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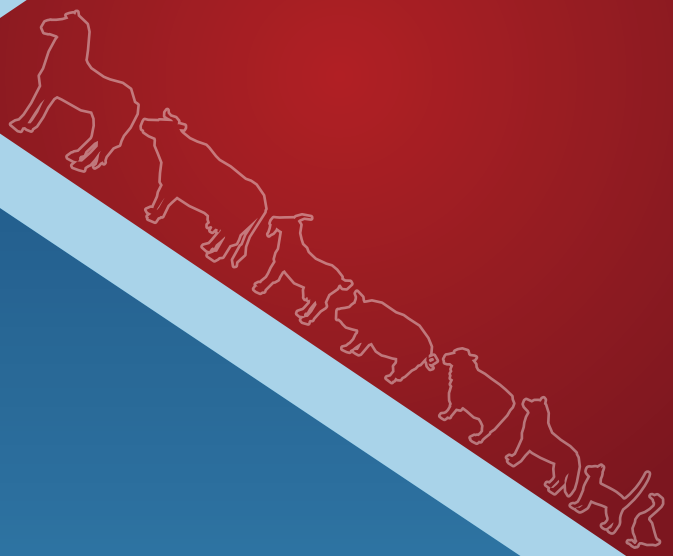

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RESEARCH ARTICLE

Comparison of Different Order and Heterogeneous Residual Variances Legendre Polynomials in Random Regression Models

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Abstract

In this study, it was aimed to estimate covariance function, covariance components, permanent environmental effect, additive genetic effect and heritability values, and comparison of models with different order and heterogeneous residual variances Legendre Polynomials in the first lactation Turkish Holstein cows more than 10 test day milk yields. For this aim, 7340 test day records of 386 Holstein Friesian cows in the first lactation raised in private dairy farm calving from 2013 to 2018 in Kırşehir-Turkey were used. The six Legendre polynomial models by random regression described as L(2,2), L(3,3), L(4,4), L(5,5), L(6,6) and L(7,7) were evaluated using first lactation test day records. Heterogeneous residual variances (RV) were modeled by considering five sub-classes. Analyzes were performed using the WOMBAT statistical package. In comparison of the models, -2LogL, Akaike Information Criterion (AIC), Bayes Information Criterion (BIC) and RV were used. Also, the compatibility of random regression models was examined in terms of eigenvalues of covariance matrices. The values of -2LogL (between 28334.16 and 26610.07), AIC (between 28356.16 and 26732.07) and BIC values (between 28432.05 and 27129.21) obtained from the study result decreased as the model order increased. As a result, it was determined that the 3rd degree Legendre polynomial model can provide sufficient compliance. However, when looking at the values for -2LogL, AIC and RV, it was determined that the L(7,7) model fits well according to other models.

Keywords: *Heritability, Permanent environmental effect, Additive genetic effect, Holstein Friesian, First lactation*

Şansa Bağlı Regresyon Modellerinde Farklı Dereceli ve Heterojen Hata Varyanslı Legendre Polinomlarının Karşılaştırılması

Öz

Bu çalışmada, 10'dan fazla test günü süt verimine sahip birinci laktasyondaki Holstein Friesian ineklerinde farklı dereceli Legendre Polinomları kullanılarak birinci test günü süt verimleri için kovaryans fonksiyonu, kovaryans bileşenleri, kalıcı çevresel etki, eklemeli genetik etki ve kalıtım derecelerinin tahmin edilmesi ve modellerin karşılaştırılması amaçlanmıştır. Bu amaçla Kırşehir-Türkiye'de 2013'ten 2018'e kadar buzağılayan özel süt çiftliğinde yetiştirilen birinci laktasyondaki 386 Holstein Friesian ineklerinin 7340 test günü kaydı kullanılmıştır. L(2,2), L(3,3), L(4,4), L(5,5), L(6,6) ve L(7,7) olarak tanımlanan rastgele regresyon ile altı Legendre polinom modeli birinci laktasyon test günü kayıtları kullanılarak değerlendirilmiştir. Heterojen hata varyansları (RV), beş alt sınıf dikkate alınarak modellenmiştir. Analizler, WOMBAT istatistik paketi kullanılarak yapılmıştır. Modellerin karşılaştırılmasında -2LogL, Akaike Bilgi Ölçütü (AIC), Bayes Bilgi Ölçütü (BIC) ve hata varyansları (RV) kullanılmıştır. Ayrıca, şansa bağlı regresyon modellerinin uyumluluğu kovaryans matrislerinin özdeğerleri açısından incelenmiştir. Çalışma sonucundan elde edilen -2LogL (28334.16 ve 26610.07 arasında), AIC (28356.16 ve 26732.07) ve BIC (28432.05 ile 27129.21 arasında) değerleri model sırası arttıkça azalmıştır. Sonuç olarak, 3. derece Legendre polinom modelinin yeterli uyumu sağlayabileceği belirlenmiştir. Ancak -2LogL, AIC ve RV değerlerine bakıldığında, L(7,7) modelinin diğer modellere göre iyi uyum gösterdiği belirlenmiştir.

Anahtar sözcükler: *Kalıtım derecesi, Kalıcı çevresel etki, Eklemeli genetik etki, Holstein Friesian, Birinci laktasyon*

INTRODUCTION

The main purpose of animal breeding is to increase productivity by choosing the better animals for the next generation. In dairy cattle breeding is usually made over milk yield records. So milk yield records of cows most important for the dairy herds [1]. At the same time sire evaluation is done mainly based on 305-day milk yield in dairy farms. However, in 305-day milk yield models, only average lactation curve of cows is considered [2]. Test day

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(TD) yields at monthly intervals are suggested for sire and cow evaluation because the test-day model highly has accuracy due to having a larger number of measurements per daughter. Today, test-day models are much more common than lactation models for genetic evaluations of production traits worldwide [3]. Test-day models provide more accurate genetic evaluations of cows and bulls due to the better definition of contemporary groups and the elimination of environmental effects [4]. Therefore, random regression models (RRM) have been developed to use of test day milk records instead of 305-d lactation milk yield [5]. The RRM is also used to estimate the genetic parameters of TD yields in dairy cows [6]. RRM also allows the evaluation of cows for parameters associated with the shape of the lactation curve simultaneously with the production level [2]. RRM has different approaches to the test day models and attempts to fit the (co)variance structure of repeated measures during the lactation curve [5]. This model assumes standard shapes of the lactation curve for all cows of the same age and season subclass, and the estimated additive genetic effects of the animals reflected the height differences of these curves [7].

Henderson [8] and Laird and Ware [9] developed the RRM. The use of linear RRM as an extension of fixed regression models has been proposed by Schaeffer and Dekkers [10]. Different models can be proposed to estimate the genetic parameters for TD yields of dairy cattle [11]. Thus, the (co) variances between records of additive genetic effects and permanent environmental effects can be described using Legendre polynomials [5]. Legendre polynomials (LP) are orthogonal and normalized and more accurate results than the conventional polynomials [5]. The use of LP has become widespread, as it provides good convergence and is more reliable than other polynomial estimates [12]. LP reduce correlation among estimated regression coefficients in comparison to other functions with the same number of parameters [11]. Olori et al.[13] reported that the critical issues in fitting an RRM include the order of the polynomial used to model the lactation curve at the fixed and random levels. Meyer [14] pointed out that higher-order polynomials are flexible and that changes in means and variances can be modeled along a continuous scale.

In the majority of studies with RRM, the number of test days is usually 10 [1,15-17]. In some studies, the test day is less than 10 [18,19], while in other studies it is more than 20 test days [20,21].

Studies with more test days investigating the use of RRM for estimating genetic parameters for TD yields in dairy cows are scarce. Therefore, the study aimed to estimate covariance function, covariance components, permanent environmental effect, additive genetic effect and heritability values, and comparison of models with different orders and heterogeneous residual variances Legendre Polynomials in the first lactation Turkish Holstein cows more than 10 test day milk yields.

Material and Methods

Data comprised of 7340 TD records of 386 Holstein Friesian cows in first lactation raised in private dairy husbandry calving from 2013 to 2018 in Kırşehir of Turkey. The cows, which produced milk yields on the day of the test, were the daughters of 76 sires and 304 dams. A total test-day milk yields from the morning and evening milking were collected with 15 days intervals from 5 to 305 days of lactation and first record was obtained up to 5 days after calving. There is a minimum of 11 TD records (0.26%) and a maximum of 21 TD (30.83%). The rate of animal records for more than 15 or more TD is 96.63%. The mean and standard deviation of milk yields on the day of the test were 30.03±7.04 kg and a coefficient of variation of 23.46%, respectively. The following RRM was used in the TDMY analysis.

$$y_{ij} = CY + CM + CS + \sum_{m=0}^{k_A-1} \alpha_{im}\phi_m(t_{ij}) + \sum_{m=0}^{k_R-1} \gamma_{im}\phi_m(t_{ij}) + e_{ij}$$

CY : Fixed effect of calving year

CM : Fixed effect of calving month

CS : Fixed effect of calving season

α_{im} : m^{th} additive genetic random regression coefficients for animal i

γ_{im} : m^{th} permanent environmental random regression coefficients for animal i

k_A : Order of fit for additive genetic random regression coefficients

k_R : Order of fit for permanent environmental random regression coefficients

t_{ij} : j^{th} test day for animal i

$\Phi_m(t_{ij})$: m^{th} Legendre polynomial evaluated for t_{ij}

e_{ij} : Random error $e \sim N(\mu, \sigma_e^2)$

The six Legendre polynomial models by random regression described as L(2,2), L(3,3), L(4,4), L(5,5), L(6,6) and L(7,7) were evaluated using first lactation TD records. In addition, the calving year, calving month and calving season were included in the model as fixed effects. Cows were assigned the six subclasses for calving year (2013-2018), four sub-classes for seasons of calving (summer, winter, spring, autumn) and twelve calving months. Heterogeneous residual variances (RV) were modeled by considering five sub-classes (5-60, 61-120, 121-180, 181-240, 241-305 days). Analyzes were performed using the WOMBAT statistical package.

In comparison of the models, -2LogL, Akaike Information Criterion (AIC) [22], Bayes Information Criterion (BIC) [23] and residual variances (RV) were used. AIC and BIC values were calculated as reported in the Takma and Akbas [24] studies. In comparison, the smallest values of AIC, -2LogL, BIC and RV criteria explain that this model fits well [24,25]. The significance of the change between different order of

Legendre polynomials models was examined by chi-square test (χ^2) [26]. Also, the compatibility of random regression models was also examined in terms of eigenvalues of covariance matrices [1,25,26].

Results

Estimates of the additive genetic effect for TDMY on different test days ranged from 0.09 to 20.10. Especially, the genetic effect in TDMY was generally greater at the beginning of lactation in L(4,4), L(5,5), L(6,6) and L(7,7) models. Genetic effects predicted at the end of lactation were high in all models, but the prediction from the L (7,7) model showed a decreasing trend *(Fig. 1)*.

Estimates of the permanent environmental effects of TDMY from the L(2,2), L(3,3), L(4,4), L(5,5), L(6,6), L(7,7) Legendre models have changed between 10.49 and 48.65 and their trends have been illustrated in *Fig. 2*. This figure shows that the estimates of permanent environmental effects are more stable at the start of lactation and increase during the rest of lactation. Also, there is a very high increase in the L (4,4) model on the last test day.

Heritability values estimates of TDMY ranged from 0.002 to 0.389. This change resembled the results obtained from the estimates of genetic effects. Heritability values estimates were lowest with the L (2,2) model at the beginning of lactation, while the highest with the L (7,7) model. On the 8th and 9th test days (110 - 125th days), while all models formed close estimates, the highest heritability value estimation at the end of lactation was found with the L (3,3) model *(Fig. 3)*.

The findings of the criteria used for comparing different orders Legendre models are given in *Table 1*. In the table seen that -2LogL, AIC and BIC values vary between 26610.07 and 28432.05, while RV value varies between 5.09 and 30.32.

The maximum log-likelihood (LogL) values and the LogL changes of models with different orders are shown in *Table 2*. As seen in the table, LogL values varied between -14167.08 and -13305.03. The highest change was observed in the L(3,3) model, while the least change was observed in the L(6,6) model.

Table 3 presents eigenvalues and their proportions in the

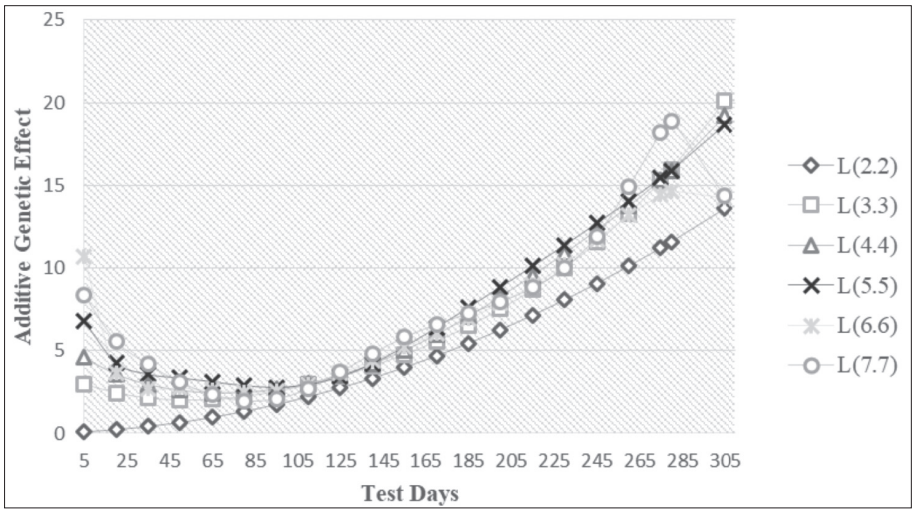
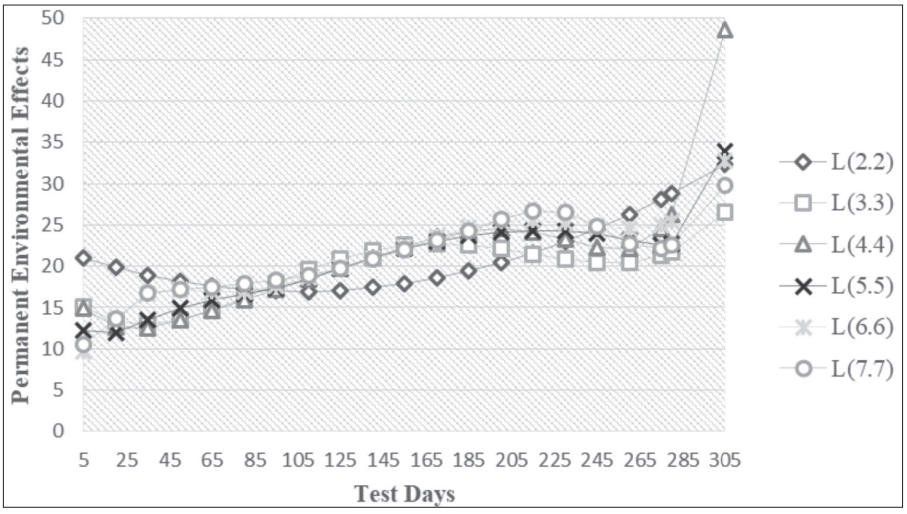


Fig 1. Estimation of additive genetic effects for TDMY from different order Legendre models

Fig 2. Estimation of permanent environmental effects for TDMY from different order Legendre models



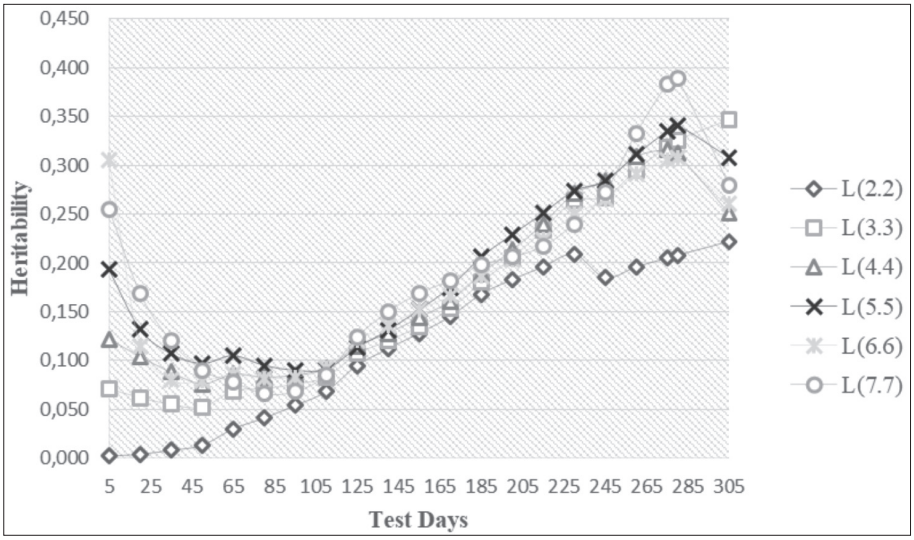


Fig 3. Estimation of heritability values for TDMY from different order Legendre models

Table 1. *Findings of the criteria used for comparing different order Legendre models*

Models	Number of Parameters	-2LogL	AIC	BIC	RV1	RV2	RV3	RV4	RV5
L(2.2)	11	28334.16	28356.16	28432,05	30.32	13.46	9.15	7.63	15.37
L(3.3)	17	27677.92	27711.92	27829.18	23.62	12.91	7.99	7.05	11.27
L(4.4)	25	27034.55	27084.55	27257.00	18.13	11.45	7.58	6.04	8.67
L(5.5)	35	26817,78	26887.78	27129.21	16.22	10.82	7.09	5.80	8.06
L(6.6)	47	26721.68	26815.68	27139.89	14.81	10.64	6.82	5.49	7.60
L(7.7)	61	26610.07	26732.07	27152.83	13.91	10.17	6.56	5.09	7.06

Table 2. *LogL values and the changes in LogL for different order models*

Models	Number of Parameters	LogL	Changes in LogL	Changes in LogL (%)	Chi-Square
L (2,2)	11	-14167.08	-	-	-
L (3,3)	17	-13838.96	328.12**	2,37	16.81
L (4,4)	25	-13517.27	321.69**	2,38	20.09
L (5,5)	35	-13408.89	108.38**	0,81	23.21
L (6,6)	47	-13360.84	48.05**	0,36	26.22
L (7,7)	61	-13305.03	55.81**	0,42	29.14

*** P<0.01*

Table 3. *Eigenvalues and their proportions in the total variance for the (co) variance of the additive genetic effect estimated from different models*

Models	1	2	3	4	5	6	7
L (2,2)	9.88 (99.99)	0.000 (0.01)					
L (3,3)	13.19 (97.07)	0.40 (2.93)	0.00 (0.00)				
L (4,4)	13.64 (94.52)	0.69 (4.81)	0.10 (0.67)	0.00 (0.00)			
L (5,5)	13.88 (91.65)	0.090 (5.94)	0.36 (2.40)	0.00 (0.01)	0.00 (0.00)		
L (6,6)	12.44 (89.75)	0.85 (6.12)	0.46 (3.28)	0.12 (0.84)	0.00 (0.01)	0.00 (0.00)	
L (7,7)	13.65 (89.75)	1.01 (6.67)	0.46 (3.02)	0.08 (0.50)	0.01 (0.04)	0.00 (0.01)	0.00 (0.00)

Table 4. *Eigenvalues and their proportions in the total variance for the (co) variance of the permanent environmental effect estimated from different order models*

Models	1	2	3	4	5	6	7
L (2,2)	36.16 (86.80)	5.50 (13.20)					
L (3,3)	30.99 (79.69)	5.60 (14.40)	2.30 (5.92)				
L (4,4)	30.46 (72.83)	7.15 (17.09)	3.29 (7.88)	0.92 (2.20)			
L (5,5)	30.10 (74.06)	5.93 (15.58)	3.13 (7.70)	1.30 (3.19)	0.19 (0.47)		
L (6,6)	31.31 (73.37)	6.28 (14.73)	3.17 (7.42)	1.47 (3.45)	0.32 (0.75)	0.12 (0.28)	
L (7,7)	30.47 (72.21)	6.01 (14.25)	3.29 (7.81)	1.53 (3.62)	0.65 (1.54)	0.18 (0.42)	0.07 (0.16)

total variance for the (co)variance of the additive genetic effect estimated from different models. One of the additive genetic effects in L(2,2), L(3,3), L(4,4) and L(5,5) models constitutes more than 90% of total eigenvalues. However, 2 of the additive genetic effects in the L(6,6) and L(7,7) models constitute more than 90% of the total eigenvalues.

The eigenvalues calculated in the (co)variance matrices of the permanent environmental effects and the proportions of these eigenvalues in the total eigenvalues estimated from different models are given in *Table 4*. For the permanent environmental effect, at least 2 eigenvalues in L(2,2) and L (3,3) models constitute more than 90% of the total eigenvalue. In other models, at least 3 eigenvalues constitute more than 90% of the total eigenvalue.

Discussion

Different methods are used in modeling repeated measurements in animals. These models explain how the feature in question changes over time. The important point on repeated measurements is the relationships between the test day yields. For this reason, the (co)variance structure between test days is important in the analysis of repeated measurements.

The random regression approach, which is suitable for repeated records and allows model-specific effects for each measurement, is the most appropriate approach to this type of information [27].

At the beginning of lactation, additive genetic effect estimates for TGSV range from 0.09 to 20.10. Other models show similar trends, except for the L(7,7) model excluding the last test days. Obtained results were found similar to the works of Takma and Akbaş [5], Peixoto et al.[16], López-Romero and Carabaño [25], Bignardi et al.[28].

Estimates of the permanent environmental effects of TDMY from the Legendre models have changed between 10.49 and 48.65. When the permanent environmental effect value is analyzed, the trend obtained from the LEG65_10 model found by Behzadi and Mehrpoor [15] were similar to this study result except L(4,4) model result. But, it is the opposite of the tendency found by Takma and Akbaş [5] study. When the results of the heritability values obtained from the study were examined, the heritability values obtained from different models varied between 0.002 and 0.305 on the first test day and the other test days it was found between 0.004 and 0.389. Takma and Akbaş [5] found their heritability estimates in the range of 0.26 to 0.57. Galiç and Kumlu [29] found between 0.12 and 0.15. Prakash et al.[17] estimated between 0.007 and 0.088 with homogeneous residual variance. Behzadi and Mehrpoor [15] estimated between 0.13 and 0.66 with the LEG65_10 model. Naderi [30] estimated heritability between 0.45 and 0.60 with RRM for milk yield. According to these studies, it can be said that the heritability estimates obtained are in a similar range except Naderi [30] study results. Meyer [14] reported that there may be erratic and implausible estimates of variance components and genetic parameters using RRM using cubic, quartic or higher-order polynomials, especially in data sets that contain fewer or less recent records than polynomial order [31]. In the study, there is a situation arising from the variation at the beginning of lactation on the first test day. It is believed that an unreasonable prediction is not encountered on other test days except the last. This situation is thought to arise from the heterogeneous error variance.

The values of -2LogL (between 28334.16 and 26610.07), AIC (between 28356.16 and 27152.83) and BIC (between 28432.05 and 27129.21) obtained from the study result decreased as the model order increased except BIC. These values were found higher than the works of Takma and Akbas [24] and Naderi [30] and lower than the works of Behzadi and Mehrpoor [15], Takma and Akbas [32] and Haiduck Padilha et al.[19].

The magnitude of eigenvalues calculated in additive genetic effects (co) variance matrices and the share of these eigenvalues in total eigenvalues, at least 1 eigenvalue in L(2,2), L(3,3), L(4,4) and L(5,5) models, in other models at

least 2 eigenvalues constitute more than 90% of the total eigenvalues. For the permanent environmental effect, at least two eigenvalues in models L(2,2) and L(3,3) and 3 eigenvalues in others constitute more than 90% of the total eigenvalues. According to the obtained findings, it was determined that the 3rd degree Legendre polynomial model can provide sufficient compliance. These findings were similar to those obtained from the works of Takma and Akbas [24]. Behzadi and Mehrpoor [15] suggested the L6,5_RV10 model. However, when looking at the values for -2LogL, AIC and heterogeneous RV, it was determined that the L(7,7) model fits well according to other models.

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Conflict of Interest

The author declares that he has no conflict of interests.

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RESEARCH ARTICLE

Computed Tomography-Based Morphometric Analysis of the Hip Bones *(Ossa coxae)* in Turkish Van Cats

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Abstract

This study was carried out to determine the osteometric measurement values of the ossa coxae by using three-dimensional (3D) modeling of the images obtained by computed tomography in Van Cats. In the study, 16 adult Van cats, 8 of which are male ones and 8 female ones, were used. Firstly, cats were anesthetized with the ketamine HCl - xylazine HCl combination. Their images were obtained by scanning through 16 cross-sectional CT devices (Somatom Sensation 16; Siemens Medical Solutions, Erlangen, Germany). Then, The scanned images of the ossa coxae in each cat were transformed into the three-dimensional structure by using MIMICS 20.1 (The Materialise Group, Leuven, Belgium) software program. Later, morphometric (12 linear), volumetric, and surface area measurements were calculated and statistical analyses were performed. In the present study, 12 of 14 measurement parameters of the ossa coxae were higher in males than in females. In the osteometric analysis, it was observed that the greatest length of one half (GL), length between the inner edges of the acetabulum (LA), the greatest breadth across the tuber ischiadicum (GBTi), and the smallest breadth across the bodies of the ischia (SBI) measurement values were significantly greater in male cats than in female cats ($P<0.05$). The volume and surface area values of the ossa coxae in van cats were found as 12.33±2.14 cm^3 in male cats and 9.81±0.71 cm^3 in female cats; 94.66±11.01 cm^2 in male cats and 83.84±7.73 cm^2 in female cats, respectively. These differences between the measurement values of male and female cats were seen to be statistically significant ($P<0.05$). As a result, basic morphometric measurement parameters of ossa coxae between both genders in adult Van cats were determined by using CT and 3D modeling programs. It is thought that the data obtained from this study may be useful in determining the sexual dimorphism of these species and also in determining the clinical applications and scientific studies to be carried out in the region.

Keywords: *Computed tomography, Morphometry, Ossa coxae, Three-dimensional reconstruction, Van cat*

Türk Van Kedilerinde Kalça Kemiklerinin *(Ossa coxae)* Bilgisayarlı Tomografi Tabanlı Morfometrik Analizi

Öz

Bu çalışma Van kedilerinde ossa coxae'nin bilgisayarlı tomografi (BT) ile elde edilen görüntülerinin üç boyutlu (3B) modellemesini yapmak, osteometrik ölçüm değerlerini belirlemek amacıyla yapıldı. Çalışmada 16 adet (8 erkek, 8 dişi) erişkin Van kedisi kullanıldı. Öncelikle ketamin HCl - xylazin HCl kombinasyonu ile anesteziye alınan kediler, 16 kesit çok sıralı BT cihazı (Somatom Sensation 16; Siemens Medical Solutions, Erlangen, Germany) ile taranarak görüntüleri elde edildi. Daha sonra, her kedinin ossa coxae'sine ait tarama görüntüleri MIMICS 20.1 (The Materialise Group, Leuven, Belgium) programı yardımıyla üç boyutlu yapıya dönüştürülerek, morfometrik (12 doğrusal), hacim ve yüzey alanı ölçümleri alındı ve istatistiki analizi yapıldı. *Ossa coxae*'ya ait 14 adet ölçüm parametresinden 12 ölçüm parametresinin erkek kedilerde dişilere göre daha yüksek olduğu belirlendi. Osteometrik analiz sonuçlarına bakıldığında, bir yarımın en büyük uzunluğu (GL), acetabulum'un iç kenarlarının uzunluğu (LA), tuber ischiadicum'ların karşıdan karşıya maximum genişliği (GBTi) ve corpus ossis ischii'lerin karşıdan karşıya minumum genişliği (SBI) ölçüm değerlerinin erkek kedilerde dişi kedilere göre istatistik olarak anlamlı bir şekilde yüksek olduğu gözlendi ($P<0.05$). Erkeklerde ossa coxae'nin hacim değeri 12.33±2.14 cm^3, dişilerde 9.81±0.71 cm^3; yüzey alanı ise erkeklerde 94.66±11.01 cm^2, dişilerde 83.84±7.73 cm^2 olarak bulundu. Erkek ve dişi kedilerinin ölçüm değerleri arasında görülen bu farklılıkların istatistik olarak önemli düzeyde olduğu görüldü ($P<0.05$). Sonuç olarak, erişkin Van kedilerinde ossa coxae'nin temel morfometrik ölçüm parametrelerinin cinsiyetler arasındaki farklılıkları BT ve 3B modelleme programı kullanılarak tespit edildi. Çalışmadan elde edilen bilgilerin, türe ait seksüel dimorfizmin belirlenmesinin yanı sıra bölge ile ilgili yapılacak olan klinik uygulamalara ve bilimsel çalışmalara fayda sağlayacağı düşünülmektedir.

Anahtar sözcükler: *Bilgisayarlı tomografi, Morfometri, Ossa coxae, Üç boyutlu rekonstrüksiyon, Van kedisi*

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Introduction

Hip bone or os coxae consists of three bones; os pubis, os ilium, and os ischii. The corpus of these three bones creates a hollow which is called the acetabulum and enables the femur to make a joint with the caput ossis femoris. The pelvis, which is formed by the ossa coxae, on sides right and left, and the symphysis pelvina, occurred by the joining of these bones at the ventral, and also the sacrum and the first few caudal vertebrae (coccygeal) from the dorsal. The cavity surrounded by the bones of the pelvis is the cavum pelvis [1,2].

When comparing ossa coxae in cats to dogs, some slight differences can be seen. For example; in cats, the ilia get slightly away toward the cranial and the wings of these bones are relatively smaller and shallower, which makes a large passage shaped cone from cavum abdominis to cavum pelvis. The ischial tubers being closer to each other has led to the pelvic being in a more rectangle shape and exit to be more narrow. However, in both species, the axis of the pelvic channel is flat and in general, this enables cats and dogs to give an easier birth [3]. In addition, pelvic bones and joints they create (articulatio sacroiliaca, articulatio coxae, symphysis pelvina), have more movement space and ability to create versatility in animals such as cats and dogs. It also has tasks such as to provide posture and locomotion of the skeleton, the birth, act of squat or lifting legs during urination in a more comfortable way, scratching the head, neck, and thorax area by means of the hind leg, to transmit the movement starting from hind leg to body, to help determining the movement and posture peculiar to breeds, protecting the organs inside and to enable functions being operated [4,5].

Today, thanks to available computer-supported technological developments in medical imagining methods and computed tomography (CT) and three-dimensional modeling, significant changes have occurred in treatments and diagnosis of various illnesses and especially in anatomy education [6]. Computed tomography and three-dimensional reconstruction programs, especially in small animals such as cats and dogs are often used as a standard imaging method in imaging complex anatomical structures such as pelvic and obtaining various morphometric measurements [7,8], in scanning, treating, and diagnosing various pathological structures such as rectum cancer, colon cancer, prostate in the pelvic cavity, lower urinary tract disorders, glandula bulbourethralis cyst, cervix and ovary carcinomas [9,10], in studies in the anthropology fields [11].

Van cat, an endemic cat breed, which lives in the Van region and is named after here is an important part of living culture in the region. It is an important cultural wealth source of the region. In recent years, they have attracted people owing to their physical appearances and especially being included in the endangered species [5,12]. There are very few studies about the osteological properties of these animals [5,13,14]. This study has been carried out to obtain morphometric measurements by making three-dimensional modeling by computed tomography of the ossa coxae in Van cat, to reveal the sexual dimorphism of these measurements and to provide an anatomical reference data in terms of pelvic morphology.

Material and Methods

In the study, a totally, 8 female and 8 male Van cats between the ages of 3 and 8 were used. The cats were obtained from the Van Cat Research and Application Center, Van Yuzuncu Yıl University, Turkey. *Ad libitium* drinking water and standard cat forage were given to the cats before the study day. The study was accepted by Van Yuzuncu Yıl University Local Ethical Committee of Animal Experiments (decree no: 2020/02 - 27.02.2020).

Van cats involved in the study were not given any food one day before the study by numbering them. A combination of ketamine (15 mg/kg, IM, Ketasol® 10% injectable) and xylazine (1-2 mg/kg, IM, Alfazyne® 2% injectable) was used for cat anesthesia on the examination day.

Available 16 cross-sectional multislice computed tomography (CT) devices (Somatom Sensation 16; Siemens Medical Solutions, Erlangen, Germany) at the Radiology Department of Medicine Faculty at Van Yuzuncu Yıl University were used for examination of cats. Cats were symmetrically placed in prone position to be "head first" on "the disposable" quilt laid out in the gantry. CT device parameters used during imagining were given in *Table 1*. CT application dose parameters and performed scanning were carried out based on standard protocols and literature [15,16]. Obtained images were stored in the DICOM form.

Later, reconstructions of these images were made by conveying them into three-dimensional modeling program MIMICS 20.1 (The Materialise Group, Leuven, Belgium). Osteometric measurements for 12 different parameters from ossa coxae whose modeling were made were obtained. Measurement points stated in the literature were used as a base for morphometric measurements [17].

Table 1. *CT device parameters*

KV/Effective mAs/Rotation time (sec) values	120/120/0.75
Gantry rotation period	420 ms
Physical detector collimation	16×0.6 mm
Section thickness	0.5 mm
Final section collimation	32×0.63 mm
Feed/rotation	6 mm
Kernel	U90u
Increment	0.5 mm
Resolution	512×512 pixels

Table 2. *Measurement points and abbreviations of the ossa coxae in Van cats*

Parameter	Abbreviation	Definition
1	GL	Greatest length of one half. Distance between the Tuber coxae and the tuber ischiadicum in one half (mm)
2	LA	Lenght between the outer edges of the acetabulum: Distance between the front outer edge and the back outer edge of the acetabulum (mm)
3	LAR	Lenght between the inner edges of the acetabulum: Distance between the front inner edge and the back inner edge of the acetabulum (mm)
4	LS	Length of the pelvic symphysis: Distance between the cranial end and the caudal end of the pelvic symphysis (mm)
5	SH	Smallest height of the shaft of ilium (mm)
6	SB	Smallest breadth of the shaft of ilium (mm)
7	LFo1	Inner lenght of the foramen obturatum (mm)
8	LFo2	Inner breadth of the foramen obturatum (mm)
9	GBTc	Greatest breadth across the tuber coxae (mm)
10	GBA	Greatest breadth across the acetabula (mm)
11	GBTi	Greatest breadth across the tuber ischiadicum (mm)
12	SBI	Smallest breadth across the bodies of the ischia (mm)
13	Volume	Volume of the ossa coxae (cm^3)
14	Surface area	Surface area of the ossa coxae (cm^2)

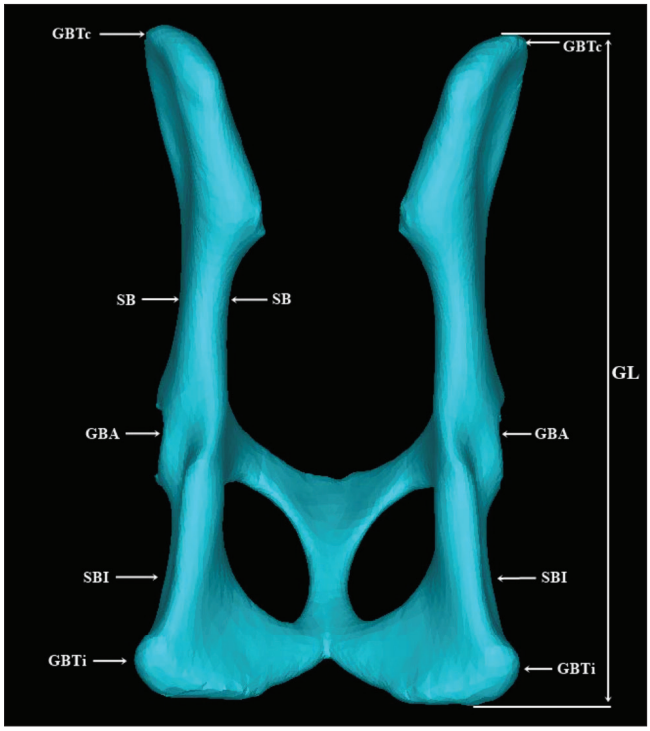

Fig 1. Measurement points on facies dorsalis of ossa coxae. GL: Greatest length of one half; GBTc: Greatest breadth across the tuber coxae; SB: Smallest breadth of the shaft of ilium; GBA: Greatest breadth across the acetabula; SBI: Smallest breadth across the bodies of the ischia; GBTi: Greatest breadth across the tuber ischiadicum

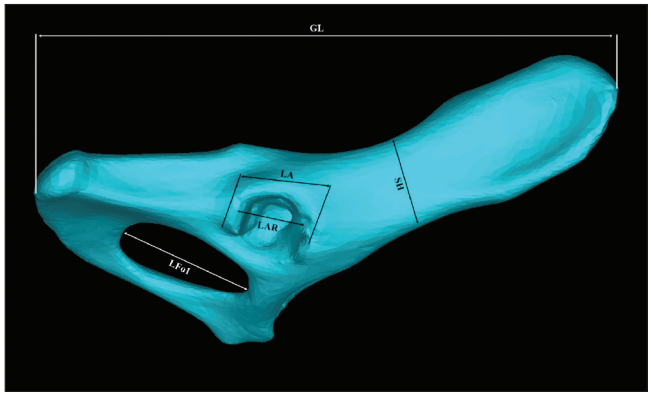

Fig 2. Measurement points on facies lateralis of os coxae. GL: Greatest length of one half; SH: Smallest height of the shaft of ilium; LA: Lenght between the outer edges of the acetabulum; LAR: Lenght between the inner edges of the acetabulum; LFo1: Inner lenght of the foramen obturatum

After morphometric measurements were completed, the surface area and volume value of ossa coxae were calculated. Definition and abbreviations of measured osteometric parameters in *Table 2*, their regions on ossa coxae in *Fig. 1*, *2*, and *3* have been presented. Nomina Anatomica Veterinaria [18] as the terminology has been used in the study. In addition, for the weight measurements of the cat that have been used in the study, digital scales (TESS®, RP-LCD, Çomak Scales, İstanbul) has been used.

