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The mandibles of the Honamli and Hair goat *(Capra hircus)*; a geometric morphometric study

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Abstract: The aim of the study was to analyze mandible of the Honamli and Hair goats according to the sex factor by geometric morphometric methods. A total of 36 adult goat mandibles including 9 males and 9 females for each breed, were used in the study. After the mandibles were photographed from the left lateral side, 10 homolog landmarks were marked. Generalized Procrustes, Relative Warp, and Principal Component Analysis were performed for the data of landmark coordinates. In the study, the first principal component explained 28.752% and 37.325% of the total shape difference in the Honamli and Hair goat, respectively. In the analysis made among goat breeds according to the sex factor, the first principal component explained 40.809% of the total shape difference in females and 30.486% of the total shape difference in males. Consequently, the Hair goat showed a significant sex difference compared to the Honamli goat. Besides, it was remarkable that male goats clustered clearly compared to female goats in terms of the breed factor.
Keywords: Geometric morphometry, Honamli goat, mandible, Principal Components Analysis.

Honamlı ve Kıl keçisi *(Capra hircus)* mandibula'sı; geometrik morfometrik bir çalışma

Özet: Çalışmada geometrik morfometrik metotlarla Honamlı ve Kıl Keçisi mandibula'sının cinsiyet faktörü ile birlikte analiz edilmesi amaçlandı. Çalışmada her ırk için 9'ar adet erkek ve dişi olmak üzere toplamda 36 adet ergin keçi mandibula'sı kullanıldı. Mandibula'lar sol lateral yönden fotoğraflandıktan sonra 10 adet homolog landmark işaretlendi. Landmark koordinat verileri General Procrustes, Relative Warp ve Temel Bileşenler analizine tabi tutuldu. Çalışmada Honamlı ve Kıl Keçisinde cinsiyetler arası yapılan karşılaştırmada birinci temel bileşen toplam şekil farklılığının sırasıyla %28,752 ve %37,325'ini açıkladı. Cinsiyet faktörüne göre keçi ırkları arasında yapılan analizde ise birinci temel bileşen dişilerde toplam şekil farklılığının %40,809'unu, erkeklerde %30,486'sını açıkladı. Sonuç olarak Kıl Keçilerinin Honamlı Keçilerine göre oldukça belirgin bir cinsiyet farklılığı göstermesi, ırk faktörü bakımından erkek keçilerin, dişi keçilere kıyasla net bir şekilde kümeleşmesi dikkat çekiciydi.
Anahtar sözcükler: Geometrik morfometri, Honamlı keçisi, mandibula, Temel Bileşenler Analizi.

Introduction

The domesticated goat, also known as *Capra Hircus*, is a species from the genus Capra belonging to the family *Bovidae* (2, 24). Being one of the first domesticated animal species along with sheep (37). Goat is an animal species that is commonly reared for its meat, milk, skin, and hair products from the first periods of mankind. This animal species can adapt to different environmental and breeding conditions and is resistant to many diseases; therefore, it is commonly reared (7, 19).

Honamli goat is a new goat species under protection that has been registered based on the communique of the Turkish Ministry of Agriculture and Forestry (34). The foreheads and legs of purebred Honamli goat reared in Taurus region are white or brown and their bodies are covered with black hairs (6). Being found in all the regions of Turkey, Hair goat is another goat breed commonly reared in the mountainous areas, forestry, and maquis shrublands like Taurus mountains (33).

Geometric morphometry is a shape analysis method that has been commonly used in recent years and is developed by subjecting geometric methods to the form difference analysis. Form is the geometric data obtained by removing variation differences from an object (38). Shape is the geometric properties which are steady based on environmental compatibility, location, and scale (31).

In geometric morphometry, homolog landmarks (LM) are used and the geometric structure of the samples are digitized at Cartesian coordinates (23). Thus, only the shape of sample is analyzed but not its form (20, 32). Slice (32) has reported that the geometric morphometry method offers more appropriate data for statistical analyses when compared to the traditional morphometric studies. There are studies in the literature using different types of geometric morphometry (8, 12, 13, 22, 36). However, there is no geometric morphometric study in Hair and Honamli goat breeds in the literature. For this reason, the aim of the study was to determine the shape differences in the mandibles of Hair and Honamli goat breeds in terms of sex factor using geometric morphometry method.

Materials and Methods

The study was approved by the Local Ethics Committee of Burdur Mehmet Akif Ersoy University (Approval number 2020/645). Mandibles of 36 (4 groups) adult Honamli and Hair goats, including 9 males and 9 females from each breed, were used in the study. The materials were removed from the skin and soft tissue and macerated by boiling.

Geometric morphometric analysis: The mandibles were photographed from a 30-cm distance on the left lateral side by focusing on the third premolar tooth (Canon EOS 650D, Japan). The images were saved in JPEG format in the computer. These images were converted into tps format using TpsUtil (Version 1.79) software (30). 10 homolog LMs (36) (Figure 1) were marked on the images through TpsDig2 (Version 2.31) (28) software so that the Cartesian coordinates of LMs were determined. Homolog LM confirmatory test was performed by using TpsSmall (Version 1.34) (27) software. Also, the slope and correlation values were determined as 0.999777 and 1.0 in Honamli goat, and 0.999694 and 1.0 in Hair goat, respectively.

As there were differences between the mandibles in terms of size, position and direction, Generalized Procrustes Analysis (superimposition-GPA) was performed (32). PAST (Version 4.02) (14) software was used for this analysis. Principal Components Analysis (PCA) was performed on the new coordinates obtained as a result of Procrustes Analysis. Thus, the degree of distinguishing the samples based on breed and sex was determined by applying Covariance Analysis between the factors (38). In addition, MorphoJ (16) software was used to indicate LM levels and directions where the shape differences were observed.

In the study, Relative Warp Analysis (RWA) was performed by TpsRelw (Version 1.70) (29) software and the consensus graphics of the groups were formed. The distribution of the groups on graphic was also tested by this analysis.

Statistical analysis: The statistical analysis of LM coordinate values based on the groups was performed by ANOVA test in PAST (Version 4.02) (14) software.

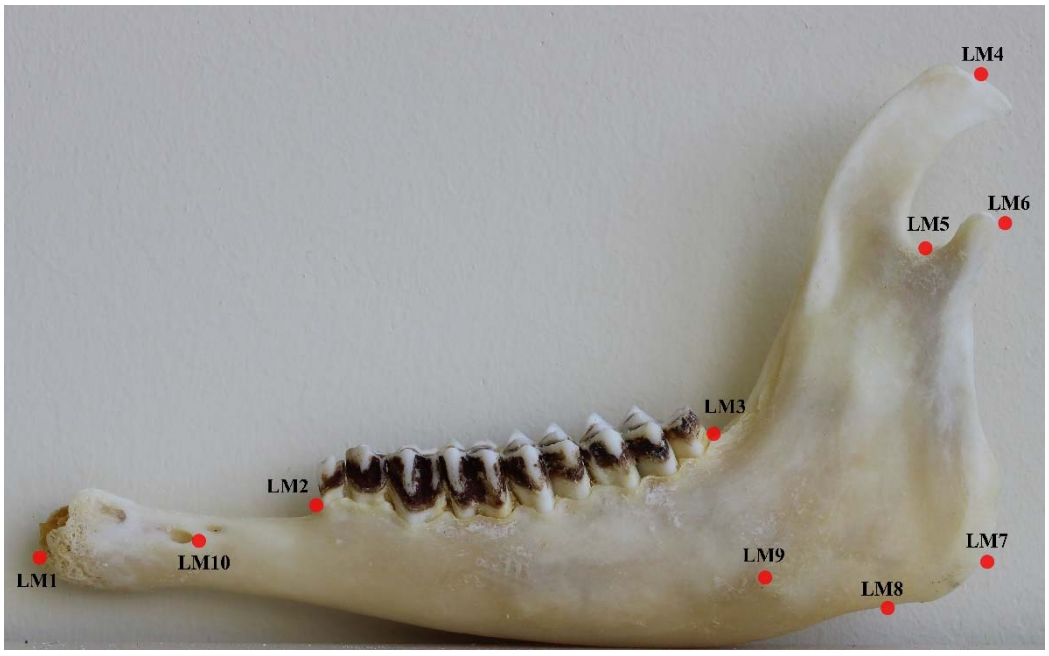

Figure 1. The view of Landmarks on mandible of the Honamli goat. LM1. Aboral cranioventral end point of alveoli dentales of I1, LM2. Cranioventral margin of P1, LM3. Caudoventral margin of M3, LM4. End-middle point of processus coronoideus, LM5. Medioventral point of incisura mandibula, LM6. Caudal end point of condylus mandibulae, LM7. Caudoventral corner of angulus mandibulae, LM8. Incisura vasorum facialium. LM9. Cranial junction point of the dorsal and ventral axes of fossa masseterica, LM10. Caudal margin of foramen mentale.

Results

Table 1 shows the results of the PCA in the study. Accordingly, the first Principal Component (PC) accounted for 28.752% and 37.325% of the total shape difference in the sex-based comparison made in Honamli and Hair goat breeds, respectively. In the analysis performed between the goat breeds based on the sex factor, the first PC accounted for 40.809% of the total shape difference in females and 30.486% of the total shape difference in males.

Figure 2 shows the results of the first PC based on the breed and sex factors. Accordingly, the sexual dimorphism in terms of the first PC was more apparent in Hair goat compared to Honamli goat (Figure 2 a, d). Also, based on the first PC, the distinction of the male Honamli and Hair goats was more apparent compared to the female counterparts (Figure 2 b, c).

Table 1. Results of the PCA, S: sex, B: breed.

PC	Honamli goat (S)		Hair goat (S)		Female goat (B)		Male goat (B)	
	Eigenvalue	Variance %	Eigenvalue	Variance %	Eigenvalue	Variance %	Eigenvalue	Variance %
1	0.000588581	28.752	0.000749422	37.255	0.000836954	40.809	0.000598768	30.486
2	0.00047459	23.183	0.000421354	20.946	0.000480911	23.449	0.000400184	20.375
3	0.000316428	15.457	0.000210353	10.457	0.000199762	97.402	0.000335132	17.063
4	0.000248741	12.151	0.00018255	90.748	0.000161586	78.788	0.000180213	91.753
5	0.000151471	73.992	0.000131645	65.442	9.99E-01	48.717	0.000137439	69.976
6	8.51E+00	41.575	9.90E+00	49.232	7.73E+00	37.685	0.000109006	55.499
7	5.56E+00	27.172	7.88E+00	39.194	7.23E+00	3.525	6.07E-01	30.895
8	3.81E+00	18.624	4.70E+00	23.353	4.66E+00	22.714	4.74E+00	24.133
9	3.04E+00	14.864	3.54E+00	17.615	2.61E+00	12.712	3.36E+00	17.126
10	2.00E+00	0.97888	2.25E+00	11.163	1.59E+00	0.77733	2.64E+00	13.463
11	1.45E+00	0.70964	1.30E+00	0.64772	1.34E+00	0.6526	1.48E+00	0.75217
12	1.01E+00	0.49278	8.77E-01	0.43618	8.84E-01	0.431	1.40E-01	0.71142
13	7.84E-01	0.38304	6.49E-01	0.32281	5.14E-01	0.25051	4.02E-01	0.20489
14	3.37E-01	0.16467	3.60E-01	0.17875	4.02E-01	0.19613	1.73E-01	0.08831
15	1.94E-01	0.094936	1.19E-01	0.059029	1.34E-01	0.065548	6.40E-02	0.032603
16	1.86E-02	0.009079	4.55E-02	0.022642	8.27E-02	0.040322	5.22E-03	0.0026602
17	3.23E-03	0.0015775	1.03E-03	0.00051376	3.74E-03	0.0018216	1.76E-05	8.98E-02

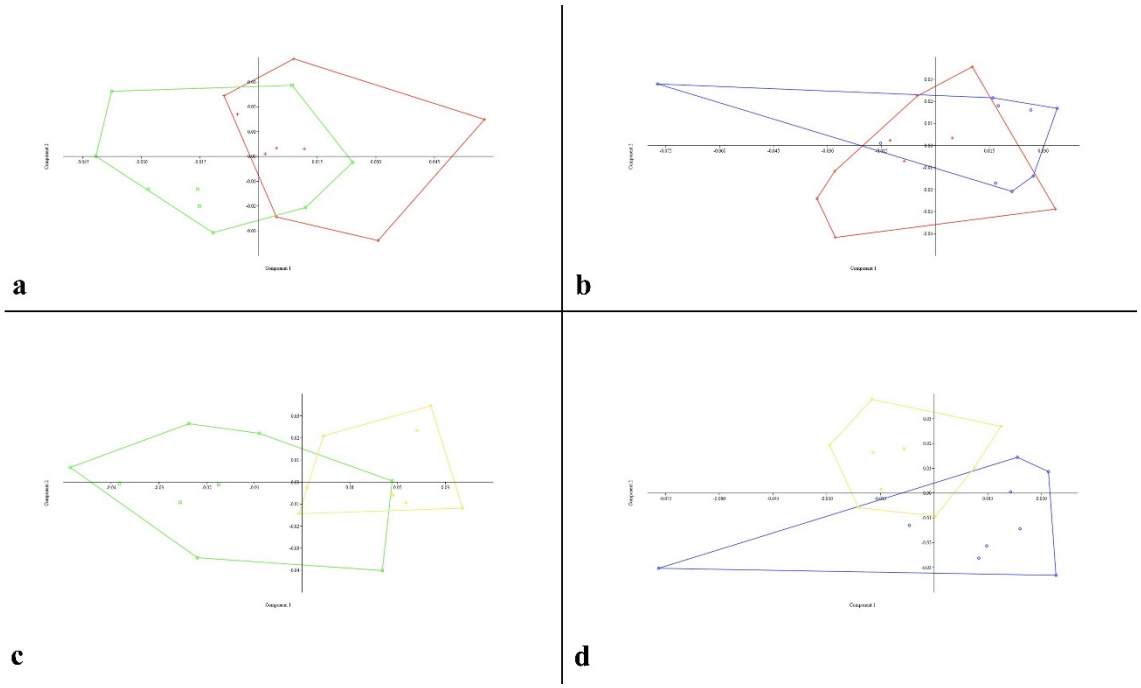

Figure 2. Graphic representation of the results obtained based on the first PC. a. Honamli goat (Sex), b. Female Honamli and Hair goat (Breed), c. Male Honamli and Hair goat (Breed), d. Hair goat (Sex).

Figure 3 shows the consensus graphics obtained as a result of RWA along with variation vectors in the study. Accordingly, vectoral variation density was determined at LM3, 4 and 9 levels in the female Honamli goat and at LM3, 4, 7, 8 and 9 levels in the male Honamli goat (Figure 3 a, c). The individual shape variation density was determined at LM1, 4, 7, 8, and 9 levels in the female Hair goat and at LM3 and 4 levels in the male Hair goat (Figure 3 b, d). Also, Figure 4 shows the graphics obtained as a result of the RWA made between the groups. According to the result of this analysis, it was remarkable that the sexual dimorphism was more apparent in Honamli goat than Hair goat (Honamli goat RWA1: 51.12%, RWA2: 26.68%, RWA3: 13.01%; Hair goat RWA1: 58.45%, RWA2: 19.11%, RWA3: 15.88%). It was observed in the breed distinction performed based on the sex factor by RWA that the male goat was diverged more apparently from the female goat (Male goat RWA1: 57.23%, RWA2: 22.18%, RWA3: 12.35%; Female goat RWA1: 53.81%, RWA2: 24.50%, RWA3: 13.72%).

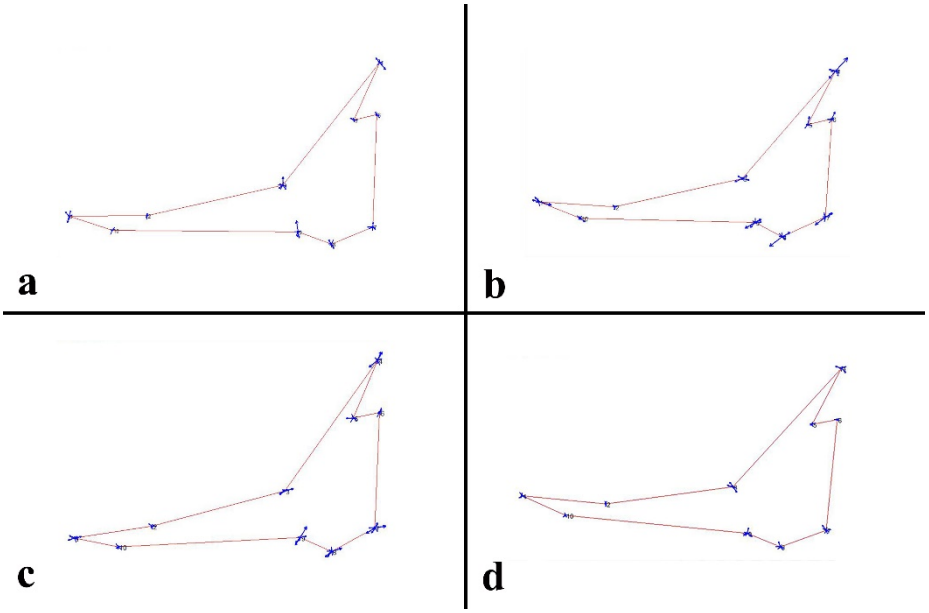

Figure 3. Consensus graphics based on groups, a. Female Honamli goat, b. Female Hair goat, c. Male Honamli goat, d. Male Hair goat.

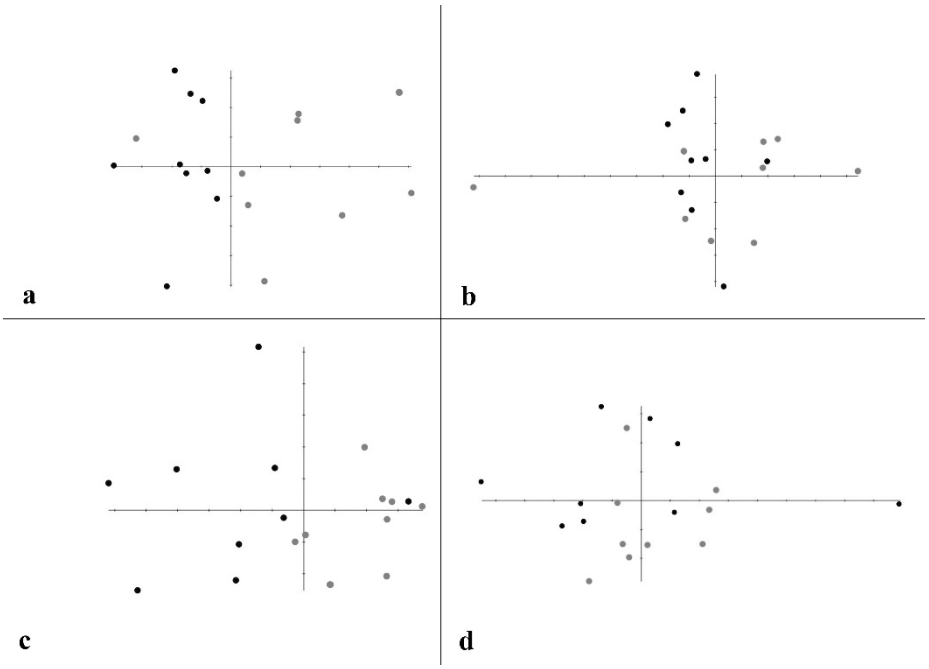

Figure 4. Graphic of RWA. a. The black points represent the female Honamli goat and the grey points represent the male Honamli goat, b. The black points represent the female Honamli goat and the grey points represent the female Hair goat, c. The black points represent the male Honamli goat and the grey points represent the male Hair goat, d. The black points represent the female Hair goat and the grey points represent the male Hair goat.

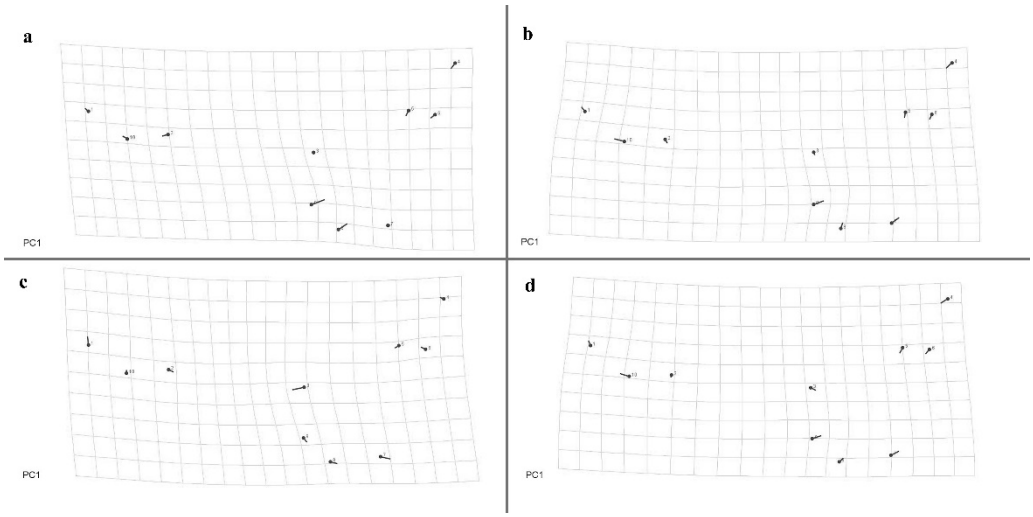

Figure 5. The lollipop representation of the shape differences occurred on the landmarks based on the first PC. a. Honamli goat (Sex), point represents female Honamli goat, b. Female Honamli and Hair goat (Breed), point represents female Honamli goat, c. Male Honamli and Hair goat (Breed), point represents male Honamli goat, d. Hair goat (Sex), point represents female Hair goat.

Table 2. Results of the statistical analysis of Landmark values.

Landmarks	Coordinates	Significant	F	P-value
	X	NS	1.777	0.17
LM1	Y	AB	8.37	0.00038
	X	C	3.961	0.01636
LM2	Y	B	9.921	0.00012
	X	C	3.103	0.03872
LM3	Y	NS	1.534	0.2245
	X	NS	0.8482	0.4822
LM4	Y	NS	1.854	0.1559
	X	NS	2.067	0.1242
LM5	Y	NS	4.979	0.0057
	X	A	4.39	0.01008
LM6	Y	NS	1.262	0.3036
	X	B	3.932	0.01597
LM7	Y	NS	2.265	0.09833
	X	A	2.937	0.04858
LM8	Y	NS	1.073	0.3737
	X	NS	0.9657	0.4198
LM9	Y	NS	1.002	0.4069
	X	NS	0.4384	0.74
LM10	Y	NS	1.186	0.3338

NS. Non-significant (P>0.05), **A.** Significant statistical difference between the male and female Honamli goat (P<0.05), **B.** Significant statistical difference between the male Honamli and Hair goat (P<0.05), **C.** Significant statistical difference between the male and female Hair goat (P<0.05).

Figure 5 shows the LM levels where shape differences occurred. Accordingly, there was a cranioventral shape difference at LM4, 5 and 6 and a caudodorsal shape difference at LM8 and 9 (Figure 5 a) in Honamli goat. Similar but less apparent shape differences were determined in Hair goat (Figure 5 d). In the comparison of breeds in terms of the sex factor, a craniodorsal shape difference at LM1, a point shape difference at LM3, an cranioventral shape difference at LM4, 5 and 6, a caudodorsal shape difference at LM7, 8, and 9 and a cranial shape difference at LM10 were observed in the female goats (Figure 5 b). A dorsal shape difference at LM1, a cranioventral shape difference at LM3 and 5, a craniodorsal shape difference at LM4 and 6, a caudoventral shape difference at LM7, 8 and 9, and a dorsal shape difference at LM10 were determined in the male goats (Figure 5 c).

Table 2 shows the data obtained as a result of the comparison of LM x and y coordinate values between the groups. Accordingly, a statistically significant difference was observed at LM1, 6 and 8 in Honamli goat and at LM2 and 3 in Hair goat (P<0.05). No statistical difference was

determined at LMs in the female goats (P>0.05). But there was a statistical difference at LM1, 2 and 7 in the male goats (P<0.05).

Discussion and Conclusion

In the study, the mandibles of two native goat breeds of Anatolia were analyzed by using geometric morphometric methods in terms of sexual dimorphism. In the literature, no study analyzing goat mandible in terms of breed and sex factors by using the geometric morphometric method was found. This is the first attempt to investigate both goat species and goat breeds. This is the most apparent limitation of this study.

Native goat breeds are a superior gene source with their high adaptation skills for the local conditions (9). Considering that goats have been a part of human life for a very long time, it is quite possible to find native animal breed bones in archaeological excavations. The data obtained from the archaeological bone material are important for fauna determination, the comparison with other historical processes, and estimation of the morphological characteristics of animals (5, 11, 21). The morphological data to be obtained in the skull and mandible of the living mammals by using geometric morphometric method may be used to reveal the phylogenetic relationships (17). Landmark-based geometric morphometry method analyzes shapes without linear deterioration by keeping their integrity (1, 25). For this reason, the data of the present study are important to provide principal formal information on the small ruminant mandible remains uncovered in the archaeological excavations in the Western Mediterranean region with high archaeological value.

Researchers commonly use the phenotypical and morphological characteristics in the distinction of animal breeds (3). Geometric morphometric analysis provides to determine shape differences which cannot be determined visually on LM coordinates. Therefore, it measures the shape change amount using the location differences of coordinates between objects (15, 35, 39). Superimposition (GPA), which is a geometric morphometric procedure, eliminates all the variations such as the location, direction, and scale of objects, which are not related to shape, by aligning coordinates according to weighted scaling factors for each "sample" (18, 26, 38). The size and direction of the movement of coordinates among different populations or samples are mapped and thus the results are interpreted (4, 10). In the present study, the shape difference between the mandibles of two native goat breeds was analyzed by using the geometric morphometric method. Based on the first PC, it was observed that the distinction of the male Honamli and Hair goats was quite apparent compared to the female counterparts. This was thought to be associated with that the male goats were preferred for breeding due to the phenotypical factors.

In their study, Yalcin et al. (36) stated that the first degree shape differences were located at LM1, 3, 8, 9, and 10. They reported an anterioventral shape difference at LM3 and 9 in Akkaraman sheep and a posteriodorsal shape difference in Wild sheep. In the same study, they stated that LM8 was posterior in Akkaraman sheep and anterior in Wild sheep. Also, they reported a posteriodorsal direction difference at LM1 and 10 in Akkaraman sheep and a anterioventral direction difference in Wild sheep (36). In the present study, the first-degree shape differences were observed at LM4, 8 and 9 in Honamli goat and at LM4, 7, 9 and 10, in Hair goat respectively. LM4 was cranioventral and LM8 and 9 were caudodorsal in Honamli goat. LM4 was cranioventral, LM7 and 9 were caudodorsal and LM10 was craniodorsal in Hair goat. This information obviously indicated the mandible differences in terms of species and breeds.

Yalcin et al. (36) reported that the difference at LM9 level was quite apparent and this may be associated with the differences such as environmental conditions and feeding habits as well as adaptation to domestication process. Also, in the present study, it was observed that the most apparent differences were at LM9 level, which is compatible with above-mentioned finding.

Yalcin et al. (36) reported that sexual dimorphism was not observed in mandible of Anatolia Wild sheep. In the present study, goat mandible was analyzed by using geometric morphometric method for the first time in terms of sexual dimorphism based on breed factor. In the study, sexual dimorphism was observed in both breeds. However, the sexual dimorphism of Hair goat in terms of the first PC was more apparent compared to Honamli goat.

Consequently, in the present study, mandibles of domesticated goats were analyzed using geometric morphometric methods based on breed and sex factors for the first time. It was remarkable that the Hair goat had quite apparent sexual dimorphism compared to the Honamli goat. Also, one of the most remarkable results of the study was that the male goats clustered clearly compared to the female goat in terms of breed factor. It is considered that the data obtained in this study would contribute to the other ruminant mandible studies by using geometric morphometric method. Also, we think that the data would be used in the distinction of the mandible remains uncovered in zooarchaeological excavations and especially considered as ovicapri (sheep-goat).

Ethical Statement

The study was approved by the Local Ethics Committee of Burdur Mehmet Akif Ersoy University (approval number 2020/645).

Conflict of Interest

The authors declared that there is no conflict of interest.

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References

1. **Adams DC, Rohlf FJ, Slice DE** (2013): *A field comes of age: Geometric morphometrics in the 21st century.* Hystrix, **24,** 7-14.
2. **Ansell WFH** (1972): Order Artiodactyla. In The mammals of Africa. An identification manual Smithsonian Institution Press, Washington.
3. **Batubara A, Noor RR, Farajallah A, et al** (2011): *Morphometric and phylogenic analysis of six population Indonesian local goat.* J Ani Sci Tech, **34,** 165-74.
4. **Bigoni L, Velemínská J, Brůžek J** (2010): *Three-dimensional geometric morphometric analysis of cranio-facial sexual dimorphism in a Central European sample of known sex.* Homo, **61**, 16-32.
5. **Clark KM** (1995): *The later prehistoric and protohistoric dog: the emergence of canine diversity.* Archaeozoologia, **7,** 9-32.
6. **Elmaz Ö, Saatçi M, Mamak N, et al** (2012): *The determination of some morphological characteristics of Honamli goat and kids, defined as a new indigenous goat breed of Turkey.* Kafkas Univ Vet Fak Derg, **18**, 481-5.
7. **Ensminger ME, Parker RO** (1986): Sheep and goat Science, Fifth Edition. Danville, Illinois: The Interstate Printers and Publishers Inc.
8. **Figueirido B, Serrano-Alarcón SJ, Palmqvist P** (2012): *Geometric morphometrics shows differences and similarities in skull shape between the red and giant pandas.* J Zool, **286,** 293-302.
9. **Food and Agriculture Organization; FAO** (2007): Global Plan of Action for Animal Genetic resources and Interlaken Declaration on Animal Genetic Resources, Rome, Italy.
10. **Frost SR, Marcus LF, Bookstein FL, et al** (2003): *Cranial allometry, phylogeography, and systematics of largebodied papionins (primates: Cercopithecinae) inferred from geometric morphometric analysis of landmark data.* Anat Rec A Discov Mol Cell Evol Biol, **275,** 1048-1072.
11. **Guintard C, Lallemand M** (2003): *Osteometric study of metapodial bones in sheep (Ovis aries, L. 1758).* Ann Anat, **185,** 573-583.
12. **Gündemir O, Özkan E, Dayan MO, et al** (2020): *Sexual analysis in turkey (Meleagris gallopavo) neurocranium using geometric morphometric methods.* Turk J Vet Anim Sci, **44**, 681-687.
13. **Gürbüz İ, Aytek Aİ, Demiraslan Y, et al** (2020): *Geometric morphometric analysis of cranium of wolf (Canis lupus) and German shepherd dog (Canis lupus familiaris).* Kafkas Univ Vet Fak Derg, **26**, 525-532.
14. **Hammer Q, Harper DAT, Ryan PD** (2001): *PAST: Paleontological Statistics Software Package for Education and Data Analysis*. Palaeontol Electron, **4,** 9.
15. **Kimmerle EH, Ross A, Slice D** (2008): *Sexual dimorphism in America: geometric morphometric analysis of the craniofacial region.* J Forensic Sci, **53,** 54-57.
16. **Klingenberg CP** (2011): *MorphoJ: an integrated software package for geometric Morphometrics*. Mol Ecol Resour, **11**, 353-357.
17. **Marcus LF, Hingst-Zaher E, Zaher H** (2000): *Application of landmark morphometrics to skulls representing the orders of living mammals.* Hystrix, **11,** 27-47.
18. **Mitteroecker P, Gunz P** (2009): *Advances in Geometric Morphometrics.* Evol Biol, **36,** 235-247.
19. **Naderi S, Rezaei HR, Pompanon F, et al** (2008): *The goat domestication process inferred from large-scale mitochondrial DNA analysis of wild and domestic individuals.* Proc Natl Acad Sci, **105,** 17659-17664.
20. **O'Higgins P** (2000): *The study of morphological variation in the hominid fossil record: biology, landmarks and geometry.* J Anat, **197,** 103-120.
21. **Onar V, Belli O** (2005): *Estimation of shoulder height from long bone measurements on dogs unearthed from the Van-Yoncatepe early Iron Age necropolis in Eastern Anatolia.* Rev Med Vet, **156,** 53-60.
22. **Önel A** (2010): Comparison of hares (Lepus europaeus Pallas, 1778) from Elazığ and Malatya by using geometric morphometrics techniques. Doktora Tezi. Fırat Üniversitesi Fen Bilimleri Enstitüsü, Elazığ.
23. **Özden B** (2008): İran küçük bal arısı (Apis florea Fabricius) populasyonlarında geometrik morfometrik analizi. Bilim Uzmanlığı Tezi, Karaelmas Üniversitesi Fen Bilimleri Enstitüsü, Zonguldak.
24. **Payne WJA, Wilson RT** (1999): An introduction to Animal Husbandry in the tropics. Blackwell Publishing Company: Oxford, UK.
25. **Rohlf FJ, Bookstein FL** (1990): Proceedings of the Michigan Morphometrics Workshop; Proceedings Michigan Morphometrics Work. University of Michigan Museum of Zoology: Ann Arbor, MI, USA.
26. **Rohlf FJ, Slice DE** (1990): *Extensions of the Procrustes method for the optimal superimposition of landmarks.* Syst Zool, **39,** 40-59.
27. **Rohlf FJ** (2017): TpsSmall Version 1.34, Ecology & Evolution. SUNY at Stone Brook, USA.
28. **Rohlf FJ** (2018): TpsDig Version 2.31, Ecology & Evolution. SUNY at Stone Brook, USA.
29. **Rohlf FJ** (2019): TpsRelw Version 1.70, Ecology & Evolution. SUNY at Stone Brook, USA.
30. **Rohlf FJ** (2019): TpsUtil program Version 1.79, Ecology & Evolution. SUNY at Stone Brook, USA.
31. **Slice DE** (2005): Modern Morphometrics. In: Physical Anthropology, Kluwer Acad./Plenum, New York.
32. **Slice DE** (2007): *Geometric morphometrics.* Annu Rev Anthropol, **36**, 261-281.
33. **Tekin ME, Öğeç M** (2017): *Konya Bölgesinde Halk Elinde Yetiştirilen Kıl Keçisi Oğlaklarının Büyüme ve Yaşama Gücü.* Lalahan Hay Araşt Enst Derg, **57**, 93-98.
34. **The Official Gazette of Turkish Republic** (2015): Communication on Supporting Principles of Animal Husbandry Practices, No: 2015/43, Num: 29535.

35. **Viscosi V, Cardini A** (2011): *Leaf Morphology, Taxonomy and Geometric Morphometrics: A Simplified Protocol for Beginners.* PLoS One, **6,** e25630.
36. **Yalcin H, Kaya MA, Arslan A** (2010): *Comparative Geometrical Morphometries on the Mandibles of Anatolian Wild Sheep (Ovis gmelini anatolica) and Akkaraman Sheep (Ovis aries).* Kafkas Univ Vet Fak Derg, **16**, 55-61.
37. **Zeder MA, Hesse B** (2000): *The Initial Domestication of goat (Capra hircus) in the Zagros Mountains 10,000 Years Ago*. Science Journal, **287**, 2254-2257.
38. **Zelditch ML, Swiderski DL, Sheets HD, et al** (2004): Geometric Morphometrics For Biologists: A Primer, Academic Press, Cambridge.
39. **Zelditch ML, Swiderski DL, Sheets HD** (2012): Geometric Morphometrics for Biologists: A Primer, Academic Press, Amsterdam.

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The effects of cage type, oviposition time and egg storage period on the egg quality characteristics of laying hens

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Abstract: Egg has valuable nutrients however; these nutrients may be affected by many factors. In this study, it was aimed to determine the effects of cage type, oviposition time and egg storage period on egg quality characteristics of laying hens. A total of 410 eggs were collected from unenriched and enriched cages in the morning and in the afternoon. After the eggs were weighed, they were stored at 21ºC and 20% relative humidity until the 30th day of storage. It was determined that unenriched cage eggs had higher in shell breaking strength, albumen ash and pH & lipid of yolk and lower egg weight loss, yolk index & yolk color compared with enriched cage eggs. Morning eggs had the highest egg weight loss and the lowest egg weight, yolk height and yolk diameter. Dry matter & protein of albumen and dry matter & lipid of yolk were lower, while protein of yolk was higher in the morning eggs than those in the afternoon. It was observed that the storage period was important factor for egg quality characteristics. The effect of oviposition time and storage period interaction on egg quality should not be neglected. Interactions between cage type and storage period and between oviposition time and storage period were found statistically significant for some chemical composition of albumen and yolk. It is concluded that A quality egg criteria are preserved up to 14 days at an average temperature of 21°C and 20% humidity.
Keywords: Cage type, egg composition, egg quality, oviposition time, storage period.

Yumurtacı tavuklarda kafes tipi, yumurtlama zamanı ve yumurta depolama süresinin yumurta kalite özelliklerine etkileri

Özet: Yumurta değerli besinlere sahiptir, fakat bu besinler birçok faktörden etkilenebilir. Çalışmada, yumurtacı tavuklarda kafes tipi, yumurtlama zamanı ve depolama süresinin yumurta kalite özelliklerine etkilerini belirlemek amaçlanmıştır. Zenginleştirilmemiş ve zenginleştirilmiş kafeslerden sabah ve öğleden sonra toplam 410 yumurta toplanmıştır. Yumurtalar tartıldıktan sonra depolamanın 30. gününe kadar 21ºC sıcaklık ve %20 nemde depolanmıştır. Zenginleştirilmemiş kafes yumurtaları, zenginleştirilmiş kafes yumurtaları ile karşılaştırıldığında daha yüksek kabuk kırılma mukavemeti, ak kül ve sarı pH & yağ değerlerine ve daha düşük yumurta ağırlık kaybı, sarı indeksi & sarı rengine sahip olduğu belirlenmiştir. Öğleden sonraki yumurtalara kıyasla sabah yumurtalarının daha yüksek yumurta ağırlık kaybı ile daha düşük yumurta ağırlığı, sarı yüksekliği ve sarı çapına sahip olduğu gözlenmiştir. Ayrıca bu yumurtalarda ak kuru madde & protein düzeyi ile sarı kuru madde & yağ düzeyi daha düşükken, sarı protein düzeyi daha yüksektir. Depolama süresinin yumurta kalite özellikleri için önemli bir faktör olduğu gözlenmiştir. Yumurtlama zamanı ve depolama süresi etkileşiminin yumurta kalitesi üzerindeki etkisi ihmal edilmemelidir. Kafes tipi ve depolama süresi ile yumurtlama zamanı ve depolama süresi arasındaki etkileşimler ak ve sarının bazı kimyasal bileşimleri için istatistik açıdan önemli bulunmuştur. A kalitede ki yumurtada aranan kriterlerin ortalama 21 °C sıcaklıkta ve % 20 nemde 14 güne kadar korunduğu sonucuna varılmıştır.
Anahtar sözcükler: Depolama süresi, kafes tipi, yumurta bileşimi, yumurta kalitesi, yumurtlama zamanı.

Introduction

The laying hen industry has moved away from unenriched cages (UEC) to housing systems that are considered more welfare-friendly systems such as enriched cages (EC). The increasing importance given to animal welfare has made consumers think that eggs obtained from alternative systems are healthier than those obtained from conventional systems. The alternative systems have focused on developing better animal welfare and behaviour for laying hens. These systems allow the birds to exhibit their natural behaviour, decrease the probability of disease & injury, increase productivity, egg quality, and food safety (11, 16).

Egg is a food containing most of the nutrients that human needs (26). Eggs are also an excellent source of protein and fat. Having the highest quality protein among animal products, they are also rich in vitamins such as A, D, E, K and B, and minerals such as iron and phosphorus (21, 27). Egg quality is important for both producers and consumers. In terms of producers, it is desired that the egg is large, clean-looking, delicious, properly shaped, and has good shell quality and internal quality.

Egg processing guidelines and quality standards are based for UEC eggs. Egg quality characteristics are affected by many factors such as genotype of the hens, diet, environmental conditions and housing system used in production and oviposition time (3, 11, 12, 16, 17, 20). However, these effects may vary with the interaction between the factors examined. Egg has a limited shelf life depending on the storage conditions (2). However, the interactions of cage systems used in the production, oviposition time and storage conditions are not fully investigated. Therefore, the current study was aimed to determine the effects of cage type (UEC and EC), oviposition time, and egg storage period on egg quality characteristics of ISA-Brown laying hens aged 57 weeks.

Materials and Methods

The eggs were obtained from ISA-Brown laying hybrids aged 57 weeks that were kept in two caging systems (UEC and EC) in the same poultry house. Twenty hens were kept in the UEC system (192 cm width, 62.5 cm depth, and 57 cm height), while 18 hens were kept in the EC system (240 cm width, 62.5 cm depth and 57 cm height). EC included the nest (48 cm width x 62.5 cm depth), scratch-pad (35 cm width x 5 cm length), perch and claw shortener (12 cm width x 3 cm length). The nesting area was separated from the other areas with blue plastic strips. Two plastic perches were used with 190 and 137 cm in length. Each cage had eight nipple type drinkers. The lighting program was 16 L:8 D during the laying period. The average poultry house temperature on the day that the eggs were collected was 25.1°C, and the average humidity was 31%. The ingredients and the chemical composition of layer diet were given in Table 1. Chemical composition of the diet was determined according to the methods described by AOAC (4).

The eggs to be used in the study were collected from the poultry house for two consecutive days from 8:30 to 11:30 am, and from 13:30 to 16:30 (2 different oviposition times). Eggs with damaged shell structure were excluded from the study. A total of 410 eggs were selected for this study, 120 eggs in the morning and 90 eggs in the afternoon from UEC and 120 eggs in the morning and 80 eggs in the afternoon from the EC system.

Table 1. Ingredients and chemical composition of the diet.

Ingredients, g/kg as fed	**Diet**
Maize	520.24
Maize DDGS, 28% CP	52.54
Wheat	34.00
Full-fat soya	154.08
Soybean meal,47% CP	9.22
Sunflower seed meal, 36% CP	118.33
Monocalcium phosphate	5.49
Calcium carbonate	93.50
Sodium bicarbonate	1.21
Salt	2.57
Methionine	1.09
Lysine sulphate	1.34
Choline chloride	0.50
Vitamin premix [a]	1.00
Mineral premix [b]	1.00
Xylanase [c]	0.38
Phytase [d]	0.50
Carophyll red	2.44
Carophyll yellow	0.57
Composition	
Metabolizable energy[e], MJ/kg	11.67
Crude protein, %	16.10
Ether extract, %	4.80
Crude fiber, %	3.90
Crude ash, %	11.20
Ca, %	3.90
P, %	0.60

[a]: Each kg contain 12000000 IU vitamin A, 5000000 IU vitamin D3, 65 g vitamin E, 3 g vitamin K3, 3 g vitamin B1, 7 g vitamin B2, 15 g calcium D-pantothenate, 4 g vitamin B6, 20 mg vitamin B12, 60 g niacin, 2 g folic acid and 0.25 g biotin. [b]: Each kg contain 25 g iron, 16 g copper, 120 g manganese, 110 g zinc, 1.25 g iodine and 0.3 g selenium. [c]: Ronozyme® WX, [d]:Ronozyme® HiPhos, [e]: Estimated using equation by Carpenter and Clegg (9).

After the eggs were weighed, they were stored at 21°C and 20% relative humidity until the last storage day. The quality characteristics of five eggs from each group were examined at the 1st, 7th, 14th, 21st, and 30th day of storage. In each storage period, egg weight, egg weight loss, shape index, shell breaking strength, shell weight, shell thickness, albumen height, albumen length, albumen width, albumen pH, yolk color, yolk weight, yolk height, yolk diameter, yolk pH, albumen index, yolk index, and the Haugh unit were determined (27, 28). Dry matter, total lipid, protein and ash values were determined (4) in the yolk and albumen samples.

Statistical analysis: Distribution, the homogeneity of variance of the data, was analyzed. Two-way ANOVA determined the effects of cage type and oviposition time on egg weight and shape index. The effects of cage type, oviposition time and storage period to all the other

examined properties were determined by using three-way ANOVA with SPSS for Windows (SPSS Inc., Chicago, IL). Tukey test was used to check the significance of the difference between the groups. P≤0.05 was taken into account statistically significant (10).

Results

The effects of cage type and oviposition time on egg weight and shape index were given in the Table 2. It was observed that the weight of the eggs collected between 08.30 and 11.30 was lower than those were collected between 13.30 and 16.30 (P<0.001). In this study, it was determined that the shape index was not affected by cage type and oviposition time. No interaction was observed between the cage type and the oviposition time in terms of egg weight and shape index.

The egg weight loss during the storage period (Table 3) was higher in eggs obtained from EC than in UEC (P<0.001). The weight loss in eggs stored at room temperature increased from 0.13 to 4.15 g and percentage of weight loss increased from 0.20 to 6.39% as storage length increased. For egg weight loss, the interaction between cage type and storage period & oviposition time and storage period were found to be statistically significant. Greater weight losses were obtained in the eggs from EC in the 14th and 21st days of storage and also

Table 2. Effects of cage type and oviposition time on egg weight and shape index of eggs.

Cage type	Oviposition time	n	Egg weight (g)	Shape index (%)
UEC		210	65.29±0.32	80.09±0.18
EC		200	66.11±0.33	79.93±0.18
	08.30-11.30	240	64.81±0.30	80.11±0.16
	13.30-16.30	170	66.60±0.35	79.91±0.19
UEC	08.30-11.30	120	64.38±0.42	80.16±0.23
	13.30-16.30	90	66.21±0.49	80.01±0.26
EC	08.30-11.30	120	65.24±0.42	80.05±0.23
	13.30-16.30	80	66.99±0.51	79.81±0.28
			P	
Cage type			0.078	0.531
Oviposition time			<0.001	0.432
Cage type X Oviposition time			0.930	0.861

UEC: unenriched cages, EC: enriched cages, Mean±SEM.

Table 3. Effects of cage type, oviposition time and storage period on egg weight loss and shell quality of eggs.

Cage type	Oviposition time	Storage period (day)	Weight loss (g)	Weight loss (%)	Breaking strength (kg/cm^2)	Shell thickness (mm)	Shell weight (%)
UEC			1.87±0.04	2.90±0.05	3.63±0.04	38.37±0.14	11.80±0.06
EC			2.05±0.04	3.10±0.05	3.50±0.05	38.24±0.15	11.65±0.06
	08.30-11.30		1.97±0.03	3.07±0.05	3.57±0.04	38.13±0.13	11.80±0.05
	13.30-16.30		1.94±0.04	2.92±0.06	3.57±0.05	38.47±0.16	11.65±0.06
		1	0.13±0.06^a	0.20±0.08^a	3.16±0.07^a	37.91±0.23	11.46±0.09^a
		7	0.91±0.06^b	1.39±0.08^b	3.12±0.07^a	38.58±0.23	11.61±0.09^{ab}
		14	1.73±0.06^c	2.64±0.08^c	3.39±0.07^a	38.15±0.23	11.58±0.09^{ab}
		21	2.86±0.06^d	4.37±0.08^d	3.36±0.07^a	38.44±0.24	11.92±0.09^{ab}
		30	4.15±0.06^e	6.39±0.08^e	4.81±0.07^b	38.45±0.22	12.05±0.09^b
				P			
Cage type			<0.001	0.006	0.035	0.550	0.068
Oviposition time			0.595	0.038	0.964	0.098	0.064
Storage period			<0.001	0.000	<0.001	0.235	<0.001
Cage type X Oviposition time			0.356	0.228	0.156	0.180	0.092
Cage type X Storage period			0.009	0.065	0.876	0.632	0.091
Oviposition time X Storage period			<0.001	0.027	0.048	0.336	0.272
Cage type X Oviposition time X Storage period			0.774	0.351	0.203	0.833	0.878

UEC: unenriched cages, EC: enriched cages, Mean±SEM, $^{a, b, c, d, e:}$ The difference among means carrying different letters in the same column is statistically significant (P<0.05).

in the eggs laid in the afternoon in the last week of storage. In the study, the breaking strength of eggs obtained from UEC was higher than those obtained from EC (P<0.05). Breaking strength of eggs increased from 3.16 to 4.81 kg/cm^2 and shell weight increased from 11.46 to 12.05% as length of storage period increased. According to the findings obtained from the study, the effects of oviposition time on breaking strength, shell thickness, and shell percentage were found to be statistically insignificant.

The effects of the cage type, oviposition time and storage period on albumen quality and Haugh unit were given in the Table 4. Only storage period affected the examined traits (P<0.001). The interaction between oviposition time and storage period on the percentage and pH of the albumen and Haugh unit were statistically significant (P<0.05).

No significant effects of the cage type on the percentage, height, and diameter of the yolk were observed (Table 5). The eggs obtained from EC had higher yolk index and darker yolk color and had lower yolk pH than those obtained from UEC (P≤0.05). There was no difference in groups of cage type and oviposition time in terms of yolk percentage. The height and diameter of yolk were lower in eggs in the morning than those in the afternoon (P<0.01). There were no significant effects of oviposition time on index, color, and pH of the yolk. The yolk percentage increased in eggs stored at room temperature for 30 days (P<0.001). While yolk diameter increased, yolk height and consequently, yolk index decreased (P<0.001). The reduction in yolk index was greater in the eggs laid in the morning than those in the afternoon as the storage period increased. The yolk pH increased in eggs stored at room temperature for 30 days (P<0.001). While the yolk color decreased in the first seven days of the storage period, it started to increase again after the 7th day (P<0.001). The interaction between oviposition time and storage period had an important effect on all the properties of yolk quality (P<0.01). The interaction between the cage type and the storage period was effective in pH and diameter of yolk (P<0.05). The interaction between cage type, oviposition time, and storage period was observed to be effective in pH, height, diameter, color, and index of yolk (P<0.01). The effects of cage type, oviposition time and storage period on chemical composition were given in the Table 6. Ash of albumen and dry matter of yolk were found as 0.75 and 46.83% & 0.71 and 46.25% in UEC and EC eggs, respectively (P<0.001). Oviposition time was found important for dry matter and protein of albumen & dry matter protein and lipid of yolk values. Storage period and interaction of oviposition time & storage period affected the composition of albumen and yolk (P<0.001).

Table 4. Effects of cage type, oviposition time and storage period on albumen quality and Haugh unit of eggs.

Cage type	Oviposition time	Storage period (day)	Albumen percentage (%)	Albumen pH	Albumen height (mm)	Albumen length (mm)	Albumen width (mm)	Albumen index	Haugh unit
UEC			61.33±0.17	9.15±0.02	4.36±0.03	107.94±0.72	87.89±0.70	4.78±0.06	54.91±0.43
EC			61.28±0.17	9.16±0.02	4.30±0.04	109.54±0.76	88.70±0.73	4.67±0.06	54.26±0.44
	08.30-11.30		61.35±0.15	9.17±0.02	4.30±0.03	108.49±0.67	87.45±0.65	4.73±0.05	54.66±0.39
	13.30-16.30		61.27±0.18	9.14±0.02	4.36±0.04	108.98±0.80	89.14±0.77	4.72±0.06	54.50±0.47
		1	63.47±0.25^{d}	8.66±0.03^{a}	7.13±0.05^{d}	90.65±0.97^{a}	73.29±0.94^{a}	8.73±0.08^{d}	82.17±0.61^{d}
		7	62.78±0.25^{c}	9.13±0.03^{b}	4.78±0.05^{c}	107.85±1.00^{b}	84.40±0.96^{b}	5.03±0.08^{c}	64.11±0.62^{c}
		14	61.44±0.25^{b}	9.22±0.03^{b}	3.40±0.05^{b}	115.14±1.16^{c}	92.38±1.12^{c}	3.34±0.09^{b}	48.10±0.62^{b}
		21	59.86±0.28^{a}	9.33±0.03^{c}	2.02±0.05^{a}	121.30±1.05^{d}	103.10±1.01^{d}	1.80±0.08^{a}	23.94±0.61^{a}
		30	58.98±0.28^{a}	9.43±0.03^{c}	-	-	-	-	-
P									
Cage type			0.837	0.632	0.244	0.126	0.421	0.170	0.290
Oviposition time			0.736	0.320	0.213	0.643	0.096	0.929	0.791
Storage period			<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Cage type X Oviposition time			0.878	0.771	0.090	0.002	0.269	0.672	0.193
Cage type X Storage period			0.358	0.807	0.907	0.323	0.475	0.833	0.892
Oviposition time X Storage period			0.004	0.038	0.083	0.305	0.231	0.203	0.034
Cage type X Oviposition time X Storage period			0.539	0.661	0.739	0.204	0.272	0.648	0.291

UEC: unenriched cages, EC: enriched cages, Mean±SEM, $^{a, b, c, d:}$ The difference among means carrying different letters in the same column is statistically significant (P<0.05).

Table 5. Effects of cage type, oviposition time and storage period on yolk quality of eggs.

Cage type	Oviposition time	Storage period (day)	Yolk percentage (%)	Yolk pH	Yolk height (mm)	Yolk diameter (mm)	Yolk index	Yolk color
UEC			26.89±0.15	6.09±0.01	13.58±0.06	43.04±0.14	31.86±0.18	9.97±0.04
EC			27.10±0.15	6.06±0.01	13.74±0.06	42.69±0.14	32.56±0.18	10.08±0.04
	08.30-11.30		26.87±0.13	6.07±0.01	13.49±0.06	42.59±0.12	32.02±0.16	10.02±0.04
	13.30-16.30		27.12±0.17	6.07±0.01	13.83±0.07	43.14±0.15	32.40±0.20	10.03±0.05
		1	25.08±0.22^{a}	5.93±0.02^{a}	15.63±0.10^{d}	40.22±0.20^{a}	38.90±0.27^{e}	10.28±0.06^{c}
		7	25.64±0.23^{a}	5.94±0.02^{a}	14.65±0.10^{c}	40.47±0.21^{a}	36.24±0.27^{d}	9.74±0.06^{a}
		14	27.00±0.23^{b}	6.00±0.02^{b}	14.30±0.10^{b}	42.50±0.21^{b}	33.68±0.28^{c}	9.83±0.06^{ab}
		21	28.29±0.26^{c}	6.09±0.02^{c}	11.72±0.11^{a}	44.45±0.23^{c}	26.43±0.30^{b}	10.02±0.07^{b}
		30	28.98±0.25^{d}	6.39±0.02^{d}	11.99±0.11^{a}	46.69±0.23^{d}	25.79±0.30^{a}	10.27±0.07^{c}
				P				
Cage type			0.315	0.027	0.070	0.068	0.006	0.050
Oviposition time			0.233	0.729	<0.001	0.005	0.132	0.814
Storage period			<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Cage type X Oviposition time			0.575	0.955	0.358	0.284	0.698	0.744
Cage type X Storage period			0.482	0.039	0.082	0.010	0.195	0.283
Oviposition time X Storage period			0.003	<0.001	<0.001	<0.001	0.003	<0.001
Cage type X Oviposition time X Storage period			0.355	0.004	0.002	0.003	0.001	0.896

UEC: unenriched cages, EC: enriched cages, Mean±SEM, $^{a, b, c, d:}$ The difference among means carrying different letters in the same column is statistically significant (P<0.05).

Table 6. Effects of cage type, oviposition time and storage period on chemical composition of eggs.

Cage type	Oviposition time	Storage period (day)	Albumen			Yolk			
			Dry matter (%)	Ash (%)	Protein (%)	Dry matter (%)	Ash (%)	Protein (%)	Lipid (%)
UEC			11.65±0.07	0.75±0.01	10.86±0.07	46.83±0.09	1.57±0.02	15.40±0.04	29.86±0.08
EC			11.57±0.07	0.71±0.01	10.81±0.07	46.25±0.09	1.57±0.02	15.49±0.04	29.16±0.08
	08.30-11.30		11.41±0.07	0.73±0.01	10.63±0.07	46.36±0.09	1.55±0.02	15.51±0.04	29.29±0.08
	13.30-16.30		11.81±0.07	0.74±0.01	11.04±0.07	46.72±0.09	1.59±0.02	15.38±0.04	29.73±0.08
		1	11.13±0.11^{a}	0.72±0.01^{a}	10.35±0.11^{a}	48.48±0.15^{d}	1.70±0.03^{b}	15.69±0.06^{c}	31.70±0.12^{d}
		7	11.11±0.11^{a}	0.69±0.01^{a}	10.38±0.11^{a}	47.19±0.15^{c}	1.68±0.03^{b}	15.47±0.06^{bc}	30.02±0.12^{c}
		14	11.69±0.11^{b}	0.71±0.01^{a}	10.94±0.11^{b}	46.35±0.15^{c}	1.52±0.03^{a}	15.42±0.06^{bc}	29.39±0.12^{b}
		21	11.93±0.11^{bc}	0.77±0.01^{b}	11.13±0.11^{bc}	46.05±0.15^{b}	1.52±0.03^{a}	15.45±0.06^{bc}	29.06±0.12^{b}
		30	12.20±0.11^{c}	0.77±0.01^{b}	11.34±0.11^{c}	44.63±0.15^{a}	1.44±0.03^{a}	15.20±0.06^{a}	28.00±0.12^{a}
					P				
Cage type			0.424	<0.001	0.660	<0.001	0.947	0.081	<0.001
Oviposition time			<0.001	0.534	<0.001	0.007	0.227	0.015	<0.001
Storage period			<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Cage type X Oviposition time			0.172	0.925	0.187	0.264	0.636	0.064	0.016
Cage type X Storage period			0.010	0.008	0.020	0.017	0.098	0.154	<0.001
Oviposition time X Storage period			<0.001	0.232	<0.001	0.024	0.052	0.002	0.001
Cage type X Oviposition time X Storage period			0.226	0.209	0.255	0.183	0.841	0.600	0.093

UEC: unenriched cages, EC: enriched cages, Mean±SEM, $^{a, b, c, d:}$ The difference among means carrying different letters in the same column is statistically significant (P<0.05).

Discussion and Conclusion

There were no significant effects of the cage type on egg weight. The results are in agreement with studies (18, 20) about the effect of the housing system on laying hens. However, Dikmen et al. (11) reported that egg weight was higher in eggs obtained from UEC than those obtained from EC. This may be due to the genotype and age of the laying hens, stocking density and differentiation of the enrichment materials.

In the poultry house where the study was conducted, ovulation in the morning eggs occurred three hours after the dark period, while in the afternoon, ovulation took place six hours after the dark period. The maturation of the yolk and the albumen accumulation in the eggs laid in the afternoon coincides with the period when the laying hens were active, and it may cause to increase egg weight. Some researchers (1, 7, 17, 23, 24) stated that eggs laid in the morning were heavier than those in the afternoon. However, Ayorinde and Olagbuyiro (5) and Tumová et al. (25) reported that oviposition time did not affect the egg weight. The difference in the studies about the effects of oviposition time on egg weight is thought to be due to the lighting program applied in the poultry house, namely the starting hours of the light and dark period. This situation can be influenced by the time of albumen formation in the oviduct coincides with the light period.

Batkowska et al. (6) described that the weight loss in eggs was higher in eggs obtained from UEC than those from EC. However, in our study, high egg weight loss was seen in EC eggs. The difference in the studies is due to the age of the laying hens. The shell quality of the eggs deteriorated with increasing hen age. The interaction between the age of the hens and the cage type may have occurred. Weight loss percentage was higher in eggs collected in the morning than those in the afternoon. This may be explained that the eggs laid in the morning had less light period during the formation of the eggshell and the lower Ca level had in the blood of the hens compared to the afternoon eggs. High egg weight losses were seen in the EC eggs during the egg storage periods. This may be due to the lower shell breaking strength in these eggs. The interaction between oviposition time and storage period was found to be statistically significant, as eggs laid in the afternoon had more weight loss during the last week of storage than those in the morning.

In the present study, the breaking strength in the UEC eggs was higher than those of EC eggs. This may be due to the increase in carbondioxide loss because of the high activity of hens in EC and the inability of calcium carbonate formation by decreasing the blood's calcium binding ability. The effect of the cage type on the shell thickness was found statistically insignificant. Tumová et al. (25) and Onbaşılar and Avcılar (17) found that shell breaking strength, shell thickness, and shell percentage in eggs laid in the afternoon were better than those in the morning. The shell formation covers approximately 20-21 hours. Calcium need for the shell formation is especially important in the dark period because there is no feed consumption in this period. In the present study, the percentage of shell increased as a result of increasing the length of storage period. This can be explained by the increase in the shell rate as a result of the evaporation of the water in the albumen and yolk over time. Breaking strength increased in eggs stored at room temperature for 30 days. The reason for this may be shell contains 0.1% water, and water decreases over time with increasing storage period. The effect of interaction between oviposition time and storage period on shell breaking strength was found statistically significant. This was due to the higher breaking strength of eggs laid in the afternoon on the 14th day of storage than those in the morning.

