and afterward.

As a result, it was observed that heat stress determined by THI data had an effect on colostrum quality and serum brix value, which is an indicator of passive transfer success in calves. In addition, in this study, it was determined that PTS directly affected the diarrhea and pneumonia recovery rates in the first 1-year period. It has been observed that there is a positive and significant relationship between heat stress and colostrum quality, but this is actually due to the increase in concentration, as in low milk-yielding animals. It has been understood that cows should be protected against heat stress during and after the postpartum period in order to reduce the incidence of disease and increase the survival rate of newborns. Measures should be taken by farms to reduce the heat stress of cows and calves. It is thought that new studies on this subject should be supported, and breeders should be educated.

Financial Support

No financial support was received.

Conflict of Interest

The authors declared that there is no conflict of interest.

Author Contributions

Design: HK and MG; Control/Supervision: HK, MG; Data Collection and / or Processing: HK, MG; Analysis and / or Interpretation: HK, MG; Literature Review: HK, MG Writing the Article: HK, MG; Critical Review: HK, MG.

Data Availability Statement

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

Ethical Statement

The present study was approved by the Animal Research Ethics Committee of the University of University of Ankara (Ethics approval number: AÜHADYEK, number 2022-21-184).

Animal Welfare

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

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Effects of common centaury (*Centaurium erythraea*) oil and laurel (*Laurus nobilis*) seed oil on full-thickness excisional skin wound healing in rats

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ARTICLE INFO

Article History
Received : 10.03.2022
Accepted : 19.10.2023
DOI: 10.33988/auvfd.1085562

Keywords
Alternative
Centaurium
Dermatology
Herbal oil
In vivo

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How to cite this article: Semiz N, Deveci MZY (2024): Effects of common centaury (*Centaurium erythraea*) oil and laurel (*Laurus nobilis*) seed oil on full-thickness excisional skin wound healing in rats. Ankara Univ Vet Fak Derg, 71 (4), 487-496. DOI: 10.33988/auvfd.1085562.

ABSTRACT

The aim of this study was to investigate the effects of common centaury (*Centaurium erythraea*) oil and laurel (*Laurus nobilis*) seed oil in a full-thickness excisional skin wound model in rats. In the present study, 18 adult male Wistar rats were divided into three groups (n=6) the control (CO) group, the common centaury oil (CCO) group, and the laurel seed oil (LSO) group. Under general anesthesia, a full-thickness excisional wound (2.25 cm²) was created on the caudal of the interscapular region on the back of the rats. Treatments were applied topically once a day in all groups. Wound area measurements revealed that the use of CCO accelerated wound healing, while the use of LSO disturbed the healing process (P≤0.001). In the histopathological results, blood vessel formation, fiber synthesis, granulation, and mononuclear cells in the wounds were higher in the CCO group than the other groups and higher in the LSO group than the CO group. Biochemical results revealed differences between groups in TP, GLU, and UREA values (P<0.05). As a result, it was determined that the topical use of common centaury oil accelerated wound healing, while laurel seed oil adversely affected wound healing in the experimental excisional full-thickness skin wound model in rats.

Introduction

Wound healing is a complex physiologic process that consists of different cell types and cellular phenomena or reactions. It includes three intricate stages of inflammation, tissue regeneration, and remodeling of new tissue (maturation). These stages may take different timescales, from several days to months or years. The aims of the treatment are to support wound healing and prevent any failure leading to nonhealing wounds (42, 45). Chronic wounds may lead to severe morbidity and mortality, especially in older individuals, particularly those with concurrent diseases such as diabetes mellitus and vascular diseases. Nonhealing wounds require high costs for treatment and significantly affect the quality of life (15, 45). Also, surgery is a potential wound complication cause (4). Nearly 234 million surgeries are performed worldwide every year, which is an important threat to public health and the economy. Although many studies have been conducted on wound healing, medical plants and herbal mixtures are needed to be further investigated considering their properties, such as being easily accessible, inexpensive, effective, and having limited adverse effects (42, 45, 51). In order to improve wound healing processes, promising therapeutics based on the active components have been used (8).

Natural product-based compounds are preferred over synthetic products to improve wound healing (7, 31). Researchers have been exploring herbal medicines and

mixtures for wound treatment for years. A wide range of herbs have been investigated so far. *Laurus nobilis Linn.* (bay) has been used traditionally, and its neuroprotective, antioxidant, antiulcerogenic, anticonvulsant, analgesic, anti-inflammatory, antimutagenic, antiviral, antibacterial, antifungal, and anticholinergic effects have been reported (1-18, 20). Common centaury herb and oil are known in traditional medicine for their tonic, sedative, digestive, antipyretic, and antipyretic properties and have been used in diabetes, hepatitis, gout, indigestion, gastritis, and inflammation. Its external use is known for inflammation, wound treatment, snake bites, and eczema-like conditions (50). There are many in vivo and in vitro studies on the medical effects of *C. erythraea*. Anti-inflammatory, antipyretic, antidiabetic, diuretic, and hepatoprotective effects have been reported in vivo studies, as have gastroprotective, antidiabetic, antioxidant, antibacterial, cytotoxic, antimutagenic, and insecticidal effects in in vitro studies (11, 25, 50).

The aim of this study was to evaluate the wound-healing activity of common centaury (*Centaurium erythraea*) oil and laurel (*Laurus nobilis*) seed oil on the excisional full-thickness skin wound model in Wistar rats in terms of wound healing, clinical, histopathological, and biochemical changes.

Materials and Methods

Chemical Analysis of Herbal Oils Used in Treatment: Common centaury (*Centaurium erythraea*) oil, Laurel (*Laurus nobilis*) seed oil, and corn (*Zea mays*) oil were purchased from a local traditional herbal oil vendor (in Türkiye). GC-MS analyses were performed to reveal the chemical components of the oils. For this purpose, each oil was diluted 1/20 with high-purity ethanol, and aromatic components were analyzed using a GC-MS (Shimadzu GCMS QP 2010 ULTRA) analyzer. Capillary column (RTX-5MS; 30 m; 0.25 mm; 0.25 m) and helium were used as carrier gas for the analysis. Column furnace, interface, ion source, and injection temperatures were adjusted to 40°C, 250°C, 200°C, and 250°C, respectively. The injection volume was 1 µl, and the split (1/5) method was used for injection. During analysis, 3 min at 40°C, 4°C/min increment from 40°C to 240°C, 10 min at 240°C, 4°C/min increment from 240°C to 260°C, and 10 min at 260°C, a total of 78 minutes of oven cycle were applied (21). Chromatograms and ingredient lists of the chemical components of the oils were obtained.

Animals and Experimental Design: The ethical approval of this study was obtained from the Local Ethics Board of Animal Experiments of Hatay Mustafa Kemal University (Decision No: 2020/04-34). Experiments were performed in accordance with the Turkish Code of the Welfare and Protection of Animals Used for Experimental and Other Scientific Purposes and Directive 2010/63/EU on the protection of animals used for scientific purposes. In total, 18 healthy adult male Wistar rats (400–550 g) were purchased from the Hatay Mustafa Kemal University Experimental Research and Application Center. One week prior to the study, the animals were taken to the study site to undergo routine health checks, and time for adaptation was provided. All rats were maintained individually in standard cages with water and food provided ad libitum on a 12:12-h light-dark cycle in a climatically controlled room. Following general anesthesia induction (Ketamine HCl 50 mg/kg and Xylazine HCl 5 mg/kg, i.m.), the dorsal neck area of each animal was shaved, disinfected, and a 1.5x1.5 cm (2.25 cm^2) sized square full-thickness excisional wound created. The animals were separated into the three experimental groups (n=6) randomly. Group 1 was treated with corn (*Zea mays*) oil as a control group (CO), group 2 with common centaury oil (CCO), and group 3 with laurel seed oil (LSO). All the treatments were applied topically, covering all wound areas (~1 ml), once a day for 14 days in total. No other drugs were used post-surgically.

Clinical Examinations and Wound Area Measurement: Before topical treatment, local examinations of wound areas and general clinical examinations of animals were conducted daily. The body weight, feed, and water consumption of each animal were determined weekly. The wound areas of each animal were photographed individually by a digital camera at 0, 1, 4, 7, 11, and 14 days after wound creation. The wound surface area (cm^2) was measured using Image J software.

Collection of Tissue: 14 days after applying the treatment, the rats were deeply anesthetized with the combination of xylazine HCl (10 mg/kg, i.p.) and ketamine HCl (100 mg/kg, i.p.) and sacrificed. The wound tissues were removed with a surgical blade and scissor. The wound samples of each animal were preserved in 10% formalin separately until histopathological analysis.

Histopatological Analysis: The specimens were fixed in 10% neutral buffered formalin for 24 hoursand dehydrated in a series of graded ethanol solutions (60, 70, 80, 90, and 100%). Following xylol treatment, embedded in paraffin wax. Paraffin-embedded sections were sequentially sliced at a thickness of 4 µm by a microtome (Leica RM2235®, Almanya), stained with hematoxylin and eosin and further evaluated under light microscopy (Olympus BX50-F4, Tokyo, Japan). Histopathological evaluation of inflammatory processes and healing in the scar tissue was scored by examining the number of blood vessels, granulation, fiber synthesis, and mononuclear cells. In this scoring, the relevant parameters were evaluated as absent/unformed (0), low (1), moderate (2), high (3), and severe (4).

DOI: 10.33988/auvfd.1085562

Biochemical Analysis: Blood samples were centrifuged (3000 rpm, 10 min), and their serums were separated. Serum samples were analyzed for ALP, AST, CRE, UREA, GLU, and TP values individually by a biochemistry autoanalyzer.

Statistical Analysis: Statistical analyses of the results were performed with the SPSS (Statistical Package for Social Sciences, 26.0) program. The sample size of the study was determined by reference to recent similar scientific studies (19, 36). Descriptives of the results were presented as mean ± standard error of mean. After the normality tests, one-way ANOVA and post-hoc Tukey tests were used to evaluate the differences between the groups. Repeated measured variance analysis following the Bonferroni test was applied for the wound area alterations by time comparisons between groups. The significance was set at $P<0.05$.

Results

GC-MS Analyses: The highest component of CCO was determined to be octadec-9-enoic acid (25.57%), LSO's oleic acid (22.44%), and CO's oleic acid (26.09%). The GC-MS analysis results of CCO, LSO, and CO used in the wound treatments are presented in Table 1 by specifying the substances with the highest ratio. GC-MS chromatogram images are presented in Figure 1.

Clinical Results and Wound Healing: Respiration and vital behaviors were observed as normal in all experimental animals. However, a stressed and mildly depressed appearance was observed in four individuals in the LSO group. The body weight, feed, and water consumption alterations of the groups are presented in Table 2. Local wound sensitivity was increased in animals in the LSO group, and the animals were observed to be irritated by LSO. The wound area results on certain days in all groups are presented in Table 3 and Figure 2. And the time-group wound area comparisons are presented in Table 4.

Table 1. Most-to-lowest-ordered components from GC-MS analysis results of the CCO, LSO and CO.

%	**Common Centaury (*Centaurium erythraea*) Oil**
25.57	Octadec-9-enoic acid
15.04	9,12-Octadecadienoic acid (Z,Z)-
10.66	Palmitic acid
8.33	Di-(9-octadecenoyl)-glycerol
7.60	Octadecanoic acid
7.58	Hexadecanoic acid, 1-(hydroxymethyl)-1,2-ethanediyl ester
25.22	Other components*
%	**Laurel (*Laurus nobilis*) Seed Oil**
22.44	Oleic acid
10.38	3H)-one, 3a,4,6a,7,8,9,9a,9b-octahydro-6-methyl-3,9-bis(methylene)-, [3aS-(3a.alp
7.07	-ethenylhexahydro-6-methyl-3-methylene-7-(1-methylethenyl)-, [3aS-(3a.alpha.,6.
6.85	1,1'-Bis(cyclooct-2-en-4-one)
6.74	Palmitic acid
6.00	Eucalyptol
5.58	Cycloisolongifolene, 8,9-dehydro-9-formyl-
34.94	Other components*
%	**Corn (*Zea mays*) Oil**
26.09	Oleic acid
14.18	9,12-Octadecadienoic acid (Z,Z)-
13.08	Tributyl acetylcitrate
9.94	Hexadecanoic acid, 1-(hydroxymethyl)-1,2-ethanediyl ester
8.29	Palmitic acid
28.42	Other components*

*Components below 5% are included in 'Other components'.

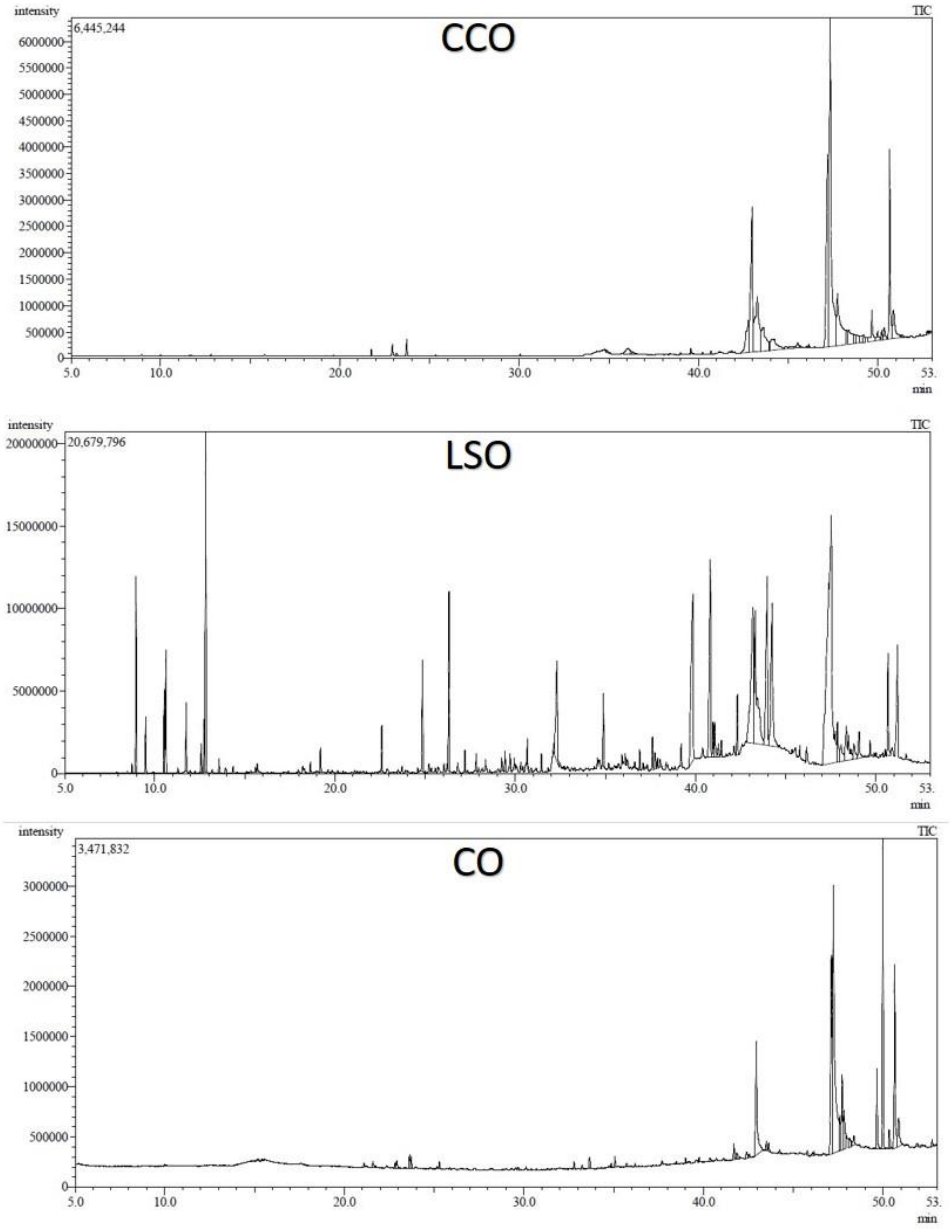

Figure 1. Chromatograms of the CCO, LSO and CO (GC-MS analysis).
CO: Corn oil, CCO: Common centaury oil, LSO: Laurel seed oil.