Shapiro-Wilk test (n<50) was used to examine whether the mean values of the ossa coxae were distributed in three-dimensional modeling and morphometric properties in Van cats by using computerized tomography images, and nonparametric tests were applied because the measurement values of the variables were not normally distributed. Descriptive statistics for measurement values in our study have been expressed as mean, standard deviation, minimum, and maximum. Mann-Whitney U test has been used in the comparisons for genders according to measurements. Spearman coefficient of correlation has been calculated to determine the relation among measurements on the condition that they are different

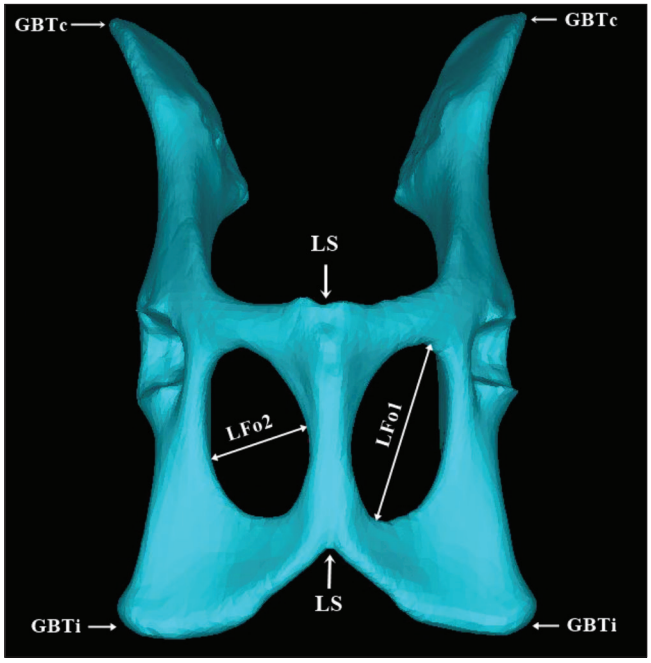

Fig 3. Measurement points on facies ventralis of ossa coxae. GBTc: Greatest breadth across the tuber coxae; GBTi: Greatest breadth across the tuber ischiadicum; LS: Length of the pelvic symphysis; LFo2: Inner breadth of the foramen obturatum

Table 3. *Descriptive statistics of the measurements of the ossa coxae in Van cats by gender*

Parameter	Gender	Mean	Std. Dev.	Min.	Max.	*P.
Age	Female	5.000	2.000	3.000	8.000	1.000
	Male	5.000	2.000	3.000	8.000	
Body Weight (W)	Female	6.284	0.280	5.810	6.630	0.027
	Male	7.075	0.702	6.300	8.050	
GL	Female	76.638	1.542	74.390	78.590	0.021
	Male	80.398	3.473	75.670	85.820	
LA	Female	10.868	0.472	10.110	11.430	0.009
	Male	11.968	0.781	10.780	13.110	
LAR	Female	9.570	0.247	9.150	9.830	0.172
	Male	9.914	0.672	8.840	11.100	
LS	Female	29.380	2.250	25.690	32.780	0.115
	Male	31.731	2.416	29.290	36.020	
SH	Female	11.628	0.390	10.920	12.030	0.248
	Male	12.255	1.074	10.560	13.610	
SB	Female	4.779	0.481	4.280	5.570	0.208
	Male	4.437	0.626	3.730	5.250	
LFo1	Female	19.763	0.882	18.470	20.860	0.999
	Male	20.009	1.295	18.140	22.630	
LFo2	Female	13.749	1.285	12.070	15.630	0.916
	Male	13.705	0.869	12.760	15.120	
GBTc	Female	41.361	3.448	36.860	47.790	0.248
	Male	43.537	3.150	38.850	47.700	
GBA	Female	38.644	1.395	36.490	40.850	0.074
	Male	40.409	1.952	37.530	43.620	
GBTi	Female	43.471	1.638	41.540	47.020	0.016
	Male	46.055	2.580	43.370	50.630	
SBI	Female	34.457	1.796	31.130	36.350	0.012
	Male	37.245	2.238	34.790	41.810	

** P<0.05; Mann-Whitney U Test*

Table 4. *Descriptive statistics of the volume and surface area measurements of the ossa coxae in Van cats by gender*

Parameter	Gender	Mean	Std. Dev.	Min.	Max	*P.
Volume	Female	9.806	0.707	8.835	10.891	0.005
	Male	12.327	2.138	9.701	15.854	
Surface area	Female	83.840	7.727	76.196	99.072	0.036
	Male	94.660	11.007	70.639	105.588	

** P<0.05; Mann-Whitney U Test*

Table 5. *Correlation between ossa coxae measurements in Van cats by gender*

↓ →		Age	Weight	GL	LA	LAR	LS	SH	SB	LFo1	LFo2	GBTc	GBA	GBTi	SBI
Age	r		0.390	0.049	-0.537	-0.295	0.488	0.098	-0.146	0.390	0.439	0.732*	0.146	-0.390	0.098
Weight	r	0.957**		0.619	0.238	-0.024	0.619	0.476	0.452	0.762*	0.571	0.095	0.452	-0.762*	-0.286
GL	r	0.878**	0.850**		0.095	0.084	0.452	0.119	-0.095	0.286	0.000	-0.190	0.071	-0.214	-0.405
LA	r	0.878**	0.826*	0.952**		0.311	0.095	0.619	0.381	0.357	0.238	-0.524	0.024	-0.119	-0.524
LAR	r	0.565	0.554	0.719*	0.731*		-0.611	0.587	0.431	-0.443	0.431	-0.719*	0.000	0.575	-0.371
LS	r	0.781*	0.826*	0.619	0.619	0.048		0.143	-0.214	0.857**	0.143	0.619	0.310	-0.762*	-0.143
SH	r	0.761*	0.783*	0.467	0.395	0.000	0.826*		0.571	0.333	0.762*	-0.190	0.333	-0.143	-0.381
SB	r	0.439	0.287	0.357	0.262	-0.228	0.476	0.539		0.119	0.500	-0.310	0.429	-0.333	0.167
LFo1	r	0.878**	0.850**	0.952**	0.857**	0.647	0.619	0.587	0.381		0.452	0.405	0.405	-0.857**	-0.333
LFo2	r	-0.195	0.036	-0.190	-0.190	-0.216	0.214	0.048	-0.571	-0.119		0.048	0.524	-0.262	-0.429
GBTc	r	0.390	0.311	0.071	0.095	-0.503	0.690	0.695	0.738*	0.119	-0.119		0.333	-0.500	0.429
GBA	r	0.878**	0.814*	0.762*	0.762*	0.347	0.810*	0.671	0.619	0.714*	-0.286	0.595		-0.476	0.071
GBTi	r	0.488	0.419	0.238	0.190	-0.263	0.571	0.731*	0.833*	0.238	-0.429	0.786*	0.571		-0.143
SBI	r	0.439	0.323	0.286	0.214	-0.204	0.452	0.575	0.929**	0.262	-0.619	0.714*	0.595	0.952**	

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

for genders. Statistical significance levels were considered as 1% and 5%. The SPSS (IBM SPSS for Windows, Ver.23) statistic packaged software has been used for calculations.

Results

Osteometric measurements from 14 parameters as well as surface area and volume of ossa coxae were taken in our study. Male and female group average of obtained morphometric measurement values and differences between genders have been detected by carrying out statistical analysis. Later, considerable differences (P<0.05) between these measurement values have statistically been reported. These evaluated measurement values have been presented in *Table 3, Table 4, Table 5* and *Fig. 4*.

Descriptive statistics of osteometric measurement values of the ossa coxae according to gender were given in *Table 3*. Accordingly, it was observed that GL, LA, GBTi, and SBI measurement values were statistically significantly higher in male cats compared to female cats (P<0.05)

Distribution of the ossa coxae morphometric measurements by gender has been given in *Fig. 4*. According to the graph in the figure, while GL, LA, LAR, LS, SH, LFo1, GBTc, GBA, GBTi, and SBI measurement values for male cats are higher; SB and LFo2 measurement values for female cats are larger.

Surface area and volume measurements of the ossa coxae of male and female Van cats have been separately performed and descriptive statistics of these measurement values have been given in *Table 4*. The volume of the ossa coxae in male cats was found to be 12.33±2.14 cm^3 whereas, in female cats, it was measured as 9.81±0.71 cm^3. The surface area of the ossa coxae was measured to be 94.66±11.01 cm^2 in male cats and 83.84±7.73 cm^2 in female cats. It was determined that these differences between the measurement values of male and female Van cats were statistically significant (P<0.05).

In *Table 5*, the relationship between ossa coxae's morphometric measurement values by gender was studied. Accordingly, a significant positive relationship in male cats was observed between age and body weight with GL, LA, LS, SH, LFo1, and GBA measurement values; between LA with LAR, LFo1, and GBA measurement values; between LS with SH and GBA measurement values, between SH and GBTi measurement values; between the SB with GBTc, GBTi, and SBI measurement values; between LFo1 and GBA measurement values; between GBTc with GBTi and SBI measurement values; between GBTi and SBI

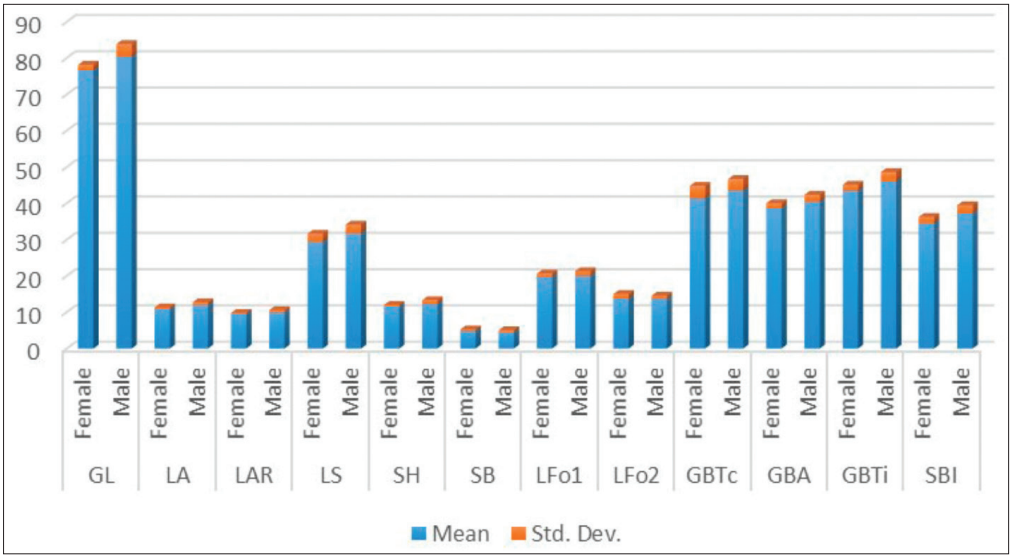

Fig 4. Distribution of the ossa coxae morphometric measurements by gender

measurement values (P<0.05). In female cats, a significant positive relationship was determined between age and GBTc measurement values, between body weight and LFo1 measurement values; between LFo1 with body weight and LS measurement values; between LFo2 and SH measurement values (P<0.05). However, a negative significant correlation was found between GBTi and body weight measurement values at 76.2%; between GBTc and LAR measurement values at 71.9%; between GBTi with body weight, LS, and LFo1 measurement values at 76.2%, 76.2%, and 85.7% (P<0.05).

Discussion

Morphometric analysis on animal bones is a common method in determining differences between genders, studying different breed among animal species, revealing morphological variations in a species, and also presents significant data for various science fields such as forensic, developmental, and evolutionary sciences [19]. A number of studies have been carried out in humans [11,20,21] and veterinary medicine field [5,19,22-27] in order to reveal differences of measurements between genders obtained from ossa coxae.

Computed tomography and three-dimensional reconstruction programs are often used in screening complex anatomical structures such as pelvic in small animals like dog and cat, obtaining various morphometric values, in the diagnosis of diseased or pathological structures in the region, and in evaluating treatment options for these structures [5,7,9]. In this study, morphometric, volumetric, surface area measurement values of the ossa coxae have been determined and differences of these values between genders have been revealed by using CT and three-dimensional modeling in adult Van cat.

In the morphological and radiological studies carried out on osteometric properties of pelvic in cats, it has been found that measurement parameters of pelvic are mostly larger in male cats compared to female cats, but pelvic angles are higher in female cats [5,19,24,28]. In our study, in parallel with this information, obtained 12 measurement parameters out of 14 as well as volumetric measurement value and surface area of the ossa coxae have been found to be higher in male cats than female ones. Distribution of these measurement parameters by genders and descriptive statistics have been given in *Fig. 4* and *Table 3*. It was observed that GL, LA, GBTi, and SBI measurement values among measurement parameters were statistically significantly higher in male cats than female ones (P<0.05). Accordingly, we can conclude that the ossa coxae in Van cats are bigger in male cats than female ones.

In a study on pelvic osteometric measurements of Retriever dogs performed by Nganvongpanit et al.[27], it has been found that GL, GBTc, GBTi, LS, LFo1, and LFo2 measurement values in the male are 161.50 mm, 88.44 mm, 105.22 mm, 47.15 mm, 29.13 mm, 23.97 mm respectively whereas in females they are 145.93 mm, 80.05 mm, 98.93 mm, 42.33 mm, 27.11 mm, 23.47. In another study on pelvic morphometric measurements of domestic cats performed by Pitakarnnop et al.[19], it has been reported that these measurement values in the male are 79.46 mm, 42.97 mm, 37.23 mm, 16.70 mm, 18.01 mm, 14.54 mm respectively whereas in females they are 74.61 mm, 39.73 mm, 39.58 mm, 18.21 mm, 18.41 mm, 14.12 mm. In the study conducted, these values in male Van cats have been detected as 80.40 mm, 43.54 mm, 46.06 mm, 31.73 mm, 20.01 mm, 13.71 mm; 76.64 mm, 41.36 mm, 43.47 mm, 29.38 mm, 19.76 mm, 13.75 mm. That, in general, there are slight differences between these values is thought to have resulted from species, age, height, breed, and body weight properties.

The age and body weight are of great importance in terms of pelvic growth or evaluating osteometric measurement parameters. In the studies carried out, it has been reported that there is a mostly positive correlation between age and body weight of male-female animals and morphometric measurements of pelvic in general [5,23,28,29]. In the study we have performed, a significant positive relationship has been observed between age and body weight with GL, LA, LS, SH, LFo1, and GBA measurement values in male cats (P<0.05). A significant positive relationship in female cats has been seen between age and GBTc measurement values; between body weight and LFo1 measurement values; whereas, a negative significant relationship has been found between body weight and GBTi measurement values at 76.2%. (P<0.05). In addition, the correlation between other measurement values of the ossa coxae in male and female Van cat has been given in *Table 5*. It is thought that together with pelvic angle measurements taken by Yilmaz et al.[5] in Van cats, osteometric measurements and correlations taken in these studies may be useful in future characterization studies including determination of external body measurements and body condition score in these cats.

In recent years thanks to technological improvements such as CT in the medical imaging field, images of desired thickness can be taken under anesthesia without causing any vital damage to the living creature, and from these images, anatomical studies can be done in the relevant structure by using various three-dimensional reconstruction programs [5,20]. Measurement values such as osteometric, volumetric, and surface area of pelvic and complex anatomical structures around it can be easily calculated using CT and various developed three-dimensional modeling programs and so diagnosis and treatment of various pathological condition activities can be evaluated by obtaining quantitative data of relevant anatomical structure [7,21,29]. In the study we conducted, the volume and surface area of the ossa coxae were calculated via a 3D modeling program (MIMICS 20.1-The MaterialiseGroup, Leuven, Belgium) using CT images in Van cats. In Van cats, the volume value of the ossa coxae in males was determined 12.33±2.14 cm^3, in females 9.81±0.71 cm^3; the surface area was determined 94.66±11.01 cm^2 in males and 83.84±7.73 cm^2 in females. It was found that these differences observed between measurement values of male and female Van cats are statistically at a significant level (P<0.05).

As a result, statistical differences of measurement parameters of the ossa coxae in Van cats between genders were found by using computed tomography and a three-dimensional modeling program. In addition, in this study, basic morphometric data were obtained that would benefit veterinarians and studies in zooarchaeology in the fields of surgery, clinical, gynecological, diagnostic imaging, and treatment, especially in anatomy education.

Conflict of Interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of paper.

Author Contributions

OY and İD planned, designed, and supervised the research procedure. OY and İD performed the anatomical analysis. OY carried out the statistical analysis. OY and İD performed the imaging stage and the CT parameters. The manuscript was written by OY and İD. OY contributed to the language editing of the final manuscript. All authors have interpreted the data, revised the manuscript for contents, and approved the final version.

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RESEARCH ARTICLE

Effects of Probiotic *(Lactobacillus farciminis)* Supplementation in Quail *(Coturnix coturnix japonica)* Rations on Growth Performance, Blood Antioxidant Capacity and Cecal Some Short-Chain Fatty Acid Concentrations

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Abstract

The purpose of this study was to investigate the effect of *Lactobacillus farciminis* supplementation in quail diets on performance, blood antioxidant capacity and the concentrations of cecal short-chain fatty acid (SCFA). A total of 180 day-old quail chicks were randomly divided into 3 groups each containing 60 chicks. Each group was randomly divided into 5 subgroups each containing 12 chicks. The chicks were fed with corn, soybean meal and full-fat soybean based rations for 35 days. While the control group was fed with basal ration, the experimental groups were fed with probiotic supplementation at 0.1 g/kg and 0.3 g/kg doses, respectively. At end of the experiment, the use probiotics in quails did not affect initial LW, final LW, LWG, FI and FCR. The increase in dietary probiotic, MDA, GSH, SOD, CAT and GPx exhibited a linear response. However, ceruloplasmin, albumin, total protein and globulin were not affected by the addition of probiotic. Significant linear responses in asetic acid, isocaproic acid and SCFA were observed with the gradued level of probiotic. However, a significant quadratic response in the caproic acid was observed. Therefore, the effective dose for caproic acid was 0.1 g/kg. There were no significant differences in acetic acid, propionic acid, butyric acid, isobutyric acid, valeric acid, isovaleric acid and BCFA concentrations for quails fed with different levels of probiotic. In conclusion, diets containing *Lactobacillus farciminis* in quail can be used to improve the antioxidant capacity and intestinal health.

Keywords: *Lactobacillus farciminis, Growth performance, Antioxidant capacity, Cecal short-chain fatty acid, Japanese quail*

Bıldırcın *(Coturnix coturnix japonica)* Rasyonlarına Probiyotik *(Lactobacillus farciminis)* İlavesinin Büyüme Performansı, Kan Antioksidan Kapasite ve Sekal Bazı Kısa Zincirli Yağ Asidi Konsantrasyonları Üzerine Etkileri

Öz

Bu çalışmanın amacı, bıldırcın diyetlerinde *Lactobacillus farciminis* takviyesinin performans, kan antioksidan kapasitesi ve sekal bazı kısa zincirli yağ asidi (SCFA) konsantrasyonları üzerindeki etkisini araştırmaktır. Toplam 180 günlük bıldırcın civcivleri rastgele olarak her biri 60 civciv içeren 3 gruba ayrılmıştır. Her grup rastgele olarak her biri 12 civciv içeren 5 alt gruba ayrılmıştır. Tüm civcivler 35 gün boyunca mısır, soya fasulyesi ve tam yağlı soya fasulyesine dayalı rasyonla beslenmiştir. Kontrol grubu (C) bazal rasyonla beslenirken, deney gruplarına bazal rasyona ek olarak sırasıyla 0.1 g/kg (P1) ve 0.3 g/kg (P2) dozlarında probiyotik ilavesi yapılmıştır. Deneme sonunda, bıldırcınlarda probiyotik kullanımı başlangıç canlı ağırlık, bitiş canlı ağırlık, canlı ağırlık artışı, yem tüketimi ve yemden yararlanma oranını etkilememiştir. Rasyona probiyotik ilavesi ile MDA, GSH, SOD, CAT ve GPx'teki artış lineer bir cevap vermiştir. Fakat seruloplazmin, albümin, toplam protein ve globulin probiyotik ilavesinden etkilenmemiştir. Kademeli artan probiyotik seviyesinde asetik asit, izokaproik asit ve SCFA'da önemli lineer tepkiler gözlenmiştir. Fakat, kaproik asitte önemli bir kuadratik yanıt gözlenmiştir. Kaproik asit için etkin doz 0.1 g/kg'dır. Farklı seviyelerde probiyotikle beslenen bıldırcınlar için asetik asit, propiyonik asit, alıcı asit, izobutirik asit, valerik asit, izovalerik asit ve BCFA konsantrasyonlarında önemli bir fark görülmemiştir. Sonuç olarak, bıldırcınlarda *Lactobacillus farciminis* içeren rasyonları antioksidan kapasite ve bağırsak sağlığını iyileştirmek için kullanılabilir.

Anahtar sözcükler: *Büyüme performansı, Lactobacillus farciminis, Japon bıldırcını, Probiyotik, Sekum kısa zincirli yağ asitleri*

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Introduction

With advances in animal nutrition and biotechnology, various feed additives are now used in poultry feed in order to increase healthy animal production. Antibiotics used for growth promoters have been produced by selected microorganisms and the development of natural metabolites [1]. The prolonged use of antibiotics as growth factor in poultry has prevented the growth of pathogenic microorganisms in the digestive tract of animals as well as beneficial microorganisms. The use of antibiotics in feeds has promoted development of resistance aganist pathogenic bacteria. In addition, residues in animal products become a risk to human health [2]. For these negative reasons, the use of antibiotics as a growth factor in animal feeds have been prohibited. After this ban, probiotics, prebiotics, enzymes, organic acids and some products such as essential oils are started to be used as an alternative feed additives to antibiotics [3].

The term "probiotic" is derived from Latin preposition and pro ("for" or "supported") and the Greek word biotikos means "for life". Probiotics are described as living microbial feed supplements that beneficially affect the host animal by improving intestinal microbial balance. They have strengthened the immune system by increasing the level of antibodies in the digestive system [4]. In the recent research, the use of probiotics as feed additives in Japanese quails was observed to improve feed bioavailability, health and immune status [5]. Probiotic bacteria have significant antioxidant abilities both *in vivo* and *in vitro* [6]. With the use of *Lactobacillus* spp. supplement for manipulation of secum fermentation in broiler, may increase some short chain fatty acid accumulation in the cecum and thus improve gastrointestinal system development and prevent disease [7]. Using *Lactobacillus acidophilus* and *L. casei* as probiotics in broiler rations may affect growth performance positively [8].

The aim of this study was to investigate the effect of probiotic in Japanese quail diets on growth performance, some blood parameters, such as superoxide dismutase (SOD), glutathione peroxidase (GPx), catalase (CAT), malon-dialdehyde (MDA) and glutathione (GSH), ceruloplasmin, albumin, total protein, globulin and cecal short-chain fatty acid (SCFA) concentrations.

Material and Methods

Animals, Experimental Design and Feed

The study was carried out with the permission of the Kafkas University Animal Experiments Local Ethics Committee (Decision No: KAU-HAYDEK/2019-055) report. A total of 180 one-day-old Japanese quails *(Coturnix coturnix japonica)* were included in the study regardless of gender. All chicks were randomly divided into 3 groups each containing 60 chicks. Each group was then randomly divided into 5 subgroups each containing 12 chicks. The animals were fed with corn, soybean meal basal ration and trial continued for 35 days *(Table 1)*. All diets were determined according to NRC [9] standards. Nutrient analyses (dry matter, crude protein, crude fibre, ether extract, ash, Ca and total P) of the feed were performed according to AOAC [10]. Birds were caged in breeding cages. Each subgroup was equipped with manual feeders and automatic nipple drinkers. Water and feed were given *ad libitum*. The house temperature was monitored thermostatically throughout the study. The temperature, which was 32-35°C on the first day, was gradually lowered and maintained at 22°C for the last two weeks. Artificial light program was implemented in accordance with commercial conditions (23 h of lighting throughout the experiment per day). The experimental diets were as follows: C, basal diet (Control; without addition probiotic); P1; 0.1 g/kg probiotic and P2; 0.3 g/kg probiotic. Probiotic (Biacton+®) used in the study were supplied from a commercial company (TARIMSAN CHEMICAL A.Ş. Istanbul, Turkey). Composition of probiotic used in the study has contained *Lactobacillus farciminis* $5x10^9$ CFU/g. In our study, we determined the probiotic doses of the groups by considering the doses recommended by the commercial product.

Table 1. *Composition of basal diets used in experiment (%)[1]*

Feed Materials	**%**
Corn	56.35
Soyben meal	36.10
Corn gluten (CP, 60%)	4.35
Limestone	1.45
Dicalciumphosphate	1.00
DL- Methionine	0.08
L-Lysine Hydrochloride	0.07
Vitamin- mineral premix	0.40
Salt	0.20
Total	100.00
The Calculated Value	
Crude protein, %	23.00
ME (kcal/kg)	2909.33
Ca, %	0.90
Total P, %	0.59
Analysis Values	
ME (kcal/kg)	2915.25
Crude protein, %	23.11
Ca, %	1.01
Total P, %	0.49

[1] As-fed basis; [2] Vitamin-mineral premix provided per kg diet: Vit. A 8.000 IU, Vit. D_3 1.000 IU, Vit. E 20 IU, Vit. K 0.5 mg, Vit. B_1 3 mg, Vit. B_2 9 mg, Vit. B_6 7 mg, Vit. B_{12} 0.03 mg, niacin 35 mg, D-pantothenic acid 10 mg, folic acid 0.55 mg, biotin 0.18 mg, Fe 100 mg, Cu 8 mg, Zn 100 mg, Mn 120 mg, I 0.7 mg, and Se 0.3 mg

Growth Performance

In the study, live weights (LW) were recorded weekly for each subgroups. Live weight gain (LWG) was determined by the difference between following weeks. Each subgroup's feed intake (FI) was recorded weekly and used for the calculation of feed conversion ratio (FCR).

Blood Antioxidant Capacity

At the end of the experiment, blood samples were taken from wing vein of the animals to the anticoagulant (EDTA) tubes. Plasma samples were taken after centrifugation of the blood samples at 3000 rpm for 15 min and stored at -20°C until the analyses were carried out. SOD, GPx and CAT antioxidant enzyme activities in plasma were determined by ELISA device (Epoch, Biotek, USA) using commercial kits (Cayman Chemical Company, USA). Whole blood reduced GSH analysis was determined colorimetrically (Epoch, Biotek, USA) according to the method of Beutler et al.[11]. MDA in plasma was determined by the method of Yoshoiko et al.[12], ceruloplasmin by the method of Colombo and Ricterich [13], and albumin and total protein levels by a commercial test kit (Biolabo, Maizy, France). The globulin value was determined by subtraction of the albumin from the total protein [14].

Cecal Short-Chain Fatty Acid Concentrations

The cecal digesta that were obtained after sacrification of the animals was used for the determination acetic, propionic, butyric, isobutyric, valeric and caproic acid with a gas chromatography (GC) (Shimadzu GC, Shimadzu Co., Kyoto, Japan), a flame ionization detector (FID) and colons (Teknokroma; TR-151035, TRB-FFAP 30m×0.53 mm×0.50 µm). At the end of the study, the cecum content were stored at -18°C and then were dissolved at + 4°C before analysis. The contents were centrifuged at 4000 rpm for 15 min at +4°C for homogenization. The supernatant was taken into a 750 µL Eppendorf tube and mixed with 150 µL ice-cold 25% metaphosphoric acid solution. After that, the tubes were kept in ice for 30 min to ensure the collapse of proteins. Subsequently, tubes were centrifuged for 10 min at 10000 rpm at +4°C. Supernatants were analyzed using GC. The analysis was performed according to Zhang et al.[15]. Helium was used for the carrier gas and the colon temperature was programmed so that it was increased stepwise from 110°C to 180°C. Also, the FID (Flame Ionization Detector) and injector block temperature was set to 250°C. The calibration curve was drawn with the Supelco Volatile Free Acid Mix, 46975-U (10 mmol/L) as a standard curve.

Statistical Analysis

The one-way analysis of varience (ANOVA) method was used for the statistical calculations of the groups and polynomial contrast test was used to determine the dose effect of the probiotic used at different levels in the groups. Statistical differences and trend analysis were considered significant at P≤0.05. The statistical analysis was done with the SPSS software package [16].

Results

The increase in probiotic did not affect initial LW, final LW, LWG, FI and FCR. The effect of probiotic in quail rations on growth performance is presented in *Table 2*.

The increase in dietary probiotic exhibited linear response (linear, P=0.000, for all except GPx; P=0.001) with MDA, GSH, SOD, CAT and GPx. However, ceruloplasmin, albumin, total protein and globulin were not affected by the addition of probiotic. Influence of probiotic on antioxidant capacity is given in *Table 3*.

The effect of the addition of probiotic in quail rations on some cecal short-chain fatty acid concentrations (µmol/g) is given in *Table 4*. Asetic acid (P=0.021), isocaproic acid (P=0.001) and SCFA (P=0.015) were linearly affected with the gradued level of probiotic. However, a significant quadratic effect in the caproic acid (P=0.003) was observed. Therefore, the effective dose for caproic acid was 0.1 g/kg. There were no significant differences in acetic acid, propionic acid, butyric acid, isobutyric acid, valeric acid, isovaleric acid and BCFA concentrations for quails fed different levels of probiotic.

Table 2. *The effect of probiotic in quail rations on growth performance*

Performance Parameters	Groups			Significance		
	Control	P1	P2			
	X±Sx	X±Sx	X±Sx	L	Q	Main Effect
Initial Live Weight, g	13.61±0.16	13.27±0.29	12.77±0.41	0.079	0.835	0.196
Final Live Weight, g	221.36±1.47	207.74±8.79	216.27±3.16	0.523	0.124	0.245
Live Weight Gain, g	207.75±1.40	194.46±9.08	203.49±3.18	0.602	0.131	0.271
Feed Intake, g	651.33±33.80	609.97±29.66	612.54±17.55	0.345	0.532	0.520
Feed Conversion Ratio	3.13±0.16	3.14±0.11	3.00±0.05	0.468	0.625	0.625

[1] The mean (x) and standard error (Sx) values of 5 replicates in each group; [2] C: Control basal ratio, P1: 0.1 g/kg probiotic added to basal ratio and P2: 0.3 g/kg probiotic added to basal ratio; [3] Polynomial contrasts: L = linear and Q = quadratic effect of supplemental probiotic

Table 3. *Influence of probiotic on antioxidant capacity*

Blood Parameters	Groups			Significance		
	Control	P1	P2			
	X±Sx	X±Sx	X±Sx	L	Q	Main Effect
MDA (µmol/L)	7.20±0.01	7.04±0.03	6.97±0.02	0.000	0.151	0.000
GSH (mg/dL)	15.26±0.10	23.68±0.87	37.12±1.54	0.000	0.068	0.000
SOD (U/mL)	22.39±1.23	29.21±1.00	37.35±1.12	0.000	0.641	0.000
CAT (nmol/min/mL)	3.36±0.04	3.28±0.02	3.16±0.01	0.000	0.616	0.002
GPx (nmol/min/mL)	33.23±0.51	37.55±1.36	39.74±0.87	0.001	0.395	0.002
Ceruloplasmin (mg/dL)	16.83±0.32	16.91±0.64	16.94±0.28	0.872	0.960	0.985
Albumin (g/dL)	2.34±0.06	2.31±0.02	2.29±0.03	0.517	0.864	0.791
Total protein (g/dL)	3.14±0.01	3.13±0.03	3.12±0.03	0.670	0.978	0.909
Globulin (g/dL)	0.79±0.06	0.82±0.04	0.82±0.02	0.716	0.859	0.918

[1] The mean (x) and standard error (Sx) values of 5 replicates in each group; [2] C: Control basal ratio, P1: 0.1 g/kg probiotic added to basal ratio and P2: 0.3 g/kg probiotic added to basal ratio; [3] Polynomial contrasts: L = linear and Q = quadratic effect of supplemental probiotic

Table 4. *The effect of the addition of probiotic in quail rations on cecal some short-chain fatty acid concentrations (µmol/g)*

SCFA Parameters	Groups			Significance		
	Control	P1	P2			
	X±Sx	X±Sx	X±Sx	L	Q	Main Effect
Acetic acid	32.73±5.57	51.99±7.76	56.01±4.98	0.021	0.338	0.047
Propionic acid	7.57±2.63	12.30±3.34	9.22±2.68	0.696	0.294	0.525
Isobutyric acid	0.42±0.04	0.52±0.11	0.44±0.06	0.881	0.369	0.650
Butyric acid	4.98±0.92	6.06±1.12	6.32±1.83	0.498	0.810	0.765
Valeric acid	0.80±0.13	0.99±0.15	0.93±0.00	0.486	0.437	0.574
Isovaleric acid	1.91±0.06	1.98±0.21	2.03±0.00	0.545	0.970	0.825
Caproic acid	0.15±0.00	0.21±0.01	0.16±0.00	0.434	0.003	0.008
Isocaproic acid	1.62±0.01	1.69±0.02	1.73±0.00	0.001	0.419	0.002
BCFA	3.13±0.20	3.49±0.28	3.39±0.11	0.408	0.397	0.492
Total SCFA	48.43±8.03	73.85±5.91	74.95±5.73	0.015	0.161	0.025

[1] The mean (x) and standard error (Sx) values of 5 replicates in each group; [2] C: Control basal ratio, P1: 0.1 g/kg probiotic added to basal ratio and P2: 0.3 g/kg probiotic added to basal ratio; [3] Polynomial contrasts: L = linear and Q = quadratic effect of supplemental probiotic

Discussion

Probiotics modulate the intestinal microbiota by reducing the number of pathogenic microorganisms, increasing the number of beneficial bacteria and nutrient absorption [17]. With the use of *Lactobacillus acidophilus* and *Bacillus subtilis* in laying hens, there is an improvement in performance, an increase in antibody production, and a decrease in blood triglyceride and cholesterol levels [18]. The addition of *Lactobacillus plantarum* in broiler chickens caused an increase in body weight, feed consumption and feed conversion rate, a decrease in the number of fecal coliforms and an increase in the number of fecal *Lactobacillus* [19]. In our study, it was observed that the use of probiotics in quails did not affect performance parameters. However, high doses are affected more than low doses, numerically. There have been many studies that are compatible with our study [20]. In the study of Huang et al.[21] they reported that the use of *Bacillus subtilis* did not affect growth performance. Another study reported that while the LWG value increased in the use of probiotics in quail diets, the FI value was not affected [18]. On the other hand, in the study of Hosseini et al.[22], they reported that the use of probiotics positively affected performance. In a study using *Lactobacillus* strains at doses of 50, 100, 150, and 200 g/ton in quail diets, the results showed that probiotic supplementation improved LWG values [23]. Jazi et al.[24], reported that feeding quails with *Bacillus subtilis* improved growth performance. In another study that used probiotics in quail with doses of 100, 150, 200 and 250 mg/kg, improved growth performance and increased feed efficiency [25]. The difference in among the results obtained

in these studies can be explained by the dose, quality, and chemical composition of the probiotic and the difference in maintenance and feeding conditions.

Probiotics have their own antioxidant enzymatic systems [26]. The culture supernatant, intact cells, and intracellular cell-free extracts of *Bifidobacterium animalis* were found to clear up hydroxyl radicals and superoxide anion as *in vitro* by improving the antioxidase activities [27]. In our study, MDA, GSH SOD, CAT and GPx values in blood plasma were linearly affected by the addition of probiotic in quail rations. Ceruloplasmin, albumin, total protein and globulin values in blood plasma among groups were not affected by the addition of probiotic in quail diets. There have been many studies that are compatible with our study [24]. In a study in pigs, the addition of *Lactobacillus fermentum* increased serum SOD, GPx and hepatic CAT and muscle SOD [28]. In another study, the use of probiotics at different doses increased in GPx activity in chicks [29]. Abdel-Moneim et al.[18] reported that serum total protein, albumin GSH, and CAT increased while MDA decreased in due to probiotics added to Japanese quail rations. In addition, probiotics were observed to promote antioxidant enzymatic activities (eg, SOD, GPx and total antioxidant status of host [30]. On the other hand, Jazi et al.[24] reported that SOD and GSH-Px activities, and MDA content in breast muscle, were not affected by the addition of *Bacilius subtilis* in quail diets. These results are consistent with our findings except for MDA. The obtained results may be influenced by dose and content differences of the probiotics used in the study and environmental conditions such as housing.

Probiotics, that can colonize the intestinal tract, have been shown to have effects on metabolic diseases such as obesity and diabetes by modulating the intestinal micro-organisms [31]. SCFA is formed as a result of bacterial fermentation in the cecum which is needded for metabolism of the intestinal epithelial cells. They stimulate cell growth and differentiation in the intestine, improving intestinal integrity, as well as reducing the digestive tract pH and preventing the growth of pathogenic micro-organisms [32]. There are a limited number of studies that have focused on the use of the probiotic on the examination of the parameters related to the concentration of SCFA in the cecum, while there is no study investigating this parameter in quail. In our study, asetic acid, isocaproic acid and SCFA were linearly affected while caproic acid were quadratically affected by the addition of probiotic in quail rations. There were no significant differences in acetic acid, propionic acid, butyric acid, isobutyric acid, valeric acid, isovaleric acid and BCFA concentrations for quails fed different amounts of probiotic. Morever, there are many studies that are compatible with our study. Researchers have reported a correlation between the secum microflora composition and the SCFA concentration [7]. Increased SCFA concentrations have been shown to have beneficial effects on energy, metabolism, microflora and immune responses [33]. Weng et al.[34] reported that in the study of correlation of variables on metabolic and microbiota with diet in case of intestinal inflammation, the value of isocaproic acid decreased. Decrease in isocaproic acid value shows that acidity in the intestine is impaired and pathogenic microorganisms are active in inflammation. In the light of these studies, the increase in secum short chain fatty acids can be interpreted as having a positive effect on gut health. In a study in pigs revealed that the usage of lactulose in the diet increased the SCFA concentration in the large intestine [35]. On the other hand, cecal fermentation was influenced by the use of probiotic in broiler diets in a study. Morever, the addition of *Bacillus licheniformis* was decreased in cecal concentrations of propionic, butyric, n-butyric and n-valeric acids [36]. The difference in among the results obtained can be explained with the dose, quality, and chemical composition of the probiotic and maintenance and feeding conditions.

In conclusion, increased dietary addition of probiotics did not affect performance. MDA, GSH, SOD, CAT and GPx exhibited a linear response. However, ceruloplasmin, albumin, total protein and globulin were not affected by the addition of probiotic. Significant linear responses in asetic acid, isocaproic acid and SCFA were observed with the gradued level of probiotic. However, a significant quadratic response in the caproic acid was observed.

Therefore, the effective dose for caproic acid was 0.1 g/kg. There were no significant differences in acetic acid, propionic acid, butyric acid, isobutyric acid, valeric acid, isovaleric acid and BCFA concentrations for quails fed with different levels of probiotic. Therefore, blood antioxidant capacity and cecal SCFA results; it has been effective in protecting quail oxidative stress and improving intestinal health.

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Author Contributions

ÖDA conceived and supervised the study. GY, ÖDA and OM collected and analyzed data. ÖDA wrote the first draft of manuscript. All authors contributed to the critical revision of the manuscript and have read and approved the final version.

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RESEARCH ARTICLE

Prediction of Immunoglobulin G in Lambs with Artificial Intelligence Methods

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Abstract

The health, mortality and morbidity rates of neonatal ruminants depend on colostrum quality and the amount of Immunoglobulin G (IgG) absorbed. Computer-aided estimates are important as measuring IgG concentration with conventional methods is costly. In this study, artificial neural network (ANN), multivariate adaptive regression splines (MARS), support vector regression (SVR) and fuzzy neural network (FNN) models were used to predict the serum IgG concentration from gamma-glutamyl transferase (GGT) enzyme activity, total protein (TP) concentration and albumin (ALB). The correlation between parameters was examined. IgG positively correlated with GGT and TP and negatively correlated with ALB ($R = 0.75$, $P<0.001$; $R = 0.67$, $P<0.001$; $R = -0.17$, $P<0.01$, respectively). IgG, GGT, and TP cut-off values were determined for mortality, healthy, and morbidity in neonatal lambs by decision tree method. IgG ≤113 mg/dL ($P<0.001$), GGT ≤191 mg/dL ($P=0.001$), and TP ≤45 g/L ($P<0.001$) were determined for mortality. IgG >575 mg/dL ($P=0.02$), GGT >191 mg/dL ($P<0.001$), and TP >55 g/L ($P<0.001$) were determined for healthy. It has been observed that the FNN is the most successful method for the prediction of IgG value with a correlation coefficient (R) of 0.98, root mean square error (RMSE) of 234.4, and mean absolute error (MAE) of 175.8.

Keywords: *Artificial neural network, Decision tree, Fuzzy neural network, Immunoglobulin G, Multivariate adaptive regression splines, Support vector regression*

Yapay Zeka Yöntemleri İle Kuzularda İmmünoglobulin G Tahmini

Öz

Yenidoğan ruminantların sağlığı, ölüm ve hastalık oranları, kolostrum kalitesine ve emilen Immunoglobulin G (IgG) miktarına bağlıdır. Konvansiyonel yöntemlerle IgG konsantrasyonunun ölçülmesi maliyetli olduğundan, bilgisayar destekli tahminler önemlidir. Bu çalışmada, gama-glutamil transferaz (GGT) enzim aktivitesi, toplam protein (TP) ve albümin (ALB) değerlerinden serum IgG konsantrasyonunu tahmin etmek için yapay sinir ağı (YSA), çok değişkenli uyarlanabilir regresyon eğrileri (MARS), destek vektör regresyonu (SVR) ve bulanık sinir ağı (FNN) modelleri kullanılmıştır. Parametreler arasındaki korelasyon incelenmiş ve serum IgG konsantrasyonunun, GGT ve TP ile pozitif, ALB ile negatif korelasyonlu olduğu görülmüştür (sırasıyla $R = 0.75$, $P<0.001$; $R = 0.67$, $P<0.001$; $R = -0.17$, $P<0.01$). Yenidoğan kuzularda ölüm, sağlıklılık ve hastalık için eşik değerler karar ağacı yöntemiyle belirlenmiştir. Ölümler için IgG ≤113 mg/dL ($P<0.001$), GGT ≤191 mg/dL ($P=0.001$) ve TP ≤45 g/L ($P<0.001$) olarak belirlenirken sağlıklılık için IgG >575 mg/dL ($P=0.02$), GGT >191 mg/dL ($P<0.001$) ve TP >55 g/L ($P<0.001$) olarak belirlenmiştir 0.98 korelasyon katsayısı (R), 234.4 hata kareler ortalamasının karekökü (RMSE) ve 175.8 ortalama mutlak hata (MAE) ile IgG değerini tahmin etmede en başarılı yöntemin FNN olduğu görülmüştür.

Anahtar sözcükler: *Bulanık sinir ağı, Çok değişkenli uyarlanabilir regresyon eğrileri, Destek vektör regresyonu, Immunoglobulin G, Karar ağacı, Yapay sinir ağı*

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Introduction

Ruminants are born agammaglobulinemic due to the placental structure. Because of this placental structure, it largely inhibits the transfer of immunoglobulins (Ig) to the fetus. Failure of passive transfer (FPT) develops when neonatal ruminants do not receive enough and sufficient quality Ig. Ruminants with FPT cannot be protected from infectious diseases, thus getting the disease more and the mortality rate increase [1,2].