There were no significant effects of the cage type and oviposition time on percentage, height, length, width, pH, and index of the albumen and Haugh unit. Results of some researches (7, 23, 25) consistent with the study. Similarly, Karkulín (15) reported that the cage type was not effective on the albumen height and Haugh unit. The percentage of albumen was decreased in eggs stored at room temperature for 30 days. Depending on the age of the hens, water constitutes about 87-89% of an albumen and the decrease in the percentage of albumen may be due to the high amount of water loss. While the length and width of albumen increased, height and index of albumen decreased in eggs stored at room temperature for 21 days. The decrease in albumen height is caused by the breakdown of ovomucin (22). In table eggs, AA quality egg should be 79 or higher, A quality 55-78, B quality 31-54, and C quality 30 or less. In our study, after 21 days of storage, the Haugh unit decreased from 82.17 to 23.94%. The decrease in the Haugh unit may be due to the decrease in albumen height because of the destruction of ovomucin and lysozyme complex. It was observed that the reduction in the Haugh unit increased in the afternoon eggs as the storage period increased. The pH of the albumen is reported between 7.6 and 8.5 (30). The increase in the albumen pH was higher in the first week of storage compared to other weeks. Immediately after ovulation, the egg starts to lose carbon dioxide from the pores of the shell, and the albumen pH rises (14). The increase in albumen pH level mainly depends on the temperature and duration of storage and the egg shell quality (25).

The increase in the percentage of the yolk with storage period may be due to the passage of water from the albumen to the yolk (2). The increase in yolk pH with storage period is due to the loss of carbon dioxide from the

egg. Stress factors in relation to the production system alter blood protein and lipid levels of hens (29). The chemical content of egg is related to the blood chemistry of hen. Albumen ash and dry matter &lipid of yolk were found lower in eggs obtained from the EC than those from the UEC. Dry matter & protein of albumen and dry matter & lipid of yolk were higher, while protein of yolk was lower in the afternoon egg than those in the morning. Dry matter, ash and protein of albumen were increased, while dry matter, ash, protein and lipid of yolk were decreased with increasing storage period at the room temperature. The dry matter concentration of albumen is also used as an indicator of egg freshness and is related to thinning or liquefaction of albumen (13). Caner and Yüceer (8) reported that dry matter of albumen increases with storage period as a result of albumen liquefaction. Interaction of cage type and oviposition time on yolk lipid, interaction of cage type and storage period on all examined albumen composition and dry matter & lipid of yolk and interaction of oviposition time and storage period on dry matter & protein of albumen and dry matter, protein & lipid of yolk were found statistically significant in the present study.

In conclusion, cage type (UEC or EC) had no significant effect on egg quality during storage conditions except weight loss, breaking strength, ash of albumen and pH, index, color, dry matter & lipid of yolk. Yolk protein was higher, while albumen protein and yolk lipid were lower in the morning eggs than those in the afternoon. No interaction was observed between the cage type and the oviposition time in terms of the examined properties except that albumen length and yolk lipid. However, the interaction between oviposition time and storage period should not be neglected in the egg quality. It should be noted that interaction among cage type, oviposition time, and storage period is also effective in some yolk properties. It is concluded that A quality egg criteria are preserved up to 14 days at an average temperature of 21°C and 20% humidity.

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Ethical Statement

This study does not present any ethical concerns.

Conflict of Interest

The authors are declared that there is no conflict of interest.

References

1. **Aksoy T, Yılmaz M, Tuna T** (2001): *The effect of oviposition time on egg quality and the possibility of estimating egg shell weight using a formula in commercial layers.* Turk J Vet Anım Sci, **25**, 811-816.
2. **Akyurek H, Okur AA** (2009): *Effect of storage time, temperature and hen age on egg quality in free-range layer hens.* J Anım Vet Adv, **8**, 1953-1958.
3. **Angelovičová M, Ševčíková V, Angelovič M, et al** (2014): *Comparison of two different breeding systems laying hens in relation to egg shell quality.* J Anim Sci Biotechno, **47**, 166-171.
4. **AOAC** (2000): Official Methods of Analysis. 17th ed. AOAC International, Maryland, USA.
5. **Ayorinde KL, Olagbuyiro B** (1991): *The influence of clutch size and oviposition time on egg weight and egg index in Hubbard layers.* Bull Anim Health Prod Afr, **39**, 251-253.
6. **Batkowska J, Brodacki A, Knaga S** (2014): *Quality of laying hen eggs during storage depending on egg weight and type of cage system (conventional vs. furnished cages).* Ann Anim Sci, **14**, 707-719.
7. **Campo J, Cigarroa F, Torres O** (2015): *Effect of oviposition time on egg quality characteristics.* World's Poult Sci J, **71**, 106.
8. **Caner C, Yüceer M** (2015): *Efficacy of various protein-based coating on enhancing the shelf life of fresh eggs during storage.* Poult Sci, **94**, 1665-1677.
9. **Carpenter K, Clegg K** (1956): *The metabolizable energy of poultry feeding stuffs in relation to their chemical composition.* J Sci Food Agri, **7**, 45-51.
10. **Dawson B, Trapp RG** (2001): Basic and Clinical Biostatistics, 3rd ed. Lange Medical Books. McGraw-Hill Medical Publishing Division, New York.
11. **Dikmen BY, Ipek A, Şahan Ü et al** (2017): *Impact of different housing systems and age of layers on egg quality characteristics.* Turk J Vet Anım Sci, **41**, 77-84.
12. **Hanusova E, Hrnčár C, Hanus A, et al** (2015): *Effect of breed on some parameters of egg quality in laying hens.* Acta Fytotechn Zootechn, **18**, 20-24.
13. **Heldman DR** (2011): *Food preservation process design.* Procedia Food Sci, **1**, 685-689.
14. **Hill AT, Hall JW** (1980): *Effects of various combinations of oil spraying, washing, sanitizing, storage time, strain, and age of layer upon albumen quality changes in storage and minimum sample sizes required for their measurement.* Poult Sci, **59**, 2237-2242.
15. **Karkulín D** (2006): Comparison of production and egg quality parameters of laying hens housed in conventional and enriched cages. Proceedings of EPC, 10-14.
16. **Ledvinka Z, Zita L, Klesalová L** (2012): *Egg quality and some factors influencing it: a review.* SAB, **43**, 46-52.
17. **Onbaşılar EE, Avcılar ÖV** (2011): *The effects of age and oviposition time on egg weight and shell quality of brown layer.* Lalahan Hayv Araşt Enst Derg, **51**, 15-19.
18. **Onbaşılar EE, Ünal N, Erdem E, et al** (2015): *Production performance, use of nest box, and external appearance of two strains of laying hens kept in conventional and enriched cages.* Poult Sci, **94**, 559-564.

19. **Onbaşılar EE, Ünal N, Erdem E** (2018): *Some egg quality traits of two laying hybrids kept in different cage systems.* Ankara Univ Vet Fak Derg, **65**, 51-55.
20. **Pohle K, Cheng HW** (2009): *Comparative effects of furnished and battery cages on egg production and physiological parameters in White Leghorn hens.* Poult Sci, **88**, 2042-2051.
21. **Réhault-Godbert S, Guyot N, Nys Y** (2019): *The golden egg: Nutritional value, bioactivities, and emerging benefits for human health.* Nutrients, **11**, 684.
22. **Short FJ, Walker AW, Elson A** (2001): *Eggshell density in furnished cages: effect of dustbath and perch provision.* Brt Poult Sci, **42**, 77-78.
23. **Tůmová E, Ebeid T** (2005): *Effect of time of oviposition on egg quality characteristics in cages and in a litter housing system.* Czech J Anim Sci, **50,** 129-134.
24. **Tůmová E, Ledvinka AZ** (2009): *The effect of time of oviposition and age on egg weight, egg components weight and eggshell quality.* Arch Geflugelkde, **73**, 110-115.
25. **Tůmová E, Zita L, Hubený M, et al** (2007): *The effect of oviposition time and genotype on egg quality characteristics in egg type hens.* Czech J Anim Sci, **52**, 26-30.
26. **Yalçın SS, Yalçın S** (2013): Poultry eggs and child health–a Review. Lohmann Inf, **48**, 3-14.
27. **Yalçın S, Yalçın S, Onbaşılar İ, et al** (2014): *Effects of dietary yeast cell wall on performance, egg quality and humoral immune response in laying hens.* Ankara Univ Vet Fak Derg, **61**, 289-294.
28. **Yalçın S, Eser H, Onbaşılar İ, et al** (2016): *Effects of dietary sepiolite on performance, egg quality and some blood parameters in laying hens.* Ankara Univ Vet Fak Derg, **63**, 25-29.
29. **Yenice G, Kaynar O, Ileriturk M, et al** (2016): *Quality of eggs in different production systems.* Czech J Food Sci, **34**, 370-376.
30. **Yenilmez F, Uruk E, Serbester U, et al** (2017): *Effects of wholesaler and consumer conditions on egg quality parameters in different seasons.* Çukurova Tar Gıda Bil Derg, **32**, 61-68.

Ankara Univ Vet Fak Derg, **68**, 337-342, 2021
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The short-term impact of the Covid-19 pandemic on livestock products and feed prices in Turkey

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Abstract: This study aimed to investigate changes in the prices of animal products (meat, milk, eggs), and feed before and during the pandemic period on a sectoral basis in Turkey. The material for the study consisted of the prices of animal products (beef, lamb and, poultry meat, eggs, and milk) and feed collected from official institutions and producer associations in the period before (December 2019-February 2020) and during (March-May 2020) the pandemic. Considering the prices of investigated animal products, there were increases at different rates in all products during the pandemic (1.82-11.57%). While the differences between producer prices of beef and broiler meat before and during the pandemic were found to be statistically significant ($P<0.05$), the changes in lamb meat and egg prices were not statistically significant. On the other hand, feed prices were found to be increased by 9.71-12.57%. It was determined that the feed, which is the most critical input of producers, showed a significant increase ($P<0.05$) during the pandemic, especially in the poultry sector. During the pandemic, the highest reduction in livestock product/feed parity was experienced in the egg sector, with -10.28%. In conclusion, examined animal product prices increased during the pandemic period, but input prices increased more than products. In addition, it was determined that the producers had to produce with lower profitability according to the product-feed parity values, especially in the egg poultry sector during the pandemic period.
Keywords: Covid 19, feed, meat, milk, prices.

Türkiye'de Covid-19 pandemisinin hayvansal ürün ve yem fiyatlarına kısa süreli etkisi

Özet: Bu çalışmada, Türkiye'de pandemi öncesi ve pandemi dönemindeki hayvansal ürün (et, süt, yumurta) ve yem fiyatlarındaki değişimlerin sektörel bazda araştırılması amaçlanmıştır. Çalışma materyalini resmi kurumlardan ve üretici birliklerinden pandemi öncesi (Aralık 2019-Şubat 2020) ve pandemi sırasında (Mart-Mayıs 2020) elde edilen hayvansal ürün (sığır eti, kuzu eti, kanatlı eti, yumurta ve süt) ve yem fiyatları oluşturmuştur. İncelenen hayvansal ürünlerin fiyatları açısından değerlendirme yapıldığında, pandemi döneminde tüm ürünlerde farklı oranlarda artış yaşanmıştır (%1,82-11,57). Pandemi öncesi ve sırasında sığır eti ve piliç eti üretici fiyatları arasındaki fark istatistiksel olarak anlamlı bulunurken ($P<0,05$), kuzu eti ve yumurta fiyatlarındaki değişiklikler istatistiksel olarak anlamlı bulunmamıştır. Diğer taraftan, girdi maliyetleri yönünden yaşanan değişiklikleri göstermesi bakımından yem fiyatları da incelenmiş ve %9,71-12,57 oranında arttığı tespit edilmiştir. Üreticilerin en önemli girdisi olan yemin pandemi döneminde özellikle kanatlı sektöründe önemli düzeyde artış ($P<0,05$) gösterdiği belirlenmiştir. Pandemi sırasında hayvansal ürün/yem paritesindeki en fazla azalma -%10,28 ile yumurta sektöründe yaşanmıştır. Sonuç olarak, pandemi döneminde hayvansal ürün fiyatları artmış ancak girdi fiyatlarında artış daha fazla olmuştur. Ayrıca üreticilerin pandemi döneminde başta yumurta tavukçuluğu olmak üzere ürün/yem paritesi değerlerine göre daha düşük karlılıkla çalışmak zorunda kaldıkları belirlenmiştir.
Anahtar sözcükler: Covid 19, et, fiyat, süt, yem.

Introduction

Covid-19, which emerged on December 12, 2019, in Wuhan Province, China, was declared a pandemic by the World Health Organization (WHO) on March 11, 2020, as it began to spread worldwide (21). The first Coronavirus case in Turkey was declared by the Ministry of Health on March 11, 2020, and the first death occurred on March 18, 2020 (7, 16). The rapid transmission of the virus through human contact, causing a significant number of deaths, has forced all countries to take radical measures. In this context; schools have been closed, domestic/international

travel restrictions have been introduced, quarantine measures have been applied, the borders of countries have been closed (import/export), rapid vaccine and drug studies have been initiated, and respiratory equipment, masks, gloves, and disinfectant production have been increased. As a result, serious decisions were made that would profoundly affect all sectors and economies.

The livestock sector has continuous production and constitutes a significant part of the food supply, which is a basic need of people. During the pandemic period, it has been observed that the demand for products such as red meat, chicken meat, eggs, and milk has increased, especially in some households (except lower income families), where people have stayed at home. For this reason, despite this sudden increase in demand for animal products in the short term, price increases (partially speculative) have been experienced in the markets since supply could not respond in the same manner because of its production structure. According to the course of the pandemic, price increases in animal products have been shaped over time according to the behavior of consumers and changes in input costs (mainly feed). While it is thought that it will take time for the markets to stabilize in the near future, it may be easier to shape the supply according to the expectations of the market in the medium and long term with supporting measures such as capacity increase, incentives, and subsidies.

In Turkey, during this period, no additional measures have been needed to increase the production of livestock by the government. Only electricity, water, and credit payments have been postponed, and advantageous credit opportunities have been offered (12, 17). In addition, the problems encountered in the transfer of animal and animal products during the pandemic caused disruptions in animal feed supply and slaughter.

The most significant barrier to reveal the economic impact of the Spanish flu experienced in the world in 1918 was the lack of economic data (5). The current study not only reveals the effects of Covid-19 on the animal product and feed prices but also provides a basis for future studies on the long-term impact of the pandemic. This study aims to investigate changes in the prices of animal products (meat, milk, eggs) and feed (beef fattening, lamb, broiler, layer, and dairy feeds) before and during the pandemic period on a sectoral basis in Turkey.

Materials and Methods

The material for the study consisted of the prices of animal products (beef, lamb, and poultry meats, eggs, and milk) and feed (beef fattening, lamb, broiler, layer, and dairy feeds) collected from official institutions (Meat and Milk Board-ESK, the National Red Meat Council-UKON, the National Milk Council-USK) and producer associations (Kaytaş) in the period before (December, January, February) and during (March, April, May) the pandemic (4, 9, 18, 19). In addition, animal products and feed prices parities for the period before and during the pandemic were calculated using the following formula:

Product / Feed Parity = Animal product sale prices (producer) / feed price.

In the study, the prices before pandemic (December 2019-February 2020) and during pandemic (March-May 2020) were compared.

Statistical analysis: Monthly mean prices of livestock products and feed were considered in the statistical analysis. Student's t-test was used for the statistical significance control of the difference between the mean of the prices before and during the pandemic, and the relationship between the variables was evaluated with the Pearson correlation coefficient. Statistical analyses were performed with the SPSS 22.0 package program.

Results

In this study, animal product and feed prices along with product/feed parities for the periods before and during the pandemic were analyzed and presented as tables (Tables 1-3) and graphics (Figures 1-4).

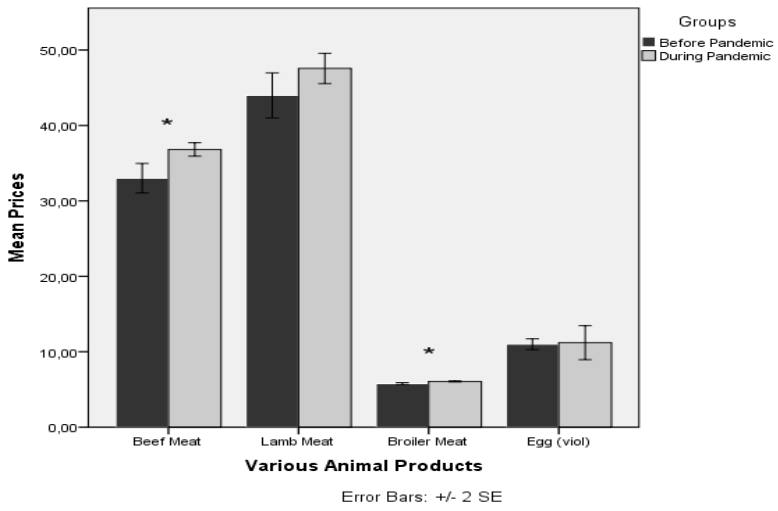

Figure 1. Changes in animal product prices before and during the pandemic period.

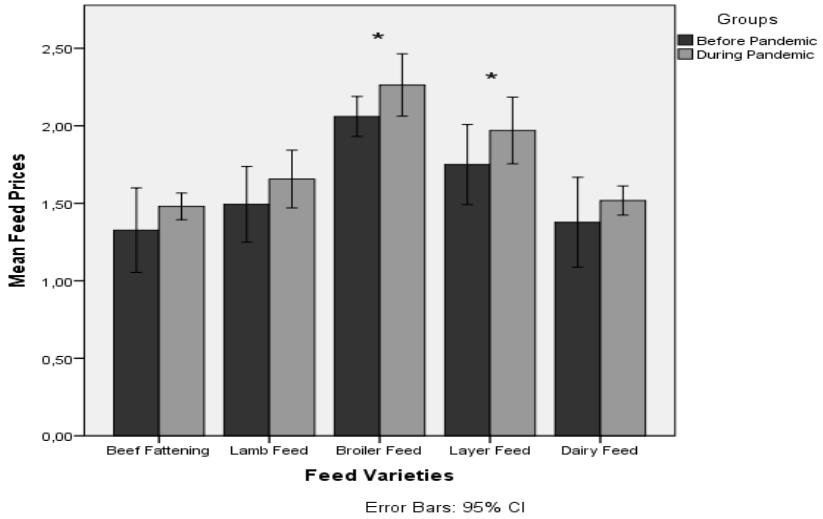

Figure 2. Changes in animal feed prices before and during the pandemic period.

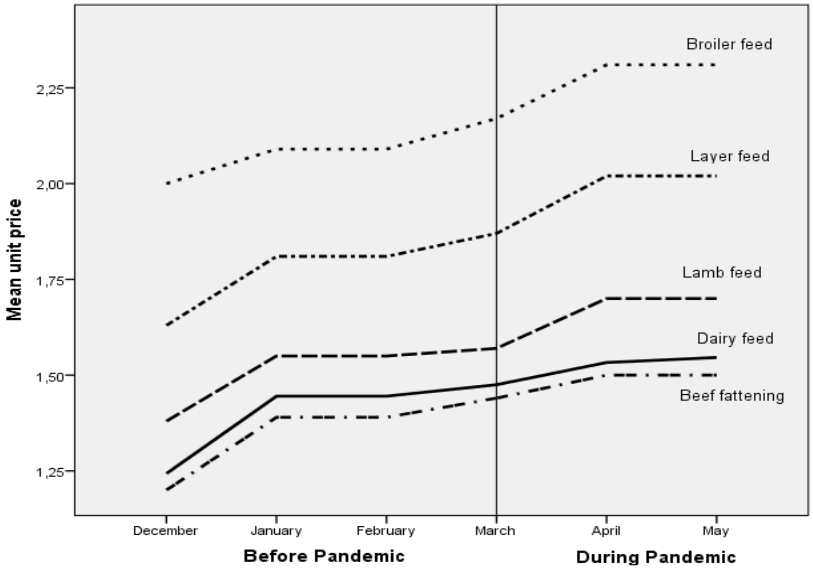

Figure 3. Changes in animal feed prices before and during the pandemic period.

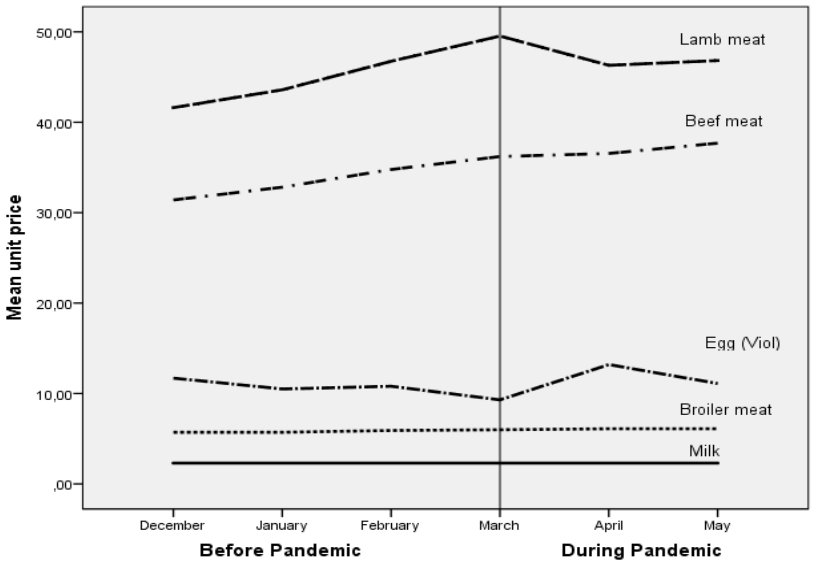

Figure 4. Changes in animal product prices before and during the pandemic period.

Table 1. Various animal product prices (TL) and changes (%) before and during the pandemic.

Variables*	Groups	Means ± Standard Error	Price Changes (%)	P
Beef meat	Before pandemic	33.00±0.98	+11.57	**0.024**
	During pandemic	36.82±0.45		
Lamb meat	Before pandemic	43.99±1.49	+8.11	0.118
	During pandemic	47.56±1.00		
Broiler meat	Before pandemic	5.78±0.07	+4.84	**0.020**
	During pandemic	6.06±0.04		
Egg**	Before pandemic	11.00±0.36	+1.82	0.874
	During pandemic	11.20±1.13		

* Since milk prices have not changed, no comparison has been made. ** Egg prices have been calculated over 30 viol.

Table 2. Prices (TL) and changes (%) in animal feed before and during the pandemic.

Variables	Groups	Means ± Standard Error	Price Changes (%)	P
Beef fattening feed	Before pandemic	1.33±0.06	+12.03	0.082
	During pandemic	1.49±0.02		
Dairy feed	Before pandemic	1.38±0.07	+10.14	0.118
	During pandemic	1.52±0.02		
Lamb feed	Before pandemic	1.49±0.06	+11.41	0.084
	During pandemic	1.66±0.04		
Broiler feed	Before pandemic	2.06±0.03	+9.71	**0.021**
	During pandemic	2.26±0.47		
Layer feed	Before pandemic	1.75±0.06	+12.57	**0.048**
	During pandemic	1.97±0.05		

Table 3. Feed parities of various animal products before and after the pandemic.

Variables	Groups	Means ± Standard Error	Parity Changes (%)	P
Beef meat/feed parity	Before pandemic	24.94±0.74	-0.16	0.972
	During pandemic	24.90±0.27		
Milk/feed parity	Before pandemic	1.68±0.09	-9.52	0.195
	During pandemic	1.52±0.02		
Lamb meat/feed parity	Before pandemic	29.48±0.68	-2.38	0.674
	During pandemic	28.78±1.39		
Broiler meat/feed parity	Before pandemic	2.81±0.04	-4.63	0.087
	During pandemic	2.68±0.04		
Egg/feed parity	Before pandemic	6.32±0.43	-10.28	0.361
	During pandemic	5.67±0.46		

Considering the prices of investigated animal products, there were increases at different rates in all products during the pandemic (1.82-11.57%). The differences between producer prices of beef and broiler meat before and during the pandemic were found to be statistically significant ($P<0.05$), and changes in lamb meat and egg prices were not statistically significant (Table 1).

On the other hand, to show the changes in terms of input costs, feed prices were also examined and were found increased by 9.71-12.57% during the pandemic period. It was determined that the feed, which is the most critical input of producers, showed a significant increase ($P<0.05$) during the pandemic, especially in the poultry sector (Table 2).

When the feed parities of various animal products are examined, it is possible to say that the parity decreases numerically out of the favor of the producer in all products. During the pandemic, the lowest parity was experienced in the egg sector, with 10.28% (Table 3).

As seen in the graph (Figure 3), a sudden rise (10-12%) in feed prices was experienced due to an increase in the exchange rate with the emergence of the first cases (March 2020) in Turkey.

Discussion and Conclusion

An animal product supply chain consists of different actors such as the producer, industry/business, transportation/logistics, storage, and retail services (2). The operation of these actors in a particular order is

essential in terms of connecting the consumers sustainably to the animal products in the market. However, measures taken such as curfew, import/export ban, inter-provincial travel restrictions) in the pandemic period in Turkey that put pressure on the sector and increased uncertainty for the future has been an obstacle for the continuity of production. On the other hand, since feed ingredients such as corn and soybeans are imported, price increases have been experienced due to the increase in foreign exchange rates and difficulties in obtaining these feedstuffs in Turkey. Also, the absence of policies that encouraged production in animal husbandry during the pandemic period raised future concerns in terms of sustainability of production. Moreover, the disruptions experienced in the logistics stages of animal products and raw materials caused deterioration in products and an increase in waste. The panic that occurred in the first days when curfew restrictions were applied in the pandemic period led to the stockpiling of basic needs by the public (including animal products), and crowding was experienced in shopping centers.

The impact of the Covid-19 pandemic on animal products and feed prices has been evaluated in some other countries (1, 3, 6, 8, 13, 14, 20), However, to the best of the present authors' knowledge, there is no study investigating the effects of the Covid-19 pandemic on animal products and feed prices in Turkey.

In all the animal products examined in this study, price increases were experienced, and beef meat (11.57%) was the most affected livestock product in Turkey during the three-month pandemic period examined. A possible reason of this increase may be due to scarcity of supplying the beef cattle and meat to the market in this period (seasonal effect), and reflection of increased feed prices on production costs. Due to lambing season, price increases in lamb meat (8.11%) has been more limited compared to beef meat during the pandemic period.

On the other hand, there was a quite limited increase in egg prices (1.82%) during the pandemic period. Due to the higher egg production capacity of Turkey and interruption in the exportation of the eggs, a market supply shortage was not experienced, which resulted in a negligible increase in egg prices. However, considering the feed prices, the poultry sector was negatively affected in this period due to the high dependence of feed ingredients (corn, soybean, etc.) on importation. When the animal product/feed parity is evaluated together, it is understood that the parity has decreased the most in the poultry sector, especially in egg poultry.

Similar with our study, price increase in meat was reported as significant and egg prices were not significantly affected during the pandemic period in Europe (1).

In Turkey, in the pandemic period, warnings such as stay-at-home and maintain social distance as well as travel restrictions and the enforcement of a partial curfew has changed consumer behavior due to the uncertainty of the future. Thus, the demand for animal products was increased at the beginning of the pandemic period. Similar effects were seen in other countries (10). Although the impact of the pandemic on the producers is not yet entirely clear, considering together the prices and parities, it is possible to say that the pandemic reduces the profitability of producers in the short term in Turkey. Considering parities, the egg and milk producers were the most affected by the pandemic in the evaluated period, whereas the impact on the red meat sector was more limited. However, feed prices in the USA between January and May 2020 (6) and slaughtered beef prices in Canada decreased by 16% (11). Although it has been calculated that the pandemic caused significant losses in animal products and feed in the USA (8), beef producers in Canada expect recovery to start in the autumn of 2020, and to return to normal in early 2021 (13).

In the present study, the changes in the prices of animal products and inputs (feed) on the producer basis during the pandemic in Turkey were evaluated. In addition, it is of importance to evaluate the effects of a pandemic on consumer behavior in terms of livestock products. Despite the increase in animal product prices during the pandemic period, the increase in demand is thought of as a disaster-specific and research-worthy consumer behavior. Because demand elasticity decreased in this period (8), the order of importance of the needs of consumers changed, and food products, especially animal products, had more priority.

Beside Covid-19 pandemic, some different factors may affect the prices of livestock products and feed such as season, exchange rates and inflation. For example, similar increase in price of beef meat (9.17%) was seen a year before in Turkey. Exchange rates and inflation were increased 12.67% and 13.9% during the pandemic period compared to before the pandemic period in Turkey, respectively (15).

It is difficult to increase the supply quantity in a short time due to the structure of production in livestock production. For example, at least 40-45 days are required for broiler meat production, 90-180 days for beef production, 120-130 days for egg production, and 150 days in lamb production. In the pandemic period, the producers' failure to respond to the increasing demand for animal products in a short time can only be explained by the production period. For these reasons, especially in extraordinary situations, such as the pandemic period, products such as broilers and eggs, that can increase supply in a shorter time, are essential. Additionally, it is thought that additional support and subsidies are needed to increase animal production. In this way, the implementation of measures that will both encourage

production and secure supply will be beneficial. The long-term effect of the pandemic on the supply of animal products clearly will be more evident in the future. The present study, demonstrating as it does the short-term effects of the pandemic on the animal product and input prices, is thought to be a basis for future studies investigating the long-term effects of the Covid-19 pandemic on animal production in Turkey.

In conclusion, animal product prices increased during the pandemic period, but input prices increased more than products. An increase in foreign exchange prices and dependency are thought to be responsible for this situation. In addition, it was determined that the producers had to produce with lower profitability compared to the product/feed parity values, especially in the egg poultry sector during this period.

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Ethical Statement

This study does not present any ethical concerns.

Conflict of Interest

The authors declared that there is no conflict of interest.

References

1. **Akter S** (2020): *The impact of COVID-19 related 'stay-at-home' restrictions on food prices in Europe: findings from a preliminary analysis.* J Food Secur, **12**, 719-725.
2. **Cullen MT** (2020): Coronavirus food supply chain under strain what to do? Available at: http://www.fao.org/3/ca8308en/ca8308en.pdf. (Accessed Jun 15,2020).
3. **Dennis EJ** (2020): Has Covıd-19 impacted price-weight relationships and value of gain? Extension Farm and Ranch Management. Available at: https://digitalcommons.unl.edu/cgi/viewcontent.cgi?article=1031&context=ageconfarmmgmt. (Accessed Apr 29, 2020).
4. **ESK** (2020): Hayvan Alım Fiyatları. Available at: https://www.esk.gov.tr/tr/11931/Alim-Fiyatlari. (Accessed Jun 10, 2020).
5. **Garrett TA** (2007): Economic effects of the 1918 influenza pandemic: Implications for a modern-day pandemic. Federal Reserve Bank of St. Louis. Available at: https://www.stlouisfed.org/~/media/files/pdfs/community-development/research-reports/pandemic_flu_report.pdf. (Accessed May 8, 2020).
6. **Gertner D, Dennis E** (2020): Impact of Covid-19 on demand for distillers grains from livestock operations. Cornhusker Economics June 3, 2020. Available at: https://agecon.unl.edu/cornhusker-economics/2020/Impact-of-COVID-19-on-Demand-for-Distillers-Grains-from-Livestock-Operations.pdf. (Accessed Jun 3, 2020).
7. **Göncüoğlu M, Ayaz N, Cengiz G, et al** (2020): *Emerging details about COVID-19 and chronology of the pandemic in Turkey*. Ankara Univ Vet Fak Derg, **67**, 323-332.
8. **Hart CE, Hayes DJ, Jacobs KL, et al** (2020): The impact of Covid-19 on Iowa's corn, soybean, ethanol, pork, and beef sectors. CARD Policy Briefs. Available at: https://www.card.iastate.edu/products/publications/pdf/20pb28.pdf. (Accessed Apr 25, 2020).
9. **Kaytaş** (2020): Yumurta Fiyatları. Available at: http://fiyatlistesi.kaytas.com.tr/Product/ListAll. (Accessed Jun 10, 2020).
10. **Lusk J** (2020): Impacts of coronavirus on food markets. Available at: http://jaysonlusk.com/blog/2020/3/16/impacts-of-coronavirus-on-food-markets. (Accessed Jun 12, 2020).
11. **McMillin D** (2020): Covid-19 pandemic sees cattle markets. Canadian cattlemen. Available at: https://www.canadiancattlemen.ca/livestock/beefcattle/covid-19-pandemic-sees-cattle-markets-plunge/. (Accessed Jun 17, 2020).
12. **Resmi Gazete** (2020): T.C. Ziraat Bankası A.Ş. ve Tarım Kredi Kooperatiflerince Tarımsal Üretime Dair Düşük Faizli Yatırım ve İşletme Kredisi Kullandırılmasına İlişkin Uygulama Esasları Tebliği. Tebliğ No: 2020/4. Available at: https://www.resmigazete.gov.tr/eskiler/2020/03/20200325-10.htm. (Accessed Apr 12, 2020).
13. **Rude J** (2020): *COVID-19 and the Canadian cattle/beef sector: Some preliminary analysis.* Cand J Agr Econ, **68**, 207–213.
14. **Seleiman MF, Selim S, Alhammad BA, et al** (2020): *Will novel coronavirus (Covid-19) pandemic impact agriculture, food security and animal sectors?* Bioscience J, **36**, 1315-1326.
15. **T.C. Merkez Bankası** (2020): Döviz kurları. Available at: https://www.tcmb.gov.tr/wps/wcm/connect/TR/TCMB+TR/Main+Menu/Istatistikler/Doviz+Kurlari. (Accessed Oct 12, 2020).
16. **T.C. Sağlık Bakanlığı** (2020): Covid-19, Türkiye'deki güncel durum. Available at: https://covid19.saglik.gov.tr/. (Accessed Apr 15, 2020).
17. **T.C. Tarım ve Orman Bakanlığı** (2020): Orman köylü ve kooperatiflerinin kredi taksitleri kovid-19 nedeniyle ertelendi. Available at: https://www.tarimorman.gov.tr/Haber/4514/Orman-Koylu-Ve-Kooperatiflerinin-Kredi-Taksitleri-Kovid-19-Nedeniyle-Ertelendi. (Accessed May 1, 2020).
18. **UKON** (2020): Kırmızı Et Alım Fiyatları. Available at: http://www.ukon.org.tr/Fiyatlar.aspx. (Accessed Jun 10, 2020).
19. **USK** (2020): Çiğ Süt Fiyatları. Available at: https://ulusalsutkonseyi.org.tr/2019-yili-cig-sut-fiyatlari-2019-2583/. (Accessed Jun 10, 2020).
20. **Weersink A, Von Massow M, McDougall B** (2020): *Economic thoughts on the potential implications of COVID-19 on the Canadian dairy and poultry sectors.* Cand J Agr Econ, **68**, 195–200.
21. **WHO** (2020): Director-General's opening remarks at the media briefing on Covid-19. Available at: https://www.who.int/dg/speeches/detail/who-director-general-s-opening-remarks-at-the-media-briefing-on-covid-19---11-march-2020. (Accessed May 25, 2020).

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The effects of Pennyroyal (*Mentha pulegium L.*) on performance, carcass and meat quality in Japanese quails (*Coturnix coturnix japonica*)

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Abstract: This study investigated the Pennyroyal in terms of its effects on performance and meat quality (color and pH) in Japanese quails in different levels. A total of one day-old Japanese quail chicks were randomly distributed to a control and two experimental groups containing three different levels of pennyroyal (0.0, 0.1 and 0.2 %). Each experimental group was divided into five repetitive groups with, ten quail chicks in each group. At the end of the experiment, the performance values and meat quality parameters (color and pH) were measured. Significant differences were found between the groups according to the growth performance of the quails (P<0.05). At the end of the study, the body weight and body weight gain of the experimental groups in which 0.1% (P1) and 0.2% (P2) pennyroyal supplemented were found to be higher than the control group. It was observed that the feed consumption increased in the P2 group compared to the control group, and the feed conversion rate decreased (P<0.05). There was no significant difference was observed in the carcass yield, heart, liver and gizzard weight (P>0.05). The brightness (L*), redness (a*), yellowness (b*) and pH values of the breast meat were not affected by the supplementation of pennyroyal (P>0.05). According to the results of the study, the supplemented of pennyroyal by 0.2% was more effective on the performance.
Keywords: Meat color, pennyroyal, performance, pH, quail.

Yarpuzun (*Mentha pulegium L.*) Japon bıldırcınlarında (*Coturnix coturnix japonica*) performans, karkas ve et kalitesi üzerine etkisi

Özet: Bu çalışmada, Yarpuzun (*Mentha pulegium L.*) farklı seviyelerde Japon bıldırcınlarında performans ve et kalitesi (renk ve pH) üzerine etkisi araştırılmıştır. Toplam 150 adet günlük yaşta Japon bıldırcın civcivi üç farklı düzeyde yarpuz içeren (%0,0, 0,1 ve 0,2) bir kontrol ve iki deneme grubuna rastgele dağıtıldı. Her deneme grubu içerisinde 10 bıldırcın bulunan beş tekerrürlü gruba ayrıldı. Deneme sonunda performans değerleri ve et kalite parametreleri (renk ve pH) ölçüldü. Bıldırcınların büyüme performansı değerlendirildiğinde gruplar arasında önemli farklılıklar bulundu (P<0,05). Çalışma sonu itibariyle %0,1 (P1) ve %0,2 (P2) yarpuz ilave edilen deneme gruplarının canlı ağırlık ve canlı ağırlık artışları kontrol grubuna oranla yüksek bulundu. Yem tüketiminin P2 grubunda kontrol grubuna oranla arttığı, yemden yararlanma oranının ise düştüğü gözlendi (P<0,05). Karkas randımanı, kalp, karaciğer ve taşlık ağırlıkları açısından anlamlı bir fark olmadığı görüldü (P>0,05). Göğüs etinin parlaklık (L*), kırmızılık (a*) ve sarılık (b*) ile pH değerleri yarpuz ilavesinden etkilenmedi (P>0,05). Çalışma sonuçlarına göre yarpuzun %0,2 düzeyinde ilavesinin performans üzerine daha etkili olduğu belirlendi.
Anahtar sözcükler: Bıldırcın, et rengi, performans, pH, yarpuz.

Introduction

Mentha pulegium L, which belongs to the *Mentha* species of the *Labiatae* family, is ubiquitous in Europe, North Africa and Central Asia and known as pennyroyal (11). It has been used as flavorant, expectorant, appetite regulator or diuretic for many years. In addition, it is widely used in the treatment of diseases such as feed poisoning, bronchitis and tuberculosis (21). In addition to these effects, it has also been reported to have aromatic regulation, antioxidant, anti-inflammatory and

antimicrobial effects due to the phenolic compounds in its structure, such as Linalool, Menthone, p-Menthan, Pulegone, Piperitenone oxide (20, 35, 36). After the prohibition of antibiotics in the breeding of poultry, pennyroyal and aromatic plants are reported to be useful due to its effects on increasing digestibility, balancing the intestinal microbial ecosystem and excreting endogenous digestive enzymes (13, 17, 40). There is a growing awareness in consumers with the development of technology in the field of feed safety, which led to a growing interest in the use of medical aromatic plants that are not harmful to human and animal health as well as their various forms (18, 22, 32). In many studies, it was found that pennyroyal and other Mentha species make positive contributions to the efficiency and shelf life of animal products (5, 16, 26).

The main aim of this study was to investigate the Pennyroyal (*Mentha pulegium L.*) in different levels supplemented to quail rations in terms of its effects on growth performance and breast meat quality (color and pH).

Materials and Methods

Animals, experimental design and feed: The experiment was carried out in the Kafkas University Research and Practice Farm. This study was approved by the Kafkas University Animal Experiments Local Ethics Committee (KAÜ-HADYEK/2020-008). A total of 150 a day old Japanese quails (*Coturnix coturnix japonica*) were used as the animal material. The study was performed with one control and two experiment groups, each of which included 50 quail chicks. All groups were divided into five subgroups, each containing 10 quail chicks. Dried and powdered pennyroyal was added to the basic ration of the control and experimental groups by 0.0%, (control) 0.1% (P1) and 0.2% (P2), respectively. The quail chicks were placed in plastic cages of 60×20×100 cm. The study was completed in a total of 35 days, including 7 days of adaptation and 28 days of feeding periods. The animals were given feed and water ad *libitum*. Throughout the study, all animals were kept at 32-33°C for the first three days, which was then fixed to 25°C by a gradual decrease of 1-2°C on a weekly. The cages were kept illuminated for 24 hours/day for 35 days. The rations were prepared in accordance with the NRC (14), and chemical analyses were carried out in accordance with the AOAC (25) (Table 1).

Feed additive: Plants (pennyroyal) were collected from Kars province (40 ° 48’21.2 "N 42 ° 53’37.8" E) in Turkey and harvested in its season. Plants’ leaves were dried at room temperature without direct exposure to the sun light. After drying, they were powdered for supplementation to the ration. The essential oil was obtained from Pennyroyal through vapor distillation method. The main active compositions of the pennyroyal leaves essential oil were determined by GC/MS and contained 13.61% of Linalool, 10.56% of p-Menthone, 6.19% of p-Menthan, 4.45% of Pulegone, 0.11% of Isopulegone, 3.07% of Piperitenone oxide, and 0.49 of Thymol phenolic compounds (35).

Table 1. Ingredients and nutrient composition of diet.

Ingredients	**%**
Corn, yellow	46.30
Soybean meal (48% CP)	32.41
Barley meal	11.00
Corn gluten meal	4.40
Wheat bran	2.12
Vegetable oil	0.60
Limestone	1.80
Dicalcium phosphate	1.12
Salt	0.30
DL-methionine	0.10
L-lysine sulfate	0.40
Vit-min mix[1]	0.25
Analyzed values	
CP (%)	24.00
ME(kcal/kg)	2901
Ca (%)	0.80
Av. P (%)	0.38

CP: Crude protein, ME: Metabolism energy, Ca: Calcium, Av. P: Available phosphorus

[1] Vit-min mix: Vit A; 15.00 IU/kg, Vit D; 3.30 IU/kg, Vit E; 25.00 IU/kg, K; 0.86%, Mg; 0.16%, S; 0.21%, Fe; 146.89 mg/kg, Mn; 121.11 mg/kg, I; 0.50 mg/kg, Zn; 110.03 mg/kg, Cu; 13.05 mg/kg.

Performance: Body weight (BW) and feed consumption (FC) were determined in all groups every week. The increase in body weight gain (BWG) and feed conversion rate (FCR) were calculated (FC/BWG) based on the differences obtained as a result of the weighing processes. At the end of the study, 10 quails were randomly selected from each group to analyze their carcass traits. The animals were not fed for 12 hours. Then, they were weighed individually and slaughtered after determining their slaughtering weight. The blood of the slaughtered animals was drained, and their plume was plucked. Subsequently, internal organs (heart, liver and gizzard) were removed to calculate the carcass and internal organ weights.

Determining the color quality and pH of meat: Breast meat samples were taken from the slaughtered animals to determine the color intensity and pH. A colorimeter (Minolta-Japan) was used to determine the color intensities of the breast meat samples (L*: brightness, a*: redness, b*: yellowness). The color intensities were determined as per the characteristics specified by the International Commission on Illumination. Average values were determined through the

measurements performed at four different parts of each sample. In order to determine the pH values of the breast meat samples, 10 grams of breast meat was added to 100 ml of purified water. Then it was homogenized and measured using a pH meter (SCHOTT L 6880).

Statistical analysis: The performance and the parameters of breast meat quality (color, pH) were evaluated on the SPSS 20.0 (IBM-USA) statistics software. The difference between the results of the groups was analyzed with one-way variance analysis (ANOVA). The Duncan, multiple comparison test, was used in the bilateral comparisons between the groups. The level of significance was determined to be at P<0.05.

Results

It was observed that pennyroyal had positive effects on performance in the quails (P<0.05). While the BW, BWG and FC values in the groups with pennyroyal supplementations tended to increase compared to the control group, the FCR had a significant decrease in the group with the supplementation of pennyroyal by 0.2% (Table 2). It was determined that pennyroyal did not have any effects on the post-slaughtering carcass yield and weights of the internal organs (P<0.05) (Table 3).

It was observed that pennyroyal did not have any statistical effects on the color of the breast meat (L*, a*, b*) and pH value (P>0.05) (Table 4).

Table 2. The effect of Pennyroyal (*M. pulegium L.*) on performance parameters.

	BW (g)				
	Groups			P values	
	Control	P1	P2	*L*	*Q*
d 7	29.20±0.73	29.00±0.17	28.98±1.39	0.982	0.939
d 21	116.05±0.59	117.70±1.46	118.70±1.69	0.185	0.848
d 35	180.81±1.97^{b}	186.05±1.45^{a}	188.88±1.18^{a}	0.003	0.542
	BWG (g/quails)				
	Groups			P values	
	Control	P1	P2	L	Q
d 7 to 21	86.85±1.01	88.69±1.40	89.73±2.00	0.207	0.832
d 21 to 35	64.76±1.84^{b}	68.36±1.07^{ab}	70.17±0.91^{a}	0.014	0.598
d 7 to 35	151.61±1.45^{b}	157.05±1.35^{a}	159.90±1.78^{a}	0.003	0.506
	FC (g)				
	Groups			P values	
	Control	P1	P2	L	Q
d 7 to 21	276.96±3.23	279.23±4.50	282.94±3.45	0.272	0.877
d 21 to 35	259.03±4.46	265.00±3.00	268.35±2.45	0.064	0.756
d 7 to 35	535.99±3.87^{b}	544.23±3.64^{ab}	551.29±3.26^{a}	0.004	0.895
	FCR (g/g)				
	Groups			P values	
	Control	P1	P2	L	Q
d 7 to 21	3.19±0.03	3.15±0.05	3.15±0.02	0.461	0.572
d 21 to 35	4.00±0.04^{a}	3.88±0.06^{ab}	3.82±0.03^{b}	0.008	0.540
d 7 to 35	3.53±0.01^{a}	3.47±0.02^{ab}	3.45±0.01^{b}	0.001	0.172

P1: 0.1% Pennyroyal, P2: 0.2% Pennyroyal, BW: Body weight, BWG: Body weight gain, FC: Feed consumption, FCR: Feed conversion rate, *L*: Linear, *Q*: Quadratic

^{a,b} Values within a column with different superscripts differ significantly at P<0.05.

Table 3. The effect of Pennyroyal (*M. pulegium L.*) on carcass parameters.

	Groups			**P values**	
	Control	P1	P2	*L*	*Q*
Carcass yield (%)	73.16±0.19	73.31±0.11	73.34±0.12	0.380	0.730
Heart (g)	1.60±0.03	1.61±0.06	1.47±0.06	0.839	0.124
Liver (g)	3.31±0.43	2.65±0.19	3.41±0.41	0.130	0.542
Gizzard (g)	2.62±0.20	2.26±0.18	2.70±0.13	0.738	0.639

P1: 0.1% Pennyroyal, P2: 0.2% Pennyroyal, *L*: Linear, *Q*: Quadratic.

Table 4. The effect of Pennyroyal (*M. pulegium L.*) on meat color and pH.

	Groups			P values	
	Control	P1	P2	*L*	*Q*
L*	45.51±1.23	45.96±1.79	45.29±1.35	0.947	0.845
a*	11.60±0.44	11.23±0.41	11.41±0.17	0.807	0.694
b*	4.85±0.19	4.75±0.14	4.72±0.17	0.725	0.911
pH	5.48±0.07	5.50±0.03	5.50±0.03	0.892	0.875

P1: 0.1% Pennyroyal, P2: 0.2% Pennyroyal, L *: Brightness, a *: Redness, b *: Yellowness, *L*: Linear, *Q*: Quadratic.

Discussion and Conclusion

The supplementation of pennyroyal on quail rations had a positive effect on performance. In the study, it was determined that the supplementation of pennyroyal in increasing levels had a positive effect on the BW, BWG and FC values, compared to the control group. It was determined that the best FCR was obtained in the group (2.86) with supplementation of 0.2% of pennyroyal. The BW and BWG results obtained were in accordance with the results of the study, in which pennyroyal was used as a supporting growth agent (33, 35). The results of the current study also had similarities with the studies on the effects of different Mentha species (*M. Longifolia* and *M. Piperita*) on BW and BWG in the poultry (3, 4). In the broiler study, in which the pennyroyal, essential oil, probiotics and antibiotics were used in different levels, the best performance values were obtained in the group with the addition of pennyroyal, thereby affecting the BWG and FCR significantly. The results of the study suggested that pennyroyal could be used as an alternative to antibiotics in poultry (2). It is believed that the phenolic compounds in pennyroyal and other Mentha species decreased the presence of pathogenic microorganisms in the intestines of the poultry, thereby showing an antimicrobial effect as well as affecting the growth performance (21, 30). The fact that pennyroyal increases feed consumption due to its appetizing effect has been mentioned in the literature (21). Similarly, there have been studies, in which the Mentha species increased feed consumption (3, 4).

Contrary to these studies, there have been studies reporting that the Mentha species do not affect the performance values (BW, BWG and FC) in the poultry (15, 19, 34). Erhan et al. (17) found that pennyroyal did not affect the body weight increase in broiler chickens, and it decreased the feed consumption, thereby improving the feed conversion rate. Ghalamkari et al. (20) stated that the addition of antibiotics and pennyroyal did not cause a difference in terms of the performance values of the broiler chickens, Arjomandi et al. (8) found that the pennyroyal mixed with probiotics decreased the feed consumption in laying hens, and Aydin and Bolukbasi (10) reported that the addition of pennyroyal in increasing levels (32.5, 65 and 130 mg/kg) did not affect the feed consumption in laying hens, which were not in compliance. It is believed that these differences might have stemmed from the region where the plant was grown, the harvest season, the breed of animal used, the dosage used and feeding conditions.

It was determined that the addition of pennyroyal did not affect the weights of carcass and internal organs (heart, liver and gizzard) in quails. The results of the present study were following the results of some other studies (1, 21, 31). Similarly, Nobakht et al. (33) reported that pennyroyal did not have any effects on the carcass yield or the weights of the internal organs. On the contrary, Shamlo et al. (39) stated that the pennyroyal extract (0. 50, 100 and 150 ppm) increased the carcass weight, carcass yield and the weight of the heart; however, it did not have any effects on the weights of the liver and gizzard. Along with this study, there are other studies in the literature stating that the carcass parameters and internal organ weights changed due to the addition of pennyroyal and aromatic plants together or separately (4, 9, 38). It was stated that pennyroyal increased digestive enzymes in the poultry, which had a positive effect on the pancreas, and could positively affect the carcass parameters particularly by the increase of the amino-acid absorption (6). The differences observed between studies might have resulted from the diversity of aromatic plant used, the interactions between additives, dosage, and differences in animals.

The unique color of the product is another significant criterion for consumers to choose any product. This color should not change during the time it remains on the shelf. The oxidation of the myoglobin and hemoglobin pigments is useful in the changing of the natural color of the meat (23). In the study, no pennyroyal-related effects were observed on the color intensities (L*, a*, b*) and pH values of the breast meat of the quails (P>0.05). The current study is in accordance with the studies, in which aromatic plants and their essential oil derivatives were used, and had no effects on the color and pH values of the meat (12, 24, 27, 37).

There are also studies determining that pennyroyal and different aromatic plants had effects on the color and pH of the meat (28, 29). It was determined that *Mentha*

piperita affected the a* and b* of the meat color intensities; however, it did not affect the L* and pH in the quails (7). In their study on the effect of thyme and pennyroyal on the broiler meat color and PH value in the broilers, Pirmohamammadi et al. (36) found that the pennyroyal affected the L* value; however, it did not have any effects on the a*, b* and pH values compared to the control group. They also stated that thyme and pennyroyal increased the pH when used combined ($P<0.05$).

As a result, it was concluded that the pennyroyal (*Mentha pulegium L.*) significantly affected the growth performance without changing the carcass, internal organs, or the meat quality (color, pH) in the Japanese quails.

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Ethical Statement

This study was approved by the Kafkas University Animal Experiments Local Ethics Committee (KAÜ-HADYEK/2020-008).

Conflict of Interest

The authors declared that there is no conflict of interest.

References

1. **Abd Al-Jaleel R** (2012): *Use of turmeric (Curcuma longa) on the performance and some physiological traits on the broiler diets.* Iraqi J Vet Sci, **36**, 51-57.
2. **Abedini A, Hassanabadi A, Afzali N, et al** (2017): *The effect of different dietary levels of Pennyroyal (Mentha Pulegium L.), probiotic and antibiotic on performance, carcass characteristics and, sellected nutrients digestibility in broiler chickens*. Arch Med Lab Sci, **3**, 15-22.
3. **Al-Ankari A, Zaki M, Al-Sultan S** (2004): *Use of habek mint (Mentha longifolia) in broiler chicken diets*. Int J Poult Sci, **3**, 629-634.
4. **Al-Kassie GA** (2010): *The role of peppermint (Mentha piperita) on performance in broiler diets*. Agric Biol J N Am, **1**, 1009-1013.
5. **Alizadeh Amoli Z, Mehdizadeh T, Tajik H, et al** (2019): *Shelf life extension of refrigerated, vacuum-packed rainbow trout dipped in an alginate coating containing an ethanolic extract and/or the essential oil of Mentha Aquatica*. Chem Pap, **73**, 2541-2550.
6. **Aminzade B, Karami B, Lotfi E** (2012): *Growth response and carcass characteristics of Japanese quail to Mentha piperita plant supplementation*. Anim Biol Anim Husb, **4**, 24-27.
7. **Aminzade B, Karami B, Lotfi E** (2012): *Meat quality characteristics in Japanese quails fed with Mentha piperita plant*. Anim Biol Anim Husb, **4**, 20-23.
8. **Arjomandi M, Nobakht A, Pishchang J, et al** (2011): *Evaluoation the effects of using of probiotic, and pennyroyal (Mentha pulegium L.) medicinal plant on perormance of laying hens*. J Appl Environ Biol Sci, **1**, 164-167.
9. **Asadi N, Husseini SD, Tohidian M-T, et al** (2017): *Performance of broilers supplemented with peppermint (Mentha piperita L.) powder*. Evid Based Compl Alt Med, **22**, 703-706.
10. **Aydin A and Bolukbasi SC** (2020): *Effect of supplementation of hen diet with pennyroyal extract (Mentha pulegium) on performance, egg quality and yolk TBARS values*. Pak J Zoology, **52**, 1045-1051.
11. **Chalchat JC, Gorunovic MS, Maksimovic ZA, et al** (2000): *Essential oil of wild growing Mentha pulegium L. from Yugoslavia*. J Essent Oil Res, **12**, 598-600.
12. **Cho JH, Kim HJ, Kim IH** (2014): *Effects of phytogenic feed additive on growth performance, digestibility, blood metabolites, intestinal microbiota, meat color and relative organ weight after oral challenge with Clostridium perfringens in broilers*. Livest Sci, **160**, 82-88.
13. **Cross D, Acamovic T, Deans S, et al** (2007): *The effects of dietary inclusion of herbs and their volatile oils on performance, dietary digestibility and gut microflora in chickens from 7 to 28 days of age*. Br Poultry Sci, **48**, 496-506.
14. **Dale N** (1994): *National research council nutrient requirements of poultry-ninth revised edition*. J Appl Poult Res, **3**, 101-101.
15. **Demir E, Kilinc K, Yildirim Y, et al** (2008): *Comparative effects of mint, sage, thyme and flavomycin in wheat-based broiler diets*. Arch Zootech, **11**, 54-63.
16. **Erhan M, Aktaş Ş, Ürüşan H** (2015): *The effects of supplementation of pennyroyal (Mentha Pulegium L) on meat fatty acid composition and shelf-life in broilers*. Ataturk Univ Vet Bilim Derg, **10**, 179-185.
17. **Erhan M, Bölükbaşı Ş, Ürüşan H** (2012): *Biological activities of pennyroyal (Mentha pulegium L.) in broilers*. Livest Sci, **146**, 189-192.
18. **Erkan N, Doğruyol H, Günlü A, et al** (2015): *Use of natural preservatives in seafood: Plant extracts, edible film and coating*. Food and Health, **1**, 33-49.
19. **Geran M, Irany M, Dehpourjoybari A** (2010): The effect of pennyroyal essential oil on performance of broilers. 1-3. Proc 5th cong of new idea in agric.
20. **Ghalamkari G, Toghyani M, Landy N, et al** (2012): *Investigation the effects using different levels of Mentha pulegium L.(pennyroyal) in comparison with an antibiotic growth promoter on performance, carcass traits and immune responses in broiler chickens*. Asian Pac J Trop Biomed, **2**, S1396-S1399.
21. **Goodarzi M and Nanekarani S** (2014): *Effects of feeding Mentha pulegium L. as an alternative to antibiotics on performance of broilers*. APCBEE Procedia, **8**, 53-58.
22. **Gümüş R, Erol HS, Imik H, et al** (2017): *The effects of the supplementation of lamb rations with oregano essential oil on the performance, some blood parameters and antioxidant metabolism in meat and liver tissues*. Kafkas Univ Vet Fak Derg, **23**, 395-401.
23. **Gümüş R, Gelen SU, Ceylan ZG, et al** (2017): *Bıldırcın rasyonuna katılan kekik uçucu yağının göğüs etinin bazı mikrobiyolojik ve fizikokimyasal özelliklerine etkisi*. FÜ Sağ Bil Vet Derg, **31**, 153-158.

24. **Hong JC, Steiner T, Aufy A, et al** (2012): *Effects of supplemental essential oil on growth performance, lipid metabolites and immunity, intestinal characteristics, microbiota and carcass traits in broilers*. Livest Sci, **144**, 253-262.
25. **Horwitz W and Latimer GW** (2005): AOAC Official methods of analysis of AOAC International. AOAC Official methods of analysis of AOAC International. AOAC International, Gaithersburg, MD, USA.
26. **Kačániová M, Petrová J, Mellen M, et al** (2019): *Impact of anise (Pimpinella anisum) and mint (Mentha piperita) essential oils to microbial activity in chicken meat*. J Microbiol Biotechnol Food Sci, **4**, 28-31.
27. **Kennedy O, Mbaba E, Iso I, et al** (2020): *Effects of turmeric rhizome powder on growth, carcass and meat quality of Japanese quails fed sorghum-soybean-based diets*. Livest Sci, **11**, 1-7.
28. **Kirkpinar F, Ünlü HB, Serdaroğlu M, et al** (2014): *Effects of dietary oregano and garlic essential oils on carcass characteristics, meat composition, colour, pH and sensory quality of broiler meat*. Br Poult Sci, **55**, 157-166.
29. **Küçükyilmaz K, Kiyma Z, Akdağ A, et al** (2017): *Effect of lavender (Lavandula Stoechas) essential oil on growth performance, carcass characteristics, meat quality and antioxidant status of broilers*. S Afr J Anim Sci, **47**, 178-188.
30. **Lee KW, Everts H, Kapperst HJ, et al** (2003): *Dietary carvacrol lowers body weight gain but improves feed conversion in female broiler chickens*. J Appl Poult Res, **12**, 394-399.
31. **Mondal MA, Yeasmin T, Karim R, et al** (2015): *Effect of dietary supplementation of turmeric (Curcuma longa) powder on the growth performance and carcass traits of broiler chicks*. SAARC J Agric, **13**, 188-199.
32. **Murbach Teles Andrade BF, Nunes Barbosa L, da Silva Probst I, et al** (2014): *Antimicrobial activity of essential oils*. J Essent Oil Res, **26**, 34-40.
33. **Nobakht A, Norani J, Safamehr A** (2011): *The effects of different amounts of Mentha pulegium L.(pennyroyal) on performance, carcass traits, hematological and blood biochemical parameters of broilers*. J Med Plant Res, **5**, 3763-3768.
34. **Ocak N, Erener G, Burak Ak F, et al** (2008): *Performance of broilers fed diets supplemented with dry peppermint (Mentha piperita L.) or thyme (Thymus vulgaris L.) leaves as growth promoter source*. Czech J Anim Sci, **53**, 169.
35. **Ölmez M** (2018): Etlik piliç karma yemlerine ilave edilen yarpuzun (Mentha pulegium L.) besi performansı, karkas kalitesi ve bazı kan parametreleri üzerine etkisi. Doktora. Atatürk Universitesi Sağlık Bilimleri Veteriner Hayvan Besleme ve Beslenme Hastalıkları, Erzurum.
36. **Pirmohamammadi A, Daneshyar M, Farhoomand P, et al** (2016): *Effects of Thymus vulgaris and Mentha pulegium on colour, nutrients and peroxidation of meat in heat-stressed broilers*. S Afr J Anim Sci, **46**, 278-284.
37. **Ri CS, Jiang XR, Kim MH, et al** (2017): *Effects of dietary oregano powder supplementation on the growth performance, antioxidant status and meat quality of broiler chicks*. Ital J Anim Sci, **16**, 246-252.
38. **Shafiei A, Salehinezhad F, Gholizadeh K, et al** (2014): *Effects of using different herbs mixture on fat percentage and other carcass traits of broilers of Chaharmahal and Bakhtiyari province*. J Bio & Env Sci, **4**, 84-91.
39. **Shamlo R, Nasr J, Kheiri F** (2014): *Effects of various levels of pennyroyal (Mentha pulegium L.) on carcass characteristics and serum cholesterol in broiler*. Res Opin Anim Vet Sci, **4**, 453-457.
40. **Şahin T, Sural T, Ölmez M, et al** (2020): *Bitkisel ekstrakt karışımlarının broylerlerde performans, karkas randımanı ve bazı iç organ ağırlıkları üzerine etkisi*. Vet Hek Der Derg, **91**, 137-146.