Table 2. Bodyweights, feed and water consumption (mean, standard error) during the experiment according to the groups (gram).

Variable	CO	LSO	CCO	P Value
Bodyweight (day 0)	505.97 ± 17.40	512.52 ± 25.98	507.13 ± 18.45	P>0.05
Bodyweight (day 14)	463.92 ± 7.60	415.00 ± 21.08	456.62 ± 17.55	P>0.05
Feed consumption (day 7)	243.75 ± 5.82	226.83 ± 17.49	234.82 ± 8.85	P>0.05
Feed consumption (day 14)	243.20 ± 6.53[a]	215.17 ± 8.74[b]	230.27 ± 7.27[a.b]	P<0.05
Water consumption (day 7)	218.33 ± 8.63	223.33 ± 26.79	204.17 ± 14.52	P>0.05
Water consumption (day 14)	236.67 ± 13.02[b]	302.50 ± 17.26[a]	224.00 ± 11.81[b]	P<0.05

a, b: shows the statistical differences between groups. CO: Corn oil, CCO: Common centaury oil, LSO: Laurel seed oil.

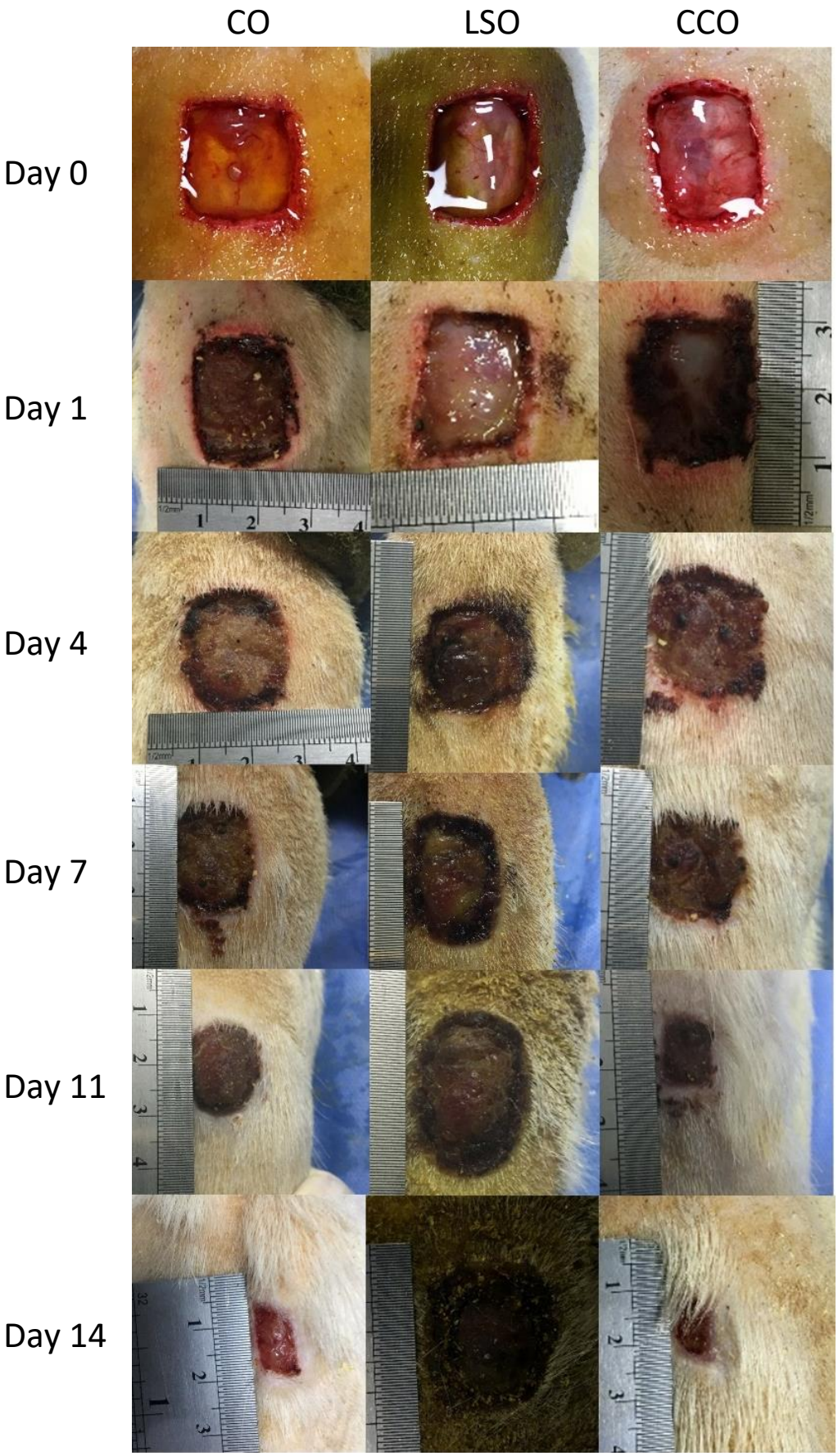

Figure 2. Photographic representation of the short-term (14 days) local clinical follow-up of skin wound healing process on different days by Control (CO: corn oil) group and experimental (LSO: Laurel seed oil, CCO: Common centaury oil) groups.

Table 3. Wound area measurements according to the groups on certain days during the experiment.

Group	Time	Mean	Std. Error
CO	Day 1	3.019	0.068
	Day 4	2.539	0.085
	Day 7	2.084	0.082
	Day 11	1.367	0.080
	Day 14	0.916	0.097
LSO	Day 1	1.983	0.068
	Day 4	2.431	0.085
	Day 7	2.064	0.082
	Day 11	1.943	0.080
	Day 14	1.304	0.097
CCO	Day 1	2.408	0.068
	Day 4	1.822	0.085
	Day 7	1.569	0.082
	Day 11	0.999	0.080
	Day 14	0.560	0.097

CO: Corn oil, CCO: Common centaury oil, LSO: Laurel seed oil.

Table 4. Wound area Time-Group comparison according to the groups on certain days during the experiment.

Time	Group Comparison	P Value	Std. Error
Day 1	CO-LSO	P<0.001	0.096
	CO-CCO	P<0.001	
	CCO-LSO	P=0.001	
Day 4	CO-LSO	P>0.05	0.120
	CO-CCO	P<0.001	
	CCO-LSO	P<0.001	
Day 7	CO-LSO	P>0.05	0.116
	CO-CCO	P=0.001	
	CCO-LSO	P=0.001	
Day 11	CO-LSO	P<0.05	0.113
	CO-CCO	P>0.05	
	CCO-LSO	P<0.001	
Day 14	CO-LSO	P<0.001	0.137
	CO-CCO	P<0.001	
	CCO-LSO	P<0.001	

CO: Corn oil, CCO: Common centaury oil, LSO: Laurel seed oil.

Table 5. Histopathological examination scores (mean, standard error) of wound tissues by groups.

Histopathological Variables	CO	LSO	CCO	P Value
Blood vessels	1.33 ± 0.21^{b}	2.50 ± 0.22^{a}	3.00 ± 0.37^{a}	P<0.05
Granulation	0.17 ± 0.17^{c}	2.67 ± 0.21^{b}	3.50 ± 0.22^{a}	P<0.05
Fiber synthesis	-	2.00 ± 0.26^{b}	2.83 ± 0.17^{a}	P<0.05
Mononclear cells	0.33 ± 0.21^{b}	2.17 ± 0.17^{a}	2.83 ± 0.31^{a}	P<0.05

a, b, c: shows the statistical differences between groups. CO: Corn oil, CCO: Common centaury oil, LSO: Laurel seed oil.

Table 6. Serum (blood) biochemistry results (mean, standard error) by groups.

Biochemical Parameters	CO	LSO	CCO	P Value
AST (GOT)	130.25 ± 4.28	179.07 ± 15.53	1168.07 ± 37.81	P>0.05
ALT (GPT)	69.17 ± 3.38	84.83 ± 3.68	72.50 ± 4.36	P>0.05
GLU	159 ± 15.75^{a}	105.67 ± 7.57^{b}	181.83 ± 10.59^{a}	P<0.05
CRE	0.87 ± 0.10	0.84 ± 0.69	0.80 ± 0.57	P>0.05
TP	$6.50 \pm 0.77^{a,b}$	6.13 ± 0.15^{b}	6.75 ± 0.12^{a}	P<0.05
UREA	64.67 ± 3.06^{b}	$70.67 \pm 3.04^{a,b}$	78.67 ± 4.18^{a}	P<0.05

a, b: shows the statistical differences between groups. CO: Corn oil, CCO: Common centaury oil, LSO: Laurel seed oil. AST: Aspartate aminotransferase, ALT: Alanine aminotransferase, GLU: Glucose, CRE: Creatinine, TP: Total protein.

Histopatological Results: In the histopathological results of the wound tissues, an increase in keratinocytes in the stratum basale layer for healing and epidermal regeneration in epithelial cells was determined in the CCO group. It was also revealed that the pilosebaceous contains epithelial stem cells that can regenerate and differentiate into basal keratinocytes and are necessary for the re-epithelialization process. Angiogenesis was observed with increased vascularization of wound healing in the dermis layer. In the LSO group, epidermis-associated hair follicles, pilosebaceous, ecrine, and apocrine glands were found to be normal, and vascularization increased. In the CO group, fiber synthesis and granulation formation were very low, but sebaceous glands and hair follicles were found in normal amounts. The histopathological scores of the wound tissues according to the groups are presented in Table 5. Histopathological microscopic images of wound tissues are presented in Figure 3.

Biochemical Results: GLU results were significantly lower in the LSO group than in the other groups. TP results were significantly lower in the LSO group than in the CCO group. UREA results were significantly higher in the CCO group than in the CO group. Serum biochemistry findings by groups are presented in Table 6.

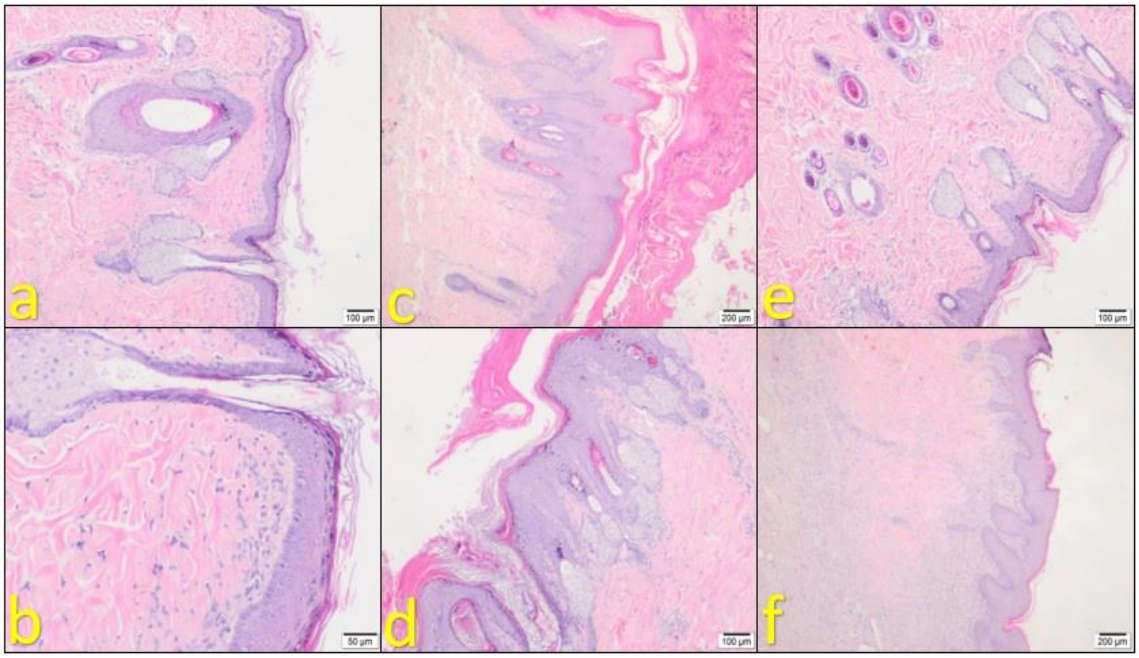

Figure 3. Histopathological (H&E) images of skin wound tissues at day 14: CO group (a, b), LSO group (c, d), and CCO group (e, f). CO: Corn oil, CCO: Common centaury oil, LSO: Laurel seed oil.

Discussion and Conclusion

Ideal wound healing is defined as the successful closure of the wound in the shortest time without any undesirable effects (10). Wound healing involves continuous, active, highly complex processes that may vary depending on many factors (22). Any effect disrupting the natural chain of wound healing may also affect the following stages, resulting in abnormal healing, chronic wounds, or scar tissue formation (13). The course of wound healing is largely associated with the antioxidant activity of the therapeutic agent used. Antioxidants accelerate wound healing by removing free oxygen radicals and increasing colloid synthesis (46).

Topical applications of anti-inflammatory and free radical scavenging products increase wound healing and protect tissues from oxidative damage (6, 21, 29, 48). Open wound treatment is frequently required in many medical branches. There are many studies trying to develop an ideal and faster open wound treatment option. Medicines, biomaterials, and methods that are thought to be more effective in open wound treatment have been tested. The use of natural products or the active substances obtained from them in wound dressings or wound care processes is increasing. In this context, wound healing studies are increasingly continuing.