The first 28 days of life, which is referred to as the neonatal period, are vital for ruminants. FPT is one of the most important factors affecting the health of ruminants in the neonatal period. FPT causes enteritis and septicemia, especially in the neonatal period, leading to death and mortality. Mortality of ruminants in the neonatal period leads to losses for both the enterprise and the economy [3-6].

Colostrum has high energy, protein and vitamin content and contains a high proportion of immunoglobulin G (IgG) [7]. Colostrum quality is directly proportional to the amount of IgG it contains [8]. Ruminants with high IgG concentrations are reported to have higher survival rates than ruminants with low IgG levels in colostrum [9,10]. IgG levels can be measured by direct radial immunodiffusion (SRID) [11] and Enzyme-linked immunosorbent assay (ELISA) [12] methods. These methods are difficult, expensive, time-consuming and incapable of testing of a large number of samples at once [2,8]. The fast, cheap and reliable prediction of the IgG concentration is required [1,12,13]. Therefore, in the present study, IgG value was predicted by different AI models. A regression is a type of supervised learning problem and it refers to the prediction of a continuous dependent variable from one or more independent variables [14,15].

Recently, regression methods have been used for a wide range of applications in civil engineering [16], environmental science [17], veterinary [18], agriculture [16], and medicine [16]. However, it is known that artificial intelligence (AI) regression models are not used sufficiently in the veterinary field [18,19].

The purposes of this study were (1) to examine the correlation between parameters: Immunoglobulin G (IgG), gamma-glutamyl transferase (GGT), total protein (TP), and albumin (ALB) in neonatal lamb, (2) to determine the most successful/appropriate AI model(s) (artificial neural network, ANN; multivariate adaptive regression splines, MARS; support vector regression, SVR; and fuzzy neural network, FNN) that predict(s) IgG (mg/dL) levels from GGT, TP and ALB values in blood, (3) to determine the cut-off point that is important for the risk of disease and death in lambs for GGT, TP, and ALB.

Material and Methods

Animals

In this study, using the hematological-immunological data (IgG, GGT, TP and ALB) obtained from the TOVAG 108O847 project [1], which was carried out with the approval numbered KAÜ-HADYEK-2008-23, serum IgG concentration predicted by artificial intelligence regression methods (ANN, MARS, SVR and FNN). In addition, cut-off values for IgG, GGT, TP and ALB were determined with the machine learning decision tree method. Briefly, 347 Akkaraman lambs on two neighboring farms with similar management practices were included in the study. The lambs were weighed before taking colostrum. After this procedure, the lambs were allowed to suck their mothers naturally for one week. During this time the lambs were not fed with supplemental colostrum. After this period, the lambs were transferred to a separate pen and their mothers were allowed to feed twice a day for three months.

Blood Sample Collections

The blood samples were taken from Akkaraman crossbred lambs (n=347). The blood samples were taken 24±1 h after birth, centrifuged at 3000 rpm for 5 min and the serum samples were stored at -20°C till analysis.

IgG and Other Test Assay

Serum IgG concentrations were measured using the ELISA kit (Bio-X Competitive ELISA Kit for Ovine blood serum IgG Assay-BIO K350, Bio-X Diagnostics, Belgium). Gammaglutamyltransferase (GGT), total protein (TP) and albumin (ALB) analyses were done using commercial spectrophotometric kit (TML, Tani Medical, Turkey). These tests were performed and the results were calculated according to the manufacturer's instruction manual.

Statistical Analysis

The prediction methodology of IgG (mg/dL) values is as follows: After the pre-processing step was applied (e.g. sigmoid normalization [20] to the dataset, 80% of the dataset was used for model training and 20% was used to test the model performance. K-fold cross validation technique was used in the model training phase in which the dataset is randomly divided into k equal size of subsamples. One of the subsample is used as the validation data for testing the model and the remaining k-1 subsamples are used as training data. This cross-validation process repeats k-times (fold). ANN, MARS, SVR and FNN models were used for the prediction of IgG values. After the IgG value was predicted by regression models, the model prediction accuracy was measured using the test dataset. The correlation coefficient (R), root mean square error (RMSE), and mean absolute error (MAE) statistical criteria were used to compare the prediction performance of the regression models.

Artificial neural networks (ANN) can be defined as a system designed to model the way the brain performs a function. ANN consists of several ways of connecting artificial nerve with one another and is usually arranged in layers. The input layer includes neurons that receive raw data from

outside. Only the input values are transmitted to the next layer without any processing. The output layer is the layer containing the neurons that transmit the outputs. The input and output layers include a single layer. The hidden layer contains hidden nodes. Hidden nodes have no direct connections to the outside world. They perform calculations and transfer information from input nodes to output nodes. The hidden layer may comprise one or more layers. Activation functions are important for ANN to learn [21]. The parameters of the developed ANN model in the study are as follows: 3 input layer (GGT, TP, ALB), 2 hidden layers and 1 output layer (IgG) was used. Logistic function was used for the activation function, backpropagation algorithm was used for training of neural networks, number of epochs was 1000 and learning rate was 0.01.

Multivariate Adaptive Regression Splines (MARS) is a version of the iterative separation method and stepwise linear regression. MARS makes no assumptions about the functional relationship between dependent and independent variables. Instead, it creates relationships between different input variables based on the basic functions and coefficient. MARS establishes a flexible regression model using basic functions corresponding to different ranges of independent variables. In other words, the basic idea is based on the method of divide-and-conquer strategy [22].

Support Vector Machine (SVM) is a kernel-based method used for classification and regression problems. Regression with support vector machines is called support vector regression (SVR). In the case of linear separation of data, the aim is to find the best hyperplane that maximizes the margin. In the case of non-linear separation of data, mapping is performed to the higher space from which the data can be parsed linearly with the help of dataset [23]. The parameters of the developed SVR model in the study are as follows: Polynomial kernel function was used with gamma=0.001 and degree=3. Cost of constraints violation was 1.

Fuzzy Neural Network (FNN) or neuro-fuzzy systems based on combining the ability of artificial neural networks (ANNs) to learn and find the most appropriate with the advantages of fuzzy logic to make decisions like a human and provide expert knowledge [24]. In FNN, ANN combines with fuzzy rule based systems (FRBS). FRBS are based on the fuzzy set theory and proposed by Zadeh [25]. FRBS are also known as fuzzy inference systems (FIS) and fuzzy models. FRBS are used for classification and regression problems. Generally, fuzzy rule based system (FRBS) architecture consists of fuzzification, knowledge base, inference, and defuzzification steps [26,27]. At the fuzzification step, input variables (crispy values) are converted to linguistic term (fuzzy sets) using membership functions. Knowledge base is composed rule base and database components. Rule base contains fuzzy If-Then rules and database includes the fuzzy sets. At the inference engine step, the fuzzy output is generated from the fuzzy inputs using by inference model (Mamdani, Takagi-Sugeno-Kang). At the defuzzification step, the fuzzy output of the inference engine is mapped into a numerical output [28,29]. In this study, method type was selected Adaptive-network-based fuzzy inference system. Takagi Sugeno Kang type fuzzy model used for linguistic rules. Max iteration was 5, step size was 0.01 and ZADEH type of implication function was used for a value representing.

In order to evaluate the prediction performance of the methods, it should be examined how much the actual value and the estimated value match. In the present study, R, RMSE and MAE statistical criteria were used to evaluate model prediction accuracy. As a result of testing the prediction methods, it is desirable that the R-value is high and the RMSE and MAE value is low. R (Eq. 1.), RMSE (Eq. 2.) and MAE (Eq. 3.) were determined as follows:

$$R = \frac{\sum_{i=1}^{n}(a_i-\bar{a})(p_i-\bar{p})}{\sqrt{\sum_{i=1}^{n}(a_i-\bar{a})^2 \sum_{i=1}^{n}(p_i-\bar{p})^2}} \quad \text{Eq. 1.}$$

$$RMSE = \sqrt{\frac{1}{n}\sum_{i=1}^{n}(a_i - p_i)^2} \quad \text{Eq. 2.}$$

$$MAE = \frac{1}{n}\sum_{i=1}^{n}|a_i - p_i| \quad \text{Eq. 3.}$$

Where n is the number of data, α is the actual value, p is the predicted value, $\bar{\alpha}$ is mean of actual values and $\bar{p}$ is the mean of the predicted values. In the study, the R programming language was used for statistical computations and AI models development.

Results

Correlation between the serum IgG concentration with GGT, TP and ALB are illustrated in *Fig. 1*. Parameters' distribution is shown in the diagonal; correlation coefficient and significance levels (P<0.05 *, P<0.01 **, P<0.001 ***) are in the upper triangle; bivariate relationships are shown in the lower triangle of *Fig. 1*. IgG was significantly and positively associated with GGT (R=0.75, P<0.001) and TP (R=0.67, P<0.001). There was an inversely and low correlation between ALB and IgG (R=-0.17, P<0.01). Also, when the distributions of the features in the dataset were examined, it was seen that variables were not normally distributed.

In this study, it was aimed to define a cut-off point for serum IgG concentration, serum TP concentration, and serum GGT activity associated with the risk of death in lambs. The decision tree method was used to determine cut-off levels. Decision tree is a machine learning method used for classification and regression. The decision tree splits the input variables (IgG, GGT, TP) from the cut points where the target variable can be grouped most homogeneously

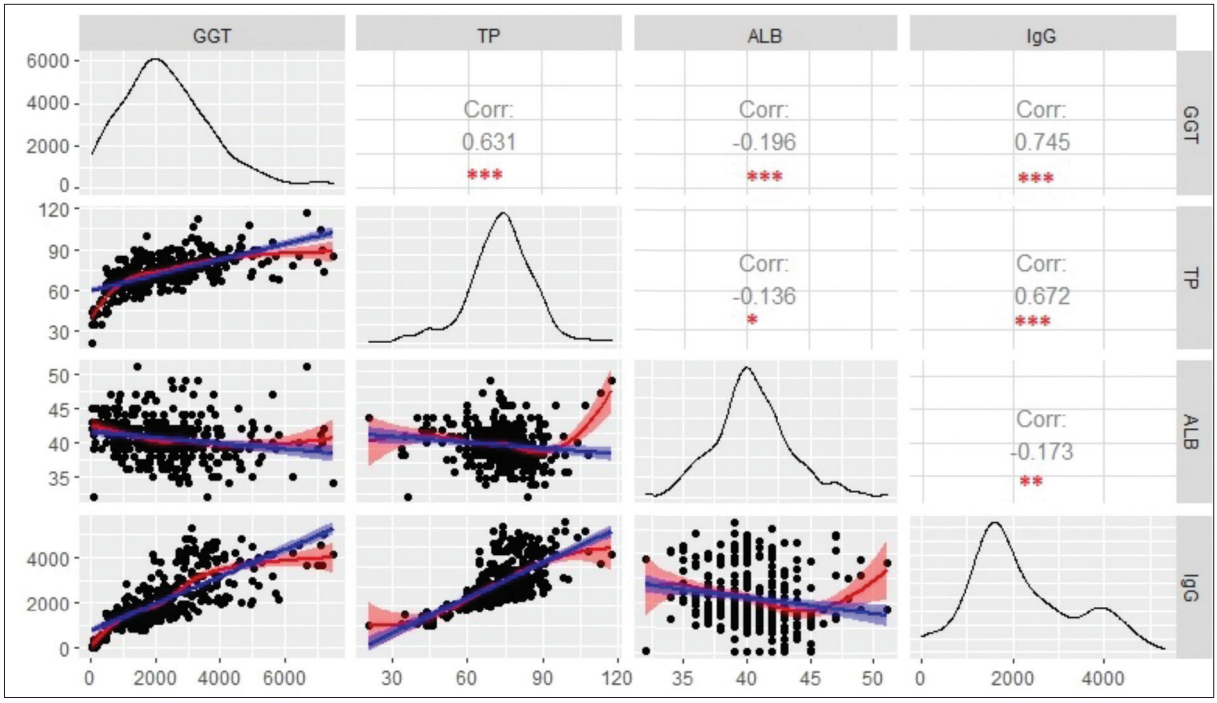

Fig 1. Correlation matrix of parameters, *** = *P<0.001*, ** = *P<0.01*, * = *P<0.05*

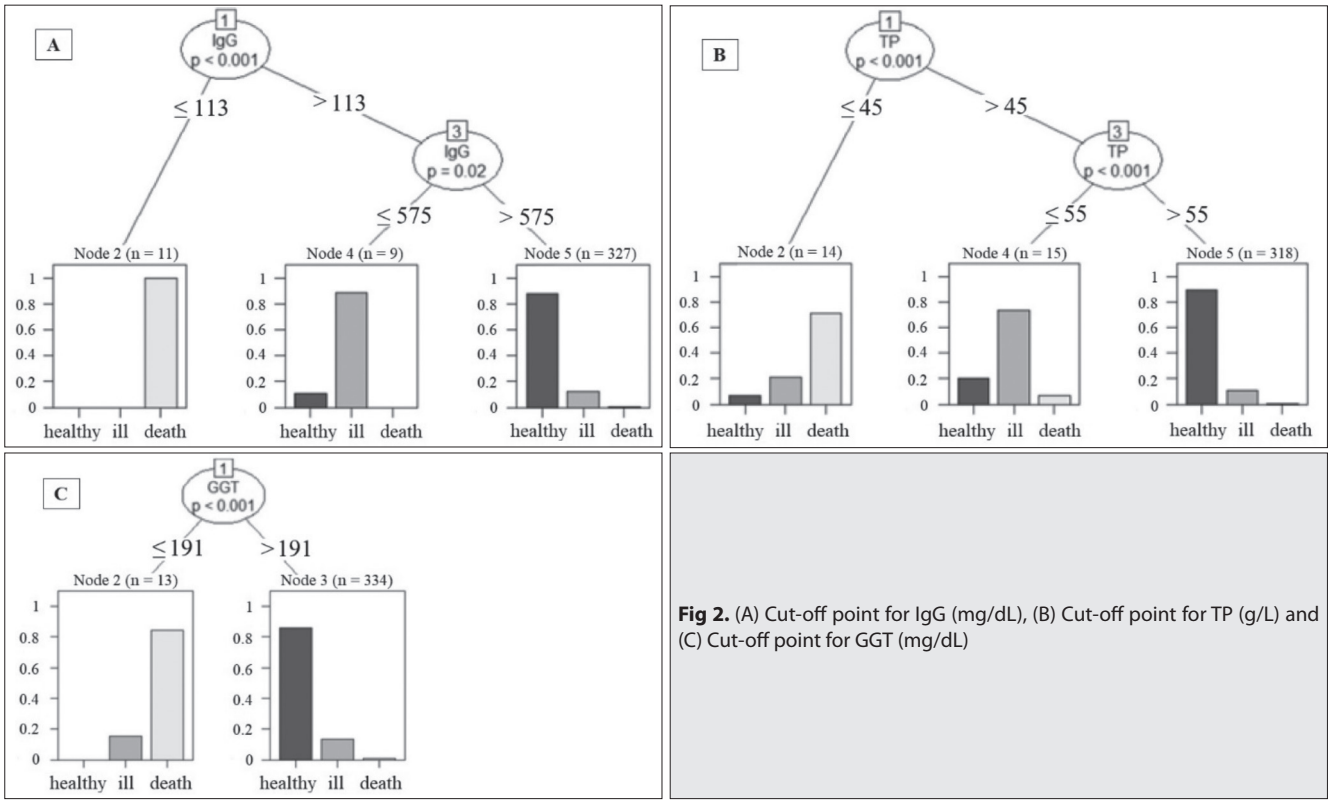

Fig 2. (A) Cut-off point for IgG (mg/dL), (B) Cut-off point for TP (g/L) and (C) Cut-off point for GGT (mg/dL)

(healthy, ill, death). The results are presented in *Fig. 2*. It was also tried to determine the threshold value for ALB, but it was not possible to find an exact range for ALB.

The IgG threshold value for lambs in the neonatal period was determined as ≤113 by the decision tree method *(Fig. 2-A)*. The mortality rate of lambs with IgG value ≤113 was 100% (11/11) (P<0.001). Lambs with IgG values were in the range of 113-575 with the morbidity rate of 89% (8/9). For the IgG value >575, the healthy, morbidity and mortality rates in lambs were 87.4% (286/327), 12% (39/327), and 0.6% (2/327) (P=0.02), respectively.

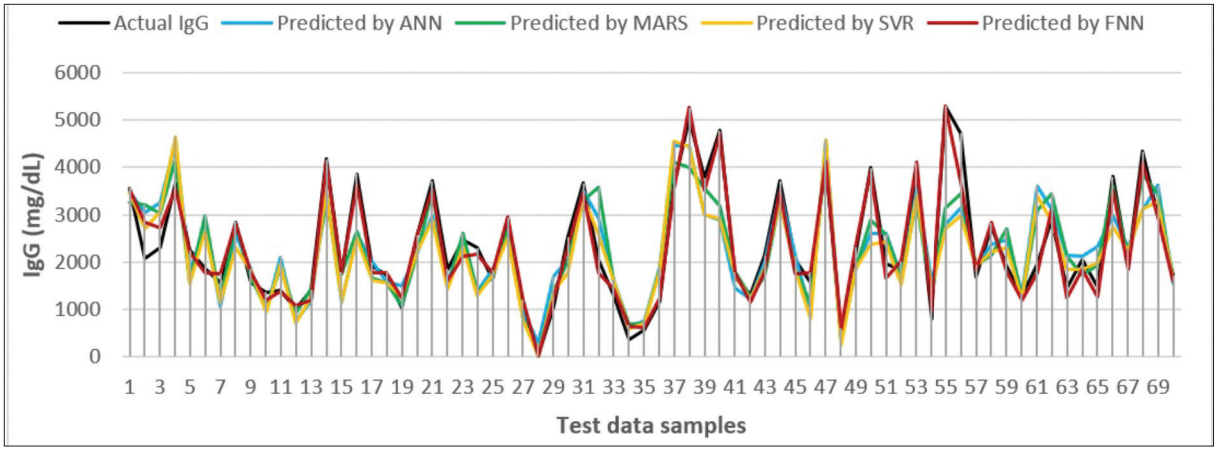

Fig 3. Comparisons of the actual IgG (mg/dL) and predicted IgG (mg/dL) with the ANN, MARS, SVR, and FNN models

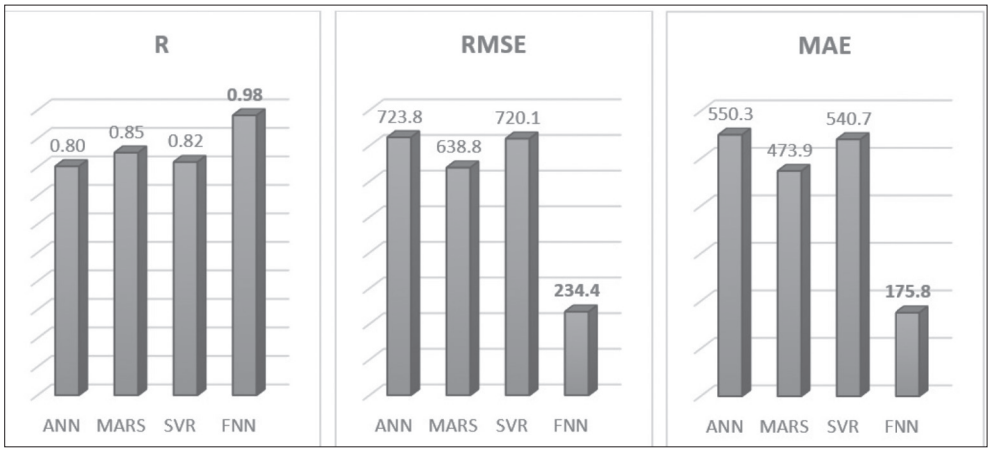

Fig 4. Prediction results of models

In neonatal lamb mortality, the threshold value for TP≤45 *(Fig. 2-B)*. For the TP value ≤45, the mortality rate was 71% (10/14) (P<0.001). The morbidity rate was 73% (11/15) when TP was in the range of 45-55 (P<0.001). In the case of TP >55, the healthy, morbidity, and mortality rates in lambs were 89% (283/318), 10.4% (33/318) and 0.6% (2/318) (P<0.001), respectively.

The GGT threshold value ≤191 was determined for the lamb's mortality *(Fig. 2-C)*. The rate of mortality was 85% (11/13), when the GGT value ≤191 (P<0.001). For the GGT value >191, the healthy, morbidity and mortality rates in lambs were 86% (287/334), 13.4% (45/334), and the 0.6% (2/334) (P<0.001), respectively.

ANN, MARS, SVR and FNN models were used to estimate IgG output value from GGT, TP and ALB input values. After the completion of the training process, the predictive performances of the models were tested with test set. Randomly selected 277 samples were used for regression models training and remaining unseen 70 samples were used to predict IgG value. For these unseen 70 samples, IgG values were estimated and compared with the actual IgG values and R, RMSE and MAE metrics were calculated. Actual IgG values and predicted IgG values by regression models are given in *Fig. 3*. The prediction performance of AI models was presented in *Fig. 4*.

When comparing the prediction accuracy of the models, the highest R-value and the smallest RMSE and MAE values are desirable. Examining *Fig. 4*, the FNN model shows superior performance than other models (R=0.98). According to R-value, the FNN model is followed by MARS (R=0.85), SVR (R=0.82) and ANN (R=0.80) respectively. Likewise, according to the statistical results of R, RMSE and MAE, the FNN model produced more successful results than other models. These statistical results indicate that the FNN model is the best for predicts the IgG (mg/dL) concentrations.

Discussion

Because of the lambs' placental structure, they are born hypogammaglobulinemic. Hence, ingestion and absorption of maternal antibodies in colostrum is necessary for providing humoral immunity in the neonatal period. This

process is denominated passive transfer and is determined by measuring serum IgG concentrations.

Numerous studies in the last thirty years have associated neonatal diseases with insufficient serum IgG, in other words with FPT in animals, thus demonstrating the importance of IgG in preventing infections and increasing growth performance in neonatal animals [3,4,30-34]. It is known that if the lambs received sufficient volume and quality of colostrum in the first 12 h of life, the adequate passive immune transfer is provided [30,35-38]. Otherwise, a secondary immunodeficiency develops, called Passive Transfer Failure (FPT).

SRID and ELISA tests are used for a measure of serum IgG concentration by directly. But these kits are costly, time-consuming and incapable of testing of a large number of samples at once. Due to these disadvantages, it is urgent and important to predict accurately the IgG value by indirectly with alternative methods. In addition to this method, the SRID or ELISA can be used as a confirmatory diagnosis. GGT and TP are highly correlated with serum IgG concentration and are used to estimate IgG concentration. These indirect methods are fast, practical, inexpensive and easier to apply in the field. Therefore, indirect tests such as GGT and TP can be used for estimation of IgG concentration and direct tests are used only as confirmatory methods. In this study, serum GGT, TP and ALB activities were used as input variables to predict the IgG output variable using ANN, MARS, SVR and FNN model. To the best of our knowledge, there is no such study in the literature that examines and compare regression models for predicting the IgG (mg/dL) value.

There is no global accepted optimal IgG threshold by the veterinary community for FPT in lambs. And it's known that limited studies in which passive transfer deficiency is indicated by the cut-off point obtained by indirect methods in lambs [30,35,39]. In this study, a cut-off point was also defined for passive transfer deficiency in lambs using decision tree algorithm.

It is known the passive immunity develops when the threshold value of IgG <1000 when SRID used, whereas the threshold value of IgG is <500 when ELISA used [35]. In the present study, the cut-off level for serum IgG concentration was determined as ≤113 mg/dL (P<0.001) for mortality in neonatal lambs (rate is 100%). IgG >575 mg/dL (P=0.02) for healthy (rate is 87.4%). IgG values 113-575 mg/dL range (P=0.02) were determined for morbidity (rate is 89%).

GGT enzyme is produced from ductile cells in mammary glands and is present in high concentration in colostrum. It has been reported that serum and plasma GGT activity may be useful in the evaluation of passive transfer status in ruminants. GGT enzyme activities significantly correlated with colostrum IgG concentrations and it is known that it can be used to determine colostrum quality [39,40]. The results of this study indicated a significant linear correlation between GGT and IgG (R=0.75, P<0.001). GGT≤191 mg/dL (P<0.001) were determined for mortality (rate is 85%). GGT>191 mg/dL (P<0.001) were determined for healthy (rate is 86%).

The measurement of TP is the appropriate method for indirect assessment of immune status because of significant correlation [41]. Although the relationship between TP and neonatal diseases in calves is frequently studied, the relationship between TP and neonatal diseases in lambs is not known and there is no STPC threshold used for disease risk [30,38,41,42]. In the present study, a significant linear association was detected between TP and IgG (R=0.67, P<0.001). The cut-off level with TP≤45 g/L (P<0.001) were determined for mortality (rate is 71%). TP>55 g/L (P<0.001) were determined for healthy (rate is 89%). TP values 45-55 g/L range (P<0.001) were determined for morbidity (rate is 73%).

Albumin, which is synthesized in the liver and constitutes 50% of plasma proteins, is reported to be the highest stored and largest amino acid carrier. A negative and low correlation was observed between ALB an IgG concentration (R=-0.17, P<0.01). In this study, the specific cut-off point for ALB could not be determined.

This study presents the following two novel contributions. First, the prediction of serum IgG concentration from blood samples (GGT, TP, and ALB) is possible using artificial intelligence methods. IgG concentration is measured by commercial kits to give an idea about the health status of ruminants. Measurement of IgG concentration with these kits is difficult, expensive, time-consuming and incapable of testing of a large number of samples at once. Because of these disadvantages of the kits, it is important to predict the IgG concentration indirectly using alternative methods. Second, it is also possible to determine the cut-off level for healthy, mortality, and morbidity for neonatal lambs. In this study, unlike other studies, cut-off values were determined using the decision tree method.

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Author Contributions

P. CİHAN, performed the proposed models, processed and analyzed the data, P. CİHAN and E. GÖKÇE, writing-original draft preparation, E. GÖKÇE, O. ATAKİŞİ, A.H. KIRMIZIGÜL and H.M. ERDOĞAN, collected hematological-immunological data. All authors discussed the results and contributed to the final manuscript.

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RESEARCH ARTICLE

Four Temperate Bacteriophages from Methicillin-resistant *Staphylococcus aureus* Show Broad Bactericidal and Biofilm Removal Activities [1]

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Abstract

The emergence of multi-drug resistance among many bacteria including zoonotic pathogens in the food chain poses a growing public health threat to humans, animals, and the environment worldwide. The inefficiency of current antibiotics to control these pathogens necessitated the development of alternative approaches, such as phage therapy, for the prevention and treatment of human and animal infections, food safety, and wastewater treatment. In this study, four temperate bacteriophages, designated as Trsa205, Trsa207, Trsa220, and Trsa222 were isolated by mitomycin C induction from methicillin-resistant *Staphylococcus aureus* (MRSA) strains. The phages were characterized based on their electron microscope morphology, burst size, host range, and biofilm removal potential. Based on their morphology, all four phages with isometric heads and long non-contractile tails belong to *Siphoviridae* family. The one-step growth curves of phages revealed that Trsa205 and Trsa207 have latent periods of about 20 min that results in a burst size of 30 and 45 virions/host cell, respectively, while Trsa220 and Trsa222 showed 25 min of latent period and produced 20 virus particles/cell. The agar-spot assay was used for phage host range determination, and biofilm removal activities were measured spectrophotometrically after crystal violet staining. It was found that at least two-thirds of 56 *S. aureus* strains (66%) could be lysed by phages when used in combination, and 20-38% by one of the phages. The four phages in combination were able to remove the *S. aureus* biofilms by 65%. Our results indicated that the newly identified bacteriophages have the potential to be used in phage therapy against multi-drug resistant *S. aureus* including MRSA and removal of biofilms.

Keywords: *Bacteriophage, Siphoviridae, Staphylococcus, Biofilm, Host range*

Geniş Bakterisidal ve Biyofilm Giderme Etkisi Gösteren Metisiline Dirençli *Staphylococcus aureus* Kaynaklı Dört Yeni Ilıman Bakteriyofajın Tanımlanması

Öz

Çoklu antibiyotik direncinin besin zincirindeki zoonotik patojenler dahil tüm dünyada birçok bakteride ortaya çıkması insanlar, hayvanlar ve çevre için artan bir halk sağlığı tehdidi oluşturmaktadır. Bu patojenleri kontrol etmek için mevcut antibiyotiklerin yetersizliği, insan ve hayvan enfeksiyonlarının tedavisi, gıda güvenliği ve atık su arıtımı için faj terapisi gibi alternatif yaklaşımların geliştirilmesini gerektirmiştir. Bu çalışmada, metisiline dirençli *Staphylococcus aureus* (MRSA) suşlarından mitomisin C indüksiyonu ile Trsa205, Trsa207, Trsa220 ve Trsa222 olarak adlandırılan dört ılıman bakteriyofaj izole edilerek tanımlanmıştır. Fajlar, elektron mikroskop morfolojisi, konak hücre başına oluşan faj sayısı, konak genişliği ve biyofilm giderme potansiyellerine göre karakterize edildi. Morfolojilerine göre, izometrik başlı ve uzun kasılmayan kuyruklu dört fajın *Siphoviridae* ailesine ait oldukları belirlendi. Fajların tek aşamalı büyüme eğrilerine göre, Trsa205 ve Trsa207'nin 20 dakikalık latent periyotlara sahip olduğu ve sırasıyla hücre başına 30 ve 45 faj partikülü oluşturduğu, Trsa220 ve Trsa222'nin ise 25 dakikalık latent periyotu takiben hücre başına 20 virüs partikülü oluşturduğu saptandı. Faj konak genişliği tayini için agar-damlatma yöntemi kullanıldı ve kristal viyole boyamadan sonra biyofilm giderme aktiviteleri spektrofotometrik olarak ölçüldü. Fajların tek başlarına kullanıldıklarında 56 *S. aureus* suşunun %20-38'unu, dört faj birlikte kullanıldığında ise tüm suşların üçte ikisini (%66) enfekte ederek lize ettiği saptandı. Dört fajın kombine kullanıldığında *S. aureus* biyofilmini %65 oranında giderebildiği gösterildi. Sonuçlarımız, yeni tanımlanan bakteriyofajların, MRSA dahil çoklu ilaca dirençli *S. aureus* suşlarına karşı ve biyofilm giderme amacıyla faj tedavisinde kullanılma potansiyeline sahip olduğunu göstermiştir.

Anahtar sözcükler: *Bakteriyofaj, Siphoviridae, Staphylococcus, Biyofilm, Konak spektrumu*

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Introduction

Antimicrobial resistance (AR) in bacteria is a growing problem worldwide today and will remain a major threat to humans and animals as well as sustainable economic growth. In a recent report by the World Health Organization (WHO), 12 bacterial species were listed as global priority pathogens (GPP) including *Staphylococcus aureus,* emphasizing the immediate need for new antimicrobial alternatives [1]. This report discusses the current antibacterial therapy options, such as phage and phage endolysin therapy against infections caused by methicillin-resistant *S. aureus* (MRSA). *Staphylococcus* species are one of the most common bacteria of healthcare-associated infections and mainly responsible for infections related to catheters, implants, and medical devices [2]. Many of the healthcare-associated infections, particularly those that are caused by *S. aureus* including MRSA, are mainly transmitted from person to person through contaminated medical devices or via direct contact with colonized healthcare workers or patients. *S. aureus* as a member of ESKAPE *(Enterococcus, S. aureus, Klebsiella pneumonia, Acinetobacter baumannii, Pseudomonas aeruginosa*, and *Escherichia coli)* organisms result in prolonged hospital stay, hospital-associated mortality, and substantial economic burden [3]. Contamination and infection of *S. aureus* is also a major problem in the agri-food sector, particularly bovine mastitis, which adversely affects animal health and the quality of the milk [4].

Staphylococcal strains participating in the biofilm structure exhibit more resistance to conventional antibacterial agents than their free-living counterparts [2]. Due to the intensive use and the emergence of antibiotic resistance globally, phage therapy has become one of the promising alternatives to treat these persistent infections [5]. The phage therapy has been performed by simply adding naturally existing bacteriophages at the infection site to kill the pathogenic bacteria. Moreover, the recent advancement of biotechnology expanded the potential applications of phage therapy and phage derived lytic proteins, such as endolysins [6]. The use of the lytic phages in the reduction of pathogenic bacteria and their biofilms either alone or in combination have been reviewed elsewhere [7,8]. However, compared to the estimated global phage population size, which is the most numerous and diverse viruses on the planet, the reported phages are very limited in numbers [9]. Phages can readily be isolated from any environmental samples that can support bacterial growth such as aquatic habitats [9], soil [10], wastewater [11] or induction of phages from lysogenized host strains [12]. Therefore, there is still potential for novel phages to be explored. The induction of prophages from various bacterial strains including *S. aureus* by treatment with mitomycin C has previously been described [13,14]. In this study, we aimed to isolate and characterize bacteriophages of lysogenic origin infecting *S. aureus* with broad host range and biofilm removal potential.

Material and Methods

Bacterial Strains, Media and Culture Conditions

A total of 56 *S. aureus* including methicillin-resistant strains isolated previously from various samples and maintained at our culture collection were included in this study. Tryptic Soy Broth (TSB) and Tryptic Soy Agar (TSA) (Lab M. UK) were used for culturing of staphylococci at 37°C if indicated otherwise.

Induction and Purification of Phages

Prophages were isolated by mitomycin C (Sigma-Aldrich, USA) induction as described elsewhere [14]. Briefly, overnight cultures of staphylococci were subcultured in fresh media containing 10 mM $CaCl_2$ and grown at OD_{600nm} = 0.5). The mid-log cultures were induced with 0.5 µg/mL mitomycin C for 4 to 6 h or until the clearance of the turbid cultures was observed. The induced cultures were centrifuged at 10.000 xg for 20 min to remove cell debris and unlysed cells. The supernatant containing the page particles filtered through a 0.2 µm filter (Sartorius Stedim Biotech GmbH, Germany) and maintained at 4°C until use. Five microliters of phage lysate were dropped onto lawns of the staphylococcal host strains. Observation of clear zone or single phage plaques on the agar surface indicated the presence of lysogenic phages. Several single phage plaques were picked from the agar plates and transferred into a microcentrifuge tube containing 100 µL SM buffer (50 mM Tris-HCl, pH 7.5; 8 mM $MgSO_4.H_2O$; 100 mM NaCl) for further enrichment using plaque assay with some modification as described elsewhere [15]. Briefly, 100 µL of phage lysate was diluted 10-fold and mixed with 100 µL of logarithmic phase *S. aureus* cultures in the presence of 1 mM $CaCl_2$ in a microcentrifuge tube and incubated for 30 min to facilitate phage attachment to their hosts. A 4 mL of soft agar (0.6%) at 49°C was added to the phage infected cells, mixed and poured over agar plates, and incubated for 12-24 h to allow phage plaques to form. The phage particles were collected from the soft agar by scraping in a centrifuge tube containing 4 mL of SM buffer. Following the centrifugation, the supernatant was filtered, treated with 1 µg/mL DNase at room temperature, and phage particles were precipitated with 10% (w/v) polyethylene glycol 8000 in 0.5 M NaCl solution at 4°C overnight [16]. The phage particles were collected by centrifugation at 10.000 x g for 15 min and resuspended in SM buffer, filtered and stored at 4°C for short term and -80°C for long term use.

Transmission Electron Microscopy

Purified and concentrated phage particles were stained with 2% uranyl acetate (pH 4.0), and examined for their morphologies under Transmission Electron Microscopy (TEM) as described elsewhere with some modifications [17].

Ten microliters of phage lysate containing 10^{10} plaque-forming unit per milliliter (PFU/mL) was added to the

formvar carbon-coated grid (Electron Microscopy Science, USA) and allowed to absorb for 4 min. The girds were then stained for 3 min by adding 10 µL of 2% uranyl acetate. Excess stain was removed and the girds were air dried for overnight. The phages were viewed at 75 kV using Jeol JEM-1010 Transmission Electron Microscope.

Host Range

The host ranges of the phages were determined by the agar- spot assay using various staphylococcal strains as previously described [17]. Briefly, 100 µL exponential phase cultures were mixed with 4 mL of 0.6% soft agar at 49°C and poured on TSA plates. After the agar solidified for about 10 min, 5 µL from each phage lysate was spotted on the agar surface and incubated for 12-24 h. The clear or turbid phage plaques were examined visually or under a stereomicroscope.

One-step Growth Curve

The one-step growth curve of each phage was determined as described by Li and Zhang [4] with slight modification. The phage lysate and early-mid culture of S. aureus TRSA 201 were mixed at a multiplicity of infection (MOI) of 0.01. Following the phage adsorption at 37°C for 30 min, infected cells were collected by centrifugation at 10,000 xg for 1 min. The infected cells were resuspended in 3 mL fresh pre-warmed TSB medium and incubated at 37°C with shaking at 160 rpm to allow the life cycles of the phages. A 100 µL of the samples were collected at every 10 min intervals (up to 2 h), diluted, and plated by a double agar overlay method and incubated for 12 to 24 h to allow the formation of single phage plaques. The latent-period (the interval between phage adsorption by the host and the beginning of lysis) and the burst size (the ratio of the final average number of phage particles liberated) of each phage were by enumerating the plaque-forming units per milliliter (PFU/mL).

Biofilm Removal Assay

A microtiter plate biofilm assay was carried out essentially by the procedure described by Soni and Nannapaneni [18]. Briefly, an overnight culture of *S. aureus* TRSA 201, a common host strain for all four phages, was adjusted to 10^6 CFU/mL in fresh TSB broth containing 0.25% w/v glucose and 200 µL was transferred into 96 wells flat-bottomed microtiter plate for biofilm formation at 37°C without agitation for 48 h. The media from the wells were removed and biofilm was rinsed two times with sterile phosphate-buffered saline (PBS) and treated with 200 µL phage particles (10^{10} PFU/mL) in SM buffer for 12 h by incubating at 37°C. The contents of the wells were removed and the biofilm was washed with PBS as described. The biofilm was stained with 200 µL of 1% (w/v) crystal violet solution for 5 min, and unbound stain was washed away. The crystal violet was solubilized and removed from the biofilm with 200 µL ethanol-acetone (80:20). The remaining biofilm after phage treatment was quantified by measuring the OD of the crystal violet in ethanol-acetone solution at 595 nm, and the percentage reduction in biofilm biomass was estimated as compared to untreated control biofilm. For each phage and combination of phages, four biofilm wells were used and experiments repeated at least three times.

Statistical Analysis

Analysis of Variance (ANVO) and Student's t-test of the SPSS software version 22 were used for the analysis of the data. P-values of <0.05 were considered statistically significant.

Results

Characterization of Phages

Four phages, Trsa205, Trsa207, Trsa220, and Trsa222 were confirmed for their lytic functions by spot and plaque assay methods *(Fig. 1-A,B)* on the lawn of *S. aureus* TRSA 201 host strain. TEM analysis of phages revealed that all four phages morphologically were similar with isometric heads measuring about 40 to 62 nm and long non-contractile tails of approximately 90 to 210 nm in length *(Table 1)*. Based on the morphology, they belong to

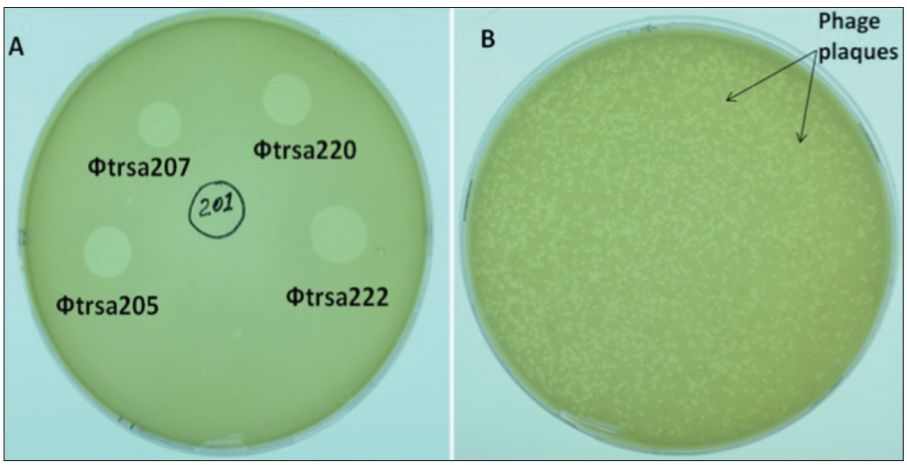

Fig 1. Lytic activity of phages (Trsa205, Trsa207, Trsa220, and Trsa222) against *S. aureus* TRSA 201 indicator strain using the agar-spot (A), and single plaque assay method for Trsa205 (B)

Table 1. Morphological characteristics of phages

Phage	Capsids Diameter (nm)	Tail Width (nm)	Tail Length (nm)
Trsa205	62.3±1.3	14.2±0.3	143.4±1.3
Trsa207	46.4±2.0	10.7±2.1	165.0±2.8
Trsa220	48.6±1.0	10.3±0.8	128.3±0.3
Trsa222	41.8±0.8	12.5±1.3	96.2±2.0

Trsa207 were about 20 min which is followed by a raise period of 30 min that results in a burst size of 45 and 30 PFU/infected cells, respectively *(Fig. 3-A)*. Phages Trsa220 and Trsa222 had a latent period of about 25 min and a raise period of 30 min that resulted in a burst size of 20 PFU/infected cell *(Fig. 3-B)*.