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Mycobiota of Konya mold-ripened (Kuflu) Tulum cheese and the diversity of *Penicillium roqueforti* isolates

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Abstract: Konya Kuflu Tulum cheese is a well-known variety of Turkish mold-ripened cheeses produced by cutting the mature Tulum cheese into pieces to allow the filamentous fungi to grow on its surface in the cool and humid atmosphere in cellars or caves. The aim of the present study was to determine the fungal flora of Kuflu cheese using 54 filamentous fungi and 8 yeasts that were isolated from 26 cheese samples. Internal transcribed spacer (ITS) sequencing indicated that 53 of the mold isolates were *Penicillium roqueforti* and 1 was *Cladosporium cladosporioides*. The yeasts were identified as *Pichia membranifaciens*, *Candida zeylanoides*, *Debaryomyces hansenii*, and *Geotrichum candidum*. Morphological examination of the *P. roqueforti* isolates on various media revealed similar phenotypes among all but two isolates; however, $(GTG)_5$ fingerprinting analysis indicated that the isolated *P. roqueforti* strains were highly similar in all but one case, which displayed a different pattern. To our knowledge, this study represents the first to conduct molecular methods for identification of fungi associated with Konya Kuflu Tulum cheese. In addition, the morphological and genetic diversities of the Turkey-originated *P. roqueforti* isolates are presented.
Keywords: Genetic diversity, $(GTG)_5$ fingerprinting, Konya Kuflu cheese, morphological diversity, *Penicillium roqueforti*.

Konya küflü Tulum peynirinin mikobiyotası ve *Penicillium roqueforti* izolatlarının çeşitliliği

Özet: Konya Küflü Tulum peyniri, olgunlaştırılmış Tulum peynirinin parçalar halinde kesilerek mağara ve mahzenlerin serin ve nemli atmosferinde peynir yüzeyinin küflendirilmesi ile elde edilen, tanınmış bir Türk küflü peynir çeşididir. Bu çalışmanın amacı, 26 peynir örneğinden izole edilen 54 filamentli fungus ve 8 maya kullanılarak Küflü peynirin fungal florasını belirlemektir. Internal transcribed spacer (ITS) dizilimi, küf izolatlarının 53'ünün *Penicillium roqueforti* ve 1'inin *Cladosporium cladosporioides* olduğunu göstermiştir. Mayalar, *Pichia membranifaciens*, *Candida zeylanoides*, *Debaryomyces hansenii* ve *Geotrichum candidum* olarak tanımlanmıştır. *P. roqueforti* izolatlarının çeşitli besiyerleri üzerindeki morfolojik incelemesi, iki izolat dışında tüm izolatlarının benzer fenotipleri olduğunu ortaya çıkarmıştır; bununla birlikte, $(GTG)_5$ parmak izi analizi, izole edilmiş *P. roqueforti* suşlarının, farklı bir patern sergileyen biri hariç, oldukça benzer olduğunu göstermiştir. Bilindiği kadarıyla, bu çalışma Konya Kuflu Tulum peyniri ile ilişkili küflerin tanımlanmasında moleküler yöntemler uygulayan ilk çalışmayı temsil etmektedir. Ayrıca, Türkiye menşeli *P. roqueforti* izolatlarının morfolojik ve genetik çeşitliliği sunulmuştur.
Anahtar sözcükler: Genetik çeşitlilik, $(GTG)_5$ parmak izi, Konya Küflü peyniri, morfolojik çeşitlilik, *Penicillium roqueforti*.

Introduction

Kuflu cheese is a popular variety of traditional Turkish mold-ripened cheese produced in Konya and its surrounding areas (23). The cheese is made largely from skimmed or partially skimmed, raw sheep or goat milk (16, 27). After the milk has been allowed to coagulate using rennet, the liquid is drained using a fabric bag to collect the curd. The curd is then cut into pieces, salted, and packed tightly into Tulum bags made of goat skin to ripen for ~3 months in caves or cellars at 6–12°C and 80–90% relative humidity, which is the traditional method of production in artisanal facilities (16, 27). In others, plastic bags are used and the cheese is ripened in cold storage rooms at 4°C (14, 27). After ripening, the cheese is cut into 5- to 6-cm blocks and left to ripen again in caves, cellars, or rooms until blue-green mold growth can be seen (16, 29). The fungal growth on the cheese is spontaneous and is composed natural flora (14, 16).

Blue cheeses of different varieties, such as Roquefort, Gorgonzola, Stilton, Cabrales, and Danablue,

are produced worldwide (5, 13). *Penicillium roqueforti* is the principal filamentous fungal species associated with blue cheeses and is an important component for the formation of the cheese's characteristic color, flavor, and texture (20). This species is used either as a mold starter or predominates spontaneously on the cheese while it ripens in caves or cellars (5). Substantial morphological, genotypic and functional diversity has been detected among *P. roqueforti* isolates (7, 12). Strain-level differences are not only important in the formation of the typical characteristic product but also to develop starter cultures for industrial cheese production.

The filamentous fungi associated with Kuflu cheese was identified in a limited number of studies using only morphological techniques (6, 16, 23). In addition, the isolated fungi have not been investigated in terms of genotypic diversity. In the present study, the fungi associated with Kuflu cheese were identified using molecular evidence. The morphological diversity of the *P. roqueforti* isolates were analyzed using different media, and their genetic diversity was assessed using $(GTG)_5$ repetitive element polymerase chain reaction (rep-PCR).

Materials and Methods

***Cheese samples*:** Twenty-six mold-ripened cheese samples were obtained from different stores and bazaars in Konya in September–October 2018. The cheeses were kept refrigerated until analyses, which were conducted within 1 week at most. This period did not cause fungal viability loss in any cheese sample.

Sample preparation: A 10-g cheese sample was homogenized in 90 mL 2% sodium citrate buffer (pH 7.5; Sigma-Aldrich, St. Louis, MO, USA, S4641) using a Stomacher (Bagmixer 400, Interscience, Saint Nom, France) (2).

Isolation of fungi: Serial dilutions from the cheese homogenate were prepared in 1/4 Ringer's solution (Merck KGaA, Darmstadt, Germany, 115525) and inoculated on potato dextrose agar (PDA, Biolife, Milano, Italy, L001929) (2, 15). After 4–5 d at 25°C, morphologically different yeasts and molds that appeared on the plate were streaked onto new PDA plates. This step was repeated twice for purification. For long-term storage, the fungi were stored in yeast extract peptone dextrose broth (YPD; 10 g/L yeast extract [Sigma-Aldrich, Y1625], 20 g/L peptone [Merck, 107212], and 20 g/L dextrose [Sigma-Aldrich, 16301]) containing 20% glycerol at -80°C.

DNA extraction: For DNA isolation, the PureLink Genomic DNA Mini Kit (Thermo Fisher Scientific, St. Louis, MO, USA, K182002) was used with some modifications. First, molds were grown in 20 mL YPD broth at 25°C and 115–120 rpm until visible growth for 1–2 d using the SI-300R Lab Companion shaker incubator (Jeio Tech, Inc, Des Plaines, IL, USA). Approximately 200 mg filtered fungal mass was mixed with 200 mg glass beads (0.1 mm in diameter; Next Advance, Troy, NY, USA, GB01) in 400 µL TEN buffer (40 mM Tris–HCl pH 8.0 [Sigma-Aldrich, T1503], 1 mM ethylenediaminetetraacetic acid [EDTA, Sigma-Aldrich, E4884], 150 mM NaCl [Isolab Laborgeräte GmbH, Eschau, Germany, 969.036]) (28). The mixture was then homogenized using TissueLyser LT (Qiagen, Hilden, Germany) with four successive operations of 2 min at 50 Hz and 2 min waiting on ice, after which 10 µL proteinase K (20 mg/mL, Thermo Fisher Scientific, EO0491) and 100 µL 10% SDS (Merck, 817034) were added to the homogenate, and the mixture was incubated at 55°C for 60 min using the MTH-100 Thermo Shaker Incubator (Hangzhou-Miu Instruments Co. Ltd., Zhejiang, China) (28). After incubating, 20 µL RNase A (10 mg/mL, Thermo Fisher Scientific, EN0531) was added and the mixture was incubated again at room temperature (22–23°C) for 20 min (28). The tubes were then centrifuged at 15000 rpm for 10 min using the Microfuge 20R (Beckman Coulter, Inc., Brea, CA, USA). The supernatant was transferred into a clean microcentrifuge tube and the same amounts of binding buffer from the kit and 96% ethanol (Merck, 159010) were added. After the mixture was transferred into the kit columns, the manufacturer's protocol was followed. The pure DNA was eluted with 70 µL elution buffer included with the kit. To isolate the DNA from the yeasts, cultures were grown in 10-mL YPD broth in glass tubes and incubated at 25°C for 1 d. After centrifugation at 13000 rpm for 10 min, the cell pellet was resuspended in 400 µL TEN buffer and the same protocol for filamentous fungi was followed.

The concentration and purity of the isolated DNA were evaluated using the BioSpec Nano spectrophotometer (Shimadzu, Kyoto, Japan).

Polymerase chain reaction and agarose gel electrophoresis: To identify the fungi, universal fungal primers ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') were used (10). The PCR reaction was set up using the following protocol: 1X buffer, 0.2 mM deoxynucleoside triphosphates (dNTP) mix, 2.0 µL 10 mM forward primer, 2.0 µL 10 mM reverse primer, ~50 ng template DNA, 2.5 U Dream Taq DNA polymerase (Thermo Fisher Scientific, EP0702), and water added to a volume of 50 µL. DNA was amplified using the T100 Thermal Cycler (Bio-Rad Laboratories, Hercules, CA, USA) under the following conditions: first denaturation of 1 min at 94°C, 34 cycles of denaturation (94°C for 30 s), annealing (52°C for 30 s), extension (72°C for 1 min), and a final extension of 10 min at 72°C.

ITS PCR reactions were purified using the GeneJet PCR purification kit (Thermo Fisher Scientific, K0701) according to manufacturer's instructions and were subjected to Sanger sequencing.

The genetic diversity of the *P. roqueforti* isolates was investigated using rep-PCR fingerprinting with $(GTG)_5$ primer (5′-GTGGTGGTGGTGGTG-3′). The PCR protocol used was the same as that for ITS PCR except that 4.0 µL single primer ($[GTG]_5$ (10 mM) was used. The PCR conditions were as follows: first denaturation of at 95°C for 7 min, followed by 30 cycles of denaturation at 90°C for 30 s, annealing at 40°C for 1 min, extension at 65°C for 8 min, and a final extension at 65°C for 16 min (17). Fingerprinting reactions were run on 0.8% agarose gel at 35 V for ~5 h and visualized using the Gel Doc EZ Imager (Bio-Rad Laboratories, Hercules, CA, USA).

Phylogenetic tree construction: The phylogenetic tree of the ITS sequences of the *P. roqueforti* isolates was constructed using the maximum likelihood method and the Kimura 2-parameter model (18). Evolutionary analysis was conducted using MEGA X with 1000 bootstraps (https://www.megasoftware.net/) (18).

Morphology analysis of P. roqueforti isolates: The morphological diversity of the *P. roqueforti* isolates was investigated using PDA, yeast extract sucrose (YES) agar (20 g/L yeast extract [Biolife, 4122202], 150 g/L sucrose [Sigma-Aldrich, S0389], 0.5 g/L $MgSO_4.7H_2O$ [Sigma-Aldrich, 63138], 1 mL/L trace element stock solution [5 g/L $CuSO_4.5H_2O$, Sigma-Aldrich, C8027], 1 g/L $ZnSO_4.7H_2O$ [Sigma-Aldrich, Z0251], and 20 g/L agar [Sigma-Aldrich, A1296]) and malt extract agar (MEA) (30 g/L malt extract [Merck, 105391], 3 g/L peptone [Merck, 107212], 15 g/L agar [Sigma-Aldrich, A1296]), and oatmeal agar (OA, Sigma-Aldrich, O3506) (8, 32). The isolates were assigned codes according to their colors on PDA using a color chart as a reference (25).

Results

Fungal diversity of Konya mold-ripened Tulum cheese: Fifty-four filamentous fungi and 8 yeasts were isolated from 26 cheese samples. ITS sequencing resulted in the identification of 53 of the filamentous fungal isolates as *P. roqueforti* and 1 as *C. cladosporioides* with a BLAST identity score of 100% (Table 1). In addition, four of the yeasts were identified as *P. membranifaciens*, two as *C. zeylanoides*, one as *D. hansenii*, and one as *G. candidum* (Table 1).

A phylogenetic tree was constructed with the ITS sequences of the reference type strains of *Penicillium* species closely related to *P. roqueforti*, namely, *P. paneum*, *P. carneum*, and *P. psychrosexualis*. All *P. roqueforti* isolates were clustered together with the *P. roqueforti*–type strain CBS 221.30^T (Figure 1).

Table 1. Identification of fungi from Konya mold-ripened Tulum cheese.

Number of isolates	Identified species	GenBank number of the reference internal transcribed spacer sequence
53	*Penicillium roqueforti*	KM115117
1	*Cladosporium cladosporioides*	AY213640
4	*Pichia membranifaciens*	NR_111195
1	*Debaryomyces hansenii*	NR_120016
2	*Candida zeylanoides*	NR_131278
1	*Geotrichum candidum*	MH443758

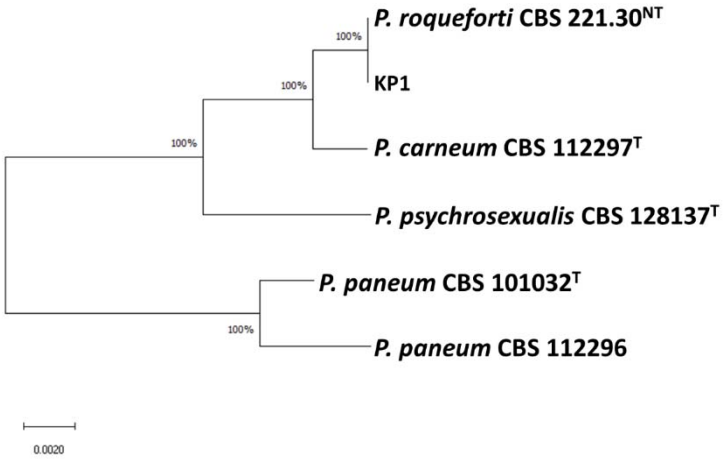

Figure 1. Phylogenetic analysis of the *Penicillium roqueforti* isolates. All isolates contained the same sequence; therefore, only the KP1 sequence is shown for simplification. The tree was constructed using the following reference sequences of closely related species: *P. roqueforti* CBS 22130^{NT} (NR_103621), *P. carneum* CBS 112297^T (NR_111551), *P. psychrosexualis* CBS 128137^T (NR_111552), *P. paneum* CBS 101032^T (NR_103620), and *P. paneum* CBS 112296 (HQ442339). In the final dataset, there were 465 positions, and the analysis involved 6 nucleotide sequences. The tree with the highest log likelihood (-765.89) is shown. Percentages show the ratio of trees in which the associated taxa were clustered together. Branch lengths were measured using the number of substitutions per site.

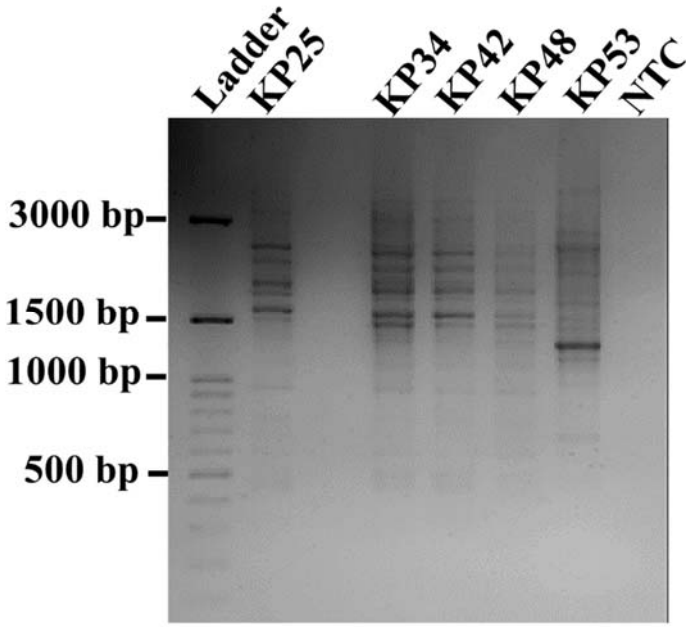

Figure 2. $(GTG)_5$ repetitive element polymerase chain reaction (rep-PCR) profiles of selected *Penicillium roqueforti* isolates. KP53 had a pattern different from that of the rest of the *P. roqueforti* isolates, as representatives KP25, KP34, KP42, and KP48 are given.
Notes: NTC = no template control in which water is used instead of DNA.

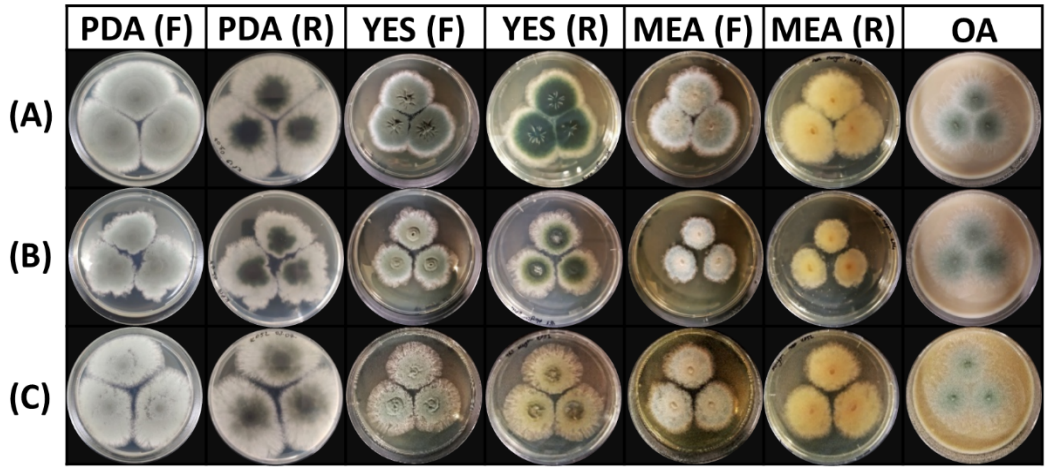

Figure 3. Morphologies of the following three representative *Penicillium roqueforti* isolates: KP19 (A), KP14 (B), and KP52 (C) on PDA, YES agar, MEA, and OA. Forward (F) and reverse (R) sides of the petri dishes are shown for all media except OA, which is very thick and not suitable for back-side appearance.

Morphological diversity of P. roqueforti isolates: The morphological features of the 53 *P. roqueforti* isolates were examined using PDA, YES agar, MEA, and OA. The isolates were mostly similar showing absinthe green on PDA according to the color chart (25) (Figure 2). Two isolates were different from the others—KP52 showed a lighter color on all media (viridine green on PDA, (25)), and KP14 had an irregular growing pattern with no difference in color from the others. The results were consistent for two different inoculations conducted at different times (data not shown).

***$(GTG)_5$ rep-PCR analysis of genetic diversity of P. roqueforti isolates*:** $(GTG)_5$ rep-PCR conducted on 53 *P. roqueforti* isolates resulted in highly similar electrophoretic patterns in all isolates except KP53. Figure 3 shows the electrophoretic pattern in isolate KP53 that is different from that of the rest of isolates, for which four representative patterns are shown. The different pattern of KP53 was reproducible and confirmed by different PCRs (data not shown).

Discussion and Conclusion

Few studies have investigated the mycobiota of Konya Kuflu cheese. Demirer (6) has isolated filamentous fungi from 10 Konya Kuflu cheese samples and identified all as *P. roqueforti* using morphological techniques. Using similar techniques, Özkalp and Durak (23) have found *Penicillium* (87.16%) to be the dominant mold microflora in 140 Konya Kuflu cheese samples and that the dominant species was *P. roqueforti* (42.91%). Hayaloglu and Kirbag (16) have isolated 158 molds from 30 Kuflu cheese samples and identified their morphological characteristics

and pigments; however, unlike the results of previous studies, most of the isolated filamentous fungi were observed to be *P. commune* (10.1%), followed by *P. verrrucosum* (9.5%) and *P. roqueforti* (8.9%). In the present study, we used ITS sequencing to identify the fungi species, and 53 out of 54 isolates were determined to be *P. roqueforti* (98.1%). Although this finding is more consistent with that of Demirer (6), two later studies found lower percentages of *P. roqueforti* (16, 23). Because starter cultures are not used in the production of Kuflu cheese, filamentous fungal profiles could be changing over time, especially according to the mycobiota of the cellars/caves in which the cheeses are ripened. The only isolate we found other than *P. roqueforti* was *C. cladosporioides*, which is a contaminant that can be found in cheeses (16).

In some cheese samples, we observed heavy growths of yeasts on PDA together with the filamentous fungi. These yeasts were isolated and identified as *P. membranifaciens*, *C. zeylanoides*, and *D. hansenii* in addition to the filamentous yeast *G. candidum*. Although yeasts of Konya Kuflu Tulum cheese had not been identified, there are reports of yeasts of other Tulum cheeses. In these studies, while *D. hansenii* was observed to be the most common yeast (21, 22), *C. zeylanoides* and *G. candidum* have also been reported (22, 33). Yeasts are an important component of the microbiota in blue cheeses and contribute to the production of their characteristic aroma, making them candidates of adjunct cultures (1, 30).

In this study, *P. roqueforti* isolates were further investigated using both morphological methods and $(GTG)_5$ rep-PCR fingerprinting analyses. The morphologies of the isolates on PDA, YES agar, MEA, and OA were mostly similar in terms of the color and texture among them, with the exception of two isolates—one with a lighter color (KP52) and the other (KP14) with an irregular growth pattern. Gillot et al. (11) have reported a high morphological diversity of *P. roqueforti*. In that study, the researchers collected 120 blue-veined cheeses from 18 different countries in addition to some non-cheese substrates to create a *P. roqueforti* collection. High morphological diversity was observed (nine morphotypes) and the most distinctive colors were observed on PDA, with color differences between light to dark greenish-gray and a texture from velvety to fascicular. In the present study, isolates were not as diverse as those in the Gillot et al. (11) study, most likely because only one cheese type was used.

The genetic diversity of P. *roqueforti* isolates has been investigated in a number of studies. For example, studies using random amplified polymorphic DNA PCR–based fingerprinting using several primers revealed very similar banding patterns among different *P. roqueforti* isolates, which indicated high genetic similarity; however, different strains can be differentiated (4, 9). Studies conducted using developed microsatellite markers allowed the different *P. roqueforti* populations throughout the world to produce and identify different types of blue cheeses (11, 26). The most recent study (7) analyzed the genomes of *P. roqueforti* isolates from different origins and found four genetically differentiated populations—Roquefort cheese isolates, non-Roquefort blue cheese isolates, silage and food spoiler group, and wood-associated isolates with other food spoilers.

In the present study, we used repetitive sequence-based PCR to analyze genetic diversity, which is also very useful for bacteria as well as fungal fingerprinting (24, 31). This technique has never been used on *P. roqueforti*. Similar to the results of previous RAPD-based studies, we observed very similar electrophoresis band patterns among the *P. roqueforti* isolates with the exception of one (KP53), which clearly showed a reproducible different pattern. The similarity of 52 out of the 53 isolates might indicate the presence of a specific strain that inhabits the cellars/rooms/caves used for Kuflu cheese ripening and that passes through the cheeses to create a nearly uniform population; however, there might be different genotypes that we were not able to identify using $(GTG)_5$. The morphologically different isolates (KP52 and KP14) had the commonly observed $(GTG)_5$ pattern among the isolates; therefore, they could not be genotypically differentiated. Additional studies using different primers or more sophisticated techniques, such as whole-genome comparisons, might provide further information on genetic diversity.

In conclusion, in the present study, 53 of 54 filamentous fungal isolates of Konya Kuflu Tulum cheese were identified as *P. roqueforti*. In addition, 8 yeast isolates were identified as *P. membranifaciens*, *C. zeylanoides*, *D. hansenii*, and *G. candidum*. With the exception of two isolates, all *P. roqueforti* isolates had similar morphologies. In addition, $(GTG)_5$ fingerprinting of *P. roqueforti* isolates revealed a nearly identical pattern for all except one isolate. In future studies, a comparison among Konya Kuflu cheese isolates and other isolates of Turkish mold-ripened cheeses, such as Erzurum Kuflu Civil, Kars Kuflu Civil, and Divle cave cheese, and among blue cheese starter strains used throughout the world would help to understand the factors that make these different types of blue cheeses unique.

Finacial Support

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Ethical Statement

This study does not present any ethical concerns.

Conflict of Interest

The authors declared that there is no conflict of interest.

References

1. **Álvarez-Martín P, Flórez AB, López-Díaz TM, et al** (2007): *Phenotypic and molecular identification of yeast species associated with Spanish blue-veined Cabrales cheese*. Int Dairy J, **17**, 961-967.
2. **Albenzio M, Corbo M, Rehman S, et al** (2001): *Microbiological and biochemical characteristics of Canestrato Pugliese cheese made from raw milk, pasteurized milk or by heating the curd in hot whey*. Int J Food Microbiol, **67**, 35-48.
3. **Belén Flórez A, Álvarez-Martín P, López-Díaz TM, et al** (2007): *Morphotypic and molecular identification of filamentous fungi from Spanish blue-veined Cabrales cheese, and typing of Penicillium roqueforti and Geotrichum candidum isolates*. Int Dairy J, **17**, 350-357.
4. **Boysen M, Skouboe P, Frisvad J, et al** (1996): *Reclassification of the Penicillium roqueforti group into three species on the basis of molecular genetic and biochemical profiles.* Microbiology, **142**, 541-549.
5. **Cantor MD, van den Tempel T, Hansen TK, et al** (2004): Blue cheese. 175-198. In: Fox PF, McSweeney PLH, Cogan TM, Guinee TP (Eds), Cheese: Chemistry, Physics and Microbiology, Academic Press, London.
6. **Demirer MA** (1974): *A study of molds isolated from certain cheeses and their ability to produce aflatoxins*. Ankara Univ Vet Fak Derg, **21**, 180-198.
7. **Dumas E, Feurtey A, Rodríguez de la Vega RC, et al** (2020): *Independent domestication events in the blue-cheese fungus Penicillium roqueforti.* Mol Ecol, **29**, 2639-2660.
8. **Frisvad JC, Samson RA** (2004): *Polyphasic taxonomy of Penicillium subgenus Penicillium. A guide to identification of food and air-borne terverticillate Penicillia and their mycotoxins*. Stud Mycol, **49**, C174.
9. **Geisen R, Cantor M, Hansen T, et al** (2001): *Characterization of Penicillium roqueforti strains used as cheese starter cultures by RAPD typing*. Int J Food Microbiol, **65**, 183-191.
10. **Glass NL, Donaldson GC** (1995): *Development of primer sets designed for use with the PCR to amplify conserved genes from filamentous ascomycetes.* Appl Environ Microbiol, **61**, 1323-1330.
11. **Gillot G, Jany J-L, Coton M, et al** (2015): *Insights into Penicillium roqueforti morphological and genetic diversity.* PloS One, **10**, e0129849.
12. **Gillot G, Jany J-L, Poirier E, et al** (2017): *Functional diversity within the Penicillium roqueforti species.* Int J Food Microbiol, **241**, 141-150.
13. **Gripon JC** (1993): Mould-Ripened Cheeses. 111-136. In: PF Fox (Ed), Cheese: Chemistry, Physics and Microbiology, Springer, Boston.
14. **Guley Z, Uysal H, Kilic S** (2015): *Lactic acid bacteria flora of Konya kuflu cheese: a traditional cheese from Konya province in Turkey*. J Microbiol Biotechnol Food Sci, **04**, 238-242.
15. **Harrigan W** (1998): Laboratory Methods in Food Microbiology. Academic Press, San Diego.
16. **Hayaloglu AA, Kirbag S** (2007): *Microbial quality and presence of moulds in Kuflu cheese.* Int J Food Microbiol, **115**, 376-380.
17. **Healy M, Huong J, Bittner T, et al** (2005): *Microbial DNA typing by automated repetitive-sequence-based PCR.* J Clin Microbiol, **43**, 199-207.
18. **Kimura M** (1980): *A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences.* J Mol Evol, **16**, 111-120.
19. **Kumar S, Stecher G, Li M, et al** (2018): *MEGA X: Molecular evolutionary genetics analysis across computing platforms*. Mol Biol Evol, **35**, 1547-1549.
20. **Metin B** (2018): Filamentous Fungi in Cheese Production. 257-275. In: Ş Öztürkoğlu Budak, HC Akal (Eds). Microbial Cultures and Enzymes in Dairy Technology. IGI Global, Hershey.
21. **Ozmen Togay S, Capece A, Sıesto G, et al** (2020): *Molecular characterization of yeasts isolated from traditional Turkish cheeses.* Food Sci Technol, **40**, 871-876.
22. **Ozturkoglu Budak S, Figge MJ, Houbraken J, et al** (2016): *The diversity and evolution of microbiota in traditional Turkish Divle Cave cheese during ripening*. Int Dairy J, **58**, 50-53.
23. **Özkalp B, Durak Y** (1998): *Investigation of mold flora in moldy cheeses of Konya and its vicinity.* Turk J Biol, **22**, 341-346.
24. **Redondo C, Cubero J, Melgarejo P** (2009): *Characterization of Penicillium Species by Ribosomal DNA Sequencing and BOX, ERIC and REP-PCR Analysis.* Mycopathologia, **168**, 11.
25. **Ridgway R** (1912): Color standards and color nomenclature. The author, Washington, D.C.
26. **Ropars J, López-Villavicencio M, Dupont J, et al** (2014): *Induction of sexual reproduction and genetic diversity in the cheese fungus Penicillium roqueforti.* Evol Appl, **7**, 433-441.
27. **Tekinşen KK, Cebirbay MA, Elmalı M** (2009): *Moldy cheese of Konya*. Akademik Gıda, **7**, 31-35.
28. **Turin, Riva, Galbiati, et al** (2000): *Fast, simple and highly sensitive double-rounded polymerase chain reaction assay to detect medically relevant fungi in dermatological specimens.* Eur J Clin Invest, **30**, 511-518.
29. **Ünsal A** (1997): Süt Uyuyunca - Türkiye Peynirleri. Yapı Kredi Yayınları, İstanbul.
30. **Van den Tempel T, Jakobsen M** (2000): *The technological characteristics of Debaryomyces hansenii and Yarrowia lipolytica and their potential as starter cultures for production of Danablu*. Int Dairy J, **10**, 263-270.
31. **Versalovic J, Schneider M, Bruijn FJ, et al** (1994): *Genomic fingerprinting of bacteria using repetitive sequence-based polymerase chain reaction.* Methods Mol Cell Biol, **5**, 25-40.
32. **Visagie CM, Houbraken J, Frisvad JC, et al** (2014): *Identification and nomenclature of the genus Penicillium.* Stud Mycol, **78**, 343-371.
33. **Yalcin S, Senses S, Ozbas Y** (2012): *Identification and enzymatic characterization of the yeasts isolated from Erzincan tulum cheese*. Mljekarstvo, **62**, 53-61.

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Three-dimensional bone modeling of forelimb joints in New Zealand Rabbit: A Micro-Computed Tomography study

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Abstract: In this study, it was aimed to obtain 3-dimensional (3D) digital and printed models of healthy forelimb joints using micro-computed tomography (µCT) technique in New Zealand Rabbit, which is frequently preferred in experimental orthopedic studies. Moreover, it was aimed to provide morphometric measurements on the shoulder and elbow joints over 3D digital models. A total of 14 adults (7 female, 7 male) New Zealand Rabbits were used in the study. After imaging the forelimbs with the µCT device, 3D digital and printed models were obtained. Biometric measurements of shoulder and elbow joints were performed over 3D digital models and the data obtained from female and male rabbits were evaluated statistically. The anatomical structure on the 3D joint models was very detailed due to the low section thickness and high detector quality. 3D printed models produced as a result of the 3D printing process were quite durable, odorless, and clean. No anatomical differences were observed between 3D printed models and 3D digital models. In this study, it is thought that the anatomical and morphometric data obtained from laboratory rabbits will contribute to scientists take part both in experimental orthopedic intervention and clinical anatomy education.
Keywords: Forelimb joints, laboratory rabbit, micro-computed tomography, morphometry, three-dimensional reconstruction.

Yeni Zelanda Tavşanı'ında ön bacak eklemlerinin üç boyutlu kemik modellenmesi: Mikro Bilgisayarlı Tomografi çalışması

Özet: Bu çalışmada, deneysel ortopedik çalışmalarda sıklıkla tercih edilen Yeni Zelanda Tavşanı'nda sağlıklı ön bacak eklemlerinin mikro bilgisayarlı tomografi (µBT) tekniği ile elde edilen görüntülerinden 3B dijital modellerinin oluşturulması, modeller üzerinde omuz ile dirsek eklemlerine ait morfometrik ölçümlerin sağlanması ve bu modellerden 3B yazıcılar kullanılarak 3B baskı modellerinin üretilmesi amaçlanmıştır. Çalışmada toplamda 14 adet (7 dişi, 7 erkek) erişkin Yeni Zelanda Tavşanı kullanıldı. Ön bacakları µBT cihazı ile görüntülenip görüntülerden 3B dijital ve baskı modelleri elde edildi. 3B dijital modeller üzerinden omuz ile dirsek eklemlerine ait biyometrik ölçümleri gerçekleştirildi. Dişi ve erkek tavşanlara ait elde edilen veriler istatistiki açıdan değerlendirildi. Kesit kalınlığının düşük ve dedektör kalitesinin yüksek olması sebebiyle 3B eklem modellerindeki anatomik yapı oldukça detaylıydı. Üç boyutlu baskılama işlemi sonucunda üretilen 3B baskı modelleri son derece dayanıklı, kokusuz ve temizdi. 3B baskı modelleri ile 3B dijital modeller arasında herhangi bir anatomik farklılık gözlenmedi. Bu çalışmada laboratuvar tavşanlarına ait elde edilen anatomik ve morfometrik verilerin hem deneysel amaçlı ortopedik girişimlerde bulunan hem de klinik anatomi eğitimlerinde rol alan bilim insanlarına katkı sağlayacağı düşünülmektedir.
Anahtar sözcükler: Laboratuvar tavşanı, mikro bilgisayarlı tomografi, morfometri, ön bacak eklemleri, üç boyutlu rekonstrüksiyon.

Introduction

The New Zealand Rabbit (*Oryctolagus cuniculus L.*) is a species frequently preferred by scientists because it is used in many experimental studies. In terms of its anatomical features, it stands out as an animal model to be used in experimental orthopedic studies for joint areas in human medicine (17, 21). Digital x-ray (DR), computed tomography (CT), magnetic resonance imaging (MRI), and micro-computed tomography (µCT) methods, which are diagnostic imaging techniques, are frequently used in the diagnosis and treatment of joint diseases. In addition, these methods used for diagnosis and treatment of diseases have an effective role in imaging healthy macro-anatomical structures. Today, these methods, which are

used very much in terms of both treatment and clinical anatomy education in human medicine, are also very useful in different areas of veterinary medicine (14).

In the late 1980s, Feldkamp and his colleagues were introduced μCT which is a unique imaging technique for the evaluation of bone morphology and microstructures in mice and other small animal models (3, 10). Today, μCT devices provide images with superior resolution. Since optical images provide real magnification in μCT, even the smallest details of bone tissue can be clearly identified (12).

A series of 2-dimensional (2D) cross-sectional images are produced showing the anatomical relationship of the important structures of the body with medical imaging methods. 2D images are digitally stored in the international standard DICOM (Digital Imaging and Communications in Medicine) file format. This file format contains the necessary data about 3D reconstructed and sectional images. 3D models of the anatomical region are produced using software programs specially developed for DICOM files. With various software, the anatomical structure of the examined area can be clearly displayed, measured, highlighted, or partitioned separately at the same time (7, 23). In addition, 3D reconstructed images are used as a guide about the anatomy of the relevant region for surgical planning (5, 13, 20). Traditionally, measurements on post-mortem anatomical samples have been the only method available to access linear, angular, and volumetric biometric data. Standard radiographic images are often used for linear biometric measurements. However, measurements on 2D radiographic images provide very limited information to researchers. Recently, accuracy and reliability of the measurements taken from 3D reconstructed models have become a curiosity for researchers. In the previous studies, 3D linear and angular anatomical measurements have been compared with measurements using digital calipers and goniometers (9). When the obtained data have been evaluated, it has been stated that the accuracy and reliability of 3D anatomical measurements are quite high (2, 8, 13, 19, 28). It is known that 3D anatomical measurements can be used safely on skeletal structures, especially in the articular regions (9). 3D printing models of desired anatomical structure can be produced by using 3D digital models. 3D printing technology is very beneficial in areas such as tissue and organ construction, creation of customized prostheses, implants, and anatomical models (29). In addition, 3D models are very valuable educational materials for students, especially in radiology and anatomy education. 3D printing allows simpler visualization of complex anatomical structures (27). It has been stated that the cost of 3D printed anatomical specimens is quite convenient compared to the cost of cadaver traditionally used in educations. Using 3D samples instead of cadavers, which are difficult to obtain, provides a serious convenience in anatomy education (1, 6, 18, 28).

In this study, it was aimed to produce 3D digital and printing models from μCT images of healthy forelimb joints in laboratory rabbits and to evaluate the joints anatomically. Furthermore, we aimed to perform 3D morphometric measurements of the shoulder and elbow joints in female and male rabbits and to evaluate the obtained data statistically.

Materials and Methods

A total of 14 adults (7 females and 7 males) New Zealand Rabbits (*Oryctolagus cuniculus L.*) were used in the study. All procedures on animals were carried out in line with the approval of the ethics committee from the Ankara University Animal Experiments Local Ethics Committee (Approval number: 2017-5-32). The forelimbs of euthanized rabbits were removed from the body by appropriate dissection method. The imaging of the forelimbs was performed with the μCT device (Super Argus PET / CT, Sedecal, Spain). The forelimbs were placed and fixed on the bed in the medio-lateral position in accordance with their anatomical postures. The scanning process was performed from the middle level of the scapula to the distal ending of the distal phalanx with a thickness of 0.12 mm. In the imaging process, 40 kV and 140 μA values were used at standard resolution. The obtained DICOM files were transferred to the 3D Slicer software (3D Slicer, 4.9.0 version, GitHub, San Francisco) and the images in transversal, frontal and sagittal planes were evaluated on the personal computer. 3D digital images of bone tissue were obtained by performing volume rendering and segmentation on the bone tissue window. Anatomical measurements of the shoulder and elbow joints were carried out over 3D reconstructed models in accordance with the relevant literatures (24, 25, 31). The measurement process for each point was performed 3 times at different times and the final value was determined as the average of the whole measurement value. 3D printed models of forelimb joints were produced using a 3D printer (Anycubic i3 Mega FDM-printer). In the 3D printing process, 3D digital joint models of bone tissue were uploaded to the computer in the 'STL' file format. Then, the printing process was started by uploading files in the “STL” format to the 3D printer. This process was basically carried out using the Polylactic Acid (PLA) polymer in x, y, z coordinates. The use of anatomical terminology is based on Nomina Anatomica Veterinaria (22).

Descriptive statistics were calculated and presented as “Mean ± Standard Error of Mean (SEM)”. Before performing the statistical analysis, data was examined with Shapiro Wilk test for normality as parametric test assumptions. Data was analyzed using the GLM for

Repeated Measures procedure of SPSS 14.01 (SPSS Inc., Chicago, IL, USA, License No: 9869264). The model included the gender as between subject factor and the side (left and right) as within subject factor, and the 2-way interaction term. Post hoc testing was carried out for the significant interaction term using simple effect analysis. A probability value of less than 0.05 was considered significant, unless otherwise noted.

Results

The anatomical structures on the 3D digital models obtained from the µCT images were quite detailed. On the 3D models of the shoulder joint, the joint cavity was quite evident, and the joint gap between cavitas glenoidalis and caput humeri was clearly observed. Tuberculum supraglenoidale and tuberculum infraglenoidale were prominent above the cranial and caudal edges of the cavitas glenoidalis, respectively. Caput humeri observed convexly on the proximal of the humerus was limited by the tuberculum minus and tuberculum majus in the craniomedial and craniolateral, respectively (Figure 1). On the 3D models of the elbow joint, the trochlea humeri was articulated with fovea capitis radii and olecranon in distal and caudal, respectively. Epicondylus medialis and epicondylus lateralis were in the form of a protrusion on the medial and lateral edges of the trochlea humeri. It was determined that incisura trochlearis supports trochlea humeri from the caudal edge and the two anatomical formations were quite compatible with each other (Figure 2). All bone structures of all joints were determined in detail on the 3D model of regio manus. Even small bone structures such as carpal bones were easily identified on the

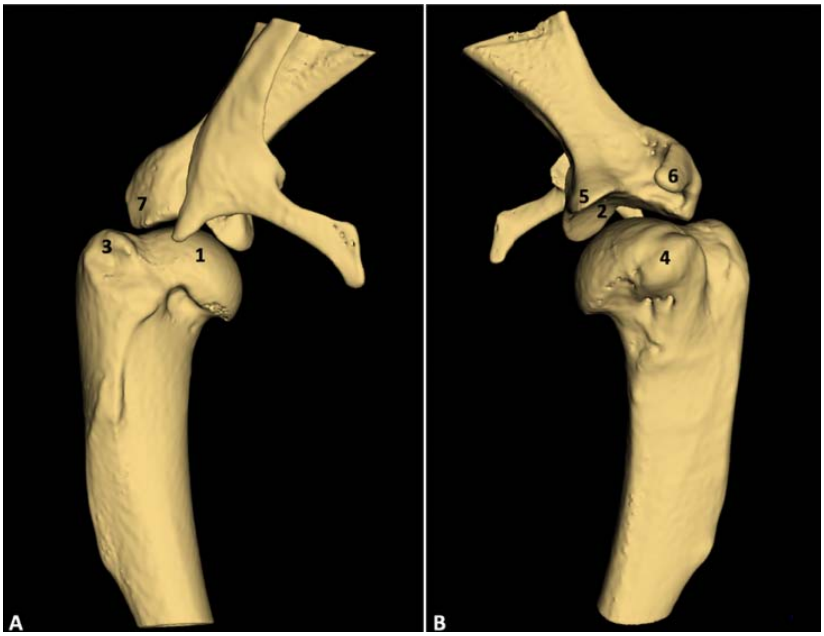

Figure 1. Lateral (A) and medial (B) view of the 3D digital model of the left shoulder joint. 1: Caput humeri; 2: Cavitas glenoidalis; 3: Tuberculum majus; 4: Tuberculum minus; 5: Tuberculum infraglenoidale; 6: Processus coracoideus; 7: Tuberculum supraglenoidale.

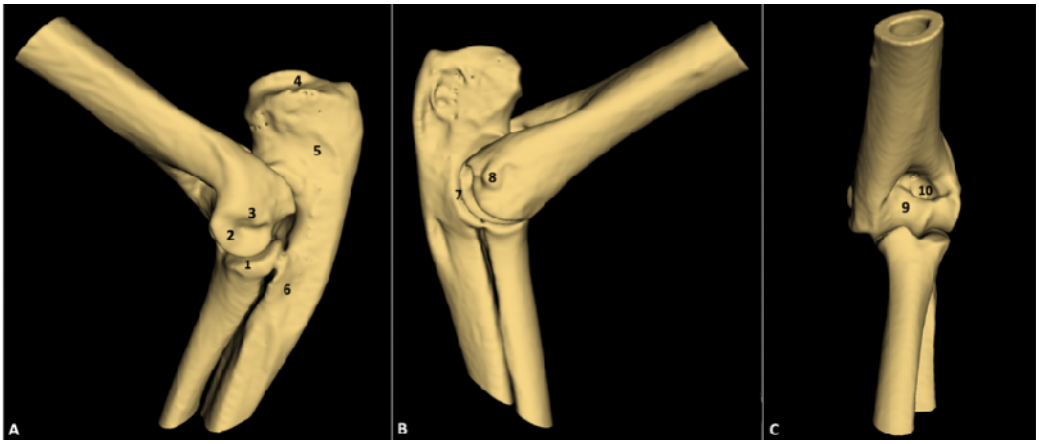

Figure 2. Lateral (A), medial (B) and cranial (C) view of the 3D digital model of the left elbow joint. 1: Fovea capitis radii; 2: Capitulum humeri; 3: Epicondylus lateralis; 4: Tuber olecrani; 5: Olecranon; 6: Corpus ulnae; 7: Incisura trochlearis; 8: Epicondylus medialis; 9: Trochlea humeri; 10: Fossa coronoidea.

3D models (Figure 3). Articulatio radioulnaris distalis was formed by the medial surface of the processus styloideus lateralis and incisura ulnaris. While the distal of the radius, os carpi radiale and os carpi intermedium were added to the form of the articulatio radiocarpeae, the distal of the ulna, os carpi ulnare, and os carpi accessorium were added to the form of the articulatio ulnocarpeae. In the distal, os carpi radiale, os carpi intermedium, and os carpi ulnare were jointed with os carpale I and os carpale II, os carpale III and os carpale IV, os carpale IV, respectively. When the joints of the carpal bones located in the distal row with the bones in the distal were examined, the bones involved in the structures of the joints were: os carpale I with os metacarpale I and os metacarpale II; os carpale II with os metacarpale II; os carpale III with os carpi centrale and os metacarpale II; os carpi centrale with os metacarpale III; os carpale IV with os metacarpale III, os metacarpale IV and os metacarpale V. On the palmar face of the articulatio metacarpophalangea, there was a pair of proximal sesamoid bones, one axial and the other abaxial. Likewise, there was a distal sesamoid bone on the palmar face of the articulatio interphalangea distalis (Figure 3).

Morphometric bone measurements of shoulder and elbow joints were provided over 3D models. Measurement points were indicated in Figure 4. The values of the measurement results were given in Table 1. There was no statistically difference between the right and left values of the female and male animals in the following measurements; the proximal width of the humerus (HPW), the lateral width of the proximal radius (PRLW), the medial width of the proximal radius (PRMW), the distal width of the humerus (HDW), the width of the processus coronoideus (PCW), the smallest depth of the olecranon (OSD), the depth of the processus anconeus (PAD). At the same time, the difference between the genders for the right and left values was statistically insignificant (P>0.05). For the width of cavitas glenoidalis (CGW), the difference between the right and left values of female and male rabbits was statistically insignificant (P>0.05). However, it was determined that the right and left values were higher in male rabbits than in females, and this difference was statistically significant (P<0.05). For the depth of tuber olecrani (TOD), the left measurement values of females and males were higher than the right measurement values. The difference between values was found statistically important (P<0.05). When the right and left measurement values were compared between the genders, it was determined that the values were quite close to each other. In the measurements of the width of epicondylus lateralis (ELW), the difference between the right and left values of the female and male was statistically insignificant (P>0.05). In addition, the fact that the left measurement value of males was higher than the females was statistically significant (P<0.05). For the width of the proximal radius (RPW), the difference between the right and left values of females and males was statistically unimportant (P>0.05). However, it was determined that right and left values were higher in male rabbits than in females, and this difference was statistically significant (P<0.05). For the width of epicondylus medialis (EMW), the difference between the right and left values in females and males was found statistically important (P<0.05). The right values for both genders were higher than the left values. However, when the right and left measurement values were compared between genders, the difference between the values was found statistically insignificant (P>0.05).

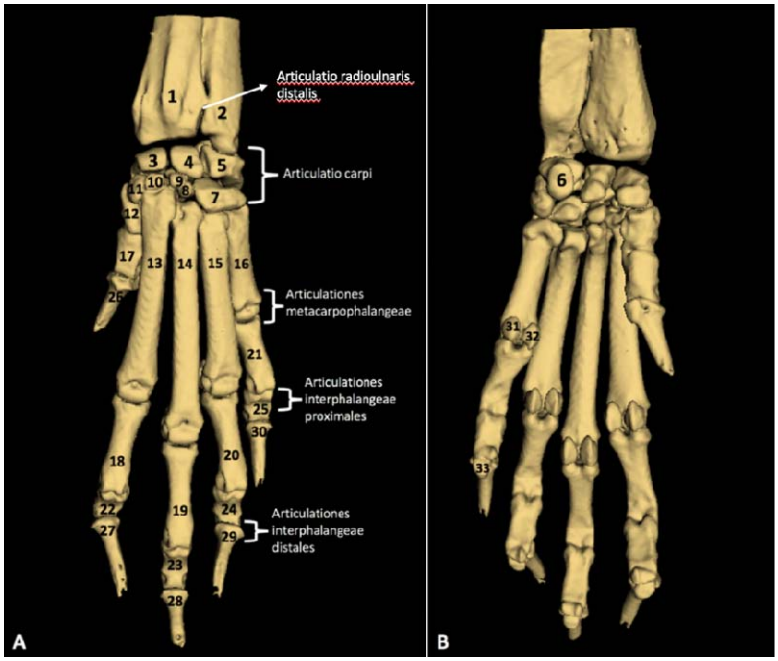

Figure 3. Dorsal (A) and palmar (B) view of the 3D digital model of the left regio manus. 1: Radius; 2: Ulna; 3: Os carpi radiale; 4: Os carpi intermedium; 5: Os carpi ulnare; 6: Os carpi accessorium; 7: Os carpale IV; 8: Os carpi centrale; 9: Os carpale III; 10: Os carpale II; 11: Os carpale I; 12: Os metacarpale I; 13: Os metacarpale II; 14: Os metacarpale III; 15: Os metacarpale IV; 16: Os metacarpale V; 17: Phalanx proximalis digiti I; 18: Phalanx proximalis digiti II; 19: Phalanx proximalis digiti III; 20: Phalanx proximalis digiti IV; 21: Phalanx proximalis digiti V; 22: Phalanx media digiti II; 23: Phalanx media digiti III; 24: Phalanx media digiti IV; 25: Phalanx media digiti V; 26: Phalanx distalis digiti I; 27: Phalanx distalis digiti II; 28: Phalanx distalis digiti III; 29: Phalanx distalis digiti IV; 30: Phalanx distalis digiti V; 31-32: Os sesamoideum proximale; 33: Os sesamoideum distale.

Table 1. Statistical data of 3D morphometric measurement values.

Measurement	Gender	Side		P		
		Right (mm)	Left (mm)	Gender (G)	Side (S)	G*S
HPW	F	13.90 ± 0.130	13.85 ± 0.081	0.591	0.503	0.902
	M	13.97 ± 0.130	13.93 ± 0.081			
CGW	F	9.13 ± 0.161^{B}	8.87 ± 0.186^{B}	0.045	0.598	0.280
	M	9.39 ± 0.161^{A}	9.48 ± 0.186^{A}			
TOD	F	8.33 ± 0.034^{a}	8.54 ± 0.082^{b}	0.966	0.036	0.304
	M	8.39 ± 0.034^{a}	8.47 ± 0.082^{b}			
ELW	F	7.29 ± 0.116^{B}	7.09 ± 0.104^{B}	0.061	0.459	0.039
	M	7.43 ± 0.116^{A}	7.53 ± 0.104^{A}			
PRLW	F	4.86 ± 0.158	5.02 ± 0.162	0.779	0.957	0.250
	M	4.96 ± 0.158	4.79 ± 0.162			
HDW	F	10.65 ± 0.116	10.57 ± 0.107	0.256	0.143	0.732
	M	10.85 ± 0.116	10.71 ± 0.107			
RPW	F	7.58 ± 0.092^{B}	7.39 ± 0.079^{B}	0.005	0.161	0.445
	M	7.81 ± 0.092^{A}	7.75 ± 0.079^{A}			
EMW	F	7.27 ± 0.101^{a}	7.11 ± 0.078^{b}	0.099	0.016	0.254
	M	7.44 ± 0.101^{a}	7.38 ± 0.078^{b}			
PRMW	F	4.49 ± 0.143	4.42 ± 0.111	0.638	0.204	0.550
	M	4.62 ± 0.143	4.43 ± 0.111			
PCW	F	6.54 ± 0.064	6.50 ± 0.086	0.204	0.574	0.933
	M	6.65 ± 0.064	6.62 ± 0.086			
OSD	F	7.90 ± 0.081	7.98 ± 0.058	0.453	0.299	0.993
	M	7.95 ± 0.081	8.03 ± 0.058			
PAD	F	8.57 ± 0.048	8.61 ± 0.044	0.904	0.674	0.102
	M	8.63 ± 0.048	8.57 ± 0.044			

^{a,b}: Different letters on the same line indicate statistically significant difference (P<0.05); ^{A,B}: Different letters on the same column indicate a statistically significant difference (P<0.05). F: Female; M: Male. HPW: Proximal width of the humerus; CGW: Width of the cavitas glenoidalis; TOD: Depth of the tuber olecrani; ELW: Width of the epicondylus lateralis; PRLW: Lateral width of the proximal radius; HDW: Distal width of the humerus; RPW: Width of the proximal radius; EMW: Width of the epicondylus medialis; PRMW: Medial width of the proximal radius; PCW: Width of the processus coronoideus; OSD: The smallest depth of the olecranon; PAD: Depth of the processus anconeus.

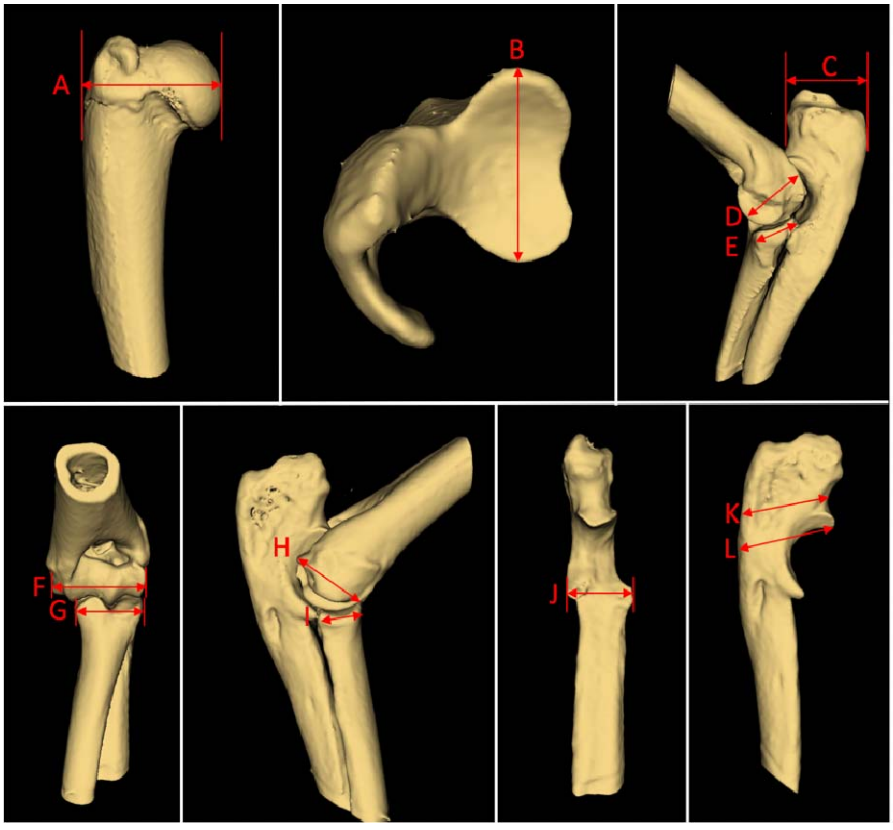

Figure 4. Morphometric measurement points taken over 3D models. A: Proximal width of the humerus (HPW); B: Width of the cavitas glenoidalis (CGW); C: Depth of the tuber olecrani (TOD); D: Width of the epicondylus lateralis (ELW); E: Lateral width of the proximal radius (PRLW); F: Distal width of the humerus (HDW); G: Width of the proximal radius (RPW); H: Width of the epicondylus medialis (EMW); I: Medial width of the proximal radius (PRMW); J: Width of the processus coronoideus (PCW); K: The smallest depth of the olecranon (OSD); L: Depth of the processus anconeus (PAD).

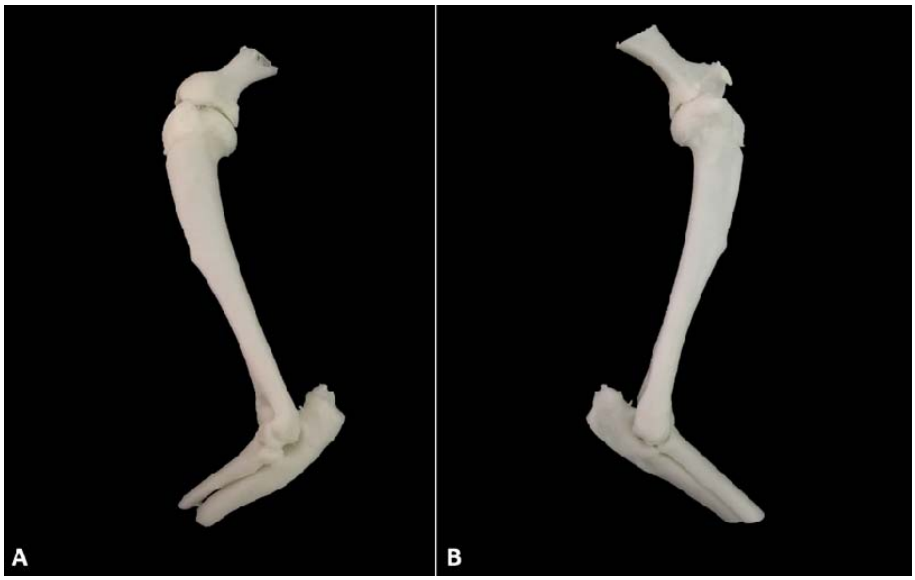

Figure 5. Lateral (A) and medial (B) view of the 3D printed model of the left shoulder and elbow joints.

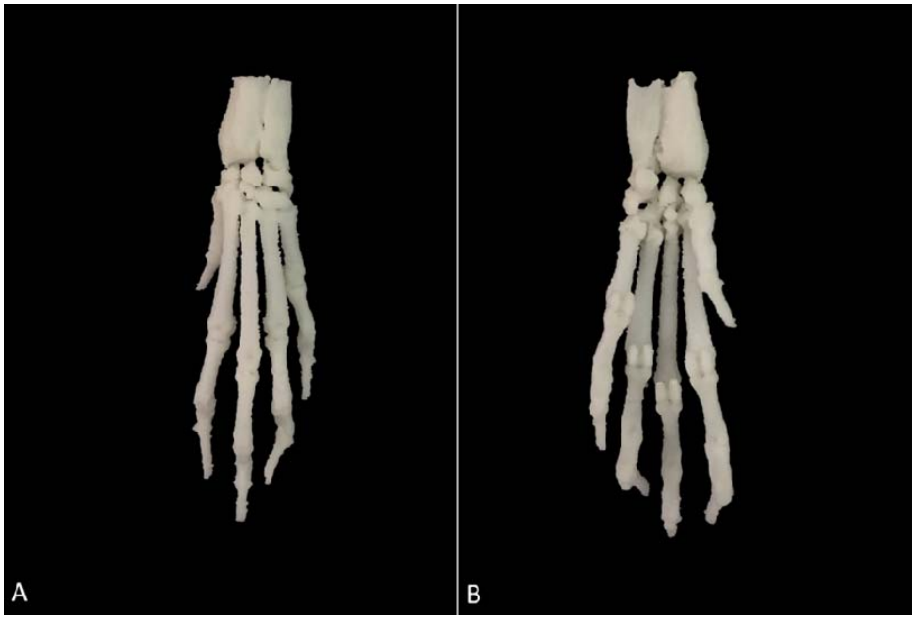

Figure 6. Dorsal (A) and palmar (B) view of the 3D printed model of the left regio manus.