Common centaury oil (CCO) is a herbal oil that contains over 230 different components, including a wide variety of fatty acids and terpenes in its chemical composition (25). Its antioxidant, antibacterial, and dermatoprotective effects have been reported (11, 24). Laurel seed oil (LSO) is a herbal oil with strong antibacterial, anti-inflammatory, and antioxidant properties (15, 18). St. John's Wort (*Hypericum perforatum*) plant extract and oil, which can be considered close to CCO, are used in many areas, especially wound healing, and there are many scientific studies about them (2, 3, 12, 21, 47, 49). To our knowledge, there are no studies investigating the effects of CCO on wound healing. Although there are many studies on LSO, there is also no study showing its effects on wound healing. Based on the knowledge of the antioxidant, antimicrobial, and dermatoprotective effects of these oils (11, 24, 37, 50), this study was planned with the hypothesis of their possible wound healing effects. Although the chemical components of the herbal oils used in our study were similar to the results of other studies (14, 25, 41), the percentages were determined to be relatively different. However, as a result of the GC-MS analysis, it is noteworthy that the LSO used in our study contains 6% eucalyptol. It is known that the content of the vegetable oils may vary according to the collection period and region. Because in previous studies eucalyptol ratios of laurel oil and LSOs have been reported to be around 30-60% (14, 41), it was considered that the proportional differences in the content of herbal oils are posibly caused by the harvesting regions. In experimental wound model studies, corn oil has been used as a control group in cases where the treatment group has a different oil-based substance (23, 44). Although some researchers prefer no treatment in the control group (48), in order to get a better oil-based substance comparison, the use of corn oil was considered necessary in our study as a negative control group.

It is noteworthy that the GLU results in the biochemical changes were significantly lower in the LSO group than the other two groups and lower in the CO group than the CCO group. Differences in GLU value are associated with stress and food consumption (32). It was considered that the changes in the GLU values showed the differences in the amount of stress caused by the application of the oils. It's known that a high TP value contributes to reducing inflammation and increasing fibroplasia (38). The fact that the TP value was significantly lower in the LSO group than the other two groups may indicate that the anti-inflammatory events are insufficient. AST, ALT, and CRE values did not differ statistically between the groups. Food and water consumption in experimental animals is an indicator for the evaluation of the wellfare and stress (5). In our study, no significant difference was found in the clinical examination, body weight, and feed consumption results of the groups. However, water consumption increased in the LSO group during the second week of wound healing. The reason was considered to be increased stress by LSO treatment irritating animals.

In a 14-day study measuring the effects of coconut oil in terms of wound closure time, antioxidants, and biomechanics, it was suggested that coconut oil has an antioxidant effect and accelerates wound healing (35). Another study suggests that *Nigella sativa* oil had stronger antioxidant properties than the *hypericum perforatum* oil and the placebo cream treatments, and *hypericum perforatum* increased wound healing via its effects on epithelialization and granulation (21). In a 14-day rat diabetic wound model study, by measuring the percentage of wound tissues macroscopically, the use of topical bitter melon oil accelerated wound healing (16). In another study of a 14-day experimental rat wound model, although black cumin (*Nigella sativa*) oil and zinc-silver cream in topical use gave poor results in macroscopic findings, zinc-silver cream gave good results in histopathological findings and physical testing. Thus, it has been suggested that it positively affects wound healing (29). Another study examined the effects of ozonated black cumin (*Nigella sativa*) oil, sesame oil, and St. John's Wort (*Hypericum perforatum*) oil on wound closure rate and healing process, both microscopically and macroscopically. Black cumin oil gave better results than other groups (12). Poljšak et al. (38) also reported similar effects and suggested that in-depth studies are needed to gain knowledge about vegetable oils' effects on the skin (38). In our study, macroscopic findings revealed LSO had an irritating effect on the skin and negatively affected wound healing. The increase in stress was at a level that would affect even food consumption. Although such a negative effect was not observed in the CO group, no effect that increased wound healing was observed. In the CCO group, on the other hand, as a result of macroscopic findings, the amount of wound closure was higher, and thus wound healing accelerated. Based on the results, it was determined macroscopically that CCO enhanced wound healing but LSO delayed it.

In the wound healing study using coconut oil, it was reported that fibroblast proliferation and neovascularization, pepsin-soluble collagen level, and cross-linking increased in the histopathological results, thus revealing the contribution to wound healing (35). The positive effect of bitter melon oil on wound healing was explained by the low inflammation findings in the histopathological examination (16). In the study showing the effects of black seed oil and sesame oil in the experimental wound model in rats, although significantly positive results were observed in macroscopic results, no significant difference was found between groups in histopathological results (12). In our study, histopathological findings revealed the scores in the CCO group were higher than the other two groups in terms of blood vessel formation, fiber synthesis, granulation, and mononuclear cells, and the LSO group had higher scores than the CO group. Additionally, macroscopic (wound area measurements and local clinical findings) and histopathological results support each other, hence showing that the CCO group had significantly better wound healing than the other two groups. However, histopathological results were better in the LSO group than in the CO group, although results that impair wound healing were observed in wound area measurements and local clinical findings in the LSO group compared to the other two groups. The reason for that was considered to be that, although it provides an anti-inflammatory effect at the cellular level, the wound healing process is adversely affected due to the irritating effect of the oil components. Because, in two different studies, the presence of six different cytotoxic components in the Laurel plant (*Laurus nobilis*) extract was identified (9, 18). On the other hand, it has been reported that Laurel plant extract obtained by different extraction methods has antioxidant effects in vitro and in vivo, but different effects may occur depending on the methods and solutions used in the extraction (26). Also, many extraction methods have been reported for medicinal purposes in wound healing (28, 31, 34). In an in vivo wound model study using *Allamanda* and *Laurus nobilis* extracts, the *Allamanda* group gave significantly better results than the *Laurus nobilis*, but still, the *Laurus nobilis* had better results than the control group (34). Although macroscopic findings for LSO in our study do not support this, microscopic findings partially support it.

In conclusion, the topical use of Common centaury (*Centaurium erythraea*) oil accelerated wound healing, while Laurel (*Laurus nobilis*) seed oil adversely affected wound healing in an in vivo experimental excisional full-

thickness skin wound model in rats by a clinical, macroscopic, histopathological, and biochemical investigation. Further investigation may provide original results for these oils if used in diseased wound models, such as infected or diabetic wounds, in future studies.

Acknowledgements

This study was derived from the MSc thesis of the first author. The authors would like to thank Assist. Prof. Dr. Ömer KIRGIZ for his help in the experimental work and the images of the study.

Financial Support

This research received no grant from any funding agency/sector.

Conflict of Interest

The authors declared that there is no conflict of interest.

Author Contributions

MZYD and NS designed the study and animal experiments. MZYD and NS carried out the animal experiments, sample collection, and analyses. MZYD contributed to the interpretation of the results and discussion. MZYD and NS wrote the manuscript.

Data Availability Statement

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

Ethical Statement

The ethical approval of this study was obtained from the Local Ethics Board of Animal Experiments of Hatay Mustafa Kemal University (Decision No: 2020/04-34).

Animal Welfare

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

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Spinal Meningothelial Meningioma in a Dog

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ARTICLE INFO

Article History
Received : 05.12.2022
Accepted : 13.09.2023
DOI: 10.33988/auvfd.1170085

Keywords
Dog,
Meningothelial meningioma
MRI
Neurosurgery

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How to cite this article: Pazarbaşılar İ, Demirtaş OB, Nazır NB, Vural S, Beşaltı Ö (2024): Spinal Meningothelial Meningioma in a Dog. Ankara Univ Vet Fak Derg, 71 (4), 497-501. DOI: 10.33988/auvfd.1170085.

ABSTRACT

The objective of this study is to report clinical, MRI, surgical, and histological findings of spinal meningothelial meningioma in a dog. The study material was a 9 years old, spayed dog with a history of progressive nonambulatory tetraparesis. The dog had intact cranial and spinal reflexes and deep pain perception. Magnetic resonance images revealed a mass located at the left side C2-C3 level, hyperintense in T1W, isointense on T2W, and well contrast enhancing on postcontrast T1. The mass was microsurgically resected and sub-gross. The dog's neurological status was improved at one week and survived for 15 months without signs of metastasis. Histological and histochemical workup revealed grade I, meningothelial meningioma. Surgical intervention for spinal meningioma can be suggested as the sole treatment in dogs.

Spinal meningiomas are the most common primary spinal tumors that have solitary structures and can be seen in any layer of meninges, especially arachnoid. These tumors are a significant cause of mortality in companion animals depending on their subtypes (4). They could be located in intradural-extramedullary or extradural (3).

Breed and sex predisposition for spinal meningioma was not reported, however, it is observed more commonly between ages five and fourteen years old (10). Computerized Tomography (CT) and Magnetic Resonance Imaging (MRI) along with histopathologic examination are the main diagnostic tool in spinal meningiomas (3). MRI is the method of choice to characterize the tumor deciding about the possibility of surgical resection. However, histological analysis is required to make a definitive diagnosis (3, 9).

Tumor classification by World Health Organization (WHO) is modified for defining grades of meningiomas in veterinary medicine. It was stated that grade 1 tumor type has a benign character, but grade 2 and grade 3 are thought to be malign. In addition to histological examination of spinal meningiomas immunohistochemistry is needed to determine for more detail (2, 9, 12). Meningiomas are stained positive for S100, Vimentin, some of the Cytokeratin (CK) markers, E-cadherin, and CD34 and are negative for GFAP, CD45/CD18, and some of the Claudin antibodies in veterinary pathology (8, 12, 14).

Treatment options for spinal meningiomas are surgical removal, chemotherapy, radiotherapy, or different combinations of them. The prognosis is related to the degree of spinal cord damage, the amount of resected tumor, and the tumor subtype (3).

This study aimed to report the clinical symptoms, diagnostic workup, surgical management, and histopathologic characteristics of cervical meningioma in a dog.

Nine years old spayed mongrel dog admitted to Ankara University Faculty of Veterinary Medicine Department of Surgery was subjected. The dog had a history starting with left hind limb ataxia, combined with

right thoracic limb ataxia in a month. Subsequently, ataxia was generalized with all limbs and more remarkable on the left side by the third month. Within four months, the patient became tetraparesis and was not able to walk for a week. The cell blood count and routine serum biochemistry were in reference limits except for high White blood cell (WBC) and neutrophilia. The dog was mentally alert and had normal cranial and spinal reflexes. The patient was in non-ambulatory tetraparesis in lateral recumbence, with patellar and withdrawal reflexes, and intact deep pain perception in all limbs. Spinal radiographs were normal. Neuroanatomical localization of the lesion was thought in C1-C5 spinal cord segments. Intervertebral disc disease, neoplasia, subarachnoid cyst, and meningomyelitis were considered among differential diagnoses. A cervical MRI was planned to narrow the list.

MRI was carried out with 0.3 Tesla. Anesthesia was induced by butorphanol (0.1 mg/kg) and diazepam (0.5 mg/kg) and it was maintained by Total Intravenous Anesthesia (TIVA) consisting of propofol with a dosage of 0.3 mg/kg/min. Images were acquired as sagittal, axial, and transverse views in T1W, T2W, and post-contrast T1W. Gadolinium dimeglumine was used at a dose of 0.1 mmol/kg body weight (BW) intravenously. The lesion was observed as a focal mass. It looked at isointense in T2W and hyperintense in T1W images (Figure 1). In the T2W image, the circumference of the lesion looked hyperintense. The lesion was located on the left side adjacent to the dorsal and left dural margins. Transverse and dorsal images of the T2W sequence showed that left-sided subarachnoid space expanded to compensate for the mass, so the mass was indicated as an intradural-extramedullary origin (Figure 1). Post-contrast T1W image revealed a broad-based attachment to the dura mater and had the appearance of as golf-tee-like mass, located intradural-extramedullary in the C2-C3 region (Figure 1). The mass extended into the intervertebral foramen along the spinal nerve, which mimics a nerve sheath tumor. For a more definitive diagnosis, surgical removal of the mass was planned because of its well-demarcated appearance.

Cephalosporin cefazolin (Eqizolin®500mg/2mL, Türkiye) was used at a dose of 20 mg/kg intravenously for prophylaxis. A transdermal fentanyl patch (Duragesic® 50 mcg/h patch, Belgium) was applied before the surgery to provide preemptive analgesia. Intravenously diazepam (Diazem® 10 mg/2 mL, Türkiye) with a dose of 0.5 mg/kg was used for premedication. Propofol (Propofol PF® %1 200 mg/20 mL, Türkiye) with a dosage of 3 mg/kg, was used to make induction of anesthesia, and the dog was intubated orolaryngeally. The anesthesia was maintained by isoflurane (Isoflurane USP® %100, USA). Constant Rate Infusion (CRI) of ketamine (Ketasol® %10, Austria) at a dosage of 0,3 mg/kg/h with 5 ml/kg/h speed was used for perioperative analgesia.

The dog was positioned in sternal recumbency with the head gently flexed in a neutral position. The surgical area was prepared for aseptic surgery. A midline incision was made over the C1-C4 spinous processes. Paraspinal muscles were separated by subperiosteal dissection. The lateral side of C2 –C3 vertebrae, intervertebral foramen, and nerve root were exposed by Gelpi retractors. Hemilaminectomy was performed by preserving nerve roots at C2-C3 intervertebral foramen level by surgical burr. Based on the swelling of the spinal cord and the purplish color change in durameter the hemilaminectomy defect was enlarged. The durotomy was carried out under the operation microscope, and the mass was revealed in greyish color, well-demarcated and it pushed the spinal cord to the right side. The mass was removed by microdissection and aspiration. Blood vessels of the meninges were obscured by electrocoagulation. The dural defect was not repaired for the possibility of recurrence. Fat graft, which was harvested subcutaneously, was placed in hemilaminectomy defect. The operating wound was closed routinely.