Antibacterial and Anti-biofilm Potential of Phages

Spot assay on a lawn of host bacteria was applied for the determination of the lytic spectrum of the phages. A total of 56 *S. aureus* isolates were infected by each phage. The phage Trsa205 showed lytic infection against 21 (37.5%) while Trsa207, Trsa220, and Trsa222 were effective against 15 (26.7%), 12 (21.4%), 11 (19.6%) of the strains tested, respectively. The combination of four phages lysed 37 (66%) of the strains *(Table 2)*.

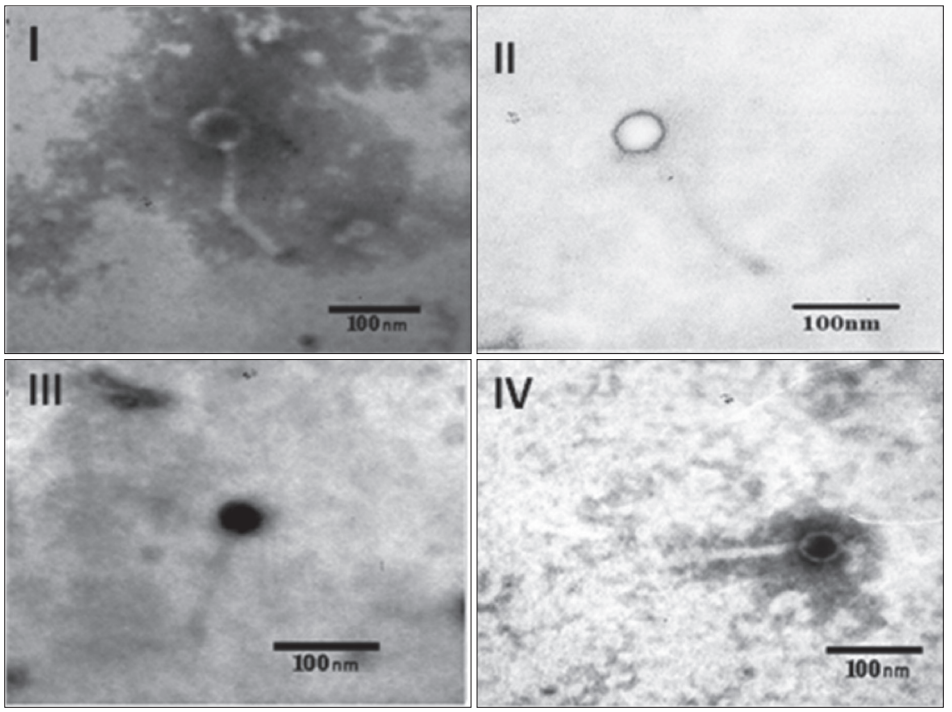

Fig 2. Transmission Electron Microscopic images of phages: I, Trsa205; II, Trsa207; III, Trsa220; IV, Trsa222; the scale bar denotes 100 nm

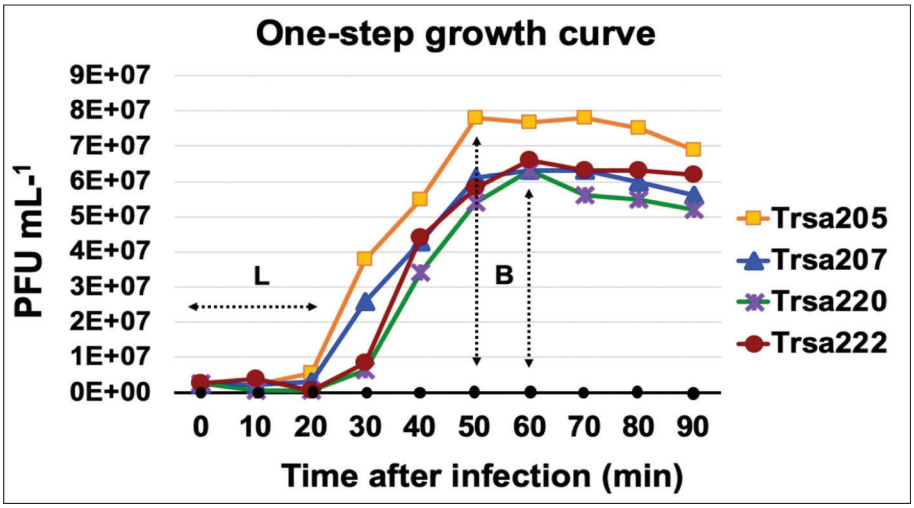

Fig 3. One-step growth curves of phages; The curves shown here is the PFU/infected cell at several time points over 90 min. L, latent periods; B, burst size

the *Siphoviridae* phage family, which is characterized by phages with a non-contractile tail *(Fig. 2)*.

One-step Growth Curve

The results obtained from the one-step growth curve experiments reviled that the latent periods for Trsa205 and

Table 2. *Host range of four phages and phage cocktail against different S. aureus strains*

No	Strains	Origin	Phages				
			Trsa 205	Trsa 207	Trsa 220	Trsa 222	Cocktail
1	*S. aureus* TRSA 1	Pus	-	-	-	-	-
2	*S. aureus* TRSA 2	Wound	+	+	+	+	+
3	*S. aureus* TRSA 3	Blood	+	-	-	-	+
4	*S. aureus* TRSA 4	Tracheal aspirate	-	-	-	-	-
5	*S. aureus* TRSA 5	Tracheal aspirate	-	-	-	-	-
6	*S. aureus* TRSA 6	Tracheal aspirate	-	-	-	-	-
7	*S. aureus* TRSA 8	Tracheal aspirate	+	+	-	-	+
8	*S. aureus* TRSA 9	Ear	-	-	+	-	+
9	*S. aureus* TRSA 10	Wound	+	-	-	-	+
10	*S. aureus* TRSA 11	Ear	-	-	-	-	-
11	*S. aureus* TRSA 12	Wound	-	-	-	-	-
12	*S. aureus* TRSA 13	Tracheal aspirate	-	-	+	-	+
13	*S. aureus* TRSA 14	Wound	-	-	-	-	-
14	*S. aureus* TRSA 15	Tracheal aspirate	+	-	-	+	+
15	*S. aureus* TRSA 16	Wound	-	-	-	-	-
16	*S. aureus* TRSA 17	Sputum	-	-	-	-	-
17	*S. aureus* TRSA 18	Urine	-	-	-	-	-
18	*S. aureus* TRSA 19	Tracheal aspirate	+	+			+
19	*S. aureus* TRSA 20	Wound	-	-	+	-	+
20	*S. aureus* TRSA 22	Wound	+	-	-	-	+
21	*S. aureus* TRSA 23	Wound	-	+	-	-	+
22	*S. aureus* TRSA 24	Tracheal aspirate	+	-	-	-	+
23	*S. aureus* TRSA 25	Tracheal aspirate	-	+	-	-	+
24	*S. aureus* TRSA 26	Wound	+	-	-	-	+
25	*S. aureus* TRSA 27	Nose swab	-	-	-	-	-
26	*S. aureus* TRSA 28	Wound	-	-	+	-	+
27	*S. aureus* TRSA 29	Wound	-	-	-	-	-
28	*S. aureus* TRSA 30	Pus	-	-	-	+	+
29	*S. aureus* TRSA 31	Tracheal aspirate	-	-	-	-	-
30	*S. aureus* TRSA 32	Wound	+	+	+	-	+
31	*S. aureus* TRSA 33	Tracheal aspirate	-	-	-	-	-
32	*S. aureus* TRSA 35	Wound	+	+	-	-	+
33	*S. aureus* TRSA 36	Blood	+	+	-	-	+
34	*S. aureus* TRSA 37	Tissue	-	-	-	+	+
35	*S. aureus* TRSA 38	Blood	-	+	-	-	+
36	*S. aureus* TRSA 39	Wound	-	-	+		+
37	*S. aureus* TRSA 40	Wound	-	-	-	-	-
38	*S. aureus* TRSA 41	Tracheal aspirate	+	-	-	-	+
39	*S. aureus* TRSA 42	Wound	-	-	-	-	-
40	*S. aureus* TRSA 43	Pus	-	-	+	-	+
41	*S. aureus* TRSA 44	Blood	-	-	-	-	-
42	*S. aureus* TRSA 196	Wound	+	+	+	+	+
43	*S. aureus* TRSA 197	Wound	-	-	-	-	-

+ clear lysis, - no lysis

Table 2. *Host range of four phages and phage cocktail against different S. aureus strains (continued)*

No	Strains	Origin	Phages				
			Trsa 205	Trsa 207	Trsa 220	Trsa 222	Cocktail
44	*S. aureus* TRSA 198	Wound	+	-	-	-	+
45	*S. aureus* TRSA 199	Tracheal aspirate	-	-	-	-	-
46	*S. aureus* TRSA 200	Tracheal aspirate	+	+	-	-	+
47	*S. aureus* TRSA 201	Sputum	+	+	+	+	+
48	*S. aureus* TRSA 202	Pus	+	-	+	-	+
49	*S. aureus* TRSA 203	Wound	-	+	-	-	+
50	*S. aureus* TRSA 204	Tracheal aspirate	+	-	-	-	+
51	*S. aureus* TRSA 205	Wound	-	-	+	-	+
52	*S. aureus* TRSA 207	Catheter	+	-	-	+	+
53	*S. aureus* TRSA 216	Blood	+	-	-	+	+
54	*S. aureus* TRSA 220	Blood	-	+	-	-	+
55	*S. aureus* TRSA 221	Blodd	-	-	-	+	+
56	*S. aureus* TRSA 222	Tracheal aspirate	-	-	-	-	-
Total number of sensitive (%) strains			**21 (37.5)**	**15 (26.7)**	**12 (21.4)**	**11 (19.6)**	**37 (66.0)**

+ clear lysis, - no lysis

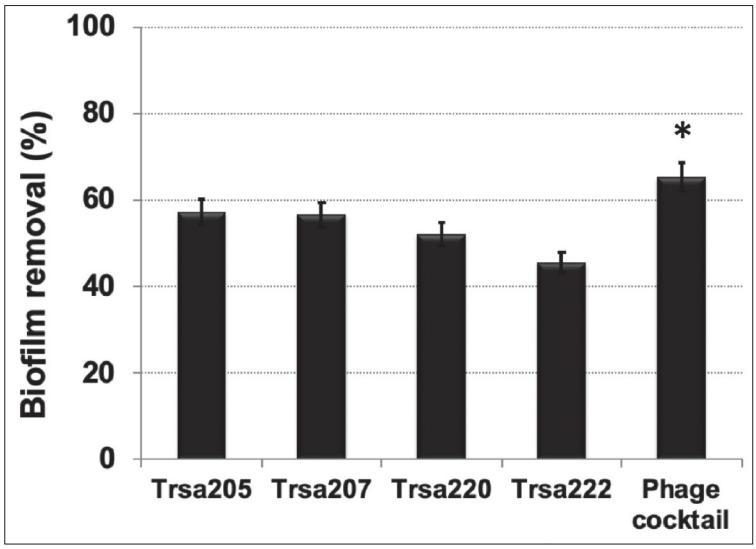

Fig 4. Biofilm removal capacity of phages as determined by 96-well microtiter plate assay. Error bars represent the SD of three independent experiments. Significant differences by two-tailed t-test (P<0.05) compared with control are marked by an asterisk (*)

To assess the biofilms removal potential of the four phages *in vitro,* 48 hour-old *S. aureus* TRSA 201 biofilms were established in 96-well polystyrene microtiter plates. Following the treatment of established biofilms with each of the four phages and phage cocktail for 12 h, remaining biofilm mass was stained with crystal violet, and the remaining biofilm was estimated by spectrophotometric measurement of the solubilized biofilm-bound crystal violet concentration. The data demonstrated that phage treatment reduced the amount of biofilm significantly relative to the control when they were applied as a cocktail *(Fig. 4)*. The amount of *S. aureus* TRSA 201 biofilm was removed for about 65% by the phage cocktail and 57%, 56%, 52%, and 45% by Trsa205, Trsa207, Trsa220, and Trsa222, respectively.

Discussion

Among coagulase-positive staphylococci *S. aureus* is one of the most commonly isolated pathogens responsible for local and systemic infections in both humans and animals. They cause healthcare-, community- and live-stock-associated infections via transferring from humans to animals and vice versa resulting in outbreaks and major economic burden [19,20].

With the increasing problem of antibiotic resistance in foodborne pathogens, alternative therapeutic approaches, such as phage and phage endolysin therapies, have attracted the interest of scientists for the treatment of these pathogenic bacteria [21,22].

Therefore, in this study, we aimed to identify and characterize new temperate phages and assess their potential to lyse *S. aureus* strains and reduce staphylococcal biofilms. Using the induction technique, four functional temperate phages were isolated and confirmed by the agar-spot and plaque assays *(Fig. 1-A,B)* since clear zones can also be produced by bacteriocins [23].

The transmission electron microscopy analysis revealed that all four phages have long tails with isometric and icosahedral heads *(Fig. 2)*. The tails of Trsa205, Trsa207, Trsa220, and Trsa222 were measured to be 143.4±1.3, 165.0 ±2.8, 128.3±0.3, and 96.2±2.0 nm, respectively *(Table 1)*. The icosahedral heads of phages were very similar in size. According to their morphological characteristics, which is one of the most frequently used methods for the classification of bacteriophages, all four phages belong to *the Siphoviridae* family in the order of *Caudovirales (Fig. 2)*. Furthermore, as they have long tails, they belong to type B phages in Bradley's classification [23]. More than 95% of the reported phages so far were assigned to the family of *Siphoviridae* in the order of *Caudovirales* and about 60% of these phages have long and flexible tails [24].

The one-step growth curve experiments were performed as the estimation of the burst size and burst period in the life cycle of phages has great importance in phage therapy [8]. The scheduling of host cell lysis by phages is crucially controlled by holin protein and any mutation in this protein can modify the lysis timing or the latent period [25]. The burst size of phage Trsa207 was relatively higher (45 PFU/mL) than those of others, which is the more preferable features of the lytic phages that can be used as an antibacterial agent. The burst size plays a crucial role in phage propagation [26] and this can increase the initial dose of the infective phages several folds in a short period of time [8,27].

Among four phages, phage Trsa205 had a broader host range with lytic activities against 37.5% of the strains tested. While phage Trsa207, Trsa220, and Trsa222, were effective against 27%, 21%, and 20% strains, respectively. The low lytic activity may be associated with lysogenicity, which required induction for the lytic activity or with superinfection immunity by resident prophages [14], as this was also confirmed in the present study, in which, the addition of Trsa205, Trsa207, Trsa220, and Trsa222 did not infect their respective host in which they were originated. The potency against *S. aureus* isolates increased when the combinations of the four phages applied. From a total of 56 staphylococcal strains tested, 37 (66%) strains were sensitive to at least one of the four phages. This highlights the use of phage cocktails that are applicable to broad staphylococcal targets including biofilms. The biofilm removal capacity of the newly identified phages was significant (66%) when used as a cocktail as compared to the individual phages and control *(Fig. 4)*.

In conclusion, due to limited therapeutic options to combat life-threatening infections, the phages of staphylococci could be used for local infections, such as wound infections caused by multidrug-resistant *S. aureus* strains including MRSA. Also, they could be used as a sanitizer for surfaces or topical agents for eliminating *S. aureus* from human and animal skin, where they serve as a reservoir for infections. Due to their inherent capacity to mediate gene transfer via transduction between bacteria, historically the lysogenic phages have not often considered for phage therapy. However, the advanced technologies including sequencing, recombination, and gene editing enable the use of temperate phages for the prevention and treatment of bacterial infections [28]. Therefore, these bacteriophages have the potential to be used not only in phage therapy but also serve as a source for endolysins which could be produced recombinantly and used as anti-staphylococcal and anti-biofilm agents.

Conflict of Interest

The authors declare that they have no conflict of interest.

Author Contributions

A.O. KILIÇ, M.A. ABDURAHMAN, and I. TOSUN, planned and designed the research. M.A. ABDURAHMAN, I. DURUKAN and M. KHORSHIDTALAB carried out experiments and helped analysis of the results. All authors contributed to writing of the final manuscript.

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RESEARCH ARTICLE

Determination of AFM$_1$ Levels of Mare's Milk and Koumiss Produced in the Highlands of the Kyrgyz Republic

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Abstract

Mare's milk and koumiss are two very important basic foodstuffs in central Asia.This study aimed to research the AFM1 contamination in mare's milk and koumiss samples collected from local producers in the highlands in Naryn, Issyk-Kul, Bishkek and Talas regions of Kyrgyz Republic. For this purpose, 75 raw mare's milk and 75 koumiss samples collected in May, June and July 2017 were analyzed using the ELISA method. AFM1 was detected in 13.3% of the 75 raw mare's milk samples with levels ranging between 6.48 and 25.45 ng/L. Approximately 53.3% of the koumiss samples were found to contain AFM1, with levels ranging between 5.93 and 18.77 ng/L. None of the AFM1 levels in the tested samples exceeded above the 50 ng/L maximum limit set by the European Union for milk and the 500 ng/kg limit set by Kyrgyz Republic. As the levels of AFM1 in the raw mare's milk and koumiss were low, it can be said that these products do not pose a serious risk in terms of public health. This study has been an important source for determining AFM1 levels in mare's milk and koumiss.

Keywords: *Aflatoxin M$_1$, Mare's Milk, Koumiss, ELISA*

Kırgızistan Yaylalarında Üretilen Kısrak Sütü ve Kımız'ın AFM$_1$ Seviyelerinin Belirlenmesi

Öz

Kısrak sütü ve kımız, Orta Asya'da çok önemli iki temel gıda maddesidir. Bu çalışma, Kırgızistan'ın Narın, İssyk-Kul, Bişkek ve Talas bölgelerindeki yerel üreticilerden toplanan kısrak sütü ve kımız örneklerinde AFM1 kontaminasyonunu belirlemek amacıyla yapılmıştır. Bu amaçla Mayıs, Haziran ve Temmuz 2017'de toplanan 75 çiğ kısrak sütü ve 75 kımız numunesi ELISA yöntemi kullanılarak analiz edilmiştir. AFM1, 6.48 ile 25.45 ng/L arasında değişen seviyelerde 75 çiğ kısrak süt numunesinin %13.3'ünde tespit edilmiştir. Kımız numunelerinin yaklaşık %53.3'ünün AFM1 içerdiği bulunmuştur ve seviyeleri 5.93 ile 18.77 ng/L arasında değişmiştir. Test edilen numunelerdeki AFM1 seviyelerinin hiçbiri, Avrupa Birliği tarafından süt için belirlenen 50 ng/L maksimum sınırı ve Kırgıziztan tarafından belirlenen 500 ng/kg sınırını aşmadı. Çiğ kısrak sütü ve kımızdaki AFM1 seviyeleri düşük olduğu için bu ürünlerin halk sağlığı açısından ciddi bir risk oluşturmadığı söylenebilir. Bu çalışma kısrak sütü ve kımızdaki AFM1 seviyelerini belirlemesi bakımından önemli bir kaynak olmuştur.

Anahtar sözcükler: *Aflatoksin M$_1$, Kısrak Sütü, Kımız, ELISA*

INTRODUCTION

Mare's milk is one of the most important foods used since the ancient times, especially by the societies of central Asia and the former Soviet Union. Important differences exist among the mare, human and cow milk in terms of composition and nutritional properties [1,2]. Crude protein content of mare's milk is higher than human milk and lower than cow's milk. On the other hand, it is categorized in the group of albuminous milks since it contains about

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50% casein and about 40% whey protein in terms of protein content [2,3]. Thanks to its high content of whey proteins, mare's milk is rich in essential amino acids, with high biological value and true digestibility (95-97%) [4,5]. Its balanced amino acid content ensures healthy growth of human body [6]. Mare's milk contains less fat than human milk and cow's milk. Compared to the cow's milk, mare's milk is richer especially in linoleic (C18: 2) and linolenic (C18: 3) acids [2]. While mare's milk shows similarity to human milk in terms of lactose content, its lactose content is significantly higher than cow's milk. Its energy content is lower than both human milk and cow's milk [2,3]. This milk is a very good source of vitamin C (428 times more than cow's milk) [3]. When its composition and some structural features are considered, the mare's milk is more suitable for infant nutrition than cow's milk [2,3,7,8].

Koumiss is a traditional product that is produced by the fermentation of mare's milk and widely consumed in the central Asian countries. The high nutritional property of koumiss comes from the mare's milk used in its production. Koumiss is fermented by lactic acid bacteria, mainly including *Lactobacillus helveticus, Lactobacillus kefiranofaciens, Lactobacillus kefiri, Streptococcus parauberis, Lactococcus lactis*, and yeasts [9,10]. For hundreds of years, koumiss has been considered not only as a kind of food but also a natural and alternative medicinal remedy [11]. Koumiss consumption helps regulate the gut environment and immune system by modulating the intestinal microflora [12]. In some countries, koumiss has been successfully applied in combination with traditional medicine in treating clinical conditions like hepatitis, chronic gastric ulcer, and tuberculosis [10].

The basic structure of aflatoxins consists of bifuran and coumarin. The toxicity of aflatoxins comes from the bifuran structure and its carcinogenicity is due to coumarins [13-15]. Aflatoxins are an important global public health problem due to their naturally occurring toxic, carcinogenic, teratogenic and mutagenic effects in the feed and food industry [16]. Aflatoxins are basically toxic secondary metabolites produced by some Aspergillus species, such as *A. flavus, A. parasiticus*, and *A. nomius* Aflatoxin M_1 (AFM_1), a metabolite of aflatoxin B_1 and found in dairy products, is classified as a Group 1 toxin [16,17].

In order to protect consumers several countries have established legislation to regulate the levels of AFM_1 in milk and dairy products. These legal limits range from 50 ng/L in many countries such as those in the EU [18] to 500 ng/L in countries such as the US. The legal limit of AFM_1 levels in milk and milk products in Kyrgyz Republic is 500 ng/L [19].

AFM_1 contamination in raw milk is an important public health problem. The AFM_1 level has been examined in milk and dairy products in the World [15,20-23]. AFM_1 levels of milks obtained from different animals (buffalo, cow, goat, and sheep) [15,21,24] and different dairy products such as yoghurt, cheese, butter, and buttermilk are researched in the literature [23,25-28]. AFM_1 contamination has been reported even in breast milk from different countries [29]. Although mare's milk and koumiss are among the main foodstuffs of central Asian societies, no studies are found in the literature to report the AFM_1 contamination of these products.

The aim of this study was to investigate the AFM_1 content of raw mare's milk and koumiss obtained from milk producers located in the highlands of Naryn, Issyk-Kul, Bishkek and Talas in Kyrgyz Republic during milking season.

Materials and Methods

Sample Collection

A total of 75 raw mare's milk and 75 koumiss samples were collected from local producers in the highlands of Naryn, Issyk-Kul, Bishkek and Talas regions of Kyrgyz Republic. The samples were collected in May, June and July, 2017, when the mare's milk production is at its highest level, from 2 highlands in Talas (Bakai-Ata, Beshtash), 4 highlands in Bishkek (Kashka Suu, Chunkurchak, Kemin, Suusamyr), 2 highlands from Issyk-kul (Jeti Oguz, Ottuk), and 3 highlands from Naryn (At-Bashi, Song-Kul, Kochkor). These pastures are located at an altitude of 2.200 m above sea level. Pastures in this region are used for grazing by cattle, horses and small ruminants. The mares were on good quality pasture from spring to autumn without any concentrated feed supplement.

Measuring the AFM_1 Levels in the Raw Mare's Milk and Koumiss Samples

The AFM_1 levels in raw mare's milk and samples koumiss were determined using an enzyme-linked immunosorbent assay (ELISA) with a minimum detection limit of 5 ng/L (RIDASCREEN Aflatoxin M_1 test kit, R-Biopharm AG, Germany). AFM_1 standards (including 0, 5, 10, 20, 40, and 80 ng/L) are contained in the test kit. The test was used according to the manufacturer's instructions. A sample was considered to be negative for AFM_1 if the levels were below the minimum detection limit of the assay. The validation variables in this study were as follows:

Limit of detection (LOD) = 5 ng/L.

Limit of quantification (LOQ) = 8.5 ng/L.

Recovery rate = 100-116%, R.

Relative standard deviation calculated under repeatability conditions (RSDr) <10%.

Statistical Analysis

The study adopted statistical methods to evaluate the incidence of AFM_1 in samples, reported as mean and standard

deviation (SD). And the data were statistically analyzed by a one way analysis of variance (ANOVA) using SPSS version 19.0 (SPSS, Inc., Chicago, IL, US). The level of confidence required for significance was set at $P \leq 0.05$.

Results

In this study a total of 75 raw mare's milk samples and 75 koumiss samples were analyzed for AFM_1 with the ELISA. The occurrence and the distribution of AFM_1 concentration in various ranges in raw mare's milk and koumiss samples are presented in *Table 1*.

Table 1 shows the frequencies of AFM_1 contamination in the raw mare's milk samples from Bishkek, Issyk-kul, Naryn and Talas. Only three samples (12%) from Bishkek and two samples (11.1%) from Issyk-kul were found to be positive for AFM_1 contamination. However, the samples obtained from Naryn (15%) and Talas (16.6%) had a relatively high rate of contamination. The raw mare's milk samples with the lowest AFM_1 contamination rate were obtained from Issyk-kul (11.1%), which was followed by Bishkek (12%), Naryn (15%) and Talas (16.6%). From the 75 raw mare's milk samples 10 (n=13%) were found to be positive for AFM_1. Among these samples, the AFM_1 content in four of them (5.3%) was less than 10 ng/L, in five of them was between 10 and 20 ng/L, and in one (1.3%) ranged between 21 and 50 ng/L. The overall mean level of AFM_1 in the raw mare's milk samples was 13.39±5.87 ng/L. The minimum and maximum AFM_1 concentration in the raw mare's milk were 6.48 and 25.45 ng/L, respectively. None of the AFM_1 levels in the tested samples exceeded above the 50 ng/L maximum limit set by the EU [18] for milk and the 500 ng/kg limit set by Kyrgyz Republic [19].

The presence and concentration of AFM_1 contamination in the koumiss samples are presented in *Table 1*. A total of 15 samples (60 %) obtained from Bishkek, 11 samples (61.1%) from Issyk-kul, nine samples (45%) from Naryn and five samples from Talas were found to be positive for AFM_1. The koumiss samples with the highest AFM_1 contamination rate were obtained from Issyk-kul (61.1%), followed by Bishkek (60%), Naryn (45%) and Talas (41.6%). A total of 40 (53.3%) koumiss samples contained AFM_1. The minimum and maximum AFM_1 concentration in the koumiss samples were 5.93 and 18.77 ng/L, respectively. The average level of AFM_1 in the koumiss samples was determined as 8.38±3.14 ng /L. Furthermore, none of the koumiss samples contained AFM_1 concentrations above the highest tolerance limit (50 ng/L) set by the EU and Kyrgyz Republic (500 ng/L).

Discussion

Kyrgyz Republic is a northern Asian country where mare's milk and koumiss are widely produced and consumed. The producers settle in the highlands after mid-March. Therefore, milk and koumiss are produced when the mares are fed with fresh grass. Mare's milk is mostly produced in May, June and July. Kyrgyz use mare's milk for the production of drinking milk and koumiss. Although the positive effects of mare's milk and koumiss consumption on health are known, no study is found in the literature that reports the AFM_1 contamination of these products. For this reason, the findings of this study are believed to be important.

In recent times, high AFM_1 contamination reported in milk and dairy products in the world has become an important public health problem. Although no study exists in the literature on AFM content of raw mare's milk and koumiss, AFM_1 contamination has been reported in different milk types and fermented dairy products such as yogurt and ayran.

Table 1. *Occurrence and distribution of AFM1 contamination in raw mare's milk and koumiss samples collected from the highlands of Kyrgyz Republic*

Samples	Location	Highlands	Samples	Positive* (n/%)	Frequency Distribution of AFM_1 Concentration (ng/L)			AFM_1 Concentration (ng/L)			Above the EU** and Kyrgyz Republic Limit***
					< 10	10-20	21-50	Min	Max	Mean ± SD	
Mares milk	Bishkek	4	25	3 (12%)	1	1	1	8.68	25.45	16.66±8.41	0
	Issyk-kul	2	18	2 (11.1%)	1	1	-	9.73	13.48	11.61±2.65	0
	Naryn	3	20	3 (15%)	-	3	-	10.81	18.25	15.43±4.04	0
	Talas	2	12	2 (16.6%)	2	-	-	6.48	7.94	7.21±1.03	0
Total		11	75	10 (13.3%)	4 (5.3%)	5 (6.6%)	1 (1.3%)	6.48	25.45	13.39±5.87	0
Koumiss	Bishkek	4	25	15 (60%)	12	3	-	5.93	16.78	8.38±3.00	0
	Issyk-kul	2	18	11 (61.1%)	10	1	-	5.97	18.77	8.04±3.70	0
	Naryn	3	20	9 (45%)	8	1	-	6.56	14.31	7.73±0.88	0
	Talas	2	12	5 (41.6%)	4	1	-	5.99	16.33	8.99±4.32	0
Total		11	75	40 (53.3%)	34 (45.3%)	6 (8%)	-	5.93	18.77	8.38±3.14	0

** A sample was considered negative when its AFM_1 concentration did not exceed 5 ng/L, which was the detection limit of the RIDASCREEN Aflatoxin M_1 test kit; ** A sample was considered to be above the EU legal limit when its AFM_1 concentration exceeded 50 ng/L, *** A sample was considered to be above the Kyrgyz Republic legal limit when its AFM_1 concentration exceeded 500 ng/L*

Contrary to this study, high AFM_1 contamination in different milk types was reported in the literature. In one of the studies that report high AFM_1 contamination in milk samples [30] reported that all 110 samples were contaminated with AFM_1 between 0.028 and 4.98 µg/L, and 29 samples (26.3%) exceeded the legal limits (0.5 µg/L). Similarly, it was reported that 80% of milk samples sold in Syria are contaminated with AFM_1 and 52% of the milk samples exceed the EU's legal limits [31]. It was reported that 73.6% of 111 UHT milk samples and 131 pasteurized milk samples (n = 242) were contaminated with AFM at concentrations ranging from 100 ng/L to 22 ng/L, and 1.8% of UHT milk samples and 59.5% of pasteurized milk samples exceeded the EU's legal limits between 2016 and 2017 in China [32]. Similarly, in a three-year (2013-2015) study conducted in China, 1.8-11% of analyzed milk samples were found to be contaminated with AFM_1 above the EU's legal limits [33]. In a study conducted in Shush, Iran, it was reported that 75% of a total of 120 raw water buffalo milk and cow's milk samples are contaminated with AFM_1 and 8% of the samples exceed the EU's legal limits [34]. In a study conducted in Turkey, it was found that all 39 organic UHT milk samples (100%) exceeded the legal limit of Turkish Food Codex [35]. Also, high AFM_1 incidences have been reported in buffalo milk samples at 7.2%, 50%, 27%, 38.7% and 52% in Southern Italy, India, Afyonkarahisar of Turkey, Ahvaz of Iran, and Ismailia of Egypt, respectively [24,36-39].

Similarly, Hashemi [40] from Iran and Assem and Mohamad [41] from Lebanon, reported AFM_1 contamination in 56% and 65% of the milk samples, respectively, with 30% and 21% exceeding the legal limits set by EU. Differences in AFM_1 levels in various countries can potentially be attributed to geographical conditions, climate, animal feeding styles (pasture feeding, hay feeding, silage feeding), animal feed and dairy processing measures taken to prevent AFM_1 contamination. Therefore, when compared to the studies that report high level of AFM_1 contamination exceeding legal limits in Syria, China, Iran, Lebanon, and Turkey, the mare's milk produced in Kyrgyz Republic can be considered to be of very high quality. The low AFM_1 concentrations found in the mare's milk produced in Kyrgyz Republic show similarity to the studies conducted in China, Qatar, and Italy. It was reported that 5.650 raw milk samples collected during four seasons in 2016 from major dairies in China were contaminated with AFM at a rate of 4.7% and only 1.1% of the samples exceeded the EU's legal limits and AFM contamination incidence was at the highest level in winter [16]. Similar to this study, it was found in another study conducted in Southern China that 62.5% of raw buffalo milk samples were contaminated with AFM_1 between 4 and 243 ng/kg, and only 5.9% of samples exceeded the EU's legal limits. Also, it was reported that buffalo dairy products (HTST milk, UHT milk) were contaminated at concentrations between 10-50 ng/kg and the samples did not exceed the EU's legal limits [15]. In a study conducted in Qatar, it was found that 85% of milk samples are contaminated with AFM_1, but no sample exceeds the legal limits (50 ng/L) [25]. Likewise, the findings of two studies recently conducted in Italy can be evaluated as similar to the findings of this study. It was reported that 12% of the cow's milk samples analyzed in Northern Italy is contaminated with AFM_1 and only one sample exceeds the EU's legal limit [36] and in a study conducted in Bologna region [42], it was reported that none of the 60% positive milk samples exceeded the EU's legal limits.

In the literature, there are few studies reporting such low level of contamination. In this study, low concentrations of AFM found in raw mare's milk are quite striking. This may be due to the mares feeding fresh grass in the pastures. Because mares are milked mostly in May, June and July. During this period, the mares are fed with fresh grass in the pasture. Accordingly, studies have reported that the frequency of AFM_1 contamination is higher in winter months when the level of feeding with dry grass is high [16,43]. During the winter months, fresh grass consumption decreases, while stored concentrate feed consumption of animals increases. The risk of aflatoxin formation due to mold growth increases in the feeds that are stored under unsuitable conditions [44].

The AFM_1 concentrations found in the koumiss samples are very low. Although no studies exist on AFM_1 contamination in the literature, there are studies conducted on fermented dairy products such as yoghurt, ayran and laban. In a study conducted in Qatar, it was determined that 76% of the yoghurt samples and 76.1% of the laban samples were contaminated with AFM_1. However, the levels in none of the samples exceeded the legal limits (50 ng/L) [25].

In most studies, higher AFM_1 contamination is reported in fermented milk products such as koumiss. Contrary to the present study, it was determined that the yoghurt and ayran samples of a study conducted in Turkey contained 95% and 87.5% AFM_1, respectively, with 20% and 13.6% of these samples displaying levels higher than the maximum limit of 50 ng/kg determined by the Turkish Food Codex [27]. In a study conducted on organic yoghurt samples in Turkey, it was reported that all 26 samples exceeded the legal limit of Turkish Food Codex, which is 50ng/kg [35]. In their study conducted in Turkey [45] reported that 89% of the yogurt samples they tested contained AFM_1 and 26% of these samples had levels higher than the maximum limits set by the EU. Similarly, a study conducted in Pakistan reported the presence of AFM_1 in 61% of the yogurt samples with 47% of the samples having levels higher than the maximum limit of 50 ng/kg set by the EU [46]. It is found that, in 95% and 33% of the yogurt samples positive for AFM_1 contamination [47,48]. In a study conducted in the South of China, AFM_1 was detected in 55.5% of the yogurt samples, all of which had levels lower than 50 ng/kg [15].

The main reasons for detecting low concentrations of AFM_1 in koumiss may include the use of milk containing low

AFM_1 in production or it may be due to the fact that this is a fermented product. It is reported that the fermentation is effective in mycotoxin decontamination and reduction of mycotoxins, and is a feasible traditional food processing technique which does not only improve useful ingredients but also contributes to the food safety [49].

As the levels of AFM_1 in the raw mare's milk and koumiss were low, it can be said that these products do not pose a serious risk in terms of public health. In this study, although AFM_1 contamination in mare's milk and koumiss is below legal limits, especially the incidence of AFM_1, which is 53.3% in kumiss samples, should not be ignored due to the carcinogenicity of AFM_1.

Conflict of Interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of paper.

Author Contributions

MAA, HÖ, FRİ and MA planned, designed, and supervised the research procedure. The samples were collected by FRİ. MAA and HÖ performed analysis. The manuscript was written by MAA and MA. MA contributed to the language editing of the final manuscript. All authors have interpreted the data, revised the manuscript for contents, and approved the final version.

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RESEARCH ARTICLE

Geometric Morphometric Analysis of Skull and Mandible in Awassi Ewe and Ram

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Abstract

The aim of the study was to analyze Awassi ewe and ram skull and mandible by geometric morphometric methods. For this purpose, different numbers of skulls and mandibles of adult Awassi sheep were used based on sexual dimorphism. The skull was analyzed using the photos taken from 17 samples from dorsal side and 16 samples from the left lateral side, and the mandible was analyzed using the photos taken from 20 samples from the left lateral side. In the comparison of Awassi sheep skull from the dorsal and lateral sides between the sexes, the first principal component accounted for 37.719% and 44.238% of the total shape difference, respectively. In mandible, the first principal component accounted for 24.92% of the total shape difference. The skull had an apparent dimorphism in both sides between the sexes but the same effect was not observed in mandible. As a result, it is considered that the results obtained would contribute to the future studies related to ruminant cranium to be performed using geometric morphometric method.

Keywords: *Geometric morphometry, Awassi sheep, Principal components analysis, Relative warp analysis*

İvesi Koyunu ve Koçunda Kafatası ve Mandibulanin Geometrik Morfometrik Analizi

Öz

Çalışmada İvesi koç ve koyun kafatası ve mandibula'sının geometrik morfometrik yöntemlerle analizi amaçlandı. Bu amaçla cinsiyet farkı gözetilerek farklı sayıda ergin İvesi koyunu kafatası ve mandibula'ları kullanıldı. Kafatası dorsal yönden 17, sol lateral yönden 16, mandibula ise sol lateral yönden 20 örnekten alınan fotoğraflardan analiz edildi. İvesi koyunu kafatasında dorsal ve lateral yönden cinsiyetler arası yapılan karşılaştırmada birinci temel bileşen toplam şekil farklılığının sırasıyla %37.719 ve %44.238'ini açıkladı. Mandibula'da ise birinci temel bileşen toplam şekil farklılığının %24.92'sini açıkladı. Kafatası her iki yönde cinsiyetler arası belirgin bir dimorfizm gösterirken, aynı etki mandibula'da gözlenmedi. Sonuç olarak elde edilen bulguların ileride planlanacak ruminantia cranium'u ile ilgili geometrik morfometrik yöntemle yapılacak çalışmalara katkı sağlayacağı düşünülmektedir.

Anahtar sözcükler: *Geometrik morfometri, İvesi koyunu, Temel bileşenler analizi, Relative warp analizi*

INTRODUCTION

Geometric morphometric method determines shape differences by landmark (LM) coordinates and measures the amount of shape changes using the location differences of objects [1]. Superimposition (General Procrustes Analysis) is one of the most important points of this method. By applying this method, variations of the objects related to their shape such as their location, direction, and scale are removed [2]. Therefore, the coordinates are aligned and the size and direction of the movement between different populations or samples are mapped [3].

Awassi Sheep's name is originated from the El Awas tribe between Tigris river and Euphrates river and it is a combined, fat-tailed sheep species and named after different names based on regions. This sheep has completely adapted to the harsh climate conditions of South-West Asia [4]. Awassi is the most common sheep species which is not Europe-origin [5].

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In recent years, geometric morphometry method analysis of cranium has started to be used on different species in the field of veterinary anatomy [6-11]. Cranium is one of the main parts of skeleton demonstrating the taxonomic relationship in animals [12]. In the literature review, the cranium in Awassi sheep is analyzed by geometric morphometric method according to gender, and no other result has been found. In this study, it was aimed to determine whether gender makes a difference on the cranium in terms of shape or not by geometric morphometric method in Awassi sheep.

Material and Methods

The permissions were obtained from Harran University Animal Experiments Local Ethics Committee (Decision no: 2020/003-01-12).

In the study, different numbers of adult Awassi ewe and ram skull and mandible were used by considering sexual dimorphism. The materials were boiled and macerated. While the skull was analyzed using the photos taken from 17 (10 ewe, 7 ram) samples from dorsal side and 16 (9 ewe, 7 ram) samples from the left lateral side, the mandible was analyzed using the photos taken from 20 (10 ewe, 10 ram) samples from the left lateral side. The photos were taken from a 30-cm distance by focusing on frontonasal suture from the dorsal side, the ventral edge of orbita from the lateral side for skull and between the second and third premolar teeth for mandible. The photos were saved in a computer in JPEG format. Firstly, the photos were converted into a tps file using TpsUtil software (Version 1.79) [13]. 10 homolog LMs [11,14] (*Fig. 1, Fig. 2,* and *Fig. 3)* were marked from all directions on the photos by TpsDig2 software (Version 2.31) [11,14,15]. The confirmation test of the homolog LMs, the cartesian coordinates of which were determined, was performed by TpsSmall (Version 1.34) [16] software. As a result of this analysis, it was determined that the slope and correlation values were 0.999412 and 1.0 and 0.999741 and 1.0 from the dorsal and left lateral sides, respectively. These values were 0.999853 and 1.0 in mandible. All these values demonstrated that LMs were accurate.