As a result of the 3D printing process, durable, odorless, clean, and realistic 3D printing models were produced. No anatomical differences were observed between the 3D printing models produced and the 3D digital models in the 'STL' file format that were loaded on the 3D printer before the printing process. Since the printing materials were quite small, the supporting texture was used in the printing process. Therefore, it was necessary to clean the support tissue consisting of thermoplastic polymer around the samples produced after printing. It was determined that the structures belonging to the bone tissue on the joint areas on the printed models retain their anatomical features. It was observed that the bone structures boundaries of all joint regions, especially the small carpal bones, were easily distinguishable from each other (Figures 5 and 6).

Discussion and Conclusion

It is possible to design 3D digital anatomical models from 2D cross-sectional images using various commercial software programs. The production mechanisms of 3D models from 2D images have been explained by using different software programs (4). With the 3D Slicer software program used in this study, reformat images were created in sagittal and frontal planes using DICOM files containing transversal μCT sectional images. Segmentation of bone tissue was performed from the sectional images. As a result of the segmentation process,

3D digital models were successfully visualized. In addition, the fact that the 3D Slicer program could be accessed free of charge from the internet environment has significantly reduced the cost of this research.

3D printing technology has many benefits in anatomy education. Among the reasons of the increase in the interest of scientists researching in the medical field to this technology are the difficulties of finding cadavers and the formaldehyde used for fixation and storage of tissues is very harmful to human health (11, 16). In this study, it is thought that the production of bone models with 3D printing technology will reduce the use of cadaver, which is constantly required in the preparation of organic bone samples. It has been stated that 3D printing models can be used safely by both educators and students, and with the help of printing models, quite complex anatomical structures can be visualized simply (16, 26). In this study, 3D printed bone models were produced from μCT images of joint regions. Especially the anatomical structures and neighbourhoods of very small carpal bones in articulatio carpi were easily determined on models. Li et al. (16) have produced 3D printing models of some cattle bones in their study. They have evaluated the effectiveness of these printed models in anatomy education with the survey on students. The vast majority of the students who participated in the survey stated that the anatomical structures in 3D printing models were not different from those of organic bone samples, and the models were odorless, light, and durable. 3D printing models obtained in this study were also found to be very light, odorless, durable, and clean. When 3D printing and digital models were compared, there was no anatomical difference between them.

It is known that 3D morphometric studies of the rabbit's forelimb skeleton structures belong only to antebrachium (24). 3D digital models of antebrachium have been carried out over CT images. In addition, Pazvant and Kahvecioğlu (25) have performed the measurements over the forelimb long bones of rabbits with the traditionally used digital caliper method. However, in the literature search, no detailed morphometric study on the shoulder and elbow joints of rabbits was found. In our research, the μCT technique was used to produce more detailed 3D anatomical models and to perform detailed biometric measurements over the 3D models. The bone tissue on the images was segmented sectionally by using the "paint" and "level tracing" commands using the 3D Slicer software program. After the segmentation process, the 3D models obtained were rotated in the desired way and the measurements of shoulder and elbow joints were easily performed.

Pazvant and Kahvecioğlu (25) have investigated the homotypic variations of the forelimb and hindlimb long bones of rabbits in their study. They have provided morphometric measurements with the help of digital calipers over the real bone samples they obtained as a result of the maceration process. In the measurements of the proximal width of the humerus, the right and left mean values of females have been determined as 13.87 mm and 13.82 mm, respectively. For the measurements of male rabbits, the right and left values have been calculated as 13.67 mm and 13.66 mm, respectively. In the measurements of the distal width of the humerus, the right mean values of the females and males have been specified as 10.67 mm and 10.75 mm, and the left mean values have been determined as 10.88 mm and 11.32 mm, respectively. In our study, it was determined that the 3D morphometric measurement data obtained from the proximal and distal width of the humerus were parallel to the data in the literature, and there was no statistically difference between the genders and the right and left data (Table 1). In the literature, it has been stated that there is no statistical difference between the genders and the right and left values in the genders for the proximal width of the radius. In our study, the difference between the right and left values of females and males was statistically insignificant. However, it was determined that the right and left values were higher in male rabbits than in females, and this difference was statistically significant (Table 1). Moreover, all measurement values for this region were found to be higher than in the literature. This differentiation is thought to be due to the difference in body mass. Morphometric values of the depth of olecranon in the related literature were similar to the values obtained in our research. Unlike the literature, the left measurement values of the male and female obtained in our study were higher than the right measurement values. The difference between data was statistically significant (Table 1).

Özkadif et al. (24) have produced 3D models of multidetector-CT images of antebrachium in rabbits and provided morphometric measurements on models. They have compared the measured values statistically between female and male animals. The difference between the right and left values of females and males has been found statistically insignificant in the measurements of the proximal width of the radius. However, the right and left measurement values of male rabbits have been higher than that of females, and this difference has been found statistically significant. In our study, 3D models with high resolution and anatomically more detailed were produced from μCT images. All the measured values of proximal width of the radius were found higher than the values specified in the literature. This differentiation is thought to be due to the fact that the bone margins are more clear in the anatomical models we have obtained and therefore there is a difference in measurement points. In parallel with the literature, it was determined that the right and left

values were higher in male rabbits than in females, and this difference was statistically significant (Table 1).

In this study, it has been determined that 3D digital anatomical models can be easily produced using special software programs, and 3D printing models can be designed simply by using 3D printers. It has been thought that 3D technology can be used in areas such as veterinary anatomy, veterinary surgery, and radiology. It has been anticipated that the use of 3D digital and printing models in anatomy education will considerably reduce the use of cadavers and harmful chemicals such as formaldehyde. It has been understood that morphometric measurements made on organic bone samples can be also easily performed on 3D models.

In conclusion, it is thought that the data obtained in this study will benefit the fields of veterinary anatomy, surgery, and radiology. It is also anticipated that this study will bring a modern approach to the field of veterinary anatomy and will be an useful resource for scientists who will research on the forelimb joints of laboratory rabbits.

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Ethical Statement

This study was approved by the Ankara University Animal Experiments Local Ethics Committee (Approval number: 2017-5-32).

Conflict of Interest

The authors declared that there is no conflict of interest.

References

1. **Bakıcı C, Akgün RO, Oto Ç** (2019): *The applicability and efficiency of 3 dimensional printing models of hyoid bone in comparative veterinary anatomy education.* Vet Hekim Der Derg, **90**, 71-75.
2. **Berco M, Rigali PH, Miner RM, et al** (2009): *Accuracy and reliability of linear cephalometric measurements from cone-beam computed tomography scans of a dry human skull*. Am J Orthod Dentofac, **136**, 1-9.
3. **Bouxsein ML, Boyd SK, Christiansen BA, et al** (2010): *Guidelines for assessment of bone microstructure in rodents using micro-computed tomography*. J Bone Miner Res, **25**, 1468-1486.
4. **Brazina D, Fojtik R, Rombova Z** (2014): *3D visualization in teaching anatomy*. Procedia Soc Behav Sci, **143**, 367-371.
5. **Estai M, Bunt S** (2016): *Best teaching practices in anatomy education: A critical review*. Ann Anat, **208**, 151-157.
6. **Fasel JHD, Aguiar D, Kiss-Bodolay D, et al** (2016): *Adapting anatomy teaching to surgical trends: a combination of classical dissection, medical imaging, and 3D-printing technologies*. Surg Radiol Anat, **38**, 361-367.
7. **Freitas EP, Noritomi PY, Silva JVL** (2011): Use of Rapid Prototyping and 3D Reconstruction in Veterinary Medicine. In: ME Hoque (Ed), Advanced Applications of Rapid Prototyping Technology in Modern Engineering. InTech, China.
8. **Gribel BF, Gribel MN, Frazao DC, et al** (2011): *Accuracy and reliability of craniometric measurements on lateral cephalometry and 3D measurements on CBCT scans*. Angle Orthod, **81**, 26-35.
9. **Jamali AA, Deuel C, Perreira A, et al** (2007): *Linear and angular measurements of computer-generated models: Are they accurate, valid, and reliable?* Comput Aided Surg, **12**, 278-285.
10. **Jiang Y, Zhao J, White DL, et al** (2000): *Micro CT and micro MR imaging of 3D architecture of animal skeleton*. J Musculoskel Neuron Interact, **1**, 45-51.
11. **Jones DG** (2019): *Three-dimensional printing in anatomy education: Assessing potential ethical dimensions*. Anat Sci Educ, **12**, 435-443.
12. **Keleş A, Alçin H** (2015): *Mikro bilgisayarlı tomografi ve endodontik araştırmalardaki yeri*. Türkiye Klinikleri J Endod-Special Topics, **1**, 32-39.
13. **Kim M, Huh KH, Yi WJ, et al** (2012): *Evaluation of accuracy of 3D reconstruction images using multi-detector CT and cone-beam CT*. Imaging Sci Dent, **42**, 25-33.
14. **Labruyere J, Schwarz T** (2013): *CT and MRI in veterinary patients: an update on recent advances*. In Pract, **35**, 546-563.
15. **Lagravere MO, Carey J, Toogood RW, et al** (2008): *Three- dimensional accuracy of measurements made with software on cone-beam computed tomography images*. Am J Orthod Dentofac, **134**, 112-116.
16. **Li F, Liu C, Song X, et al** (2018): *Production of accurate skeletal models of domestic animals using three-dimensional scanning and printing technology*. Anat Sci Educ, **11**, 73-80.
17. **Martini L, Fini M, Giavaresi G, et al** (2001): *Sheep model in orthopedic research: A literature review*. Comp Med, **51**, 292- 299.
18. **Mitsouras D, Liacouras P, Imanzadeh A, et al** (2015): *Medical 3D printing for the radiologist*. Radiographics, **35**, 1965- 1988.
19. **Moreira CR, Sales MAO, Lopes PML, et al** (2009): *Assessment of linear and angular measurements on three-dimensional cone- beam computed tomographic images*. Oral Surg Oral Med Oral Pathol Oral Radiol Endod, **108**, 430-436.

20. **Murgitroyd E, Madurska M, Gonzalez J, et al** (2015): *3D digital anatomy modelling - Practical or pretty?* The Surgeon, **13**, 177-180.
21. **Naff KA, Craig S** (2012): The Domestic Rabbit, Oryctolagus Cuniculus: Origins and History. In: MA Suckow, KA Stevens, PP Wilson (Eds), The Laboratory Rabbit, Guinea Pig, Hamster and Other Rodents. Academic Press, London.
22. **Nomina Anatomica Veterinaria** (2017): Prepared by the international committes on veterinary gross anatomical nomenclature and authorized by the general assambly of the world association of veterinary anatomists (6th ed.). Hanover, Germany, Ghent, Belgium, Columbia, MO, Rio de Janeiro, Brazil: The Editorial Committee.
23. **Özkadif S, Eken E** (2015): *Contribution of virtual anatomic models to medical education*. Atatürk Üniversitesi Vet Bil Derg, **10**, 46-54.
24. **Özkadif S, Eken E, Beşoluk K, et al** (2015): *Three-dimensional reconstruction of New Zealand rabbit antebrachium by multidetector computed tomography*. Iran J Vet Res, **16**, 205-209.
25. **Pazvant G, Kahvecioğlu KO** (2009): *Tavşanlarda ön ve arka bacak uzun kemiklerinin homotipik varyasyonları üzerinde araştırmalar*. İstanbul Üniv Vet Fak Derg, **35**, 23-39.
26. **Preece D, Williams SB, Lam R, et al** (2013): *"Let's Get Physical": Advantages of a physical model over 3D computer models and textbooks in learning imaging anatomy*. Anat Sci Educ, **6**, 216-224.
27. **Pujol S, Baldwin M, Nassiri J, et al** (2016): *Using 3D modeling techniques to enhance teaching of difficult anatomical concepts*. Acad Radiol, **4**, 507-516.
28. **Remzi OA, Caner B, Okan E, et al** (2019): *Accuracy and Reliability of Measurements Obtained from 3-Dimensional Rabbit Mandible Model: A Micro-Computed Tomography Study*. Acta Vet-Beograd, **69**, 192-200.
29. **Rengier F, Mehndiratta A, Tengg-Kobligk H, et al** (2010): *3D printing based on imaging data: review of medical applications*. Int J Cars, **5**, 335-341.
30. **Ventola CL** (2014): *Medical applications for 3D printing: Current and projected uses*. Pharm Ther, **39**, 704-711.
31. **Von Den Driesch A** (1976): A Guide to the Measurement of Animal Bones from Archaeological Sites. Pea Body Museum Bulletin 1. Harward University, Massachusetts.

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Behavioural assessment of three chicken genotypes under free-range, semi-intensive, and intensive housing systems

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Abstract: The present study evaluated the effects of housing systems (free-range, semi-intensive, and intensive) on the behaviour of chickens over 10 weeks period (7-16 weeks of age. A total of 360 birds were selected and subjected to different housing systems. A Randomized Complete Block Design (RCBD) considered the following: 3 genotypes (RNN, BNN, and NN) × 2 sexes (30 cockerels and 30 pullets = 60 / genotype) × 3 housing systems (free-range, semi-intensive, and intensive) = 18 experimental units with 20 birds per unit = 360 birds. Regarding behavioural response, male birds under the intensive system were more aggressive and showed more sitting and standing behaviour followed by semi-intensive and free-range systems. Jumping, running, walking and wing flapping behaviours were higher in semi-intensive birds followed by free-range and intensive systems. Regarding females, RNN and BNN chicken revealed higher running behaviour than NN. In terms of housing systems, birds reared in the intensive system were more aggressive and showed an increased frequency of sitting and standing behaviours followed by semi-intensive and free-range systems. Birds under the free-range system spent most of their time in feeding and wing flapping followed by semi-intensive and intensive housing systems. Jumping, running, and walking was more pronounced in the semi-intensive system followed by a free-range and intensive system. It was concluded that RNN and BNN chickens expressed more natural behaviours under semi-intensive and free-range systems than NN chickens; hence, crossbred chickens could be reared under such types of environments to achieve their maximum genetic potential.

Keywords: Behavioural response, chicken, crossbred, housing system, purebred.

Introduction

In developing countries, the popularity of backyard type chicken meat is increasing day by day reason behind its unique flavour and texture which is regarded as a greater delicacy than commercial lines. The local market for indigenous chicken has increased substantially, which stimulated its export potential. Therefore, changing the housing system of these chickens from an extensive backyard to a semi-intensive or intensive system could increase the income for small scale rural poultry farmers (1, 8). In Pakistan, there are three indigenous breeds viz., Desi (non-descript), Naked Neck, and Aseel. Among these, Naked Neck has greater production potential as the other two breeds are very late maturing, slower growing, and produce fewer and lighter eggs. The Naked Neck has the potential to be developed as a dual-purpose breed and kept by farmers for both egg and meat production due to its better survivability in adverse rural conditions. The Naked Neck gene is also known to have the potential to alleviate heat stress in birds (19). Rhode Island Red is originally bred in Adamsville (Rhode Island) and is an American chicken breed popular for its better meat and egg production. The black-breasted red Malay cock was considered as one of the foundation sires of this breed and officially displayed at Smithsonian Institution as the father of Rhode Island Red (1). Black Australorp is a highly successful commercial line, originating from Australia via selective breeding with Black Orpington (1, 2). It is a dual-

purpose breed having dark textured meat and is a good producer of brown eggs (23). This breed holds a unique record of producing 364 eggs in 365 days (5). Crossbreeding is a useful technique to exploit genetic variation and is generally termed as the mating of two individuals having different genetic makeup (21). Crossbreeding increases heterozygosity in the population (15), with the main objective being to produce offspring that has desirable qualities of both parent lineages (18). Crossbreeding is generally helpful not only as it creates combinations of desirable characters, but is also produces heterosis or hybrid vigour. It is also responsible for rapid change in population with the introduction of new breeds (13).

Behaviour is an overt expression of what's going on internally, inside the bird, or as a result of external circumstances. It is a reaction to environmental changes that largely depends upon both genetic and non-genetic factors. Consequently, a better understanding of environmental influence could be helpful for indemnification and quantification of welfare. Environment and housing conditions are generally considered as important factors that directly influence chicken behaviour. Therefore, environmental enrichment may influence a bird's behaviour and provide a large number of behavioural opportunities. Chicken behaviour depends on the housing environment (6).

The free-range system is generally considered as effective from the bird's welfare point of view because it allows the bird to perform and exhibit natural behaviours and provide additional space for exercise. A free-range system results in more favourable meat quality traits especially chemical composition although this system has some demerits, such as exposure to pathogens and adverse weather conditions (7). Farmers might avoid this system for several reasons that include lack of appropriate shelter, weight reduction of young birds being outside. Furthermore, the free-range system is associated with a reduction in weight gain and poor feed conversion efficiency in meat-type birds (3).

The housing system may influence the health-promoting activities of birds which ultimately affects the properties of meat and eggs but the significance of genotype should not be undervalued when selecting birds for any housing environment. Backyard chicken genotypes perform substantially better in the free-range system; however, a better understanding of how different housing systems can influence the behaviour of different genotypes is still required. Therefore, the objective of this study was to investigate the behavioural response of three chicken genotypes under free-range, semi-intensive, and intensive housing systems. We hypothesized that different chicken genotypes behave differently in alternative housing environments.

Materials and Methods

This study was conducted at the Department of Poultry Production, the University of Veterinary and Animal Sciences, Ravi Campus, Pattoki, Pakistan. This city experiences a normally hot and humid tropical climate with temperatures ranging from 13 ℃ in winter and + 45 ℃ in summer.

Ethics: The care and use of birds were by the laws and regulations of Pakistan and was approved by the Committee of Ethical Handling of Experimental Birds (No. DR/124), the University of Veterinary and Animal Sciences (UVAS), Lahore, Pakistan.

Population Size: A baseline population of pure Naked Neck (NN), Rhode Island Red (RIR), and Black Australorp (BAL) already maintained at Indigenous Chicken Genetic Resource Centre (ICGRC), UVAS, Pakistan comprising 450 birds (90 male + 360 female), 150 from each breed (30 males + 120 females). RIR and BAL males were crossed with NN females and their progeny were selected for this experimentation termed as crossbreds. Besides, NN males are also crossed with NN females and their progeny were selected for this experimentation and named as purebred. A total of 480, day-old chicks comprising 160 from each genotype (RIR × NN = RNN, BAL × NN = BNN, and NN × NN = NN) were brooded at a well ventilated open-sided house with standard managemental conditions till 6th weeks of life. Birds were provided by ration formulated according to the recommendation of Leeson and Summers (10), and daily bird's allowance was increasing as per requirement. In the brooding period, birds were vaccinated against Newcastle Disease and Infectious Bronchitis according to the schedule of the local area. After 6 weeks, when birds were acclimatized to the environment experimentation was started and lasted 10 weeks.

During the growing phase (7-16 weeks), 360 birds were selected and subjected to different housing systems. A Randomized Complete Block Design (RCBD) considered the following: 3 genotypes (RNN, BNN, and NN) × 2 sexes (30 cockerels and 30 pullets = 60 / genotype) × 3 housing systems (free-range, semi-intensive, and intensive) = 18 experimental units with 20 birds per unit = 360 birds.

Free-range, semi-intensive and intensive system: All the experimental birds were maintained in an open-sided shed (6.1m L × 6.1m W × 3.66m H) oriented east to west. Birds were reared on the floor having a bedding material of rice husk at stocking density (0.19m^2 per bird). Freshwater was provided through a manual drinker. A patch of fertile land measuring (10m L × 2.99m W; stocking density = 0.23m^2 / bird) located in front of the shed was used as a range area. Seasonal leguminous and non-leguminous plants were grown in the range area. In the ranging area, two rows were made by using fishing

nets (one for free-range and the other for semi-intensive). Fresh *ad libitum* water was ensured through manual drinkers (Henan Lima Machinery Co., Ltd., China). For the protection of the birds, a 2.44 m high wire-mesh enclosure was installed which surrounded the range area. In free-range and semi-intensive systems, birds were given access to vegetation from 06:00 AM to 06:00 PM and 06:00 AM to 12:00 PM, respectively (Figure 1, 2). The free-range group was offered with a 50% grower ration in the evening.

In an intensive housing system, birds were maintained in the battery cage system (FACCO, Poultry Equipment-C3) situated in a well-ventilated poultry shed and were fed commercial grower ration as per the recommendation of Leeson and Summers (10). The daily allowance was increased corresponding to their growth and requirement (Table 1, 2, and 3).

Table 1. Nutrient composition of experimental ration for different phases.

Nutrients	Starter (0-6 weeks)	Grower (7-16 weeks)
CP (%)	16	14
ME (Kcal/Kg)	2900	2850
Ca (%)	0.90	0.87
Av. P (%)	0.40	0.38
Lysine (%)	0.80	0.70
Methionine (%)	0.33	0.30
Na (%)	0.19	0.19

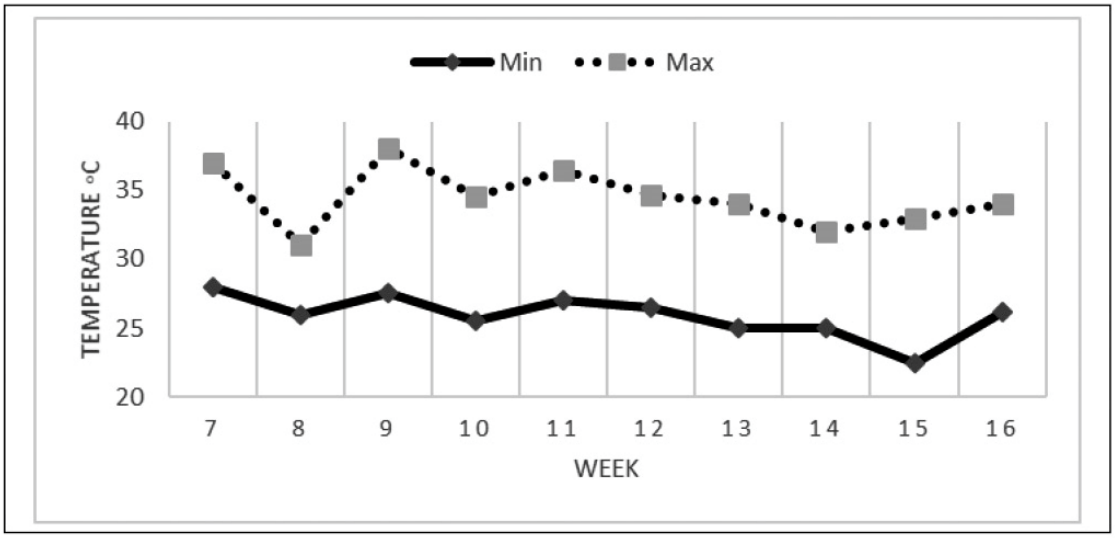

Figure 1. Trend of weekly temperature during experimentation.

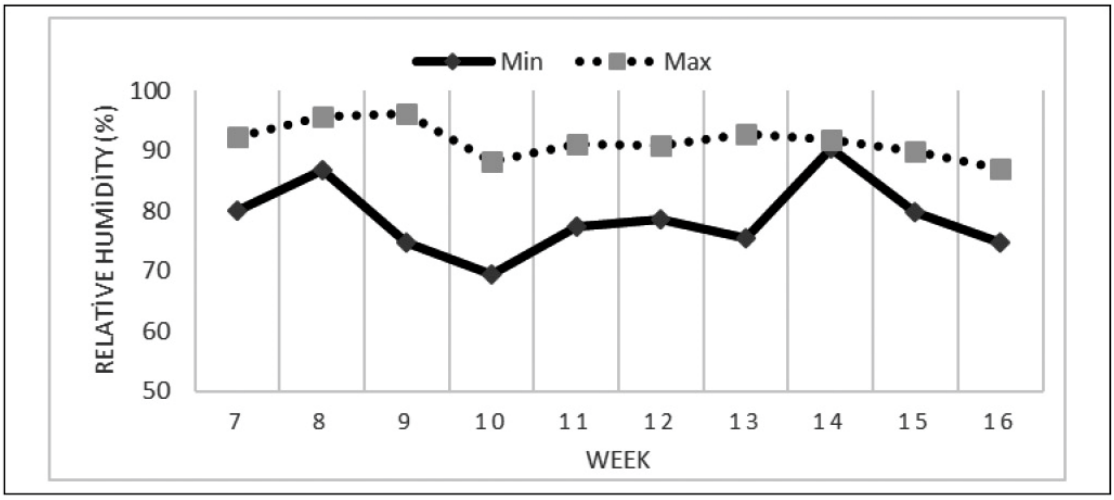

Figure 2. Trend of weekly relative humidity during experimentation.

Table 2. Weekly feed allowance (g/bird) in the growing phase (7-16 weeks).

Age (Week)	Housing System		
	Free Range	Semi-intensive	Intensive
7	0	12	24
8	0	14	28
9	0	15	30
10	0	15	30
11	0	17	34
12	0	18	36
13	0	19	38
14	0	19	38
15	0	20	40
16	0	21	42

Table 3. Composition of Experimental Rations (Starter and Grower).

Feed Ingredient (%)	Starter (0-6 weeks)	Grower (7-16 weeks)
Corn	60.63	62.55
Soybean Meal	33.34	32.33
Fish Meal	2.00	0.00
Soybean Oil	2.00	3.00
DCP	1.50	1.70
NaCl	0.30	0.30
Methionine	0.23	0.12
Total	100	100

Table 4. Ethogram of behavioural pattern[1].

Behaviour	Definition
Walking	The bird moves at least two steps in succession. This may or may not include scratching with feet.
Jumping	Movement of bird in a rebound by leaping with all feet off the ground.
Running	An activity of wing-assisted running.
Feeding / Foraging	The bird's head is located inside the feeder / towards forage and carrying out pecking, manipulating, or ingesting feed once or repeatedly.
Standing	The feet are in contact with the ground. No other body part is touching the floor. The body posture is in the upright position.
Sitting	The ventral part of the bird is in contact with the ground. Legs are bent at the knee with the fibula and tibia touching the ground.
Aggressiveness	A response that delivers somewhat unpleasant, giving, or receiving peck forcefully, the beak being above the receiver's head.
Dust Bathing	Dustbathing bouts of bird either in free-range or semi-intensive, squatting down in the substrate, with the use of wings, head, neck, and legs performing sequential vertical wings shaking.
Wing Flapping	Wings are extended horizontally from the body such that can be seen between the underside of the wing and the surface of the bird's body.

[1] Modified from Costa et al. (4) and Mohammed et al. (12).

Parameters evaluated: Each experimental bird was wing tagged. The observation was conveyed weekly, between 11:00 AM to 01:00 PM and the time spent in each behaviour was noted (Table 4). The behaviour repertoire was recorded according to the focal animal sampling method adopted by Rehman et al. (16). Before visual scanning, at least a half-hour elapsed that allowing the birds to redistribute normally as the observers moved across the replicates. During the observer training period, birds were habituated to the presence of the observers and the beep sounds of the stopwatch. Birds were randomly selected (3 birds per replicate) and marked on the neck and tail feather with a white colour stain for identification. All four observers were positioned near birds, avoiding any interruption in their activities. The birds were observed for 15 minutes by using stopwatches fixed at 5 seconds beeps and the behavioural range was recorded individually (time spent in each behaviour). After each observation, the observers paused for 5 minutes, allowing the next focal bird to regain its normal position. The behaviour of chicken was included walking, jumping, running, feeding, standing, sitting, aggressiveness, dust bathing, and wing flapping, and behaviour occurrence proportions were calculated. Ethogram of the behavioural pattern are presented in Table 4 (4, 12).

Statistical analysis: The experiment was set up as an RCBD with the following model:

$$Y_{ijk} = \mu + \beta_i + \tau_j + (\beta \times \tau)_{ij} + \epsilon_{ijk}$$

Where,

Y_{ijk} = Observation of dependent variable recorded on j^{th} Housing System in i^{th} Block

μ = Overall population mean

β_i = Effect of i^{th} Block (i = 1, 2, 3)

τ_j = Effect of j^{th} Housing System (j = 1, 2, 3)

$(\beta \times \tau)_{ij}$ = Interaction between the block and housing system

ϵ_{ijk} = Residual error of k^{th} observation on j^{th} treatment in i^{th} block NID ~ 0, σ^2

The effect of different chicken genotypes and housing systems on behavioural traits were evaluated through Factorial ANOVA using General Linear Model Procedures in SAS software (version, 9.1.3). For pair viz comparison of means, the Tukey Kramer test (22) was applied and differences were considered statistically significant at $P \leq 0.05$. All the data were represented as least square mean ± standard errors.

Results

The behavioural pattern differed among genotypes and the interactions between genotypes and housing systems (Tables 5, 6, 7, and, 8). Regarding males, significant differences were observed among different housing systems in terms of aggression, dust bathing, feeding, jumping, running, sitting, standing, walking, and wing flapping. Birds under the intensive-system were more aggressive following by semi-intensive and free-range systems (P<0.001). Dust bathing was more pronounced in free-range birds than the semi-intensive systems (15.15 vs. 12.43 %; P<0.001). Birds were spending most of the time feeding in the free-range system, followed in semi-intensive and intensive systems (P<0.001). Jumping (P<0.001) and running (P<0.001) behaviours were more frequent in the semi-intensive system followed by free-range and intensive systems.

Table 5. Effect of genotype and housing system on male behavioural traits (7-16 weeks[1]).

Trait[2]	Genotype			P-value	Housing System			P-value
	RNN (n = 60)	BNN (n = 60)	NN (n = 60)		FR (n = 60)	SI (n = 60)	I (n = 60)	
WAK	13.29 ± 1.38	13.21 ± 1.38	13.25 ± 1.38	0.431	13.17 ± 0.05^{b}	26.27 ± 0.06^{a}	0.31± 0.00^{c}	<0.001
JMP	0.74 ± 0.07	0.74 ± 0.07	0.73 ± 0.07	0.467	0.92 ± 0.01^{b}	1.25 ± 0.01^{a}	0.04 ± 0.00^{c}	<0.001
RUN	7.82 ± 0.78	7.92 ± 0.80	7.84 ± 0.78	0.199	8.89 ± 0.04^{b}	14.69 ± 0.05^{a}	0.00 ± 0.00^{c}	<0.001
FD	18.09 ± 0.30	18.06 ± 0.32	18.10 ±0.31	0.797	21.28 ± 0.04^{a}	17.30 ± 0.05^{b}	15.67 ± 0.04^{c}	<0.001
STD	14.30 ± 1.19	14.25 ± 1.20	14.22 ± 1.19	0.286	9.11 ± 0.03^{b}	6.51 ± 0.02^{c}	27.14 ± 0.05^{a}	<0.001
SIT	13.54 ± 1.09	13.55 ± 1.08	13.58 ± 1.09	0.713	8.42 ± 0.03^{b}	6.92 ± 0.02^{c}	25.33 ± 0.05^{a}	<0.001
AGR	1.72 ± 0.08	1.71 ± 0.08	1.72 ± 0.08	0.804	1.12 ± 0.01^{c}	1.51± 0.01^{b}	2.52 ± 0.02^{a}	<0.001
DB	13.79 ± 0.22	13.81 ± 0.23	13.79 ± 0.22	0.964	15.15 ± 0.04^{a}	12.43 ± 0.04^{b}	--	<0.001
WF	10.66 ± 0.92	10.66 ± 0.92	10.73 ± 0.93	0.397	15.08 ± 0.04^{b}	16.28 ± 0.05^{a}	0.69 ± 0.00^{c}	<0.001

$^{a-c}$ Means in a row with no common superscript differ significantly at P ≤ 0.05.
[1]Values are least square mean ± standard error, average of each behaviour repertoire from 7-16 weeks
[2]Traits are presented as percentage of time spent in different behavioural activities
RNN = Rhode Island Red × Naked Neck; BNN = Black Australorp × Naked Neck; NN = Naked Neck; FR = Free Range; SI = Semi Intensive; I = Intensive; WAK = Walking; JMP = Jumping; RUN = Running; FD = Feeding; STD = Standing; SIT = Sitting; AGR = Aggressiveness; DB = Dust Bathing; WF = Wing Flapping.

Table 6. Interaction effects (genotype × housing system) on male behavioural traits (7-16 weeks[1]).

Trait[2]	RNN			BNN			NN			P-value
	FR (n = 20)	SI (n = 20)	I (n = 20)	FR (n = 20)	SI (n = 20)	I (n = 20)	FR (n = 20)	SI (n = 20)	I (n = 20)	
WAK	13.27 ± 0.06^{b}	26.29 ± 0.11^{a}	0.31 ± 0.01^{c}	13.12 ± 0.10^{b}	26.19 ± 0.10^{a}	0.31 ± 0.00^{c}	13.14 ± 0.10^{b}	26.31 ± 0.11^{a}	0.31 ± 0.01^{c}	<0.001
JMP	0.94 ± 0.02^{b}	1.25 ± 0.02^{a}	0.04 ± 0.00^{c}	0.92 ± 0.01^{b}	1.25 ± 0.02^{a}	0.04 ± 0.00^{c}	0.90 ± 0.02^{b}	1.25 ± 0.01^{a}	0.04 ± 0.00^{c}	<0.001
RUN	8.87 ± 0.05^{b}	14.60 ± 0.10^{a}	0.00 ± 0.00^{c}	8.90 ± 0.09^{b}	14.85 ± 0.09^{a}	0.00 ± 0.00^{c}	8.91 ± 0.08^{b}	14.60 ± 0.08^{a}	0.00 ± 0.00^{c}	<0.001
FD	21.22 ± 0.06^{a}	17.28 ± 0.07^{b}	15.76 ± 0.06^{c}	21.35 ± 0.07^{a}	17.30 ± 0.08^{b}	15.54 ± 0.09^{c}	21.27 ± 0.08^{a}	17.32 ± 0.10^{b}	15.72 ± 0.08^{c}	<0.001
STD	9.18 ± 0.04^{b}	6.52 ± 0.03^{c}	27.18 ± 0.07^{a}	9.09 ± 0.06^{b}	6.46 ± 0.05^{c}	27.21 ± 0.08^{a}	9.07 ± 0.06^{b}	6.56 ± 0.03^{c}	27.03 ± 0.09^{a}	<0.001
SIT	8.44 ± 0.06^{b}	6.82 ± 0.04^{c}	25.36 ± 0.10^{a}	8.40 ± 0.07^{b}	6.97 ± 0.05^{c}	25.29 ± 0.07^{a}	8.41 ± 0.06^{b}	6.98 ± 0.03^{c}	25.36 ± 0.10^{a}	<0.001
AGR	1.11 ± 0.02^{c}	1.50 ± 0.02^{b}	2.56 ± 0.05^{a}	1.12 ± 0.02^{c}	1.51 ± 0.02^{b}	2.49 ± 0.04^{a}	1.13 ± 0.02^{c}	1.53 ± 0.02^{b}	2.50 ± 0.04^{a}	<0.001
DB	15.11 ± 0.07^{a}	12.47 ± 0.08^{b}	--	15.19 ± 0.08^{a}	12.42 ± 0.08^{b}	--	15.16 ± 0.05^{a}	12.41 ± 0.06^{b}	--	<0.001
WF	15.02 ± 0.07^{b}	16.26 ± 0.12^{a}	0.69 ± 0.01^{c}	15.04 ± 0.06^{b}	16.26 ± 0.09^{a}	0.68 ± 0.01^{c}	15.18 ± 0.09^{b}	16.32 ± 0.08^{a}	0.69 ± 0.01^{c}	<0.001

$^{a-c}$ Means in a row with no common superscript differ significantly at P ≤ 0.05.
[1] Values are least-square mean ± standard error, an average of each behaviour repertoire from 7-16 weeks
[2]Traits are presented as the percentage of time spent in different behavioural activities
RNN = Rhode Island Red × Naked Neck; BNN = Black Australorp × Naked Neck; NN = Naked Neck; FR = Free Range; SI = Semi-Intensive; I = Intensive; WAK = Walking; JMP = Jumping; RUN = Running; FD = Feeding; STD = Standing; SIT = Sitting; AGR = Aggressiveness; DB = Dust Bathing; WF = Wing Flapping.

Table 7. Effect of genotype and housing system on female behavioural traits (7-16 weeks[1]).

Trait[2]	Genotype			P-value	Housing System			P-value
	RNN (n = 60)	BNN (n = 60)	NN (n = 60)		FR (n = 60)	SI (n = 60)	I (n = 60)	
WAK	13.09 ± 1.29	13.11 ± 1.29	13.14 ± 1.29	0.657	14.77 ± 0.04^{b}	24.34 ± 0.05^{a}	0.23 ± 0.00^{c}	<0.001
JMP	0.89 ± 0.08	0.93 ± 0.09	0.91 ± 0.09	0.770	1.25 ± 0.04^{b}	1.45 ± 0.03^{a}	0.03 ± 0.00^{c}	<0.001
RUN	6.66 ± 0.66^{a}	6.65 ± 0.65^{a}	6.52 ± 0.65^{b}	0.047	7.77 ± 0.07^{b}	12.06 ± 0.05^{a}	0.00 ± 0.00^{c}	<0.001
FD	17.58 ± 0.54	17.56 ± 0.54	17.60 ± 0.54	0.806	23.10 ± 0.05^{a}	16.47 ± 0.04^{b}	13.17 ± 0.04^{c}	<0.001
STD	12.45 ± 1.18	12.48 ± 1.19	12.45 ± 1.18	0.740	6.39 ± 0.03^{b}	5.72 ± 0.02^{c}	25.30 ± 0.04^{a}	<0.001
SIT	11.55 ± 0.90	11.53 ± 0.90	11.57 ± 0.91	0.709	7.30 ± 0.03^{b}	6.02 ± 0.02^{c}	21.33 ± 0.05^{a}	<0.001
AGR	1.53 ± 0.06	1.54 ± 0.07	1.51 ± 0.06	0.496	1.04 ± 0.01^{c}	1.36 ± 0.01^{b}	2.18 ± 0.02^{a}	<0.001
DB	14.84 ± 0.25	14.78 ± 0.23	14.80 ± 0.24	0.767	16.28 ± 0.05^{a}	13.34 ± 0.05^{b}	--	<0.001
WF	4.33 ± 0.37	4.29 ± 0.36	4.29 ± 0.36	0.571	6.53 ± 0.03^{a}	5.99 ± 0.04^{b}	0.39 ± 0.00^{c}	<0.001

$^{a-c}$ Means in a row with no common superscript differ significantly at $P \leq 0.05$.
[1]Values are the least-square mean ± standard error, the average of each behaviour repertoire from 7-16 weeks.
[2]Traits are presented as the percentage of time spent in different behavioural activities
RNN = Rhode Island Red × Naked Neck; BNN = Black Australorp × Naked Neck; NN = Naked Neck; FR = Free Range; SI = Semi Intensive; I = Intensive; WAK = Walking; JMP = Jumping; RUN = Running; FD = Feeding; STD = Standing; SIT = Sitting; AGR = Aggressiveness; DB = Dust Bathing; WF = Wing Flapping.

Table 8. Interaction effects (genotype × housing system) on female behavioural traits (7-16 weeks[1]).

Trait[2]	RNN			BNN			NN			P-value
	FR (n = 20)	SI (n = 20)	I (n = 20)	FR (n = 20)	SI (n = 20)	I (n = 20)	FR (n = 20)	SI (n = 20)	I (n = 20)	
WAK	14.73 ± 0.08^{b}	24.32 ± 0.07^{a}	0.22 ± 0.01^{c}	14.79 ± 0.08^{b}	24.30 ± 0.09^{a}	0.23 ± 0.01^{c}	14.79 ± 0.08^{b}	24.38 ± 0.08^{a}	0.23 ± 0.01^{c}	<0.001
JMP	1.27 ± 0.07^{ab}	1.38 ± 0.05^{ab}	0.03 ± 0.00^{c}	1.28 ± 0.07^{ab}	1.46 ± 0.06^{a}	0.03 ± 0.00^{c}	1.21 ± 0.07^{b}	1.51 ± 0.06^{a}	0.03 ± 0.06^{c}	<0.001
RUN	7.84 ± 0.10^{b}	12.15 ± 0.10^{a}	0.00 ± 0.00^{c}	7.90 ± 0.12^{b}	12.05 ± 0.07^{a}	0.00 ± 0.00^{c}	7.57 ± 0.11^{b}	11.98 ± 0.06^{a}	0.00 ± 0.00^{c}	<0.001
FD	23.12 ± 0.09^{a}	16.48 ± 0.07^{b}	13.14 ± 0.05^{c}	23.02 ± 0.08^{a}	16.51 ± 0.06^{b}	13.14 ± 0.11^{c}	23.16 ± 0.09^{a}	16.42 ± 0.07^{b}	13.22 ± 0.06^{c}	<0.001
STD	6.45 ± 0.05^{b}	5.70 ± 0.03^{c}	25.31 ± 0.07^{a}	6.34 ± 0.04^{b}	5.73 ± 0.03^{c}	25.36 ± 0.08^{a}	6.40 ± 0.05^{b}	5.72 ± 0.03^{c}	25.24 ± 0.08^{a}	<0.001
SIT	7.30 ± 0.06^{b}	6.02 ± 0.03^{c}	21.35 ± 0.09^{a}	7.30 ± 0.05^{b}	6.04 ± 0.02^{c}	21.25 ± 0.08^{a}	7.31 ± 0.04^{b}	6.02 ± 0.03^{c}	21.38 ± 0.08^{a}	<0.001
AGR	1.03 ± 0.02^{c}	1.38 ± 0.01^{b}	2.18 ± 0.04^{a}	1.05 ± 0.02^{c}	1.36 ± 0.02^{b}	2.21 ± 0.04^{a}	1.06 ± 0.01^{c}	1.34 ± 0.02^{b}	2.14 ± 0.03^{a}	<0.001
DB	16.40 ± 0.08^{a}	13.29 ± 0.07^{b}	--	16.17 ± 0.09^{a}	13.40 ± 0.10^{b}	--	16.28 ± 0.08^{a}	13.32 ± 0.09^{b}	--	<0.001
WF	6.58 ± 0.06^{a}	6.03 ± 0.08^{b}	0.39 ± 0.00^{c}	6.52 ± 0.05^{a}	5.98 ± 0.07^{b}	0.39 ± 0.00^{c}	6.50 ± 0.05^{a}	5.98 ± 0.08^{b}	0.39 ± 0.00^{c}	<0.001

$^{a-c}$ Means in a row with no common superscript differ significantly at $P \leq 0.05$.
[1]Values are the least-square mean ± standard error, the average of each behaviour repertoire from 7-16 weeks.
[2]Traits are presented as the percentage of time spent in different behavioural activities
RNN = Rhode Island Red × Naked Neck; BNN = Black Australorp × Naked Neck; NN = Naked Neck; FR = Free Range; SI = Semi Intensive; I = Intensive; WAK = Walking; JMP = Jumping; RUN = Running; FD = Feeding; STD = Standing; SIT = Sitting; AGR = Aggressiveness; DB = Dust Bathing; WF = Wing Flapping.

Sitting (P<0.001) and standing (P<0.001) behaviours were more pronounced in the intensive system birds followed by free-range and semi-intensive systems. Birds under semi-intensive spent most of their time in walking (P<0.001) and wing-flapping behaviour following by free-range and intensive systems. Interactions were significant (P<0.001) between genotypes and housing systems regarding walking, jumping, running, feeding, standing, sitting, aggressiveness, dust bathing, and wing flapping.

Regarding females, a significant difference was observed regarding running behaviour among different genotypes. RNN and BNN chicken different and revealed more intense running behaviour than NN (6.66, 6.65 vs. 6.52%; P=0.047). In terms of housing systems, significant

differences were observed regarding aggressiveness, dust bathing, feeding, jumping, running, sitting, standing, walking, and wing flapping. Birds reared under the intensive systems were more aggressive followed by semi-intensive and free-range systems (P<0.001). Dust bathing was more pronounced in free-range birds than the semi-intensive systems (16.28 vs. 13.34 %; P<0.001). Birds under the free-range system spent most of their time feeding (P<0.001) and wing flapping (P<0.001) followed by semi-intensive and intensive housing systems. Jumping (P<0.001), running (P<0.001), and walking (P<0.001) were more pronounced in the semi-intensive system followed by a free-range and intensive system. Sitting (P<0.001) and standing (P<0.001) behaviour were higher in birds under intensive system followed by free-range and semi-intensive systems. Interactions were significant (P<0.001) between genotypes and housing systems regarding walking, jumping, running, feeding, standing, sitting, aggressiveness, dust bathing, and wing flapping.

Discussion and Conclusion

The present study aimed to explore the genetic potential of different chicken genotypes under alternative production systems and this was successful as the behaviour of different genotypes was astonishing when subjected to different housing systems. Free-range birds spent more time in dust bathing than semi-intensive chickens and this could be due to infrequent behaviour which is initiated when birds given access to the ample space for movement and birds have the opportunity to find material for its cleaning such as dust. Moreover, the reason for this result may be the length of time the birds were spending outside the facility. Bathing is usually done in the afternoon, and the birds from the semi-intensive system were not outside at that time. According to RSPCA (17) domestic chicken has intrinsic motivation for cleaning its feathers. This corresponds to the findings of Appleby et al. (2) who reported that an intensive system is not suitable for the birds because it restricts the expression of natural behaviours like dust bathing.

Feeding behaviour is more pronounced in free-range birds followed by a semi-intensive and intensive system. It might be due to the variety of stimuli in the range area which provokes foraging behaviour in the birds. Furthermore, due to ample space in the free-range system, it provides lots of opportunities for the birds to initiate their explorative behaviour. Similar findings were also suggested that foraging behaviour increased in the commercial broiler with the provision of the free-range area as compared to confinement (14). Moreover, Shimmura et al. (20) supported to enhance the feeding behaviour of commercial layers when given access to a free-range system.

Jumping, running walking, and wing-flapping behaviour was more pronounced in semi-intensive birds followed by the free-range and intensive system. The more likely explanation of these behaviours is because when birds are provided with an enriched environment or outdoor access it promotes comfort level, reduces stress, and stimulating activities. This corresponds to the findings of Irfan et al. (9) who found increased immobility in turkey (*Meleagris gallopavo*) maintained in confinement than free-range birds. Moreover, Mench et al. (11) reported that the frequency of leg stretching, perching, and wing flapping of broiler chicken increased when given access to an outdoor range. Similarly, an enhanced walking behaviour of Aseel chicken was recorded when subjected to part-time free-range access (16).

In this study, increased aggressive, sitting, and standing behaviour in birds with an intensive housing system might be attributed to the higher stocking density that restricts the bird's activity and stimulates short duration behaviour such as aggression. This corresponds to the findings of Rehman et al. (16) who found enhance sitting and standing behaviour of Aseel chicken varieties reared under confinement. On an overall basis, the behavioural repertoire did not differ among RNN, BNN, and NN genotype throughout the experimental period. However, another study (16) reported a higher frequency of aggressive behaviour in crossbred chicken (cross between Thai native male "Luang Hang Khao or White-tailed Yellow breed" and ISA Brown Commercial layer type female).

It was concluded that RNN and BNN chickens expressed more natural behaviours under semi-intensive and free-range systems than NN chickens; hence, crossbred chickens could be reared under such types of environment to achieve their maximum genetic potential.

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Ethical Statement

The care and use of birds were by the laws and regulations of Pakistan and 101 was approved by the Committee of Ethical Handling of Experimental Birds

(No. 102 DR/124), the University of Veterinary and Animal Sciences (UVAS), Lahore, Pakistan.

Conflict of Interest

The authors declared that there is no conflict of interest.

References

1. **Anonymous** (2011): South African show poultry organisation breed standards. Available at: http://www.saspo.org.za. (Accessed September 06, 2020).
2. **Appleby MC, Mench JA, Hughes BO** (2004): Poultry Behaviour and Welfare. 118-175. In: Perceptions of Welfare. CABI Publishing, Oxfordshire, UK.
3. **Bogosavljević-Bošković S, Rakonjac S, Dosković V, et al** (2012): *Broiler rearing systems: A review of major fattening results and meat quality traits.* Worlds Poult Sci J, **68**, 217-228.
4. **Costa LS, Pereira DF, Bueno LGF, et al** (2012): *Some aspects of chicken behaviour and welfare*. Braz J Poult Sci, **14**, 159-164.
5. **Dohner JV** (2010): Black Australorp chickens: Heritage poultry breeds. Available at: https://www.motherearthnews.com/homesteading-and-livestock/raising-chickens/black-australorp-heritage-poultry-zeylaf. (Accessed September 06, 2020).
6. **El-Deek A, El-Sabrout K** (2019): *Behaviour and meat quality of chicken under different housing system*. Worlds Poult Sci J, **75**, 1-9.
7. **Fanatico AC, Pillai PB, Cavitt LC, et al** (2006): *Evaluation of slower growing broiler genotypes grown with and without outdoor access: sensory attributes*. Poult Sci, **85**, 337-343.
8. **Huo X, Na-Lampang P** (2016): *Effects of stocking density on feather pecking and aggressive behaviour in Thai crossbred chickens*. Agric Nat Res, **50**, 396-399.
9. **Javid A, Ashraf M, Mahmud, A, et al** (2016): *Time-budgets of turkeys (Maleagris gallopavo) reared under confinement and free range rearing systems*. Pak J Zool, **48**, 1951–1956.
10. **Leeson S, Summers JD** (2005): Commercial Poultry Nutrition. 3rd Edition. Nottingham University Press, Nottingham, England.
11. **Mench JA, Garner JP, Falcone C** (2001): Behavioural activity and its effects on leg problems in broiler chickens. 152–156. In: Proceedings of 6th European Symposium Poultry Welfare Zollikofen, Switzerland.
12. **Mohammed AA, Jacobs JA, Murugesan GR, et al** (2018): *Effect of dietary synbiotic supplement on behavioural patterns and growth performance of broiler chickens reared under heat stress*. Poult Sci, **97**, 1101-1108.
13. **Momoh OM, Nwosu CC** (2008): *Genetic evaluation of growth traits in crosses between two ecotypes of Nigerian local chicken*. Livestock Res Rural Dev, **20**, 580-690.
14. **Ponte PIP, Rosado CMC, Crespo JP, et al** (2008): *Pasture intake improves the performance and meat sensory attributes of free-range broilers*. Poult Sci, **87**, 71-79.
15. **Razuki WM, Mukhlis SA, Jasmin FH, et al** (2011): *Productive performance of four commercial broiler genotypes reared under high ambient temperatures*. Int J Poult Sci, **10**, 87-92.
16. **Rehman MS, Mahmud A, Mehmood S, et al** (2018): *Assessing behaviour in Aseel pullets under free-range, part-time free-range, and cage system during growing phase*. Poult Sci, **97**, 725-732.
17. **RSPCA** (2016): The Welfare of Layer Hens in Cage and Cage-Free Housing Systems. RSPCA Australia, Deakin West ACT 2600, Australia.
18. **Saadey SM, Galal A, Zaky HI, et al** (2008): *Diallel crossing analysis for body weights and egg production traits of two native Egyptian and two exotic chicken breeds*. Int J Poult Sci, **7**, 64–71.
19. **Shafiq M, Mahmud A, Hussain J, et al** (2018): *Comparative production performance of four different Naked Neck chicken phenotypes in Pakistan*. J Anim Plant Sci, **28**, 33-37.
20. **Shimmura T, Suzuki T, Hirahara S, et al** (2008): *Pecking behaviour of laying hens in single-tiered aviaries with and without outdoor area*. Brit Poult Sci, **49**, 396–401.
21. **Siwendu NA, Norris D, Ng'Ambi JW, et al** (2012): *Heterotic and combining ability for body weight in a diallel cross of three chicken genotypes*. Trop Anim Health Prod, **20**, 23-25.
22. **Tukey JW** (1953): The problem of multiple comparisons. The Collected Works of JOHN W. Tukey VIII. Multiple Comparisons. Chapman and Hall, New York.
23. **Van Marle-Köster E, Hefer CA, Nel LH, et al** (2008): *Genetic diversity and population structure of locally adapted South African chicken lines: Implications for conservation*. South Afr J Anim Sci, **38**, 271-281.

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Determination of antimicrobial and anti-quorum sensing activities of water and ethanol extracts of propolis

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Abstract: Quorum Sensing is a special system between bacteria to communicate with the signal molecules. Propolis is a resin collected from plants by honey bees. The present study examined anti-quorum sensing and antimicrobial activities of water and ethanol extracts of propolis. The antimicrobial activities of the propolis extracts were studied against *Escherichia coli* ATCC 25922 Gram (-), *Listeria monocytogenes* ATCC 7644, *Streptococcus mutans* CNCTC 8/77, *Staphylococcus aureus* ATCC 25923 Gram (+), *Candida albicans* ATCC 10239 (yeast) by the well diffusion method. Anti-quorum sensing activity, violacein inhibition activity, and anti-swarming activity were studied using *Chromobacterium violaceum* CV026 and C. *violaceum* ATCC 112472, and *Pseudomonas aeruginosa* (PA01) respectively. Both propolis extracts were detected antibacterial activity against Gram (+) bacteria and Candida albicans. However, antibacterial activity against Gram (-) bacteria were not observed. While the antimicrobial activity of water extract propolis was determined more effectively than ethanol extract, anti-quorum sensing and anti-swarming activities less effective. It was also detected that both extracts display violacein inhibition. It was revealed that the antimicrobial, anti-quorum sensing, anti-swarming activity depended on the dose of propolis extracts. Thirty-six phenolic compounds were analysed of propolis extract by HPLC-DAD. The amount and presence of phenolic compounds in the ethanol extract were more than the water extract apart from benzoic acid, cinnamic acid, syringic acid, (±) epigallocatechin, vitexin, rutin, and trans-chalcone. Consequently, the study put forward that the phenolic compounds of propolis have antibacterial, anti- quorum sensing, violacein inhibition, and anti-swarming activities changing depend on directly the extraction solvent and phenolic compounds.

Keywords: Antimicrobial activity, anti-quorum sensing activity, anti-swarming activity, propolis, violacein inhibition activity.

Propolisin sulu ve etanol ekstresinin antimikrobiyal ve anti-çoğunluk algılama aktivitesinin belirlenmesi

Özet: Çoğunluğu algılama sistemi bakterilerin sinyal molekülleri ile kendi aralarında iletişim kurmasını sağlayan özel bir sistemdir. Propolis bal arılarının bitkilerden topladığı reçinedir. Bu çalışmada etanol ve su ekstrakt propolisin antimikrobiyal ve anti-çoğunluk algılama aktivitesi incelendi. Propolis ekstraktlarının antimikrobiyal aktivitesi *Escherichia coli* ATCC 25922, *Listeria monocytogenes* ATCC 7644, *Streptococcus mutans* CNCTC 8/77, *Staphylococcus aureus* ATCC 25923 ve *Candida albicans* ATCC 102392'e karşı disk difüzyon yöntemi ile çalışıldı. Anti-çoğunluk algılama, violacein inhibisyon ve kaynaşma önleyici aktiviteleri sırasıyla *Chromobacterium violaceum* CV026, *C. violaceum* ATCC 112472 ve *Pseudomonas aeruginosa* PA01 ile çalışıldı. Propolis ekstraktlarının Gram (+) bakteriler ve *Candida albicans* üzerine antimikrobiyal etkisi olmasına rağmen Gram (-) bakterilerde herhangi bir etki tespit edilememiştir. Sulu ektrakt propolisin antibakteriyel aktivitesi etanol ekstrakt propolise göre daha yüksek iken anti-çoğunluk algılama ve kaynaşma önleyici aktivitesi daha az olduğu tespit edildi. Violacein inhibisyon aktivitesi ise her iki propolis ekstraktında da tespit edildi. Propolis ekstraktlarının antibakteriyel, anti-çoğunluk algılama ve kaynaşma önleyici aktiviteleri doza bağımlı olarak değiştiği görüldü. Propolis ekstraktlarında otuz altı fenolik bileşik HPLC-DAD ile analiz edildi. Benzoik asit, sinnamik asit, şiringik asit, epigallokateşin, viteksin rutin ve kalkon dışındaki tüm fenolik bileşikler hem miktarı hem de yaygınlığı etanol ekstrakt propoliste daha yüksek olduğu belirlendi. Sonuç olarak bu çalışma propolisin ekstraksiyon solventine ve içeriğindeki fenolik bileşiklere bağlı olarak antimikrobiyal, anti-çoğunluk algılama, violacein inhibisyon ve kaynaşma önleyici aktivitelerinin olduğunu ortaya koydu.

Anahtar sözcükler: Antimikrobiyal aktivite, anti-çoğunluk algılama aktivitesi, kaynaşma önleyici aktivitesi, propolis, violacein inhibisyon aktivitesi.

Introduction

Honeybees collect the propolis from resin and bud exudates of plants (3, 17, 30). Propolis is used as a building and protection material by honey bees, which fills holes and cracks, repairing of honeycombs in the hive, narrowing the beehive entrance against harmful creatures, and cold (30). The chemical composition of propolis is affected primarily by vegetation, that is the source of propolis, and secondly, by seasons as well as by altitudes both of which affect the vegetation (33). The biological activities of propolis are dependent on its chemical constituents, mainly phenolic compounds (3). Propolis has a vigorous antimicrobial activity known as a natural antibiotic. Many researches revealed the antibacterial effect of propolis on many bacteria by using different solvents and techniques (7). Antibacterial activity of propolis has found to be high against Gram-positive bacteria and weak against Gram-negative bacteria (7, 25, 30). Propolis has antibacterial activity on many resistant bacteria such as methicillin-resistant *Staphylococcus aureus* and vancomycin-resistant enterococci, which are dangerous for human and animal health (2, 21, 31). Studies show that the antibacterial activity of propolis is due to inhibition of protein synthesis and disruption of cytoplasmic membrane integrity and permeability of bacteria (7). The bacteria have developed resistance to many antibacterial agents thanks to genetic mutation, quorum sensing (QS) activities, and changing of the antibacterial target (10).

The QS is a cell-to-cell communication mechanism dependent on cell density, which allows bacterial populations to regulate the expression of genes and release chemical signal molecules called auto-inducers (10). Bacteria producing the auto-inducer may change their phenotype so that their metabolism and other activities can be successful in the new environment (10). The auto-inducers are responsible for biological behaviors which are biofilm formation, virulence symbiosis, sporulation, competence, motility, conjugation, and antibiotic production in the different bacteria using N-acyl homoserine lactones (AHL) and oligopeptides (1, 10, 26, 32). Previous studies have determined the anti-QS activity of many natural products. Phenolic compounds, terpenoids, and oils found in natural products which are responsible for the anti-QS activity (1, 32). Some studies have also determined the anti-QS activity of propolis, which containing various phenolic components (5, 16). Chemical composition of propolis may change primarily depending on seasons, altitudes, and climate zone, which are pre-collecting factors that directly concerning vegetation around apiaries (33). Secondly, the chemical composition of propolis is affected significantly by the extraction method and its solvent, which are post-collecting factors (15, 34).

The present study was aimed to determine and compare the anti-QS and antimicrobial activities of the water and ethanol extracts of propolis, which collected from Bursa in Turkey. Moreover, it was studied antimicrobial and anti-QS activities related to the content of phenolic compounds in propolis.

Materials and Methods

Chemical and Reagents: Biomonitor strains used in the anti-QS studies were growth on Luria–Bertani broth. Other bacteria were fed on Brain Heart Infusion Broth (BHIB), Sabouraud Dextrose Broth (SDB), Nutrient Broth, Luria Bertani Broth, and Mueller Hinton Broth over agar (Merck). 24-well cell culture plates (Greiner Bio-One, Kremsmünster, Austria, sterile, with lid), N-hexanoyl-DL-homoserine lactone (C6-HSL, ≥97%, Sigma-Aldrich), Tryptone (Sigma-Aldrich), kanamycin sulfate (Sigma-Aldrich), D-(+)-glucose (≥99.5%, Sigma-Aldrich), sodium chloride (Sigma-Aldrich), N-decanoyl-DL-homoserine lactone (C10-HSL, ≥97%, Sigma-Aldrich), proteose peptone (Sigma-Aldrich), dimethyl sulfoxide (DMSO, Sigma-Aldrich), were used in anti-QS activities. The minimal inhibitory concentration (MIC) of the propolis extracts against the strains was determined using a 96-well microplate reader (Greiner Bio-One, sterile, PP, U-bottom). HPLC grade methanol, ethanol, formic acid, was purchased from Merck (Darmstadt, Germany). The phenolic compounds were used as standards in LC-MS/MS analysis which galangin, rutin trihydrate, kaempferol, quercetin hydrate, quercitrin, *p*-coumaric acid, *trans*-chalcone, caffeic acid phenethyl ester (CAPE), *trans*-ferulic acid, cinnamic acid, luteolin, pinocembrin, caffeic acid, and gallic acid were purchased from Sigma-Aldrich), *m*-coumaric acid was purchased from Fluka, protocatechuic acid, *trans*-isoferulic acid, daidzein, rosmarinic acid, syringic acid, (±)-catechin, (±)naringenin, 3-4 dimethoxycinnamic acid, apigenin, benzoic acid, ellagic acid dihydrate, emodin, pinobanksin, vitexin, (±)epicatechin, (±)epigallocatechin, isorhamnetin, chrysin, methyl syringate, naringin, and myricetin were purchased from Santa-Cruz biotechnology. Ultrapure water was obtained from ELGA® LabWater, Purelab flex.