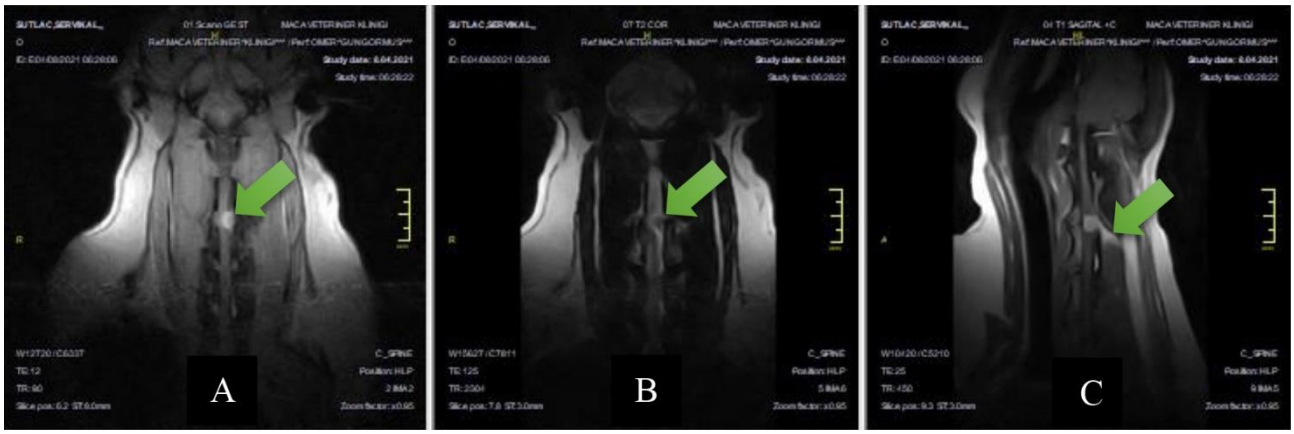

Figure 1. (A) Dorsal postcontrast T1W hyperintense image and (B) Dorsal T2W isointense image show a round-shaped mass on the left side at the C2-C3 vertebrae (green arrow). (C) Sagittal postcontrast T1W MR image shows well contrast enhancement and golf-tee sign located intradural-extramedullary at the C2-C3 vertebrae (green arrow).

DOI: 10.33988/auvfd.1170085

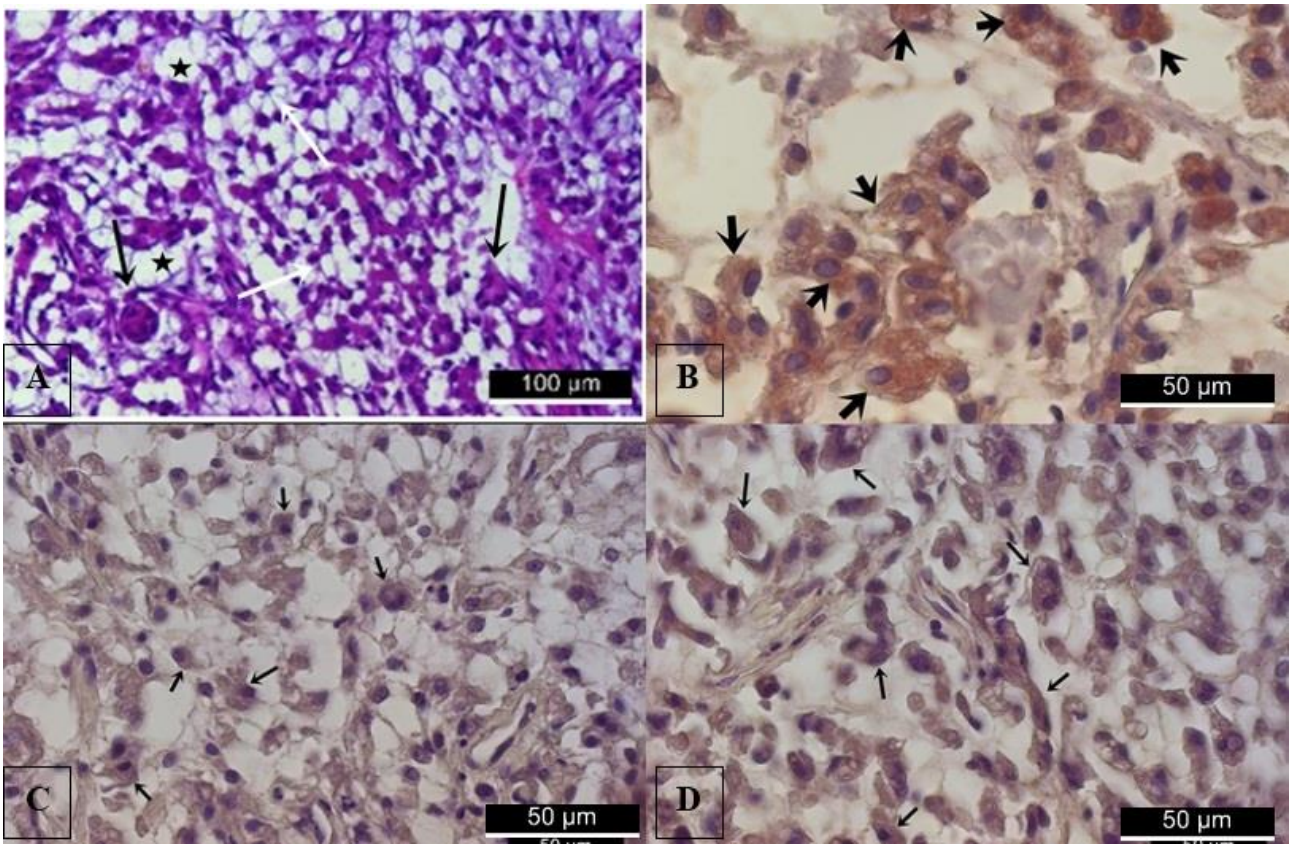

Figure 2. **A:** Histopathological image, meningothelial cells with round/oval basophilic nuclei and abundant eosinophilic cytoplasm with cytoplasmic extensions formed small clusters (black arrows) or a web-like pattern (white arrows). The myxomatous substance is seen in between (black stars) (HE); **B:** CK 8 positive meningoendothelial cells (black arrows), Strept ABC-P; **C:** S-100 positive meningeal cells (black arrows), Strept ABC-P; **D:** Vimentin positive meningeal cells (black arrows), Strept ABC-P.

In the postoperative period, fluid therapy (dose of 2 ml/kg/h) was continued until the patient recovered from anesthesia. Transdermal fentanyl patch had been used as postoperative analgesia for 3 days. Afterward, tramadol with a dose of 2 mg/kg was administered every 8 hours PO to make pain management for two weeks after the surgery. Prednisolone at a dose of 0.5 mg/kg, PO, BID for two weeks and amoxicillin and clavulanic acid at a dose of 25 mg/kg PO, BID for one week were recommended just after the surgery.

The retrieved sample was fixed in 10% formaldehyde, routinely processed, and embedded in paraffin blocks. Then, 5 µm thick sections were taken (Leica RM2125, Deer Park, Illinois, USA) and stained with Hematoxylin and Eosin (HE), using an automatic slide stainer (Leica Autostainer XL, Deer Park, Illinois, USA). Immunohistochemically, the sections were stained with StreptAvidin-Biotin Complex Peroxidase (Strept ABC-P) method by using S-100 (1:150, ThermoScientific, Waltham, Massachusetts, USA), Vimentin (1:500 ScyTek, West Logan, Utah, USA) and Cytokeratin 8 (CK8) (1:50 Novocastra, Newcastle, UK) antibodies according to the kit procedure (UltraVisionDetection System Large Volume Anti-Polyvalent, Thermo Scientific, Waltham, Massachusetts, USA). Control sections were treated with normal Mouse IgG serum (Ready to use, Novocastra, Newcastle, UK). Afterward, all of the sections were examined under the light microscope (Leica DM 4000, Deer Park, Illinois, USA) and photographs (Leica MC170, Deer Park, Illinois, USA) were taken from appropriate sites.

Uniform meningothelial cells with large, round/oval, normochromic basophilic nuclei, and wide eosinophilic cytoplasm were seen (Figure 2A). These cells tend to come together as lines or small clusters in which the cytoplasmic borders could not be distinguished, with a blue-colored mucinous material between them. There was no visible mitotic activity. Immunohistochemically, these neoplastic cells were markedly stained positive with S-100, vimentin, and CK 8 antibodies at homogenous brownish color unlike the control sections (Figure 2B-2D). It was diagnosed as a grade 1 endothelial subtype in the light of findings.

The reported case introduces a meningioma that mimics a nerve root tumor in MRI causing nonambulatory tetraparesis. The location of the mass led us to consider a

nerve sheath tumor rather than a meningioma in MRI. However, it was definitively diagnosed as meningothelial meningioma by histopathologic and immunohistochemical examination. Immunohistochemical confirmation with S100, CK8, and Vimentin was carried out. The high-grade positivity of mentioned biomarkers was found to correlate with the literature as spinal meningothelioma (7, 15, 16).

Nerve sheath tumors and meningiomas are the most common intradural spinal neoplasias in dogs, like in humans (5, 6). Meningiomas may account for up to 65% of canine primary spinal cord tumors (8). Differentiation of both tumors can be challenging in some cases by MRI (11, 13, 16).

MRI findings of spinal meningioma in humans and dogs as iso to hypointense on T1W images and slightly hyperintense on T2W images relative to the spinal cord. Dural tails are observed as a result of post-contrast T1W images (8, 13). High signal intensity on T1W in human meningioma is considered related to; intra-extra tumor hemorrhage, high lipid content in tumor cells, mild calcification, and high cellular density. Hyperintensity on the T1W image was also reported in a case with cranial meningioma in a dog (6). In our case, T1W hyper-intense and T2W isointense appearance relative to the spinal cord was observed (Figure 1). Additionally, a round-shaped mass was surrounded by hyperintense circumference in the T2W image, and an extension of the tumor along the nerve roots through the intervertebral foramen was seen. This unusual MRI finding was not confirmed by the histological examination regarding to possible causes of human beings. Meanwhile T1 hyperintense and T2 isointense with circumferential hyperintense lesions are thought to be related to hemorrhage and exposed hemosiderin. Extension of the lesion to the intervertebral foramen was reported before and they should be considered in MRI evaluation (8).

Presumptive diagnosis is often possible based on tumor characteristics and location using advanced imaging techniques; however, for definitive diagnosis histological examination is required (1, 3). Additionally, there are no guidelines or criteria for grading the malignancy potential of meningiomas in canine practice, so WHO classification is used basically. Treatments of canine spinal meningiomas consist of medical management and cytoreductive surgery with/without radiation therapy. Survival times after surgery as a sole treatment vary from 4 to 47 months (10, 16). In human grade, I meningioma, surgery is the method of choice for the treatment and results are satisfactory and recurrence postoperatively is low which is found to be approximately 3–15% (5). The case presented in this study is in the same line with grade I.

There was limited information about the surgical technique for removing meningioma, especially at the spine. The location, suitable exposure of the surgical area for manipulation on tumor removal, the method of hemostasis, microsurgical skill, etc. are crucial for a successful surgery. Resection with meticulous hemostasis by electrocoagulation was thought to have a crucial role of one year surviving without neurologic signs, and improvement in neurological status in a relatively short term, which was one week, was thought to be associated with low surgical morbidity on neural tissues. Neurologic improvement in one week after the surgery and remaining silent for 15 months was found successful. The patient was followed up with a detailed neurologic examination over a routine period of 2 months. During that period, there did not exist any proprioceptive deficit or delayed spinal reflexes. Although the surgical technique was not detailed in the veterinary literature, we think that sub-gross total removal of the tumor with minimal trauma is crucial because the outcomes after surgical resection for spinal meningioma remain more unclear compared to cranial meningioma because of the fewer reported cases. The interval between realizing first clinical signs and admission time is reported as about 1 month (13). In the presented case, the first clinical signs were noticed by the owner four months ago, and progressive deterioration resulted in non-ambulatory tetraparesis.

These kinds of studies should be done with more dogs to come up with a result and make a specific tumor classification which helps the management to become easier. Surviving with seamless clinical signs for 15 months can be considered as a clue to suggest surgical intervention for canine meningioma as a sole treatment.

Financial Support

This research received no grant from any funding agency/sector.

Ethical Statement

This study does not present any ethical concerns.

Conflict of Interest

The authors declared no conflict of interest

Author Contributors

Case examination, evaluation of clinical findings, interpretation of MRI images, and surgical intervention were done by IP, NBN, and OB. Histopathologic evaluation was done by OBD and SV. The article was written, accordingly. All authors have read and agreed to the submitted version of the manuscript.

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Management of inflammatory bowel disease and lymphangiectasia in a dog with octreotide and tranexamic acid

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ARTICLE INFO

Article History
Received : 10.03.2023
Accepted : 10.01.2024
DOI: 10.33988/auvfd.1263493

Keywords
Dog
Inflammatory bowel disease
Lymphangiectasia
Octreotide
Tranexamic acid

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How to cite this article: Kaçar Y, Küpeli ZA, Canatan U, Özyiğit Ö, Gül Satar NY, Temizel EM (2024): Management of inflammatory bowel disease and lymphangiectasia in a dog with octreotide and tranexamic acid. Ankara Univ Vet Fak Derg, 71 (4), 503-509. DOI: 10.33988/auvfd.1263493.

ABSTRACT

In dogs, inflammatory bowel disease (IBD) is a well-defined form of intestinal disease. Most cases are associated with lymphangiectasia. A 2-year-old, American Staffordshire Terrier male dog, presented with progressive diarrhea for more than 3 months. Clinical findings, laboratory findings, ultrasound images of the dog and also, histopathological evaluation of punch biopsy samples from the intestines were compatible with IBD and lymphangiectasia. The treatment of the dog was started with the treatment protocol containing octreotide (10 µg/dog per day, BID, sc.) and tranexamic acid (10 mg/kg, BID, im.) which was used for the first time in dogs with IBD and lymphangiectasia. Fecal consistency and defecation frequency returned to normal on the 15th day, also at the end of the first month of therapy hypoalbuminemia began to normalize, ascites completely disappeared and the thickness of the mucosal layer began to normalize, and the patient began to gain weight. In the controls performed at the end of the second month, it was determined that the patient's clinical signs and all laboratory results improved. To our knowledge, these results suggest that the combination of octreotide and tranexamic acid can effectively and without any side effects be used for the treatment of IBD in dogs for the first time. This case report, it is aimed to present a successful treatment method using octreotide and tranexamic acid in a dog with intestinal lymphangiectasia related to IBD and to raise awareness among veterinarians in patients with similar clinical findings.

Inflammatory Bowel Disease (IBD) is a complex disease that is characterized by cellular infiltration within intestinal layers (1, 2, 5, 11, 23, 28). Lymphocytic, lymphoplasmacytic enteritis, eosinophilic gastroenteritis, and mixed inflammation are the most frequently encountered types of IBD in dogs (1, 5). Malabsorption and chronic protein-losing enteropathy may result from prominent lymphocyte and plasmacyte infiltration in the lamina propria. The most frequently encountered complication is intestinal lymphangiectasia, which is characterized by the dilation of lymph vessels (18). Enlargement of the lacteals results in decreased lymph absorption in the lamina propria and subsequent loss of protein and other nutrients into the intestinal lumen (28). Diarrhea is the most common symptom in dogs with intestinal lymphangiectasia and IBD but also weight loss, ascites, peripheral edema, vomiting, anorexia, hydrothorax, chylothorax, flatulence, lethargy, borborygmus, hematemesis, melena, and abdominal pain can be seen in these dogs (1, 8, 11, 17).