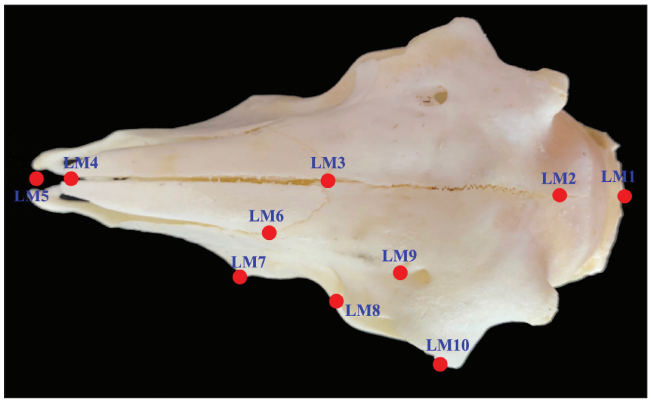

Fig 1. View of dorsal landmarks on skull. LM1: External occipital protuberance, LM2: Junction of sutura coronalis and sutura interfrontalis, LM3: Junction of sutura interfrontalis, sutura internasalis and frontonasal suture, LM4: Anterior edge of sutura internasalis, LM5: Anterior edge of fissura interincisiva, LM6: Fissura nasomaxillaris, LM7: Tuber faciale, LM8: Medial angle of orbita, LM9: Foramen supraorbitale, LM10: Posterio-ventral corner of margo supraorbitalis

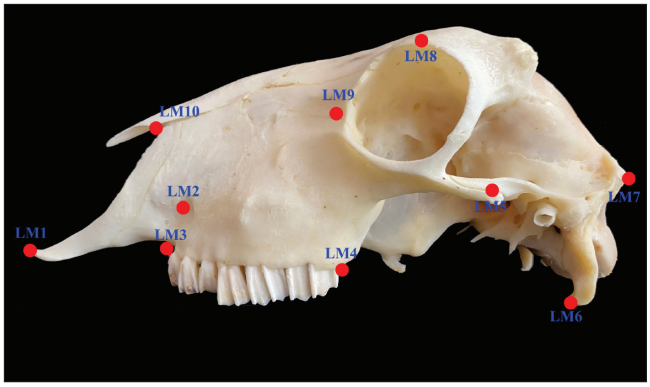

Fig 2. View of lateral landmarks on skull. LM1: Anterior edge of os incicivum, LM2: Foramen infraorbitalis, LM3: Anterio-dorsal edge of PM1, LM4: Caudal edge of M3, LM5: Middle point of arcus zygomaticus, LM6: Ventral edge of processus jugularis, LM7: External occipital protuberance, LM8: Middle point of margo supraorbitalis, LM9: Fossa lacrimalis externa, LM10: Fissura nasomaxillaris

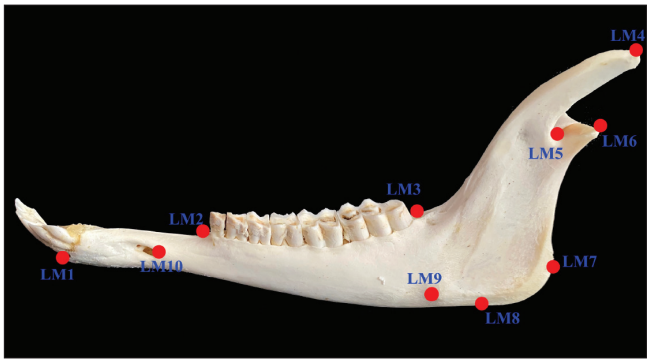

Fig 3. View of landmarks on mandible. LM1. Aboral anterioventral end point of alveoli dentales of L1, LM2. Anterioventral edge of P2, LM3. Posterioventral edge of M3, LM4. End-middle point of Processus coronoideus, LM5. Medioventral point of Incisura mandible, LM6. Posterior end point of Condylus mandible, 7. Posterioventral corner of angulus mandible, LM8. Incisura vasorum facialium. LM9. Anterior junction point of the dorsal and ventral axes of fossa masseterica, LM10. Posterior margin of Foramen mentale

As there are differences between the mandible in terms of size, position and direction etc., General Procrustes Analysis (superimposition) were performed [17]. PAST (Version 4.02) [18] software was used for this analysis. Principal components analysis (PCA) was performed through the same software on the new coordinates obtained as a result of Procrustes analysis. Thus, the degree of diverging of the samples based on sex was determined using covariance analysis [2]. In addition, MorphoJ software was used to demonstrate shape differences at which LM levels and directions. In this software, the average shapes were determined with their differences based on sex using discriminant function analysis.

In the study, relative warp analysis (RWA) was performed by TpsRelw (Version 1.70) [19] software and the consensus graphics of the groups were formed. Also, distribution of the groups on graphic was also tested by this analysis. The statistical analysis of LM coordinate values based on the groups was performed by 2-t test in PAST (Version 4.02) software.

Results

Skull Analysis

Table 1 shows the results related to the skull principal components analysis in the study. Accordingly, in the

Table 1. *Values obtained as a result of the principal component analysis*

PC	Skull Dorsal		Skull Lateral		Mandible	
	Eigenvalue	% Variance	Eigenvalue	% Variance	Eigenvalue	% Variance
1	0.00165069	37.719	0.00105427	44.238	0.000412877	24.92
2	0.00121854	27.844	0.000346344	14.533	0.000306917	18.525
3	0.000456679	10.435	0.000243111	10.201	0.000230935	13.939
4	0.000340601	7.7829	0.000198678	8.3366	0.000208795	12.602
5	0.000233552	5.3368	0.000156905	6.5838	0.000134089	8.0934
6	0.000130795	2.9887	0.000151965	6.3765	8.93702E-05	5.3942
7	0.000105017	2.3997	7.0557E-05	2.9606	7.58173E-05	4.5762
8	9.10019E-05	2.0794	4.94674E-05	2.0757	6.44643E-05	3.8909
9	5.55349E-05	1.269	4.20146E-05	1.7629	4.64634E-05	2.8044
10	3.87545E-05	0.88556	2.14639E-05	0.90063	3.78018E-05	2.2816
11	3.1854E-05	0.72788	2.09338E-05	0.87839	2.06527E-05	1.2466
12	1.02154E-05	0.23343	1.39763E-05	0.58645	1.40721E-05	0.84936
13	8.47604E-06	0.19368	6.2137E-06	0.26073	7.50213E-06	0.45281
14	4.16989E-06	0.095284	4.54143E-06	0.19056	3.92625E-06	0.23698
15	3.69238E-07	0.0084373	2.76267E-06	0.11592	2.1843E	0.13184
16	2.8442E-09	6.4992E-05			8.97427E	0.054167
17					1.46284E	0.00088294
18					2.39165E	1.4436E-11
19					1.20709E	7.2857E-13

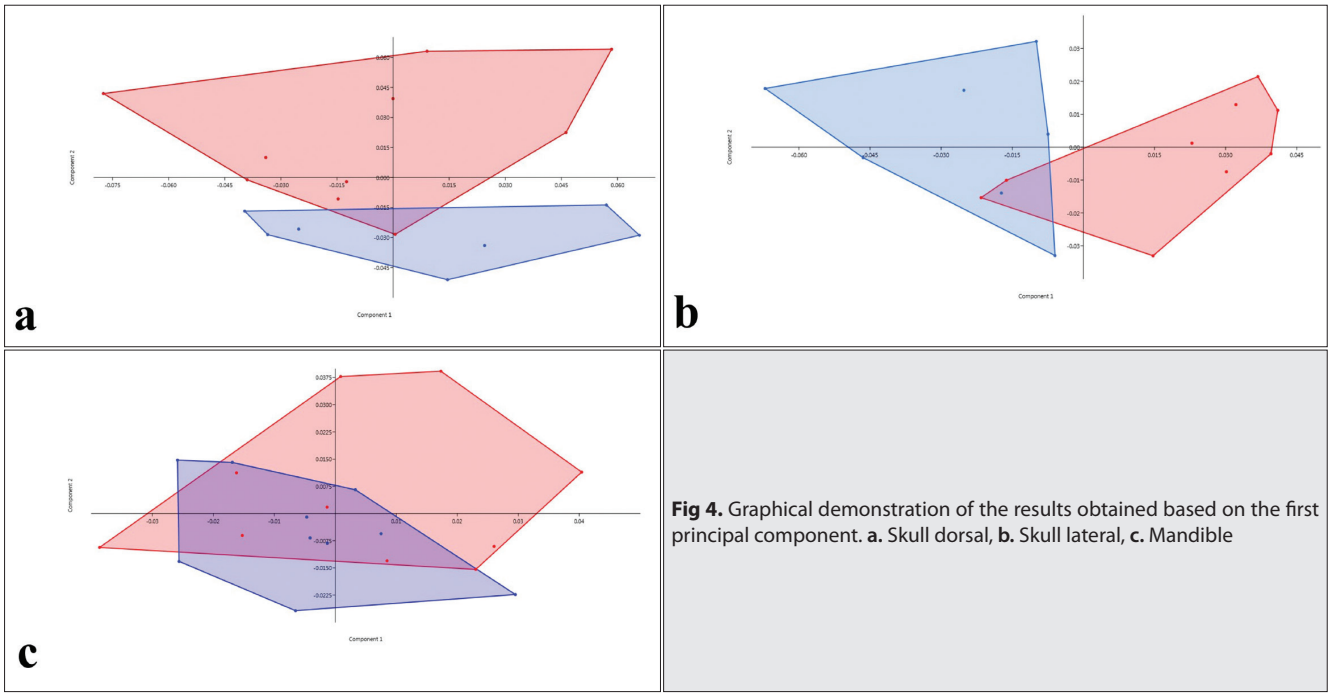

Fig 4. Graphical demonstration of the results obtained based on the first principal component. **a.** Skull dorsal, **b.** Skull lateral, **c.** Mandible

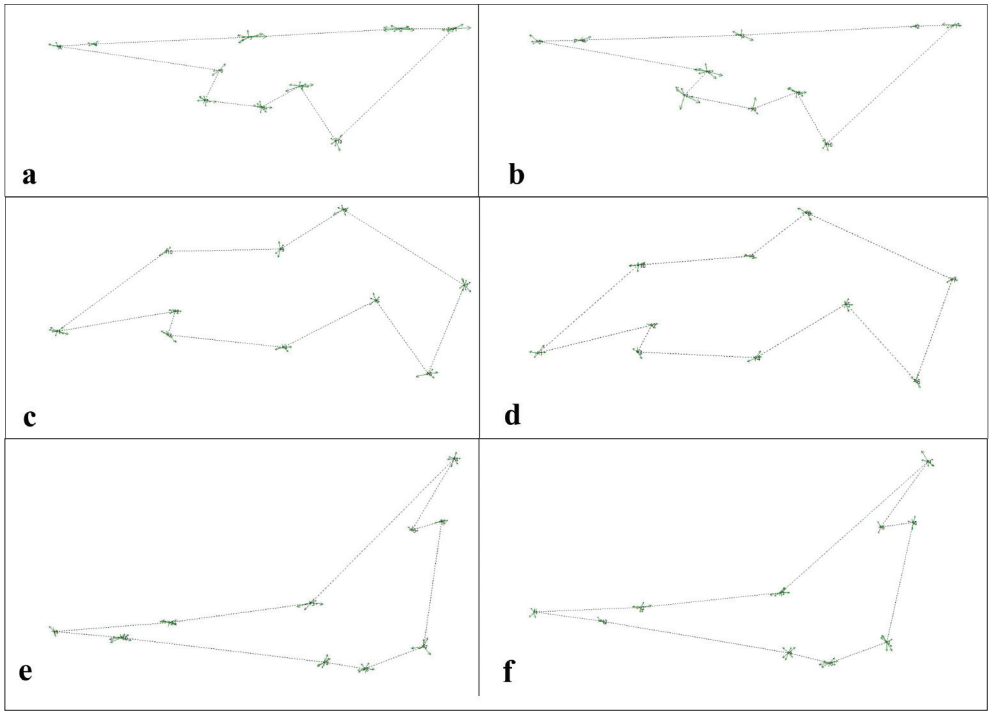

Fig 5. Consensus graphics based on groups, **a.** Skull ewe dorsal, **b.** Skull ram dorsal, **c.** Skull ewe lateral, **d.** Skull ram lateral, **e.** Ewe mandible, **f.** Ram mandible

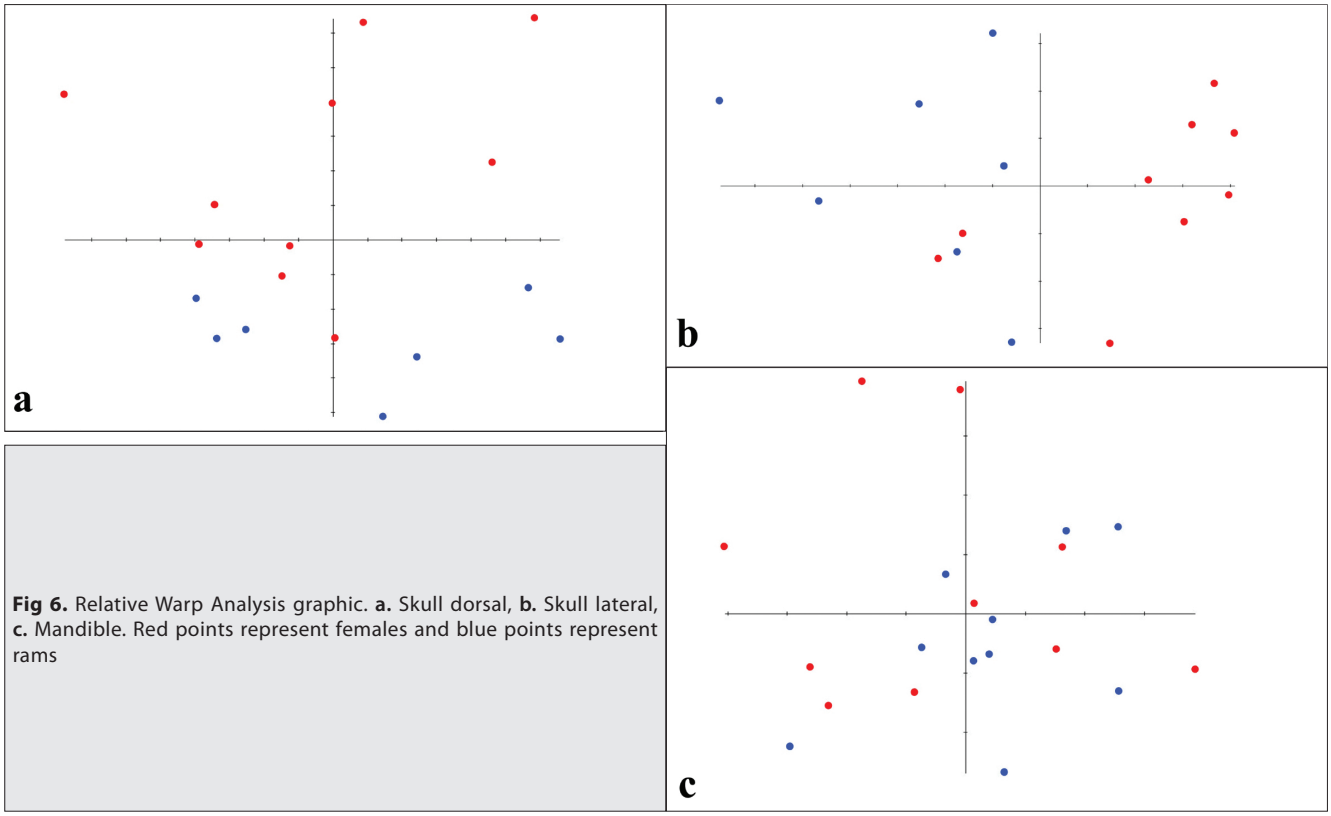

Fig 6. Relative Warp Analysis graphic. **a.** Skull dorsal, **b.** Skull lateral, **c.** Mandible. Red points represent females and blue points represent rams

comparison of Awassi ewe and ram skull from the dorsal and lateral sides between the sexes, the first principal component accounted for 37.719% and 44.238% of the total shape difference, respectively. The graphics in *Fig. 4-a,b* shows the sexual dimorphism in terms of the first principal component. Accordingly, it was observed that the crania of the ewe and ram individuals formed groups apparently in both dorsal and lateral side.

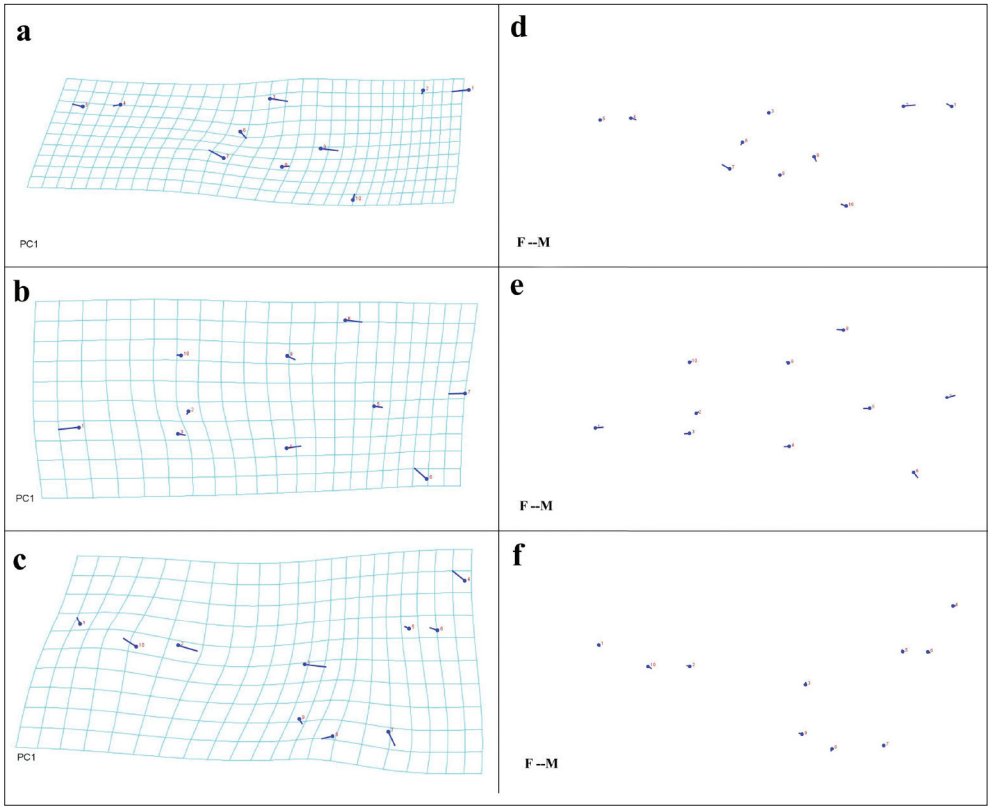

Fig 7. The lollipop demonstration of the shape differences occurring on the landmarks based on the first principal component and the discriminant function analysis. **a, d.** Skull dorsal, **b, e.** Skull lateral, **c, f.** Mandible. Point represents ewes

Fig. 5-a,b,c,d show the consensus graphics obtained as a result of relative warp analysis along with change vectors in the study. Accordingly, vector intensity was determined at LM2, 3 and 9 levels from the dorsal side and at LM1, 6 and 7 levels from the lateral side in the Awassi ewe. Vector intensity was determined in the Awassi ram at LM6 and 7 levels from the dorsal side and at LM1 and 8 levels from the lateral side. Also, *Fig. 6-a,b* shows the graphics obtained as a result of the between-groups relative warp analysis. As a result of this analysis, it was observed that the ram differentiates from ewe under the x axis from the dorsal side and on the left part of y axis from the lateral side (Dorsal RWA1: 37.75%, RWA2: 27.85%, RWA3: 10.44%, Lateral RWA1: 44.26%, RWA2: 14.53%, RWA3: 10.20%).

Fig. 7-a,b,d,e show in terms of PCA1 and sexes at which LM levels shape differences occurred in the study. From the dorsal side, a shape difference was determined from anteriodorsal side at LM1 and LM7 and from caudoventral side at LM4 and 9 in the ram compared to the ewe. From the lateral side, a shape difference was determined from caudal side at LM1 and LM7, from caudoventral side at LM6 and from anterior side at LM4 and 8 in the ram compared to the ewe. In the comparison of LMs performed in terms of coordinate value, a statistically significant difference was determined at LM2, 4, 9 and 10 dorsally and at LM1, 5, 7 and 8 laterally between the sexes ($P<0.05$).

Mandible Analysis

Table 1 shows the results of the principal components analysis related to mandible in the study. Accordingly, in the comparison between the sexes in mandible of Awassi sheep, the first principal component accounted for 24.92% of the total shape difference. The graphics in *Fig. 4-c* shows the sexual dimorphism in terms of the first principal component. Accordingly, it was observed that the mandibles of ewe and ram individuals were not separated with apparent borders.

Fig. 5-e,f show the mandible consensus graphics obtained as a result of relative warp analysis along with change vectors in the study. Accordingly, the vector intensity in the ewe and ram was at similar LMs. Also, *Fig. 6-c* shows the graphics obtained as a result of the between-groups relative warp analysis. The result of this analysis indicated that the discrimination was not apparent between the ewe and ram individuals as in the principal components analysis (RWA1: 24.92%, RWA2: 18.53%), RWA3: 13.94%).

Fig. 7-c shows at which LM levels shape differences occurred in mandible in the study. A shape difference was determined from caudal side at LM2 and 3, from caudoventral at LM10 and from the anteriodorsal side at LM9 in the male sheep compared to the ewe. In the comparison of LMs in terms of coordinate value, no statistical difference was observed between sexes ($P>0.05$).

Discussion

In the study, the shape difference of skull and mandible in Awassi sheep breed with a common breeding potential was analyzed using geometric morphometric methods based on sex factor. There are classic morphometric studies in the literature conducted on sheep skull and mandible related to sexual dimorphism [20,21]. However there is only one study examining sheep skull using geometric morphometric analysis [9].

Jaslow [22] stated that horn affected the cranial morphology and craniometry significantly in wild sheep [23]. Also, in the present study, LMs were selected cautiously from both lateral and dorsal sides for geometric morphometric method in order to minimize the effect of horn.

Sexual dimorphism is one of the most interesting phenotypic variation sources in animals and plants. Sexual differences in morphological characteristics in animal taxonomy are a general phenomenon. The most apparent one of this phenomenon is the body size difference based on sex [23]. There are important results of size difference based on sex for ecology, behavior, generation mobility and evolution [24]. Although it is stated in the literature [25] that sexual dimorphism is significant in sheep, it is important to analyze the points apart from horn and the shape of mandible based on sheep breeds, especially in skull. Abbasabadi et al.[21] stated that there was no sexual dimorphism in skull of Zell sheep using classic morphometric method, Pares-Casanova et al.[26] stated that there was no sexual dimorphism in skull of Gwembe-Dwarf goat using classic morphometric method and Pares-Casanova [9] expressed that there was no sexual dimorphism in skull of Fardasca sheep using geometric morphometric method. Pares-Casanova et al.[27] stated in their study conducted by geometric morphometric method that the skull of White Rasquera goats included sexual dimorphism. Therefore, in the present study, it was observed that skull of Awassi sheep had an apparent differentiation from the lateral side compared to the dorsal side. In their study, Yalçın et al.[11] reported that gender dimorphism was not observed in mandible of Anatolia Wild sheep. Likewise, in the present study, the grouping based on gender in the mandible was not apparent.

Pares-Casanova [5] used the crania of 16 ewe and 2 ram individuals of native sheep breed in his study. A limited number of male individual materials was remarkable. In the present study, 17 samples (10 ewe, 7 ram) were used from the dorsal side and 16 samples (9 ewe, 7 ram) were used from the lateral side. Also, the geometric morphometric analysis of mandible was performed.

Pares-Casanova [9] reported in his study that the first three principal components (PC) accounted for 63.68% (PC1: 30.43%, PC2: 18.77%, PC3: 14.47%) of the total shape variation in Fardasca sheep skull. In the present study, it was determined that the first three principal components explained 68.97% (PC1: 44.14%, PC2: 14.53%, PC3: 10.2%) of the total shape variation from the lateral side in skull of Awassi sheep.

The data obtained from the archaeological bone residues is important as it allows the estimation of the morphological characteristics of animals, determination of fauna or other socio-economic comparisons [27-30]. The morphological data to be obtained in the skull and mandible of the living mammals through geometric morphometric method may be used to reveal the phylogenetic relations [31]. For this reason, the information obtained in the present study is important as they provide basic shape information for the small ruminant skull or mandible remains to be found in the archaeological excavations especially in Mesopotamia region including Gobeklitepe.

In their study, Yalçın and Kaya [14] compared Akkaraman and Anatolian Wild Sheep crania by taking 13 LM from the dorsal side using a geometric morphometric method. It was stated in this study that the first and second principal components accounted for 58.55% and 11.75% of the total shape difference, respectively. In the present study, the first and second principal components accounted for 37.72% and 27.84% of the total shape difference from dorsal side between sexes in Awassi sheep, respectively. These rates showed that skull of Awassi sheep had an apparent difference from the dorsal side based on sex.

Yalçın et al.[11] reported in their study that the difference in mandibles at LM9 level is quite apparent and this may be associated with the differences such as environmental conditions and feeding habits as well as adaptation to the domestication process. In the present study, although it was observed that the most apparent differences in mandible were at levels of LM2, 8, 9 and 10, the differences were quite limited.

Consequently, Awassi sheep skull and mandible were analyzed in this study using geometric morphometric methods in terms of presence of sexual dimorphism. In the study, it was an important finding that Awassi sheep crania formed groups considerably as ram and ewe from both the dorsal and lateral sides in principal components and relative warp analyses. However, the mandibles did not exhibit an apparent difference in the same analyses based on sex. It is considered that the data found in this study would contribute to the possible ruminant skull and mandible studies by using geometric morphometric method. Also, we think that these data would be useful in the distinction of the cranium remains uncovered in zooarcheological excavations and, especially assessed as ovicapri (sheep-goat).

Author Contributions

Demircioğlu İ and Demiraslan Y designed and directed

the study. Dayan MO, Demiraslan Y and Gürbüz İ conducted geometric morphometric application. Demircioğlu İ and Demiraslan Y provided Awassi sheep skulls. Demircioğlu İ, Demiraslan Y and Gürbüz İ and Dayan MO cowrote the overall paper.

Conflicts of Interest

The authors declare no conflict of interest.

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RESEARCH ARTICLE

In Vitro Effect of *Pelargonium sidoides* on Promastigote Forms of *Leishmania infantum* and *Leishmania tropica*

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Abstract

Leishmaniasis is recognized as a neglected disease by the World Health Organization (WHO). New treatment modalities are needed for the treatment of leishmaniasis due to the limited number of drugs that can cause toxic side effects. Therefore, studies are being carried out on herbal extracts, which can be potential candidates for the treatment. *Pelargonium sidoides* a perennial herb originating in Africa, is used to treat infectious diseases. The aim of this study was to perform *in vitro* investigation of the direct effect of *P. sidoides* commercially available root extract (EPs 7630) on promastigotes of *Leishmania infantum* and *Leishmania tropica*. For this purpose, *L. infantum* and *L. tropica* strains were grown on NNN medium and then transferred into RPMI 1640 medium supported by 10% fetal bovine serum. After mass growing, the promastigotes were placed into 96-well plates with *L. infantum* as $5x10^4$ and L. tropica as $1.5x10^5$. EPs 7630 was diluted at a concentration of 400, 200, 100 and 50 µg/mL. Afterwards, EPs 7630 was added and then counted by hemocytometry at 24, 48, 72, and 96 h. The calculations were done after the experiments repeated three times. Comparison with the control group and liposomal amphotericin B showed that EPs 7630 had no inhibitory effect on the growth of *Leishmania* promastigotes at the concentrations of 50 and 100 µg/mL, a partial inhibitory effect at 200 µg/mL, and an inhibitory effect at 400 µg/mL. It was concluded that identifying the substance(s) responsible for the antileishmanial effect of *P. sidoides* extract, conducting toxicity studies, and improving the results of these studies in *in vivo* models may be useful as steps for future clinical studies.

Keywords: *Pelargonium sidoides, Leishmaniasis, in vitro, Antileishmanial agent*

Pelargonium sidoides'in *Leishmania infantum* ve *Leishmania tropica* Promastigot Formlarına *İn Vitro* Etkisi

Öz

Leishmaniasis Dünya Sağlık Örgütü (DSÖ) tarafından ihmal edilmiş bir hastalık olarak kabul edilmektedir. Leishmaniasis tedavisinde kullanılan ilaçların sınırlı, maliyeti yüksek, toksik ve yan etkileri bulunması sebebiyle yeni tedavi yöntemleri geliştirilmeye ihtiyaç duyulmaktadır ve bu kapsamda bitkisel ekstraktlar üzerinde çalışmalar yapılmaktadır. Afrika kökenli çok yıllık bir bitki olan *Pelargonium sidoides* birçok hastalığı tedavi etmek için kullanılmaktadır. Bu çalışmada *Leishmania infantum* ve *Leishmania tropica* promastigotları üzerinde ticari olarak mevcut *P. sidoides* kök ekstraktının (EPs 7630) doğrudan etkinliğinin *in vitro* olarak araştırılması amaçlanmıştır. Bu amaçla *L. infantum* ve *L. tropica* suşları NNN besiyerinde üretildikten sonra %10 fetal sığır serumu eklenen RPMI 1640 besiyerine aktarıldı. Çoğaltıldıktan sonra promastigotlar, *L. infantum* için $5x10^4$ ve *L. tropica* için $1.5x10^5$ olacak şekilde 96 oyuklu plakalara yerleştirildi. Daha sonra, EPs 7630'un 400, 200, 100 ve 50 µg/mL konsantrasyonları ile 24, 48, 72 ve 96 saat inkübe edildi. EPs 7630'un *L. infantum* ve *L. tropica* suşlarının promastigotları üzerine etkinliği hemositometri yöntemi ile sayılarak belirlendi. Hesaplamalar, deneyler üç kez tekrarlandıktan sonra yapıldı. Kontrol grubu ve lipozomal amfoterisin B ile karşılaştırıldığında EPs 7630'un 50 ve 100 µg/mL konsantrasyonlarda *Leishmania* promastigot üremesi üzerinde inhibe edici etkisinin olmadığı, 200 µg/mL'de düşük inhibitör etkili olduğu ve 400 µg/mL'de etkili olduğu saptanmıştır. EPs 7630'un anti-leishmanial etkisinden sorumlu madde veya maddelerin incelenmesi, toksisite çalışmalarının yapılması ve bu çalışma sonuçlarının *in vivo* modellerle geliştirilmesinin klinik çalışmalara basamak olması açısından yararlı olabileceği düşünülmüştür.

Anahtar sözcükler: *Pelargonium sidoides, Leishmaniasis, in vitro, Anti-leishmanial ajan*

INTRODUCTION

Leishmaniasis is a disease caused by protozoan parasite *Leishmania* spp., which is transmitted by the bite of the insect vector female sand fly *(Phlebotomus* spp./*Lutzomyia* spp.). Leishmania is a genus in the order of Kinetoplastida and in the family of Trypanosomatidae [1,2]. Leishmaniasis is seen in 97 countries in the tropical and subtropical regions

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of America, Africa, Asia, and Europe [3]. There are four main clinical forms of leishmaniasis: Visceral leishmaniasis (VL), cutaneous leishmaniasis (CL), mucocutaneous leishmaniasis (MCL) and Post-Kala-Azar Dermal Leishmaniasis (PKDL) [4]. It is estimated that there are seven hundred thousand to 1.5 million new cases of leishmaniasis in the world each year causing 26.000 to 65.000 deaths [5]. Leishmaniasis is accepted as a neglected tropical disease by World Health Organization (WHO) [6]. Pentavalent antimony compounds, amphotericin B and liposomal amphotericin B, miltefosine, paromomycin, and pentamidine are used in the treatment of different clinical forms of leishmaniasis [7]. Today, antimony compounds accepted as the gold standard in the treatment of leishmaniasis [8]. The very limited number of drugs and the development of resistance pose big problems in the treatment of leishmaniasis. New treatment modalities are being investigated because of the toxicity potential, high cost, and drug resistance of existing drugs [9]. Therefore, in recent years, herbal extracts have been emphasized as an alternative treatment option [10].

In this study, we aimed to conduct an *in vitro* investigation to understand the efficacy of *Pelargonium sidoides* root extract (EPs 7630) on promastigotes of *Leishmania infantum* and *Leishmania tropica*.

Material and Methods

Supply and Cultivation of Leishmania spp. Strains

In this study, *Leishmania infantum* strain (confirmed patient's isolate) was obtained from the National Parasitology Reference Laboratory of the General Directorate of Public Health belonging to Turkish Ministry of Health and *L. tropica* strain (EP 200) from Ege University Faculty of Medicine, Department of Medical Parasitology.

The NNN medium was prepared with a mixture of 3.5 g Bacteriological Agar (Oxoid, UK) and 2.5 g sodium chloride (Carlo Erba Reagents, Italy) in 230 mL distilled water in a 500 mL bottle and then heated to dissolve the agar. The solution was sterilized for 20 min at 121°C in an autoclave device (ALP, Japan) and cooled to 55°C in a hot water bath (Nüve ST30, Turkey). Then, 0.6 mL of penicillin G, 0.6 mL streptomycin sulfate, and 70 mL of defibrinated horse blood, which was aseptically collected, were added to the medium. Thus, final concentrations were penicillin G 200 IU/mL and streptomycin sulfate 200 µg/mL in the medium. The medium was then dispensed in a volume of 4.5 mL into sterilized conical centrifuge tubes (ISOLAB GmbH, Germany), and the medium was allowed to freeze in a 10° inclined position and stored at +4°C.

The *Leishmania* strains supplied were inoculated on NNN Medium and left to incubate at 24°C. After mass growing of promastigotes, they were transferred from NNN medium to cell culture flasks containing RPMI 1640 (Sigma R8755-1L, Germany) supported by 10% Fetal Bovine Serum, HEPES and 80 µg/mL gentamicin. Flasks were checked for viability of promastigotes under an inverted microscope (Leica S40/0.45, Germany). It was observed that the *L. infantum* promastigotes reached the logarithmic phase on the third day and *L. tropica* promastigotes on the second day. Accordingly, *L. infantum* promastigotes were included in the study on the third day and *L. tropica* promastigotes on the second day.

Prior to the study, the initial promastigote counting was done on a Thoma counting slide (Witeg, Germany). Thus, using the Hemocytometer Sidekick application, *L. infantum* was counted as $5x10^4$ cells and *L. tropica* as $1.5x10^5$ cells. The IC_{50} value was calculated using AAT Bioquest software *(https://www.aatbio.com/tools/ic50 calculator)*.

Herbal Extract

In this study, the EPs 7630 (Dr. Willmar Schwabe, GmbH&Co. KG, Germany) standard solution was used as a root liquid extract of *P. sidoides*. The main stock solution at a concentration of 800 µg/mL was diluted into herbal extract solutions at 400, 200 and 100 and 50 µg/mL concentrations. Lipo-somal Amphotericin B (L-AmB) (Gilead Sciences, Inc., USA), known to be effective on *Leishmania* species, was used as a positive control in the present study. The dilution was made in conical centrifuge tubes to prevent *Leishmania* promastigotes from being damaged during pipetting.

In this study, 96-well flat-bottom cell culture plates (Deltalab S.L., Spain) were used. Nine study groups were designed. Firstly, 100 µL of media containing promastigotes from previously prepared cell culture flasks were placed in all wells. Different concentrations of *P. sidoides* and L-AmB were added to each well based on the study groups. Only RPMI 1640 medium was added to control-1 group and a solvent to control-2 group. The distribution and quantities among the groups are summarized in *Table 1*.

The cell culture plates were wrapped in a plastic film (Parafilm, 3M) and left to incubate in a 24°C incubator (Nüve N500, Turkey).

In this study, *P. sidoides* herbal extract was studied in triplicate for all groups on *L. infantum* and *L. tropica* strains.

Hemocytometry

Samples at a volume of 10 µL were taken from each well at 24, 48, 72, and 96 h, respectively, from cell culture plates incubated at 24°C were counted on Thoma chamber (Witeg, Germany) and calculated and recorded using the Hemo-cytometer Sidekick application. Comparison of efficiency between groups was performed using one-way analysis of variance (ANOVA).

Table 1. *Distribution of concentrations of P. sidoides, L-AmB, and solvent by groups*

Groups		*Leishmania infantum* (Initial count: 5x10⁴)	*Leishmania tropica* (Initial count: 1.5x10⁵)
1	Ps	50 µg/mL	50 µg/mL
2	Ps	100 µg/mL	100 µg/mL
3	Ps	200 µg/mL	200 µg/mL
4	Ps	400 µg/mL	400 µg/mL
5	Control 1	Promastigote medium	Promastigote medium
6	Control 2	Promastigote medium + solvent (11 µL/mL 70% ethanol)	Promastigote medium + solvent (11 µL/mL 70% ethanol)
7	L-AmB	12.5 µg/mL	12.5 µg/mL
8	L-AmB	25 µg/mL	25 µg/mL
9	L-AmB	50 µg/mL	50 µg/mL

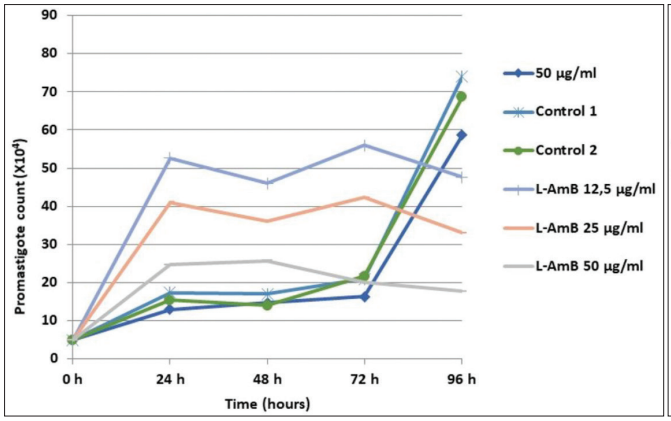

Fig 1. The effects of EPs 7630 on *Leishmania infantum* promastigotes at 50 µg/mL

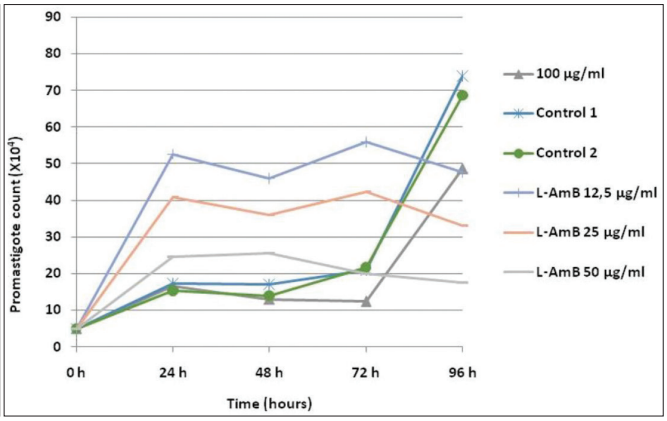

Fig 2. The effects of EPs 7630 on *Leishmania infantum* promastigotes at 100 µg/mL

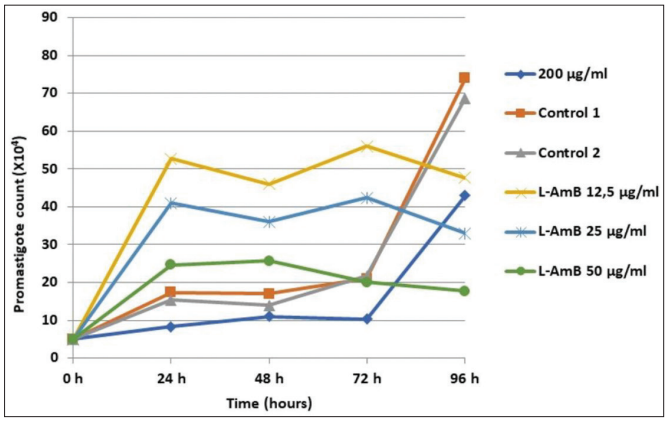

Fig 3. The effects of EPs 7630 on *Leishmania infantum* promastigotes at 200 µg/mL

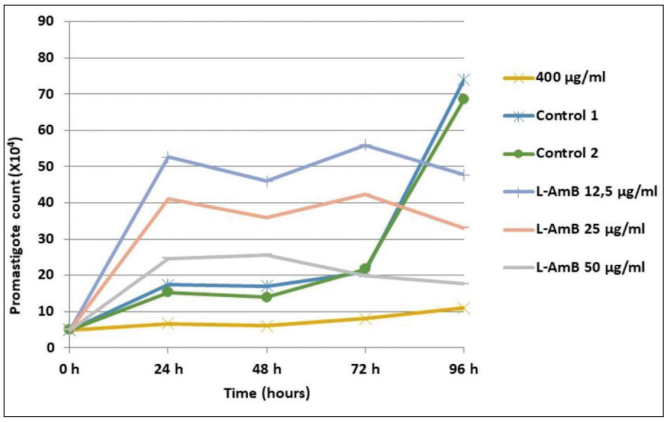

Fig 4. The effects of EPs 7630 on *Leishmania infantum* promastigotes at 400 µg/mL

Results

Samples taken from the wells at 24, 48, 72, and 96 h were counted, followed by determination of the growth rates.