Propolis Collection and Extraction: Propolis samples were collected in Bursa by using a plastic propolis trap in May, July, and September 2015 (Civan Incorporation, Bursa, Turkey). Firstly, the propolis samples were removed from traps than were stored at -20 ºC until extraction. A coffee bean grinder was used to create powder all propolis samples to homogenization after it was frozen at -20 ºC (Delonghi Kg49, Hampshire, UK). Preparation of ethanol extract propolis (EEP); five grams of the homogenize propolis were weighed and added to 50 ml 70% ethanol/water (v/v) in an Erlenmeyer flask (100 ml) (33). Preparation of water extract propolis

(WEP); five grams of the homogenize propolis were weighed and added to 50 ml ultra-pure water (ELGA® LabWater, Purelab flex) in an Erlenmeyer flask (100 ml). Both extracts were shaken with using a shaker (Nüve SL-35, Turkey) for an hour after followed by ultrasonication (Bandelin Sonorex RK100, Germany) for 30 minutes. The mixtures were filtered by using filter papers after waited one hour in the freezer at +4 ºC to remove the wax and bee part (Watman No: 1, UK). EEP and WEP were stored at +4 ºC until the experimental study. 5 ml extract was poured into glass tubes, which was tared before and evaporated using a vacuum concentrator (Jouan, RC 10-10) for HPLC-DAD analyses and determination of percent of resin (33). EEP and WEP resin determined as 313 mg/ml, 112 mg/ml respectively. Both propolis extracts were adjusted to 100 mg resin/ ml for anti-quorum sensing activity assays.

Validation of HPLC analyses method: Thirty-six phenolic compounds were injected as single and mixed to the HPLC-DAD to the determination of retention times, calibration curves, and other validation parameters (28). The phenolic compounds chromatogram was presented in Figure 1. Calibration curves were plotted by of 2, 4, 8, 16, and 32 µg/mL concentration of mixed phenolic compounds. All calibration curves of components showed good linearity (R2 between 0.997-0945). The limit of detection (LOD) and limit of quantification (LOQ) ranged from 0.09 to 1.6 µg/ml and 0.27 to 4.8 µg/ml, respectively. The relative standard deviations values of precision test, coefficient of variation were less than 2.9% and 4.7%. Concentration of 8, 16, and 32 µg / mL mixed phenolic compound were spiked in propolis extracts to determine the recovery. The spiked propolis samples are analyzed five different days as five times for recovery, accuracy, and repeatability of the method. In the recovery test results of accuracy ranged from 80% to 108% and RSD values less than 3.2%.

Analysis of HPLC-DAD: Ethanol and water extract dry propolis firstly was diluted as a 1/40 ratio with its extraction solvent, and then filtered through a polyvinyl difluoride (PVDF) syringe filter (Millipore Millex-HV, 0.45 µm). Finally, 5 µL was injected into the HPLC-DAD system (n=3) (22). The EEP and WEP-analysis were performed using high-performance liquid chromatography (Shimadzu Kyoto, Japan). HPLC system was equipped with a pump (LC-20AD), auto-sampler (SIL 20 AC), detector (SPD-M20A), for separation was used by column intersil ODS (4.6 x 150 mm ID, 5 µm). The UV wavelength was set at 270-360 nm. The column was eluted using a linear gradient as follows: mobile phase A (0.1% formic acid in water) and mobile phase B (acetonitrile) with a flow rate of 1 mL/min. The gradient elution was applied; 3–10% B at 0,02–3 min, 10-13% B at 3-30 min, 13-16% B at 30-60 min, 16-17% B at 60-70 min, 17-18% B at 70-80 min, 18-30% B at 80-120 min, 30-35% B at 120-135 min, 35-40% B at 135-170 min, 40-45% B at 170-172 min, 45-60% B at 172-182 min, 60-90% B at 182-202 min, 90% B at 202-203 min, 90-30% B at 203-205 min and 30-3% B at 205-206 min. The column temperature was set at +30 ºC.

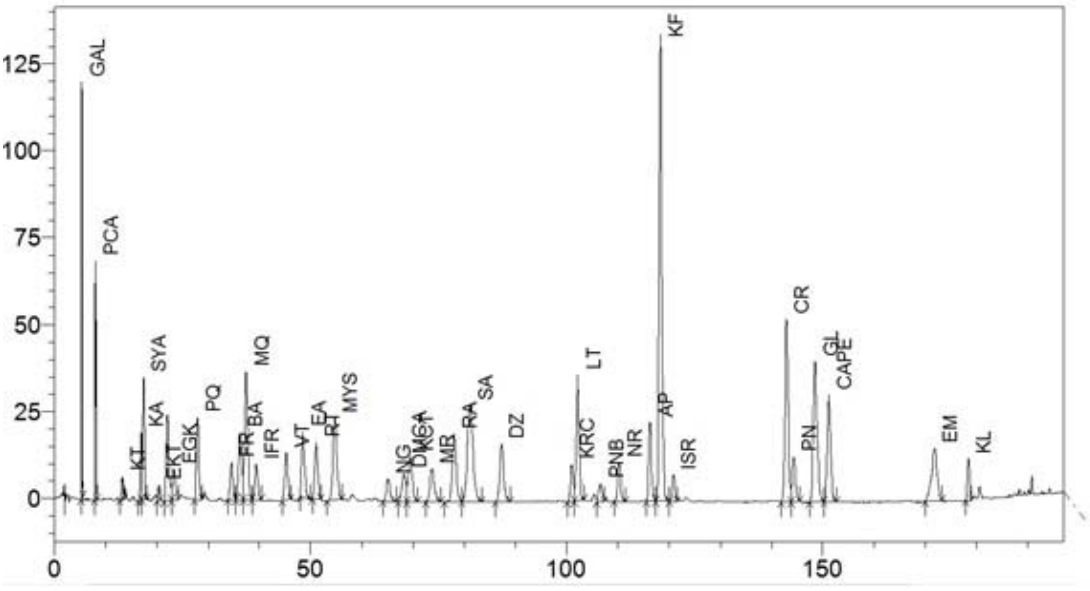

Figure 1. The HPLC-DAD chromatogram of mixed phenolic compounds in 270 nm.

Gallic acid (GAL), protocatechuic acid (PCA), (±)-catechin (KT), caffeic acid (KA), syringic acid (SYA), (±)epicatechin (EKT), (±)epigallocatechin (EGK), *p*-coumaric acid (PQ), *trans*-ferulic acid (FR), benzoic acid (BA), *m*-coumaric acid (MQ), *trans*-isoferulic acid (IFR), vitexin (VT), ellagic acid (EA), rutin (RT), methyl syringate (MYS), naringin (NG), 3-4 dimethoxycinnamic acid (DMCA), quercitrin (KCT), myricetin (MR), rosmarinic acid (RA), cinnamic acid (SA), daidzein (DZ), quercetin (KRC), luteolin (LT), pinobanksin (PNB), (±)naringenin (NR), apigenin (AP), kaempferol (KF), isorhamnetin (ISR), chrysin (CR), pinocembrin (PN), galangin (GL), caffeic acid phenethyl ester (CAPE) emodin (EM)and *trans*-chalcone (KL).

Antimicrobial activity

Microorganisms and conditions for cultivation: *Staphylococcus aureus* ATCC 25923, *Listeria monocytogenes* ATCC 7644, *Escherichia coli* ATCC 25922, and *Candida albicans* ATCC 10239 were obtained from the American Type Culture Collection. *Streptococcus mutans* CNCTC 8/77 was obtained from the Czechoslovak Collection of Type Cultures Institute of Hygiene and Epidemiology. The above-mentioned bacteria, except *Streptococcus mutans*, were cultured in Nutrient Broth (NB) at 37 ± 0.1˚C; *Streptococcus mutans* were cultured in Brain Heart Infusion Broth (BHIB) at 37±0.1°C, *Candida albicans* cultured in Sabouraud Dextrose Broth (SDB) at 28 ± 0.1°C. Inoculate was prepared by adjusting the turbidity of the medium to match the 0.5 Mcfarland Standard. Dilutions of the suspension in 0.1% peptone (w/v) solution in sterile water were inoculated on NB, BHIB, SDB to check the viability of the preparation. The cultures of bacteria were maintained in their appropriate agar slants at 4°C throughout the study and used as stock cultures.

Agar Well Diffusion Methods: The antimicrobial activity of propolis extracts was studied by the well diffusion method (18, 34). The test microorganisms were activated in the above media at appropriate temperatures for 24 h. Petri dishes containing 20 mL of Muller Hinton agar were prepared previously and inoculated with 0.1 ml of 24 h broth culture of test microorganisms. After this stage, the plates were kept in the refrigerator for 30 min. Then, the holes with a diameter of 6 mm have punched aseptically with a sterile cork borer, and a volume (50 µL) of propolis extracts at desired concentration (25, 50, 100 mg/mL) were introduced into the wells. The petri dishes were incubated at 37 °C for 24 h. Then the diameter of the inhibition zone was measured with calipers in mm. The antimicrobial activity was determined by measuring the clear zone around the wells.

Anti-quorum sensing activity assay

Bacterial strains, growth media, and conditions: The biomonitor strains *Chromobacterium violaceum* CV 026, *Chromobacterium violaceum* ATCC 12472, and *Pseudomonas aeruginosa* PA01 were used for the assay of QSI effects of the propolis extracts. CV 026 (growth with Kanamycin at 20 mg/L) and CV 12472 cultures were grown in Luria Bertani (LB) broth at 30 °C with shaking. PA01 cultures were grown on LB agar (LBA) plates at 37 °C.

Determination of MIC: The MIC of propolis extracts was determined against biosensor strains (CV12472, CVO26, and PAO1) by broth microdilution method (35). MIC is defined as the minimum concentration of propolis extracts at which there was no visible growth of test strain. The test medium was Mueller–Hinton Broth (MHB) and the density of bacteria was 5×105 colony-forming units (CFU)/mL. Cell suspensions (200 µL) were inoculated into the wells of 96-well microtitre plates in the presence of propolis extracts with different final concentrations (0.78, 1.56, 3.125, 6.25, 12.5, 25, 50, 100 mg/mL). The wells containing only MHB and MHB with inoculum were employed as negative and positive controls, respectively. The inoculated microplates were incubated at 37 °C for 24 h. The lowest concentration of the tested samples, which did not show any visual growth of tested organisms after macroscopic evaluation, was determined as MIC, which was expressed in mg/mL. Each assay was performed in triplicate for all microorganisms. Sub-MICs were selected for the assessment of anti-QS activity in the above-mentioned strains.

Anti-Quorum Sensing (Anti-QS) Activity Assay: The Anti-QS activity of propolis extracts using CV 026 was assayed by the agar well diffusion method (14). In the presence of approximately a standardized amount of natural C_6-AHL. Briefly, LB agar plates were spread with 0.1 mL of approximately diluted (*c.* $2.5x10^6$ CFU mL^{-1}) freshly grown cultures, and 6 mm diameter wells were cut and varying amounts (50 µl) of appropriately diluted propolis extracts in ethanol were loaded along with natural C_6-AHL. Plates were incubated for 18-24 h at 28 °C to check the inhibition of pigment production around the well. Growth inhibition, if any, was also recorded.

Violacein Inhibition Assay: The propolis extracts were subjected to the qualitative analysis to find out their QSI potentials against *C. violaceum* ATCC 12472 (20). Overnight culture (10 µl) of *C. violaceum* (adjusted to 0.4 OD at 600 nm) was added into wells of sterile microtiter plates (MTP) containing 200 µl of LB broth and incubated in the presence and absence of sub-MIC concentrations of propolis extracts. These MTPs were incubated at 30 °C for 16 h and observed for the reduction in violacein pigment production. The percentage of violacein inhibition was calculated by following the formula: percentage of violacein inhibition= (control OD_{585} -test OD_{585} / controlOD_{585}) ×100.

Anti-swarming Activity Assay: The anti-swarming potential of propolis extracts was performed by following the method specified by Packiavathy et al., 2012 (23). For this assay, 5 µl (0.4 OD at 600 nm) overnight culture of PA01 were inoculated at the center of the swarming agar medium consisting of 1% peptone, 0.5% NaCl, 0.5% agar and 0.5% of filter-sterilized D-glucose with increasing concentrations of propolis extracts (25, 50 and 100 µg/ml). The plates were then incubated at 30 °C in an upright position for 16 h. The reduction in swimming and swarming migration was recorded by measuring the swim and swarm zones of the bacterial cells after 16 h.

Statistical analysis: The results were presented as mean±SD of three independent experiments. Statistical differences were determined by one-way ANOVA followed by Dunnett's test and unpaired t-test using Graph pad Prism software. Differences were considered significant at P≤0.05.

Results

Antimicrobial activities of propolis extracts against test microorganisms were conducted by well diffusion method, and antimicrobial results were given in Table 1 (P<0.05). Well diffusion test results reveal that both propolis extracts had an antimicrobial effect against Gram-positive strains and *C. albicans*. However, the propolis extracts had no antimicrobial effect against *E. coli,* which was a Gram-negative strain. While the highest antimicrobial effects were determined that inhibition zone of 20 mm against *L. monocytogenes* of WEP, 16 mm inhibition zones against *S. aureus* of EEP (Table 1). Both of the propolis extracts were found weak antimicrobial activity against *S. mutans* that inhibition zone between 6-9 mm. WEP showed higher antibacterial activity than EEP. While both extracts showed inhibition effect against the Gram-positive bacteria and yeast, non-effect against the Gram-negative bacteria. It was revealed that the antimicrobial effect of both propolis extracts varied depend on the dose.

MIC results were obtained from the broth microdilution method of propolis extracts against biomonitor strains used for anti-QS tests were presented in Table 2. The lowest MIC concentration against *C. violaceum* CV026 was detected in EEP as 1.56 mg/mL. However, the WEP against the same strain showed inhibition at a concentration of 50 mg/ml. The MIC concentrations against *C. violaceum* CV12472 were detected in EEP 12.5 mg/ml, and in WEP 25 mg/ml, while the MIC against *P. aeruginosa* PA01 in both extracts was determined above 100 mg/mL. Moreover, anti-quorum sensing and violacein pigment inhibition tests were conducted using MIC and sub-MICs propolis extracts.

The sub-MICs ranges were selected for anti-QS screening using CV026 strain and agar well diffusion method adopted in the presence of C_6-AHL. The anti-QS activity test results of propolis extracts were given in Table 3 and Figure 2 (P<0.05). While the anti-QS activity of the EEP measured at the concentration of 1.56 mg/ml with an inhibition zone of 14.17±1.61 mm and the concentration 0.78 mg/ml with an inhibition zone of 11.5±0.5 mm, the anti-QS activity of WEP was measured at 50 mg/ml concentration with an inhibition zone of 12.67±1.15 mm. Anti-QS inhibition zones were observed decreased proportionally depending on the dose of both extracts.

Table 1. Antimicrobial activities of propolis extracts.

Concentration Microorganisms	**WEP**			**EEP**		
	mg/mL					
	100	**50**	**25**	**100**	**50**	**25**
	Inhibition zones (mm±**SD**)					
L. monocytogenes ATCC 7644	20±0.41[a]	18±0.81[b]	17±0.0[c]	15±0.47[a]	13±0.41[b]	11±0.0[c]
S. mutans CNCTC 8/77	9±0.47	7±0.0	NI	7±0.41	6±0.0	NI
S. aureus ATCC 25923	18±0.41[a]	16±0.0	15±0.0	16±0.47[a]	15±0.0	13±0.47
E. coli ATCC 25922	NI	NI	NI	NI	NI	NI
C. albicans ATCC 10239	15±0.47	13±0.0[b]	12±0.0[c]	15±0.47	11±0.0[b]	10±0.0[c]

NI: No inhibition, WEP: Water extract propolis, EEP: Ethanol extract propolis, SD: Standard deviation
[a,b,c] Values within a row with same superscripts differ significantly at P<0.05 on the same concentration.

Table 2. MIC result of the propolis extracts against biosensor strains.

	Microorganisms		
	***C. violaceum* CV026**	***C. violaceum* CV12472**	***P. aeruginosa* PA01**
		mg/mL	
WEP	50	25	>100
EEP	1.56	12.5	>100

WEP: water extract propolis, EEP: ethanol extract propolis.

Table 3. Anti-QS activity of propolis extracts against *Chromobacterium violaceum* CV026.

	Antimicrobial Activity	Anti-QS activity
WEP (mg/mL)	**Inhibition zones (mm)±SD**	
50	NI	12.67±1.15
40	NI	11.5±0.5
30	NI	11.0±0.5
20	NI	10.67±0.58
EEP (mg/mL)		
1.56	10.5±0.5	14.17±1.61
0.78	9.33±0.76	11.5±0.5
0.39	8.5±0,5	10.17±0.29
0,195	8.0±0	9.67±0.29
C10HSL	NI	32.17±1.61
Ethanol	8.17±0.29	8.5±0.5

WEP: water extract propolis, EEP: ethanol extract propolis, SD: standard deviation, NI: No inhibition.

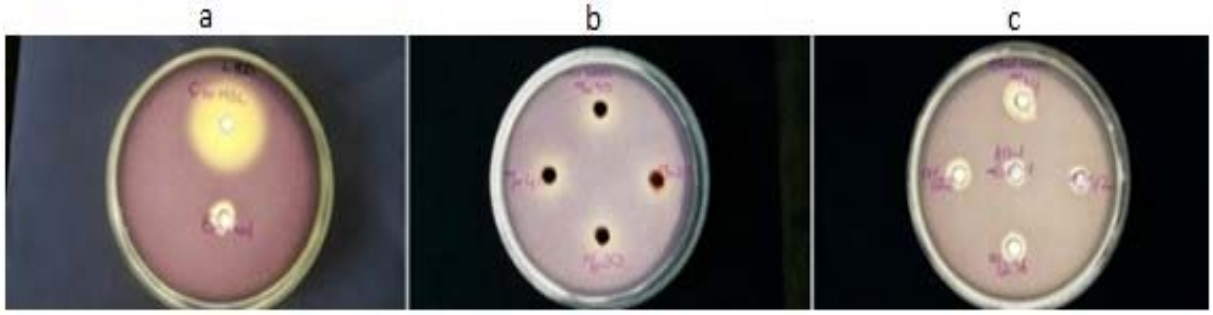

Figure 2. Anti-QS activity by propolis extracts against biosensor strain CV026 using agar well diffusion method.
a: C_{10}HSL control, b: water extract propolis plate, c: ethanol extract propolis.

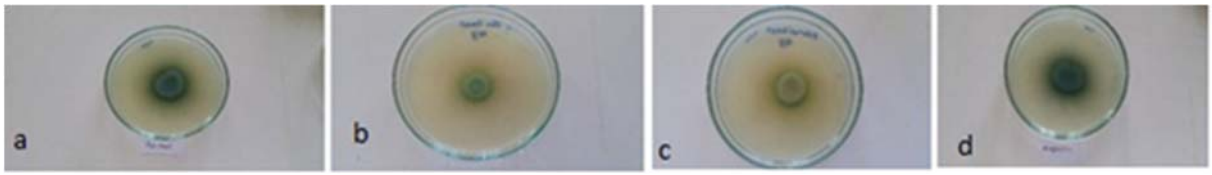

Figure 3. Swarming motility inhibition of propolis extracts in PA01.
a: control group, b. water extract propolis (25 µg/mL). c. ethanol extract (25 µg/mL) d. Ampicillin (10 µg/mL).

The violacein pigment inhibition tests of propolis extracts against CV 12472 strain were determined by the microplate dilution method using MIC and sub-MIC (Table 4, $P<0.05$). Although both extracts inhibited the production of violacein pigment 100% in MIC, EEP was observed to more inhibit the production of violacein pigment compare to WEP, according to Sub-MIC (Table 4).

Inhibition of swarming motilities of WEP and EEP were determined using strain. Results were presented in Table 5 and Figure 3 ($P<0.05$). The propolis extracts performed swarming motility inhibition between 39.13% and 13.04%, depending on concentration according to results. EEP showed higher rates of swarming inhibition activity than WEP (Table5).

HPLC analysis revealed that the number of phenolic compounds in the EEP was more than the WEP apart from benzoic acid and cinnamic acid. Some phenolic compounds were not determined in both of propolis extracts which gallic acid, m-coumaric acid, methyl syringate, naringin, quercetin, myricetin, rosmarinic acid, and daidzein (Table 6). While syringic acid, (±)epigallocatechin, vitexin, rutin, and trans-chalcone were only determined in WEP, catechin, caffeic acid, ellagic acid, pinobanksin, naringenin, and emodin were determined only in EEP. The present study generally indicated that the phenolic compounds in propolis more soluble in EEP.

WEP inhibition zones were determined to be larger than EEP when the inhibition zones of the antimicrobial activity was examined (Table 1). The reason for the more antibacterial activity of WEP to EEP was thought to be due to phenolic compounds which are more soluble in water or only in WEP. Although the antibacterial activity of EEP was weaker compared to WEP, anti-quorum sensing, violacein pigment inhibition, and swarming motility inhibition activity was determined higher than WEP. The phenolic compounds which are more soluble in ethanol or only consist of EEP may have caused the high anti-quorum sensing, violacein pigment inhibition, and swarming motility inhibition activity than the WEP (Table 2, 3, 4, and 5).

Table 4. Concentration-dependent inhibition of violaceum by propolis extracts in *C. violaceum* CV12472.

Propolis concentration	Reduction in the absorbance of violacein (%)	
(mg/mL)	WEP±SD	EEP±SD
MIC	100±0.01	100±0.01
MIC/2	72.24±0.01	85.94±0.03
MIC/4	46.77±0.02	81.65±0.03
MIC/8	41.95±0.08	69.17±0.01
MIC/16	36.47±0.08	67.46±0.01
MIC/32	20.57±0.07	39.01±0.003
MIC/64	5.54±0.06	26.93±0.01

WEP: water extract propolis, EEP: ethanol extract propolis, SD: standard deviation.

Table 5. Swarming motility inhibition in *Pseudomonas aeruginosa* PA01.

	Swarming motility inhibition (%)	
Extract concentration (µg/mL)	WEP±SD	EEP±SD
100	30.43±0.62[a]	39.13±0.35[a]
50	26.09±0.82[b]	34.78±0.43[b]
25	13.04±0.33[c]	30.43±0.22[c]
Ampicillin (10 µg/mL)	26.09±0.41	
Kanamycin (10 µg/mL)	No	
Ethanol	No	

WEP: water extract propolis, EEP: ethanol extract propolis, SD: standard deviation
[a,b,c] Values within a row with same superscripts differ significantly at $P<0.05$ on the same concentration.

Table 6. Phenolic Compounds in Propolis Samples Analysis (HPLC-DAD).

Phenolic Compounds	R time (min)	WEP (mg/kg)	EEP (mg/kg)
Gallic acid	5.338	ND	ND
Protocatechuic acid	7.996	14.61	15.50
(±)-Catechin	13.232	ND	12.91
Caffeic acid	16.832	ND	1021. 71
Syringic acid	17.376	2.22	ND
(±)epicatechin	20.196	6.82	31.02
(±)Epigallocatechin	23.248	3.91	ND
p-Coumaric acid	27.822	17.76	265.77
trans-Ferulic acid	34.456	24.60	141.52
Benzoic acid	36.224	197.21	130.90
m-Coumaric acid	37.280	ND	ND
trans-Isoferulic acid	39.265	302.02	425.38
Vitexin	45.043	5.63	ND
Ellagic acid	48.294	ND	30.93
Rutin	50.900	3.74	ND
Methyl syringate	54.603	ND	ND
Naringin	64.914	ND	ND
3-4 Dimethoxycinnamic acid	68.032	527. 82	1946.41
Quercitrin	69.248	ND	ND
Myricetin	73.486	ND	ND
Rosmarinic acid	78.344	ND	ND
Cinnamic acid	80.945	189.22	142.10
Daidzein acid	87.101	ND	ND
Quercetin	100.798	22.26	78.31
Luteolin	102.368	2.74	16.36
Pinobanksin	106.423	ND	2228.45
(±)Naringenin	110.070	ND	50.07
Apigenin	116.113	82.41	251.58
Kaempferol	118.147	ND	100.72
Isorhamnetin	120.681	27.07	173.34
Chrysin	142.719	618.54	1472.02
Pinocembrin	144.153	357.77	1859.83
Galangin	148.185	1095.85	3655. 08
CAPE	150.990	69.14	3319.89
Emodin	171.582	ND	44.67
trans-Chalcone	178.308	77.33	ND

ND: non-determined, R time: retention time, WEP: water extract propolis, EEP: ethanol extract propolis.

Discussion and Conclusion

Researchers have studied the effectiveness of propolis on Gram-negative and Gram-positive bacteria (2, 24, 27, 31). Results of the present study are similar to previous studies which showed tht propolis has an antibacterial activity gram-positive bacteria and yeast, while it doesn't have any effect on gram-negative bacteria (4, 12, 19, 24). However, previous studies investigating the anti-QS activity of propolis with different methods were quite limited. Lamberte et al. (16) determined the ethanol extract propolis has anti-QS activity against *Chromobacterium violaceum* ATCC 12472 and *Pseudomonas aeruginosa* PAO1. While Lamberte et al. (16) reveal that low doses prevented biofilm formation than the high doses, the present study was increasing the dose-dependent. Savka et al. (29) found the violacein inhibition on *Chromobacterium violaceum* CV026 of propolis, which has higher in pinocembrin and flavonoid. Similarly, in the present study was found violacein inhibition higher in EEP that contains more pinocembrin and flavonoids than WEP. Gemiarto et al. (11) determined the higher antibacterial activity and anti QS activity than was detected in the present study, while similar results were obtained for violacein inhibition. Bulman et al. (6) revealed that violacein inhibition on *Chromobacterium violaceum* (CV026), and anti-swarming activity on *Pseudomonas aeruginosa* (PAO1) of the propolis. Kasote et al. (13) found an antifungal activity of propolis which has high content pinocembrin and also violacein inhibition activity of propolis, which has high content caffeic acid. Although a significant difference was observed in the present study between EEP (1859.83 mg/kg) and WEP (357.77 mg/kg) in terms of pinocembrin level, no significant difference was found in antifungal activity. Similarly, in the present study, EEP that has high levels of caffeic acid was found to be higher in the inhibition of violacein. De Marco et al. (8) did not report the anti-swarming activity of *P. aeruginosa* of 85% ethanol extract propolis. While the amount of pinocembrin, galangin, and chrysin in the propolis was found to be higher than the present study, the CAPE was lower. Moreover, Djais et al. (9) have found no effect of propolison biofilm formation on *S. mutans* (ATCC 25175).

In the present study, it was determined that propolis has an antimicrobial activity due to dose and extraction solvent against Gram-positive bacteria and yeasts. Numerous studies have demonstrated that propolis possesses a marked antibacterial and moderate antifungal activites. However, both propolis extracts did not show any activity against Gram-negative bacteria. It is generally recognized that Gram-positive bacteria are more susceptible to the antibacterial activity of propolis than Gram-negative bacteria. Anti-quorum sensing, violacein inhibition, and anti-swarming activity of propolis were also determined depending on the dose and extraction solvent. Although EEP had lower antibacterial activity when compared with WEP, anti-quorum sensing, violacein inhibition, and anti-swarming activity were found out higher than WEP. The results of this study show that the phenolic compounds in propolis have a direct effect on antibacterial activity, anti-quorum sensing, violasein inhibition and anti-swarming activity depending on the extraction solvent.

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Ethical Statement

This study does not present any ethical concerns.

Conflict of Interest

The authors declared that there is no conflict of interest.

References

1. **Alvarez MV, Moreira MR, Ponce A** (2012): *Antiquorum Sensing and Antimicrobial Activity of Natural Agents with Potential Use in Food*. J Food Saf, **32**, 379-387.
2. **Astani A, Zimmermann S, Hassan E, et al** (2013): *Antimicrobial activity of propolis special extract GH 2002 against multidrug-resistant clinical isolates*. Die Pharmazie, **68**, 695-701.
3. **Banskota AH, Tezuka Y, Kadota S** (2001): *Recent progress in pharmacological research of propolis*. Phytother Res, **15**, 561-571.
4. **Boonsai P, Phuwapraisirisan P, Chanchao C** (2014): *Antibacterial activity of a cardanol from Thai Apis mellifera propolis*. Int J Med. Sci, **11**, 327.
5. **Bulman Z, Hudson A, Savka MA** (2011). *Propolis, a product of the bee hive, has an antagonistic affect on quorum-sensing regulated bioluminescence, gene transcription and motility*. The FASEB Journal, **25**, 948.
6. **Bulman Z, Le P, Hudson AO, et al** (2011): *A novel property of propolis (bee glue): anti-pathogenic activity by inhibition of N-acyl-homoserine lactone mediated signaling in bacteria*. J Ethnopharmacol, **138**, 788-797.
7. **De Castro S** (2001): *Propolis: biological and pharmacological activities. Therapeutic uses of this bee-product*. Annu Rev Biomed, **3**, 49-83.
8. **De Marco S, Piccioni M, Pagiotti R, et al** (2017): *Antibiofilm and antioxidant activity of propolis and bud poplar resins versus Pseudomonas aeruginosa*. eCAM, **2017**.
9. **Djais AA, Putri N, Jemmy J, et al** (2019): *Effect of Propolis on Streptococcus mutans Biofilm Formation*. Pesqui Bras Odontopediatria Clin Integr, **19**, 5221.
10. **Dong Y-H, Zhang L-H** (2005): *Quorum sensing and quorum-quenching enzymes*. J Microbiol , **43**, 101-109.
11. **Gemiarto AT, Ninyio NN, Lee SW, et al** (2015): *Isoprenyl caffeate, a major compound in manuka propolis, is a*

quorum-sensing inhibitor in Chromobacterium violaceum. Anton Leeuw Int J G, **108**, 491-504.

12. **Gonsales G, Orsi R, Fernandes Júnior A, et al** (2006): *Antibacterial activity of propolis collected in different regions of Brazil*. J Venom Anim Toxins Incl Trop Dis, **12**, 276-284.
13. **Kasote D, Ahmad A, Chen W, et al** (2015): *HPTLC-MS as an efficient hyphenated technique for the rapid identification of antimicrobial compounds from propolis*. Phytochemistry Letters, **11**, 326-331.
14. **Koh KH, Tham F-Y** (2011): *Screening of traditional Chinese medicinal plants for quorum-sensing inhibitors activity*. J Microbiol Immunol Infect, **44**, 144-148.
15. **Kubiliene L, Laugaliene V, Pavilonis A, et al** (2015): *Alternative preparation of propolis extracts: comparison of their composition and biological activities*. BMC Complement Altern Med, **15**, 156.
16. **Lamberte LE, Cabrera EC, Rivera WL** (2011): *Activity of the Ethanolic Extract of Propolis (EEP) as a Potential Inhibitor of Quorum Sensing-Mediated Pigment Production in Chromobacterium violaceum and Virulence Factor Production in Pseudomonas aeruginosa*. Philipp Agric Sci, **94**, 14-22.
17. **Lotfy M** (2006): *Biological activity of bee propolis in health and disease*. Asian Pac J Cancer Prev, **7**, 22-31.
18. **Magaldi S, Mata-Essayag S, De Capriles CH, et al** (2004): *Well diffusion for antifungal susceptibility testing*. Int J Infect Dis, **8**, 39-45.
19. **Majiene D, Trumbeckaite S, Pavilonis A, et al** (2007): *Antifungal and antibacterial activity of propolis*. Curr Nutr Food Sci, **3**, 304-308
20. **McLean RJ, Pierson III LS, Fuqua C** (2004): *A simple screening protocol for the identification of quorum signal antagonists*. J Microbiol Methods, **58**, 351-360.
21. **Noori A, Al-Ghamdi A, Ansari MJ, et al** (2012): *Synergistic effects of honey and propolis toward drug multi-resistant Staphylococcus aureus, Escherichia coli and Candida albicans isolates in single and polymicrobial cultures*. Int J Med Sci, **9**, 793.
22. **Nyandwi R, Kılıç AS, Çelik M, et al** (2019): *Determination and Quantification of Gallic Acid in Raw Propolis by High-performance Liquid Chromatography–Diode Array Detector in Burundi*. East Afr J Sci, **1**, 43-48.
23. **Packiavathy IASV, Agilandeswari P, Musthafa KS, et al** (2012): *Antibiofilm and quorum sensing inhibitory potential of Cuminum cyminum and its secondary metabolite methyl eugenol against Gram negative bacterial pathogens*. Food Res Int, **45**, 85-92.
24. **Pobiega K, Kraśniewska K, Derewiaka D, et al** (2019): *Comparison of the antimicrobial activity of propolis extracts obtained by means of various extraction methods*. J Food Sci Technol, **56**, 5386-5395.
25. **Przybyłek I, Karpiński TM** (2019): *Antibacterial properties of propolis*. Molecules, **24**, 2047.
26. **Rasmussen TB, Givskov M** (2006): *Quorum-sensing inhibitors as anti-pathogenic drugs*. Int J Med Microbiol, **296**, 149-161.
27. **Santos HCD, Vieira DS, Yamamoto SM, et al** (2019): *Antimicrobial activity of propolis extract fractions against Staphylococcus spp. isolated from goat mastitis*. Pesqui Vet Bras, **39**, 954-960.
28. **Sariyev R, Çayci M, Oruç HH** (2019): *Azerbaycan Propolislerinin Fenolik Madde İçerikleri ve Kalite Açısından Değerlendirilmesi.* J Res Vet Med, **38,** 1-6.
29. **Savka MA, Dailey L, Popova M, et al** (2015): *Chemical composition and disruption of quorum sensing signaling in geographically diverse United States propolis*. eCAM, **2015**.
30. **Sforcin JM, Bankova V** (2011): *Propolis: is there a potential for the development of new drugs?* Journal of Ethnopharmacology, **133**, 253-260.
31. **Silva JC, Rodrigues S, Feás X, et al** (2012): *Antimicrobial activity, phenolic profile and role in the inflammation of propolis*. Food ChemToxicol, **50**, 1790-1795.
32. **Song X, Xia Y-X, He Z-D, et al** (2018): *A review of natural products with anti-biofilm activity*. Curr Org Chem, **22**, 789-817.
33. **Sorucu A, Oruç HH** (2019): *Determination of biologically active phenolic compounds in propolis by LC–MS/MS according to seasons and altitudes*. J Food Meas Charac, **13**, 2461-2469.
34. **Volpi N, Bergonzini G** (2006): *Analysis of flavonoids from propolis by on-line HPLC–electrospray mass spectrometry*. J Pharm Biomed Anal, **42**, 354-361
35. **Wayne P** (2011): Clinical and laboratory standards institute. Performance standards for antimicrobial susceptibility testing.

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Genomic prediction and association analyses of energy corrected milk yield in dairy cows

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Abstract: Energy balance plays a critical role in the maintenance of metabolism for producing milk yield (MY) in dairy cows. In recent years, there has been increasing interest in genetic and genomic analyses of MY. In contrast to MY there is much less information about genomic evaluation of energy corrected milk yield (ECMY). The purpose of this paper is to detect associated single nucleotide polymorphisms (SNPs) with ECMY and genomic prediction (GP) of ECMY using different genomic models with special reference to underlying genetic architecture of ECMY. In this study we used published data of 773 Holstein cows with phenotypic observations for ECMY and dairy farm information with 62410 SNPs. One interesting finding is that some short chromosomes as such chromosomes 5 (included 28446 SNP) and 29 (included 12776 SNP) had higher effects sizes compared with the rest of the genome. A possible explanation for these results may be related with the existence of major genes at the chromosome 5. The GP results showed that ECYM and residuals of ECYM, had the accuracies from a 10-fold cross validations as 0.6422 and 0.3529 respectively. It was found that ECMY could be used for GP due to moderate accuracies. Taken together, dairy farm effects suggest an impact for accuracies of GP.
Keywords: Energy corrected milk yield, genome wide association analyses, genomic selection, milk yield.

Süt sığırlarında enerjice düzeltilmiş süt veriminin genomik tahmin ve ilişki analizleri

Özet: Süt sığırlarında, süt verimi (SV) için enerji dengesi ile metabolizmanın korunması önemlidir. SV için genetik ve genomik analizlerine olan ilgi son yıllarda önem kazanmıştır. Enerjice düzeltilmiş süt verimi (EDSV) konusunda ise SV'den farklı olarak daha az araştırma bulunmaktadır. Bu çalışmanın amacı EDSV'ye sebep olabilecek tek nükleotid polimorfizmlerini (TNP) belirlemek ve bunlar üzerinden farklı genomik modeller kullanarak genomik tahminler (GT) yapmaktır. Bu çalışmada daha önceden yayınlanmış bir veri seti kullanılarak, 773 Holstayn ineğe ait EDSV gözlemleri ile 62410 TNP ve çiftlik bilgileri incelenmiştir. Beşinci kromozom gibi kısa bir kromozomda (28446 TNP) ve 29. kromozomda (12776 TNP) GT için genomun diğer bölgelerine göre daha yüksek etki büyüklükleri belirlenmiştir. Bu durum 5. kromozomda yer alan major bir gen ile açıklanabilir. GT sonuçları EDSV ve EDSV kalıntıları ile elde edilmiş ve 10 katlı çapraz sorgulama ile 0,6422 ve 0,3529 doğruluk oranları bulunmuştur. Bu da ECYM'nin GT modellerinde orta doğrulukta kullanılabileceğini göstermiştir. Bu çalışmada; çiftlik etkilerinin GT doğruluklarında bir etkiye sahip olduğu gösterilmiştir.
Anahtar sözcükler: Enerjice düzeltilmiş süt verimi, genom tabanlı ilişki analizi, genomik seleksiyon, süt verimi.

Introduction

Energy balance plays a critical role in the maintenance of metabolism for producing milk yield (MY) in dairy cows. For instance, energy deficit postpartum is a common condition which has a considerable impact on the productional and functional traits in dairy cows (10). In recent years, there has been increasing interest in genetic and genomic analyses of MY. In contrast to MY there is much less information about genomic evaluation of energy corrected milk yield (ECMY) (4, 10). Genomic ECMY findings should make an important contribution to the field of animal breeding and husbandry by genomic selection of superior animals in shorter generation intervals.

Genome wide association studies (GWAS) are fast becoming a key instrument for detecting associated genes with the phenotypes based on molecular markers as such single nucleotide polymorphisms (SNPs). A considerable amount of literature has been published on GWAS of MY in dairy cows. Previous GWAS research has established

that various loci are correlated with MY. Jiang et al. (9) conducted a GWAS for various milk production traits using 294.079 first-lactation Holstein cows and detected strong genomic signal from chromosome 14 (DGAT1 gene) in association with MY. Han et al. (7) studied and detected the effects of nucleobindin 2 (NUCB2) gene on milk production traits using Chinese Holstein cattle. Iung et al. (8) investigated the impact of tropical condition to GWAS of Brazilian Holstein population for milk production traits and detected various genomic signals from Microsomal glutathione S-transferase 1 (MGST1), ATP-binding cassette super-family G member 2 (ABCG2), (Diacylglycerol O-Acyltransferase 1) DGAT1 and progestagen-associated endometrial protein (PAEP) genes. Lopdell et al. (13) analyzed the data from Holstein, Jersey, and crossbred populations and detected Colony Stimulating Factor 2 Receptor Subunit Beta (CSF2RB) gene in connection with milk production traits. Wang et al. (25) carried out a GWAS based on Chinese Holstein population and detected genomic signals from 7 SNPs for MY. The research to date has tended to focus on MY rather than ECMY. To date there is only one study that has investigated the ECYM in GWAS (8). Hence the use of ECMY in genomic prediction (GP) has not yet been investigated. The present research explores, for the first time, GP of ECMY with different genomic models.

Li et al. (11) examined the genetic correlations among ECMY, dry matter intake and body weight using different cattle breeds and concluded that the genetic correlations varied over lactations and showed similar patterns within each breed. ECMY is a principal determining factor of energy balance, compared with dry matter intake (10). This indicates a need to understand the genomic evaluation of ECMY by predicting associated SNPs and/or genes.

The purpose of this paper is to detect associated SNPs with ECMY (4) and genomic prediction of ECMY using different genomic models with special reference to underlying genetic architecture of ECMY.

Materials and Methods

In this study we used published data of (4). The GWAS analyses included 773 Holstein cows with phenotypic observations for ECMY and dairy farm information. The 773 cows had 624100 SNPs obtained from Illumina BovineHD BeadChip. The details of the dataset could be found at (4).

Population stratification, or systematic genotypic differences due to sources of variations may lead to false positive signals in GWAS. We used linear mixed model for correction of population stratification as was implemented in GenABEL (1) using genomewide rapid association using mixed model and regression (GRAMMAR-gamma) (11,21) approach in R software (17).

The linear mixed model used as

$$y = Xb + Za + e \qquad (1)$$

where y contains the observations, b is the dairy farm a is the additive genetic effect, matrices X and Z are incidence matrices, and e is a vector containing residuals.

$$Var\begin{pmatrix} a \\ e \end{pmatrix} \sim N\left[\mathbf{0};\begin{pmatrix} \mathbf{A}\sigma_a^2 & \mathbf{0} \\ \mathbf{0} & \mathbf{I}\sigma_e^2 \end{pmatrix}\right],$$

For the random effects, it is assumed that A is the coefficient of coancestry obtained from genotype of animals; I is an identity matrix, σ^2_a is the additive genetic variance and σ^2_e is the residual variance. In GWAS the huge number of hypothesis may cause the inflated number of false positive genomic signals (22). One advantage of the false discovery rate (FDR) approach is that it avoids the problem of false positive genomic signals by increasing significance levels to 0.05/(number of SNPs).

Different from major SNPs effects used in model (1) (27) defined sparse and larger variances to model SNPs effects as "Bayesian sparse linear mixed models", BSLMM, (15) used mixture of two normal distributions and additional random effects to have more flexible model compared with other Bayesian models.

We used BSLMM for prediction of SNP effects;

$$y_i = farm + \sum_{j=1}^{n}(z_{ij}\alpha_j\delta_j) + e_i \qquad (2)$$

where y_i is the phenotypes of the *i*th animal, z_{ij} is an indicator variable (small or major effects from the two normal distributions) for the *i*th animal, *j*th SNP locus and *k*th allele, α_j is marker locus effects, δ_j is indicating if SNP has effect or not and e_i is the residual for animal *i*. To see if the various assumptions regarding genetic architecture of the ECMY gave different results, the number of mixtures increased. Different from model (2) BayesR (15) assumed a mixture of four normal distributions for the SNP effects to be predicted (assumed to be 0.00001, 0.0001, 0.001,0.01 of the genetic variances). For each phenotype the Markov Chain Monte Carlo (MCMC) algorithm were run for 1.000.000 samples and first 2000 samples discarded as burn in period. We collected each 10th samples from each realization of the MCMC as thinning period.

One of the most well-known model for assessing polygenic effects in GP is to use of genomic relationship matrix in (1) where a refers to animals termed as genomic best linear unbiased prediction (GBLUP) (17). We used GBLUP, BayesR and BSLMM for prediction of phenotypes using known genotypes based on their breeding values (BV) or small gene effects (ALPHA) (27).

The whole genomic dataset was partitioned by reference and validation set. ECMY measurements of the validation set were assumed to be missing. Phenotypes of the validation set were predicted using the information from the reference set. A random sample of reference set (2/3 of the animals, n=517) was used to create predictive equations. This procedure was repeated 10 times. Correlation coefficient between the predicted and realized phenotypes of the validation animals was calculated over 10 replications.

Results

The main aim of this study was to detect gene variants that associated with ECMY using 624100 SNPs and 773 cows. In order to investigate population stratification we used a multi-dimensional scaling (MDS) analysis (1). Figure 1 presents an overview of stratification by genotypes of cows based on top two genomic principal components using identity by descent information over MDS analysis. As shown in Figure 1 the main cow population are closely related but still separate clusters exist in the MDS plot (Figure 1).

The mean heterozygosity for a SNP was 0.3552 (0.1396) and for a cow was 0.3587 (0.014). GWAS assume homogeneous populations for contrasting frequencies of the SNPs to detect putative genomic associations. By employing single regression models (SRM) with correction for the population stratifications may lead to valid results of the GWAS. In order to take population stratification into account for SRM, we predicted genomic relationship matrix (1) and conducted the association analyses using the function "gamma" (SRM) as was implemented in GenABEL package (1). Table 1 shows the GWAS results of the SRM with false discovery rate for multiple hypothesis testing correction. The genomic inflation factor found to be 1.025 with standard error of 0.000041. The estimated genomic heritability of ECMY was 0.8541.

The most significant SNP on chromosome 5 (Table 1) was within the QTLs of milk fat percentage, milk protein percentage and milk fat yield (18). The second QTL on the same locus of chromosome 5 (Table 1) associated with body weight (58.3-70.8 Mbp) (19). Other significant SNP was BovineHD1700005467 (Table 1) whose importance in milk palmitoleic acid content has been defined (20) at the vicinity of chromosome 17 at 17.1-22.4 mega base pairs. The SNP on chromosome 6 (BovineHD0600008918) was identified in association with body weight in cattle at 35.56 Mb (2).

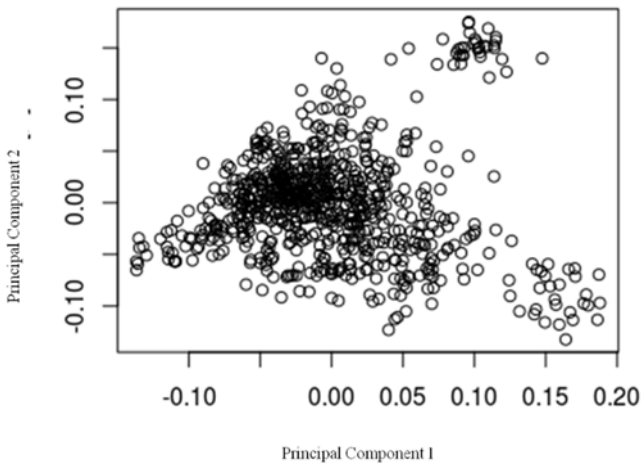

Figure 1. Multi-dimensional scaling analyses of genotypes.

Table 1. Top SNPs of the single regression model with GRAMMAR-gamma correction analyses of ECMY for false discovery rate of 0.05.

Marker	Chromosome	P value	BP	False Discovery Rate
BovineHD0500016776	5	1.11E-10	59905836	0.0000693
BovineHD3000016842	30	6.46E-10	58003830	0.000202
BTB-01179030	14	1.27E-09	60164356	0.000264
BovineHD1700005467	17	4.72E-09	18948241	0.000736
BovineHD0600008918	6	2.25E-08	31792754	0.002808
BovineHD1800014456	18	5.21E-08	49004334	0.005419
BovineHD1500019853	15	6.38E-08	68743862	0.005688
BovineHD0800008984	8	1.42E-07	29615889	0.011078
BovineHD0200029838	2	6.37E-07	103773772	0.04013
BovineHD1300000094	13	6.43E-07	599841	0.04013
BovineHD1300000090	13	7.16E-07	584808	0.040623

In order to assess the genetic architecture of ECMY, different effect sizes of the SNPs effects were used by BayesR model. This was done because the SRM model only assumed SNPs with major effects for the ECMY. Table 2 presents the summary statistics for the top ten SNPs obtained by BayesR. Table 3 provides the breakdown of genetic variance according to chromosomes. The number of SNPs associated with ECMY changed considerably among chromosomes. Highest proportion of the total variance is explained by chromosome 5 (Table 1).

Table 4 compares the correlation coefficients for GP using different models for ECYM and corrected ECYM for dairy farm effects. On average correlations were shown to have similar results for different models for ECYM and the residuals of ECYM. However, from the Table 4, it can be seen that corrected ECYM resulted in the lowest correlations compared with ECYM. Data for the MY and residuals of MY in Table 1 can be compared with the ECYM and residuals of ECMY which shows similar trends over different GP models. However, the results of the BayesR resulted in the highest correlations for MY (0.3529) and the lowest for the residuals of MY (0.0268).

Table 2. Top ten SNPs of the BayesR model

SNP	CHR	BP	PROP
BovineHD2900006632	29	23294478	0.044745
BovineHD0500016776	5	59905836	0.038795
BovineHD0800008984	8	29615889	0.034092
BovineHD1800010522	18	34509354	0.013303
Hapmap24310-BTA-162764	15	3335649	0.010461
BovineHD2400005028	24	19198046	0.010436
BovineHD0300012765	3	41843197	0.009673
BovineHD1400019682	14	70036249	0.008425
BovineHD0200000556	2	1929907	0.007427
BovineHD0900018960	9	68547030	0.006995

Table 3. Sum of SNPs effects and number of SNPs obtained over chromosomes from BayesR.

Chromosome	Sum of SNP effects	Number of SNPs by BayesR	Number of SNPs in the map file
1	0.05208	434	38338
2	0.04452	371	32162
3	0.04147	319	29400
4	0.0372	310	29010
5	0.07194	327	28446
6	0.03432	312	30057
7	0.03096	258	26839
8	0.05824	224	22970
9	0.04256	304	25829
10	0.03341	257	25933
11	0.0296	296	27589
12	0.0246	205	21635
13	0.0185	185	16974
14	0.03444	164	17576
15	0.0357	238	21066
16	0.02412	201	20006
17	0.02544	212	19259
18	0.03553	187	17088
19	0.01925	175	16312
20	0.02472	206	18517
21	0.01925	175	17586
22	0.0194	194	15981
23	0.01104	138	13311
24	0.03111	183	15421
25	0.01062	118	11520
26	0.01287	143	13380
27	0.01359	151	11639
28	0.01742	134	11625
29	0.05658	138	12776
30	0.01815	165	15822

Table 4. Pearson correlations of Genomic predictions obtained by different models for ECYM and residuals of ECYM.

Method	ECMY	Residuals of ECMY	MY	Residuals of MY
BayesR	0.6422	0.5046	0.3529	0.0268
BSLMM_BV	0.6275	0.5418	0.2405	0.0541
GBLUP_BV	0.6275	0.5475	0.2429	0.0692
BSLMM_ALPHA	0.6276	0.5422	0.2399	0.0536
GBLUP_ALPHA	0.6244	0.5475	0.2429	0.0694

Discussion and Conclusion

To date only one study have used ECMY as phenotype in GWAS (24). However a strong relationship between ECMY and productional and functional traits has been reported in the literature. An initial objective of the study was to identify SNPs in associated with ECMY. ECMY, as derived trait from MY was used as a response variable in GWAS for the current study. However it is not uncommon to use derived and/ or standardized errors as phenotypes in GWAS. In recent years, there has been an increasing amount of literature on deregressed estimated breeding values (DEBV) as phenotypes in GWAS. A significant analysis and discussion on the subject was presented by (16) and (20). A recent study by (12) involved a GWAS using DEBV for MY in buffalo.

MDS plot detected genotypic clusters using principal components analyses (Table 1). We used SRM model with genotypic relationship matrix to take this relationship into account for GWAS of ECMY. The result of this SRM analyses indicate that there are various genomic signals in association with ECMY (Table 1), particularly from chromosomes of 5, 14 and 30. This finding broadly supports the work of other studies in this area linking ECMY with energy metabolism over MY and body weight. It is somewhat surprising that no gene was detected at vicinity of the chromosome 14 SNP of BTB-01179030 (Table 1). This outcome is contrary to previous studies which have suggested that strong genomic signals for MY from various loci of chromosome 14.

In accordance with the present results, previous studies have demonstrated that complex phenotypes could be explained by genes with small to major effects (15). It was hypothesized that the SNPs could be distributed into four classes according to their effects sizes on the ECMY. As can be seen from the Table 2 that the strongest genomic signal was found to be at chromosome 29 BayesR. These results reflect those of (12): who also found a genomic signal from the similar location for milk protein yield. There was a significant negative linear relationship between number of chromosomes and number of SNPs per chromosomes (Table 3) similar to the other organisms. Contrary to expectations, this study did not find a strict linear relationship between chromosomal sizes, detected number of SNPs and sum of SNPs effects (Table 3) (24). One interesting finding is that some short chromosomes as such chromosomes 5 (included 28446 SNP) and 29 (included 12776 SNP) had higher effects sizes compared with the rest of the genome. A possible explanation for these results may be related with the existence of major genes at the chromosome 5 (Table 1).

GP results of Table 4 is revealing in several ways. The results showed that ECYM and residuals of ECYM using BayesR, had the higher accuracies from a 10-fold cross validations as 0.6422 and 0.3529 respectively. In reviewing the literature, no data was found on the GP of ECMY.

With respect to the research question, it was concluded that ECMY could be used for GP due to moderate accuracies in Table 4. In this study (as environmental and genetical factors) dairy farm effects were found to cause of inflation for accuracies of GP (Table 4). In accordance with the present results, previous studies have demonstrated the effect of environmental factors (dairy farm) for the GP (19, 23, 26). These findings contribute in several ways to our understanding genomics of ECMY and provide a basis for GP studies or pathway/ gene investigations by detected SNPs (Table 1-3).

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Ethical Statement

This study does not present any ethical concerns.

Conflict of Interest

The authors declared that there is no conflict of interest.

References

1. **Aulchenko YS, De Koning DJ, Haley C** (2007): *Genomewide rapid association using mixed model and regression: a fast and simple method for genomewide pedigree-based quantitative trait loci association analysis*. Genetics, **177**, 577-585.
2. **Bennewitz J, Reinsch N, Guiard V, et al** (2004): *Multiple quantitative trait loci mapping with cofactors and application of alternative variants of the false discovery rate*

in an enlarged granddaughter design. Genetics, **168**, 1019-1027.

3. **Buttchereit N, Stamer E, Junge W, et al** (2011): *Genetic relationships among daily energy balance, feed intake, body condition score, and fat to protein ratio of milk in dairy cows*. J Dairy Sci, **94**, 1586-1591.
4. **Clancey E, Kiser JN, Moraes JG, et al** (2019): *Genome-wide association analysis and gene set enrichment analysis with SNP data identify genes associated with 305-day milk yield in Holstein dairy cows*. Anim Genet, **50**, 254-258.
5. **Gebreyesus G, Buitenhuis AJ, Poulsen NA, et al** (2019): *Multi-population GWAS and enrichment analyses reveal novel genomic regions and promising candidate genes underlying bovine milk fatty acid composition*. BMC Genom, **20**, 178.
6. **Habier D, Fernando RL, Garrick DJ** (2013): *Genomic BLUP decoded: a look into the black box of genomic prediction*. Genetics, **194**, 597-607.
7. **Han B, Yuan Y, Li Y, et al** (2019): *Single nucleotide polymorphisms of NUCB2 and their genetic associations with milk production traits in dairy cows*. Genes, **10**, 449.
8. **Iung LHS, Petrini J, Ramírez-Díaz J, et al** (2019): *Genome-wide association study for milk production traits in a Brazilian Holstein population*. J Dairy Sci, **102**, 5305-5314.
9. **Jiang J, Ma L, Prakapenka D, et al** (2019): *A Large-Scale genome-wide association study in U.S. holstein cattle*. Front Genet, **14**, 412.
10. **Krattenmacher N, Thaller G, Tetens J** (2019): *Analysis of the genetic architecture of energy balance and its major determinants dry matter intake and energy-corrected milk yield in primiparous Holstein cows*. J Dairy Sci, **102**, 3241-3253.
11. **Li B, Fikse WF, Løvendahl P, et al** (2018): *Genetic heterogeneity of feed intake, energy-corrected milk, and body weight across lactation in primiparous Holstein, Nordic Red, and Jersey cows*. J Dairy Sci, **101**, 10011-10021.
12. **Liu JJ, Liang AX, Campanile G, et al** (2018): *Genome-wide association studies to identify quantitative trait loci affecting milk production traits in water buffalo*. J Dairy Sci, **101**, 433-444.
13. **Lopdell TJ, Tiplady K, Couldrey C, et al** (2019): *Multiple QTL underlie milk phenotypes at the CSF2RB locus*. Genet Sel Evol, **51**, 1.
14. **McClure MC, Morsci NS, Schnabel RD, et al** (2010): *A genome scan for quantitative trait loci influencing carcass, post-natal growth and reproductive traits in commercial Angus cattle*. Anim Genet, **41**, 597-607.
15. **Moser G, Lee SH, Hayes BJ, et al** (2015): *Simultaneous discovery, estimation and prediction analysis of complex traits using a Bayesian mixture model*. PLoS Genet, **11**, e1004969.
16. **Ostersen T, Christensen OF, Henryon M, et al** (2011): *Deregressed EBV as the response variable yield more reliable genomic predictions than traditional EBV in pure-bred pigs*. Genet Sel Evol, **43**, 38.
17. **R Development Core Team** (2013): A language and environmental for statistical computing. R Foundation for Statistical Computing; Vienna, Austria.
18. **Saatchi M, Schnabel RD, Taylor JF, et al** (2014): *Large-effect pleiotropic or closely linked QTL segregate within and across ten US cattle breeds*. BMC Genom, **15**, 442.
19. **Schultz NE, Weigel KA** (2019): *Inclusion of herdmate data improves genomic prediction for milk-production and feed-efficiency traits within North American dairy herds*. J Dairy Sci, **102**, 11081-11091.
20. **Song H, Li L, Zhang Q, et al** (2018): *Accuracy and bias of genomic prediction with different de-regression methods*. Animal, **12**, 1111-1117.
21. **Svishcheva GR, Axenovich TI, Belonogova NM, et al** (2012): *Rapid variance components-based method for whole-genome association analysis*. Nat Genet, **44**, 1166-1170.
22. **Tam V, Patel N, Turcotte M, et al** (2019): *Benefits and limitations of genome-wide association studies*. Nat Rev Genet, **20**, 467-484.
23. **Tiezzi F, de Los Campos G, Gaddis KP, et al** (2017): *Genotype by environment (climate) interaction improves genomic prediction for production traits in US Holstein cattle*. J Dairy Sci, **100**, 2042-2056.
24. **Veerkamp RF, Coffey MP, Berry DP, et al** (2012): *Genome-wide associations for feed utilisation complex in primiparous Holstein-Friesian dairy cows from experimental research herds in four European countries*. Animal, **6**, 1738-49.
25. **Wang D, Ning C, Liu JF, et al** (2019): *Short communication: Replication of genome-wide association studies for milk production traits in Chinese Holstein by an efficient rotated linear mixed model*. J Dairy Sci, **102**, 2378-2383.
26. **Yao C, De Los Campos G, VandeHaar M, et al** (2017): *Use of genotype × environment interaction model to accommodate genetic heterogeneity for residual feed intake, dry matter intake, net energy in milk, and metabolic body weight in dairy cattle*. J Dairy Sci, **100**, 2007-2016.
27. **Zhou X, Carbonetto P, Stephens M** (2013): *Polygenic modeling with Bayesian sparse linear mixed models*. PLoS Genet, **9,** e1003264.

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Decontamination of *Salmonella* Typhimurium with chitosan and lactic acid on broiler carcasses

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Abstract: *Salmonella* Typhimurium is frequently isolated from chicken meat. The main purpose of current study was to analyze the decontamination of *S.* Typhimurium by using different concentrations of chitosan, lactic acid and chitosan and lactic acid combination on broiler carcasses. *S.* Typhimurium was inoculated to broiler carcasses at 10^8 cfu/mL in eight different study groups. Then, contaminated carcasses were treated with 1%, 2% lactic acid and 0.1%, 0.05% chitosan for 5, 10, 15 min. Also, effects of the combination of chitosan and lactic acid (0.05% chitosan- 1% lactic acid, 0.01% chitosan- 1% lactic acid) were analyzed for 5, 10, 15 min. Carcasses samples treated with chitosan and lactic acid were analysed for survival of *S.* Typhimurium on the 0, 3 and 7 days of storage time. Lactic acid (1%, 2%), combination of chitosan and lactic acid (0.05% chitosan + 1% lactic acid and 0.01% chitosan and 1% lactic acid) were detected to have antimicrobial effect on *S.* Typhimurium inoculated into carcasses ($P<0.05$). There is no difference between the working groups in terms of implementation time ($P>0.05$). According to the study, it was found that the combination of lactic acid and chitosan is the most effective method against *S.* Typhimurium in poultry carcasses. As a result, it is thought that the decontaminant agents which preferred in the study can be used in various applications in the poultry industry.
Keywords: Broiler carcass, Chitosan, Lactic acid, *Salmonella* Typhimurium.