The diagnosis of IBD and intestinal lymphangiectasia can be made by clinical signs, biochemical examinations, ultrasonography, and histopathologic examination (1, 4, 11, 17, 18, 27). Dogs

with intestinal lymphangiectasia related to IBD usually need long-term treatment and they may have a poor prognosis in many cases (18). Conventional treatment of IBD and intestinal lymphangiectasia in dogs have consisted of resolution of the underlying disease, dietary modification (low-fat diet especially long-chain triglyceride restriction), and pharmacological therapy (immunosuppressive drugs, NSAID, etc.) (1, 5, 11, 18, 23). However, since these drugs, used in the treatment of IBD with lymphangiectasia have limited effectiveness, the search for more effective drugs continues today.

Octreotide is a synthetic somatostatin analogue, and it affects many organ systems, especially the gastrointestinal tract (10, 22). It has been suggested that octreotide promotes the relaxation of intestinal smooth muscles, decreases gastrointestinal motility, and reduces secretory diarrhea (antisecretory effect) by enhanced water and electrolyte absorption, reduces jejunal secretions, and alters ion transport in the gastrointestinal tract (10, 14, 17, 22). Also, it has been stated that octreotide reduces the perception of gastric, colonic, and rectal distension in IBD patients (14). In addition, it is commonly used in controlling lymphatic leakage in humans and a dramatic decrease in lymphatic output can occur (25).

Tranexamic acid is also used in children with IBD. In this disease, increased fibrinolytic activity can increase protein loss from the intestinal area. Tranexamic acid reduces the fibrinolytic activity, so with this property, it may contribute to the treatment of the disease (15, 20).

Here we present a case report of the dog with intestinal lymphangiectasia related to IBD which was successfully treated with octreotide and tranexamic acid.

Case description: A 2-year-old, 23 kg body weight, male American Staffordshire Terrier was referred due to diarrhea lasting for more than 3 months to Bursa Uludağ University Faculty of Veterinary Medicine, Department of Internal Medicine. Anamnestically the dog was castrated a few months ago, and diarrhea and vomiting were worsening gradually after the operation. It was also reported that the dog has lost about 10 kg weight during this time. It has been noted that abdominal enlargement was revealed in the dog for 1-2 weeks. The dog had a good appetite and was fed commercial dog food (Enjoy®, adult dog food, Lider Petfood Industry and Trade Ltd. Co, Türkiye). It was stated that despite the many therapeutic efforts, could not be recovered including ampicillin sulbactam, enrofloxacin, gentamicin, ranitidine, metoclopramide, and probiotic treatments. In addition, all vaccinations and internal-external parasitic (anthelmintic and parasitic) applications were performed routinely. All physiological parameters were within reference limits (P:88/Bpm, R:36/min, T:38.5/^{0}C, CRT:2 sec., Lymph Nodes: Normal), on the other hand, abdominal palpation revealed fluctuation with tension and moderate abdominal pain (Table 1, Figure 1 and Figure 2). The fecal consistency was scored based on a 5-point scale (1= very hard to 5= watery diarrhea) (16) during the treatment period. According to the owner's description and our clinical examination, fecal scores were described as 5. The dog's stool had a yellowish-colored and oily appearance but didn't contain any undigested food.

Table 1. Pre-treatment (Day 0), during treatment (Day 0-60), and after treatment (Day 180) haemogram results.

Parameter	Day 0	Day 15	Day 30	Day 60	Day180	References (26)
WBC (10^3 /mm3)	16.44	31.15	15.10	15.84	8.91	6.0-17.0
Lymphocyte (10^3 /mm^3)	0.96	0	2.73	2.89	2.28	1.0-4.8
Monocyte (10^3 /mm^3)	0.02	0	0.46	1.37	0.58	0.1-1.3
Neutrophil (mm^3)	15.13	31.00	11.82	11.38	5.66	3.0-13.5
Eosinophil (10^3 /mm^3)	0.33	0.16	0.07	0.17	0.30	0.1-1.2
Basophil (mm^3)	0.00	0.00	0.00	0.00	0.10	0.0-0.1
nRBC (10^6 / mm^3)	4.36	2.41	7.45	3.41	3.44	0.0-99.9
RBC (10^6 /mm^3)	7.84	4.40	6.32	7.27	7.97	5.5-8.5
HGB (g/dL)	21.00	11.60	15.00	18.60	18.90	12.0-18.0
HCT (%)	56.10	30.70	44.10	47.80	53.00	37.0-55.0
MCV (fL)	71.60	69.90	69.90	65.80	66.50	60.0-77.0
MCHC g/dL)	37.40	37.70	34.00	38.90	35.60	32.0-36.0
PLT (mm^3)	663.000	482.000	473.000	331.000	353.000	200.0-500.0

WBC: White blood cells; nRBC: Nucleated red blood cells; RBC: Red blood cells; HGB: Hemoglobin; HCT: Hematocrit; MCV: Mean corpuscular volume; MCHC: Mean corpuscular hemoglobin concentration; PLT: Platelet count.

DOI: 10.33988/auvfd.1263493

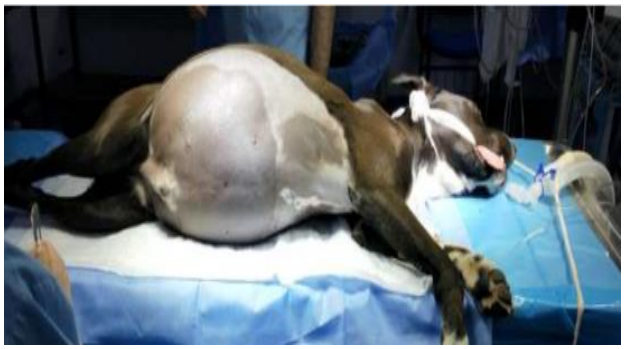

Figure 1. Abdominal distention (dilatation with ascites fluid).

Figure 2. Ascites fluid in the abdomen (10 L).

Diagnostic procedures: The haemogram and serum biochemistry results are presented in Table 1 and Table 2. There were no parasites in the fecal examination and the occult blood test was negative. Also, there was no abnormality in the results of the radiographic, electrocardiographic, and echocardiographic examinations. The anechoic-free fluid (ascites) in the abdomen was observed in the ultrasonographic examination. In the ultrasonographic examination of the intestines, it was found that the total wall thickness increased (0.68 cm), especially the mucosal layer was 0.56 cm. Since it is known that the normal total wall thickness of the duodenum in dogs is 0.53 cm or less (7), it was determined that the mucosal layer thickness was increased in this dog (Figure 3). Also, rivolta test of the abdominal fluid (ascites) that was taken by abdominocentesis was determined as transudate. Also, there were no abnormalities detected on the urinalysis of the dog. On the other hand, the patient's TLI value which was measured by using a commercial assay (Canine TLI-RIA, Siemens Medical Solutions, Malvern, PA) was found to be lower than the references. Based on clinical signs and laboratory findings, the patient was diagnosed with inflammatory bowel disease and exocrine pancreatic insufficiency.

All treatment protocols applied to the dog are summarized in Table 3. Also, a fat-restricted but protein-rich diet was applied to the dog with the initiation of the first treatment protocol. Despite these first two treatment protocols, the patient's clinical and laboratory findings have not improved, so exploratory laparotomy was performed. In the exploratory laparotomy, approximately 10 liters of free fluid (ascites) was drained from the abdomen (Figure 2). On the inspection, thickening was observed in some areas of the duodenum and jejunum. Punch biopsy samples of approximately 3 mm in size, including all intestinal layers, were taken from this thickened area of the duodenum and jejunum. Also, punch biopsy samples were taken from the pancreas. Biopsy samples were sent to the Department of Pathology for histopathological examination.

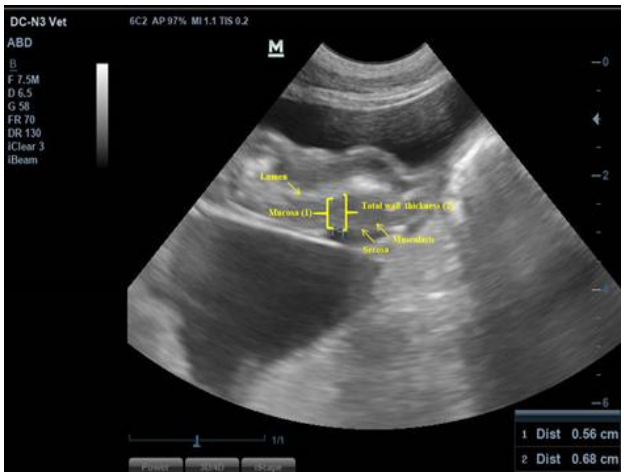

Figure 3. Pre-treatment US appearance of duodenum.

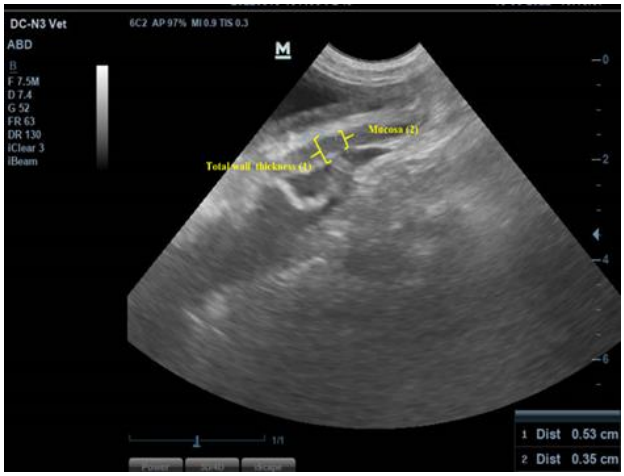

Figure 4. Post-treatment US appearance of duodenum.

Table 2. Pre-treatment (Day 0), during treatment (Day 0-60) and after treatment (Day 180) serum biochemistry results.

Parameter	Day 0	Day 15	Day 30	Day 60	Day 180	References (26)
Total protein (g/dL)	2.60	-	3.7	6.1	6.60	5.1-7.8
Albumin (g/dL)	1.20	1.30	2.3	3.3	3.30	2.6-4.3
Globulin (g/dL)	1.40	-	1.4	2.8	3.30	2.3-4.5
Alb/Glob	0.86	-	1.64	1.17	1.00	0.9-1.9
Alkaline phosphatase (U/L)	57.00	125.00	-	100.5	59.00	10-150
Glucose (mg/dL)	129.00	112.00	-	113.00	101.00	60-125
Total bilirubin (mg/dL)	0.20	0.40	-	0.20	0.20	0.0-0.4
Inorganic phosphorus (mg/dL)	3.20	-	-	3.40	3.80	2.9-5.3
Total cholesterol (mg/dL)	<50.00	-	-		254.00	112-328
Gamma-glutamyl-transferase (GGT) (U/L)	<10.00	20.00	-	<10.00	<10.00	0.0-10.0
Alanine-aminotransferase (ALT) (U/L)	127.00	219.00	-	98.00	96.00	5-60
Calsium (mg/dL)	7.20	-	-	9.5	11.60	7.5-11.3
Creatinine (mg/dL)	0.51	-	0.26	0.56	0.80	0.4-1.8
Blood urea nitrogen (mg/dL)	9.80	-	19.60	20.1	15.50	7-27
Ammonia (NH_3) (mol/L)	-	54.00	-	38.00	-	40-70

Table 3. Treatment protocols which applied to the dog.

Initial treatment protocol	Re-admission treatment protocol	Treatment protocol after definitive diagnosis
fat-restricted but the protein-rich diet	fat-restricted but the protein-rich diet	fat-restricted but the protein-rich diet
sulfasalazine (Salazopyrin®, Pfizer; 20 mg/kg, TID, po.)	sulfasalazine (Salazopyrin®, Pfizer; 20 mg/kg, TID, po.)	sulfasalazine (Salazopyrin®, Pfizer; 40 mg/kg, TID, po.)
methylprednisolone (Prednol®, Mustafa Nevzat; 2 mg/kg, BID, po.)	methylprednisolone (Prednol®, Mustafa Nevzat; 3 mg/kg, BID, po.) cyclosporine (Sandimmun neoral®, Novartis; 5 mg/kg, BID, po.)	methylprednisolone (Prednol®, Mustafa Nevzat; 2 mg/kg, BID, po.)cyclosporine (Sandimmun neoral®, Novartis; 10 mg/kg, BID, po.)
metronidazole (Flagyl®, Sanofi; 20 mg/kg, BID, po.) clarithromycin (Klasid®, İE; 20 mg/kg, BID, po.)	amoksiciline+clavulanic acid (Amoklavin®, Deva; 20 mg/kg, BID, po.)	amoksiciline+clavulanic acid (Amoklavin®, Deva; 20 mg/kg, BID, po.)
famotidine (Famodin®, Sandoz; 1mg/kg, BID, po.)	famotidine (Famodin®, Sandoz; 1mg/kg, BID, po.)	famotidine (Famodin®, Sandoz; 1mg/kg, BID, po.),
pankreatin (Pankreoflat®, Recordati; 3 x1 tablet/day)	pankreatin (Pankreoflat®, Recordati; 3 x1 tablet/day)	probiotic complex (Reflor®, Biocodex; 1x1 kps, po.)
hepatic supplement (Hepatiale forte®, Vetexpert; 1x2 tablet, po.) probiotic complex (SynbioCure®, Yeniçağ; 1 chassis /day)	probiotic complex (Reflor®, Biocodex; 1x1 kps, po.)	antidiarrheal agent (Kaopektin®, Alke; 5 ml BID, po.)
antidiarrheal agent (Kaopektin®, Alke; 5 ml BID, po.)	antidiarrheal agent (Kaopektin®, Alke; 5 ml BID, po.)	octreotide (Sandostatin®, Novartis; 10 µg/dog/ day, BID, sc.)
		tranexamic acid (Transamine®, TEVA; 10 mg/kg, BID, im.)

After laparotomy and histopathological examination of the samples, the definitive diagnosis was intestinal lymphangiectasia and IBD, and a treatment protocol was re-arranged as in Table 3. At control after 1 month, the frequency of vomiting and defecation was decreased, and clinical and laboratory examinations were repeated. In the examination, it was revealed that the fecal score was 3/5. In the ultrasonographic evaluation, it was determined that no free fluid in the abdomen, kidney, and liver echogenicity was normal, and the total intestinal wall thickness in the duodenum was 0.53 cm (Figure 4) (mucosal layer 0.35 cm) was thinner than before (0.56 cm). In this control, the dog's weight was found to be 26 kg (it gained about 3 kg). Afterward, the treatment was

continued in the same way for 1 more month and terminated. At the end of the treatment, abdominal distension (ascites) and other clinical problems (vomiting, diarrhea, etc.) were completely resolved, and the dog was 29 kg by gaining 3 more kg. In the controls which were made during and up to 6 months after the treatment protocol, it was determined that the disease did not recur and there was no problem.