At the end of 96 h of incubation at 24°C, it was found that *P. sidoides* did not have a significant inhibitory effect on the growth of *L. infantum* promastigotes at concentrations of 50 and 100 µg/mL compared to the control groups ($P>0.05$) *(Fig. 1* and *Fig. 2)*.

At the end of 96 h of incubation, *P. sidoides* was observed to significantly inhibit growth of *L. infantum* at a concentration of 200 µg/mL and 400 µg/mL compared to the control groups ($P<0.05$) *(Fig. 3* and *Fig. 4)*.

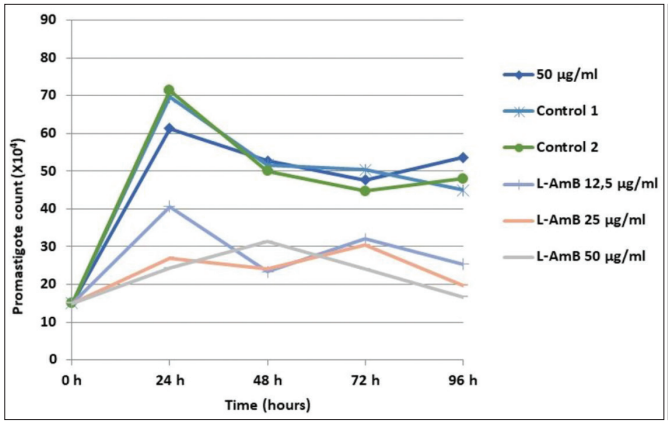

Fig 5. The effects of EPs 7630 on *Leishmania tropica* promastigotes at 50 µg/mL

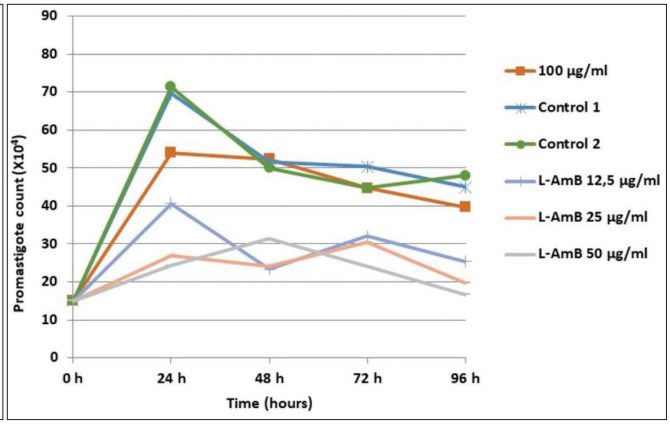

Fig 6. The effects of EPs 7630 on *Leishmania tropica* promastigotes at 100 µg/mL

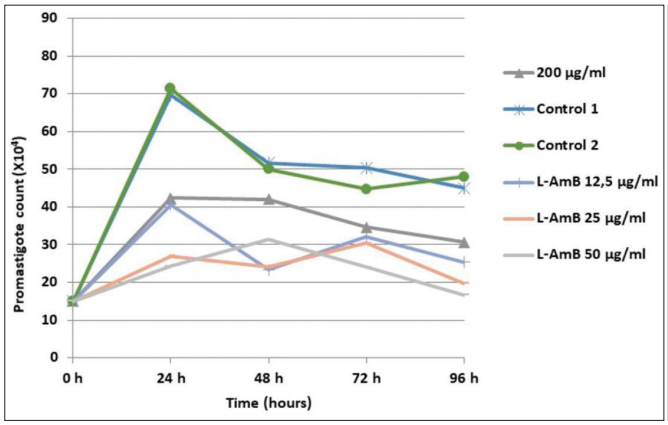

Fig 7. The effects of EPs 7630 on *Leishmania tropica* promastigotes at 200 µg/mL

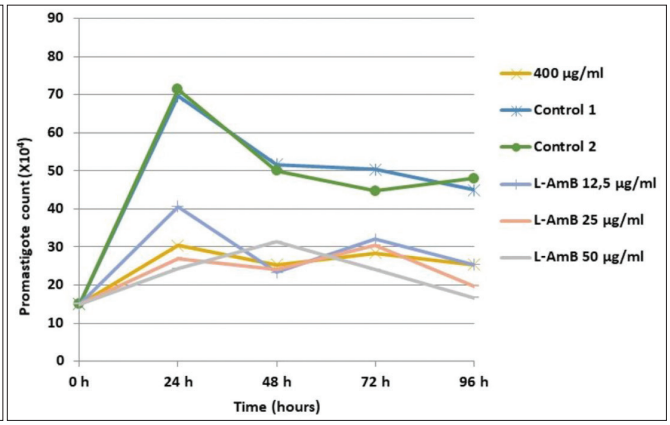

Fig 8. The effects of EPs 7630 on *Leishmania tropica* promastigotes at 400 µg/mL

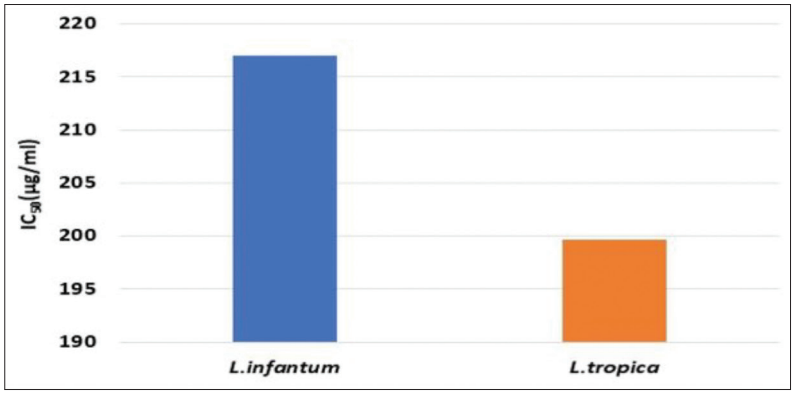

Fig 9. IC_{50} values of EPs 7630 on *L. infantum* and *L. tropica* promastigotes

At the end of 96 h of incubation at 24°C, it was found that *P. sidoides* extracts did not have a significant inhibitory effect on the growth of *L. tropica* promastigotes at concentrations of 50 and 100 µg/mL compared to the control and positive control groups (P>0.05) *(Fig. 5* and *Fig. 6)*.

It was observed that EPs 7630 significantly inhibited the growth of *L. tropica* at concentrations of 200 µg/mL and 400 µg/mL at the end of 96 h of incubation (P<0.05) *(Fig. 7* and *Fig. 8)*.

It was calculated that EPs 7630 had an IC_{50} = 217.0018 µg/mL on *L. infantum* and IC_{50} = 199.6707 µg/mL on *L. tropica* promastigotes *(Fig. 9)*.

The contribution of the solvents (ethanol) used for the dissolution of *P. sidoides* to the inhibitory effect on promastigotes was analyzed and no significant inhibitory effect was found (P>0.05).

Discussion

Leishmaniasis is a major public health problem especially in Asian, African and Latin American countries [1]. In recent years in Turkey, there has been persistent leishmaniasis cases after migration of Syrian refugees [11]. The limited number of drugs such as pentavalent antimony compounds and amphotericin B are used in the treatment of leishmaniasis. The cytotoxic effects of these drugs have led to the emergence of new drug searches.

Herbal medicines are extensively used in many countries. *P. sidoides* is a perennial herb found in the Eastern Cape Province of South Africa and the Lesotho highlands. It is used by people living in those area to treat various diseases, including diarrhea, colic, gastritis, tuberculosis, cough, liver disorders, menstrual complaints, and gonorrhea [12]. It has been reported that the antiviral effect of EPs 7630 is related to the production of interferons, which has been reported on viruses such as influenza, parainfluenza, respiratory syncytial virus (RSV), rhinovirus, coxsackie, and coronavirus [13].

Many different herbal materials have been used experimentally for the treatment of *L. donovani* and *L. tropica* [14-19].

The mechanism of anti-leishmanial action of L-AmB is thought to be drug-binding to *Leishmania* sp. ergosterol precursors causing degradation of the parasite membrane. Several *in vitro* studies have reported the effectiveness of L-AmB against *Leishmania* species. Piñero et al.[20] evaluated the *in vitro* activity of L-AmB compared to amphotericin B on different strains of *L. infantum* isolated from HIV (+) patients and noted a higher efficiency of L-AmB on promastigotes. In our study, we compared 400 µg/mL *P. sidoides* and 50 µg/mL L-AmB as a positive control on *L. infantum* and *L. tropica* promastigotes, and reported higher effectiveness of *P. sidoides* compared to L-AmB at 96 h ($P<0.05$) *(Fig. 4)*.

EPs 7630 is widely used in phytotherapy practice for therapeutic purposes. Various scientific studies have been conducted on its antibacterial and antiviral effects. In the present study, we investigated concentration-dependent *in vitro* inhibitory effect of EPs 7630 on *L. infantum* and *L. tropica* promastigotes. Ethanolic standardized extract of *P. sidoides* roots (EPs 7630) used in our experiments. When it was compared to control 1 and control 2 group (only solvent), *P. sidoides* was significantly inhibitory effect on *L. infantum* and *L. tropica* promastigotes at concentrations of 200 µg/mL and 400 µg/mL (IC_{50} = 217.0018 µg/mL and IC_{50} = 199.6707 µg/mL respectively) ($P<0.05$).

It has been reported in some studies that *P. sidoides* may not be directly effective on microorganisms. However, it has been determined that the plant is effective by disrupting the receptors and enzymes that microorganisms bind to the host cell [12]. Thäle et al.[21] investigated the effects of induced nitric oxide production on *L. major* in infected macrophages and showed that EPs 7630 alone had a lower anti-infective effect compared to its combination with IFN-gamma. In the same study, they noted that a single dose of 10 µg/mL EPs 7630 did not have any direct effect on the viability of promastigotes after 48 h. However, in our study, we found a significantly higher efficacy at higher concentrations, especially at 400 µg/mL, at 96 h. The effect of *P. sidoides* on *Leishmania* species may be mediated by the active substances, epigallocatechin and gallocatechin [22]. Also, cytotoxic effect of EPs 7630 was investigated and reported that neither EPs 7630 nor phenolic compounds, including benzoic and cinnamic acid derivatives, hydrolysed tannins and C-glycosylflavones, exhibited any cytotoxic effects [23,24]. Based on these studies, the LD_{50} value of EPs 7630 of >1000 µg/mL eliminated the expectation of any cytotoxic effect at the concentrations in our study.

As a conclusion, we detected direct antileishmanial activities of EPs 7630 against *L. infantum* and *L. tropica* promastigotes. Herbal sources gain importance in treatment applications because of their easy tolerability and fewer side effects compared to synthetic drugs. Although the screening and purification of bio-compounds from multi-molecular plant extracts requires a lot of time, planning, and cost, there is hope for further advancement in this area to effectively treat patients. According to studies, phyto-therapeutics provide a broad and promising perspective for new, safe and effective antileishmanial agents [25]. This study determined the direct efficacy of EPs 7630 solution against *Leishmania* promastigotes. It has been concluded that it may be an alternative treatment option in the future due to its lower toxic effects compared to many drugs in routine clinical practice.

Further studies are needed to identify the substance(s) responsible for the antileishmanial effect of *P. sidoides* and to perform combined with other antileishmanial compounds. Development of further *in vivo* models may be useful as a stepping stone for future clinical studies.

Acknowledgments

We would like to express our very great appreciation to Dr. Cahit Babür and Prof. Dr. Seray Töz for providing the *Leishmania* strains of this research work.

Conflicts of Interest Statement

The authors declare that there is no conflict of interest.

Author Contributions

MH and EB designed the project. MH and EB provided *Leishmania infantum* and *L.tropica* strains. NG and MH carried experiments. EB, NG, FK and MH performed statistical analysis of data and wrote the article.

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RESEARCH ARTICLE

Response of Probiotics and Yeast Added in Different Doses to Rations of Anatolian Merino Lambs on Fattening Performance, Meat Quality, Duodenum and Rumen Histology [1]

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Abstract

This study investigated the effects of dietary probiotics *(Lactobacillus reuteri* E81 [LRE], *Lactobacillus rhamnosus* GG [LRG]), yeast *(Saccharomyces cerevisiae* S81 [SCS]), and their combined supplementation on fattening performance (BW, DWG, FI, and FCR), meat quality, and rumen and duodenum histology in lambs. The study material comprised ninety 2.5-month-old Anatolian Merino lambs, and the trial was conducted for 70 days. Nine trial groups, each composed of 10 animals, were established. This study demonstrated that, when compared to the control group, the best fattening performance was achieved in the lambs that received 600 ppm of dietary LRE. Neither visceral organ weights nor rumen and duodenum histology was affected in the groups that received the tested feed supplements. Of the meat colour parameters investigated, the L* value was observed to have increased in the groups that were given feed supplements, excluding Groups LRE-600 and SCS-300. It was determined that the probiotic supplements had no effect on the a* and b* colour parameters, but affected the meat pH value. In conclusion, the assessment of the effects of different doses of dietary probiotics, yeast, and probiotic-yeast combinations on performance parameters, visceral organ weights, and meat quality in Anatolian Merino lambs showed that the best results were achieved in the group that received 600 ppm of LRE alone.

Keywords: *Probiotic, Yeast supplementation, Anatolian Merino lamb performance, Feed additive, Meat quality, Histology*

Anadolu Merinos Kuzularının Rasyonlarına Farklı Dozlarda İlave Edilen Probiyotiklerin ve Mayanın Besi Performansı, Et Kalitesi, Rumen ve Duodenum Üzerine Yanıtı

Öz

Yapılan çalışmada, kuzu rasyonlarına probiyotik *(Lactobacillus reuteri* E81 [LRE], *Lactobacillus rhamnosus* GG [LRG]), maya *(Saccharomyces cerevisiae* S81 [SCS]) ve karışımlarının ilavesinin besi performansı (CA, GCAA, Yem Tüketimi ve YYO) et kalitesi, rumen ve duodenum histolojisi üzerine etkisi araştırılmıştır. Araştırmada 2.5 aylık 90 adet Anadolu Merinosu koyun kullanılmış, çalışma 70 gün sürmüştür. Deneme her grupta 10'ar hayvan olacak şekilde 9 farklı gruptan (Kontrol, LE-300, LE-600, LR-300, LR-600, SC-300, SC-600, MİX-300 ve MİX-600) oluştu. Araştırma sonunda besi performansı üzerine kontrol grubuna kıyasla en iyi sonuçlar *L. reuteri* E81 600 ppm katkılı grupta elde edilmiştir. İç organ ağırlığı ve duodenum ile rumen histolojisi üzerine katkılı grupların etkisi olmazken, et renk parametreleri üzerine kontrol grubuna kıyasla L* parametresinde LRE 600 ve SCS 300 dışındaki gruplarda artış gözlenmiştir. Probiyotik uygulamasının, a* ve b* renk parametreleri üzerine etkisi olmazken, et pH değeri üzerinde oldukça etkili olduğu tespit edilmiştir. Sonuç olarak kuzu rasyonlarına ilave edilen probiyotik, maya ve karışımlarının besi performansı (CA, GCAA ve YYO), iç organ ağırlıkları ve et kalitesi üzerine en iyi sonuçlar *L. reuteri* E81 600 ppm gruplarda elde edilmiştir.

Anahtar sözcükler: *Probiyotik, Maya takviyesi, Anadolu Merinos kuzu performansı, Yem katkı maddesi, Et kalitesi, Histoloji*

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Introduction

The majority of the proteins required by the human body must be acquired by the consumption of animal proteins. The rapid growth of the global population hinders the access of some people to the proteins they need. Although there is continual research on how to increase the quantity and quality of food products in line with consumer preferences, a supply and demand equilibrium has not yet been established. Furthermore, the food safety approach, from the farm to the fork, requires the maintenance of the health of food-producing animals [1,2].

The gastrointestinal tract of newborn ruminants does not contain any microorganisms and is sterile. The rumen microbiota starts to form immediately after birth. As the animal grows, a microbial ecosystem containing very high concentrations of bacteria develops in the rumen [3]. The ruminal microbiota is very sensitive in the neonatal period and can be easily harmed by several factors. Changes in the rumen microbial environment can cause performance and health problems in most ruminants [4]. In this context, the incorporation of feed supplements in the ration of newborn lambs is aimed at enabling weight gain and supporting rumen development at the time of weaning [5]. For many years, antibiotics were used for these purposes, as growth promoters at sub-therapeutic doses. However, through legislative regulations, the European Union (EU) has banned the use of chemotherapeutic agents for these purposes, necessitating the development of new feed additives and alternative feeding and animal health strategies [6]. Listed among the newly developed alternative feed additives, probiotics are products that contain viable microorganisms, which effectively increase intestinal health by regulating the balance of intestinal microflora when received in sufficient amounts [7]. Research has shown that depending on the type and dose, probiotics increase performance [8,9], maintain microbial balance in the gastrointestinal tract [10], strengthen immune function [11], reduce stress [12], and increase food digestibility [13], intestinal microflora modulation [14], pathogen inhibition [15], immunomodulation and intestinal mucosal immunity [16]. Meat quality is affected by several parameters such as pre- and post-slaughter conditions, glycogen deposition [17], sex, breed, weight, and diet [18]. It is considered that probiotics may affect meat quality via their effects on animal health. However, the effect of probiotic supplementation on meat quality remains unclarified [19,20].

The objective of this study was to investigate the effect of different doses (300 ppm and 600 ppm) of dietary probiotics *(Lactobacillus reuteri* E81 [LRE], *Lactobacillus rhamnosus* GG [LRG]), yeast *(Saccharomyces cerevisiae* S81 [SCS]), and combined probiotic + yeast supplementation on fattening performance (body weight [BW], daily weight gain [DWG], feed conversion ratio [FCR], and feed intake [FI]), visceral organ weights, rumen and duodenum histology (villus height [VH], villus width [VW], intestinal crypt depth [CD], tunica muscularis width [TMW], papilla ruminis height [PRH], papilla ruminis width [PRW], lamina propria width [LPW]), meat colour parameters, and pH value in lambs.

Material and Methods

Ethical Approval

This study was conducted according to the approval (dated 18.07.2019 and numbered 2019/12) of the Local Ethics Board for Animal Experiments of the Directorate of the Central Veterinary Control and Research Institute.

Lambs, Diet, and Experimental Design

The animal material of this study comprised 90 male Anatolian Merino weanling lambs, which were 2.5 months old (24.98±6.02) and raised under intensive breeding conditions at a private farm located in the central district of the Bayburt province. The pedigree and breeding records of the farm were regularly inspected. This study was conducted for a 56-day period that began after a fourteen-day acclimatization period of the animals at the Food, Agriculture, and Livestock Research and Application Centre of Bayburt University. After being weaned, lambs of the almost similar with each other body weight were assigned to nine groups, each including 10 animals Control (C = basal diet), LRE-300 (basal diet + 300 ppm *L. reuteri* E81), LRE-600 (basal diet + 600 ppm *L. reuteri* E81), LRG-300 (basal diet + 300 ppm *L. rhamnosus* GG), LRG-600 (basal diet + 600 ppm *L. rhamnosus* GG), SCS-300 (basal diet + 300 ppm *S. cerevisiae* S81), SCS-600 (basal diet + 600 ppm *S. cerevisiae* S81), MIX-300 (basal diet + 300 ppm L. reuteri E81 + 300 ppm *L. rhamnosus* GG + 300 ppm *S. cerevisiae* S81), and MIX-600 (basal diet + 600 ppm *L. reuteri* E81 + 600 ppm *L. rhamnosus* GG + 600 ppm *S. cerevisiae* S81). Excluding those included in the control group, the animals were given a daily amount of feed that contained feed supplements. Each day, at the same time (08:00 pm), the feed remaining in the feeders was weighed, and then replaced with new feed. The lambs were provided with a basal lamb ration in concentrated pellet form, the nutrients, and energy value of which are presented in *Table 1*. The basal ration, purchased from a private feed mill in the Balıkesir province, contained added probiotic *(L. reuteri* E81 [LRE], *L. rhamnosus* GG [LRG]), yeast *(S. cerevisiae* S81 [SCS], ($4x10^{10}$ CFU/g), or combined probiotic + yeast supplements, which were produced at the Food Engineering Department of Bayburt University. The feed used in this study was analyzed following the analysis methods of the AOAC [21].

Performance Parameters

To monitor the effects of the probiotic and yeast supplements added to the lamb ration, and to determine the weight gain of the lambs, each animal was weighed individually,

Table 1. *Nutrient content of the basal diet ration (%)*

Raw Material	Lamb Ration Content
Barley	30
Corn	24
Soybean Meal	10
Wheat Bran	4
Cottonseed Meal	13
Molasses	8
Sunflower Meal	8
Premix	0.05
Salt	0.95
Dicalcium Phosphate	3
Dry Matter (%)	88
Crude Protein (%)	14
Crude Cellulose (%)	13
Crude Oil (%)	4.2
Ash (%)	9
ME (MJ/kg)	12,14

The vitamin and mineral premix provided the following (per kg): 4.000.000 IU vit. A, 800.000 IU vit. D_3, 5.000 IU vit. E, 400 mg vit. B_2, 2 mg vit. B_{12}, 5.000 mg vit. B_3, 1.000 mg D-pantothenic acid, 20.000 mg choline, 50 mg Co, 5400 mg Fe, 185 mg I, 6.900 mg Mn, 800 mg Cu, 6400 mg Zn, 14 mg Se

every 7 days, before being given feed in the morning. The average daily feed intake of the groups was designated by weighing the feed remaining in the feeders each morning, calculating the amount of feed consumed per week, and dividing the weekly amount of feed consumed by 7. The feed conversion ratio (FCR) was determined based on the proportion of daily FI to daily weight gain (DWG).

Visceral Organ Weights and Meat Quality Parameters

At the end of the experiment, a total of 27 lambs, including 3 randomly selected animals from each group, were sacrificed at the laboratory of the Food, Agriculture and Livestock Research and Application Centre of Bayburt University. The visceral organs of the sacrificed animals were weighed on a precision balance accurate to 0.001 g.

Meat quality parameters were investigated in 27 carcasses, including 3 from each group. Analyses were performed at the Food Engineering Department of Bayburt University on brisket and fat samples taken from carcasses aged 24 h.

The color parameters of the brisket and fat samples were taken from the lamb carcasses were determined using a colorimeter (CR-400, Minolta Co, Osaka, Japan). Colour saturation was determined according to the CIELAB space, based on three-dimensional colorimetry data, published by the International Commission on Illumination (CIE). Accordingly, colour saturation was assessed as follows: a*; a* = + 60 red, a*= - 60 green; b*; b* = + 60 yellow, b* = - 60 blue and L*; L* = 0 black, L* = 100 white (darkness/lightness). The pH values of the meat samples were determined by homogenizing 10 g of meat in 100 mL of distilled water with a laboratory homogenizer and using a pH-meter (Jenco Electronics 6173, Taiwan) calibrated with buffered solutions (pH 4.0 and pH 7.0).

Histomorphology

For histological analysis, at the end of the study period, three animals, randomly chosen from each group, were slaughtered. Tissue samples were taken from the duodenum and rumen and were fixed in 10% buffered formalin solution (saline). The tissues were dehydrated through a graded series of alcohol, cleared with xylene, and embedded in paraffin. Sections were cut at the 4-µm thickness and were stained with hematoxylin-eosin. The villus height (VH), villus width (VW), intestinal crypt depth (CD) and tunica muscularis width (TMW) values of the duodenum, and the papilla ruminis height (PRH), papilla ruminis width (PRW), lamina propria width (LPW), and tunica muscularis width (TMW) values of the rumen were measured in randomly selected five different areas of the duodenum and rumen specimens, using an oculometer at 10x and 20x magnification under a light microscope fitted with a stage micrometer. Each group was photographed using an Olympus BX-43 research microscope with an image analysis system (DP72-BSW). The nomenclature used in this study conforms to the Nomina Histologica.

Statistical Analysis

Firstly, a normality test was performed, and it was determined that the data were distributed normally. Variables are presented as means with standard errors. For data on rumen and duodenum histology, the non-parametric Kruskal-Wallis test was used since the number of samples did not provide any normality distribution. The Mann-Whitney U test with Bonferroni's correction was used as a post hoc test One-way ANOVA was utilized to determine the differences between the nine diet groups for FI, FCR, BW, and average DWG performances and meat colour. Duncan's multiple comparison test was performed for group means with a significance level of 0.05 using the IBM SPSS Statistics v25 software.

Results

The effects of the different doses of dietary probiotics, yeast, and combined probiotic + yeast supplementation on the performance of the lambs are shown in *Table 2* and *Table 3*. Data analysis demonstrated that, when compared to the control group, the best results for BW, DWG, and FCR were achieved in the groups that received 300 ppm and 600 ppm of dietary *L. reuteri* E81, and the results obtained in these two groups were found to be statistically significant ($P<0.05$). Nevertheless, no statistically significant difference was detected between the groups for feed intake and daily feed intake (FI) ($P>0.05$) *(Table 2, Table 3)*. Statistical analysis of visceral organ weight data showed

Table 2. *The effects of dietary probiotic, yeast, and combined probiotic and yeast supplementation on the fattening performance of lambs (Mean±SEM)*

Parameters	Control	LRE 300 ppm	LRE 600 ppm	LRG 300 ppm	LRG 600 ppm	SCS 300 ppm	SCS 600 ppm	Mix 300 ppm	Mix 600 ppm	P
BW (kg)	31.08±1.72^{dc}	38.44±1.69^{ab}	40.63±2.79^{a}	28.65±1.93^{ed}	36.25±1.95^{abc}	27.98±1.02^{ed}	18.97±1.15^{f}	34.50±2.21^{cb}	25.06±1.40^{e}	**
DWG (g)	0.27±0.03^{bc}	0.35±0.04^{ab}	0.41±0.04^{a}	0.28±0.03^{bc}	0.28±0.02^{bc}	0.16±0.02^{d}	0.17±0.02^{d}	0.33±0.02^{ab}	0.22±0.02^{dc}	**
FI (kg)	11.03±0.45	10.67±0.70	9.51±0.70	10.95±0.34	11.21±0.58	10.56±0.59	11.17±0.47	10.84±0.62	10.89±0.57	NS
FCR (kg/kg)	6.33±0.69^{b}	4.79±0.54^{bc}	3.26±0.26^{c}	6.00±0.64^{bc}	5.74±0.34^{bc}	10.19±0.30^{a}	10.09±1.75^{a}	4.81±0.34^{bc}	7.61±0.99^{ab}	**

*Means within the same column showing different superscripts are significantly different (P<0.05), * Significant at the 0.05 level, ** Significant at the 0.01 level,* ***NS:*** *Not significant (P>0.05),* ***SEM:*** *Standard error of the mean (Lactobacillus reuteri E81 [LRE]), Lactobacillus rhamnosus GG [LRG]), yeast (Saccharomyces cerevisiae S81 [SCS]),* ***BW:*** *Body Weight,* ***DWG:*** *Daily Weight Gain,* ***FI:*** *Feed Intake,* ***FCR:*** *Feed conversion ratio*

Table 3. *The effects of dietary probiotic, yeast, and combined probiotic and yeast supplementation on weekly weight gain (kg) in lambs (Mean±SEM)*

Weeks	Control	LRE 300 ppm	LRE 600 ppm	LRG 300 ppm	LRG 600 ppm	SCS 300 ppm	SCS 600 ppm	Mix 300 ppm	Mix 600 ppm	SEM
Week 1	24.93±1.72^{b}	31.14±3.11^{a}	31.39±5.14^{a}	21.28±3.57^{cd}	29.21±3.13^{a}	25.90±1.49^{b}	19.43±2.97^{d}	24.30±3.85^{cb}	14.91±1.30^{e}	**
Week 2	26.92±2.12^{c}	34.41±2.74^{a}	33.64±4.56^{a}	22.90±3.58^{ed}	30.70±2.82^{b}	28.01±2.21^{bc}	21.41±3.36^{e}	25.20±3.76^{cd}	15.90±1.40^{f}	**
Week 3	27.90±2.57^{c}	35.51±2.79^{a}	36.55±3.57^{a}	25.41±3.44^{c}	32.40±2.56^{b}	30.80±1.80^{b}	22.8±3.07^{d}	26.21±3.85^{c}	17.20±1.38^{e}	**
Week 4	29.80±3.09^{c}	38.00±2.50^{a}	40.01±3.24^{a}	27.30±3.77^{c}	34.60±2.24^{b}	33.00±1.59^{b}	24.50±2.87^{d}	27.50±3.68^{c}	17.80±1.38^{d}	**
Week 5	31.30±3.74^{d}	40.40±2.25^{b}	43.63±2.48^{a}	28.70±3.71^{e}	37.20±1.49^{c}	35.60±1.31^{c}	25.60±2.88^{f}	28.20±3.64^{e}	19.00±1.48^{h}	**
Week 6	32.70±4.67^{d}	43.51±3.36^{b}	48.12±2.66^{a}	31.51±4.27^{e}	39.40±2.13^{c}	38.20±2.23^{c}	26.50±2.91^{f}	29.00±3.78^{e}	20.20±3.78^{h}	**
Week 7	35.20±5.58^{d}	46.50±4.34^{b}	51.09±3.59^{a}	34±5.38^{d}	41.40±2.64^{c}	40.30±2.97^{c}	28.50±3.81^{e}	30.30±4.18^{e}	22.00±2.31^{f}	**
Week 8	37.60±6.23^{c}	49.01±4.78^{a}	53.37±4.57^{a}	36.00±6.82^{cd}	43.50±4.39^{b}	42.30±5.10^{b}	30.20±4.52^{e}	31.81±4.92^{de}	23.40±2.84^{f}	**

*Means within the same column showing different superscripts are significantly different (P<0.05), * Significant at 0.05 level, ** Significant at 0.01 level,* ***NS:*** *Not significant (P>0.05),* ***SEM:*** *standard error of the mean (Lactobacillus reuteri E81 [LRE]), Lactobacillus rhamnosus GG [LRG]), yeast (Saccharomyces cerevisiae S81 [SCS])*

Table 4. *The effects of dietary probiotic, yeast, and combined probiotic and yeast supplementation on lamb meat quality (Mean±SEM)*

Parameters	Variables	Control	LRE 300 ppm	LRE 600 ppm	LRG 300 ppm	LRG 600 ppm	SCS 300 ppm	SCS 600 ppm	Mix 300 ppm	Mix 600 ppm	P
Meat	L*	48.31±4.94^{ab}	51.53±5.05^{a}	49.0±3.21^{ab}	49.64±4.43^{a}	49.40±3.18^{a}	49.00±3.07^{ab}	51.12±1.62^{a}	50.00±2.57^{a}	45.99±3.25^{b}	*
	a*	22.20±1.92	20.30±3.41	20.46±2.21	22.28±2.27	22.70±2.36	22.02±2.25	22.97±1.77	23.07±3.21	23.13±3.64	NS
	b*	11.32±3.46	9.32±4.50	7.96±2.39	10.25±2.39	10.62±2.67	8.66±3.39	8.80±3.19	8.55±3.01	8.22±1.82	NS
Fat	L*	84.12±1.29^{ab}	79.94±1.62^{bc}	78.48±3.19^{c}	83.50±3.62^{ab}	84.46±4.14^{ab}	85.49±1.02^{a}	85.78±4.09^{a}	86.13±4.21^{a}	82.16±3.55^{abc}	**
	a*	4.25±1.95^{bcd}	7.14±0.91^{a}	5.24±1.63^{abc}	4.99±1.69^{bcd}	3.78±1.45^{dc}	5.27±0.88^{abc}	3.01±1.23^{d}	3.65±1.56^{dc}	5.99±0.82^{ab}	**
	b*	7.84±1.41	8.97±2.36	7.44±3.01	7.73±1.62	7.29±2.73	8.45±1.21	6.57±1.25	7.06±1.58	8.73±1.97	NS
pH		6.12±0.06^{b}	5.91±0.10^{bc}	5.87±0.09^{c}	5.98±0.08^{bc}	6.02±0.10^{bc}	6.01±0.21^{bc}	6.00±0.04^{bc}	5.96±0.08^{bc}	6.47±0.17^{a}	**

*Means within the same column showing different superscripts are significantly different (P<0.05), * Significant at 0.05 level, ** Significant at 0.01 level,* ***NS:*** *Not significant (P>0.05).* ***SEM:*** *Standard error of the mean (Lactobacillus reuteri E81 [LRE]), Lactobacillus rhamnosus GG [LRG]), yeast (Saccharomyces cerevisiae S81 [SCS])*

that combined dietary probiotic + yeast supplementation did not affect the skin, pluck (liver/heart/lungs), spleen and adrenal gland weights, and ruminal pH value *(Table 5)*.

The results obtained for meat colour parameters revealed that, when compared to the control group, the L* value was higher in the treatment groups, excluding Groups LRE-600 and SCS-300, but no effect was observed on the a* and b* colour parameters. The investigation of fat tissue colour parameters revealed an increase in the L* and a* values in Groups SCS-300, SCS-600, and MIX-300, a decrease in the same parameters in Groups LRE-300, LRE-600, and MIX-600, and no effect on the b* value in any of the treatment groups *(Table 4)*.

In the present study, the histological values of the duodenum histology were measured under a microscope. When compared to the control group, the treatment groups (A, B, C, Mix) showed no significant difference for villus height, villus width, and crypt depth *(Table 6)*. However, the width of the tunica muscularis had decreased in the control group *(Fig. 1-1a)*, and increased in Groups A, B, C, and Mix. The highest level of increase was detected in Group B 600 *(Fig. 1-1b)* and Mix 300 *(Fig.1-1c)*. There was no significant difference between the A, B, C, and Mix groups for the rumen measurements. However, the width of the tunica muscularis had decreased in the control group *(Fig. 1-1a)*, and increased in Groups A, B, C *(Fig. 1-3a)*, and Mix. The highest level of increase was detected in Group B 600

Table 5. *The effects of dietary probiotic, yeast, and combined probiotic and yeast supplementation on visceral organ weights (kg) and ruminal pH value in lambs (Mean±SEM)*

Parameters	Control	LRE 300 ppm	LRE 600 ppm	LRG 300 ppm	LRG 600 ppm	SCS 300 ppm	SCS 600 ppm	Mix 300 ppm	Mix 600 ppm	P
Head	2.51±0.48	2.67±0.29	2.83±0.12	2.71±0.30	2.67±0.25	2.67±0.29	2.50±0.61	2.48±0.22	2.02±0.11	NS
Feet	1.60±0.54	1.35±0.16	1.35±0.19	1.57±0.40	1.76±0.21	2.00±0.01	1.41±0.59	1.61±0.33	1.15±0.33	NS
Skin	5.00±1.00	6.00±1.00	6.33±0.57	6.33±0.57	6.00±1.00	5.66±1.52	5.00±2.00	5.16±1.25	4.33±0.57	NS
Pluck	1.76±0.48	1.96±0.39	2.23±0.11	2.10±0.22	1.75±0.49	2.24±0.46	1.78±0.39	1.97±0.30	1.92±0.86	NS
Liver	0.87±0.36	0.98±0.15	1.17±0.09	1.05±0.25	1.05±0.46	1.13±0.31	0.80±0.10	1.03±0.17	0.96±0.55	NS
Lungs	0.52±0.40	0.73±0.19	0.85±0.11	0.81±0.13	0.75±0.13	0.75±0.12	0.63±0.07	0.73±0.07	0.74±0.28	NS
Heart	0.19±0.01	0.22±0.06	0.21±0.05	0.22±0.02	0.19±0.04	0.24±0.03	0.15±0.01	0.41±0.42	0.21±0.03	NS
Kidneys	0.12±0.04	0.13±0.02	0.14±0.02	0.13±0.02	0.12±0.03	0.15±0.04	0.11±0.02	0.13±0.03	0.13±0.05	NS
Adrenal Glands	0.11±0.08	0.10±0.01	0.15±0.05	0.13±0.08	0.19±0.07	0.14±0.09	0.08±0.02	0.09±0.02	0.18±0.17	NS
Ruminal pH	5.24±0.14	5.46±0.04	5.35±0.31	5.61±0.86	5.49±0.25	5.57±0.13	5.40±0.35	5.66±0.52	5.58±0.63	NS
Spleen	0.09±0.01	0.09±0.01	0.12±0.02	0.09±0.01	0.09±0.01	0.10±0.01	0.10±0.02	0.11±0.03	0.11±0.03	NS

*Means within the same column showing different superscripts are significantly different (P<0.05), * Significant at 0.05 level, ** Significant at 0.01 level,* ***NS:*** *Not significant (P>0.05),* ***SEM:*** *Standard error of the mean (Lactobacillus reuteri E81 [LRE]), Lactobacillus rhamnosus GG [LRG]), yeast (Saccharomyces cerevisiae S81 [SCS])*

Table 6. *Intestinal morphology of the trial groups (Mean±SEM)*

Groups	VH	VW	CD	TMW
Control	1120.00±151.33	104.00±2.17	128.00±4.00	220.00±24.33^{a}
LRE300 ppm	1012.00±97.08	88.00±10.58	108.00±20.79	336.00±13.86^{bc}
LRE600 ppm	980.00±40.00	88.00±10.58	112.00±8.00	372.00±36.00^{bc}
LRG300 ppm	892.00±56.43	96.00±12.00	84.00±6.93	328.00±38.16^{bc}
LRG600 ppm	920.00±40.0	93.33±10.91	80.00±4.00	428.00±14.42^{c}
SCS300 ppm	980.00±40.00	72.00±0.00	108.00±12.00	356.00±28.00^{bc}
SCS600 ppm	864.00±13.86	88.00±10.58	92.00±10.58	280.00±34.18^{ab}
MIX300 ppm	972.00±12.00	96.00±0.00	84.00±6.93	380.00±58.92^{bc}
MIX600 ppm	900.00±120.00	112.00±8.00	104.00±4.00	400.00±8.00^{c}
P	NS	NS	NS	*

NS: *P>0.05, * P<0.05,* ^{a,b,c} *Means within the same column showing different superscripts are significantly different (P<0.05),* ***VH:*** *villus height,* ***VW:*** *villus width,* ***CD:*** *intestinal crypt depth,* ***TMW:*** *tunica muscularis width (Lactobacillus reuteri E81 [LRE], Lactobacillus rhamnosus GG [LRG]), yeast (Saccharomyces cerevisiae S81 [SCS])*

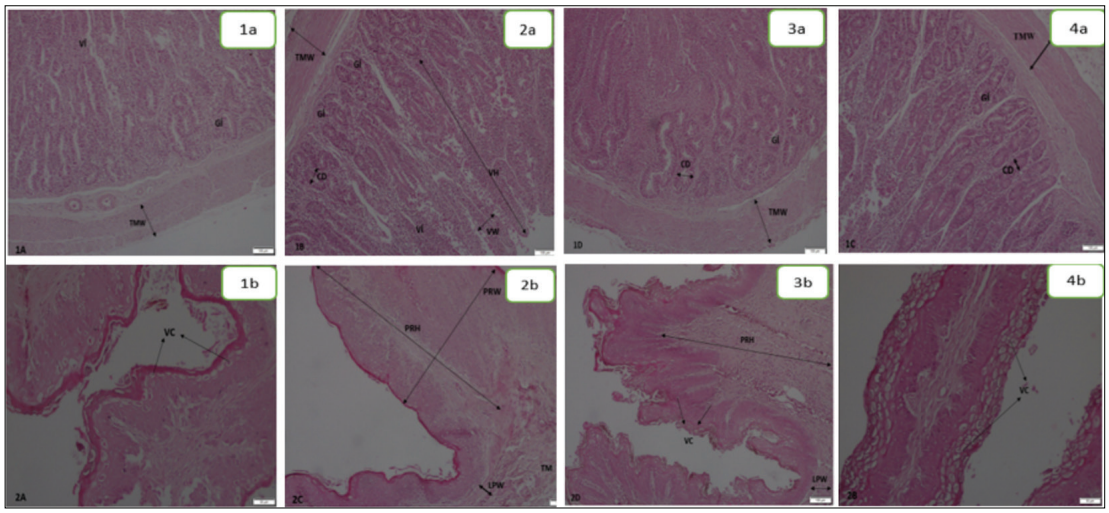

Fig 1. Photomicrographs of the duodenum (1a, 2a, 3a, 4a). TMW (Tunica muscularis width), GI (Glandula intestinalis), CD (Crypt depth). Haematoxylin-eosin, bar = 100 µm. Photomicrographs of the rumen (1b, 2b, 3b, 4b). *Black arrows:* VC (Vesicular cells). Haematoxylin-eosin, bar = 50 µm

Table 7. *Rumen morphology of the trial groups (Mean±SEM)*

Groups	PRH	PRW	LPW	TMW
Control	1540.00±530.28	560.00±66.57^{bc}	224.00±8.00	1000.00±105.83
LRE300 ppm	1360.00±80.00	516.00±12.00^{b}	240.00±0.00	976.00±112.00
LRE600 ppm	1360.00±80.00	464.00±8.00^{ab}	172.00±16.00	1072.00±136.00
LRG300 ppm	1440.00±334.07	452.00±22.27^{ab}	260.00±639.40	828.00±114.47
LRG600 ppm	1516.00±68.00	656.00±64.00^{c}	260.00±64.00	1008.00±72.00
SCS300 ppm	1400.00±160.00	520.00±8.00^{b}	108.00±24.00	1196.00±148.00
SCS600 ppm	1340.00±163.71	556.00±76.00^{bc}	188.00±22.27	1104.00±120.00
MIX300 ppm	1372.00±191.17	332.00±17.44^{a}	272.00±73.43	1024.00±209.04
MIX600 ppm	1420.00±100.00	376.00±8.00^{a}	304.00±52.00	920.00±260.00
P	NS	*	NS	NS

NS: *P>0.05,* ****** *P<0.05,* ^{a,b,c} *Means within the same column showing different superscripts are significantly different (P<0.05),* ***PRH:*** *papilla ruminis height,* ***PRW:*** *papilla ruminis width,* ***LPW:*** *lamina propria width,* ***TMW:*** *tunica muscularis width (Lactobacillus reuteri E81 [LRE]), Lactobacillus rhamnosus GG [LRG], yeast (Saccharomyces cerevisiae S81 [SCS])*

(Fig. 1-2a) and Mix 300 *(Fig.1- 4a)*. There was no significant difference between the A, B, C, and Mix groups for the rumen measurements. However, the papilla ruminis width (PRW) *(Table 7)* had significantly decreased in Groups Mix 300 *(Fig. 1-4b)* and Mix 600, and significantly increased in Group B 600 *(Fig. 1-2b)* with a moderate increase in the other groups *(Fig. 1-3b)*. Furthermore, it was ascertained that while only a few vesicular cells were observed in the rumen in the control group *(Fig. 1-1b)*, the number of these cells had significantly increased in all treatment groups, especially in the mix group *(Fig. 1-4b)*.