Broiler karkaslarında *Salmonella* Typhimurium'un kitosan ve laktik asit ile dekontaminasyonu

Özet: *Salmonella* Typhimurium, tavuk etlerinden en sık izole edilen patojenden biridir. Bu çalışmada, çeşitli konsantrasyonlarda kullanılan kitosan, laktik asit, kitosan ve laktik asit kombinasyonunun *S.* Typhimurium ile kontamine olmuş etlik broyler karkaslarına etkileri araştırılmıştır. *S.* Typhimurium broiler karkaslarına 10^8 kob/mL inokule edilerek 8 farklı grup oluşturulmuştur. Daha sonra kontamine edilen karkaslar %1, %2 laktik asit, %0,05, %0,1 kitosan, laktik asit ve kitosan kombinasyonları ile 5, 10 ve 15 dakika boyunca muamele edilmiştir. Kitosan ve laktik asit ile muamele edilmiş karkas örneklerin, 0., 3. ve 7. günlerde analizleri yapılmıştır. Karkaslara inokule edilen *S.* Typhimurium üzerine laktik asit (%1, %2), kitosan ve laktik asit kombinasyonunun (%0,05 kitosan +%1 laktik asit ve %0,01 kitosan ve %1 laktik asit) antimikrobiyal etkiye sahip olduğu tespit edilmiştir ($P<0,05$). Uygulama süresi açısından çalışma grupları arasında fark olmadığı gözlendi ($P>0,05$). Bu çalışmada, laktik asit ve kitosan kombinasyonunun kanatlı karkaslarında *S.* Typhimurium'a karşı en etkili yöntem olduğu doğrulanmıştır. Sonuç olarak çalışmada kullanılan dekontaminant ajanların kümes hayvanı endüstrisindeki çeşitli uygulamalarda kullanılabileceği düşünülmektedir.
Anahtar sözcükler: Broiler karkas, Kitosan, Laktik asit, *Salmonella* Typhimurium**.**

Introduction

Meat and meat products consumed in the diet contribute significantly to the intake of energy, protein, and micronutrients (13). Poultry meat is frequently preferred around the world because of its high protein ingredient, balance of polyunsaturated fatty acids (n-6, n-3), low fat and cholesterol content, affordable price compared to red meat (10, 49). Therefore, poultry meat constitutes 30% of the meat consumed in the world (17).

Poultry meat is an important reservoir for pathogenic bacteria and is often associated with foodborne diseases (9, 20, 21, 53). *Salmonella* spp. are potential zoonotic agents that can pose a danger to society (62). Based on reported studies, *Salmonella enterica* serovars are among the most important foodborne pathogens (4). Among the *Salmonella* serotypes, *S.* Typhimurium and *S.* Enteritidis are the two most common serotypes, and *S.* Typhimurium is known as the most dominant isolated serotype

worldwide (23, 67). Therefore, it is important to keep *S.* Enteritidis and *S.* Typhimurium separate from other *Salmonella* serotypes as they are specified in zoonosis control legislation and have differences in epidemiology (3, 32). Also, *Salmonella* spp. can infect a variety of animals such as sheep, cattle, poultry and pigs (59). *Salmonella* spp. can be transmitted to humans by way of contact with infected animals, polluted water and the environment. However, cases in humans are mostly via contaminated food products (19, 25). The consumption of poultry meat and egg are the most important source of *Salmonella* spp. for humans (37, 64).

In addition to general hygiene rules, organic chemicals are used as a decontamination fluid in the poultry and meat industry to destroy or reduce pathogenic microorganisms on the carcass surface (7, 44, 45). Organic acids have been found to be effective in reducing foodborne pathogens such as *Escherichia coli* O157:H7, *S.* Typhimurium and *Listeria monocytogenes* on carcass surfaces (34, 57). Among the organic acids, lactic acid (LA) is frequently used as a decontamination agent in broiler carcasses. Many studies have been carried out on lactic acid application to control pathogenic bacteria in broiler carcasses. (1, 47, 48).

Chitosan obtained by deacetylation of chitin is a naturally sourced polycationic biopolymer in the form of a powder that is insoluble in water, high viscosity, non-toxic, non-antigenic, soluble in organic acids (50, 66). Due to the various properties of chitosan, it is widely used in biotechnology, pharmacy, medicine, veterinary medicine, water treatment, textile, agriculture, cosmetics and food industries (5, 12, 29, 43, 57). It has been stated in various sources that chitosan can be used as an alternative in storage foods and increasing their shelf life (46, 52). Chitosan is a potential protective additive for foods with its antimicrobial effect on foodborne pathogen bacteria, mold and yeast (6, 11, 39). Chitosan can be dissolved in organic acids. Therefore, the use of chitosan alone or in combination with other organic acids is among the strategies that can be preferred in reducing the microbial load on the animal carcass surface (14, 40).

In the present study, antimicrobial effect of chitosan, lactic acid, chitosan and lactic acid combination used in various concentrations on broiler carcasses contaminated with *S.* Typhimurium were investigated.

Materials and Methods

Preparation of bacterial strain: The stock strain used in this study were, *S.* Typhimurium (ATCC 14028). Cultures were incubated in 10 mL Tryptic Soy Broth (Oxoid, UK) for at 35ºC at 24 h. The microbial density was set to 0.5 McFarland turbidity standard approximately bacteria density of 10^8 cfu/mL^{-1}. The prepared strain mixture was used for broiler carcass contamination within 30 min.

***Contamination of broiler carcasses with S.* Typhimurium:** In this present study, broiler carcasses (1.2-1.4 kg) saled from the local market were used. A total of 144 broiler carcasses were used in the study. Except the control group each experimental group, were contaminated with 10^8 cfu/mL^{-1} bacterial culture. The broiler carcasses were kept in bacterial culture for 30 min for adhesion.

Decontamination agents, groups and analysis: In the current study, chitosan (Sigma, US), lactic acid (Sigma, US) and their combinations were used. Contaminated carcasses were treated with 1%, 2% lactic acid and 0.1%, 0.05% chitosan for 5, 10, 15 min. Also, effects of the combination of chitosan and lactic acid (0.05% chitosan- 1% lactic acid, 0.01% chitosan- 1% lactic acid) were anaylsed with negative and positive control. The exposure times of the carcasses to decontaminant fluids were subdivided into 5, 10, 15 min. Microbiological analyzes were performed in these groups on days 0, 3 and 7. After each experiment, the carcass samples were kept in a refrigerator at 4°C. Chemical decontaminants which used in carcass decontamination and their prepared concentrations are shown in Table 1.

Table 1. Decontamination agents and concentrations used in broiler carcasses.

Decontaminants	Concentrations
Lactic acid	1% and 2%
Chitosan solution	0.1% and 0.05%
Combination of chitosan ve lactic acid	0.05% + 1% and 0.01% + 1%

Analysis procedure of broiler carcasses: Microbiological analysis of chicken carcasses decontaminated with *S.* Typhimurium were performed on days 0, 3 and 7. Firstly, chicken carcasses were washed with sterile distilled water in sterile bags and rinsed with peptone water (Biolife, Italy) in order to find out whether there was *Salmonella* contamination. Afterwards freshly processed broiler carcasses were rinsed with decontaminant fluids, and the rinses were serially diluted 10-fold with 0.1% peptone water. The samples were then spread on xylose lysine deoxycholate agar (XLD) (Merck, Germany) and brilliant-green phenol-red lactose sucrose (BPLS) agar (Merck, Germany) by spread plate technique (0.1 ml). After incubation, 1 - 2.5 mm in diameter, central black periphery red colonies in XLD agar and 1-1.5 mm in diameter, pink red colonies were considered as *Salmonella* suspects. Suspicious colonies were inoculated into triple sugar iron (TSI) (Merck, Germany) agar and

lysine iron agar (LIA) (Merck, Germany) at 37ºC for overnight. In the end, serologically colonies with suspected *Salmonella* spp. were confirmed by testing with *Salmonella* antiserum (Difco 2264-47-2, US) (18).

Statistical analysis: The obtained data were statistically evaluated by the use of SPSS 25 statistical package. The variables were expressed as mean ± standard deviation and median (Maximum-Minimum) percentage and frequency values. The suitability of the data to the analysis of variance in the factorial order was evaluated with the multivariate normal distribution and Box-M Test of Homogeneity of Variances. Means were compared by a factorial analyese of variance. Parametric tests (analysis of variance in factorial order) that did not meet the prerequisites, the data were reevaluated by box cox data transformation and the obtained data were processed by analysis of variance in factorial order. Corrected LSD Test was used for Multiple comparisons. The significance level was expressed as $P<0.05$.

Results

Negative and Positive Control Group Results: In this study, *Salmonella* negative broiler carcasses were used. In the positive control group, contaminated broiler carcasses with *S.* Typhimurium were determined as 6.60, 6.81, and 6.88 log cfu/mL on days 0, 3, 7, respectively. The negative control group results were detected as negative for the presence of *Salmonella*.

Effects of lactic acid (1% and 2%) application to broiler carcasses: Based on our results, compared with the control group, it was observed that the count of *S.* Typhimurium was 4.16, 4.35, 4.77 log cfu/mL at 5, 10, 15 min on day 0; 5.94, 4.42, 4.27 log cfu/mL at 5, 10, 15 min on day 3; 6.22, 5.83, 4.12 log cfu/mL at 5, 10, 15 min on day 7, respectively. The reduction levels of *S.* Typhimurium were determined as 2.44, 2.25, 1.83 log cfu/mL at 5, 10, 15 min on day 0, 0.87, 2.39, 2.54 log cfu/mL at 5, 10, 15 min on day 3, 0.66, 1.05, 2.76 log cfu/mL at 5, 10, 15 min on day 7, respectively.

In the experimental groups containing 2% lactic acid, the count of *S*. Typhimurium was found 4.05, 3.82, 3.45 log cfu/mL at 5, 10, 15 min on day 0; 3.90, 3.67, 3.33 log cfu/mL at 5, 10, 15 min on day 3; 3.60, 3.93, 3.43 log cfu/mL at 5, 10, 15 min on day 7, respectively. The reduction levels of *S.* Typhimurium were observed as 2.55, 2.78, 3.15 log cfu/mL at 5, 10, 15 min on day 0; 2.91, 3.14, 3.48 log cfu/mL at 5, 10, 15 min on day 3; 3.28, 2.95, 3.45 log cfu/mL at 5, 10, 15 min on day 7, respectively. In accordance with the results of the statistical analysis, it was observed that there was a significant difference between all groups compared to the control group ($P<0.05$), but there was no significant difference between them according to the application times.

Effects of Chitosan (0.05% and 0.1%) application to broiler carcasses: The decrease in the levels of 0.05% and 0.1% chitosan applications of *S.* Typhimurium in 5 min, 10 min and 15 min in comparison with the positive control is shown in Table 2.

Effects of lactic acid and chitosan combinations application to broiler carcasses: The results of *S.* Typhimurium of the experimental groups with the control group containing 0.01% chitosan, 1% lactic acid and 0.05% chitosan and 1% lactic acid combination solution are given in Table 3.

Table 2. Effects of 0.05% and 0.1% chitosan on *S.* Typhimurium.

Concentration of chitosan	*S.* Typhimurium counts (log cfu/mL)								
	5 min			10 min			15 min		
	Day 0	Day 3	Day 7	Day 0	Day 3	Day 7	Day 0	Day 3	Day 7
0.05%	6.53	6.46	7.32	6.29	6.22	6.3	6.57	6.74	6.05
0.1%	6.31	4.44	6.52	6.44	6.42	6.74	6.43	6.38	6.47

Table 3. Effects of Lactic Acid and Chitosan Combinations on *S.* Typhimurium.

Concentration of lactic acid and chitosan combinations	*S.* Typhimurium counts (log cfu/mL)								
	5 min			10 min			15 min		
	Day 0	Day 3	Day 7	Day 0	Day 3	Day 7	Day 0	Day 3	Day 7
0.01% chitosan and 1% lactic acid	1.99	4.82	1.99	1.99	3.14	3.53	1.96	3.78	1.99
0.05% chitosan and 1% lactic acid	1.99	5.38	6.17	1.99	6.17	6.16	1.99	3.93	6.12

Discussion and Conclusion

Poultry meat is a significant reservoir for *Salmonella* spp. (22). The main reason for the occurrence of foodborne salmonellosis in humans originate from contaminated chicken meat consumption (2, 16). Causes of *Salmonella* spp. contamination of poultry meat include slaughtering *Salmonella*-positive flocks in the slaughterhouse, the slaughter equipment, scalding, plucking, evisceration, and cross contamination and lack of personal hygiene (8, 51). *Salmonella* species are most common in fresh broiler meat among the various meat and meat product categories (pork, turkey, beef and ready-to-eat foods prepared from these meats and ground beef) (15). According to the previous studies, one of the predominant serotypes in chicken meat is *S*. Typhimurium (31, 61, 65). It has been assigned as a result of the studies that this situation creates an important potential for public health.

Despite the application of many preservation methods, foodborne infections still cannot be completely prevented. Accordingly, various decontamination methods are used to reduce or completely eliminate the number of pathogenic microorganisms in broiler carcasses to be consumed. Various chemicals are used for decontamination and organic acids take an important place among them (58). Organic acids used as decontamination liquids are frequently applied to the surfaces of various meats and carcasses. Organic acid applications are cheap, simple, fast and effective (24). Among the organic acids, acetic acid and lactic acid have been the most commonly accepted carcass decontaminant fluids (58). Bactericidal or bacteriostatic effect can be obtained on the carcass surface with various organic acids by spraying or dipping on the carcass (55). In addition, the use of organic acids is generally recognized as safe (GRAS) for meat and poultry products by the U.S. Food and Drug Administration (38). The researchers reported that the effect of lactic acid on bacteria was related to the concentration of lactic acid used, the temperature of the lactic acid solution, the method of application, the processing time and the pH-value (54, 57). The use of natural substances such as chitosan together with organic acids for decontamination is increasing day by day. In this study, the inhibitory effects of lactic acid, chitosan, and combinations of chitosan with lactic acid at different doses, different times and different storage days on *S*. Typhimurium were examined. The efficacy and antimicrobial effects of lactic acid in poultry have been studied in many studies. Xiong et al. (63) found that 1% and 2% LA sprayed on chicken skin at room temperature for 30 s reduced *S.* Typhimurium counts by 2.2 log cfu/mL. In a similar study, Li et al. (33) applied 1% lactic acid to chicken carcasses contaminated with *S*. Typhimurium by spraying method for 90 s. As a result of the application, a unit reduction of 1.6 log cfu/mL was observed. In one study, as a result of the treatment of *S*. Typhimurium with 1% and 2% lactic acid for 3 min, a decrease of 1 log and 3.3 log cfu/cm^2 was observed in the count of microorganisms, respectively (41). Mulder et al. (45), in their study on *S*. Typhimurium inoculated into chicken carcasses, they used 0.5% and 1% lactic acid in dipping water at room temperature for 10 min. The result of the application, 1-2 log cfu/mL reduction was detected. In other studies, it has been observed that varying amounts of lactic acid are quite effective on *S.* Typhimurium (28, 36, 57). This study investigated the effect of 1% and 2% lactic acid against *S.* Typhimurium in chicken carcasses. The most effective method in this study was obtained in 2% lactic acid applications for 15 min. According to other studies, the results are similar despite the differences in concentration and application time.

Chitosan which does not show toxic effects, is very effective against foodborne pathogens with its antibacterial and antifungal effects (60). Although the antimicrobial effect of chitosan is not known exactly, it may change the structure of the cytoplasmic membrane due to its cationic feature (42). Moreover, chitosan may bind to DNA and inhibit RNA synthesis (35). The antimicrobial effect of chitosan depends on the type of microorganism, properties of chitosan, physical form of chitosan and environmental factors (30). There are few studies on the effect of chitosan against *Salmonella* spp. in poultry (14, 39, 40). The effect of chitosan on *Salmonella* spp. and *S.* Typhimurium in broiler chickens was investigated by using it alone or in combination with organic acids. In the study conducted by El-Khawas et al. (14), the effect of chitosan (0.5%, 1%, 2%), lactic acid (1%) and combinations of lactic acid (1%) and chitosan (2%) on *S*. Typhimurium was investigated in chicken fillets cooled at 4°C. As a result, they found that the effect of chitosan 2%, lactic acid 1% and chitosan-lactic acid combination applications were approximately 1.5 log cfu/mL reduction. When the study and our results are compared, the effect of lactic acid is in line with our results; however, it was observed that the effect of chitosan was higher than in our study. The reason for this is thought to be due to the concentration used and the solvent differences. In this study, it was concluded that dissolving chitosan with distilled water reduces its effectiveness. In one study, skin samples were dipped into solution containing 10^6 cfu/mL of *S*. Typhimurium for 30 s. Afterwards, skin samples dipped into a solution containing 0.5% chitosan for 30 s. Chitosan has been seen to reduce the number of *S.* Typhimurium after 24 hours (40). When compared with our results, it is seen that the results are parallel according to the applications of chitosan at different densities, different solvents and times. Based on the results, it was observed that the combination of chitosan and lactic acid at different

concentrations made a significant difference compared to the control group. However, it was found that the difference between two different chitosan and lactic acid combination (0.05% + 1% and 0.01% + 1%) applications and the application times (5 min, 10 min, 15 min) was not significant. In a different study, it has been reported that by adding chitosan to the diet of broiler chickens for 7 days, it significantly reduces the rate of *S.* Typhimurium (39). According to the researches, it has been understood that taking derivatives such as chitosan and oligochitosan with diet has a prebiotic effect and supports the growth of beneficial bacteria and helps regulate the immune system (26, 27).

Poultry meat is an important food due to its nutritious properties and economic advantage compared to red meat in Turkey. However, depending on the cutting process, hygienic conditions cannot be fully provided. Consumption of broiler carcasses contaminated with *S.* Typhimurium can pose a danger to human health as well as adversely affect the poultry industry. Therefore, the use of food preservation methods to reduce or eliminate the microorganism count is important in broiler carcasses. The antimicrobial effects of organic acids and chitosan can be used when hygiene practices are inadequate to reduce microbial contamination in broiler carcasses. According to our results, it was found that the combinations of lactic acid and chitosan (0.05% chitosan + 1% lactic acid and 0.01% chitosan and 1% lactic acid) are the most effective method against *S*. Typhimurium in poultry carcasses. The combination of chitosan with lactic acid can be effective for use as a decontamination solution in broiler carcasses. The decontaminants used in the study can be used in the poultry industry to reduce the number of microorganisms and increase the shelf life of the products.

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Ethical Statement

This study does not present any ethical concerns.

Conflict of Interest

The authors declared that there is no conflict of interest.

References

1. **Agırdemır O, Yurdakul O, Keyvan E, et al** (2020): *Effects of various chemical decontaminants on Salmonella Typhimurium survival in chicken carcasses.* Food Sci Technol (Campinas).
2. **Allerberger F** (2016): *Poultry and human infections*. Clin Microbiol Infect, **22**, 101-102.
3. **Anonymus** (2014): Salmonella ve Belirlenmiş Diğer Gıda Kaynaklı Zoonotik Etkenlerin Kontrol Altına Alınması Hakkında Yönetmelik. Avalaible at: https://www.resmigazete.gov.tr/eskiler/2014/03/20140327.pdf. (Accessed Sept 24, 2020).
4. **Antunes P, Mourão J, Campos J, et al** (2016): *Salmonellosis: the role of poultry meat.* Clin Microbiol Infect, **22**, 110-121.
5. **Aranaz I, Acosta N, Civera C, et al** (2018): *Cosmetics and cosmeceutical applications of chitin, chitosan and their derivatives*. Polymers, **10**, 213.
6. **Bhoir SA, Jhaveri M, Chawla SP** (2019): *Evaluation and predictive modeling of the effect of chitosan and gamma irradiation on quality of stored chilled chicken meat.* J Food Process Eng, **42**, e13254.
7. **Bolder NM** (1997): *Decontamination of meat and poultry carcasses*. Trends Food Sci Technol, **8**, 221-227.
8. **Cardinale E, Tall F, Cisse M, et al** (2005): *Risk factors associated with Salmonella enterica subsp. enterica contamination of chicken carcases in Senegal*. Br Poult Sci, **46**, 293-299.
9. **Castañeda-Gulla K, Sattlegger E, Mutukumira AN** (2020): *Persistent contamination of Salmonella, Campylobacter, Escherichia coli, and Staphylococcus aureus at a broiler farm in New Zealand.* Can J Microbiol, **66**, 171-185.
10. **Cavani C, Petracci M, Trocino A, et al** (2009): *Advances in research on poultry and rabbit meat quality*. Ital J Anim Sci, **8**, 741-750.
11. **Chang SH, Chen CH, Tsai GJ** (2020): *Effects of chitosan on Clostridium perfringens and application in the preservation of pork sausage*. Mar Drugs, **18**, 70.
12. **Cheung RCF, Ng TB, Wong JH, et al** (2015): *Chitosan: an update on potential biomedical and pharmaceutical applications.* Mar Drugs, **13**, 5156-5186.
13. **De Smet S, Vossen E** (2016): *Meat: The balance between nutrition and health. A review*. Meat Sci, **120**, 145-156.
14. **El-Khawas KM, Mashat BH, Attala OA, et al** (2020): *Control of Salmonella and Escherichia coli in chilled chicken fillets using chitosan and lactic acid.* CYTA J Food, **18**, 445-450.
15. **European Food Safety Authority and European Centre for Disease Prevention and Control-EFSA/ECDC** (2018): *The European Union summary report on trends and sources of zoonoses, zoonotic agents and food-borne outbreaks in 2017*. EFSA Journal, **16**, e05500.
16. **Foley SL, Nayak R, Hanning IB** (2011): *Population dynamics of Salmonella enterica serotypes in commercial egg and poultry production*. Appl Environ Microbiol, **77**, 4273-4279.
17. **Food and Agriculture Organization-FAO** (2006): Databases: Food Balance Sheets. Avalaible at: http://faostat.fao.org. (Accessed Sept 24, 2020).
18. **Food and Agriculture Organization-FAO** (2016): Bacteriological Analytical Manual, Chapter 5 Salmonella.

Avalaible at: http://www.fda.gov/Food/FoodScienceResearch/LaboratoryMethods/ucm070149.html. (Accessed Sept 24, 2020).

19. **Firouzabadi A, Saadati D, Najimi M, et al** (2020): *Prevalence and related factors of Salmonella spp. and Salmonella Typhimurium contamination among broiler farms in Kerman province, Iran*. Prev Vet Med, **175**, 104838.
20. **Goncuoglu M, Ormanci FSB, Uludag M, et al** (2016): *Prevalence and antibiotic resistance of Salmonella spp. and Salmonella Typhimurium in broiler carcasses wings and liver*. J Food Saf, **36**, 524-531.
21. **Gücükoğlu A, Çadırcı Ö, Gülel GT** (2020): *Serotyping and antibiotic resistance profile of Listeria monocytogenes isolated from organic chicken meat*. Kafkas Univ Vet Fak Derg, **26**, 499-505.
22. **Heredia N, García S** (2018): *Animals as sources of food-borne pathogens: A review*. Anim Nutr, **4**, 250-255.
23. **Herikstad H, Motarjemi Y, Tauxe RV** (2002): *Salmonella surveillance: a global survey of public health serotyping*. Epidemiol Infect, **129**, 1-8.
24. **Hinton MH, Corry JEL** (1999): The decontamination of carcass meat. 285-296. In: R Richardson (Ed), Poultry Meat Science. Oxon, Cabi Publishing.
25. **Hoelzer K, Switt AIM, Wiedmann M** (2011): *Animal contact as a source of human non-typhoidal salmonellosis*. Vet Res, **42**, 34.
26. **Huang RL, Yin YL, Wu GY, et al** (2005): *Effect of dietary oligochitosan supplementation on ileal digestibility of nutrients and performance in broilers*. Poult Sci, **84**, 1383-1388.
27. **Huang RL, Deng ZY, Yang C et al** (2007): *Dietary oligochitosan supplementation enhances immune status of broilers.* J Sci Food Agric, **87**, 153-159.
28. **Hwang C, Beuchat LR** (1995): *Efficacy of selected chemicals for killing pathogenic and spoilage microorganisms on chicken skin*. J Food Prot, **58**, 19-23.
29. **Kabanov VL, Novinyuk LV** (2020): *Chitosan application in food technology: A review of rescent advances*. Food Systems, **3**, 10-15.
30. **Kong M, Chen XG, Xing K** (2010): *Antimicrobial properties of chitosan and mode of action: a state of the art review*. Int J Food Microbiol, **144**, 51-63.
31. **Kuang X, Hao H, Dai M** (2015): *Serotypes and antimicrobial susceptibility of Salmonella spp. isolated from farm animals in China*. Front Microbiol, **6**, 1-11.
32. **Lim YH, Hirose K, Izumiya H, et al** (2003): *Multiplex polymerase chain reaction assay for selective detection of Salmonella enterica serovar Typhimurium.* Jpn J Infect Dis, **56**, 151-155.
33. **Li Y, Slavik MF, Walker JT, et al** (1997): *Pre-chill spray of chicken carcasses to reduce Salmonella Typhimurium.* J Food Sci, **62**, 605-607.
34. **Loretz M, Stephan R, Zweifel C** (2010): *Antimicrobial activity of decontamination treatments for poultry carcasses: a literature survey*. Food Control, **21**, 791-804.
35. **Luo Y, Wang Q** (2013): *Recent advances of chitosan and its derivatives for novel applications in food science*. J Food Process & Bev, **1**, 1-13.
36. **Madushanka DNN, Jayaweera TSP, Jayasinghe JMCS et al** (2018): *Decontaminating effect of organic acids and natural compounds on broiler chicken meat contaminated with Salmonella Typhimurium*. Asian Food Sci J, **3**, 1-9.
37. **Magdy OS, Moussa IM, Hussein HA, et al** (2020): *Genetic diversity of Salmonella enterica recovered from chickens farms and its potential transmission to human*. J Infect Public Health, **13**, 571-576.
38. **Mani-López E, García HS, López-Malo A** (2012): *Organic acids as antimicrobials to control Salmonella in meat and poultry products*. Food Res Int, **45**, 713-721.
39. **Menconi, A., Pumford, N. R., Morgan, et al** (2014): *Effect of chitosan on Salmonella Typhimurium in broiler chickens*. Foodborne Pathog Dis, **11**, 165-169.
40. **Menconi A, Hernandez-Velasco X, Latorre JD et al** (2013): *Effect of chitosan as a biological sanitizer for Salmonella Typhimurium and aerobic gram negative spoilage bacteria present on chicken skin.* Int J Poult Sci, **12**, 318-321.
41. **Mohamed HM, Abdel-Naeem HH** (2018): *Enhancing the bactericidal efficacy of lactic acid against Salmonella typhimurium attached to chicken skin by sodium dodecyl sulphate addition*. LWT-Food Sci Technol, **87**, 464-469.
42. **Moradi M, Tajik H, No HK, et al** (2010): *Potential inherent properties of chitosan and its applications in preserving muscle food.* J Chitin Chitosan, **15**, 35-45.
43. **Morin-Crini N, Lichtfouse E, Torri G et al** (2019): *Applications of chitosan in food, pharmaceuticals, medicine, cosmetics, agriculture, textiles, pulp and paper, biotechnology, and environmental chemistry*. Environ Chem Lett, **17**, 1667–1692.
44. **Morshedy AEMA, Sallam KI** (2009): *Improving the microbial quality and shelf life of chicken carcasses by trisodium phosphate and lactic acid dipping.* Int J Poult Sci, **8**, 645-650.
45. **Mulder RWAW, Van der Hulst MC, Bolder NM** (1987): *Research note: Salmonella decontamination of broiler carcasses with lactic acid, L-cysteine, and hydrogen peroxide*. Poult Sci, **66**, 1555-1557.
46. **No HK, Meyers SP, Prinyawiwatkul W, et al** (2007): *Applications of chitosan for improvement of quality and shelf life of foods: a review*. J Food Sci, **72**, 87-100.
47. **Okolocha EC, Ellerbroek L** (2005): *The influence of acid and alkaline treatments on pathogens and the shelf life of poultry meat*. Food Control, **16**, 217-225.
48. **Park SH, Choi MR, Park JW, et al** (2011): *Use of organic acids to inactivate Escherichia coli O157: H7, Salmonella Typhimurium, and Listeria monocytogenes on organic fresh apples and lettuce*. J Food Sci, **76**, 293-298.
49. **Petracci M, Bianchi M, Mudalal S, et al** (2013): *Functional ingredients for poultry meat products*. Trends Food Sci Technol, **33**, 27-39.
50. **Rabea EI, Badawy MET, Stevens CV, et al** (2003): *Chitosan as antimicrobial agent: applications and mode of action*. Biomacromolecules, **4**, 1457-1465.
51. **Rasschaert G, Houf K, De Zutter L** (2007): *Impact of the slaughter line contamination on the presence of Salmonella on broiler carcasses*. J Appl Microbiol, **103**, 333-341.
52. **Rocha MAM, Coimbra MA, Nunes C** (2017): *Applications of chitosan and their derivatives in beverages: a critical review*. Curr Opin Food Sci, **15**, 61–69.
53. **Rossler E, Olivero C, Lorena PS, et al** (2020): *Prevalence, genotypic diversity and detection of virulence genes in*

thermotolerant Campylobacter at different stages of the poultry meat supply chain. Int J Food Microbiol, **326**, 108641.

54. **Smulders F** (1995): Preservation by microbial decontamination; the surface treatment of meats by organic acids. 253-282. In: GW Gould (Ed), New methods of food preservation. Blackie Academic and Professional, London.
55. **Sofos JN, Smith GC** (1998): *Nonacid meat decontamination technologies: model studies and commercial applications*. Int J Food Microbiol, **44**, 171-188.
56. **Şenel S, McClure SJ** (2004): *Potential applications of chitosan in veterinary medicine.* Adv Drug Deliv Rev, **56**, 1467-1480.
57. **Tamblyn KC, Conner DE** (1997): *Bactericidal activity of organic acids against Salmonella Typhimurium attached to broiler chicken skint*. J Food Prot, **60**, 629-633.
58. **Theron MM, Lues JF** (2007): *Organic acids and meat preservation: a review*. Food Rev Int, **23**, 141-158.
59. **Trongjit S, Angkititrakul S, Tuttle RE, et al** (2017): *Prevalence and antimicrobial resistance in Salmonella enterica isolated from broiler chickens, pigs and meat products in Thailand–Cambodia border provinces*. Microbiol Immunol, **61**, 23-33.
60. **Vargas M, González-Martínez C** (2010): *Recent patents on food applications of chitosan*. Recent Pat Food Nutr Agric, **2**, 121-128.
61. **Wajid M, Awan AB, Saleemi MK, et al** (2018): *Multiple drug resistance and virulence profiling of Salmonella enterica serovars Typhimurium and Enteritidis from poultry farms of Faisalabad, Pakistan*. Microb Drug Resist, **25**, 133–143.
62. **Witkowska D, Kuncewicz M, Żebrowska JP, et al** (2018): *Prevalence of Salmonella spp. in broiler chicken flocks in northern Poland in 2014-2016*. Ann Agric Environ Med, **25**, 693.
63. **Xiong H, Li Y, Slavik MF et al** (1998): *Spraying chicken skin with selected chemicals to reduce attached Salmonella Typhimurium*. J Food Prot, **61**, 272-275.
64. **Yang X, Huang J, Zhang Y, et al** (2020): *Prevalence, abundance, serovars and antimicrobial resistance of Salmonella isolated from retail raw poultry meat in China.* Sci Total Environ, **713**, 136385.
65. **Yildirim Y, Gonulalan Z, Pamuk S, et al** (2011): *Incidence and antibiotic resistance of Salmonella spp. on raw chicken carcasses*. Food Res Int, **44**, 725–728.
66. **Zargar V, Asghari M, Dashti A** (2015): *A review on chitin and chitosan polymers: structure, chemistry, solubility, derivatives, and applications.* Chem Bio Eng Reviews, **2**, 204-226.
67. **Zhao X, Hu M, Zhang Q, et al** (2020): Prevalence and antimicrobial resistance of Salmonella isolated from broilers in Shandong, China. Avalaible at: https://doi.org/10.21203/rs.3.rs-17378/v1. (Accessed Sept 24, 2020).

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Examining the morphometric features of bulbus oculi in Van cats by using computed tomography and magnetic resonance imaging

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Abstract: This study was conducted to obtain the morphometric and volumetric measurements of bulbus oculi of Van cats, growing around the city of Van in Turkey and named after here, by using computed tomography (CT) and magnetic resonance imaging (MRI), and to reveal the biometric differences of these measurement values between the sexes. A total of 16 adult Van cats including 8 females and 8 males were used in the study. The animals were anesthetized with the combination of xylazine and ketamine. The anesthetized animals were scanned by using CT and MRI devices and their images were obtained. Then, the morphometric and volumetric measurements of bulbus oculi were calculated from these images using the software (Syngo CT Software) in the workstation and their statistical analysis was performed. Upon the examination of the morphometric and volumetric analysis results, it was determined that while W (bodyweight) and ACL (Left Anterior Chamber) values were higher in male cats, DVLL (Dorsoventral length of the left lens) value was higher in female cats. These differences between the sexes were statistically significant ($P<0.05$). The volumetric measurement values of bulbus oculi and lens were determined to be averagely 4.60 ± 0.27 cm^3 and 0.67 ± 0.09 cm^3, respectively. In conclusion, the statistical differences of biometric values of bulbus oculi between male and female Van cats were determined by using CT and MRI. It is thought that the present study would contribute to the ophthalmological applications and the students receiving anatomy education.

Keywords: Bulbus oculi, computed tomography, magnetic resonance imaging, morphometry, Van cat.

Van kedilerinde bulbus oculi'nin bilgisayarlı tomografi ve manyetik rezonans görüntüleme ile morfometrik özelliklerinin incelenmesi

Özet: Bu çalışma, Türkiye'de Van ili yöresinde yetiştirilen ve ismini buradan alan Van kedilerinde bulbus oculi'nin bilgisayarlı tomografi (BT) ve manyetik rezonans görüntüleme (MRG) ile morfometrik ve volümetrik ölçülerini elde etmek ve bu ölçüm değerlerinin cinsiyetler arasındaki biyometrik farklılıklarını ortaya koymak amacıyla yapıldı. Çalışmada 8 dişi, 8 erkek olmak üzere toplam 16 adet erişkin Van Kedisi kullanıldı. Hayvanlar ketamin-ksilazin kombinasyonu ile anesteziye alındı. Anestezi altındaki hayvanlar BT ve MRG cihazı ile taranarak görüntüleri elde edildi. Daha sonra bu görüntülerden iş istasyonundaki yazılım programı (Syngo CT Software) kullanılarak bulbus oculi'nin morfometrik ve volümetrik ölçümleri hesaplandı ve istatistiki analizi yapıldı. Morfometrik ve volümetrik analiz sonuçlarına bakıldığında, W (vücut ağırlığı) ve ACL (sol ön kamera) değerlerinin erkek kedilerde; DVLL (sol lens'in dorsoventral uzunluğu) değerinin ise dişi kedilerde daha yüksek olduğu tespit edildi. Cinsiyetler arasındaki bu farkların istatistik olarak önemli düzeyde olduğu gözlendi ($P<0,05$). Bulbus oculi ve lens'e ait volümetrik ölçüm değerleri sırasıyla ortalama $4,60 \pm 0,27$ cm^3 ve $0,67 \pm 0,09$ cm^3 olarak belirlendi. Sonuç olarak, Van kedilerinde bulbus oculi'nin biyometrik değerlerinin istatistiksel olarak erkek ve dişiler arasındaki farklılıkları BT ve MRG kullanılarak tespit edildi. Sunulan çalışmanın bu kedilerle ilgili oftalmatolojik uygulamalara ve anatomi eğitimi alan öğrencilere katkı sağlayacağı düşünülmektedir.

Anahtar sözcükler: Bilgisayarlı tomografi, bulbus oculi, manyetik rezonans görüntüleme, morfometri, Van kedisi.

Introduction

Van cats have attracted great interest in recent years due to its unique physical characteristics and especially being included in the endangered species. Being growing around the city of Van in Turkey and named after here, Van cats are known for their eye colors (their eyes may be amber or blue or they have heterochromia), triangular-shaped head, soft coat on their bodies, fluffy tail, love of swimming and grooming, intelligence, and superior

learning ability. Van cats are an important source of the cultural wealth of the region (21).

The eye is a sense organ that can receive and process light stimuli from the surrounding environment and transmit them to the visual cortex of the central nervous system by converting them into an electrical signal by anatomic and physiological mechanisms (10). Being the organ of sight, the eye consists of bulbus oculi (the eyeball) and organa oculi accessoria (accessory organs of the eye)**.** Bulbus oculi, which has an active role in sight, forms the optic structure of the eye. Bulbus oculi, which is situated in orbit, has a three-layered structure including tunica fibrosa bulbi (sclera and cornea), tunica vasculosa bulbi (choroidea, corpus ciliare, and iris), and tunica interna bulbi (retina) (9). Bulbus oculi varies among animal species and breeds in terms of shape and size. In general, when the domestic mammals are listed based on their body sizes, cats have the biggest bulbus oculi, which is followed by the dog, horse, cattle, and pig, respectively (18).

The technological advancements in the computer-aided medical imaging fields such as computed tomography and magnetic resonance imaging as well as three-dimensional modeling and various software programs enable to determine of the features of many anatomic structures in human or animal bodies, and evaluation easily any pathological change in these structures (2, 39). These imaging methods provide a perfect morphological detail in imaging of the eye and relevant (peripheral) complex anatomic structures particularly in small pets such as cats and dogs (7, 22). Also, the ocular and periocular benign and malignant aggressive tumors, orbital neoplasia, various inflammation cases, cystic structures, traumas, fractures, and foreign bodies in the region can be identified easily by means of CT, MRI, and the three-dimensional reconstruction software. Briefly, they are commonly used in diagnosing eye and orbital diseases and evaluating the diagnosis and treatment activities in veterinary medicine (7, 38).

Various studies concerning the anatomic and morphometric features of bulbus oculi have been conducted on horses (13, 24), cattle (13, 27), pigs (13), camels (16), sheep (8, 11), goats (23), various dog breeds (4, 25, 30, 37), and cats (3, 5, 19) from domestic mammals. However, no study has been found on the bulbus oculi in Van cats in the literature reviews. This study was conducted to provide the imaging of anatomic structures of bulbus oculi of Van cats by using computed tomography and magnetic resonance imaging, obtain its morphometric and volumetric measurements, and reveal the biometric differences of these measurement values between the sexes.

Materials and Methods

Animal materials: In the study, a total of 16 adult Van cats (between 3 and 8 years old, the average bodyweight (W) 4.91 kg for males and 3.60 kg for females), eight females and eight males, were used. The cats were obtained from Van Yüzüncü Yıl University Van Cat Research and Application Center. Ad libitum cat feed and drinking water were provided to these cats until the day before the study. This study was approved by the Van Yüzüncü Yıl University Animal Experiments Local Ethics Committee.

Anesthesia: The cats included in the study were numbered, and were not provided any feed starting the day before the study. Then, the cats were anesthetized with xylazine (1 - 2 mg / kg, IM, Alfazyne® 2 % injectable) - ketamine (15 mg / kg, IM, Ketasol® 10 % injectable) combination.

Imaging with computed tomography: For the computed tomography (CT) tests of the Van Cats, a 16-section multi-sequential computed tomography (CT) device (Somatom Sensation 16; Siemens Medical Solutions, Erlangen, Germany) was used in the Department of Radiology of Medicine Faculty of Van Yüzüncü Yıl University. A disposable sheet was placed onto the gantry of the device, and the cats were laid onto this sheet head-first in a prone position to achieve symmetry. The device parameters for the CT scan were adjusted as follows: Section thickness, 0.5 mm; physical detector collimation, 16 × 0.6 mm; feed/rotation, 6 mm; kernel, U90u; final section collimation, 32 × 0.63 mm; KV/rotation time (sec) values/effective mAs, 120/ 0.75/120; gantry rotation period, 420 ms; increment, 0.5 mm; resolution, 512 × 512 pixels. Scanning and dosage parameters were performed in accordance with standard protocols found in published literature (15, 28). The images obtained were recorded in the DICOM format.

Magnetic resonance imaging (MRI): The Van cats being ready for the study were laid in prone position under anesthesia and they were scanned by MRI. The T1 - weighted sagittal data obtained from the MRI device was used for image analysis. MRI parameters were adjusted as follows; the field of view of 16 cm, the repetition time of 19.2 milliseconds, echo time of 9.5 milliseconds, flip angle of 30°, resolution of 512 x 512 pixels, the resolution range of 0.31 x 0.31 mm^2, and the section thickness of 1 mm. The obtained axial images were transferred to the workstation (Leonardo, Siemens Medical Solutions, Erlangen, Germany) for processing in DICOM format.

Measurements: Then, using the software in the workstation (Syngo CT Software) on these obtained images, the morphometric measurements obtained from the axial, and coronal sections were obtained from CT measurements and the morphometric measurements obtained from sagittal sections were obtained from T1-

weighted MRI images as they were more demonstrative. On the other hand, the volumetric measurements were obtained by sectioning both bulbus oculi and lens from the outer border in all sections respectively from anterior to posterior in axial CT sections, obtaining colored images by means of the special software and then calculating the common volumetric data of all the sections. Figure 1, 2, 3, and 4 shows the morphometric and volumetric measurement points of the bulbus oculi of Van cats. All measurements were made from the widest part of the bulbus oculi and lens. The morphometric and volumetric measurements of the points on the bulbus oculi were determined in Table 1.

Table 1. The measurement points of the bulbus oculi and the abbreviations.

Abbreviation	Explanation
ABR	Axial (anterior- posterior) length of the right bulbus oculi (cm)
ABL	Axial (anterior- posterior) length of the left bulbus oculi (cm)
MLBR	Mediolateral length of the right bulbus oculi (cm)
MLBL	Mediolateral length of the left bulbus oculi (cm)
DVBR	Dorsoventral length of the right bulbus oculi (cm)
DVBL	Dorsoventral length of the left bulbus oculi (cm)
ALR	Axial (anterior- posterior) length of the right lens (cm)
ALL	Axial (anterior- posterior) length of the left lens (cm)
MLLR	Mediolateral length of the right lens (cm)
MLLL	Mediolateral length of the left lens (cm)
DVLR	Dorsoventral length of the right lens (cm)
DVLL	Dorsoventral length of the left lens (cm)
ACR	Right Anterior Chamber (Anterior-posterior distance of the anterior chamber, from the cornea to the rostral border of the lens) (cm)
ACL	Left Anterior Chamber(Anterior-posterior distance of the anterior chamber, from the cornea to the rostral border of the lens) (cm)
PCR	Right Posterior Chamber (Anterior-posterior distance of the posterior chamber, from the caudal aspect of the lens to internal surface of the bulbus oculi of the posterior segment) (cm)
PCL	Left Posterior Chamber (Anterior-posterior distance of the posterior chamber, from the caudal aspect of the lens to internal surface of the bulbus oculi of the posterior segment) (cm)
VLR	Volumetric value of the right lens (cm^3)
VLL	Volumetric value of the left lens (cm^3)
VBR	Volumetric value of the right bulbus oculi (cm^3)
VBL	Volumetric value of the left bulbus oculi (cm^3)

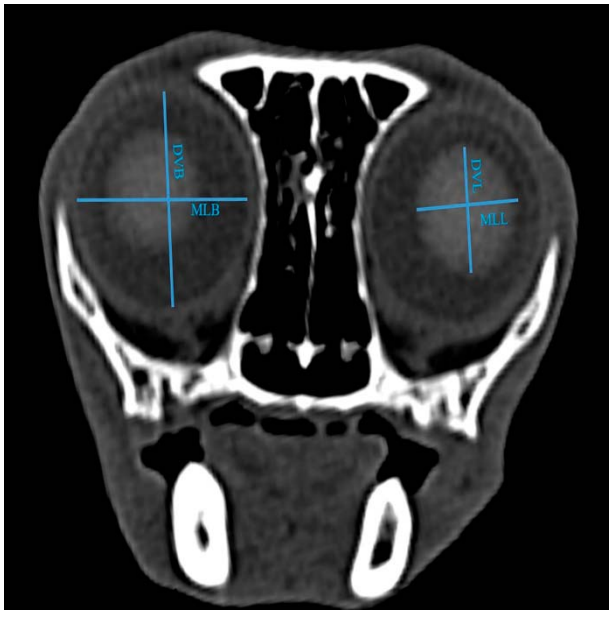

Figure 1. Measurement points of the bulbus oculi in Van cats (axial CT image). DVB: Dorsoventral length of the bulbus oculi; MLB: Mediolateral length of the bulbus oculi; DVL: Dorsoventral length of the lens; MLL: Mediolateral length of the lens.

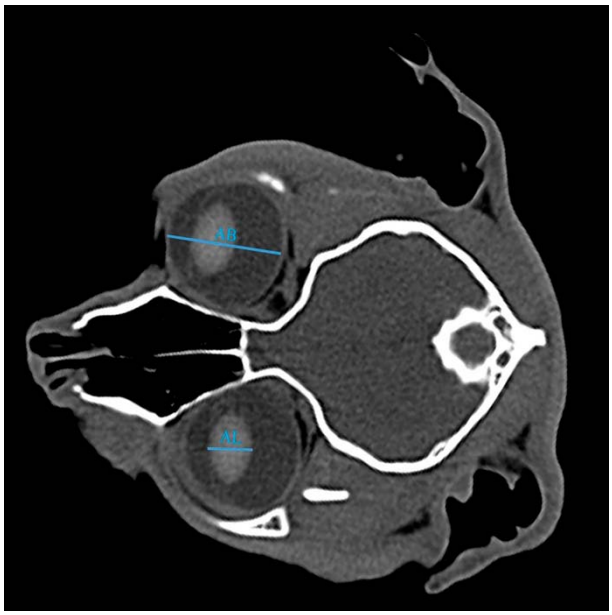

Figure 2. Measurement points of the bulbus oculi in Van cats (coronal CT image). AB: Axial (anterior - posterior) length of the bulbus oculi**;** AL: Axial (anterior - posterior) length of the lens.

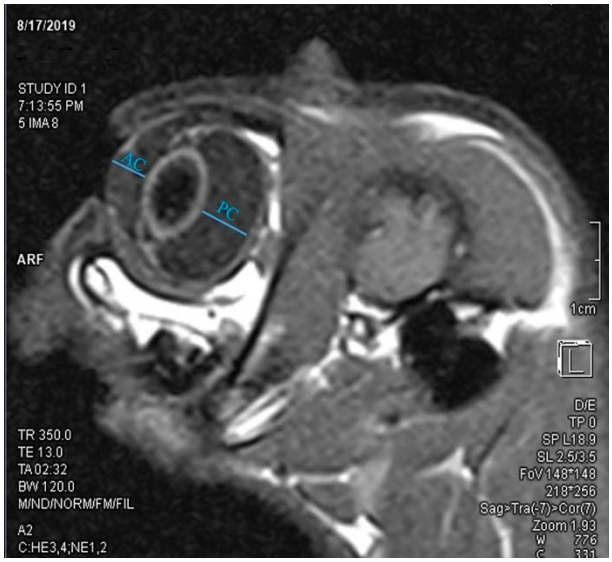

Figure 3. Measurement points of the bulbus oculi in Van cats (sagittal T1-weighted MRI image). AC: Anterior Chamber; PC: Posterior Chamber.

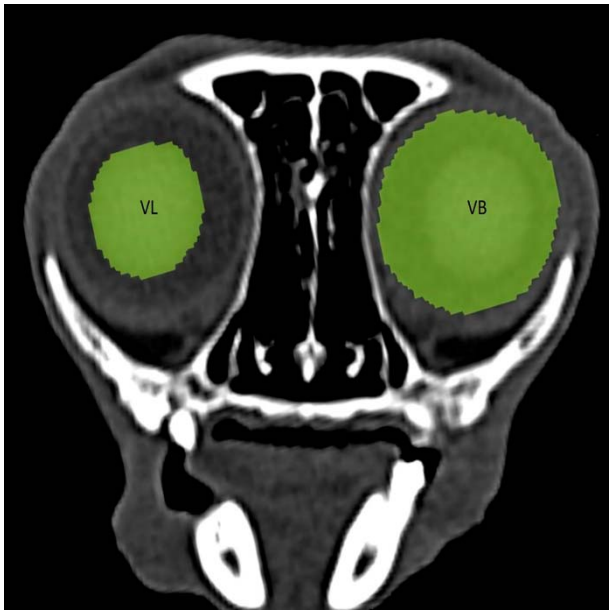

Figure 4. The process of calculating the volume value of the lens (VL) and the bulbus oculi (VB) in Van cats.

Nomina Anatomica Veterinaria was used as terminology in the study (20). Digital scales (TESS®, RP - LCD) were also used for bodyweight (W) measurements.

Statistical analysis: Whether measurement averages in this study were normally distributed or not was controlled via the Shapiro-Wilk test ($n < 50$). Since it was found that measurements of the variables were not normally distributed, nonparametric tests were used. For the sample size in the study; G*Power statistics program (ver.3.1.9.4) was used. In the calculation, the power of the test was 95 % and the type-1 error was 5 %. Accordingly, it was appropriate to have a minimum of 8 cats in each group. Descriptive statistics for measurements in the study are communicated as mean, standard deviation, minimum, and maximum. Gender-related comparisons of the measurements were made through the Mann-Whitney U test. The correlation between measurements, excluding gender-related comparisons, was determined by Spearman correlation coefficients. In measurements, the level of statistical significance (α) was deemed to be 5 %. For all measurements, SPSS (IBM SPSS for Windows, Ver. 23) statistical package program was used.

Results

The morphometric and volumetric measurement values obtained from bulbus oculi by using CT and MRI were analyzed statistically in order to determine the differences between male and female groups in terms of continuous variables. Statistically significant differences between the measured values of the bulbus oculi were recorded ($P<0.05$). Tables 2-4 show the measured values.

Table 2. Morphometric and volumetric measurement values of the bulbus oculi according to gender.

	Gender	N	Median	Mean	Std. Dev.	Min.	Max.	*P
A	Male	8	4.50	5.00	2.00	3.00	8.00	1.000
	Female	8	4.50	5.00	2.00	3.00	8.00	
W (kg)	Male	8	5.00	4.91	0.60	4.10	6.00	0.002
	Female	8	3.40	3.60	0.48	3.10	4.30	
VLR	Male	8	0.70	0.68	0.11	0.47	0.79	0.399
	Female	8	0.64	0.65	0.08	0.52	0.76	
VLL	Male	8	0.73	0.68	0.11	0.50	0.77	0.171
	Female	8	0.63	0.62	0.09	0.44	0.71	
ALR	Male	8	0.97	0.96	0.04	0.86	0.99	0.288
	Female	8	0.95	0.94	0.05	0.83	0.99	
MLLR	Male	8	1.12	1.12	0.05	1.05	1.20	0.833
	Female	8	1.12	1.10	0.06	1.01	1.17	
DVLR	Male	8	1.07	1.08	0.04	1.03	1.16	0.092
	Female	8	1.14	1.15	0.09	0.99	1.27	
ALL	Male	8	0.97	0.95	0.06	0.84	1.03	0.792
	Female	8	0.96	0.94	0.05	0.85	0.99	
MLLL	Male	8	1.10	1.11	0.04	1.05	1.18	0.596
	Female	8	1.13	1.13	0.07	1.02	1.23	
DVLL	Male	8	1.09	1.08	0.03	1.03	1.11	0.013
	Female	8	1.13	1.15	0.08	1.00	1.28	
VBR	Male	8	4.72	4.69	0.12	4.49	4.81	0.248
	Female	8	4.52	4.57	0.43	4.07	5.44	
VBL	Male	8	4.70	4.70	0.13	4.54	4.86	0.172
	Female	8	4.46	4.53	0.40	4.16	5.34	
ABR	Male	8	2.19	2.20	0.09	2.05	2.38	0.156
	Female	8	2.15	2.14	0.08	2.00	2.23	
MLBR	Male	8	2.11	2.08	0.08	1.93	2.16	0.102
	Female	8	2.04	2.01	0.10	1.81	2.10	
DVBR	Male	8	2.09	2.07	0.06	1.94	2.13	0.636
	Female	8	2.09	2.07	0.11	1.86	2.21	
ABL	Male	8	2.19	2.19	0.10	2.01	2.33	0.493
	Female	8	2.16	2.16	0.08	2.05	2.28	
MLBL	Male	8	2.07	2.06	0.07	1.97	2.14	0.429
	Female	8	2.05	2.02	0.10	1.83	2.13	
DVBL	Male	8	2.10	2.09	0.03	2.04	2.13	0.460
	Female	8	2.07	2.05	0.09	1.88	2.13	
ACL	Male	8	0.46	0.46	0.03	0.40	0.49	0.026
	Female	8	0.41	0.42	0.02	0.39	0.46	
ACR	Male	8	0.44	0.44	0.04	0.39	0.51	0.167
	Female	8	0.42	0.42	0.03	0.39	0.48	
PCL	Male	8	0.78	0.78	0.03	0.74	0.83	0.314
	Female	8	0.79	0.80	0.03	0.78	0.86	
PCR	Male	8	0.79	0.80	0.04	0.75	0.88	0.205
	Female	8	0.77	0.77	0.04	0.71	0.84	

*P<0.05; Mann-Whitney U test.

Table 3. Correlation between the morphometric and volumetric measurement values of the bulbus oculi in the male cats. (n=8).

		A	W(kg)	VLR	VLL	ALR	MLLR	DVLR	ALL	MLLL	DVLL	VBR	VBL	ABR	MLBR	DVBR	ABL	MLBL	DVBL	ACL	ACR	PCL	PCR
W (kg)	r	.957**																					
VLR	r	-.321	-.297																				
VLL	r	-.300	-.245	.840**																			
ALR	r	.500	.405	-.728*	-.850**																		
MLLR	r	.244	.335	.337	.122	-.122																	
DVLR	r	.712*	.614	-.552	-.466	.552	.180																
ALL	r	.515	.373	-.648	-.798*	.957**	.012	.614															
MLLL	r	.350	.442	.383	.200	-.125	.903**	.000	.000														
DVLL	r	.450	.565	-.049	-.100	.200	.854**	.479	.258	.725*													
VBR	r	.049	.084	-.169	-.220	.244	.690	.419	.371	.464	.805*												
VBL	r	.195	.156	-.265	-.366	.390	.619	.407	.575	.537	.659	.881**											
ABR	r	.785*	.705	-.594	-.761*	.749*	.252	.699	.795*	.270	.393	.252	.491										
MLBR	r	.172	.151	.800*	.687	-.331	.216	-.295	-.289	.405	.012	-.299	-.287	-.271									
DVBR	r	.638	.596	.315	.049	.233	.491	.096	.313	.712*	.368	.012	.228	.470	.681								
ABL	r	.750*	.638	-.543	-.750*	.800*	.268	.700	.871**	.275	.400	.317	.561	.982**	-.209	.515							
MLBL	r	.344	.422	.564	.565	-.184	.383	-.211	-.181	.626	.344	-.060	-.060	-.127	.855**	.729*	-.098						
DVBL	r	.667	.715*	.287	.296	.086	.542	.218	.127	.716*	.617	.193	.217	.248	.673	.806*	.272	.897**					
ACL	r	.864**	.733*	-.488	-.580	.642	.217	.806*	.733*	.235	.346	.193	.434	.945**	-.127	.497	.938**	-.061	.329				
ACR	r	.302	.278	-.112	-.050	-.164	.528	.420	.037	.478	.403	.430	.577	.408	-.266	.086	.352	-.167	.093	.485			
PCL	r	.683	.659	-.036	-.317	.317	.405	.180	.371	.610	.268	-.071	.238	.743*	.240	.814*	.708*	.323	.482	.687	.344		
PCR	r	.515	.464	-.770*	-.945**	.847**	-.036	.458	.807*	.000	.147	.120	.359	.886**	-.500	.223	.859**	-.337	-.061	.721*	.130	.587	

**P<0.01; *P<0.05; r: Spearman's rho Nonparametric Correlations Coefficients.

Table 4. Correlation between the morphometric and volumetric measurement values of the bulbus oculi in the female cats. (n=8).

		A	W(kg)	VLR	VLL	ALR	MLLR	DVLR	ALL	MLLL	DVLL	VBR	VBL	ABR	MLBR	DVBR	ABL	MLBL	DVBL	ACL	ACR	PCL	PCR
W(kg)	r	.390																					
VLR	r	.195	.190																				
VLL	r	.442	.407	.922**																			
ALR	r	.025	.192	-.192	-.048																		
MLLR	r	.393	-.072	.347	.542	.193																	
DVLR	r	.519	-.120	.542	.679	.182	.897**																
ALL	r	-.123	-.036	-.719*	-.482	.476	.241	-.048															
MLLL	r	.663	-.036	.443	.596	.145	.777*	.933**	-.108														
DVLL	r	.687	.024	.539	.687	.139	.747*	.939**	-.187	.988**													
VBR	r	.342	.071	.476	.671	-.060	.862**	.819*	.132	.731*	.731*												
VBL	r	.488	.167	.524	.743*	-.060	.886**	.843**	.072	.755*	.766*	.976**											
ABR	r	.146	.119	-.190	-.048	.898**	.287	.313	.395	.395	.347	.048	.024										
MLBR	r	.642	.241	-.289	-.176	-.133	-.079	-.055	-.097	.248	.188	-.193	-.096	.145									
DVBR	r	.732*	-.048	-.071	.108	-.323	.323	.434	.024	.635	.599	.429	.452	-.048	.615								
ABL	r	-.195	.286	-.095	.108	.659	.371	.145	.683	-.072	-.060	.310	.286	.405	-.554	-.429							
MLBL	r	.859**	.240	-.108	.084	-.223	.193	.248	-.078	.500	.464	.156	.263	.036	.903**	.850**	-.479						
DVBL	r	.810*	.168	-.395	-.157	-.006	.120	.139	.223	.343	.307	.012	.120	.132	.806*	.778*	-.263	.904**					
ACL	r	-.691	.036	.048	.042	.115	.164	-.104	.388	-.339	-.345	.277	.169	-.060	-.756*	-.602	.699	-.758*	-.733*				
ACR	r	-.148	.482	.277	.273	.370	.115	-.024	-.127	-.030	-.042	-.036	.000	.398	.030	-.542	.277	-.194	-.376	.323			
PCL	r	-.182	.457	.304	.313	.440	.070	-.077	-.032	-.274	-.217	-.114	-.025	.165	-.359	-.774*	.571	-.466	-.440	.436	.732*		
PCR	r	.196	.192	-.299	-.205	.548	-.169	.006	.175	.265	.229	-.156	-.216	.755*	.394	.240	-.036	.259	.295	-.345	.091	-.293	

**P<0.01; *P<0.05; r: Spearman's rho Nonparametric Correlations Coefficients.

Table 2 shows morphometric and volumetric measurements of the bulbus oculi according to sex. Accordingly, it was determined that W and ACL values were higher in the male cats compared to the female cats. In addition, DVLL values were higher in the female cats compared to the male cats. These differences were statistically significant (P<0.05). No statistically significant difference was determined between the other average measurement values of bulbus oculi based on sex (P>0.05).

Table 3 shows the correlation between the morphometric and volumetric measurement values of bulbus oculi in the male cats. Accordingly, a positive significant correlation was determined between age with W, DVLR, ABR, ABL, and ACL measurement values, between bodyweight with DVBL and ACL measurement values; between VLR with VLL and MLBR measurement values; between ALR with ALL, ABR, ABL, and PCR measurement values; between MLLR with MLLL and DVLL measurement values; between DVLR with ACL measurement value; ALL with ABR, ABL, ACL, and PCR measurement values; between MLLL with DVLL, DVBR, and DVBL measurement values; between DVLL and VBR measurement value; between VBR and VBL measurement value; between ABR with ABL, ACL, PCR measurement values; between MLBR with MLBL measurement value; between DVBR with MLBL, DVBL, and PCL measurement values; between ABL with ACL, PCL, and PCR measurement values; between MLBL and DVBL measurement value; between ACL and PCL measurement value (P<0.05). On the other hand, there was a negative significant correlation between VLR with ALR and PCR measurement values; between VLL with ALR, ALL, ABR, ABL, and PCR measurement values (P<0.05).

Table 4 shows the correlation between the morphometric and volumetric measurement values of bulbus oculi in the female cats. Accordingly, a positive significant correlation was determined between age with DVBR, MLBL, and DVBL measurement values; between VLR and VLL measurement value; between VLL and VBL measurement value; between ALR and ABR measurement value; between MLLR with DVLR, MLLL, DVLL, VBR, and VBL measurement values; between DVLR with MLLL, DVLL, VBR, and VBL measurement values; between MLLL with DVLL, VBR, and VBL measurement values, between DVLL with VBR and VBL measurement values; between VBR and VBL measurement value; between ABR and PCR measurement value; between MLBR with MLBL, DVBL, and ACL measurement values; between DVBR with MLBL and DVBL measurement values; between MLBL and DVBL measurement value; and between ACR and PCL measurement value (P<0.05). However, a negative significant correlation was determined between VLR and ALL measurement value; between DVBR and PCL measurement value; between ACL with MLBL and DVBL measurement values (P<0.05).