In veterinary medicine, mainly prednisone, prednisolone, sulfasalazine, olsalazine, azathioprine, cyclophosphamide, cyclosporine, and metronidazole were used in dogs with IBD up to date (1, 18, 23). Also, in recent years, there have been studies on the use of probiotics, mesenchymal stem cells, fecal microbiota transplantation, and therapeutic helminths in the treatment of IBD (3, 6, 21). In the treatment of IBD, considering the possible lymphangiectasia and clinical consequences in addition to the above-mentioned conventional treatments, additional medical options may play an important role in the success of the treatment.

It has been reported that in patients with IBD, lamina propria inflammatory cell infiltrates may lead to lymphangiectasia by blocking the flow in the mesenteric lymph nodes. It has also been suggested that the increased vascular permeability associated with IBD may contribute to mucosal edema and lymphatic dilatation (12). In this case, marked clinical signs, and histopathological changes were determined to be compatible with IBD and lymphangiectasia (Figure 5 and Figure 6). Therefore, also the correction of lymphangiectasia is important in the treatment of IBD. Octreotide is is highly effective in preventing lymphatic leakage (17). Indeed, studies in children (15) and adults (25) with lymphangiectasia have shown that the use of octreotide is effective. It has also been reported that people with chylothorax also prevent chylous fluid accumulation by narrowing the lymphatic vessels (19). In humans, tranexamic acid is also used as part of IBD and lymphangiectasia therapy protocols. Increased fibrinolytic activity may promote intestinal protein loss in humans (15, 20). It has been observed that protein-rich fluid excretion and so hypoproteinemia is prevented resulting in the narrowing of the enlarged lymphatic vessels and reduction of chylous leakage by using octreotide in addition to the conventional treatment. Therefore, tranexamic acid in patients with IBD has been commonly used to prevent intestine protein loss. In this case, the tranexamic acid and octreotide combination improved diarrhea and ascites after 1 month.

There are no known standardized dosage and usage duration of octreotide in dogs (15, 22). It is reported that octreotide (long-acting formulation) was used in a dog with osteosarcoma at a dose of 60 mg/per dog three times 21 days apart (13), but in the treatment of insulinoma, this drug was used at a dose of 2µg/kg (24). This paper used the octreotide dose at 10 µg/day with clinical success for 2 months. (22).

There are not many reports about the side effects of octreotide in dogs. It is well tolerated in humans and the primary side effect is pain in the local injection site and some gastrointestinal symptoms like anorexia and nausea (10). In dogs, octreotide is a well-tolerated drug except for sterile injection site abscessation (13). In this case, no local or systemic drug-related side effects were observed in the dog during the 2-month treatment protocol. Although it has been reported that long-term use of octreotide may lead to the formation of gallstones and liver damage, no adverse effects on liver functions and gallbladder were observed in the presented case (9). In this case, liver biomarkers (ALT, AST, BUN, ALB) and abdominal ultrasonography were regularly evaluated during the two-month treatment period. All biochemical, hematologic, and ultrasonographic findings in terms of 6 months were within reference limits (Table 1 and Table 2).

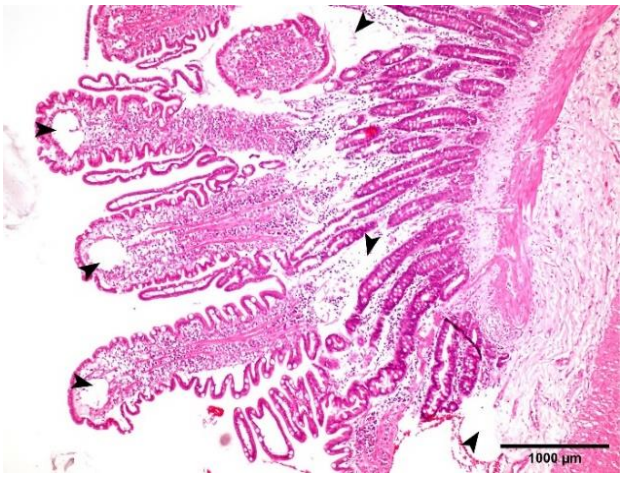

Figure 5. Jejenum-Lymphangiectasia. Lacteal dilatation and oedema in the villi and lamina propria (arrowheads).

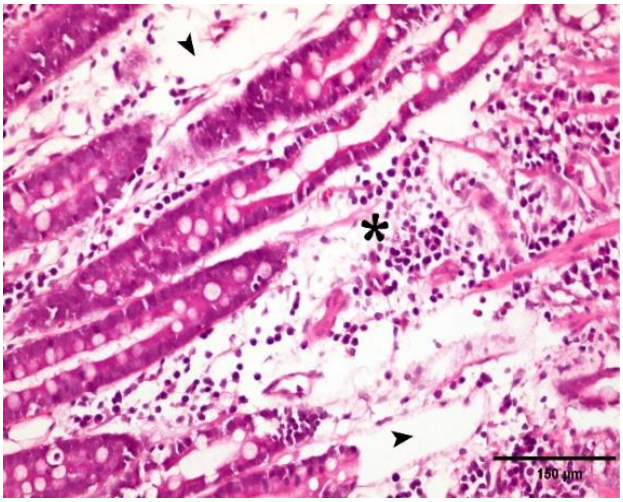

Figure 6. Jejunum-Lymphangiectasia. Inflammatory cell infiltration from a large number of mononuclear series in lamina propria (asterisk). Lacteals are dilated (arrowheads). HEx40.

In the veterinary literature, the effectiveness of octreotide and tranexamic acid in a dog with IBD and lymphangiectasis is demonstrated for the first time. Further clinical research is needed to determine the relationship between octreotide and IBD in dogs.

Financial Support

This research received no grant from any funding agency/sector.

Conflict of Interest

The authors declared that there is no conflict of interest.

Author Contributions

YK and EMT carried out all clinical and laboratory examinations. UC and NYG performed the radiological examinations and exploratory laparotomy operation. ZAK and ÖÖ performed the histopathological evaluations. All authors contributed to the writing of the manuscript.

Data Availability Statement

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

Ethical Statement

This study does not present any ethical concerns.

Animal Welfare

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

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A Comprehensive Outlook on Cultured Meat and Conventional Meat Production

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ARTICLE INFO

Article History
Received : 28.01.2024
Accepted : 30.07.2024
DOI: 10.33988/auvfd.1425678

Keywords
Cultured meat
Conventional meat
Meat industry
Meat production

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How to cite this article: Peker A, Orkan Ş, Aral Y, İplikçioğlu Aral G (2024): A Comprehensive Outlook on Cultured Meat and Conventional Meat Production. Ankara Univ Vet Fak Derg, 71 (4), 511-522. DOI: 10.33988/auvfd.1425678.

ABSTRACT

Proponents present cultured meat as a viable alternative to traditional animal-based meat production to meet the increasing demands of the growing population. This review aims to compare this subject across various dimensions, such as resource requirements, nutritional aspects, cost structure, consumer acceptance, and market trends, by focusing on recent publications. Cultured meat can be produced by applying existing cell culture practices and bio-manufacturing methods to produce tissue or dietary proteins suitable for human consumption. Studies have shown that cultured meat has some advantages over conventional meat in issues such as the environment and animal meat-related diseases. Cultured meat is a promising but early-stage technology with significant technical challenges in terms of production costs and optimized methodology. Cultured meat cannot completely achieve the texture, taste, and nutritional values of conventional meat. Religious beliefs, price, ethical values, and regional factors are important considerations in consumers' perceptions of cultured meat. Currently, the level of research conducted on aspects such as consumer acceptance, cost, texture, taste, and other characteristics closely resembling conventional meat will directly influence its entry into the market, its success in the market, and its acceptance by consumers. There is a need for further research and analysis with the joint participation of academic and sectoral stakeholders to address all technical, social, and economic dimensions.

Introduction

The world population is currently over 8 billion, and the United Nations states that this number will exceed 9 billion by 2050. With population growth, the world's need for food will increase. By 2050, the world will need 70% more food to meet demand due to limited resources and arable land problems. By 2030, experts anticipate the annual worldwide meat production to reach 465 million tons (81). Every year, the world raises and slaughters almost 70 billion animals to meet the growing demand for meat (26).

In the conventional meat production system, new alternatives for meat have been developed for many reasons, such as public health, animal welfare, and negative environmental effects. Cultured meat is obtained from tissues and cells in a laboratory. It is also known for concepts such as cultured meat, in-vitro meat, lab-grown meat, synthetic meat, clean meat, and cell meat. Mark Post made a hamburger with cultured meat for the first time in 2013. After this event, interest in cultured meat, investments, and research on this subject increased considerably. A report estimated the cultured meat market to be valued at $1.64 million in 2021, with a projected compound annual growth of 95.8% from 2022 to 2030 (4). In another report it is stated that the cultured meat market will reach 1.66 billion dollars in 2031 and 11.13 billion dollars in 2041 (25).

Cultured meat is a promising technology, but it is still in its infancy, and its industrial production faces many obstacles (19). Consumer perception, the nutritional structure of meat, and excess production costs are some of these difficulties. According to research, the three most

important factors in consumer perception of cultured meat are price, texture, and taste (18).

The following are important points for the proper development of the cultured meat industry: i) to learn more about cultured meat and to expand the technology as much as possible; ii) to improve product quality; iii) to reduce production costs; iv) to ensure product safety; and v) to improve regulatory systems and provide good market access (57).

What is cultured meat?

There has been a tendency towards cultured meat due to the effects of conventional livestock farming on the environment, the attitudes of some people towards animal slaughter, future population growth, and the need for food. Producing cultured meat eliminates the need to slaughter animals (65). Harvest mature muscle cells cultured from myo-satellite cells on a substrate in a liquid medium under mechanical stimulation as the basic methodology (82). It was originally referred to as in vitro, but in 2011, the term "cultured meat" gained popularity due to culturing techniques. In 2015, it was called "clean meat," and this attracted more attention from consumers (14). Although many different definitions, such as "lab-grown meat," "cell-based meat," "in vitro meat," and "clean meat," are used for cultured meat, the production methods are largely the same. This method of meat production is quite different from conventional animal husbandry and is advocated by some circles, such as politicians and scientists. Today, there are many companies working to produce cultured meat products and sell them in the near future (73).

Cultured meat can be a technological, economic, and cultural revolution and has significant future potential. It creates a solution to the negative impact of conventional livestock farming on natural resources such as air, water, and soil (63). It also significantly reduces animal food-borne diseases (29). Cultured meat production under sterile conditions significantly reduces the risk of contamination (73).

Production Methods for Cultured Meat

When producing cultured meat, the main goal is to reconstruct the complex structure of the animal musculature using a small number of cells. Cultured meat production generally consists of 5 stages: collecting a cell or tissue sample from a living animal, cell-banking, growth, harvest, and food processing (33).

Researchers take a biopsy from a live animal. Stem cells, which have the ability to multiply by cutting this muscle part but can also transform into different cell types such as muscle cells and fat cells, are released (65). Studies have reportedly shown that fetal bovine serum (FBS) is the ideal choice for a culture medium because it can support the growth of over a trillion cells, which naturally merge to create myotubes (6). However, this is not acceptable for vegans and vegetarians. To accelerate lab-grown meat production, researchers maintain the cells in a controlled environment that replicates the temperature found within an animal's body, like that of a cow (6).

Researchers use tissue and cell culture techniques to produce cultured meat. 3D printing and nanotechnology can also produce cultured meat in later stages. However, large-scale production of cultured meat requires technological advances in areas such as tissue engineering and bioreactors (78).

Embryonic stem cells and induced pluripotent stem cells (iPSCs) are required for ***the cell*** culture method. This method involves the isolation and cultivation of stem cells and adipocytes. Attached to a carrier or scaffold, these stem cells differentiate into more differentiated myotubes to form myofibers (45). One can gather, prepare, cook, and eat the resulting myofibers as ground meat or emulsified products (10). In essence, stem cells mimic the in vitro maturation phases of muscle fibers. Collagen, a naturally occurring and edible polymeric biomaterial, was used in the development of the scaffold, which enables the intricate structuring of cultured meat through 3D tissue culture (42, 54). Mechanical stretching of the scaffold aids in supplying nutrients to developing muscle cells (20). Thus, cell culture production aids in the development of tender, boneless meat (9).

Researchers first used the tissue culture technique to produce goldfish meat in vitro (7). The culture medium replicated the in vivo environment, making the cultured tissues resemble fresh fish fillets. However, the inability to precisely replicate the in vivo environment as well as the scarcity of blood and nutrients caused the growing cells to eventually turn necrotic. Various tissue culture and tissue engineering techniques proposed solutions in the following years (100).

Compared to other approaches, 3D printing is a novel and more advanced tissue engineering process. In addition to imitating the cellular structure of the muscles with 3D printers, it will also provide appropriate vascularization to carry blood to the whole organ (9). There are numerous types of 3D printers, including extrusion, inkjet, and laser-assisted bioprinters. While inkjets are the cheapest, laser-assisted ones have the highest resolution and are the most expensive. Microextrusion printers are slow and inexpensive (11).

The goal of nanotechnology, which is still in its early stages of development, is to create, test, and change materials with novel properties at the nanoscale (100 nm in diameter) (85). The ability to produce cultured meat using nanotechnology is critical for in vitro meat production, since these tiny molecules can improve the meat's color, flavor, and texture (94). In order to increase

the performance of biomaterials in various meat products, nanomaterials such as Poly lactic-co-glycolic acid (PLGA) nanoparticles, biopolymeric chitosan nanoparticles, and capsicum oleoresin nanoparticles have been employed (68).

Naturally occurring meat nanofibers affect the texture and color of the meat after cooking. Consequently, using nanotechnology to produce cultured meat may be successful. In addition, the packaging of meat products makes considerable use of nanotechnology (78). Manufacturers use a packaging film that distributes nanoclays over a polyamide-6 (PA6) matrix to package meat products. The hardness of meat products is increased, and the O_2 barrier qualities are improved with this nanoclay packaging film (61). Furthermore, meat science and technology may benefit from nanoscience interventions in areas such as increased sensory acceptance, improved nutrient bioavailability, targeted delivery of bioactive substances, and improved antimicrobial effects of preservatives (78).

Resource Requirements

The traditional livestock production system has a significant impact on the environment in terms of gas emissions, land, water, and greenhouse energy use. The impact of livestock on the emission of the three most important greenhouse gases, which are CO_2, CH_4, and N_2O, is 9%, 39%, and 65%, respectively. 15–24% of global greenhouse gas emissions are traced back to the global livestock production system, according to data from 2021. A huge portion of this percentage is caused by deforestation to create grazing land for livestock; however, livestock contributions to greenhouse gas emissions vary across nations and continents, as is evident (81).