Discussion

The present study was aimed at determining the possible advantages that probiotic and yeast feed supplements may offer in increasing post-weaning life expectancy, fattening performance, and the marketability of animals, through the investigation of the effects of different doses (300 ppm and 600 ppm) of dietary probiotics *(L. reuteri* E81 and *L. rhamnosus* GG), yeast *(S. cerevisiae* S-81), and combined probiotic + yeast supplementation *(Table 2, Table 3)*. Some studies have reported that, when incorporated in lamb feed, probiotics increase feed intake, improve growth performance [22-24], support the improvement of rumen ecology, regulate digestion, and thereby, increase feed intake [25-29]. On the other hand, other studies suggest that the supplementation of ruminant rations with probiotics does not affect fattening performance [5,18,30,31]. In the present study, data analysis showed that, when compared to the control group, the best results for BW, DWG, and FCR were achieved in the groups that received 300 ppm and 600 ppm of dietary *L. reuteri* E81, and the results obtained in these two groups were found to be statistically significant (P<0.05). Nevertheless, no statistically significant difference was observed between the groups for feed intake and daily feed intake (FI) (P>0.05) *(Table 2, Table 3)*. These results are in agreement with the results of some research reports [22-24] but do not concur with the results of other reports [5,18,30,31]. Such differences in research data have been attributed to differences in the feed provided to the animals, the type and dose of the probiotics added to the ration, and the feeding strategies followed by farmers [5].

In ruminants, the rumen, as the site of fermentation and hydrophilic reactions, plays an important role in the utilization of nutrients through microbial digestion. Digested plant polymers (cellulose, lignin, and hemicellulose) provide basic energy compounds and play an important role in ruminant nutrition. For optimum fattening performance, the aim is to maintain a healthy and balanced microbial environment in the rumen [32,33]. Previous research has shown that visceral organ weights vary with weight gain and age [34]. While some research indicates that the visceral organ weights of lambs are not affected by dietary probiotic supplementation [35-39], other research suggests that probiotic supplements increase visceral organ weights [18]. In this study, combined dietary probiotic + yeast supplementation was observed not to have any impact on the skin, pluck (liver/heart/lungs), spleen and adrenal gland weights, and ruminal pH value *(Table 5)*. It has been reported that, when compared to the control group, lambs given dietary probiotics presented with improved height and width measurements of the rumen papilla, whereas no effect was observed on the height of the duodenal, jejunal and ileal villi [40]. In another study, probiotic supplementation positively affected the ruminal epithelium by reducing the thickness of the stratum corneum [41]. It has been stated that the addition of probiotics to feeds affects the histology and morphology of the ruminal papilla by increasing the amount of short-chain fatty acids in the rumen [41,42]. Duodenal values were measured under a microscope. The treatment groups (A, B, C, Mix) showed no significant difference from the control group for villus height, villus width, and crypt depth *(Table 6)*. However, the width of the tunica muscularis had decreased in the control group *(Fig. 1-1a)*

and increased in Groups A, B, C *(Fig. 1-3a)*, and Mix. The highest level of increase was detected in Groups B 600 *(Fig. 1-2a)* and Mix 300 *(Fig. 1-4a)*. No significant difference was observed between Groups A, B, C, and Mix for the rumen measurements. However, the papilla ruminis width (PRW) *(Table 7)* had significantly decreased in Groups Mix 300 *(Fig. 1-4b)* and Mix 600 and significantly increased in Group B 600 *(Fig. 1-2b)* with a moderate increase in the other groups *(Fig. 1-3b)*. Furthermore, it was observed that while few vesicular cells were present in the rumen in the control group *(Fig. 1-1b)*, the number of these cells had significantly increased in all of the treatment groups, especially in Group Mix *(Fig. 1-4b)*. The differences in research results are attributed to differences in the sheep breeds and types and doses of probiotics used in these studies.

Lamb meat is a high-quality, lean, easily digestible, and nutritious food product. Therefore, consumers who prefer lean meat tend to purchase lamb meat. As an integral component of meat, fat has a considerable effect on meat's sensory properties [43]. Meat colour is determined by the amount of myoglobin and hemoglobin and the level of lipid oxidation in muscle tissue [44]. Previous research on the effects of dietary probiotic supplements on meat quality have shown that probiotics increase the water catch capacity of meat, and thereby maintain the juiciness and increase the quality of meat [30,45,46]. Similarly, research carried out on lambs has also demonstrated an increase in the water catch capacity of lamb meat as a result of dietary probiotic supplementation [47]. Lower water holding capacity causes a lighter meat colour. The results of previous research on meat colour parameters vary, in that while it has been indicated that, in comparison to the control group, probiotic feed supplements decrease the L* value, increase the a* value, and do not affect the b* value [43], it has also been suggested that dietary probiotic supplementation does not affect meat colour parameters [26,48]. The results obtained in the present study demonstrated that, when compared to the control group, the L* value was higher in the treatment groups, excluding Groups LRE-600, Mix 600, and SCS-300, but no effect was observed on the a* and b* colour parameters. pH is an important parameter for meat quality and is affected by glycolysis level and lactic acid formation under pre- and post-slaughter conditions [17]. Volatile fatty acid formation in the rumen can also affect glycogen deposition and thereby, may ultimately affect the pH value [49]. In the present study, probiotic feed supplementation showed a significant effect on meat pH value ($P<0.01$). As shown in *Table 4*, the lowest pH value was determined to be 5.87±0.09 in Group LRE- 600. A pH value above 6.0 may cause some quality problems [50]. While the pH value was above 6.0 in the control group, the highest pH value was determined in the Group Mix-600. Probiotic supplementation is used to regulate the intestinal flora, reduce the stress of animals, balance the ruminal pH [51], improve rumen fermentation, and increase feed intake [52]. In this study, although the ruminal pH did not change with dietary probiotic supplementation, Group LRE-600 presented with the highest level of weight gain and the lowest meat pH value. Probiotic supplementation may affect glycogen accumulation. The investigation of fat tissue colour parameters in the present study revealed an increase in the L* and a* values in Groups SCS-300, SCS-600, and MIX-300, a decrease in the same parameters in Groups LRE-300, LRE-600, and MIX-600, and no effect on the b* value in any of the treatment groups *(Table 4)*. While these results agree with some literature [43], they contradict the results of other reports [26,48]. These differences in research results are attributed meat colour differences caused by growth and fat content, which eventually bring about differences in meat colour parameters.

In conclusion, this study on the effects of the addition of different doses (300 ppm and 600 ppm) of probiotics *(L. reuteri* E81 [LRE], *L. rhamnosus* GG [LRG]), yeast *(S. cerevisiae* S81 [SCS]), and probiotic + yeast combinations to lamb rations has demonstrated the best fattening performance results (BW, DWG, and FCR) to have been achieved in the treatment group that received 600 ppm of *L. reuteri*, with no effect of the tested supplements on duodenum and rumen histology and visceral organ weights. Also, the meat pH value had improved in the group given 600 ppm of *L. reuteri* E81. Today, the increasing global population has increased the demand for animal products. Thus, the livestock industry is striving to lower production costs, reduce feed consumption, improve fattening performance, and produce high-quality products that meet consumer preferences. In this context, it is apparent that further research should be carried out on the potential of dietary probiotic and yeast supplementation in ruminants.

Disclosure Statement

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this article.

Author Contributions

Conceptualization: TE, BB, AV, DE; Data curation: TE, BB; Formal analysis: KM, TE; Investigation: TE, BB, DE, KA, GM; Methodology: TE, BB, AV, DE, KA, KM, GM; Duodenum and Rumen Histology: TS; Project administration: TE, BB, AV, DE, KA, KM, GM; Writing-original draft: TE, BB, AV, DE, KA, KM, GM.

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RESEARCH ARTICLE

Fisheries and Aquaculture in Veterinary Medical Education in Turkey: History and Recent Developments

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Abstract

This study aims to depict the historical background and recent developments of education on fisheries, aquaculture, and aquatic animal diseases in veterinary faculties in Turkey. Data collected by verbal communication with the deanships of veterinary faculties in Turkey and the exploration of their web sites, as well as original documents obtained first-hand from the archives of the Ankara, Ondokuz Mayıs, Bursa Uludağ, Erciyes, Harran, and Aksaray Veterinary Faculties, constituted the main material of this study. Data were assessed by means of the content analysis. In Turkey, while fisheries and aquaculture were first included in the veterinary curriculum in the 1940s as a joint lecture, under the name of Honeybee and Fish Diseases", the first department was established in 1967 within the Ankara University Veterinary Faculty. In the following years, counterpart departments were established within the veterinary faculties in Elazığ and İstanbul. However, after the reorganisation of higher education in 1981, it was decided to close down those departments. The significant advances of the aquaculture sector by the end of the 20th century, requiring the employment of veterinarians in this sector, and the inclusion of this field in the acquis of the European Union led to relevant lectures being reincluded in the curricula of veterinary faculties in Turkey and relevant departments being established within these faculties. It has been determined that, today, while 5 veterinary faculties continue with related education and training activities and academic research under the tutelage of departments of fisheries, aquaculture, and aquatic animal diseases, 21 veterinary faculties with no counterpart departments have included lectures on fisheries, aquaculture, and aquatic animal diseases in their curricula. It is considered that in order to improve the aquatic animal health status and to meet the increasing demand for veterinary human resources of the sector in Turkey, education and research opportunities offered by veterinary educational institutions need to be increased, and the authorities and responsibilities of the different occupational stakeholder groups involved in fisheries and aquaculture should be clearly demarcated in the legislation.

Keywords: *Aquaculture, aquatic animal health, Turkey, Veterinary education, Veterinary history*

Türkiye'de Veteriner Hekimliği Eğitiminde Su Ürünleri ve Balıkçılık: Tarihsel Süreç ve Son Gelişmeler

Öz

Bu çalışma, Türkiye'de veteriner fakültelerinde balıkçılık, su ürünleri ve sucul hayvan hastalıklarına ilişkin eğitim-öğretim faaliyetlerinin tarihsel gelişimini ortaya koymak ve bugünkü durumunu değerlendirmek amacıyla gerçekleştirilmiştir. Çalışmanın ana materyalini, Türkiye'deki veteriner fakültelerinin dekanlıklarıyla yapılan sözlü görüşmeler ve kurumsal web sitelerinin taranması yoluyla toplanan veriler ile Ankara, Ondokuz Mayıs, Bursa Uludağ, Erciyes, Harran ve Aksaray Üniversiteleri Veteriner Fakültelerinin Arşivlerinden sağlanan ilk elden kaynaklar oluşturmuştur. Ulaşılan veriler, içerik analizine tabi tutulmuştur. Türkiye'de veteriner hekimliği eğitim-öğretiminde su ürünleri ve balıkçılıkla ilgili ilk dersin "Arı ve Balık Hastalıkları" adıyla 1940'lı yıllarda müfredat programına alındığı belirlenmiş, 1967 yılında ilk kürsü Ankara Üniversitesi Veteriner Fakültesinde kurulmuştur. İzleyen yıllarda Elazığ ve İstanbul'da bulunan veteriner fakültelerinde de ilgili kürsüler açılmış ancak 1981 yılında yükseköğretimde gerçekleştirilen reorganizasyonun ardından, bu kürsülerin kapatılmasına karar verilmiştir. Yirminci yüzyılın sonunda önemli bir gelişim gösteren su ürünleri yetiştiriciliğinde, veteriner hekimlerin istihdamına duyulan gereksinim ve Avrupa Birliği müktesebatında konuya gösterilen hassasiyet, Türkiye'de veteriner fakültelerinde bu alana ilişkin derslerin tekrar müfredata konulmasını ve ilgili anabilim dallarının açılmasını sağlamıştır. Bugün beş veteriner fakültesinde bulunan Su Ürünleri ve Hastalıkları Anabilim Dallarında eğitim-öğretim ve akademik çalışmalar devam etmekte olup, anabilim dalı olmayan 21 veteriner fakültesinde su ürünleri ve hastalıklarına ilişkin derslerin verildiği belirlenmiştir. Türkiye'de sucul hayvan sağlığının iyileştirilmesi ve sektörde gün geçtikçe artan veteriner hekim talebinin karşılanması için, veteriner hekimliği eğitim kurumlarında bu disipline ilişkin eğitim ve araştırma olanaklarının artırılması ve bu alanla ilgili farklı meslek gruplarının yetki ve sorumluluklarının mevzuatta açıkça belirtilmesi gerektiği düşünülmüştür.

Anahtar sözcükler: *Su ürünleri yetiştiriciliği, Sucul hayvan sağlığı, Türkiye, Veteriner hekimliği eğitim-öğretimi, Veteriner hekimliği tarihi*

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Introduction

The increased use of aquatic animals as pets, experimental animals, and/or food products has increased the need to improve their health, living conditions, and welfare [1,2]. Veterinary education and training on aquatic animal health are observed to be limited on a global scale, and veterinary practitioners are found to be dissatisfied with their level of competence in this area. In many countries, veterinarians are not the only occupational group entitled to maintain aquatic animal health and treat aquatic animals. This bestows the right for practitioners of non-veterinary professions to practice on aquatic animals [3]. Nevertheless, as a result of being perceived as an occupational group responsible for the health of all animals, veterinarians are expected to be equipped with the knowledge and skills to maintain the health of aquatic animals. In order to meet this expectation, lectures on aquatic animals should be included in the undergraduate and postgraduate curricula of veterinary faculties [1].

In Turkey, education in the field of fisheries and aquaculture was first introduced within the scope of the discipline of zoology, and as of the mid-20th century [4,5], two hydrobiology research institutes, subordinated to the science faculties of İstanbul University-Cerrahpaşa and Ege University, were established [6,7]. These institutes pioneered the training of reputable scientists in the fields of hydrobiology and fish biology and served as research centres for the first academic studies on biological oceanography, ichthyology, and limnology in Turkey [4]. In the meantime, fisheries and aquaculture lectures were included in the curricula of veterinary faculties, which over the course of time led to the establishment of departments specific to this field.

This study aims to depict the historical background and recent progression of training activities in the field of fisheries, aquaculture, and aquatic animal diseases in veterinary medical education in Turkey.

Material and Methods

Relevant information was obtained from the web pages of veterinary faculties and by verbal communications with their dean ships. In addition, the historical data of the study were mainly derived from original documents belonging to the archives of the Ankara University Veterinary Faculty and five other veterinary faculties (the universities of Ondokuz Mayıs, Bursa Uludağ, Erciyes, Harran, and Aksaray), each of which have a separate Department of Aquaculture and Aquatic Animal Diseases. Data were analysed with the aim of providing a retrospective overview. Additional explanatory information and the identification tags of the original documents are detailed in the footnotes of the manuscript.

Results

Based on the data accessible to the authors, the first step taken in the field of aquaculture and fisheries within the scope of veterinary medical education was the delivery of a course of lectures entitled "Honeybee and Fish Diseases" at the Yüksek Ziraat Enstitüsü (YZE) in Ankara. This course of lectures was delivered by the Department of Parasitology and Helminthology to fifth-year students during the 1947-1948 academic year.[1] This course continued to be delivered after the subordination and transfer of the Veterinary Faculty of the Higher Agricultural Institute (YZE) to Ankara University in 1948.[2] The first department specific to fisheries and aquaculture was established on 16 May 1967 under the name of "Aquaculture, Fisheries, and Game Animals Department" within the Ankara University Veterinary Faculty (AUVF), which was the only veterinary school in Turkey until the 1970s. In the same year that the department was established, the first Turkish veterinarians specialising in the field of aquaculture and fisheries started to be trained [8].[3] During the same period, the skeleton crew of the department was also formed. This team organised several study visits for educational, investigational, and research purposes. It was ascertained that, by virtue of these visits and observations, the academic staff of the department gave priority to aquaculture research in both the seas and some lakes and state farms of Turkey.[4]These efforts were aimed at increasing access to protein sources and creating a new business area in Turkey, and they were complemented by the decision to print brochures, categorised as "community publications", to raise public awareness [9,10].[5]

In the 1970s, efforts were also made for the delivery of lectures and the establishment of departments specific to fisheries and aquaculture at veterinary faculties other than the AUVF. Hence, the Veterinary Faculty established in Elazığ in 1970, under the subordination of Ankara University[6], had a Department of Aquaculture, Fisheries, and Game Animals among its 16 departments [11]. In 1977, a Fisheries and Aquaculture Department was established within the İstanbul University-Cerrahpaşa Veterinary Faculty, which was founded in 1972 [12]. Furthermore, Bursa University, which instituted education and training activities in 1978, established an Aquaculture and Fisheries Unit within the Animal Production and Improvement Department of its Veterinary Faculty [13]. The academic staff of the Department of Aquaculture, Fisheries, and Game Animals of the AUVF also delivered lectures at some of

[1] The Exam Protocol dated June 23, 1948 of the Veterinary Faculty of YZE
[2] The Personal File of Professor Hasan Şükrü Oytun, Archive of the AUVF Deanship; Law No. 5234, Official Gazette, No. 6951, 1948
[3] The Personal File of Professor Zihni Erençin, Archive of the AUVF Deanship
[4] The Personal Files of Gülten Köksal, Orhan Erdem, Selçuk Seçer, Metin Timur and Fikri Aydın, Archive of the AUVF Deanship
[5] The official letter numbered 3365 and dated June 28, 1972 of the Aquaculture, Fisheries, and Game Animals Department addressed to the AUVF Deanship; The official letter numbered 27-74 and dated September 9, 1974 of the Editorial Board of Journal of AUVFaddressed to the AUVF Deanship
[6] In 1975, it was agreed on the separation of Elazığ Veterinary Faculty from Ankara University, and the subordination of the Faculty to Fırat University. For further information see the Law on the Establishment of Four Universities published in the Official Gazette dated April 11, 1975 and numbered 15205

the veterinary faculties of other Turkish universities on a periodic basis.[7]

The enactment of the Higher Education Law[8] in 1981 brought about fundamental changes in the Turkish university system, such that the academic organisation and curricula of these higher education institutions, including veterinary faculties, were restructured. The Aquaculture, Fisheries, and Game Animals Departments were closed down the same year, and the lectures delivered by these departments were designated as elective courses.[9] The academic staff of the departments were appointed to various other faculties and departments [14],[10]and some served as the basic education staff of the newly established Fisheries and Aquaculture Collegiate Schools [15], which were converted to faculties in 1992.[11]

Education and training activities related to fisheries and aquaculture were relaunched within veterinary faculties in the 1990s. First, lectures on fish diseases, fish parasitology, fish immunology, sperm storage and artificial insemination, vaccination of aquatic animals, processing of aquaculture and fisheries products, and hygiene rules were introduced into the postgraduate education curricula. Subsequently, the course of lectures on aquatic animals and their diseases was reintroduced into the undergraduate education curricula. Despite the extended implementation of these actions, the lack of individual specific departments within veterinary faculties and the inefficient education of veterinarians on fish diseases raised the issue of re-establishing relevant departments within veterinary faculties.[12,13] The first institution to agree upon the establishment of a Department of Aquatic Animal Diseases in 2003 was the Faculty of Veterinary Medicine of Ondokuz Mayıs University (OMU), and the department became operational in 2005.[14]

The main factors that led to the establishment of the department included the highest levels of fishery captures and aquaculture production belonging to the Black Sea region; Turkey being a member of the Black Sea Economic Cooperation Programme; the high numbers of streams, rich flora and fauna, and endemic fish and other aquatic animal populations of the region; fisheries and aquaculture having an important place in the European Union (EU) acquis; and the existing institutions falling behind in the diagnosis and treatment of aquatic animal diseases.[12]

In the ensuing years, similar attempts were made in other veterinary faculties, such that counterpart departments were established within veterinary faculties subordinated to the universities of Bursa Uludağ (2010), Erciyes (2012), Harran (2012), and Aksaray (2014).[13,15,16] Today, a total of 14 academic staff, including 5 professors, 4 associate professors, 3 assistant professors, and 2 research assistants, are appointed to these departments. These departments have been determined to deliver 5 compulsory courses and 12 elective courses, which are presented in *Table 1*, to undergraduate students.[16]

The curricula of the 21 veterinary faculties with no counterpart departments have been ascertained to include 21 compulsory and 21 elective courses *(Table 2)*. Some departments have made efforts to launch not only undergraduate but also postgraduate education and training programmes. The first step towards this purpose was taken by the FVM of Bursa Uludağ University in 2011, followed by the initiation of postgraduate education in the FVM of Ondokuz Mayıs University in 2014.[17] To date, 5 masters and 3 doctorate students have graduated from this programme.

Discussion

Following the establishment of the Department of Aquaculture, Fisheries, and Game Animals in 1967 within the AUVF, activities in this field mainly focussed on aquaculture studies.[3,4] Aquaculture studies conducted as part of the scientific research of the department[3,4,5] were performed not only for academic purposes but also with an aim to extend aquaculture and fisheries throughout Turkey and to contribute to the national economy. Besides this, the launch of an aquaculture and fisheries specialisation programme and, thereby, the education and training of the

[7] The decision numbered 118/2497 and dated November 30, 1976 of the AUSenate; The official letter numbered 1065 and dated March 3, 1978 of the AUVF Deanship addressed to the AU Rectorate; The decision numbered 3900 and dated November 7, 1978 of the AUSenate; the official letter numbered 897 and dated April 13, 1978 of Bursa University Veterinary Faculty Deanship adreesed to the AUVF Deanship; The official letter numbered 7081 and dated December 22, 1979 of the AUVF Deanshipaddressed to the Aquaculture, Fisheries, and Game Animals Department; the decision numbered 79-9/2b and dated March 29, 1979 of the Administrative Board of the Bursa University Veterinary Faculty; the decision numbered 588 and dated November 4, 1982 of the Administrative Board of AUVF

[8] Law No. 2547, Official Gazette, No. 17506, 1981

[9] The offiicial letter numbered 226/2960 and dated August 18, 1982 of the Higher Education Council adressed to AUVF Deanship

[10] The official letter numbered 9687 and dated November 29, 1982 of AUVF Deanship addressed to the AU Rectorate; The official letter numbered 9436 and dated November 17, 1982 of AUVF Deanship; The decision numbered 35 and dated September 6, 1983 of AUVF Board

[11] Law No. 2809, Official Gazette, No. 21281, 1992

[12] The Justification of the Establishment of the Department of Aquatic Animal Diseases under Ondokuz Mayıs University, Veterinary Faculty, 2003

[13] The Justification of the Establishment of the Department of Aquaculture, Fisheries and Aquatic Animal Diseases under Aksaray University, Veterinary Faculty, 2014

[14] The official letter numbered 026938 and dated December 12, 2003 of the Higher Education Council adressed to OMU Rectorate; The official letter numbered 076-516 and dated March 12, 2009 of OMU Deanship addressed to the OMU Rectorate

[15] At the Erciyes, Aksaray and Harran Veterinary Faculties, this department was named as "the Department of Aquaculture, Fisheries and Aquatic Animal Diseases". Official letter of Harran University, Veterinary Faculty dated 17.03.2011 and numbered 2011-01; official letter of Aksaray University, Veterinary Faculty dated 10.06.2014 and numbered 04-011

[16] Relevant information was obtained from the web pages of the previously mentioned veterinary faculties and by verbal communication with the administrative and academic staff of these faculties

[17] The official letter numbered 2418 and dated April 12, 2011 of the Higher Education Council adressed to Bursa Uludağ University Rectorate;The official letter numbered 65475 and dated November 7, 2014 of the Higher Education Council adressed to OMU Rectorate

Table 1. *Undergraduate courses offered by the Departments of Aquaculture and Aquatic Animal Diseases in Turkish faculties of veterinary medicine*

Veterinary School	Undergraduate Courses	Compulsory/Elective	Midterm (Theoretical/Practical)
Ondokuz Mayıs University	Aquaculture and Aquatic Animal Diseases	C	5 (1/2)
	Aquarium Fish Breeding and Diseases	E	5 (1/0)
Bursa Uludağ University	Aquatic Animal Diseases	C	5 (2/2)
	Ichthyology	E	4 (2/0)
	Aquaculture Production and Biotechnology	E	6 (2/0)
Erciyes University	Aquatic Animal Diseases	C	9 (1/2)
	Aquaculture	E	4 (1/2)
	Ecology	E	5 (1/0)
Harran University	Aquatic Animal Diseases	C	7 (2/0)
	Aquarium Fish Diseases	E	7 (1/0)
Aksaray University	Aquatic Animal Diseases	C	6 (2/1)
	Seafood Processing Technology	E	7 (1/0)
	Aquaculture	E	4 (1/0)
	Fish Farming	E	8 (1/0)
	Fish Reproduction	E	8 (1/0)
	Aquarium Fish Diseases	E	4 (1/0)

first specialist veterinarians on fisheries and aquaculture[3,4] can be considered as pioneering steps in this field in Turkey.

Despite the rapid development of fisheries and aquaculture throughout the world in the following years [16,17], the termination of specialisation training and the closing down of the Aquaculture, Fisheries, and Game Animals Departments pursuant to the higher education regulation [8,9] enacted in 1981 are still considered controversial. Both the need to employ veterinarians in the aquaculture sector, which had displayed major advances by the end of the 20th century, and the ongoing EU accession process of Turkey, requiring legislative and administrative actions, rekindled the attempt to institutionalise education, training, and academic organisation in this field within several veterinary faculties. Urdes et al.[17] pointed out significant economic losses being caused and further development being prevented by emerging diseases and pathogens in the aquaculture sector, and they highlighted the need to employ veterinarians so as to ensure both public health and the health of aquatic animals. The need for veterinary services in the aquaculture sector has also been underlined by several international organisations, including the OIE and FAO, and the employment of veterinarians in this sector has been clearly detailed in EU legislation[18] [16,17]. In this context, in view of several factors, including, among others, the geographical location, natural water resources, and physical conditions of Turkey, Aquatic Animal Diseases Departments having been established in only five *(Table1)* out of 28 veterinary faculties is considered unsatisfactory given the ongoing EU alignment process. Mean while, the fact that the curricula of the remaining 21 veterinary faculties without counterpart departments include lectures related to aquaculture and fisheries *(Table2)*, although delayed, could be considered as a positive development at first glance. Indeed, Iatridou et al.[2] reported the delivery of aquatic animal disease lectures at 74 out of 77 veterinary education institutions belonging to the European Association of Establishments of Veterinary Education (EAEVE), which is the official accreditation authority of veterinary schools in Europe, showing the importance attached to this field in veterinary medical education.

It can be said that the number and the status quo of aquaculture and fisheries lectures included in the curricula of Turkish veterinary faculties are much better than the number of specific departments established. In fact, while Turkish veterinary faculties with Aquaculture and Aquatic Animal Diseases Departments have been determined to offer 5 compulsory and 12 elective courses *(Table 1)*, the remaining 21 veterinary faculties with no counterpart departments include 21 compulsory and 21 elective courses in their curricula *(Table 2)*. On the other hand, it has been reported that 36 out of the 74 veterinary education institutions belonging to the EAEVE in 2018 offered a lecture solely devoted to this subject, while the remaining 38 faculties provided related information within other lectures. Courses on aquaculture and aquatic animal diseases have been reported to be compulsory in 42 of those institutions, elective in 9 institutions, and semi-compulsory/semi-elective in the remaining 23 faculties [2].

[18] Council Directive 91/67/EEC of 28 January 1991 concerning the animal health conditions governing the placing on the market of aquaculture animals and products (OJ No: L 46/1, 19.02.1991); Council Directive 2006/88/EC of 24 October 2006 on animal health requirements for aquaculture animals and products there of, and on the prevention and control of certain diseases in aquatic animals. (OJ No: L 328/14, 24.11.2006); Commission Decision 2008/946/EC of 12 December 2008 implementing Council Directive 2006/88/EC as regards requirements for quarantine of aquaculture animals. (OJ No: L 337/94, 16.12.2008); Regulation (EU) 2016/429 of The European Parliament and of the Council of 9 March 2016 on transmissible animal diseases and amending and repealing certain acts in the area of animal health ('Animal Health Law'). (OJ No: L 84/1, 31.03.2016)

Table 2. *Courses given by veterinary faculties with no counterpart departments*

Veterinary School	Undergraduate Courses	Compulsory/Elective	Midterm (Theoretical/Practical)
Ankara University*	Aquatic Animal Diseases	C	8 (2/2)
	Aquarium Fish Diseases	E	7 (1/0)
	Fish Diseases and Pathology	E	7 (1/0)
	Biotechnology Applications in Fish Reproduction	E	8 (1/0)
Fırat University	Aquatic Animal Diseases	C	8 (3/0)
	Ornamental Animal Diseases	E	9 (1/0)
İstanbul University-Cerrahpaşa	Fish Diseases	C	6 (1/0)
	Drug Use in Honeybees, Fish, and Exotic Animals	E	8 (1/0)
	Aquatic Animal Nutrition	E	6 (1/0)
	Fish Pathology	E	6 (2/0)
	Hygiene and Technology in Seafood Processing	E	8 (1/0)
Selçuk University	Aquatic Animals	C	6 (1/0)
Van Yüzüncü Yıl University	Aquatic Animal Diseases	C	10 (1/2)
Kafkas University	Aquatic Animal Diseases	C	6 (1/0)
Aydın Adnan Menderes University	Bacterial Fish Pathogens and Identification	C	5 (1/1)
	Aquatic Animal Medications	C	8 (1/0)
	Aquatic Animal Diseases	C	8 (1/1)
Dicle University	Aquatic Animal Diseases	C	5 (1/1)
	Fish Farming	E	7 (1/1)
	Fish Nutrition	E	2 (1/0)
	Reproduction and Artificial Insemination in Bees, Fish, Laboratory Animals, and Wild Animals	E	4 (1/0)
Burdur Mehmet Akif Ersoy University	Aquatic Animal Diseases	C	8 (1/1)
Kırıkkale University	Fish Diseases	E	5 (1/0)
Mustafa Kemal University	Aquatic Animal Diseases	C	9 (1/0)
Afyon Kocatepe University	Aquatic Animal Diseases	C	6 (1/0)
	Hygiene and Examination of Water	E	4 (1/0)
	Aquaculture	E	5 (1/0)
	Diseases and Pathology of Aquatic Animals	E	6 (1/0)
	Hygiene and Technology in Seafood Processing	E	8 (1/0)
Atatürk University	Aquatic Animal Diseases	C	6 (1/0)
Balıkesir University	Fish Diseases and Aquaculture	E	4 (3/0)
Cumhuriyet University	Aquatic Animal Diseases and Treatment	C	7 (2/0)
Bingöl University	Aquatic Animal Diseases	C	9 (1/2)
	Fish Diseases and Pathology	E	6 (2/0)
Tekirdağ Namık Kemal University	Aquatic Animal Diseases	C	8 (1/2)
Siirt University	Aquatic Animal Diseases	C	6 (1/0)
	Aquaculture	E	6 (1/0)
Çukurova University	Aquaculture and Aquatic Animal Diseases	C	7 (3/1)
Kastamonu University	Aquaculture and Aquatic Animal Diseases	C	5 (1/0)
	Industrial Aquaculture	E	6 (1/0)
Dokuz Eylül University	Aquatic Animal Diseases	C	6 (1/2)
	Aquaculture Production and Biotechnology	E	6 (1/0)

** The same courses are included in both the Turkish and English curricula of this faculty*

These numbers for undergraduate education differ for postgraduate education. Today, while two veterinary faculties subordinated to Bursa Uludağ University and Ondokuz Mayıs University are known to deliver postgraduate training in this field[17], only 30 out of the 69 veterinary education institutions belonging to the EAEVE have been reported to offer postgraduate education on aquatic veterinary medicine [2].The fact that the postgraduate education and

training programmes implemented by Turkish veterinary faculties are few in number has been attributed to the limitations of the criteria set by the Higher Education Council for the implementation of doctorate and master's degree programmes. Despite the organisational efforts initiated for the implementation of undergraduate and postgraduate aquaculture and aquatic animal diseases programmes at veterinary faculties in the last few years, the questionable infrastructure, human resources, and education quality of the increasing number of veterinary faculties [18,19] have rendered the topic more controversial than ever.

On the other hand, despite these steps recently taken in the field of veterinary education, the number of veterinarians employed in the public and private sectors for the maintenance of the health of aquatic animals remains low. This is attributed to the diversity of occupational groups working in this area; the fact that the tasks, authorities, and responsibilities of these occupational groups have not been clearly demarcated; and the existence of very few diagnostic laboratories offering services in this area [19,20]. Altun et al.[15] drew attention to a similar situation is observed in the existing academic veterinary institutions in Turkey, such that while no veterinarian is employed by 3 out of the 5 Departments of Aquatic Animals and Aquatic Animal Diseases, some veterinary faculties with no counterpart departments have appointed non-veterinary occupational groups for the delivery of the relevant lectures. Although the fisheries and aquaculture legislation in force stipulates veterinarians as the only occupational group responsible for the health of all animals, including aquatic animals, the sufficiency of the education, training, and research infrastructure of the existing veterinary educational institutions remains another issue of concern.

In conclusion, it has been determined that, despite aquaculture and fisheries-related lectures having started to be given in Turkish veterinary faculties in 1948 and a Department of Aquaculture, Fisheries, and Game Animals having been established in1967, today only 5 veterinary faculties accommodate departments specific to this field, and 26 veterinary faculties deliver relevant lectures with no counterpart departments. In the past decade, not only has the need for the employment of veterinarians to ensure both public health and aquatic animal health increased with the growth of the fisheries and aquaculture sector, but this area has also gained a strong position in the EU acquis. In order to meet the demands for veterinary human resources within the sector in Turkey, as an EU candidate country, the aquaculture and fisheries-related efforts within veterinary educational institutions should be further strengthened via the improvement of the education, training, and research opportunities and infrastructure, and the authorities and responsibilities of the different occupational groups involved in fisheries and aquaculture should be clearly demarcated in the legislation.

Conflict of Interest

The authors declare that there is no conflict of interest.

Statement of Author Contributions

B. Melikoğlu Gölcü and A. Uyguntürk conceived the ideas of the study. B. Melikoğlu Gölcü wrote the manuscript. A. Uyguntürk and A. Ünsal Adaca made substantial contributions to the collection of the historical and current information. All authors revised and read the final manuscript.

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RESEARCH ARTICLE

Inhibitory Effect and Mechanism of Oyster Enzymatic Hydrolysate on Lung Metastasis in the Subcutaneous Lewis Lung Cancer Model in Mice

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Abstract

In order to investigate the inhibitory effects of oyster enzymatic hydrolysate (OEH) on the metastasis of Lewis lung cancer and to evaluate its mechanism, daily gavage of low(LOEH), medium(MOEH) and high (HOEH) doses of OEH for 5 weeks was administered based on the subcutaneous Lewis lung cancer model in C57BL/6J male mice, the volume and weight of subcutaneous tumor were measured, the lung metastatic nodules were counted, the number of tumor-associated-macrophages (TAMs; CD11b+F4/80+), the expression of E-cadherin, Vimentin, microRNA-21 and microRNA-218 in subcutaneous tumor were measured. It was found that OEH treatment significantly decreased the subcutaneous tumor weight for the MOEH and HOEH groups (P=0.013, P=0.007) and significantly inhibited lung metastasis in a dose-dependent manner ($\chi 2$=13.16, P=0.004). The expression of E-cadherin showed a statistical increase at high dose, while the expression of Vimentin and the number of TAMs in subcutaneous tumor was significantly decreased at all OEH doses (P<0.05). The expression of microRNA-21 was significantly decreased in the group of MOEH (P=0.013) and HOEH (P=0.013), and the expression of microRNA-218 was significantly increased in all group with OEH treatment (P<0.05). In conclusion, OEH significantly reduced the growth of subcutaneous tumors and incidence of lung metastases in a dose-dependent manner. Its anti-tumorigenic activity might be explained by its ability to inhibit epithelial mesenchymal transition by reducing the number of TAMs, and down-regulate microRNA-218 as well as up-regulate microRNA-21 expression.

Keywords: *Oyster enzymatic hydrolysate (OEH), Lung metastasis of Lewis lung cancer, Vimentin, Tumor- associated-macrophages (TAMs), microRNA-21, microRNA-218*

Farelerde Subkutan Lewis Akciğer Kanseri Modelinde İstiridye Enzimatik Hidrolizatının Akciğer Metastazı Üzerine Önleyici Etkisi ve Mekanizması

Öz

İstiridye enzimatik hidrolizatının (OEH) Lewis akciğer kanserinin metastazı üzerine inhibitör etkisinin araştırılması ve inhibitör mekanizmanın aydınlatılması amacıyla subkutan Lewis akciğer kanseri modeline göre C57BL / 6J erkek farelerde 5 hafta boyunca OEH'in düşük (LOEH), orta (MOEH) ve yüksek (HOEH) dozlarının günlük gavajı uygulandı. Subkutan tümörün hacmi ve ağırlığı ölçüldü, akciğer metastatik nodüller sayıldı, tümör ilişkili makrofajlar (TAM; CD11b+F4/80+) sayıldı, deri altı tümör yapılarda E-kaderin, Vimentin, microRNA-21 ve microRNA-218 ekspresyonu ölçüldü. OEH'in MOEH ve HOEH sağaltım gruplarının subkutan tümör ağırlığını önemli ölçüde azalttığı (P=0.013, P=0.007) ve doza bağlı olarak akciğer metastazını önemli ölçüde engellediği ($\chi 2$=13.16, P=0.004) saptandı. E-kaderin ekspresyonu, yüksek dozda istatistiksel bir artış gösterirken, tüm OEH gruplarında Vimentin ekspresyonu ve subkutan tümördeki TAM hücre sayısında önemli ölçüde azalma saptandı (P<0.05). MikroRNA-21 ekspresyonu MOEH (P=0.013) ve HOEH (P=0.013) gruplarında önemli ölçüde azalırken ve microRNA-218 ekspresyonunda OEH tedavisi alan tüm gruplarda anlamlı bir artış saptandı (P<0.05). Sonuç olarak, OEH, deri altı tümörlerin gelişimi ve akciğer metastaz insidansını doza bağlı olarak önemli ölçüde azaltmıştır. Anti-tümörojenik aktivitesi, TAM'ların sayısını azaltarak epitelyal mezenkimal geçişi inhibe etme kabiliyeti ve mikroRNA-218'in ekspresyonunu azaltma ve mikroRNA-21'in ekspresyonunu artırma özelliği ile açıklanabilir.