Discussion and Conclusion

The medical imaging methods such as A-mode Ultrasonography, B-mode Ultrasonography, Computed tomography, and magnetic resonance imaging are commonly used in imaging eye and the relevant complex anatomic structures, obtaining morphometric and volumetric measurement values from these images, and interpreting pathological conditions (19, 30, 31, 36). This study is the first attempt to determine the morphometric and volumetric values of the right-left bulbus oculi using CT and MRI in Van cats and reveal the biometric differences of these values between males and females.

No statistically significant differences were observed in the ocular biometric measurement values obtained from the various planes of the right and left eyes of some animals such as domestic cats (36), Persian cat (19), mesocephalic and dolichocephalic dogs (6), Saanen goats (29), Guinea pigs (40), rabbits (34), and Striped Owls (32). In this study, it was observed that ACL measurement values were higher in the male cats compared to the female cats. In addition, DVLL measurement values were higher in the female cats compared to the male cats. These differences were statistically significant (P<0.05). No statistically significant difference was determined between the other mean measurement values of the right and left bulbus oculi based on sex (P>0.05). It was observed that other findings of bulbus oculi other than ACL and DVLL measurement values were compatible with the literature data. In addition, in the study by Schiffer et al. (31), no statistical difference was found between the right and left axial bulbus oculi length measurement values and they reported based on the differences between sexes that this measurement value was significantly longer in male dogs compared to female dogs.

Gilger et al. (12), determined that the mean values of axial bulbus oculi length, anterior chamber depth, and axial lens thickness were 20.91 ± 0 .53 mm, 5.07 ± 0.36 mm, and 7.77 ± 0.23 mm, respectively, in the ocular biometric measurements performed in 20 mixed-breed cats with clinically normal characteristics. Vosough et al. (36), determined that the mean values of axial bulbus oculi length, anterior chamber depth, axial lens thickness, and posterior chamber depth of the right and left eyes were approximately 17.1 ± 0.1 mm, 3.9 ± 0.1 mm, 5.0 ± 0.1 mm, and 7.0 ± 0.1 mm, respectively, in the ocular morphometric examinations performed with 6 healthy female domestic shorthair cats. Mirshahi et al. (19), determined that these values were 20.7 ± 1.0 mm, 4.1 ± 0.7 mm, 7.7 ± 0.5 mm, and 8.2 ± 0.4 mm, respectively, in

the ocular morphometric examinations of 40 healthy Persian cats. In this study, it was observed that these values were approximately 21.7 ± 0.1 mm, 4.3 ± 0.03 mm, 9.6 ± 0.8 mm, and 7.8 ± 0.3 mm, respectively. It was considered that these differences observed between the morphometric measurement results of the mean values of axial bulbus oculi length, anterior chamber depth, axial lens thickness, and posterior chamber depth were due to the age, length, and breed characteristics of cats.

The studies have revealed that there is a correlation between body length and ocular size in humans (26) and dogs (6). However, in the present study, the ocular morphometric characteristics were assessed with bodyweight rather than body length. Accordingly, a positive, statistical correlation was determined between bodyweight and DVBL and ACL measurement values in male cats. Also, no significant correlation was observed between the ocular parameters and bodyweight in females. However, Hollis et al. (13), reported that there was a positive correlation between bodyweight and all the ocular structures except for lens and anterior chamber depth in horses.

In the study by Larsen (17), it was stated that the size of the eye developed and increased approximately until the age of 13 in humans. Also, Ribeiro et al. (29), reported that all the ocular parameters increased until the age of 549 days in Saanen goats. In the study conducted by Tuntivanich et al. (35), during the postnatal development period of dogs by using ultrasound they determined that the axial bulbus oculi increased until the 52nd week. In the study conducted by Mirshahi et al (19), with Persian cats, they determined that there was a positive correlation between age and posterior chamber depth. In the present study, a positive significant correlation was determined between age and W, DVLR, ABR, ABL, and ACL measurement values in the male cats and between age and DVBR, MLBL, and DVBL measurement values in the female cats. Also, Tables 3 and 4 show the correlation between the other measurement values of ocular structures in male and female Van cats.

Eye volume is quite important in terms of some ocular diseases such as microphthalmus, buphthalmus (congenital glaucoma), and macrophthalmus (1). Especially in pets such as cats and dogs, eye volume can be calculated using various software developed from CT and MRI images (1, 3, 14, 30). In this study, the volumetric measurement values of bulbus oculi and lens in healthy Van cats were determined to be averagely 4.60 ± 0.27 cm^3 and 0.67 ± 0.09 cm^3 , respectively.

Using computed tomography and magnetic resonance imaging, the images with the appropriate thickness of the relevant anatomic structure can be obtained without giving any bodily harm to animals under anesthesia (33, 39). Additionally, imaging various anatomic structures morphologically, interpreting them and the morphometric and volumetric measurements of these anatomic structures can be easily performed (7, 22, 38). Due to these properties, these imaging methods are increasingly and commonly used in the field of veterinary anatomy recently together with the computer-aided technological developments (2, 39).

Consequently, the statistical differences between the male and female Van cats in terms of bulbus oculi biometric values were determined using CT and MRI. It is predicted that this study would guide the ophthalmological applications related to Van cats and the students receiving anatomy education. Also, it is thought that this study would significantly contribute to the scientific studies about the eye in Van cats.

Financial Support

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

Ethical Statement

This study was approved by Van Yüzüncü Yıl University Animal Experiments Local Ethics Committee (the decision no: 2020 / 02 and date: 27. 02. 2020).

Conflict of Interest

The authors declared that there is no conflict of interest.

References

1. **Acer N, Sahin B, Ucar T, et al** (2009): *Unbiased estimation of the eyeball volume using the Cavalieri principle on computed tomography images.* J Craniofac Surg, **20**, 233-237.
2. **Brenton H, Hernandez J, Bello F, et al** (2007): *Using multimedia and web 3D to enhance anatomy* teaching. Comput Educ, **49**, 32-53.
3. **Chandrakumar SS, Zur Linden A, Owen M, et al** (2019): *Computed tomography measurements of intraocular structures of the feline eye*. Vet Rec, **184**, 651.
4. **Chiwitt CHL, Baines SJ, Mahoney P, et al** (2017): *Ocular biometry by computed tomography in different dog breeds.* Vet Ophthalmol, **20**, 411-419.
5. **Collins SP, Matheson JS, Hamor RE, et al** (2013): *Comparison of the diagnostic quality of computed tomography images of normal ocular and orbital structures acquired with and without the use of general anesthesia in the cat.* Vet Ophthalmol, **16**, 352-358.
6. **Cottrill NB, Banks WJ, Pechman RD** (1989): *Ultrasonographic and biometric evaluation of the eye and orbit of dogs*. Am J Vet Res, **50**, 898-903.
7. **Daniel GB, Mitchell SK** (1999): *The eye and orbit*. Clin Tech Small Anim Pract, **14**, 160-169.
8. **Demircioğlu İ, Yılmaz B** (2019): *İvesi koyunlarında (Ovis aries) bulbus oculi'nin morfometrik yapısının incelenmesi*. Dicle Üniv Vet Fak Derg, **12**, 108-111.

9. **Dursun N** (2008): Veterinary Anatomy III. Medisan Publisher, Ankara, Turkey.
10. **Dyce KM, Sack WO, Wensing CJG** (2010): Textbook of Veterinary Anatomy. 332-345. 4th Edition, Saunders Elsevier Inc, Missouri, United States.
11. **Fornazari GA, Montiani-Ferreira F, de Barros Filho IR, et al** (2016): *The eye of the Barbary Sheep or Aoudad (Ammotragus lervia): Reference values for selected ophthalmic diagnostic tests, morphologic and biometric observations.* Open Vet J, **6**, 102-113.
12. **Gilger BC, Davidson MG, Howard PB** (1998): *Keratometry, ultrasonic biometry, and prediction of intraocular lens power in the feline eye*. Am J Vet Res, **59**, 131-134.
13. **Hollis AR, Dixon JJ, Berlato D, et al** (2019): *Computed tomographic dimensions of the normal adult equine eye.* Vet Ophthalmol, **22**, 651-659.
14. **Igbinedion BO, Ogbeide OU** (2013): *Measurement of normal ocular volume by the use of computed tomography*. Niger J Clin Pract, **16**, 315-319.
15. **Kalra MK, Maher MM, Toth TL, et al** (2004): *Strategies for CT radiation dose optimization*. Radiology, **230**, 619-628.
16. **Kassab A** (2012): *Ultrasonographic and macroscopic anatomy of the enucleated eyes of the Buffalo (Bos bubalis) and the One-Humped Camel (Camelus dromedarius) of different ages*. Anat Histol Embryol, **41**, 7-11.
17. **Larsen JS** (1971): *The sagittal growth of the eye. II: ultrasonic measurement of the axial diameter of the lens and the anterior segment from birth to puberty*. Acta Ophthalmol, **49**, 427-440.
18. **Liebich HG, König HE** (2007): Eye (organum visus). 571-591. In: HE König, HG Liebich (Ed), Veterinary Anatomy of Domestic Mammals: Text Book and Colour Atlas. 3rd Edition, Schattauer, Germany.
19. **Mirshahi A, Shafigh SH, Azizzadeh M** (2014): *Ultrasonographic biometry of the normal eye of the Persian cat.* Aust Vet J, **92**, 246-249.
20. **Nomina Anatomica Veterinaria** (2017): Prepared by the international committees on veterinary gross anatomical nomenclature and authorized by the general assembly of the world association of veterinary anatomists (6th Edition). The Editorial Committee Hanover (Germany), Ghent (Belgium), Columbia, MO (U.S.A.), Rio de Janeiro (Brazil).
21. **Odabasioglu F, Ates CT** (2000): Van Cats. 1st Edition, Selcuk University Printing Office, Konya, Turkey.
22. **Ohlerth S, Scharf G** (2007): *Computed tomography in small animals-basic principles and state of the art applications*. Vet J, **173**, 254-71.
23. **Olopade JO, Kwari HD, Agbashe IO, et al** (2005): *Morphometric study of the eyeball of three breeds of goats in Nigeria*. Int J Morphol, **23**, 377-380.
24. **Patruno M, Perazzi A, Martinello T, et al** (2017): *Morphological description of limbal epithelium: searchingfor stem cells crypts in the dog, cat, pig, cow, sheep and horse. Vet Res Commun,* **41**, 169-173.
25. **Paunksnis A, Svaldenienė E, Paunksnienė M, et al** (2001): *Ultrasonographic evaluation of the eye parameters in dogs of different age.* Ultragarsas, **9**, 48-51.
26. **Pereira GC, Allemann N** (2007): *Ocular biometry, refractive error and correlation with height, age, gender and years of formal education.* Arq Bras Oftalmol, **70**, 487-493.
27. **Potter JT, Hallowell DG, Bowen IM** (2008): *Ultrasonographic anatomy of the bovine eye*. Vet Radiol Ultrasound, **49**, 172-175.
28. **Prokop M** (2003): *General principles of MDCT.* Eur J Radiol, **45**, S4-S10.
29. **Ribeiro AP, Silva ML, Rosa JP, et al** (2009): *Ultrasonographic and echobiometric findings in the eyes of Saanen goats of different ages*. Vet Ophthalmol, **12**, 313-317.
30. **Salgüero R, Johnson V, Williams D, et al** (2015): *CT dimensions, volumes and densities of normal canine eyes*. Vet Rec, **176**, 386.
31. **Schiffer SP, Rantanen NW, Leary GA, et al** (1982): *Biometric study of the canine eye, using A-mode ultrasonography.* Am J Vet Res, **43**, 826-830.
32. **Squarzoni R, Perlmann E, Antunes A, et al** (2010): *Ultrasonographic aspects and biometry of Striped owl's eyes (Rhinoptynx clamator).* Vet Ophthalmol, **13**, 86-90.
33. **Thrall DE** (2007): Principles of computed tomography and magnetic resonance imaging. 20-77. In: Textbook of Veterinary Diagnostic Radiology. 5th Edition, Saunders Elsevier, St. Louis.
34. **Toni MC, Meirelles AÉ, Gava FN, et al** (2010): *Rabbits'eye globe sonographic biometry*. Vet Ophthalmol, **13**, 384-386.
35. **Tuntivanich N, Petersen-Jones SM, Steibel JP, et al** (2007): *Postnatal development of canine axial globe length measured by B-scan ultrasonography.* Vet Ophthalmol, **10**, 2-5.
36. **Vosough D, Shojaei B, Molazem M** (2009): *Magnetic resonance imaging of feline eye.* Iran J Vet Res, **10**, 66-69.
37. **Williams DL** (2004): *Lens morphometry determined by B-mode ultrasonography of the normal and cataractous canine lens.* Vet Ophthalmol, **7**, 91-95.
38. **Wisner ER, Zwingenberger AL** (2015): Atlas of Small Animal CT and MRI: Orbit. 69-85. Willey-Blackwell Publishing, USA.
39. **Yilmaz O, Soyguder Z, Yavuz A, et al** (2020): *Three-dimensional computed tomographic examination of pelvic cavity in Van Cats and its morphometric investigation.* Anat Histol Embryol, **49**, 60-66.
40. **Zhou X, Qu J, Xie R, et al** (2006): *Normal development of refractive state and ocular dimensions in guinea pigs*. Vision Res, **46**, 2815-2823.

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Short Communication / *Kısa Bilimsel Çalışma*

Methicillin-resistant *Staphylococcus aureus* isolated from nonhealing wound in a canary bird (*Serinus canaria domestica*)

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Abstract: Little is known about the presence of methicillin-resistant *Staphylococcus aureus* (MRSA) in a canary bird (*Serinus canaria domestica*). Although MRSA in a canary bird was previously reported, to the best of our knowledge, this is the first full description of the isolation and antibiotic resistance pattern of MRSA in this pet bird. A swab was taken from the nonhealing wound on the lateral thigh of a four-year-old, caged, housed alone, male canary bird. After the identification of *Staphylococcus aureus*, the antibiotic susceptibility profile of the isolate was obtained by the disk diffusion test. According to the resistance to Oxacillin and Cefoxitin, the isolate was identified as MRSA. The *mecA* gene was confirmed by PCR. The bird was treated by offering drinking water medicated with an injectable enrofloxacin formulation at 200 mg/L over 10 days period. Two weeks after therapy, intensive contraction of the wound was observed with a size reduction. A week later, the complete epithelization of the wound defect was verified. In this study, we could not confirm the source of infection in a canary bird, but we believe that transmission was from wild birds when the cage was putting out in order to allow the bird to sunbathe or more likely *via* contact with the owner. The results of this study underline the necessity for further investigations on the epidemiological role of canary birds as potential reservoirs of MRSA.

Keywords: Antimicrobial resistance, canary bird, exotic birds, *mecA*, pet birds.

Staphylococcus aureus (S. aureus) is a well-known pathogen in humans and many animal species causing a wide range of infections (5). It is mainly recognized as an important cause of morbidity and economic loss in production animals, but it is also very important in companion animals (13, 15). Pet birds, including canary birds (*Serinus canaria domestica*), are common pets, and they can be infected with *S. aureus* in different forms, including pneumonia, air sac inflammation, pericarditis, and diarrhea. Moreover, *S. aureus* can cause death in canaries kept in aviaries (5). The most important zoonotic diseases associated with birds include chlamydophilosis, salmonellosis, tuberculosis, and highly pathogenic avian influenza (2). Numerous other bacteria also have zoonotic potential, such as *Campylobacter* spp, *Borrelia burgdorferi*, *Pasteurella* spp, *Klebsiella* spp, *Yersinia* spp, *Pseudomonas* spp, and *Escherichia coli* (2, 6). In recent decades, methicillin resistance of *S. aureus* has become an emerging problem in human and veterinary medicine (1). After the first isolation of methicillin resistant *S. aureus* (MRSA) from the milk of mastitic cow in 1972, MRSA has been increasingly found in many animals (8). The evidence that transfers of methicillin resistant *S. aureus* strains can occur between animals and humans (8, 11) increase the zoonotic potential of different animals as a source of MRSA infection in humans. Due to their close contact with humans, MRSA in household pets has potentially significant implications for human health, especially because of evidence of MRSA colonization in clinically normal animals (20). Despite the importance of MRSA in pets, there is very limited information about MRSA in exotic birds. So far, a few cases in parrots have been described (3, 14, 19) and only one report of MRSA in canary birds, without any detailed case description (9).

The purpose of this report is to describe isolation of MRSA from an unhealing wound in a household canary bird (*Serinus canaria domestica*).

A four-year-old, caged, housed alone, male canary bird (*Serinus canaria domestica*) was referred to the Surgery Department of Veterinary Faculty University of Sarajevo with a history of the nonhealing wound of unknown etiology, progressing in size despite undertaken, by veterinarian prescribed, topical therapy. The previous therapy consisted of wound irrigation twice daily for seven days with the 0.1% povidone-iodine antiseptic solution. Physical examination revealed a full-thickness wound in the right lateral thigh, approximately 7 to 10 mm in diameter, presented with some portions of the granulation tissue, exudate at the wound surface, and the redness of the surrounding skin. A wound swab was taken and immediately transfer to the laboratory for bacteriological examination. The swab was inoculated onto blood agar, MacConkey's, and bromocresol purple lactose agar and incubated aerobically for 24 hours at 37 °C. Preliminary identification of the isolate was performed by colony morphology, hemolysis, Gram stain, catalase test, tube coagulase test, and fermentation of maltose on bromocresol purple maltose agar (10). The final identification of *S. aureus* was done by polymerase chain reaction (PCR) using oligonucleotide primers described by Sasaki et al. (17).

Antimicrobial susceptibility testing of MRSA isolate was performed by a disc diffusion method following the guidelines of the Clinical Laboratory Standards Institute (4). The following antimicrobial disks (Conda) were used: Amoxicillin (25 μg), Amoxicillin-Clavunalic acid (20/10 μg), Ampicillin-Sulbactam (10/10 μg), Cefoxitin (30 μg) Chloramphenicol (30 μg), Enrofloxacin (5 μg), Clindamycin (2 μg), Erythromycin (15 μg), Gentamycin (10 μg), Oxacillin (1 μg) and Penicillin G (1 IU). For detection of *mecA* and *mecC* (*mecA*$_{LGA251}$) (methicillin resistance) genes in the isolate, PCR was performed as described previously (18). Once the antibiogram results were obtained, topical treatment with 0.1% povidone-iodine antiseptic solution was discontinued, and oral application with enrofloxacin, was applied by offering drinking water medicated with an injectable enrofloxacin formulation at 200 mg/L over 10 days period. Written informed consent was obtained from the owner for all procedures undertaken.

According to the results of preliminary identification, the isolate was identified as coagulase positive staphylococci. *S. aureus* was finally identified by PCR. The *S. aureus* isolate was found to be resistant to Penicillin G, Amoxicillin, Amoxicillin-Clavunalic acid, Ampicillin-Sulbactam, Cefoxitin, Erythromycin, and Oxacillin. The isolate was intermediately susceptible to Clindamycin and susceptible to Chloramphenicol, Enrofloxacin, and Gentamycin. Due to resistance to Oxacillin and Cefoxitin, the isolate was identified as MRSA. The presence of the *mecA* gene was confirmed by PCR.

After the 4th day of antibiotic therapy, wound healing was evident by the absence of the exudate at the wound surface, a visible proliferation of the granulation tissue over the wound defect, and reduction of the redness of the surrounding skin. Two weeks after systemic antibiotic therapy was introduced, intensive contraction of the wound was observed with a concentric reduction in size. A week later, the complete epithelization of the wound defect was verified.

MRSA is well known emerged pathogen in human medicine since 1970s and has been increasingly reported as an emerging problem in veterinary medicine during the past 20 years (8). The prevalence of MRSA isolates in humans varies markedly between geographic areas. In veterinary medicine, distribution also depends on animal species. In recent years, MRSA has been identified in various animals, mainly in horses, food-producing animals, and increasingly in companion animals (8, 20). According to the European Food Safety Authority, the greatest risk to human infections are pigs, veal calves, and broilers. There are still no specific studies that examined the risk of human infection or colonization from small animals (1). However, the importance of small animals as a possible source of zoonotic infections in humans has substantially increased in proportion to the importance and number of pets in the life of a modern man. In the last two decades, isolation of MRSA has been increasingly reported from different household pets, mainly in dogs and cats, as well as in some exotic pets such as hamsters, rabbits, turtle, and bat (11, 14, 19). Unlike other pets, MRSA findings in household pet birds are less common. There are only a few reports that confirm the presence of MRSA in parrots (14, 19), with only one full clinical description (3). Recently, Loncaric et al. (9) have reported the genetic diversity of MRSA strains isolated from companion animals in Austria, including one strain isolated from a canary bird. However, to the authors' knowledge, this study is the first full description of the isolation and antibiotic resistance pattern of MRSA from the household canary bird. Unfortunately, in this study we could not confirm the source of infection in the canary bird; however, it could be *via* contact with the owners, or with the wild birds. Namely, putting the cage outside in order to allow the bird to sunbathe, could be a condition favoring contact between pet and wild birds (2). Among wild passerines, MRSA has been detected in several species, including chaffinch (*Fringilla coelebs*) (12), spotted flycatcher (*Muscicapa striata*), robin (*Erithacus rubecula*) and common blackbird (*Turdus merula*) (7). Transfer of MRSA strains between animals and humans

and *vice versa* has been reported several times (8, 16), so the infection from the humans to the canary bird in this study can not be excluded. A recent study by Loncaric et al. (9) has demonstrated that the majority of MRSA isolates recovered from small companion animals are human-related, which supports our theory that the isolate of MRSA in this study could be of human origin.

The results of this study strongly imply that a canary bird can carry MRSA and underline the need for further investigations on the epidemiological role of pet birds as potential reservoirs of MRSA. Also, this research confirms the importance of antibiogram testing in the prevention of antibiotic resistance in bacteria of animal origin. Furthermore, this is the first report of MRSA isolated from animals in Bosnia and Herzegovina.

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Ethical Statement

This study does not present any ethical concerns.

Conflict of Interest

The authors declared that there is no conflict of interest.

References

1. **Andreoletti O, Budka H, Buncic S, et al** (2009): *Assessment of the Public Health significance of meticillin resistant Staphylococcus aureus (MRSA) in animals and foods Scientific Opinion of the Panel on Biological Hazards*. EFSA J, **993**, 1-73.
2. **Boseret G, Losson B, Mainil JG, et al** (2013): *Zoonoses in pet birds: Review and perspectives.* Vet Res, **44**.
3. **Briscoe JA, Morris DO, Rankin SC, et al** (2008): *Methicillin-resistant Staphylococcus aureus-associated dermatitis in a Congo African grey parrot (Psittacus erithacus erithacus).* J Avian Med Surg, **22**, 336–343.
4. **CLSI** (2012): Performance Standards for Antimicrobial Susceptibility Testing; Twenty-Second Informational Supplement. CLSI document M100-S22. Wayne, PA: Clinical and Laboratory Standards Institute.
5. **Devriese LA, De Herdt P, Desmidt M, et al** (1994): *Pathogenic staphylococci and staphylococcal infections in canaries*. Avian Pathol, **23**,159–162.
6. **Evans EE** (2011): *Zoonotic Diseases of Common Pet Birds: Psittacine, Passerine, and Columbiform Species*. Vet Clin Exot Anim, **14**, 457–476.
7. **Kutkowska J, Turska-Szewczuk A, Kucharczyk M et al** (2019): *Methicillin-resistant Staphylococcus aureus and glycopeptide-resistant enterococci in fecal samples of birds from South-Eastern Poland.* BMC Vet Res, **15,** 1–8.
8. **Leonard FC, Markey BK** (2008): *Meticillin-resistant Staphylococcus aureus in animals: A review*. Vet J, **175**, 27–36.
9. **Loncaric I, Lepuschitz S, Ruppitsch W et al** (2019): *Increased genetic diversity of methicillin-resistant Staphylococcus aureus (MRSA) isolated from companion animals.* Vet Microbiol, **235**,118–126.
10. **Markey B, Leonard F, Archambault M et al** (2013): Staphylococcus species. 105-115. In: Clinical Veterinary Microbiology. Mosby Elsevier.
11. **Morgan M** (2008): *Methicillin-resistant Staphylococcus aureus and animals: Zoonosis or humanosis?* J Antimicrob Chemother, **62**, 1181–1187.
12. **Paterson GK, Larsen AR, Robb A et al** (2012): *The newly described mecA homologue, mecALGA251, is present in methicillin-resistant Staphylococcus aureus isolates from a diverse range of host species.* J Antimicrob Chemother, **67**, 2809–2813.
13. **Pomba C, Rantala M, Greko C et al** (2017): *Public health risk of antimicrobial resistance transfer from companion animals*. J Antimicrob Chemother, **72**, 957–968.
14. **Rankin S, Roberts S, O'Shea K et al** (2005): *Panton valentine leukocidin (PVL) toxin positive MRSA strains isolated from companion animals.* Vet Microbiol, **108**, 145–148.
15. **Rubin JE, Ball KR, Chirino-Trejo M** (2011): *Antimicrobial susceptibility of Staphylococcus aureus and Staphylococcus pseudintermedius isolated from various animals*. Can Vet J, **52**, 153–157.
16. **Rutland EB, Weese JS, Bolin C et al** (2009): *Human-to-dog transmission of methicillin-resistant Staphylococcus aureus.* Emerg Infect Dis, **15**, 1328–1330.
17. **Sasaki T, Tsubakishita S, Tanaka Y et al** (2010): *Multiplex-PCR method for species identification of coagulase-positive staphylococci.* J Clin Microbiol, **48**, 765–769.
18. **Stegger M, Andersen PS, Kearns A et al** (2012): *Rapid detection, differentiation and typing of methicillin-resistant Staphylococcus aureus harbouring either mecA or the new mecA homologue mecALGA251.* Clin Microbiol Infect, **18**, 395–400.
19. **Walther B, Wieler LH, Friedrich AW et al** (2008): *Methicillin-resistant Staphylococcus aureus (MRSA) isolated from small and exotic animals at a university hospital during routine microbiological examinations*. Vet Microbiol, **127**, 171–178.
20. **Weese JS, van Duijkeren E** (2010): *Methicillin-resistant Staphylococcus aureus and Staphylococcus pseudintermedius in veterinary medicine*. Vet Microbiol, **140**, 418–429.

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Case Report / *Olgu Sunumu*

Progressive respiratory distress caused by a laryngeal histiocytic sarcoma in a European rabbit (*Oryctolagus cuniculus*)

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Abstract: Histiocytic sarcoma (HS) represents a rare type of malignant disease, characterized by neoplastic proliferation of interstitial dendritic cells (iDCs). In rabbits, there are only two previous reports of HS with pulmonary and cutaneous primary localizations. A 2-year-old, male, European rabbit (*Oryctolagus cuniculus*) with a clinical history of chronic respiratory distress and progressive weight loss was necropsied. Postmortem examination revealed a gray-white, well-demarcated ovoid mass that deformed the ventral region of the larynx. Similar nodules were identified in both lungs. Histologically, the laryngeal mass and pulmonary nodules were composed of dense sheets of neoplastic histiocytes with marked atypia and multinucleated neoplastic cells. Immunohistochemically, the neoplastic cells were intensely positive for Iba1, CD1a, and vimentin, occasionally for E-cadherin, and negative for multi-cytokeratin. A diagnosis of laryngeal HS with multiple pulmonary metastases was made. To the authors' knowledge, laryngeal histiocytic tumor has not been previously reported in rabbits.

Keywords: Histiocytic sarcoma, larynx, pulmonary metastases, rabbit.

Histiocytic disorders are well-recognized in dogs and cats, and include canine cutaneous histiocytoma, cutaneous and pulmonary Langerhans cell histiocytosis (LCH), cutaneous and systemic reactive histiocytosis, histiocytic sarcoma, feline progressive histiocytosis, and dendritic cell leukemia (9).

Histiocytic sarcoma (HS) represents a rare type of malignant disease, characterized by neoplastic proliferation of interstitial dendritic cells (iDCs), while the hemophagocytic type originates from macrophages (9). In rabbits, there are two previous reports of HS with pulmonary and cutaneous primary localizations (7, 8). A disseminated HS with hemophagocytosis was also previously described in a rabbit (6). These tumors have variable biologic behavior, although the malignant counterparts have a poor prognosis (4).

To our knowledge, there is no previous report on primary laryngeal HS in domestic rabbits. In the present study, we describe the pathological, histological, and immunohistological features of a primary laryngeal HS with multiple pulmonary metastases in a domestic rabbit.

A 2-year-old, male, European rabbit (*Oryctolagus cuniculus*) was referred to the Emergency Department due to chronic respiratory distress and progressive weight loss lasting for two weeks. On physical examination cachexia, tachypnea, sneezing, coughing, and swelling of the ventral side of the larynx were observed. A fine-needle aspiration of the laryngeal mass was performed. Due to the poor prognosis, rapidly deteriorating body condition, and owner's financial limitation, the animal was euthanized and a postmortem examination was performed. Tissue samples from both laryngeal mass and pulmonary nodules were collected for histological and immunohistochemical examination. Tissue samples were fixed in 10% neutral buffered formalin, processed routinely, embedded in paraffin wax, cut into 3 µm sections, and stained with

hematoxylin and eosin (H&E). For immunohistochemistry, primary antibodies for vimentin (clone SRL33), Iba-1 (polyclonal), CD1a (clone MTB1), E-cadherin (clone 36B5) and multi-cytokeratin (clone AE1/AE3) were used. The samples were automatically processed using Leica Bond-Max system. Negative controls for each sample were prepared by replacing the primary antibody with mouse immunoglobulin G1 negative control. Infiltrating macrophages within the neoplastic mass (for Iba1 and CD1a) and skin, including epidermis and dermis (for multi-cytokeratin, E-cadherin, and vimentin) sampled from the same rabbit served as internal positive controls.

The cytological examination revealed small groups of pleomorphic round cells with distinct borders, abundant pale cytoplasm, high nuclear:cytoplasmic (N:C) ratio and pleomorphic nuclei with 1-2 prominent nucleoli (Figure 1). Binucleated and multinucleated neoplastic cells were occasionally seen. Consequently, a presumptive cytological diagnosis of a round cell tumor was made.

Postmortem examination revealed severe atrophy of the muscle tissue, absence of subcutaneous and retroperitoneal fat, serous atrophy of the adipose tissue at the base of the heart (cachexia), and presence of approximately 20 ml of serous fluid within the peritoneal cavity. A gray-white, well-demarcated, ovoid mass, measuring 1.4x0.9x1.6 cm in diameter deformed and effaced the ventral region of the larynx, including the thyroid and cricoid cartilages, bulged on the ventral laryngeal surface, and partially obliterated the laryngeal lumen. The intraluminal part of the mass showed extensive necrosis, hemorrhages, and ulceration. Moreover, small nodular lesions, ranging from 4 to 10 mm in diameter were randomly distributed in both lungs, mostly affecting the cranial lobes (Figure 2). No similar findings were identified in any other organ.

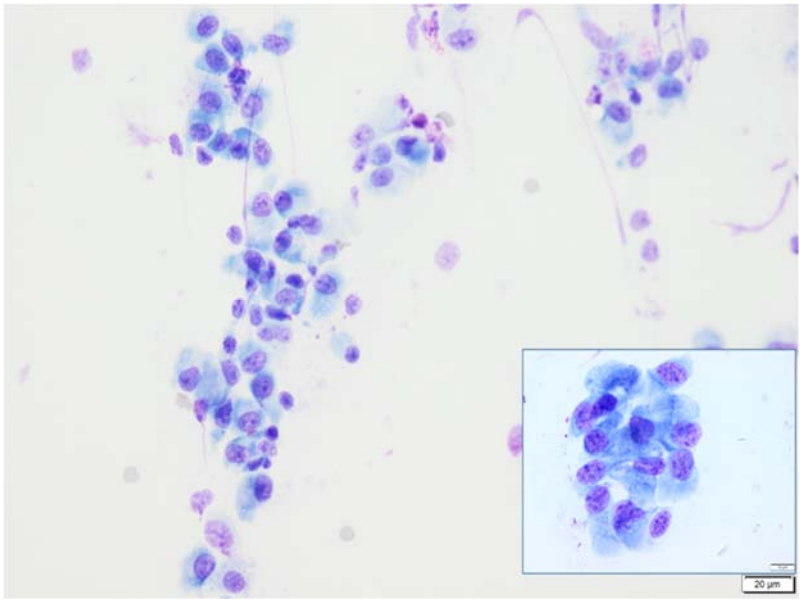

Figure 1. Photomicrograph of a fine-needle aspiration cytology from the laryngeal mass of 2-year-old domestic rabbit. Note many individualized and groups of pleomorphic round cells with abundant pale blue cytoplasm and pleomorphic nuclei. The inset shows atypical cells characterized by marked anisokaryosis and multinucleated cells (central area). Diff-Quick stain. Bar=20 µm.

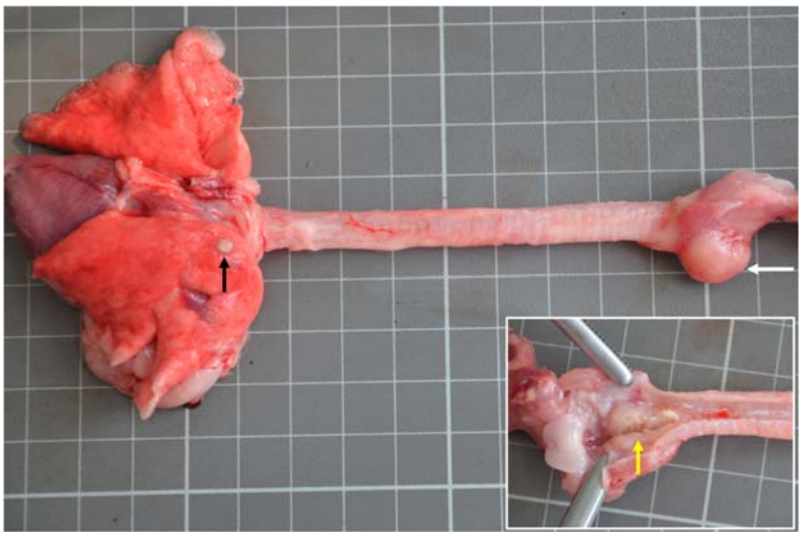

Figure 2. Gross features of the laryngeal histiocytic sarcoma and pulmonary metastases in a 2-year-old domestic rabbit. The laryngeal lumen is partially obliterated by an ulcerated neoplastic mass, infiltrating the laryngeal wall (the inset, yellow arrow) and bulging on the laryngeal serosa (white arrow). The pulmonary parenchyma is affected by randomly distributed and variably sized nodules (black arrow).

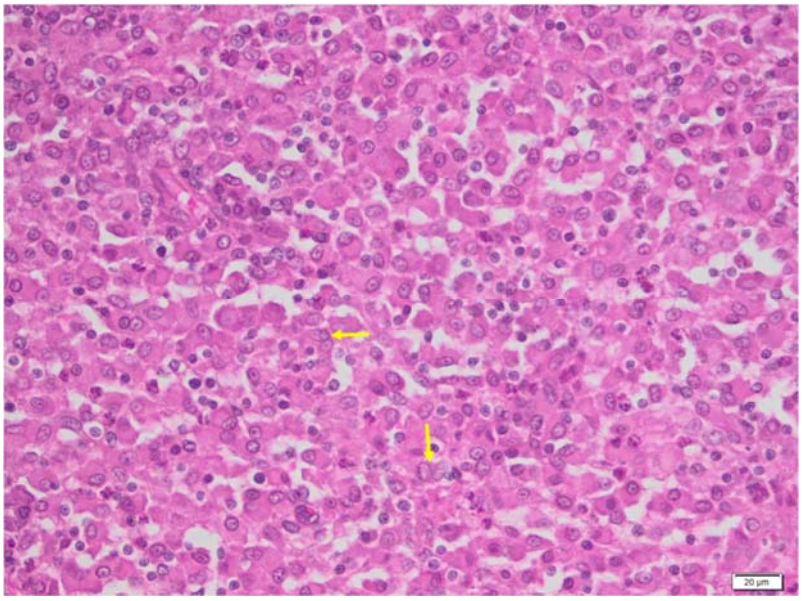

Figure 3. The laryngeal mass is composed of dense sheets of round to polyhedral cells with variably distinct borders, abundant eosinophilic cytoplasm, and oval to irregular nuclei; scattered binucleated neoplastic cells (yellow arrows) are also seen. H&E stain. Bar=20 µm.

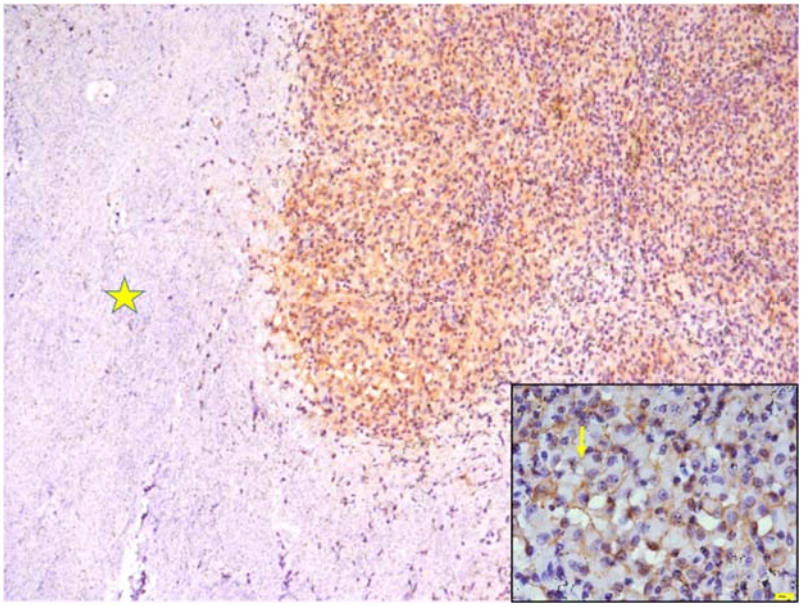

Figure 4. The neoplastic mass is diffusely immunopositive for Iba1, while the necrotic areas are negative (yellow star). Neoplastic cells showing diffuse and intense membranous immunoreactivity (the inset, yellow arrow). IHC. Bar=10 µm.

Histologically, the laryngeal mass extended from the mucosa to the serosa, infiltrating and effacing the ventral laryngeal cartilages and muscle tissue. The unencapsulated tumor was densely cellular, composed of pleomorphic cells arranged in sheets, and separated by a scant fibrovascular stroma (Figure 3). The cells were polygonal to round with variably distinct borders, moderate amounts of pale eosinophilic cytoplasm and high N:C ratio. The nuclei were pleomorphic, round, oval or indented with finely granular chromatin and 1-2 prominent nucleoli. Binucleated and multinucleated neoplastic cells were also observed. Mitotic figures varied from 1 to 5/high power field. Additionally, small areas of necrosis, hemorrhages, and mild infiltration of lymphocytes and heterophils were noticed. The pulmonary nodules showed similar histological features.

Immunohistochemically, the neoplastic cells showed an intense and diffuse reaction for Iba-1 (Figure 4), CD1a, and vimentin, and occasionally for E-cadherin. The neoplastic cells were negative for multi-cytokeratin. Based on the cytological, histological, and immunohistochemical results, the tumor was diagnosed as histiocytic sarcoma with multiple pulmonary metastases. The immunohistochemical results of pulmonary metastases were similar to those of the primary laryngeal tumor.

Respiratory disorders are the second most common health disorder in domestic rabbits after gastrointestinal diseases. Dyspnea is a very common clinical finding in rabbits and is frequently associated with various infections of the respiratory tract. Pasteurellosis is the most important respiratory disease affecting domestic rabbits,

but other bacteria, including *Bordetella bronchiseptica* and *Staphylococcus* spp. are significant opportunistic pathogens (3). In our case, physical examination and cytological evaluation of the laryngeal mass excluded a subcutaneous abscess caused by a bacterial infection.

Spontaneous neoplasia in lagomorphs are rare, accounting between 0.5% and 2.7% (13). Similarly to humans and other species, the incidence of tumors in rabbits increases with age, being up to 8.4% after 2 years of age (13).

Primary laryngeal tumors are rarely encountered among all animal species. In canine and feline patients, laryngeal tumors account only for 0.02% in dogs and 0.14% in cats (14). In dogs, laryngeal tumors are mostly represented by squamous cell carcinoma (14), rhabdomyosarcoma (11) and chondroma (10), while in cats squamous cell carcinoma and lymphoma (12) are the most commonly encountered laryngeal tumors.

In rabbits, laryngeal and tracheal tumors are considered uncommon causes of progressive dyspnea. A recent report described two cases of airway obstruction caused by laryngeal osteochondroma and tracheal adenocarcinoma (2). To the authors' knowledge, laryngeal histiocytic neoplastic diseases have not been previously reported in rabbits.

Histiocytic disorders, as neoplastic or inflammatory processes, are well documented in humans, dogs, and cats. In dogs, HS complex includes two forms, localized and disseminated HS, characterized by infiltration of neoplastic histiocytes with marked cytological atypia, multinucleated cells, and high mitotic index (9). Localized HS affects the skin and subcutis, periarticular tissues, spleen, lungs, lymph nodes, and bone marrow (9). The present case was diagnosed as localized laryngeal HS, with multiple pulmonary metastases, showing similar histological and immunohistochemical features as those described in other cases of pulmonary (8) and cutaneous (7) HS in rabbits. Regarding the behavior of localized HS, our findings are in agreement with previous studies (1, 7), demonstrating local tissue invasion and metastases.

Cells of origin for HS are iDCs, which usually express some specific leukocyte surface molecules, including CD1a, MHC class II, and CD11c/CD18 in dogs (1) and Iba1, vimentin, E-cadherin, CD204, and Ki67 in rabbits (7, 8). Immunohistochemistry is extensively used for identification of the origin of HS, but the immunohistochemical differentiation between the localized and disseminated forms of the disease is still debatable (1). In rabbits, due to the fact that there are only a limited number of reported cases of HS, a complete immunophenotype characterization is not yet established. In our case, the definitive diagnosis of HS was made based on morphological features and the expression of vimentin, Iba1, of vimentin, Iba1, and CD1a.

Despite unclear etiopathogenesis of the HS in animals, some previous studies demonstrated various deletions of the tumor suppressor genes (CDKN2A/B, RB1, and PTEN) in certain dog breeds (5). No studies concerning the etiology of HS in domestic rabbits are available.

To conclude, HS is a rare and very aggressive tumor that can affect domestic rabbits, characterized by progressive respiratory distress and poor prognosis if localized in larynx. Therefore, laryngeal HS should be included in the list of differential diagnoses for progressive respiratory disorders in domestic rabbits. Predisposing factors for HS have not been identified in domestic rabbits.

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Ethical Statement

The current study is not an experimental part on living animals, therefore any approval from the ethic committee was not required.

Conflict of Interest

The authors declared that there is no conflict of interest.

References

1. **Affolter VK, Moore PF** (2002): *Localized and disseminated histiocytic sarcoma of dendritic cell origin in dogs*. Vet Pathol, **39**, 74-83.
2. **Bertram CA, Klopfleisch R, Muller K** (2019): *Tracheal and laryngeal tumors in two domestic rabbits* (*Orytolagus cuniculus*). J Exp Pet Med, **29**, 142-146.
3. **Deeb B, DiGiacomo RF** (2000): *Respiratory diseases of rabbits*. Vet Clin North Am Exot Anim Pract, **3**, 465-80.
4. **Fulmer AK, Mauldin GE** (2007): *Canine histiocytic neoplasia: an overview*. Can Vet J, **48**, 1041-1050.
5. **Hedan B, Thomas R, Motsinger-Reif A, et al** (2011): *Molecular cytogenetic characterization of canine histiocytic sarcoma: a spontaneous model for human histiocytic cancer identifies deletion of tumor suppressor genes and highlights influence of genetic background on tumor behavior*. BMC Cancer, **11**, 201, 1-14.
6. **Ishimori M, Michishita M, Yoshimura H, et al** (2017): *Disseminated histiocytic sarcoma with hemophagocytosis in a rabbit*. J Vet Med Sci, **79**, 1503-1506.
7. **Karim MR, Izawa T, Pervin M, et al** (2017): *Cutaneous histiocytic sarcoma with regional lymph node metastasis in*

a Netherland dwarf rabbit (*Oryctolagus cuniculus*). J Comp Pathol, **156**, 169-172.

8. **Leissinger M, Brandao J, Wakamatsu N, et al** (2013): *Pulmonary histiocytic sarcoma in a rabbit*. Vet Clin Pathol, **42**, 364-367.
9. **Moore PF** (2014): *A review of histiocytic diseases of dogs and cats*. Vet Pathol, **51**, 167-184.
10. **Ramirez GA, Altimira J, Vilafranca M** (2015): *Cartilaginous tumors of the larynx and trachea in the dog: literature review and 10 additional cases (1995-2014)*. Vet Pathol, **52**, 1019-1026.
11. **Sanspoux F** (2016): *A laryngeal rhabdomyosarcoma in a dog*. Vet Rec Case Rep, **4**, 1-4.
12. **Taylor SS, Harvey AM, Barr FJ, et al** (2009): *Laryngeal disease in cats: a retrospective study of 35 cases*. J Feline Med Surg, **11**, 954-962.
13. **Weisbroth SH** (1994): Neoplastic Diseases. 259-292. In: PJ Manning, DL Ringler, CE Newcomer (Eds), The Biology of the Laboratory Rabbit. 2nd ed., Academic Press, San Diego.
14. **Wilson DW** (2017): Tumors of the Respiratory Tract. 467-498. In: DJ Meuten (Ed), Tumors in Domestic Animals. 5th ed., John Wiley & Sons Inc., Ames.

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Case Report / *Olgu Sunumu*

Complete uterine prolapse in a cat

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Abstract: In this case, it was presented a case of complete uterine prolapse in a cat following a third parturition at the age of 18 months. Upon physical examination complete uterine prolapse was diagnosed due to a mass protruding from the vulva which included both uterine horns. After the invagination and eversion were corrected and the uterus was relocated to its anatomical position, ovariohysterectomy was performed. Sutures were removed ten days after the operation. Following shrinking of uterus by using the hypertonic fluid and repositioning, ovariohysterectomy is a suitable and reliable option for cats with complete uterine prolapse.
Keywords: Cat, ovariohysterectomy, parturition, uterine prolapse.

Bir kedide tam uterus prolapsusu

Özet: Bu olguda, üçüncü doğumunu takiben tam uterus prolapsusu şekillenen 18 aylık bir kedi sunulmaktadır. Fiziksel muayenede, her iki kornu uteriyi içeren bir kitlenin vulvadan dışarı çıkması sebebiyle tam uterus prolapsusu tanısı konuldu. İnvaginasyon ve eversiyon düzeltilip uterus anatomik pozisyonuna getirildikten sonra ovaryohisterektomi yapıldı. Operasyondan 10 gün sonra dikişler alındı. Hipertonik sıvı kullanılarak uterus küçültüldükten ve yeniden pozisyon verildikten sonra, ovariohisterektomi tam uterus prolapsusu olan kediler için uygun ve güvenilir bir seçenektir.
Anahtar sözcükler: Doğum, kedi, ovariohisterektomi, uterus prolapsusu.

Uterine prolapse, rarely observed and infrequently reported in queens (10, 12), usually occurs before and during, or immediately after parturition/abortion or within 48 hours following parturition/abortion as an emergency situation in primiparous (10) and multiparous cats (1, 9). The complication can be seen in two forms: a complete or bilateral form in which both uterine horns prolapse, or a unilateral form in which one uterine horn with or without the uterine body prolapses (10, 12). Although definitive cause of uterine prolapse is unknown, the possible factors include excessive relaxation and stretching of the pelvic muscles, excessive dilatation of the cervix, uterine atony due to metritis, incomplete separation of placental membranes, severe tenesmus, mesovarium weakness, and rupture of the mesometrium (1, 10, 11). Treatment options are determined according to the degree of tissue necrosis or ischemia, that is, the severity of tissue damage. In the treatment, amputation of the prolapsed uterus, manual reduction and repositioning, oxytocin injection, local hyperosmotic fluid massage (50% dextrose), manual reduction with laparotomy and ovariohysterectomy (OHE) are used alone or in combination (1, 4, 10, 11). In this case report, the treatment protocol for complete uterine prolapse in a cat after parturition is described.

This case involved an 18-month-old calico cat presented to our clinic with complaints of a persistent vaginal mass, weakness, loss of appetite, and poor general condition. Information obtained from the anamnesis indicated that the cat gave birth to three live kittens three days earlier. The owner assumed that the mass emerging from the vulva was indicative of continuing parturition was and the cat was left alone. The cat had given birth to healthy kittens twice previously without experiencing dystocia.

A direct diagnosis was made after observing the protrusion of the bicornuate uterus from the vulva. The uterus was damaged, lacerated, and desiccated (Figure 1). Upon physical examination, it was determined that the cat

weighed 3.1 kg, with a body temperature of 39.3°C, heart rate 144 bpm, respiratory rate 24/min, dehydration rate 7%, and capillary refill time of three sec. The cat had leukocytosis and anemia, however, serum biochemical parameters were within reference values (Table 1). Abdominal ultrasonography revealed that the kidneys were pulled caudally and moved away from their normal anatomical position, and the bladder was in place.

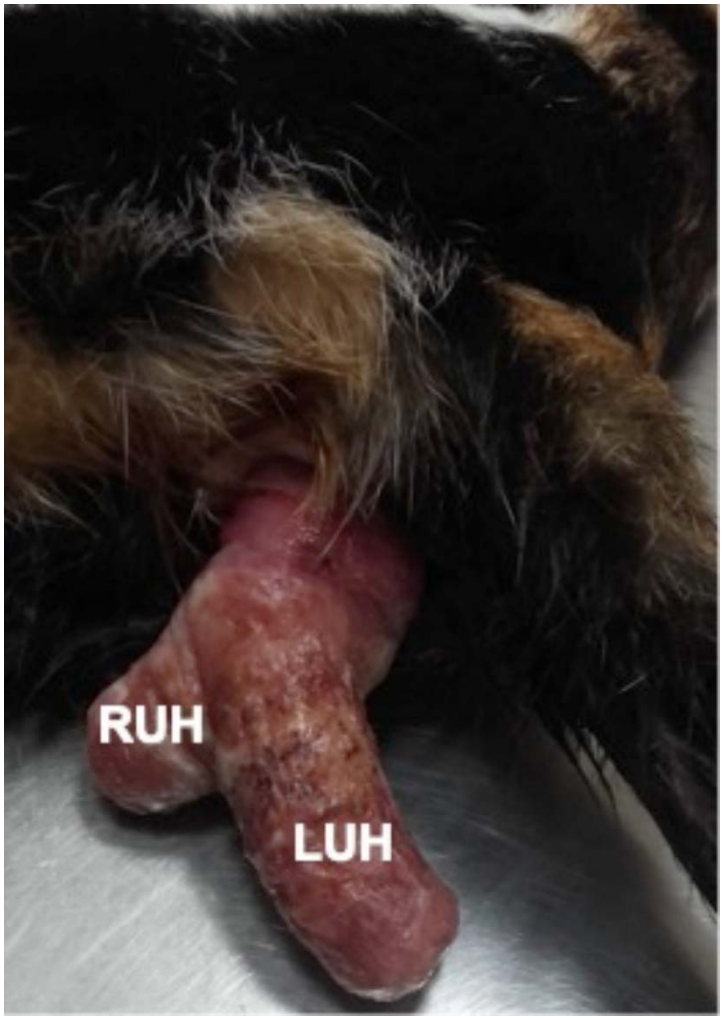

Figure 1. The appearance of the prolapsed uterus. The right uterine horn (RUH) and the left uterine horn (LUH).

Table 1. Hematological and serum biochemical values.

Hematological			Serum Biochemical		
Parameters	**Value**	**Reference Range**	**Parameters**	**Value**	**Reference Range**
WBC ($x10^9$/L)	20.63	6-17	ALP (U/L)	15	1-100
RBC ($x10^{12}$/L)	2.94	5.5-8.5	ALT (U/L)	37	16-60
HGB (g/dL)	4.4	11.0-19.0	CREA (mg/dL)	0.77	0.9-2.2
HCT	0.118	0.3-0.45	BUN (mg/dL)	12.5	19-34
PLT ($x10^9$/L)	168	117-460			
MCV (fL)	40	39-55			
MCH (pg)	14.8	13-17			
MCHC (g/L)	370	300-360			

White blood cell (WBC), red blood cell (RBC), hemoglobin (HGB), hematocrit (HCT), platelet (PLT), main corpuscular volume (MCV), main cell hemoglobin (MCH), main corpuscular hemoglobin concentration (MCHC), alkaline phosphatase (ALP), alanine transaminase (ALT), creatinine (CREA), blood ure nitrogen (BUN).

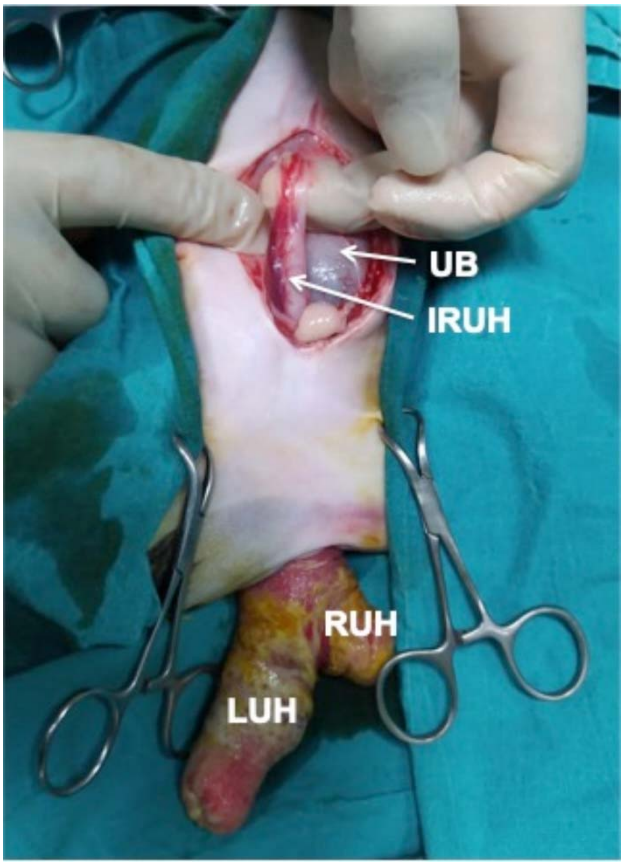

Figure 2. Invaginated right uterine horn (IRUH) was observed above the urine bladder (UB), right uterine horn (RUH), left uterine horn (LUH).

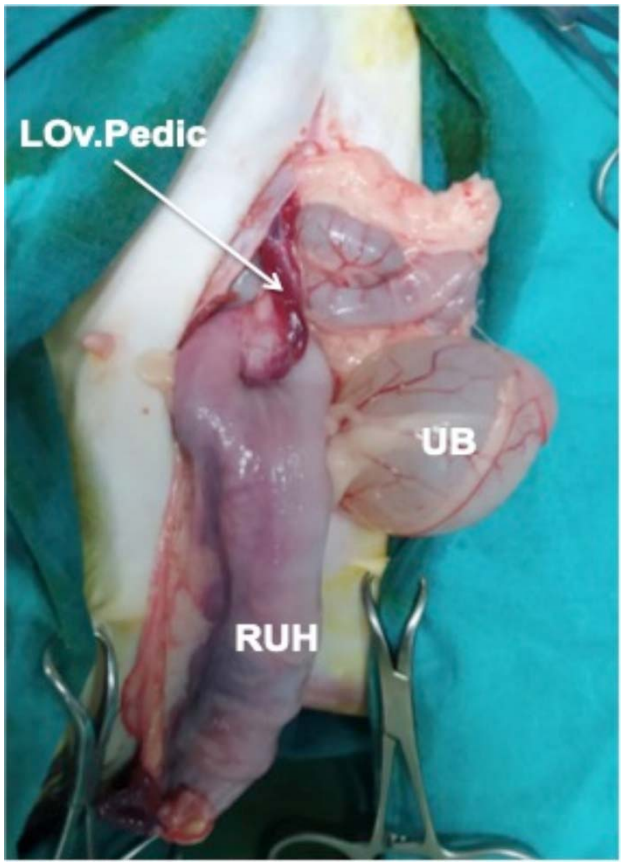

Figure 3. The view after correcting the invaginated right uterine horn. As a result of eversion of the left uterine horn, only the ovary and its pedicle (LOv.Pedic) were visible, right uterine horn (RUH), urinary bladder (UB).

It was decided to perform OHE after the prolapsed uterus was repositioned to its anatomical position in the abdomen by laparotomy. The cat was treated preoperatively with intravenous Lactated Ringer's solution (Polifleks®; Polifarma, Turkey and supportive maintenance therapy solutions (Duphalyte®; Zoetis, Spain 10 ml/kg/hour), and amoxicillin and clavulanic acid combinations (Amoklavin®; Deva, Turkey) at the dose of 20 mg/kg twice daily perorally. The prolapsed uterus was washed with saline solution and kept under a compress of hyperosmotic fluid (20% dextrose solution; Dekstroz; Polifarma, Turkey) for 25-30 min to facilitate repositioning by reducing uterine edema. Anesthesia was induced with propofol (Fresenius®; Kabi, Turkey 1%, 6 mg/kg/iv) and maintained with isoflurane (Forane®; Piramal, USA) and 100% O_2 after intubation. Intra-operative analgesia was managed with a constant-rate infusion (CRI) of a combination of saline, ketamine (Ketasol®; İnterhas, Turkey and butorphanol (Butomidor®; İnterhas, Turkey) (500 ml + 35 mg + 12 mg, respectively) at a rate of 10 ml/kg/hour. The surgery was performed by midline incision as a non-complicated OHE. During the procedure, it was determined that the right ovary was displaced to the caudo-dorsal side of the abdomen and the pedicle was stretched. On the other hand, the left ovary, accompanied by a severely stretched pedicle was found in a deep caudal position but with no pedicle rupture (Figure 2). The right horn was partially invaginated and the left horn was everted with the corpus uteri (Figure 3). First, the right cornu uteri was carefully retracted and the right side invagination was corrected. Then the left uterine horn was grasped and its eversion was corrected and both horns were brought to their normal anatomical position. During the correction of the invagination and the eversion processes, no resistance to pulling the uterine tissue or caudal reproductive tract (vulva or vagina) was encountered. Finally, a routine OHE was performed. In the postoperative period, fluid therapy and antibacterial treatment were continued for a week. Urination started within the first postoperative hour and the cat began to eat and drink the following day. The skin sutures were removed ten days later and hospitalization ended.

As a periparturient complication, prolapse uteri is rarely encountered in cats (12). According to Extrand and Linde-Forsberg (3), uterine prolapse cases are responsible

for 0.6% of maternal dystocia (3). At the same time, it is thought that predisposition factors such as dystocia, severe uterine and abdominal contractions, insufficient placental separation, a continuation of postpartum tenesmus, and loosening or rupture of uterine ligaments due to recurrent pregnancies play a crucial role in such cases (1, 4, 5, 12). Besides, body condition score (BCS) may also be an important factor. For example, uterine prolapse incidence is higher in overweight women (8) and poor BCS has an important effect on uterine or vaginal prolapse in cows (6). In this case, the patient's history was not remarkable but examination and operative findings were sufficient for diagnosis. Therefore, although it is hard to determine the exact cause of the prolapse, we can provide information about the patient's condition. Because of the absence of dystocia findings in the history and the absence of placenta or fetal structures in uterus we focused on two points as reasons for the cat's current situation: the relationship between the cat's age and the number of parturitions, and the BCS of the cat. In the present case, the cat's third parturition at 18 months of age suggests that the cat probably conceived during the first estrus cycle and the parturition-conception intervals were very short. Besides, considering the 3.1 kg body weight, the cat had a relatively weak BCS (13). Therefore, we suggest that conceiving at an early age, repeated pregnancies and parturitions, accompanied by a weak BCS triggered the prolapse by weakening the uterine suspensory ligaments and pelvic ligaments.