The meat production system needs 15.500 m^3/ton of water, while the chicken production system requires 3.918 m3/ton of water to function (41), which increases the stress on water resources and the environment. In contrast with conventionally produced beef, lamb, pork, and chicken, cultured meat production emits significantly less greenhouse gas and uses less land, water, and energy by 78–96%, 99%, 82–96%, and 7–45%, respectively (89).

***Land Usage*:** Compared to conventional meat production, cultured meat causes 99% less land use (89). But there are also opposing views on this. As a source of conventional livestock manure, organic matter, nitrogen, and phosphorus, it contributes significantly to maintaining the carbon content and fertility of the soil. Livestock feed production requires 2.5 billion hectares of land, roughly 50% of the world's agricultural area; however, 1.3 billion of these hectares are pastures unsuitable for agriculture, benefiting only livestock (55). It may not be accurate to compare cultured meat to conventional meat based on land use. This comparison excludes the variety of environmental services and the effects of livestock farming methods, such as greenhouse gas (GHG) release, water use, plant and animal biodiversity (74).

Greenhouse Gas: The share of carbon dioxide and nitrous oxide emissions, particularly methane, originating from ruminants' digestive tracts is quite large in world greenhouse gas emissions. While some of the studies conducted on this subject showed that cultured meat was advantageous (89), others were inconclusive (53). Fossil energy used to heat the culture cells in cultured meat production releases carbon dioxide (20). Some studies indicate that cultured meat production will have less impact on global warming in the first stage compared to conventional farming, but this will not happen in the long term. Because carbon dioxide accumulates in the atmosphere for a longer time than methane (50).

Water Usage: In some studies, if we compare cultured meat with conventional meat in terms of water consumption, it is seen that cultured meat consumes 82–96% less water (89). However, although it is said that 15,000 liters of water are used to produce 1 kg of beef, 95% of this amount consists of water used to grow plant products and plants to be used in animal feeding. And in fact, it is widely accepted that 550–700 liters of water are needed to produce 1 kg of beef (27). The quality of the water used by firms that produce cultured meat is another issue. This is because chemical compounds in the water may have leaked into the environment. However, this may not occur if the situation is highly controlled (20).

Nutritional Aspects

Conventional meat consists of a number of different parts, including muscle, fatty tissue, connective tissue, and bones. Meat is a good source of critical nutrients and bioactive compounds such as vitamin B_{12} and heme iron, as well as protein, amino acids, fatty acids, minerals, and vitamins including Zn, Se, K, Na, Mg, creatine, and vitamins (A, B-complex, and D) (86, 99). In meat, the typical protein content is around 22%. Meat's amino acid composition varies depending on the animal; for instance, beef has more of the important amino acids valine, lysine, and leucine than lamb and pork (1). Additionally, a number of variables, including age, the presence of connective tissue, etc., have an impact on the amino acid and protein content of meat. The age of the animal and the amount of connective tissue are both inversely correlated with the amino acid and protein content of the meat. According to research, meat's nutritional value decreases when meat's concentrations of valine, isoleucine, phenylalanine, arginine, and methionine rise with animal

age while falling with an increase in connective tissue (24).

Beef also has unsaturated fatty acids like oleic, linoleic, and arachidonic acids (1). The most beneficial and important component of beef is polyunsaturated fatty acid, also known as omega-3 fatty acid. Saturated fatty acids further significantly increase the nutritional value of beef, while extreme consumption can lead to cardiovascular diseases (CVD) (23). Minerals such as Fe, Zn, Se, K, Na, and Mg, in addition to vitamins A and B complex, are related to the nutritional value of meat. Meat is the sole source of heme and vitamin B_{12}. Thus, iron and vitamin B_{12} make up the majority of meat's nutritional value. Specific types of gut-colonizing bacteria produce vitamin B_{12}, making it exclusive to animal products, while iron in meat exists as Fe_2, a highly accessible form of heme (23).

Cultured meat could not accurately replicate many characteristics of conventional meat, including protein content, amino acid structure, protein digestibility, fat content, vitamin-mineral content, texture, and color. More studies should be done on cultured meat in order to make cultured meat similar to conventional meat and thus create a positive perception of customer preference and ensure market entry and success. The following section focuses on the nutritional analysis of cultured meat.

Protein, Vitamin, and Mineral: More research is required since there is a lack of knowledge about the factors impacting cultured meat's protein concentration, amino acid composition, and protein digestibility (59). However, morphological findings indicate that the present culturing procedures produce in vitro meat with the majority of cytoskeletal proteins in the same range as conventional meat. Although the protein content of in vitro meat has not yet been established (64, 102), Two methods to promote or monitor the synthesis of sarcomeric proteins include electrical stimulation and scaffolding that can keep the muscle fibers under tension; however, they are pricy, inefficient, and only partially scalable (102). Changing the lipid composition of the medium can control the ratio of saturated and polyunsaturated fatty acids in cultured meat, although one should consider potential effects on rancidity (10, 20). The media may need to supplement with vitamins such as vitamin B_{12} and minerals such as iron, zinc, and selenium since cultured muscle cells cannot produce them. In order for these vitamins and minerals to enter the cells, there must be transport systems and binding proteins in the medium (66, 102). Genetically altered animal cells can enhance the nutritional profile of the meat produced. For instance, Stout et al. (83) demonstrated how to create prokaryotic enzymes in primary bovine and immortalized murine muscle cells to synthesize synthetic carotenoids (phytoene, lycopene, and carotene). It remains unclear how these chemicals absorb into cultured meat (32, 83). Beyond the vitamins and minerals included in cultured meat, it is unclear whether any supplements given by the growing medium will also benefit human health (20).

Textural aspects: More fundamental research is required to assess the impact of embryonic or neonatal isoforms of actin and myosin on potential protein deterioration during or after cell harvesting. Therefore, it is difficult to predict how the texture will change, as well as the rate and scope of the tenderization process (32, 102). Cultured meat can produce steaks and whole slices of meat. Processed meats like ready-made sausage and hamburger patties can also be made using cultured meat. The first option is the most challenging due to the thickness of the desired result, the lack of blood, the limitations on oxygen and nutrient delivery over the entire structure during differentiation, and the difficulties in generating the characteristic texture of conventional meat. Thin sheets of cultured cells measuring a few hundred microns, which have already been successfully generated, can be used for the latter. Electrical and/or mechanical stimulation can increase the size and length of immature myofibers, enhancing their structure and leading them to resemble more mature muscle fibers by creating more mature myofibrillar proteins (32). It is still unknown how well these processes work at producing myofibers that can take on the role of the meat proteins found in fresh and processed meats (32).

Taste and Odor Properties: It is one of the most difficult stages to compare the taste of cultured meat with conventional meat because meat consists of many components (18). In addition to the Maillard reaction products that occur when conventional meat is heated, the breakdown of lipids, peptides, and amino acids and the interactions between these molecules also have an impact on meat odor (84). Flavor precursors occur postmortem in conventional meat, so it is unknown how these characteristics will manifest in cultured meat (32). In addition, the lack of adipocytes in growing muscle fibers in vitro may limit the sensory qualities of the cultured meat produced (40, 43). Fat has an important role in the aroma, juiciness, and tenderness of meat, and various methods have been reported to improve this condition in cultured meat. Co-growth of myoblasts and preadipocytes can increase the ratio of intramuscular fat (73). Carotenoids reduce lipid oxidation and by adding carotenoids to cultured meat, sensory properties and shelf life can be increased (83). At the product manufacturing step, fat and flavoring agents can be added to the cultured meat, taking client preferences into account (32, 102). Techniques such as heating mushroom protein hydrolysates or combining defatted soybeans with soy sauce hydrolysates can make cultured meat smell like traditional meat (101).

DOI: 10.33988/auvfd.1425678

Color Properties: Cultured meat is colorless because it contains very little myoglobin. One can alter the color of cultured meat to resemble that of normal meat by directly incorporating myoglobin or hemoglobin into the medium or by adding ingredients like beetroot juice or saffron (though these may impact the flavor) (43, 77). Metmyoglobin and hemoglobin were added to the culture medium in one study, and as a result, it was found that culture meat grown in the medium with the additional metmyoglobin had a hue that resembled beef when cooked (77). To increase the color properties of cultured meat, hemoglobin can be acquired by extracting it from animal blood, plant tissue, or by synthesizing it utilizing microbial cells. However, these procedures require a lot of time and labor, or they are unsuitable for scaling up when biosynthesis is involved (101). However, GMOs can boost microbial production (13). European Food Safety Authority (EFSA) received a dossier for an application to produce soy hemoglobin in 2019. However, compared to animal hemoglobin, soybean hemoglobin is distinct in terms of both structure and function. The use of food-grade microbial strains and properly purified hemoglobin requires special attention (101).

Economic Impact

It is very important to examine cultured meat from an economic perspective. The cost of cultured meat may have an impact on inequality, and it is believed that traditional breeders and enterprises that provide animal food may suffer as a result (12, 82).

Agricultural Employment: Although people working in the agricultural sector in the EU account for only 4.4% of total employment (30), this rate is much higher in less developed countries (72). Cultured meat may eventually replace conventional meat (97). New employment opportunities will arise with cultured meat, but people engaged in agriculture generally have lower education levels (30) and, due to the technical nature of cultured meat production, highly educated people are required to work in this sector. Therefore, people engaged in traditional animal husbandry may lose their jobs to a great extent. However, traditional producers can provide limited and high-quality service in the meat market (12). They may adopt agroecology concepts or use biotechnologies such as cloning, genetic modification, etc. to improve sustainability. Alternatively, they can produce products such as biofuel, etc., for human consumption (47).

Consumer Inequality: Some researchers on cultured meat are concerned that inequality between the poor and the rich may increase further (22, 12, 82). They proposed an alternative perspective, indicating that while the affluent may prefer conventional meat, cultured meat could be more appealing to the underprivileged (12). In contrast, Cole and Morgan (22) thought that rich people could consume cultured meat, but poor people would still have to kill animals to consume meat. Although cultured meat's cost has drastically dropped recently, experts anticipate its initial market price to exceed that of conventional beef (36). Purdy (67) suggests that in the initial phase, restaurants may only be able to sell cultured meat at high prices. According to Fountain (31), in the later stages, the price of cultured meat may decrease much more and be sold cheaper than conventional meat. Furthermore, if the cost of cultured meat falls below that of conventional meat, the meat industry may reach a turning point (15).

Economic Impacts on Developing Countries: Agriculture and animal husbandry play an important role in low-income countries such as South Asia and some African countries. The livestock sector accounts for approximately 40% of global agricultural gross domestic product (GDP) and approximately 30% of agricultural GDP in the developing world (98). 1.3 billion poor people in the world live in developing countries and depend on livestock for their livelihood (69). Livestock farming is a very important sector in these countries, as it can meet food, income, and employment needs. Livestock farming acts as a buffer to reduce the impact of instability in crop production on maintaining the availability of food produced for human consumption and thus maintaining a stable food supply (58). Livestock farming serves as an insurance policy or bank account in many developing countries (60). As a result, cultured meat may negatively affect the livestock industry, especially in developing countries. In these countries, cultured meat will have an impact on both exports and employment. Although cultured meat does not necessarily indicate animal production elimination, it will affect the sustainability of livestock farming. Furthermore, due to cultured meat production, exports of conventional meat to developed countries may decrease significantly, causing some economic problems (43).

Cost Structure: The price is probably the most important consideration when purchasing cultured meat. Although the cost of cultured meat decreases in later stages compared to the first cultured hamburger patty, it is still more expensive than conventional meat. In addition, since the technologies used to produce cultured meat change every day, it becomes difficult to calculate the cost. Garrison et al. (34) calculated the cost of large-scale cultured meat production. The goal of their study was to determine how much it would cost to produce in vitro meat in a large-scale production facility that produces 540,000 kg of product per year. In this study, in addition to basic costs such as culture media, bioreactors, and labor (these

three elements account for 80% of the total cost), costs such as employment and transportation were also calculated in detail. The expected production cost was calculated at $34.9 million annually and $95,688 per day. Calculating the cost of producing one kg of cultured meat revealed it to be $63.69, with 59% of this cost attributed to culture medium and labor costs. Culture media contributes $19.7 to the production cost, while labor contributes $17.7. The production facility's maintenance and repair costs account for 8.6% of the total cost, and their contribution to the production price of 1 kg of meat is $5.47. Costs such as water, electricity, transportation, packaging, and borrowed operating capital have a relatively lower rate (2.88%, $1.83/kg). Bioreactors constitute 28% of the total cost and contribute $17.8 to the price of one kg of cultured meat. Building and property rentals, cold storage, information and technology infrastructure, and insurance account for less than 2% of the total cost and contribute $1.26 per kg. Garrison et al. (34) suggested sensitivity scenarios after the cost analysis. The first scenario (SC1) involves a 30% change in individual production costs, with all other variables being constant. The facility will operate 365 days a year, that is, with zero interruption. As a result, a 30% change in growth medium will have an impact on the total cost of $5.88, and the price of 1 kg of cultured meat will be $57.8 instead of $63.69. The impact of SC1 on total cost is most sensitive to the costs of growth media, labor, bioreactors, and processing equipment. The second scenario (SC2) was made about what the total cost would be if 36.5 days (10%) of the year were required for maintenance and repair activities in the facility and stand-in-place cleaning. As a result of this 10% increase in time, the production cost of 1 kg of meat increases by $4.22, bringing the price to $67.91. Furthermore, fixed costs such as building rentals, computer infrastructure, bioreactors, individual labor costs, working capital interest, processing equipment, cold storage, and insurance all increase. Because these costs are normally spread over a smaller number of production days per year, For example, the total cost of labor and extra rights increases by $1.96, from $17.65 to $19.62 per kilogram. In SC3, we calculate the impact of both the changes in SC1 and SC2 on the total cost, which includes a 10% increase in total outage days over the year and a 30% change in individual production costs. As a result of the 36.5-day increase in the days the facility is closed, the additional costs will be the same as SC2, but each production cost will have additional changes depending on the interpretation of each cost change. For example, the positive assumption they make about the cost of technology and cultural media in SC1 may be unreasonably high. In this case, a 30% change in the growth medium should be considered an increase in total cost. The 30% decrease in labor costs ($5.89) should be seen as reducing the total cost when considering labor and their extra rights. Various studies have been conducted to reduce the cost of production of cultured meat, and one of them is the potential scenario Specht (80) studied to reduce the cost of cell culture media. His scenario reports that reducing the cost of the culture medium can decrease the cost of 1 kg of cultured meat from $63 to $44.09 (80). If they reduce the bioreactor and labor costs by 25% in addition to the culture media costs in Specht's scenario, the price of one kg of meat is $35.09; if they reduce it by 50%, it will be $26.1; and if they reduce it by 75%, it will be $17.1 (all remaining costs are assumed to be constant). Even with considerable price decreases in these scenarios, simply reducing the cost of one factor that contributes significantly to the total cost remains insufficient to compete with the price of conventional meat; substantial savings must be achieved across all main costs (34).