Uterine prolapse requires urgent intervention because of the possibility of life-threatening situations such as hypovolemic or septic shock due to rupture of the uterine vein or uterine mucosa or to maintain fertility by preventing further damage to the uterus (10). In this case, prolapse had occurred immediately after complete parturition, according to anamnesis. Considering the three days between the time of development of the case and the time of admission to our clinic, the prolapsed uterus and the patient's general condition were better than expected. However, the mucosa of the prolapsed uterus was observed to be dried, wrinkled, and lacerated, and the general condition of the cat had started to deteriorate. When evaluating the hemogram, we thought the cause of mild leukocytosis could result from bacterial contamination. Still, the cause of anemia was the pregnancy and early postpartum period rather than infection. Thus, the anemia was considered normal. According to Kimura and Kotani (7), RBC and HCT values tend to decline at a moderate level after mid-pregnancy in dogs (7). Also, in this case hemogram results, were similar to those reported by Uçmak et al. (5). Treatment options of should be selected according to the degree of uterine damage (10). Based on the literature, OHE is primary surgery in many cases (2, 5, 10, 11). In uterine prolapse in cats, there are two main OHE procedures. The surgery would be completed in one step by routine OHE after repositioning the prolapse by laparotomy (10), or amputation of the prolapsed uteri would be done after ovariectomy (2). In this case, we preferred the first option. After the correction of invagination and eversion, and the uterus was brought to its anatomical position, an OHE was performed. The hypertonic solution, which was administered to the prolapsed tissue, reduced the mass volume and made insertion easier. Although no information was provided about the operation time and the advantages of the method in previous publications, the procedure we followed, in this case, required less labor than the two-stage process (2). Also, we think a shorter duration of anesthesia has advantages, such as reducing the risks of complications such as hypovolemia, hypothermia, infection, or death. As a treatment option, the repositioning procedure and bringing the uterus to the anatomical position without OHE could be attempted; however, we preferred the OHE due to lacerations in the uterine mucosa, the risk of infection, and the possibility of recurrence in later deliveries in addition to the request of the owner.

This case showed that uterine prolapse could continue to exist for up to three days without life-threatening consequences. Undoubtedly, this case is an individual situation, and uterine prolapse typically requires urgent intervention because it is life-threatening. In such cases, OHE is a suitable and reliable option that can be performed after quickly repositioning the uterus made possible by keeping it in hypertonic fluid before surgery.

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Ethical Statement

This work involved the use of non-experimental animal only (including owned). Established internationally recognized high standards ('best practice') of individual veterinary clinical patient care were followed. Ethical approval from a committee was therefore not necessarily required.

Conflict of Interest

The authors declared no potential conflicts of interest concerning the research, authorship, and/or publication of this article.

References

1. **Bigliardi E, Di Ianni F, Parmigiani E, et al** (2014): *Complete uterine prolapse without uterine mucosal eversion in a queen*. J Small Anim Prac, **55**, 235-237.
2. **Deroy C, Bismuth C, Carozzo C** (2015): *Management of a complete uterine prolapse in a cat.* J Feline Med Surg Open Reports, 1-4.
3. **Ekstrand C, Linde-Forsberg C** (1994): *Dystocia in the cat: A retrospective study of 155 cases.* J Small Anim Pract, **35**, 459-464.
4. **Feldman EC, Nelson RW** (2004): Periparturient diseases. 808. In: Feldman EC and Nelson RW (Eds), Canine and Feline Endocrinology and Reproduction. 3th ed., Saunders, St Louis.
5. **Günay Uçmak Z, Uçmak M, Çetin AC, et al** (2018): *Uterine prolapse in a pregnant cat.* Turk J Vet Anim Sci, **42**, 500-502.
6. **Hadush A, Abdella A, Ragassa F** (2013): *Major prepartum and postpartum reproductive problems of dairy cattle in central Ethiopia.* J Vet Med Anim Health, **5**, 118-123.
7. **Kimura T, Kotani K** (2018): *Perinatal veterinary medicine–related evaluation in hematological and serum biochemical profiles of experimental beagles throughout pregnancy and parturition.* Animal Model Exp Med, **1,** 282-294.
8. **Myers DL, Sung VW, Richter HE, et al** (2012): *Prolapse symptoms in overweight and obese women before and after weight loss.* Female Pelvic Med Reconstr Surg, **18**, 55-59.
9. **Nöthling JO, Knesl O, Irons P, et al** (2002): *Uterine prolapse with an interesting vascular anomaly in a cheetah: A case report.* Theriogenology, **58**, 1705-1712.
10. **Özyurtlu N, Kaya D** (2005): *Unilateral uterine prolapse in a cat*. Turk J Vet Anim Sci, **29**, 941-943.
11. **Sabuncu A, Evkuran Dal G, Enginler SÖ, et al** (2017): *Feline unilateral uterine prolapse: A description of two cases.* J Fac Vet Med Istanbul Univ, **43**, 67-70.
12. **Sharma A, Sharma R, Kumari A, et al** (2019): *Ovariohysterectomy followed by hysteropexy of gravid uterine prolapse in a female cat - a case report.* Vet Res Int, **7**, 76-78.
13. **Verbrugge A** (2009): Nutritional modulation of carbohydrate metabolism in domestic cats (felis catus). PhD Thesis, Ghent University, Belgium.

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Review / *Derleme*

Rumen physiology: microorganisms, fermentation and manipulation

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Abstract: Ruminants are unique mammals that can convert the energy in roughage to edible products for humans. Hence, rumen fermentation has been excessively on the scope of researchers for long years. Advances in rumen fermentation are a vital concern to provide food with good quality for the growing population of man. This review focuses on physiology of rumen fermentation and the recent advances in the field.
Keywords: Fermentation, manipulation, methane, rumen, ruminant.

Rumen fizyolojisi: mikroorganizmaları, fermantasyonu ve manipülasyonu

Özet: Ruminantlar, kaba yemlerin içerdiği enerjiyi insanların değerlendirebileceği ürünlere dönüştürebilen benzersiz memelilerdir. Bu nedenle, rumen fermantasyonu yıllardan beri bilim insanlarının yoğun bir şekilde ilgisini çekmiştir. Rumen fermantasyonun geliştirilmesi nüfusu hızla artan insanlığın kaliteli gıda ihtiyacının karşılanmasında hayati öneme sahiptir. Bu derlemede rumen fermantasyon fizyolojisi ve bu alandaki son gelişmeler ele alınmıştır.
Anahtar sözcükler: Fermantasyon, manipülasyon, metan, rumen, ruminant.

Introduction

Plants capture and collect solar energy in their structural components and seeds via synthesizing organic compounds. The biological process of this energy conversion from the sun to the biological compounds is called photosynthesis (52, 78). It is the only way for life to survive on the earth with a few exceptions such as; some microorganisms gather energy from the oxidation of some inorganic matter instead of the sun and several rare sea creatures live on the ocean floor next to the hydrothermal vent. The most common organic compound synthesized via sunbeam is cellulose in the world (79). The dilemma about the cellulose is that neither man nor any other mammalian can degrade the cellulose properly except ruminants that diverge from monogastric mammalians with their unique continuous fermentation capability. Mammals other than ruminants cannot benefit from the chemical bond energy collected in the beta-1,4-glucosidic bonds of cellulose found in plant cell walls due to the lack of the cellulose-degrading enzymes in their gastrointestinal tract (10).

In ruminants as pregastric mammals, the rumen, reticulum, and omasum are pregastric compartments before the glandular stomach. The rumen, found only in ruminants, is a vast fermentation compartment in which nutrients are continuously digested by microorganisms. The reticulum filtrates the well-confined particles from larger ones and liquid to deliver throughout the omasum. After this, further digestion processes occur in the abomasum. The rumen is a highly developed continuous fermentation environment with up to 200 liters volume where synchronized contractions mix the rumen content at a constant temperature (~ 37-39 °C) and pH (between 5.5 and 7.0) and where plenty of microorganisms that each species has a different mission on the fermentation process (79). These properties of the rumen provide ruminants a unique advantage in degrading cellulose, thereby converting low-quality roughage to meat and milk. Hence, humans can use solar energy captured and converted into cellulose and subsequently degraded and resynthesized as digestible and healthy compounds through rumen fermentation. Ruminants' genetic revolution advanced

humans to use roughage as quality feed. Therefore, humanity is dependent on ruminants' continuous fermentation system until developing a method to convert roughage into digestible compounds conventionally. Therefore, understanding the ruminal fermentation mechanism is essential to meet the food demand of rapidly growing population of humans. This review aims to expand the process of ruminal fermentation in consideration of new literature to develop novel methods to enhance livestock performance and mitigate the environmental impacts of ruminants.

The rumen as a continuous fermenter

In mammals, the digestive system following the stomach is very similar, whereas, in the ruminants, the stomach is quite different from the other species. The ruminant digestive system consists of forestomaches; the rumen, reticulum, omasum, and abomasum, which is the equivalent of the stomach, found in monogastric animals. The rumen is a fermentation sac with a volume of up to 200 liters. According to Russell et al. (79), calculating the total volume of the rumen capacity of the world's domesticated ruminants, the rumen is the "world's largest commercial fermentation process" with 100 billion liters of total volume.

In the rumen, the enzymes of microorganisms dramatically alter the composition of the forage via the fermentation processes. Fermentation is done enzymatically and mechanically by symbiotic microorganisms in the rumen, not by the ruminant's own enzymes. This phenomenon provides ruminants the advantage of benefiting from nutrients such as cellulose, hemicellulose, lignin, and pectin, which are the most abundant in nature and other mammals and humans cannot digest or digest poorly (70). Robert E. Hungate pointed out rumen microbial ecosystem for the first time and revealed that the microbial interrelation with each other and the host is essential for other inventions to advent food and various sectors (41). Hungate has been recognized as the father of modern anaerobic microbial ecology due to his studies developing systematic anaerobic culture methods (59).

Ruminants are born functionally monogastric. Consequently, fermentation occurs by the consumption of forages which leads to a mature rumen function under the effect of volatile fatty acids (VFAs) released as the end products of fermentation, and hormones such as insulin, insulin-like growth factor IGF-1, and epidermal growth factor EGF (74). The rumen is covered with the stratified squamous epithelium with leaf-like papillae. However, the absorption of VFAs, nitrogenous compounds and minerals, and bicarbonate secretion occurs in the rumen (28, 83). The rumen and the reticulum perform the same function indeed. The reticulum anatomically shapes differently since covered with honeycomb-like internal mucosa to perform its critical function of transferring the digested, confined, and denser particles into the omasum. Therefore, the reticulum acts functionally in coordination with the rumen, ensuring that only the digested particles of the rumen content are allowed to be transferred to the omasum via its two-stage motility synchronized with rumen and omasum (17). The critical function of the rumen is; (*i*) to ensure optimal conditions such as temperature, pH, and osmotic pressure which all of them are required for effective fermentation and (*ii*) provide the nutritional presence required by microorganisms to maintain the fermentation and (*iii*) ensure the microorganism adsorption onto particles by stirring. Indeed, in this symbiotic relationship between ruminants and microorganisms, forages are digested by microorganisms, as the rumen provides the optimum conditions for increasing the microbial population by removing VFAs that lead to lower pH in the rumen if VFAs' levels are high (28).

The rumen consists of two parts in which the feed is collected according to the particle size and specific weight. Dorsal sac where gas and roughages with a lower specific weight due to being rougher and undigested are collected and ventral sac where confined and digested roughage with higher specific weight, are sedimented. Rumen pH ranges from 5.5 to 7.0, depending on the feed composition. The temperature in the rumen is between 38-40 °C, which is a proper temperature for rumen microorganisms providing effective fermentation (74).

The feed ruminant consumed, digested by rumen microorganisms and degraded into its essential components. Consequently, novel organic compounds and vitamin B_{12} are synthesized to form the structural components for the growth of the microbial population. Profile of rumen microorganisms varies depending on the content of the diet and correspondingly, an alteration in the composition of rumen microorganisms results in both intermediate and end products generated by fermentation and digestion of feed in the rumen (30, 95). The rumen content differs in free-living ruminants compared to the ruminants in the intensive stock farming. High-yield breeds are used in the intensive farming due to economic concerns. Hence, the feed with a high proportion of concentrates meaning high in digestible carbohydrates, raw proteins, and energy, is provided for high yield (63). Ruminants consume carbohydrates, cellulose, hemicellulose, lignin, pectin, vegetable wax, cutin, suberin, and starch. They also consume short-chain carbohydrates such as soluble sugars, protein, non-protein nitrogen compounds, fats, minerals, vitamins, bicarbonate, and phosphate comes in water and saliva. In the process of ruminal fermentation, fat, cellulose, hemicellulose, pectin, starch,

and soluble sugars are converted into VFAs by microorganisms called microbial degradation. As a result, the structural elements of the forage converted to VFAs, carbon dioxide, and hydrogen in the rumen (26). VFAs are essential for supply approximately 80% of the animal's energy needs. VFAs synthesized in rumen consist of roughly acetic acid (45-70%), propionic acid (15-40%), butyric acid (5-20%), and iso-acids (2-5%) derived from protein degradation (22, 47). The most critical volatile fatty acids for the ruminant are acetic and propionic acids. Acetic acid is used by cells as a direct energy source and is converted into long-chain fatty acids to form milk fat. Propionic acid is used in glucose synthesis in the liver. Butyric acid mainly provides the energy requirements of the rumen epithelium. There are other products such as formic acid, lactic acid, CO_2, ethanol, and ammonia in the rumen. Hydrogen produced by microorganisms and retained by reducing NAD^+, $NADP^+$, and FAD^+, is released into the rumen by re-oxidation of these co-factors, thus fermentation continuity is maintained. However, when hydrogen density is high in the rumen, the synthesis of volatile fatty acids is affected due to suppressed re-oxidation of NADH, NADPH, and FADH (58). Methanogens use hydrogen and CO_2 found in the rumen in the production of methane (CH_4). The entire methane is excreted with eructation. Although methane formation is an undesirable phenomenon due to energy loss, it is essential to remove hydrogen from rumen content and necessary for the continuity of fermentation (60).

Approximately 12% of the feed's gross energy is lost due to methane synthesis (46). Such losses are considerable for intensive production systems. In addition, microorganisms convert the raw protein taken with feed into amino acids, peptides, a small number of volatile fatty acids, and ammonia (84). A significant amount of ammonia is used as a source of nitrogen to synthesize protein-building compounds for the growth of microorganisms. Some ammonia is also absorbed from the rumen and converted into urea in the liver. A little proportion of urea is then moved back to the rumen through rumen epithelium, while the other part is brought back to the rumen by saliva. Another notable amount of ammonia is also excreted with urine through the kidneys and discarded. The latter is considerable nitrogen loss as up to 25% of nitrogen taken with nutrients is excreted in this way. However, this excretion rate decreases in feeding with poor quality roughages (48).

Rumen microorganisms and their role in ruminal fermentation

Rumen harbors microorganisms, bacteria, protozoa, fungi, bacteriophages, and even parasites. The total microbial biomass consists of bacteria (~ 40-50%), protozoa (40%), and fungi (8%) (88). Although bacteriophages and parasites represent a negligible percentage, they still have potential effects on the microbial diversity and consequently the performance of rumen fermentation (29, 31). Even though various factors influence the microbial diversity of the rumen, the most critical factor is the composition of feed, which alters the rumen environment by influencing the microbial diversity (50). Indeed, all biochemical flow is based on the composition of feed, the diversity of rumen microorganisms, and the environmental conditions of the rumen unless an external factor that might affect the rumen. It has known that the rumen environment has a strong adaptation capability to the feed provided and the other external changes due to the rumen microorganisms with their high adaptation and the buffering capacity of rumen and saliva. Thus, although it is easy to alter the rumen fermentation and microbial diversity by manipulating the composition of feed and adding some additives, the rumen microorganisms still adapt to the new conditions a while after providing different feed and additives (58). Therefore, discovering a persistent method to manipulate rumen fermentation has recently been an essential purpose for researchers.

Rumen bacteria

Rumen bacteria can be divided into four essential subpopulations according to their place into the rumen: 1) those attached to the rumen epithelial cells which are less than 1% of the total rumen microbes, 2) those attached to feed particles (~ 70-80% of the total rumen bacteria) (50), 3) those suspended in the ruminal fluid (~ 20-25% of total rumen bacteria) (56), and 4) those attached to the surface of protozoa or fungal sporangia (97). It is estimated that more than 3.000 species of bacteria inhabited the rumen (2). A considerable proportion of the rumen bacteria consists of obligate anaerobe (3). It is thus principal to maintain the rumen O_2 concentration below 0.5% to survive the rumen anaerobic bacteria. Facultative anaerobes, which represent a small amount of the bacterial population, are essential to maintain the rumen environment absolutely anaerobe conditions by consuming oxygen arisen from feed intake. Those bacteria require a small amount of oxygen to maintain their vitality. Gram-negative bacteria form the majority of the rumen bacterial population. On the other hand, ruminants fed with concentrates show a rapid increase in the number of Gram-positive bacteria (9). The changes in feed composition consequently cause alterations in the composition of rumen microorganisms. Furthermore, sudden changes in feed composition may have adverse effects on the protozoa and bacterial species, which may lead to several pathological problems. The higher proportion of the concentrates in the feed increases

especially the Gram-positive rumen bacteria, methanogenic archaea with greater methane production capacities compared to the other species, hyper-ammonia producing bacteria that produce ammonia 20 folds faster than the other species, and lactic acid producers (12). Gram-positive bacteria also produce butyrate, acetate, lactate, format, and hydrogen, whereas Gram-negative bacteria produce propionate and succinate, which are much more suitable for ruminants (22, 92). Fiber-associated bacteria degrade plant ingredients and have an essential role in rumen fermentation. Rumen bacteria are also classified according to their function (14).

Cellulolytics: Rumen environment's essential bacteria are the cellulolytic bacteria such as *Fibrobacter succinogenes, Butyrivibrio fibrisolvens, Ruminococcus flavefaciens, Ruminococcus albus, Clostridium cellobioparum, Clostridium longisporum, Clostridium lochheadii,* and *Eubacterium cellulosolvens* (14). *Bacteroides (Fibrobacter) succinogenes, Ruminococcus albus, and Ruminococcus flavefaciens* that Robert Hungate (40) isolated for the first time are the most important among the cellulolytic bacteria of the rumen. The latter three species have different binding sides and specificities. Thus, they do not compete for the adhesion side of the plants. Cellulolytic bacteria of the rumen require adhering to cellulose for their cellulolytic activity. These species have a wide range of fibrolytic enzymes called glycoside hydrolases (GHs) for cellulose degradation (13).

Hemicellulolytics: *Prevotella ruminicola, Eubacterium xylanophilum, Eubacterium uniformis* are non-cellulose fiber degrading bacteria of the rumen (14), and they have more than a hundred glycoside hydrolases (13, 93). Most of the cellulolytics are also capable of hemicellulose degradation (65).

Pectinolytics: *Treponema saccharophilum* and *Lachnospira multiparus* (14), mainly degrade the pectin. *Butyrivibrio fibrisolvens*, *Bacteriodes ruminicola*, *Lachnospira multiparus, Succinivibrio dextrinosolvens,* and *Streptecoccus bovis* are also pectinolytic. The three groups of bacteria above are the largest group within the bacterial population in the rumen. As fiber degraders, their population increases in roughage feed conditions, and their end products are substantially acetic acid and H_2. These three species together with the amylolytics are called primary rumen bacteria. The other bacterial groups are called seconders due to using the end products of the primary ones (22,85).

Amylolytics: *Streptococcus bovis, Ruminobacter amylophilus, Prevotella ruminicola* (14), and *Butyrivibrio fibrisolvens* (19) are mainly amylolytic bacteria. *Succinivibrio dextrinosolvens, Succnivibrio amylolytica, Selenomonas ruminantium, Bifidobacterium ruminantium, Lactobacillus acidophilus, Lactobacillus casei* and *Lactobacillus fermentum* are saccarolytic. On the other hand, rumen bacteria do not function strictly toward a single biochemical flow. Rather, a combination of different functions, as can be seen in the above-mentioned bacteria, each species has their own function such as *Prevotella ruminicola* and *Butyrivibrio fibrisolvens* are hemicellulolytic and pectinolytic, respectively. Amylolytic bacteria in the rumen are more resistant to lower pH values, while *S. bovis* is the most resistant to pH 4.5 (87). Amylolytics' growth is promoted when ruminant is fed with proportionally higher grain feed. Amylolytics are rapid-growing species with their growth rate (doubling time) from 15 minutes to 4 hours compared to cellulolytics. These species produce propionic acid, lactic acid, succinic acid, format, and CO_2 as end products. They are associated with the digestion of starch and soluble sugar (85).

Acetogens: A small proportion of rumen bacteria is described as acetogens because of their reductive acetogenesis capability from CO_2 and H_2. Acetogens in the rumen are *Acetitomaculum ruminis* and *Eubacterium limosum,* and they are expected to be more. Although their reductive acetogenesis capability can be proved *in vitro,* acetogens have not functioned as alternative H_2 sink in the rumen. Acetic acid synthesis yields less energy than methane synthesis. Hence, acetogens cannot thermodynamically compete with methanogens. Nevertheless, further studies are needed on whether the acetogens can be used as an alternative H_2 sink to mitigate methane production in the rumen with different conditions (45).

Proteolytics: *Prevotella ruminicola, Ruminobacter amylophilus, Clostridium bifermentans* and *Colostridium proteoclasticum* are the most common proteolytics (14). Although *Bacteroides ruminicola* is essential for proteolytic activities in rumen, it seems that there is a synergism between species such as *Butyrivibrio fibrisolvens, Selenomonas ruminantium,* and *Streptococcus bovis* to enhance this activity according to the variety of the bacterial population. Proteolytic activity represents a major loss of dietary amino acids for ruminant (91).

Acid and urea utilizers: *Megasphaera elsdeni* and *Anaerovibrio lipolytica* have a critical role when the rumen lactic acid production is high due to the high grain content feed (51). *Megasphaera elsdeni* also utilizes urea to ammonia (NH_3) and CO_2 (14).

Lipolytic: *Anaerovibrio lipolytica* ferments the glycerol to propionic acid and succinic acid; fructose, ribose, and lactic acid to acetic acid, propionic acid, and CO_2. The critical importance of *Anaerovibrio lipolytica* arise from its lactic acid utilizing capability in the ruminants fed with high grain feed. All fermentation processes produce a small amount of H_2 (85).

Rumen methanogens

Recent studies revealed that rumen methanogens constitute 2.8 to 4% of ruminal microorganisms (44) and belong to domain archaea (62). More than 90% of rumen archaea are member of genera; *Methanobrevibacter* (more than 60%), *Methanomicrobium* (up to 15%), and rest of the rumen archaea referred to rumen cluster C approximately 16% or *Thermoplasmatales* that function in the rumen is unknown. Although they are a small number of rumen microorganisms, the effects of rumen methanogen archaea on rumen fermentation are significant as they are the main CH_4 producers in the rumen. Interestingly, a considerable proportion of methane production in the rumen is attributed mainly not to the *Methanobrevibacter* or *Methanomicrobium*, which constitutes roughly 75% of the rumen archaea. Instead, it is attributed to the rumen cluster C archaea even though they are only 16% of rumen archaea, and their fundamental function and biochemical flow in the rumen has remained unknown yet (59).

Rumen protozoa

In addition to the bacteria, the obligate or facultative anaerobic protozoa with various species of ciliates and flagellates form another group of rumen microorganisms. Ciliata accounts for nearly half of the rumen biomass, while flagellates are much less. Some researchers consider protozoa for digestive functions in the rumen (74). In contrast, other researchers reported them as valuable as a nitrogen source to compensate the nitrogen-poor feed out of season only for wild ruminants fed primarily with poor quality forage (96). Protozoa digest nutrients, bacteria, fungi, and other protozoa. They serve a pivotal role in preventing rumen acidosis via rapidly removing carbohydrates that can be easily fermented. Moreover, they eliminate toxic compounds from plants and reduce the risk of heavy metal poisoning. However, protozoa are not essential for regular digestive functions in the rumen (65).

Rumen fungi

All fungi detected so far are obligatorily anaerobic with a narrow temperature optimum between 33-41 °C (74). The species of fungi, which belong to the genus *Neocallimastix*, *Piromyces*, *Orpinomyces*, *Caecomyces*, and *Anaeromyces* are inhabited in the rumen. Fungi vary broadly with nutrition and reach the highest amount of ruminal population 12 hours after feeding. Their mass increases to 8% of the total biomass in roughage feeding (88). Rumen fungi are crucial in the digestion of cellulose. In the absence of fungi, there is a significant decrease in the digestion of cellulose in the rumen. Fungi also facilitate bacterial colonization onto plant structure by degrading the plant cell wall and stimulating digestion. Some of the fungal species have reportedly shown proteolytic activity. Low rumen pH inhibits fungal reproduction. High levels of readily digestible sugars also reduce fungal generation. This explains the growing population of fungi in roughage-rich feeding (69).

Rumen viruses

Bacteriophages are viruses that infect rumen bacteria. Their number range from $2x10^7$ to $1x10^8$ per ml of rumen fluid. Although more than 100 different bacteriophages have been identified to date, this number can be expected to expand to a large number of bacteriophage species, given that bacteriophages are unique to all bacterial species. Biotechnological methods can develop bacteriophages specific to the type of bacteria, and there may be a potential for suppression of bacteria whose rate of reproduction in the rumen is to be decreased (49).

Rumen parasites

The first study that empirically demonstrates disease-driven increases in methane (CH_4) yield in livestock reveals interesting results. Gastrointestinal parasite infestations increase methane yield (g CH_4/kg of DMI) by up to 33% (29).

Manipulation of ruminal fermentation

A significant amount of greenhouse gases around the world is released due to agricultural activities that play a critical role in food production and the economy. However, global warming and loss of productivity due to greenhouse gas emissions arising from these activities have still been debated. While animal products account for 40% of the world's agricultural products, a significant proportion of this production comes from ruminants. Ruminants produce meat and milk, which are precious nutrients for humans, by digesting the plant's structural components that humans cannot digest. Humans provide life safety and healthy feed resources for the ruminants the whole year and ruminants provide essential nutrients for humans. This mutualistic cooperation with ruminants raised the world's ruminant population enormously while humans supply essential nutrients such as milk and meat (82). However, due to fermentation, ruminants release greenhouse gases such as methane (CH_4) and nitrous oxide (N_2O) which are essential contributors to global warming. In addition, the formation of nitrous oxide is significant during the processing of agricultural land used in ruminant nutrition. Since the 2000s, scientists have performed numerous studies to develop new methods for reducing methane gas emissions in livestock. In those studies, scientists have proposed various methods such as the

addition of ionophores, organic acids and plant essential oils to feed, immunization, modification of feed composition, rumen defaunation, alternative hydrogen (H_2) sinks, modification of the microbial rumen distribution, and animal breed replacement with the minimal methane-producing breeds (35).

Modification of the feed composition

The composition of the feed given to ruminants significantly affects the production of methane. Methane production can be reduced by up to 90% with the modification of the feed composition. Fermentation performance decreases with poor-quality roughage, lacking in vitamins, minerals, proteins, and energy, thus increases methane production. However, supplementation of minerals and nitrogen sources improves fermentation performance and consequently decreases methane production. Fresh roughage reduces methane production. Fresh alfalfa, oats, sorghum instead of hay and replacing the 30% wheat straw with fresh sorghum reduces methane production by 33% (37). Feeding with roughage rich in tannins, low fiber, a high rate of dry matter, and a shorter digestion time in rumen reduces methane production (4). Feeding with fast degradable roughage, slowly digestible starch instead of faster one, legumes instead of meadow, silage instead of fresh or dry grass, and even opting for corn silage with relatively slow degradable starch instead of meadow silage reduces methane production by up to 28% (6). Fermentation of starch promotes the production of propionic acid compared to feeds with low starch content. Propionic acid production decreases methane production by allowing the greater use of metabolic H_2 and suppressing the protozoa that are important H_2 suppliers for methanogens, by reducing rumen pH. Sugar digestion, on the other hand, leads to more methane production than starch. Since sugar can be dissolved in water, it is quickly fermented in the rumen and is mainly used to produce butyric acid. Butyric acid increases methane production when rumen pH is high and adequate metabolic H_2 is present (15). Methane production can be reduced by up to 90% when the concentrate feeds rate increases to 90%. On the other hand, in this case, the risk of subacute ruminal acidosis (SARA) should be considered. Feed with a ratio of 90% concentrates is not sustainable in ruminants (54).

Supplementing oil to the feed

Although the addition of oil to the feed varies according to the amount, form, and feed composition of the oil, it has been reported that methane production decreases by 5.6% for every 1% added oil (4). As a supplement it reduces the methane synthesis by up to 21% via hydrogenation of fatty acids by utilizing the H_2 present in the rumen, suppressing methanogenic archaea and cellulolytic bacteria, and decelerating the digestion of fibrous nutrients (24, 64). Long-chain fatty acids, especially linoleic acid, have a toxic effect due to the impairment of the cell integrity of Gram-positive bacteria such as *F. succinogenes*, *R. albus*, and *R. flavefaciens* (55).

Supplementing organic acid to the feed

Organic acids are not recommended for livestock since they are expensive and difficult to apply in grazing ruminants. However, researchers reported that organic acids (fumarate and malate) reduce the synthesis of ruminal methane. Organic acids increase the synthesis of propionic acid by using H_2 as an alternative biochemical pathway instead of methane synthesis. Thus, methane synthesis is reduced due to the lack of H_2 in the rumen (43, 61). It has been reported that methane gas production decreases to a negligible level of 1-2% with an 80-90% concentrate proportion of feed. However, in this case, the risk of subacute ruminal acidosis (SARA) arises. Further reduction in rumen pH may be prevented by an alternative H_2 sink created via organic acid supplementation. In this case, feeding with high concentrates, which radically reduces the methane synthesis, may become sustainable (1).

Supplementing antibiotics to the feed

Supplementing the feed with the ionophore group of antibiotics to increase the ruminant's yield also significantly decreased the greenhouse gas synthesis unto the 2000s. Ionophores reduce the H_2 synthesis by up to 30% via suppressing H_2 producers such as Gram-positive bacteria and ciliates rather than methanogen archaea (57). Although ionophores increase ruminant’s yield, they are not valuable for reducing methane production in advanced enterprises where ruminants feed with concentrates with high protein and energy to meet the high nutrition needs. In addition, the inhibition effect of the ionophore antibiotics on methane production is not constant (34). Although ionophores generally significantly reduce greenhouse gas emissions that arise from enteric fermentation, they have been banned in European Union Countries and Turkey since 2006 due to various concerns such as developing resistant microorganisms and food residues (23). Although it is not prohibited in other countries, the livestock and agriculture sector is forced to take alternative and even more effective measures against ionophores due to the reaction worldwide against the antibiotic additives (77).

Probiotic addition to the feed

Oeztuerk (68) reported a decrease in acetic acid/propionic acid (A/P) rate and improved fermentation performance in an *in-vitro* study 0.7% alive

Saccharomyces cerevisiae supplemented to the ruminant feed. It is reported that the addition of yeast to the diet reduces methane production by increasing the synthesis of propionic acid, reducing the number of protozoa, and increasing animal yield (11, 67, 72, 73). Lila et al. (53) reported that yeast supplementation accelerates the synthesis of acetic acid by acetogens and consequently suppresses methane synthesis through the consumption of metabolic H_2 in the rumen. Adding yeast to the feed also contributes to the stabilization of the rumen pH, thus ruminants fed with concentrates become more resistant to subacute rumen acidosis (SARA).

Adding enzymes to the feed

Enzymes such as cellulase and hemicellulase added to the feed are the concentrated fermentation products and accelerate fiber digestion. The low fiber content or easily digestible fibers in feed reduces methane production. Since the acceleration of fiber digestion shortens the duration of the ingesta's stay in the rumen, it reduces methane production (4). Accelerating fiber digestion also reduces the A/P ratio (27). On the other hand, enzyme supplementation has not affected fiber digestion in some studies. The effect of enzyme supplementation on fiber digestibility varies according to the composition of the feed. Therefore, it does not seem possible to recommend a single enzyme formula (4).

Alternative H_2 sinks

Adding unsaturated fatty acids, nitrates, and sulfate, organic acid precursors to the feed, reduces methane production by allowing H_2 to be consumed in an alternative and more competitive biochemical pathway. Although adding nitrates and sulfates reduces methane production, it is not a suitable way to recover energy lost by methane synthesis (90). In other H_2 sinks, the bio-energy potential of H_2 is used by the ruminant. However, adding short-chain fatty acids may utilize a very small proportion of H_2 from the rumen environment (20). Microbial biosynthesis is also the alternative way to use H_2 (42).

Adding secondary plant metabolites

Plant extracts are organic compounds that humans have used in various pathological situations for thousands of years. Plant extracts are not structural compounds of the plant. Secondary plant metabolites are the reproductive and defense system components, protecting the plant against insects, harmful animals, microorganisms, other plants, and even harmful sunrays. These metabolites consist of tannins, saponins, flavonoids, sulfurous organic compounds, and essential oils with inhibitor activity for many microorganisms. These compounds with selective antibacterial effects on Gram-positive bacteria significantly reduce methane synthesis in the rumen (75). Antimicrobial activity of plant extracts has not been well documented yet. However, some researchers reported that plant extracts affect microbial cells in several sites. Not all of these mechanisms are separate targets; some are affected as a consequence of another mechanism being targeted (7). However, it seems that all mechanisms are directly or indirectly connected to the primary effect of essential oils on the bacterial envelopes (81).

Impair the cell membrane: Plant extracts impair the phospholipid bilayer of the cell membrane. Essential oils could affect the biosynthesis of lipids, including unsaturated fatty acids, thus modifying the cell membrane structure due to the hydrophobic characteristic. EOs in the bacterial cell decrease the level of unsaturated fatty acids that are generally responsible for the membrane fluidity. For instance, thymol, carvacrol, and eugenol can increase saturated fatty acids and decrease C18 unsaturated fatty acids (66). Thymol binds membrane proteins by hydrophobic bonding and hydrogen bonding, and thus changes the permeability of the cell membrane. An important characteristic of plant extracts and their components is their hydrophobicity, which enables them to partition in the lipids of the bacterial cell membrane and mitochondria, disturbing the structures and rendering them more permeable (7).

Leakage of ions and other cell contents: Plant extracts may occur a leakage of ions and cell content. Ions and cell content leakage can be tolerated until a certain amount. On the other hand, extensive loss of cell contents or the exit of critical molecules and ions will lead to death (7).

Coagulation of the cytoplasmic content: It has been reported that cinnamon and oregano extracts exhibit a wide range of significant abnormalities including coagulation of the cytoplasmic content of *E. coli* and *S. aureus* (5).

Modulation of ion channels: Plant extracts studied in excitable cells and researches revealed that plant extracts affect ion channels and consequently lead to several actions including even death in cell, depending on the ion channel function (21).

Decreasing the cytoplasmic pH: A decrease in cytoplasmic pH (pH_{int}) and cell wall disruption was observed in cells treated with plant extracts, suggesting a possible mechanism of antibacterial action (32). The hypothesis of carvacrol mechanism of action on the cell membrane suggests that carvacrol binds H^+ and diffuses through the cytoplasmic membrane to the cytoplasm where it dissociates, releasing its proton. In the cytoplasm, carvacrol attaches a potassium ion (or another ion), which will be then transported across the cytoplasmic membrane to the external environment. Once outside the cytoplasmic

membrane a proton is again fixed on carvacrol, which releases the potassium ion. In its protonated (undissociated) form, carvacrol is ready to diffuse again across the cytoplasmic membrane and dissociates, releasing the proton into the cytoplasm (80). This proton transport mechanism is in accordance with the decrease in cytoplasmic pH (pH_{int}).

Compromising the genetic material of the cell: Plant extracts may compromise the genetic material of the cell. Thus, lead to mutations or death of the cell (36).

Cell lysis: Eeugenol, the main component of clove EO, has shown a broad antibacterial activity against both Gram-positive and Gram-negative bacteria. Eugenol can bind to proteins, inactivate enzyme activities (i.e. glycolytic enzymes), and cause cell lysis (18).

Plant extracts, which were commonly used until the middle of the twentieth century, have lost their popularity in developed countries after the widespread production of synthetic drugs that are economical, more specific, effective, and easily applicable. Until the 1980s, while most societies in other countries were still trying to provide treatment with secondary plant metabolites, interest in natural organic compounds in Western countries began to rise again, and it became debatable whether they were safer in terms of their side effects. However, since plant extracts contain numerous complex organic compounds, it is hard to collect them separately and reveal effective doses relative to synthetic compounds. With the increasing interest in plant extracts for half a century, the number of studies revealing the medical effectiveness of these plants is increasing rapidly (33). Plant extracts, tannins, saponins, and essential (etheric, volatile) oils can be added separately or mixed in different proportions. Nonetheless, producing commercial feed additives from plant extracts requires further researches to cope with challenges such as the rapid adaptation of ruminal microorganisms to the plant extracts, and their unpleasant smell.

Modifying the ruminal microbial distribution

It is possible to reduce methane production by regulating the distribution of microorganisms in the rumen through halogenated methane analogs (70), competitive microorganisms, specific microorganisms targeting rumen microorganisms, or immunization (25). One option is specifically to target methanogens by the antibiotics (64), bacteriocins (8, 69, 86), or bacteriophage (39). Another is to decrease H_2 production so that less H_2 is available for methane formation (45). It has been reported that methane production can be reduced with vaccines developed against methanogenic archaea (70). However, the variety of methane-microorganisms in the rumen is not limited to a particular strain. Instead, it varies according to the region and the composition of the feed. Therefore, it is pretty challenging to develop a vaccine, which can be used worldwide (94). McAllister and Newbold (58) reported that developing a vaccine targeting cell membrane components of methanogens could reduce methane production. The same researchers proposed reducing methane production by supplementing bacteriophages and microorganisms that produce bacteriocin and directing hydrogen to microorganisms that utilize H_2 to synthesize different fermentation products from methane such as propionic and acetic acid (58). Acetogenesis is, $4H_2 + 2CO_2 \rightarrow CH_3COOH + 2H_2O$ and $\Delta G = -8.8$ kJ. On the other hand methanogenesis is, $4H_2 + CO_2 \rightarrow CH_4 + 2H_2O$ and $\Delta G = -67.4$ kJ. As can be seen in the biochemical flow, Gibbs Energy Changes (ΔG) indicates the less energy demand in methane synthesis. Negative ΔG indicates the energy release. Two different H_2 sinks given above suggest that methane synthesis provides more electron flow, which means more ATP, can be synthesized in the methanogen archaea (89). Acetogenesis, on the other hand, almost occurs without ATP production. Therefore, acetogens cannot compete with methanogens thermodynamically. However, further research is needed for these proposed methods. If acetogens successfully synthesized acetic acid from H_2 and CO_2 in the rumen instead of methane production, the animal's energy gain would be higher by up to 4-15%. This method is still not available in the field, as successful results have not yet been achieved in the hydrogen competition of acetogens with acetogenesis (38).

On the other hand, halogenated methane analogs such as aminochloral, chloroform, trichloroethylene, chloralhydrate, alpha-cyclodextrin, trichloroacetamide, 9, 10-anthraquinone, 2-bromoethane sulfonic acid, and bromochloromethane are useless as well as have some harmful side effects. In addition, methanogens can develop resistance to such chemicals. Therefore, their use in ruminant nutrition is not sustainable (70). A decrease in the number of protozoa in the rumen reduces methane production because methanogens on the surface of ciliates or in their endosymbionts are removed and H_2 production by protozoa is altered (38, 47). Although removing protozoa from the rumen reduces methane gas production, it is not the most effective option concerning the deterioration of fermentation in the rumen and a decrease in the animal's performance (38).

Improving the animal breed

Excluding low-yielding animals, keeping animals more efficient, and produce less methane gas are the recommended breeding methods for mitigating methane gas production. Since methane production is directly proportional to the number of animals, separating low-yielding animals from the herd and replacing them with more efficient animals reduces the amount of methane

production per unit yield. Many studies aimed at reducing methane production have revealed different results. Therefore, some researchers have suggested that animals produce less methane gas should be bred since this may be due to phenotypic and genetic properties, although some researchers report that this may not be the efficient solution (16, 25, 76). Thus, this should be further investigated whether methane production is related to animal breeds.

Conclusions

Humans, although are unique among the Earth's life forms in their ability to use language and high cognitive skills, they unfortunately, are not able to digest cellulose that is the most abundant plant structural component synthesized by the plants via biochemical conversion of the sunbeam. Ruminants, with their unique digestive system, on the other hand, can convert the energy in roughage to edible products for humans. Hence, rumen fermentation has been excessively on the scope of researchers for long years. Advances in rumen fermentation are a vital concern to provide food with good quality for the growing population of man. Ruminants digest roughage with microbial fermentation process by the microorganisms inhabited in the rumen. Nevertheless, agriculture contributes to nutrient and air pollution in several ways and livestock holds the bigger proportion of this contribution. The main pollutants of interest in relation to ruminant production systems are nitrogen (N) from nitrate leaching and ammonia emissions/deposition, minerals particularly phosphorus (P), greenhouse gases (GHG; carbon dioxide, CO_2; methane, CH_4 and nitrous oxide, N_2O), particulate matter and volatile organic compounds. Considering the rapid growth in the human population and, thus, the increasing number of ruminants to meet the amount of quality food demand, it is expected that greenhouse gas production and environmental pollution will be at a tremendous rate soon. Although the ionophore group antibiotics supplemented in the ruminant feed help reduce methane emissions, they have been banned in EU countries and Turkey since 2006. On the other hand, supplementing ruminant feed with ionophores leads to inconvenience although it is not restricted in other countries. Therefore, researchers have been performing intensive studies to reduce methane gas and nitrous oxide synthesis in the rumen for the last two decades. Various methods have been suggested that reportedly reduce methane production, such as modifying the feed composition, modifying rumen microorganisms via supplementing the feed with additives and microorganisms. Of these methods, plant extracts, which researchers have recently been studying intensively, present promising results in reducing methane production. Since plant extracts are a mixture of numerous organic compounds, each compound should be isolated and studied individually and in combination to reveal its effectiveness in methane mitigation. Organic compounds discovered with antimicrobial activities to decrease methane production in the rumen will also shed light on humanity's fight against various infections.

Conflict of Interest

The authors declared that there is no conflict of interest.

References

1. **Asanuma N, Iwamoto M, Hino T, et al** (1999): *Effect of the addition of fumarate on methane production by ruminal microorganisms in vitro.* J Dairy Sci, **82**, 780-787.
2. **Attwood GT, Kelly WJ, Altermann EH, et al** (2008): *Application of rumen microbial genome information to livestock systems in the postgenomic era.* Aust J Exp Agric, **48**, 695-700.
3. **Bansal S, Goel G** (2015): Commercial application of rumen microbial enzymes. 281-291. In: AK Puniya, R Singh, DN Kamra (Eds), Rumen Microbiology: From Evolution to Revolution. Springer, New Delhi.
4. **Beauchemin KA, Kreuzer M, O'mara F, et al** (2008): *Nutritional management for enteric methane abatement: a review*. Austr J Exp Agric, **48**, 21-27.
5. **Becerril R, Gómez-Lus R, Goni, P, et al** (2007): *Combination of analytical and microbiological techniques to study the antimicrobial activity of a new active food packaging containing cinnamon or oregano against E. coli and S. aureus.* Analytical and Bioanalytical Chemistry, **388**, 1003-1011.
6. **Benchaar C, Pomar C, Chiquette J** (2001): *Evaluation of diet strategies to reduce methane production in ruminants: A modelling approach.* Can J Anim Sci, **81**, 563-574.
7. **Burt, S.** (2004): *Essential oils: their antibacterial properties and potential applications in foods-a review.* Int J Food Microbiol, **94**, 223-253.
8. **Callaway TR, Edrington TS, Rychlik JL, et al** (2003): *Ionophores: their use as ruminant growth promotants and impact on food safety*. Curr Issues Intest Microbiol, **4**, 43-51.
9. **Castillo-González AR, Burrola-Barraza ME, Domínguez-Viveros J, et al** (2014): *Rumen microorganisms and fermentation.* Arch Med Vet, **46**, 349-361.
10. **Cecava MJ** (1995): Rumen physiology and energy requirements. 3-24. In: T Petty, M Cecava (Eds), Beef Cattle Feeding and Nutrition. Academic Press, California.
11. **Chaucheyras F, Fonty G, Bertin G, et al** (1995): *Effects of live Saccharomyces cerevisiae cells on zoospore germination, growth, and cellulolytic activity of the rumen anaerobic fungus, Neocallimastix frontalis MCH3*. Curr Microbiol, **31**, 201-205.
12. **Chen G, Russell JB** (1989): *More monensin-sensitive, ammonia-producing bacteria from the rumen*. Appl Environ Microbiol, **55**, 1052-1057.
13. **Chesson A, Forsberg CW** (1997): Polysaccharide degradation by rumen microorganisms. 329-381. In: PN Hobson, CS Stewart (Eds), The rumen microbial ecosystem. Springer, Dordrecht.

14. **Choudhury PK, Salem AZM, Jena R, et al** (2015): Rumen Microbiology: An Overview, 3-16. In: AK Puniya, R Singh, DN Kamra (Eds), Rumen Microbiology: From Evolution to Revolution. Springer, New Delhi.
15. **Chung YH, He ML, McGinn SM, et al** (2011): *Linseed suppresses enteric methane emissions from cattle fed barley silage, but not from those fed grass hay.* Anim Feed Sci Technol, **166**, 321-329.
16. **Clark H, Pinares-Patiño C, De Klein C** (2005): Methane and nitrous oxide emissions from grazed grasslands. 279-293. In: DA McGilloway (Ed), Grassland: A Global Resource, Wageningen Academic Publishers, Wageningen.
17. **Clauss M, Hofmann RR, Streich WJ, et al** (2010): *Convergence in the macroscopic anatomy of the reticulum in wild ruminant species of different feeding types and a new resulting hypothesis on reticular function.* J Zool, **281**, 26-38.
18. **Cobellis G, Trabalza-Marinucci M, Yu Z** (2016): *Critical evaluation of essential oils as rumen modifiers in ruminant nutrition: A review.* Sci Total Environ, **545**, 556-568.
19. **Cotta MA** (1988): *Amylolytic activity of selected species of ruminal bacteria*. Appl Environ Microbiol, **54**, 772-776.
20. **Czerkawski JW** (1986): An Introduction to Rumen Studies. Pergamon Press, Oxford.
21. **De Araújo DAM, Freitas C, Cruz JS** (2011): *Essential oils components as a new path to understand ion channel molecular pharmacology*. Life Sci, **89**, 540-544.
22. **Demirtaş A, Pişkin İ** (2013): Isırgan otu (Urtica dioica L.), papatya (Matricaria chamomilla L.) ve hayıt meyvesi (Vitex agnus-castus L.) ekstraktlarının normal koşullarda ve asidoz koşullarında rumen mikrobiyal fermentasyonuna in vitro etkileri. Doktora Tezi. Ankara Üniversitesi Sağlık Bilimleri Enstitüsü, Ankara.
23. **Demirtas A, Musa SAA, Pekcan M, et al** (2020): *Effects of cleavers (Galium aparine) and yarrow (Achillea millefolium) extracts on rumen microbial fermentation in in-vitro semi-continuous culture system (Rusitec).* Kafkas Univ Vet Fak Derg, **26,** 385-390.
24. **Doreau M, Ferlay A** (1995): *Effect of dietary lipids on nitrogen metabolism in the rumen: a review.* Livest Prod Sci, **43**, 97-110.
25. **Eckard RJ, Grainger C, De Klein CAM** (2010): *Options for the abatement of methane and nitrous oxide from ruminant production: a review*. Livest Sci, **130**, 47-56.
26. **Ellis JL, Dijkstra J, Kebreab E, et al** (2008): *Aspects of rumen microbiology central to mechanistic modelling of methane production in cattle*. J Agric Sci, **146**, 213-233.
27. **Eun JS, Beauchemin KA** (2007): *Assessment of the efficacy of varying experimental exogenous fibrolytic enzymes using in vitro fermentation characteristics*. Anim Feed Sci Technol, **132**, 298-315.
28. **Faverdin P** (1999): *The effect of nutrients on feed intake in ruminants.* Proc Nutr Soc, **58**, 523-531
29. **Fox NJ, Smith LA, Houdijk JGM, et al** (2018): *Ubiquitous parasites drive a 33% increase in methane yield from livestock.* Int J Parasitol, **48**, 1017-1021.
30. **Gencoglu H, Turkmen II** (2006): *Effects of forage source on chewing and rumen fermentation in lactating dairy cows*. Rev Med Vet, **157**, 463.
31. **Gilbert RA, Klieve AV** (2015): Ruminal Viruses (Bacteriophages, Archaeaphages). 121-141. In: AK Puniya, R Singh, DN Kamra (Eds), Rumen Microbiology: From Evolution to Revolution. Springer, New Delhi.
32. **Gonelimali FD, Lin J, Miao W et al** (2018): *Antimicrobial properties and mechanism of action of some plant extracts against food pathogens and spoilage microorganisms.* Front Microbiol, **9**, 1639.
33. **Greathead H** (2003): *Plants and plant extracts for improving animal productivity*. Proc Nutr Soc, **62**, 279-290.
34. **Guan H, Wittenberg KM, Ominski KH et al** (2006): *Efficacy of ionophores in cattle diets for mitigation of enteric methane.* J Anim Sci, **84**, 1896-1906.
35. **Gür G, Öztürk H** (2021): *Ruminantlarda metan salinimini azaltma stratejileri.* Veteriner Farmakoloji ve Toksikoloji Derneği Bülteni, **12**, 43-54.
36. **Hammer KA, Carson CF** (2011): Antibacterial and antifungal activities of essential oils. 255 – 293. In: G Bergsson, H Hilmarsson, H Thormar, (Eds), Lipids and Essential Oils. John Wiley & Sons, New Delhi.
37. **Haque N, Saraswat ML, Sahoo A** (2001): *Methane production and energy balance in crossbred male calves fed on rations containing different ratios of green sorghum and wheat straw.* Indian J Anim Sci, **71**, 797-799.
38. **Haque MN** (2018): *Dietary manipulation: a sustainable way to mitigate methane emissions from ruminants.* J Anim Sci Technol, **60**, 15.
39. **Hegarty RS, Klieve AV** (1999): *Opportunities for biological control of ruminal methanogenesis.* Aust J Agric Res, **50**, 1315-1320.
40. **Hungate RE** (1950): *The anaerobic mesophilic cellulolytic bacteria*. Bacteriol Rev, **14**, 1-49.
41. **Hungate RE** (1960): *I. Microbial ecology of the rumen*. Bacteriol Rev, **24**, 353-364.
42. **Hungate RE, Mah RA, Simesen M** (1961): *Rates of production of individual volatile fatty acids in the rumen of lactating cows.* Appl Microbiol, **9**, 554-561.
43. **Jalc D, Ceresnakova Z** (2002): *Effect of plant oils and malate on rumen fermentation in vitro.* Czech J Anim Sci **47**, 106-111.
44. **Janssen PH, Kirs M** (2008): *Structure of the archaeal community of the rumen*. Appl Environ Microbiol, **74**, 3619-3625.
45. **Joblin KN** (1999): *Ruminal acetogens and their potential to lower ruminant methane emissions.* Aust J Agric Res, **50**, 1307-1314.
46. **Johnson KA, Johnson DE** (1995): *Methane emissions from cattle*. J Anim Sci, **73,** 2483-2492.
47. **Kamra DN, Agarwal N, Chaudhary L, et al** (2010): Methane emissions by livestock in India and mitigation strategies. 75 - 78. In: N.E. Odongo, M. Garcia & G.J. Viljoen (Eds), Sustainable Improvement of Animal Production and Health. Food and Agriculture Organization of the United Nations, Rome.
48. **Karcol J, Kasarda R, Šimko M** (2017): *Effect of feeding of different sources of NPN on production performance of dairy cows*. Acta Fytotech Zootech, **19**, 163-166.
49. **Klieve AV, Bauchop T** (1988): *Morphological diversity of ruminal bacteriophages from sheep and cattle.* Appl Environ Microbiol, **54**, 1637-1641.

50. Krehbiel CR (2014): *Invited review: applied nutrition of ruminants: fermentation and digestive physiology.* Prof Anim Sci, **30**, 129-139.

51. Kung JRL, AO Hession (1995): *Preventing in vitro lactate accumulation in ruminal fermentations by inoculation with Megasphaera elsdenii.* J Anim Sci, **73**, 250-256.

52. Lambers H, Chapin FS, Pons TL (2008): Photosynthesis. In: Plant Physiological Ecology. Springer, New York, NY.

53. Lila ZA, Mohammed N, Yasui T, et al (2004): *Effects of a twin strain of Saccharomyces cerevisiae live cells on mixed ruminal microorganism fermentation in vitro*. J Anim Sci, **82**, 1847-1854.

54. Lovett DK, Lovell S, Stack L, et al (2003): *Effect of forage/concentrate ratio and dietary coconut oil level on methane output and performance of finishing beef heifers.* Livest Prod Sci, **84**, 135-146.

55. Maia MR, Chaudhary LC, Figueres L, et al (2007): *Metabolism of polyunsaturated fatty acids and their toxicity to the microflora of the rumen.* Antonie Van Leeuwenhoek, **91**, 303-314.

56. McAllister TA, Bae HD, Jones GA, et al (1994): *Microbial attachment and feed digestion in the rumen.* J Anim Sci, **72,** 3004-3018.

57. McAllister TA, Cheng KJ, Okine EK, et al (1996): *Dietary, environmental and microbiological aspects of methane production in ruminants.* Can J Anim Sci, **76**, 231-243.

58. McAllister TA, Newbold CJ (2008): *Redirecting rumen fermentation to reduce methanogenesis.* Aust J Exp Agric, **48**, 7-13

59. McSweeney C, Mackie R (2012): *Commission on genetic resources for food and agriculture. microorganisms and ruminant digestion: state of knowledge, trends and future prospects.* Background Study Paper (FAO), **61**, 1-62.

60. Membrive CMB (2016): Anatomy and Physiology of the Rumen. 1-38. In: D Millen, M De Beni Arrigoni, R Lauritano Pacheco (Eds), Rumenology. Springer, Cham.

61. Mohammed N, Lila ZA, Ajisaka N, et al (2004): *Inhibition of ruminal microbial methane production by β-cyclodextrin iodopropane, malate and their combination in vitro*. J Anim Physiol Anim Nutr, **88**, 188-195.

62. Morgavi DP, Forano E, Martin C, et al (2010): *Microbial ecosystem and methanogenesis in ruminants.* Anim, **4**, 1024-1036.

63. Nagaraja TG, Towne G, Beharka AA (1992): *Moderation of ruminal fermentation by ciliated protozoa in cattle fed a high-grain diet.* Appl Environ Microbial, **58**, 2410-2414.

64. Nagaraja TG, Newbold CJ, Van Nevel CJ, et al (1997): Manipulation of ruminal fermentation. 523-632. In: PN Hobson, CS Stewart (Eds), The rumen microbial ecosystem. Springer, Dordrecht.

65. Nagaraja TG (2016): Microbiology of the rumen. 39-61. In: D Millen, M De Beni Arrigoni, R Lauritano Pacheco (Eds), Rumenology. Springer, Cham.

66. Nehme R, Andrés S, Pereira RB, et al (2021): *Essential oils in livestock: From health to food quality.* Antioxidants, **10**, 330.

67. Newbold CJ, McIntosh FM, Wallace RJ (1998): *Changes in the microbial population of a rumen-simulating fermenter in response to yeast culture.* Can J Anim Sci, **78**, 241-244.

68. Oeztuerk H (2009): *Effects of live and autoclaved yeast cultures on ruminal fermentation in vitro.* J Anim Feed Sci, **18**, 142-150.

69. Orpin CG, Joblin KN (1997): The Rumen Anaerobic Fungi. 140-195. In: PN Hobson, CS Stewart (Eds), The rumen microbial ecosystem. Springer, Dordrecht.

70. Öztürk H (2007): *Küresel isınmada ruminantların rolü*. Vet Hek Der Derg, **78**, 17-21.

71. Öztürk H (2008): *Effects of inulin on rumen metabolism in vitro*. Ankara Univ Vet Fak Derg, **55**, 79-82

72. Öztürk H (2008): *Ruminant beslemede probiyotik mayalar*. Vet Hekim Der Derg, **79**, 37-42.

73. Öztürk H, Salgirli Demirbas Y, Aydin FG, et al (2015): *Effects of hydrolyzed and live yeasts on rumen microbial fermentation in a semi-continuous culture system (Rusitec),* Turkish J Vet Anim Sci, **39**, 556-559.

74. Öztürk H (2019): Veteriner fizyoloji. Ankara Nobel Tıp Kitabevleri, Ankara.

75. Patra AK (2012): An overview of antimicrobial properties of different classes of phytochemicals. 1-32. In: AK Patra (Ed), Dietary phytochemicals and microbes. Springer, Dordrecht.

76. Pinares-Patiño CS, Ulyatt MJ, Lassey KR, et al (2003): *Persistence of differences between sheep in methane emission under generous grazing conditions.* J Agric Sci, **140**, 227-233.

77. Ribeiro Pereira LG, Machado FS, Campos MM, et al (2015): *Enteric methane mitigation strategies in ruminants: a review*. Rev Colomb de Cienc Pecu, **28**, 124-143.

78. Romero E, Augulis R, Novoderezhkin V, et al (2014): Quantum *coherence in photosynthesis for efficient solar-energy conversion.* Nat Phys, **10**, 676-682.

79. Russell JB, Muck RE, Weimer PJ (2009): *Quantitative analysis of cellulose degradation and growth of cellulolytic bacteria in the rumen.* FEMS Microbiol Ecol, **67**, 183-197.

80. Saad NY, Muller CD, Lobstein A (2013): *Major bioactivities and mechanism of action of essential oils and their components.* Flavour Fragr J, **28**, 269-279.

81. Sabo, VA, Knezevic P (2019): *Antimicrobial activity of Eucalyptus camaldulensis Dehn. plant extracts and essential oils: A review.* Industrial Crops and Products, **132**, 413-429.

82. Shimojo M, Bungo T, Imura Y, et al (2000): *Basic avoidance of food competition among ruminants, non-ruminants and humans-a simple analytic description.* J Fac Agric Kyushu Univ, **44**, 293-298.

83. Steele MA, Penner GB, Chaucheyras-Durand F (2016): *Development and physiology of the rumen and the lower gut: targets for improving gut health*. J Dairy Sci, **99**, 4955-4966.

84. Stern MD, Varga GA, Clark JH, et al (1994): *Evaluation of Chemical and Physical Properties of Feeds That Affect Protein Metabolism in the Rumen.* J Dairy Sci, **77**, 2762-2786.

85. Stewart CS, Flint HJ, Bryant MP (1997): The rumen bacteria. 10-72. In: PN Hobson, CS Stewart (Eds), The rumen microbial ecosystem. Springer, Dordrecht.

86. Teather RM, Forster RJ (1998): *Manipulating the rumen microflora with bacteriocins to improve ruminant production*. Can J Anim Sci, **78**, 57-69.
87. Therion JJ, Kistner A, Kornelius JH (1982): *Effect of pH on growth rates of rumen amylolytic and lactilytic bacteria.* Appl Environ Microbiol, **44**, 428-434.
88. Thirumalesh T, Krishnamoorthy U (2013): *Rumen microbial biomass synthesis and its Importance in Ruminant Production.* Int J Livest Res, **3**, 5-26.
89. Ungerfeld, EM (2013): *A theoretical comparison between two ruminal electron sinks.* Front Microbiol, **4**, 319.
90. Van Zijderveld SM, Gerrits WJJ, Dijkstra J, et al (2011): *Persistency of methane mitigation by dietary nitrate supplementation in dairy cows*. J Dairy Sci, **94**, 4028-4038.
91. Wallace RJ (1985): *Synergism between different species of proteolytic rumen bacteria*. Curr Microbiol, **12**, 59-63.
92. Wendy JU, Ruth B, Margaret LD, et al (2015): Biology and diseases of ruminants (sheep, goats, and cattle). 623-694. In: Anderson C, Glen O, Kathleen RPC, Mark TW, James GF (Eds), Laboratory animal medicine (Third Edition). Elsevier Publishing Press, Boston.
93. White BA, Lamed R, Bayer EA, et al (2014): *Biomass utilization by gut microbiomes.* Annu Rev Microbiol, **68**, 279-296.
94. Wright ADG, Kennedy P, O'neill CJ, et al (2004): *Reducing methane emissions in sheep by immunization against rumen methanogens.* Vaccine, **22**, 3976-3985.
95. Xu J, Hou Y, Yang H, et al (2014): *Effects of forage sources on rumen fermentation characteristics, performance, and microbial protein synthesis in midlactation cows.* Asian-Australas J Anim Sci, **27**, 667-673.
96. Yokoyama MT, Johnson KA (1988): Microbiology of the rumen and intestine. 125-144. In: DC Church (Ed), The ruminant animal: digestive physiology and nutrition. Prentice Hall, New Jersey.
97. Zhou M, Chen Y, Guan, LL (2015): Rumen bacteria. 79-95. In: AK Puniya, R Singh, DN Kamra (Eds), Rumen Microbiology: From Evolution to Revolution. Springer, New Delhi.

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