Alternative Production and Consumption Locations: Garrison et al. (34) found the cost of producing 1 kg of cultured meat to be $63, and production and consumption in this study were assumed to be in California (USA). However, countries like China and India can reduce this cost even further. More than half of the world's population lives in Asia (4.7 billion), and according to 2019 data, 2.83 billion of them live in China and India (90). In these countries, which may be advantageous compared to the USA in terms of labor costs, China may have 4% lower manufacturing labor costs than the United States, but cultured meat production can also appeal to low-income segments. It can also produce cultural media at a lower cost. However, they have to import bioreactors (34). As China's low labor costs approached US prices, companies turned to India. The minimum wage in India is 37% lower than in China (35). But transportation is expensive in India. Additionally, for some businesses, there are regulations that will make labor costs more expensive (8). Countries such as China and India may produce cultured meat at a much lower cost compared to the USA, but this reduction may not be as high as 30% (34). In Garrison et al.'s (34) study, it is estimated that the restaurant or supermarket price of cultured meat, which costs $63, will be over $100. Bioreactors, culture media, and labor costs alone total over $55. To produce at a lower cost, reducing the cost of the culture medium requires new technologies and innovations. Many countries do not approve cultured meat for human consumption, and when they do, it appears that it will be much more expensive than conventional meat.

Market Trends: Cultured beef patties, which were produced for the first time in 2013 from cultured meat, attracted the attention of investors and the media, and the

number of companies entering this sector has increased, especially in recent years. In one study, at least one of 32 cultured meat establishments had 25% interest in beef, 22% in chicken and duck meat, and 9% in pork and seafood (such as fish and shrimp). In addition, as an alternative to pet food, 2 businesses are looking into mouse meat, and 1 company is looking into kangaroo and horse meat. Of these 32 businesses, 40% are located in North America, 31% in Asia, 25% in Europe and 3% in Australia (19). Investors publicly disclosed almost 320 million US$ in cultured meat enterprises in 2015 and early 2020. Approximately 242.29 million US$ was allocated for beef and pork production, and 49.5 million USD was allocated for seafood. Many businesses can easily transition from producing animal products to poultry production, engaging in both simultaneously. While business-to-consumer is still the dominant business model, other business-to-business models have begun to take hold, including those that produce growth factors and media for cell culture, cell lines, cell production, or using fats as ingredients (19).

Consumer Perspective

The success of cultured meat depends heavily on consumer perception selection, since consumer perception is a very essential factor in product selection (5). Due to this, numerous studies have been done to determine how customers feel about cultured meat.

In a study conducted by Wilks and Phillips (96), in which 673 people participated, 65.3% of the participants stated they would try cultured meat, 32.6% were willing to consume it regularly, 47.7% expressed they would rather consume cultured meat over soy-based meat alternatives, and 31.5% stated that they want to replace conventional meat with cultured meat. 11% of the 533 participants in a different research study said they would prefer cultured meat to conventional or plant-based meat (79). According to Hocquette et al. (39), 19.2% of 817 participants were eager to eat cultured meat. According to Bryant and Barnett (16), 66.4% of the 1,185 US participants would try cultured meat, 48.9% would routinely consume it, and 55.2% would choose it above conventional meat. According to Dupont and Fiebelkorn (28), 56.4% of participants (63.2% of males and 53.3% of females) were willing to eat the cultured meat burger. 54% of the 525 Italian participants were willing to sample cultured meat (51). A study by Weinrich et al. (95) found that 30% of respondents claimed they would like to routinely eat cultured meat.

In addition to this research, demographic patterns have a significant impact on how individuals feel about cultured meat. According to Wilks and Phillips (96), males, those with low incomes, and liberals were more enthusiastic about cultured meat. Males are more likely to ingest cultured meat, according to Slade's (79) research, which also found that younger and better educated people are likewise more likely to do so.

According to research, cultured meat consumption was more prevalent among males, younger people, and urban residents than it was among females, older people, and rural residents (88). Mancini and Antonioli (51) found that young, well-educated individuals knowledgeable about cultured meat and willing to reduce their meat consumption are the likely consumers of cultured meat.

Consumers largely opposed eating lab-raised meat due to its unnaturalness, safety, healthiness, flavor, and texture. The most prevalent misconception about cultured meat is that it is artificial. One of the elements impacting how cultured meat is perceived in comparison to conventional meat, according to Marcu et al. (52), is "natural and artificial." The notion that cultured meat is an artificial product appears to be a significant barrier to society's acceptance of cultured meat (48). According to Wilks and Phillips (96), there is general agreement that cultured meat is "unnatural" in comparison to conventional meat. Consumers in three European Union nations initially strongly reject and worry about the unnaturalness of cultured meat upon learning about it. Consumers acknowledged potential societal benefits on a global scale, but saw few direct personal benefits from cultured meat after consideration (92).

In a study conducted with participants from America, India, and China, 64.6% of the participants were willing to try cultured meat, 49.1% were willing to consume it regularly, and 48.5% were willing to consume conventional meat instead. And with these results, they concluded that cultured meat can replace conventional meat to a significant extent (16). However, in a study, one-third of the participants answered ‘’I don't know’’ and concluded that educated consumers in different countries would not routinely consume cultured meat (39). However, more research and education are crucial to altering how people view engineered meat.

Food safety is another common problem with cultured meats. Laestadius and Caldwell (48) reported some concerns that cultured meat may cause cancer and that cancerous cells may develop through cell proliferation. Hocquette (40) reported that these cells are unlikely to harm consumers as they die during digestion. In their study, O'Keefe et al. (56) demonstrated that consumers would only consider consuming cultured meat if its safety was confirmed. Verbeke et al. (92) stated in their study that people would prefer the safe to the unsafe.

Another common negative situation among consumers against cultured meat is the lack of flavor, appearance, and texture of conventional meat in cultured meat (88). Similar to this, Verbeke, Marcu, et al. (92) revealed that participants believed that cultured meat

would taste terrible in their study. According to Hocquette et al. (39), just 23.6% of respondents thought cultured meat would be delectable, 39% disagreed, and 37.5% were unsure. In Slade's (79) study of 533 participants, nearly 90% thought that cultured meat tasted worse than conventional meat, although most thought it was superior to plant-based meat replacements.

Cost is another negative perception. Cultured meat would be more expensive than conventional meat (93). In the study of Wilks and Phillips (96), participants stated that they expected cultured meat to be cheaper. According to O'Keefe et al. (56), study participants thought that cultured meat should be more affordable to get wider adoption. Consumers may be influenced by price competition in real life, as a substantially lower price is a significant predictor of choice for cultured meat (79). Cultured meat's association with increased wealth disparities has been documented (12, 22, 82).

According to Bonny et al. (12), cultured meat will appeal to individuals with lower incomes, while wealthier people will still eat conventional meat. On the other hand, people worry that cultured meat, which is significantly more expensive than conventional meat, may allow the wealthy to consume meat without moral consequences, leaving only the poor to kill animals for a living (22). Cultured meat was initially only available in restaurants at exorbitant rates (67). Pricing is one factor that prevents customers from choosing cultured meat, which has the potential to have a significant impact on consumer behavior. The current price of cultured meat makes it possible to view cultured meat consumption as a luxury. Cultured meat production efficiency improvements may lead to it becoming more affordable than conventional meat in the future (31). But as cultured meat consumption increases, it may lose its opulent and prestigious structure.

Another important consideration is the ethical implications of cultured meat. According to Hocquette et al. (39), the majority of participants did not think that cultured meat would resolve issues with animal welfare in the livestock business. However, Wilks and Phillips (96) argued that cultured meat is ethical compared to conventional meat. In addition, due to the absence of the nervous system, cultured cells and cultured meat are believed to be painless, although animal biopsies to remove cells may increase concerns about animal welfare. Because it is a painless process, some scientists consider cultured meat to be vegetarian (17).

The goal of cultured meat is to produce meat with a lot fewer animals than traditional methods. Indeed, some vegetarians and vegans who want to cut back on their meat consumption for ethical reasons may find this to be appealing (42). One of the debates on this issue is the use of fetal bovine serum (FBS) when cultured meat is processed. In addition, some vegans avoid meat consumption because of its taste. Some vegans consider eating meat that can be produced without causing animal suffering. In the current situation, animals that are grown with or without pain are needed to produce cultured meat; that is, animals continue to be used to produce cultured meat (3).

Consumers also express negative attitudes related to religion. For Jews, it is a matter of debate whether cultured meat is kosher. Some Jews debate whether cultured meat can be considered Kosher, as they question if the cells can maintain their original identity regardless of the animal source (46). Islamic terms consider in vitro meat as Halal only if the stem cell comes from an animal slaughtered Halal and no blood or serum is used during the process (37). For Hindu consumers, the lack of animals to continue their rituals is a matter of concern (20).

In addition, attitudes toward cultural meat differ from country to country. For example, healthy nutrition has emerged as the most important factor for consumer acceptance in China (49). In a study conducted in Spain, the United Kingdom, the Dominican Republic, and Brazil, it was revealed that the cultural meat acceptance rate was 42% in Spain, 20% in the United Kingdom, 15% in the Dominican Republic, and 11.5% in Brazil. Researchers found that individuals with a traditional mindset are less accepting of new things (49).

Age is another important factor in the acceptance of cultured meat. Reports show that individuals aged 65 and above in Europe exhibit a greater interest in cultured meat, whereas young people globally demonstrate a higher acceptance rate (95). Furthermore, political orientation can be helpful when one wants to consume cultured meat. According to a study, liberals are more open to eating cultured meat than conservatives. Environmental issues and animal welfare also influenced liberals. Young people and city dwellers were also found to be influential among liberals (97).

It has been observed that education and socioeconomic status are also effective in influencing attitudes towards meat consumption. Low-income consumers in the United States found cultured meat more acceptable (96), while high-income individuals in New Zealand found it more acceptable (14).

Future Prospect

Meat and other animal products have always been important in human nutrition (2). Although meat production has tripled in the last 50 years (71), the demand for cultured meat production may increase due to reasons such as the increase in the costs of resources such as land, energy, and water, the fact that the world population will increase much more, and the need to increase production by at least 70% to meet the increasing demand (19). In addition to these reasons, the traditional livestock

industry's negative effects on the environment are another reason why people are interested in cultured meat. Another possible negative effect of traditional meat (especially red meat) is that it may negatively affect human health. There is increasing evidence that traditional meat can be linked to causes such as the emergence of chronic diseases in humans and an increased risk of early death (75). As a result, consumers have a common desire to produce more animal products that are environmentally sound, compatible with global food security, and more economical (21).

In the search for alternative meat, recent technologies have led to an increase in plant-based meat production. However, despite plant-based meats, traditional meat consumption is still important for many people. These problems in conventional meat production and the inability of plant-based meats to meet the required demand have led scientists to develop cultured meat production. Cultured meat seems to face some problems in its current state (70).

The consumer's perception of cultured meat currently consists of many factors, such as ethical values, nutritional content of meat, political opinion, education level, age, socioeconomic factors, and product familiarity (78). The majority of society is willing to ingest cultured meat, despite the fact that there are numerous opposing views regarding it in the current context (91). Consumers have various concerns for multiple reasons. The perception of unnaturalness caused by naming cultured meat as "in vitro," "synthetic," or "laboratory grown" (70) and the concern that various rituals will disappear (e.g., Thanksgiving turkeys) (15). However, because of its several advantages, including reducing animal suffering (38), providing protein to low-income populations, and enhancing animal welfare, it has great potential in the future (91).

Although the future of cultured meat looks good, it should not be forgotten that new technological developments are a risk, and moving away from traditional animal husbandry may have negative consequences (62). For example, rapid cell growth and division can increase the risk of mutations and potentially cancerous cells (82). However, Hocquette (40) asserts that while malignant cells may form in cultured meat, these cells will be dormant and unable to multiply until the user consumes that product. This means that it is unlikely to cause long-term harm to the consumer, although this is something that requires further investigation.

Traditional meat producers are often at risk of respiratory diseases and infections (75). In addition, there are negative consequences, such as people working in this sector having higher levels of stress and their mental health being negatively affected (75). It has been reported that cultured meat can prevent pathogens from passing from animals to humans and may be effective in preventing pandemics in the future (21, 43).

A switch to cultured meat could harm small-scale local meat producers and farmers, as well as widen the gap between rural and urban areas (76). Tubb and Seba (87) reported that plant-based meat alternatives could be five times cheaper than conventional meat by 2030, with a market shift of up to 40%. They also reported that the value of farmland could decrease by up to 80% in the United States alone. While cultured meat may soon be available on the market (70), there is also a view that plant-based alternatives could dominate the market at least until 2030 (44).

Another obstacle to the future of cultured meat is that it requires advanced production training and has a highly technical structure. To ensure cultured meat becomes as common as conventional meat in the future (82), we must seek answers to questions regarding the role of traditional producers and businesses in this sector, the long-term social, political, economic, ethical, and environmental effects of cultured meat, how information about cultured meat should be communicated, and how its proper location should be determined. Cultured meat's potential is great, but the industry's future remains complex and uncertain (62).

Conclusion

Reasons such as its negative effects on the environment, the challenge of meeting future food demand as the population grows, and concerns about animal welfare may lead to the replacement of conventional animal husbandry with alternatives in the future. Today, meat alternatives produced from plant-based proteins and cultured meat can be produced, although not on a large scale.

Cultured meat has great potential in the future in many aspects, such as having fewer negative effects on the environment and using fewer resources than conventional meat, being able to eliminate food-borne zoonotic diseases, and animal welfare. However, problems such as cost, production difficulty, nutritional content, physical properties of meat such as texture, taste, color, and smell, and the perspective of some consumers on cultured meat also need to be overcome.

There are people who are very prejudiced about cultured meat, which is still not well known, and this may make it difficult to consume cultured meat as a meat alternative in the future. Practices such as increasing awareness about cultured meat, regulating costs, increasing the production scale, developing production methods and using new technologies in production, and determining marketing strategies according to countries may be important for cultured meat to take its place in the market as a meat alternative and its success in the market.

DOI: 10.33988/auvfd.1425678

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.

Author Contributions

YA conceived and planned the concept and content of the review. AP, GİA, and ŞO contributed to the review and interpretation of the data. ŞO took the lead in writing the manuscript.

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ISSN 1300-0861 • E-ISSN 1308-2817 Volume 71 • Number 4 • Year 2024

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