Anahtar sözcükler: *Oİstiridye enzimatik hidrolizatı (OEH), Lewis akciğer kanserinin akciğer metastazı, Vimentin, Tümör ilişkili makrofajlar (TAM), MikroRNA-21, MikroRNA-218*

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Introduction

Small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC) are the main pathological types of lung cancer, for which NSCLC accounts for 85-90% [1]. As the most common cancer in the world, its rates of incidence and mortality are the highest of the malignant tumors, with increasing tendency each year [2]. It is highly invasive, and most patients have metastasis, with 80% of lung cancer patients in the middle and late stage, at the time of diagnosis [3]. Despite the use of surgery, chemotherapy, radiotherapy, molecular targeting and other comprehensive treatments, drug resistance and recurrence are common, and the 5-year survival rate is less than 14% [4].

The recurrence and metastasis of lung cancer have been shown to be closely related to epithelial mesenchymal transition (EMT). EMT can change the phenotype of tumor cells, so that they can obtain stronger invasion and metastasis ability [5]. Down regulation of E-cadherin expression and up regulation of Vimentin expression are important features of EMT [6]. EMT may also be induced by tumor-associated-macrophages (TAMs), which are the most abundant cells in the tumor immune microenvironment and have been shown to reduce the expression of E-cadherin and other epithelial adhesion proteins and increase the expression of Vimentin [7]. Therefore, TAM and EMT play important roles in the occurrence and development of lung cancer.

Studies have suggested that microRNA-218 (miR-218) and microRNA-21 (miR-21) can regulate the proliferation and invasion of tumor cells, thus affecting the prognosis, survival, rate of recurrence and metastasis in lung cancer patients. MiR-218 is down-regulated in NSCLC, which leads to the proliferation of lung cancer cells, increases the probability of recurrence and metastasis, and reduces the survival period for lung cancer patients [8-10]. MiR-21 promotes the invasion and metastasis of NSCLC cells by inhibiting the expression of PTEN and other tumor suppressor genes [11]. Therefore, the down-regulation of miR-218 expression and up-regulation of miR-21 expression are associated with poor prognosis in lung cancer.

Oyster is not only a kind of seafood with tender meat, fresh taste and high nutritional value, but also a common Chinese medicine in the Chinese Pharmacopoeia [12]. Oyster meat is rich in protein, amino acid, taurine, bioactive peptide, fatty acid, glycogen, vitamin and inorganic salt [13], it is often used to enhance immune function, reduce blood glucose and blood lipid and anti-tumor and so on [14-17]. Oyster bioactive peptide, a component of oyster enzymatic hydrolysate (OEH), has been shown to improve cellular immune function and inhibit the growth of malignant tumors [18]; however, it is not clear whether it can inhibit the metastasis of lung cancer, and its mechanism has not been fully elucidated. Mouse model of Lewis lung cancer (LLC) is the most widely used lung cancer model of the same origin, LLC cell line is an adenocarcinoma and maintains high tumorigenicity and lung metastasis in C57BL/6 mice [19,20], and the lung metastasis can be determined after subcutaneous inoculation of LLC cells in C57BL/6 mice for 17-21 days [21]. Therefore, in order to explore the inhibitory effects of OEH on the lung metastasis from subcutaneous tumor cells and to evaluate its mechanism, we established the subcutaneous Lewis lung cancer model in mice [20,21] and treated tumor-bearing mice with OEH at low, medium and high doses. The weights of subcutaneous tumor were measured, lung metastatic nodules were observed and counted, and the percentage of TAMs (CD11b + F4/80+) cells in subcutaneous tumor were detected by flow cytometry. Furthermore, the expression of E-cadherin, Vimentin, miR-21 and miR-218 in subcutaneous tumor was detected by qRT-PCR. Our results provide an experimental basis for the application of OEH in clinical adjuvant treatment of tumors.

Material and Methods

Preparation of OEH

A thousand gram fresh oyster meat *(Crassostrea rivularis)* (Beijing zhongshihaishi biotechnology Co., Ltd) were crashed with a blender, fix the volume to 3 L with distilled water, and put it in boiling water bath for 1 h, then lower it to room temperature, and centrifugated with 3000 g for 5 min. Add 500 mL distilled water to the precipitate and stir well, adjust the pH value to 7.0 with NaOH, put it in the water bath, and raise the temperature to 50°C. Neutral protease (Novozyme Biotechnology Co., Ltd) was added at an amount of 20.000 U per 100 g of raw materials, and after 1 h of enzymolysis, papain (Novozyme Biotechnology Co., Ltd) was added at an amount of 32.000 U per 100 g of raw materials, and the enzymolysis continued for 2 h. The enzyme was then inactivated at 100°C for 15 min. After cooling, centrifugated 3000 g for 10 min and the supernatant was taken to filter by 100 mesh sieve and dry by spray dryer, and dried powder of oyster hydrolysate was obtained.

Animal

Forty-eight SPF-grade 6-week-old male C57BL/6J mice, weighing 20±2 g, were purchased from the comparative medical center of Yangzhou University, they were housed under a natural photoperiod (12 hlight:12 h dark) with suitable temperature (20-25°C), and *ad libitum* food and drinking water. All the experiments that involve animals were approved by the Experimental Animal Management Committee and Experimental Animal Ethics Committee of Yangzhou University (Yzu DWLL-201804-003) and was conducted in compliance with the principles stated in the Guide for the Care and Use of Laboratory Animals (NIH) [22].

Establishment of the Lewis Lung Cancer Mouse Model

The LL/2 mouse Lewis lung cancer cell line (American strain Preservation Center; provided by Shanghai Jining Industrial Co., Ltd) was cultured incomplete Dulbecco Modified Eagle Medium (DMEM, Sigma-Aldrich) containing

10% fetal bovine serum (FBS, Hyclone) at 37°C in a 5% CO_2 incubator. At confluency, the cells were passaged1:3. Cells in logarithmic growth phase were digested with 0.25% pancreatin (Beyotime Biotechnology Co., Ltd.), centrifuged for 5 min (1000 r/min), and resuspended in normal saline. The percentage of living cells, as determined by Trypan blue (Beyotime Biotechnology Co., Ltd.) staining, was more than 95%. The cell concentration was adjusted to 6×10^6 cell/mL, and then 0.2 mLwas inoculated subcutaneously into the right forelimb armpits of C57BL/6J mice [23]. The entire process was strictly sterile and was completed within 40 min.

Grouping and Administration

After 24 h, the mice were weighed, numbered, and randomly divided into the control group (C), low dose OEH intervention group (LOEH), medium dose OEH intervention group (MOEH), and high dose OEH intervention group (HOEH) (12 mice per group). In group C, 0.9% normal saline was given every day after lung cancer cells were inoculated. For the LOEH, MOEH and HOEH groups, OEH was intragastric administered at doses of 0.8 g/kg, 1.8 g/kg and 2.5 g/kg per day for 5 weeks [24].

Sample Collection

The mice were sacrificed after anesthesia, which was performed with 1% pentobarbital sodium according to the body weight (0.05 mL/10 g). The whole subcutaneous tumor was quickly removed, weighed, and divided into three parts. One part was quickly submerged into 10% formaldehyde solution for fixation to diagnose subcutaneous tumor by observing its histological structure; one part was put into a cryopreservation tube, quickly submerged into liquid nitrogen, and stored at −80°C for testing, the other part was processed as a tumor cell suspension for testing the corresponding index. Then, the whole lung tissue was taken and placed in Bouin fixative solution to observe the lung metastasis from subcutaneous tumor by counting the pulmonary surface nodules [25] and Hematoxylin-eosin (HE) staining [26].

Determination of Indicators

Determination of OEH composition: The contents of total protein, acid soluble protein and total sugar in OEH were determined by Kjeldahl method(QSY-IIKjeldahl nitrogen determinator, Beijing Qiangsheng analytical instrument manufacturing center), three chloroacetic acid method and the reagent method (22100 UV spectrophotometer, Unocal Shanghai Instruments Co., Ltd), the composition and content of free amino acids were determined by amino acid automatic analysis(835-50 amino acid analyzer, Hitachi Limited), ash and water were determined by burning method and atmospheric drying method, and the distribution range of molecular weight was determined by high performance liquid chromatography (HPLC)(LC-20a high performance liquid chromatograph, Shimadzu, Kyoto, Japan) [27].

Identification of peptides from OEH: Nexera X2 Ultra high performance liquid chromatography and triple quadrupole mass spectrometer system (Shimadzu, Kyoto, Japan) were used to identify the peptides from OEH. The mass spectrometry conditions are as follows. Ionization mode: ESI, positive ion mode; ion spray voltage: +4.5 KV; atomization gas flow rate: 3.0 L/min of nitrogen; heating gas flow rate: 10 L/min of nitrogen; dry gas flow rate: 10 L/min of nitrogen; DL temperature: 250°C; heating module temperature: 400°C; ion source temperature: 300°C; scanning mode: multi reaction monitoring (MRM); residence time: 100 ms; delay time: 3 ms [28].

Calculation of the survival rate: The survival and death of mice were recorded every day. After 5 weeks, the survival rate of mice in each group was calculated by the formula: survival rate (%) = (survival number of mice in each group/total number of mice in each group) × 100% [29].

Calculation of the tumor inhibition rate: After the establishment of the subcutaneous Lewis lung cancer model, subcutaneous tumor formation was observed each week. The length diameter (L) and width diameter (W) of the tumor were measured with a Vernier ruler, and the tumor volume was calculated with the formula, $V = 1/2 \times L \times W^2$. The average tumor weights for each group were measured and used to calculate the tumor inhibition rate as follows: the tumor inhibition rate (%) = (1 − average tumor weight of experimental group/average tumor weight of control group) × 100% [25].

Calculation of the number of pulmonary surface nodules and the rate of metastasis inhibition: After the lung tissue was fixed in Bouin fixative solution for 24 h, it was soaked in absolute ethanol for 24 h, and the number of pulmonary metastases (visualized as white nodules) was recorded [25].

The lung metastasis rate was calculated as follows: lung metastasis rate = (number of lung metastasis/number of samples in each group) × 100%. The inhibition rate of lung metastasis was calculated as follows: Lung metastasis inhibition rate (%) = (mean number of pulmonary surface metastasis nodules in the model group - mean number of pulmonary surface metastasis nodules in the treatment group)/mean number of pulmonary surface metastasis nodules in the model group × 100% [25].

HE staining: For histological analysis, the subcutaneous tumor and lung tissues were embedded in paraffin, sliced at a thickness of 5 μm and stained with HE. Then histological structure of subcutaneous tumor and lung metastases were observed under light microscope (Olympus, Japan). The histopathological scores were graded according to the severity of the tumor cells affected lung tissue in each HE-stained section, as follows: 1 = minimal (<1%); 2 = slight (1-25%); 3 = moderate (26-50%); 4 = moderate/severe (51-75%); and 5 = severe/high (76-100%) [30,31].

Detection of E-cadherin and Vimentin mRNA expression in

subcutaneous tumor: The mRNA expression of E-cadherin and Vimentin in subcutaneous tumor was detected by qRT-PCR. RNA was isolated from 100 mg subcutaneous tumor tissues with 2 mL RNAiso Plus (Takara Biotechnology Co., Ltd). After extracting the total RNA, a NanoDrop ND-3300 micro spectrophotometer was used to detect the 260/280 absorbance ratio as a measure of RNA purity. The genomic DNA was removed according to the instructions of the cDNA synthesis kit (Takara Biotechnology), and then cDNA was synthesized using a 2720 thermal cycler (USA). The cDNA was stored at −20°C. Amplification was performed on the ABI 7500 thermocycler with the following procedure: pretreatment at 50°C for 2 min, pre-denaturation at 95°C for 10 min, denaturation at 95°C for 15 s, and annealing at 60°C for 60 s, with 40 cycles in total. At the end of the program, the CT value of the target gene and the internal reference (GAPDH) were standardized to calculate the relative expression of the target gene. The SYBR prime script TM RT Master Mix Kit was purchased from Novozin Biotechnology Co., Ltd. Primers for E-cadherin, Vimentin and GAPDH were designed and synthesized by Shanghai Bioengineering Co., Ltd. The primer sequences are shown in *Table 1*.

Detection of TAM (CD11b+F4/80+) cells in subcutaneous tumor: F4/80/CD11b antibody (BD Company) was added to 100 µL of cell suspension, which was incubated in the dark for 20 min. The cells were centrifuged at 300 g for 5 min and resuspended in 100 µL PBS, and then 0.5 µL DAPI was added. The cells were then incubated for 15 min, and 300 µL PBS was added. The amount of TAMs (CD11b+F4/80+) in lung cancer tissue was detected by Cyto FLEX flow cytometry.

Detection of miR-21 and miR-218 expression in subcutaneous tumor: miRNA was isolated using the rapid tissue Cell miRNA extraction kit, and then cDNA was synthesized using the HG TaqMan miRNA reverse transcription kit. Gene amplification was performed on the ABI 7500 with the following program: 15 min at 95°C, 10 s at 95°C and 60 s at 60°C, with 40 cycles. The CT value of the target gene was standardized to the internal reference (RNU6B), and the relative expression was calculated bythe $2^{-\Delta\Delta CT}$ method. The rapid tissue Cell miRNA extraction kit, HG TaqMan miRNA reverse transcription kit, and HGSYBR Green quantitative PCR kit were provided by Haiji Biotechnology Co., Ltd.

Statistical Analysis

SPSS 20.0 statistical software was used to process the data. The results are expressed as mean ± SD. One-way ANOVA and multiple comparison or Chi-square test were used for C, LOEH, MOEH and HOEH groups, with $P<0.05$ indicating significant difference.

Results

Composition of OEH

As shown in *Table 2*, the total protein content of OEH is 57.23%, acid soluble protein content is 52.12%, and acid soluble protein accounts for 91.07% of the total protein content. The total sugar content was 25.89%, and the free amino acid content was 6.16%. Because small peptide and free amino acids can be dissolved in acid solution [19], the content of small molecule oligopeptides in OEH is about 45.96%. The relative molecular weight was less than 1000 Da in OEH was 91.92%, indicating that the main components of OEH not only contain small molecular oligopeptides, but also rich in oyster polysaccharides and other substances.

The Sequences of Peptides in OEH

To identify the peptide sequences, OEH was subjected to chromatography and mass spectrometer system. OEH is composed of a large number of short peptides, and HPLC-MS/MS spectra processing identified 20 major peptide sequences. *Fig. 1* indicates several peptides with an arginine residue at the C-terminusor N-terminus, whose sequences include Ile-Arg, Arg-Ile and Val-Arg. The molecular mass of Ile-Arg, Arg-Ile and Val-Arg are 287.4, 287.4 and 273.3Da, respectively.

OEH Mediates Dose-Dependent Inhibition of Subcutaneous Tumor Growth in the Lewis Lung Cancer Model in Mice

To evaluate the ability of OEH to inhibit subcutaneous tumor growth in the Lewis lung cancer mouse model, we measured the tumor volume and weight and observed their histological structure in each group of mice. Though there was a trend of volume reduction with increasing OEH, the decrease was not statistically significant ($F=1.168$, $P=0.348$); however, OEH had a significant effect on the weight of subcutaneous tumors in tumor-bearing mice ($F=3.635$, $P=0.032$) *(Table 3)*. The reduction was most obvious at the HOEH dose ($P=0.007$; 36% inhibition rate), with progressively less reduction at the MOEH dose ($P=0.013$;

Table 1. *Sequence of RT-PCR specific primers*

Oligo Name	Orientation	Sequence (5'to3')	%GC	TM
E-cadherin	Forward	TATGATGAAGAAGGAGGTGGAGA	43.48	54.37
	Reverse	AACACCAACAGAGAGTCGTAAGG	47.83	56.56
Vimentin	Forward	AGTATGAAAGCGTGGCTGCC	55	58.50
	Reverse	AGCTTCCTGTAGGTGGCGAT	55	58.50
GAPDH	Forward	GGTTGTCTCCTGCGACTTCA	60	62.62
	Reverse	TGGTCCAGGGTTTCTTACTCC	50	60.47

RT-PCR: *Real time quantitative PCR;* ***GC:*** *Guanine (G) and cytosine (C) content;* ***TM:*** *Melting Temperature;* ***GADPH:*** *Glycerol 3 phosphate dehydrogenase*

Table 2. *Basic composition and molecular weight distribution of OEH*

Basic Composition		Molecular Weight Distribution	
Composition	**Proportion (%)**	**Molecular Weight (Da)**	**Proportion (%)**
Total protein	57.23	5000-10000	0.4477
Acid soluble protein	52.12	3000-5000	0.8412
Free amino acids	6.16	2000-3000	1.2510
Total sugar	25.89	1000-2000	5.4109
Ash	10.46	150-1000	65.8831
Water	6.28	1-150	26.0374

29% inhibition rate) and the LOEH dose (P=0.082; 22% inhibition rate). Consistently, the tumors in the MOEH and HOEH groups were visibly smaller than those in the C and LOEH groups *(Fig. 2-A)*. We also assessed the 5-week survival rate but observed no detectable effect ($\chi^2 = 2.254$, P=0.521). As shown in *Fig. 2-B*, the tumor cell density in group C was high, the outline was clear, the nucleus was large and deep staining, the nucleoplasm ratio was imbalanced, and the nuclear heteromorphism was obvious, with dual or multinucleus visible. The morphology of tumor tissues in the LOEH, MOEH, and HOEH groups changed significantly, the density of tumor cells was reduced, the proportion of deep stained nuclei was relatively reduced, and the nuclei appeared shrinkage or fragmentation. Spotty or patchy necrosis was appeared in tumor tissues, especially in MOEH and HOEH groups. These results suggest that OEH mediates dose-dependent inhibition of subcutaneous tumor growth by promoting the death of tumor cell.

OEH Treatment Decreases the Rate and Histopathological Scores of Lung Metastasis in the Lewis Lung Cancer Model in Mice

To further evaluate the effect of OEH in the Lewis lung tumor model, we assessed pulmonary surface nodule formation as a measure of metastasis. As shown in *Table 4*, OEH had a significant inhibitory effect on the lung metastasis in tumor bearing mice (χ^2=13.16, P=0.004; F=8.795, P=0.001). The rate and histopathological scores of lung metastasis gradually decreased with increasing OEH dose, which suggests dose dependence. The number of nodules also decreased gradually with increasing OEH dose, but the differences were not statistical (F=1.147, P=0.356; *Fig. 3-A)*. However, HE staining of lung sections showed that the lesion scores in group MOEH or HOEH had significantly fewer than that in group C (P=0.001, P=0.000) *(Fig. 3-B)*. Collectively, these results suggest that OEH may decrease lung metastasis or/and reduce the growth of lung metastases.

OEH Can Modulate the mRNA Expression of Vimentin and E-cadherin in Subcutaneous Tumor

We further assessed the effects of OEH on the expression of Vimentin and E-cadherin as indicators of EMT. As shown in

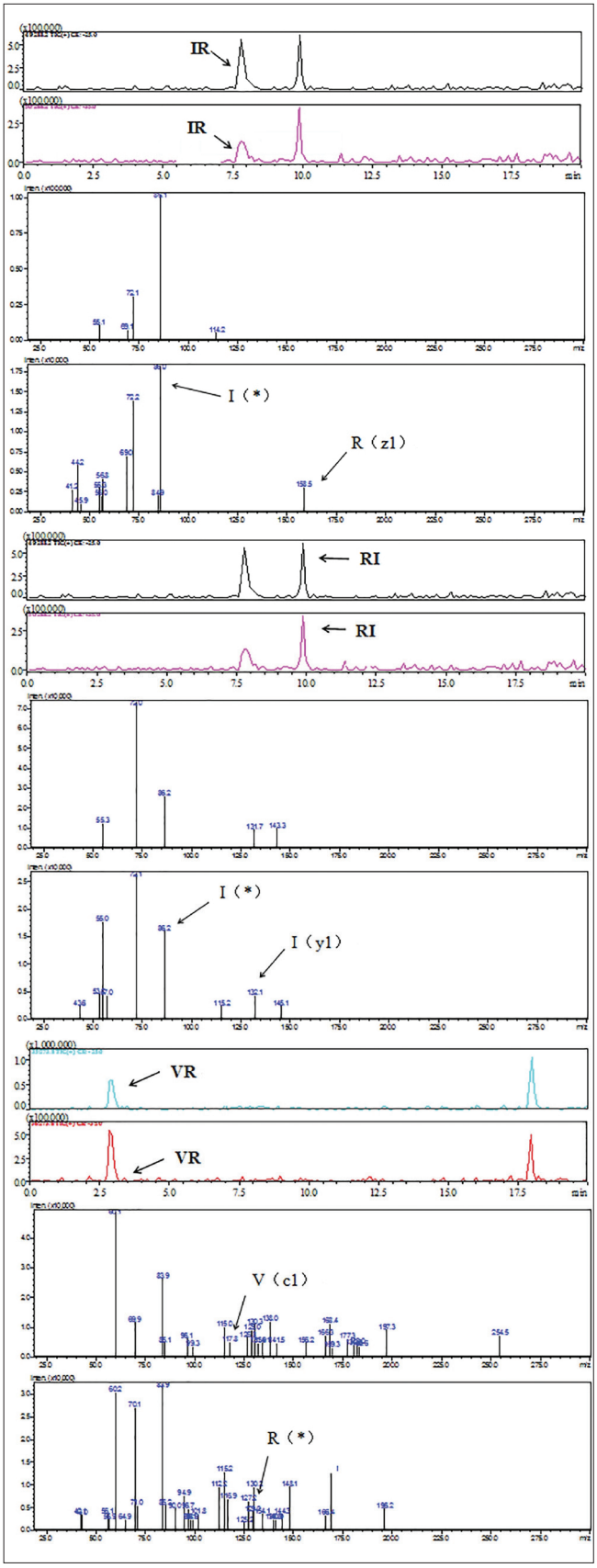

Fig 1. The map of sequence Ile-Arg, Arg-Ile and Val-Arg. HPLC-MS/MS analysis to reveal the structure of peptides with an arginine residue at the C-terminus or N-terminus, whose sequences

Table 3. *Changes in the volume, weight of subcutaneous tumor and tumor inhibition rate in OEH-treated mice (M ± SD)*

Group	n	Volume (mm^3)	Weight (g)	Tumor Inhibition Rate (%)	5-week Survival Rate (%)
C	5	5131.80±2979.03	11.12±1.19	-	42
LOEH	5	4180.90±1018.11	8.70±1.63	22	42
MOEH	8	3821.31±1325.35	7.88±2.42*	29	67
HOEH	5	3074.50±1434.92	7.10±2.50**	36	42

*One-way ANOVA and multiple comparisons or Chi-square test were carried out for groups C, LOEH, MOEH and HOEH, compared with group C. * P<0.05, ** P<0.01. C: control; OEH: oyster enzymatic hydrolysate; LOEH: low doses of OEH; MOEH: medium doses of OEH; HOEH: high doses of OEH*

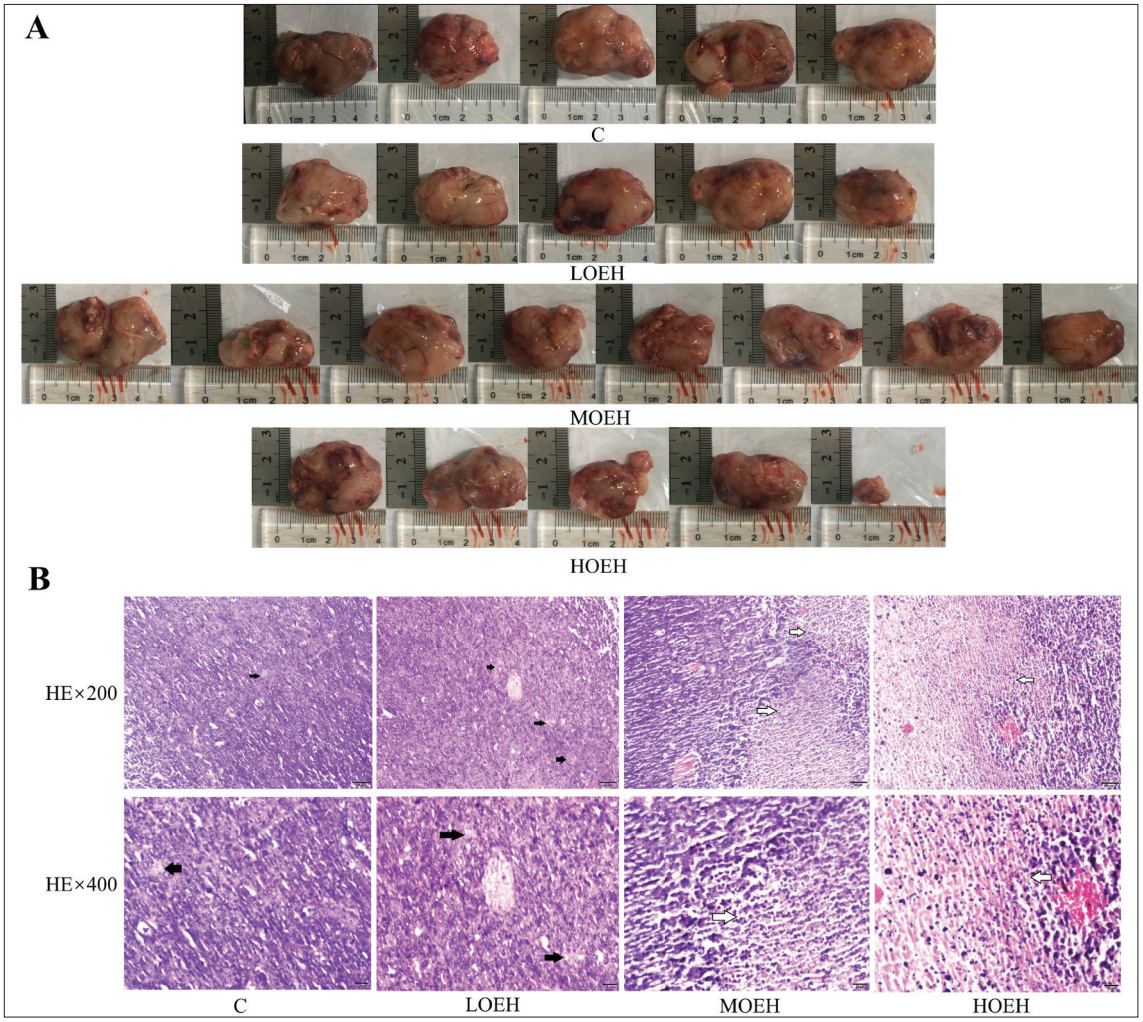

Fig 2. Subcutaneous tumors (A) and their histological structure (B, HE staining, ×200 and ×400) from each group. ↗: Spotty necrosis; ⇨:Patchy necrosis. C: control; OEH: oyster enzymatic hydrolysate; LOEH: low doses of OEH; MOEH: medium doses of OEH; HOEH: high doses of OEH

Table 5, OEH mediated a statistical increase in the expression of E-cadherin mRNA in subcutaneous tumor (F=3.318, P=0.042) and a statistical decrease in the expression of Vimentin mRNA (F=5.609, P=0.006). Compared with group C, the expression of Vimentin mRNA was decreased significantly in the LOEH, MOEH and HOEH groups (P=0.001, P=0.010, P=0.007), however the expression of E-cadherin mRNA was significantly increased only in HOEH group (P=0.014). These results suggest that OEH causes a decrease in Vimentin expression and increase in E-cadherin expression, which might suggest that OEH inhibits EMT.

OEH Treatment Reduce the Counts of TAMs (CD11b + F4/80 + cells) in Subcutaneous Tumor

To further evaluate a potential role for EMT mediating OEH-dependent decrease in tumor growth and metastasis, we assessed the effect of OEH treatment on CD11b + F4/80 + TAMS *(Fig. 4-A)*. As shown in *Fig. 4-B*, OEH mediated an overall significant decrease in the number of CD11b + F4/80 + cells in subcutaneous tumor (F=3.144, P=0.049). Nevertheless, the number of CD11b + F4/80 + cells in the LOEH, MOEH and HOEH group were decreased significantly (P=0.014; P=0.015;

Table 4. *Changes in the lung metastasis inhibition rate, the number of pulmonary surface nodules, and the 5-week survival rate in OEH-treated mice (M ± SD)*

Group	n	Lung Metastasis Rate (%)	Number of Nodules	Lung Metastasis Inhibition Rate (%)	Histopathological Scores
C	5	100.00	3.60±3.21	-	3.20±0.45
LOEH	5	100.00	3.20±1.64	11	2.40±0.55
MOEH	8	87.50	2.25±1.49	36	1.75±0.71**
HOEH	5	20.00**	1.20±2.68	67	1.20±0.84**

*Chi-square test or One-way ANOVA and multiple comparisons were carried out for groups C, LOEH, MOEH and HOEH, compared with group C. ** P<0.01. C: control; OEH: oyster enzymatic hydrolysate; LOEH: low doses of OEH; MOEH: medium doses of OEH; HOEH: high doses of OEH*

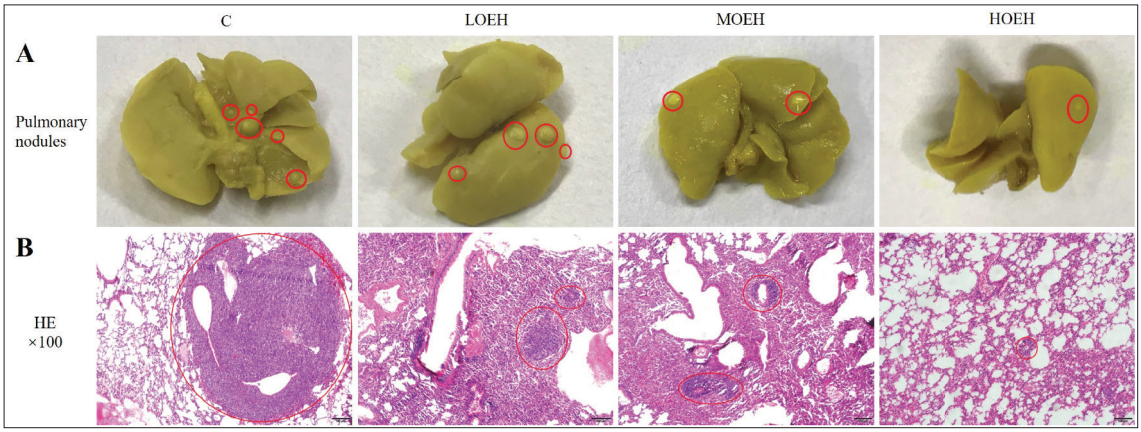

Fig 3. The pulmonary surface nodules (A) and their histological structure (B, HE staining, x100) in each group. The gray and white nodules in the red circle are metastatic tumor. C: control; OEH: oyster enzymatic hydrolysate; LOEH: low doses of OEH; MOEH: medium doses of OEH; HOEH: high doses of OEH

Table 5. *Expression of E-cadherin and Vimentin mRNA in subcutaneous tumor of mice (M ± SD)*

Group	n	E-cadherin	Vimentin
C	5	0.90±0.36	1.09±0.44
LOEH	5	1.08±0.44	0.22±0.06**
MOEH	8	0.96±0.64	0.52±0.44*
HOEH	5	1.74±0.28*	0.42±0.22**

*One-way ANOVA and multiple comparisons were carried out for groups C, LOEH, MOEH and HOEH, compared with group C. * P<0.05, ** P < 0.01. C: control; OEH: oyster enzymatic hydrolysate; LOEH: low doses of OEH; MOEH: medium doses of OEH; HOEH: high doses of OEH*

Table 6. *Expression of miR-21 and miR-218 in subcutaneous tumor of mice (M ± SD)*

Group	n	miR-21	miR-218
C	5	0.96±0.36	1.05±0.16
LOEH	5	0.64±0.25	2.52±0.89*
MOEH	8	0.49±0.28*	2.84±1.04**
HOEH	5	0.43±0.34*	2.83±1.47*

*One-way ANOVA and multiple comparisons were carried out for groups C, LOEH, MOEH and HOEH, compared with group C. * P<0.05, ** P<0.01. C: control; OEH: oyster enzymatic hydrolysate; LOEH: low doses of OEH; MOEH: medium doses of OEH; HOEH: high doses of OEH*

P=0.045) than that in the C group of mice. Therefore, OEH treatment with all dose can decreases the percentage of TAMs (CD11b + F4/80 + cells) in subcutaneous tumor.

Expression of miR-21 and miR-218 is Modulated in Subcutaneous Tumor of Mice with OEH Treatments

To evaluate the potential roles of miR-21 and miR-218 as mediators of OEH-dependent tumor reduction, we measured their expression in group C and each of the treatment groups. OEH had an overall significant effect on the expression of miR-21 and miR-218 in subcutaneous tumor of tumor bearing mice as determined one-way ANOVA (F=3.251, P=0.045; F=3.743, P=0.029). Furthermore, after multiple comparison, the expression of miR-21 significantly decreased in the group of MOEH (P=0.013) and HOEH (P=0.013) *(Table 6)*. And the expression of miR-218 was significantly increased in the group of LOEH (P=0.034), MOEH (P=0.006) and HOEH (P=0.012). These results are consistent with a potential role for miR-21 and miR-218 in mediating the tumor-protective effects of OEH.

Discussion

Oyster enzymatic hydrolysate (OEH) is obtained by enzymolysis, concentration, and freeze-drying of oyster meat. In this study, we found that the content of total protein, total sugar and small molecule oligopeptides in OEH were 57.23%, 25.89% and 45.96%, the relative molecular weightless than 1000 Da was 91.92% in OEH, indicating that the main components of OEH contain not only small molecular oligopeptides, but also rich in oyster polysaccharides. It has been proved that oligopeptides with molecular weight less than 1000 Da are mostly dipeptides and tripeptides [27],

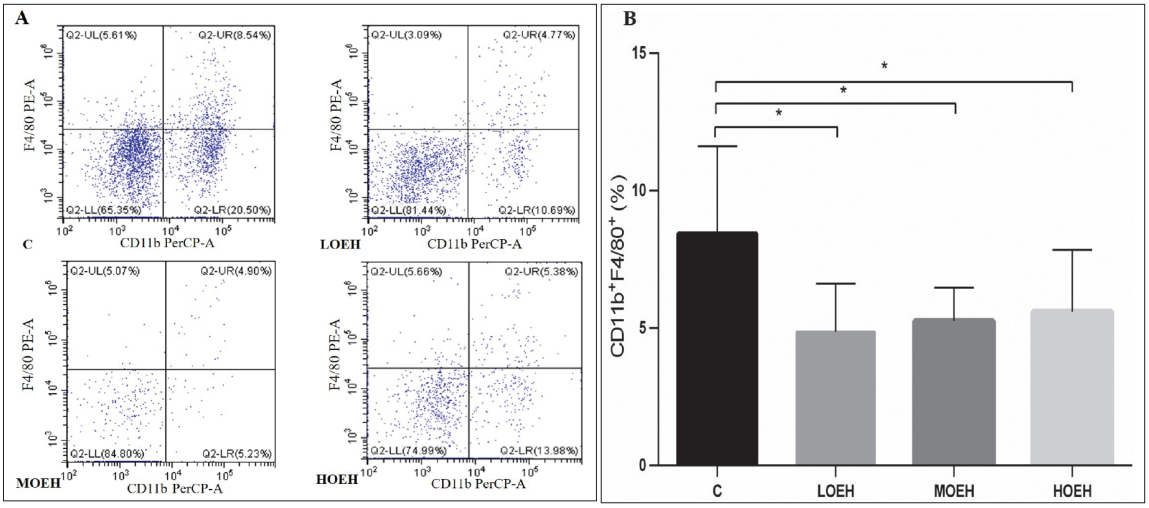

Fig 4. Distribution map (A) and percentage of CD11b+F4/80+ cells in subcutaneous tumor of mice in each group (B). The cells in UR region are CD11b + F4/80 + cells. One-way ANOVA and multiple comparisons were carried out for groups C, LOEH, MOEH and HOEH, compared with group C. * P<0.05. C: control; OEH: oyster enzymatic hydrolysate; LOEH: low doses of OEH; MOEH: medium doses of OEH; HOEH: high doses of OEH

which have faster absorption rate compared with proteins and free amino acids [12,32,33], and oyster polysaccharides can be absorbed without digestion [16]. Therefore, it can be inferred that OEH has not only higher biological activity, but also higher absorption rates than Natural Oyster Protein. At the same time, dipeptides containing arginine were isolated from OEH, and L-arginine supplementation can enhance the immune function of tumor patients, reduce the synthesis of tumor protein, and inhibit the growth of tumor [34,35]. Although the bioactive peptide of oyster has many physiological functions, there are few studies on its antitumor effect. The natural low molecular polypeptide BPO-L, which is extracted from the oyster, has been shown to effectively inhibit the proliferation [36], and change the malignant morphology and ultrastructural characteristics [37] of human lung adenocarcinoma A549 cells.

In this study, we found that increasing OEH doses show a trend of reduction in volume and weight of subcutaneous tumor in tumor-bearing mice, though the changes in tumor volume were not statistically significant, and only the tumor weight reductions in the MOEH (29% decrease) and HOEH (36% decrease) groups were statistically significant. In the meantime, HE staining of tumor tissues showed that the density of tumor cells, nuclear heteromorphism reduced and necrosis area of tumor cells increased gradually with the increase of OEH doses. Because the growth of tumor is related to the proliferation, apoptosis and necrosis of tumor cells, the supplement of OEH can promote the necrosis of tumor cells to inhibit the growth of subcutaneous tumors. Our previous studies confirmed that OEH treatment can effectively improve the function of T cells and NK cells, and increase the apoptosis and necrosis of Lewis lung cancer cells implanted subcutaneously, which can effectively inhibit the growth of subcutaneous implanted tumor [38]. However, whether OEH can inhibit the proliferation of tumor cells remains to be further studied. Furthermore, the lung metastasis rate was decreased significantly with increasing OEH dose. Although the number of lung metastasis nodules had not statistically decrease. HE staining of lung sections showed that the lesion scores in group MOEH or HOEH had significantly fewer than that in group C. Therefore, OEH can inhibit lung metastasis from subcutaneous tumor cells to a certain extent, and/or reduce the growth of lung metastases. However we found that OEH treatment cannot effectively improve the 5-week survival rate of tumor bearing mice, its reason is unclear, which needs further study.

The invasion and metastasis of lung cancer mainly involve a series of rather complex processes, such as cell adhesion, matrix degradation and cell movement [39]. Among them, EMT can change the phenotype of tumor cells, so that they have a strong ability of invasion and metastasis [40]. On the one hand, E-cadherin is an adhesion factor that can inhibit the invasion and metastasis of tumor cells. Decreased E-cadherin expression results in a decrease in the adhesion between homologous cells [41]. On the other hand, Vimentin is a marker of stromal cells and can maintain and promote stromal transformation and invasion of tumor cells. Increased expression of Vimentin marks the occurrence of the EMT process [42]. Therefore, the expression of E-cadherin and Vimentin provides an important reference value for the evaluation of tumor malignancy, invasion depth, prediction of metastasis and prognosis [43]. Studies have also shown that TAMs can promote the growth and metastasis of lung cancer cells, whereas inhibition of the aggregation of TAMs in tumors can improve the prognosis of lung cancer [44-48]. In this study, we observed that OEH supplementation with all dose could significantly reduce TAMs and Vimentin mRNA expression in subcutaneous tumor, though only increase of E-cadherin mRNA expression at high dose was statistically significant. Therefore, it is possible that the inhibition of tumor growth and invasion by OEH is mediated by inhibition of EMT.

MicroRNAs are a group of noncoding RNAs that regulate gene transcription and protein translation at the post transcriptional level and play important roles in cell proliferation, differentiation and apoptosis [49,50]. The expression of miR-218 has been shown to be down-regulated or absent in lung cancer cells [51,52]. MiR-218 inhibits the invasion and migration of lung cancer cells by inhibiting the expression of Robo1 and can promote apoptosis of A549 cells by negatively regulating the expression of its target gene, SFMBT1 [53]. On the other hand, the high expression of miR-21 in NSCLC is closely related to NSCLC cell proliferation, angiogenesis, invasion and metastasis [54,55], as well as poor prognosis in NSCLC [56], for which it serves as a potential molecular target for lung cancer diagnosis, metastasis and prognosis. Inhibition of miR-21 can induce apoptosis and inhibit proliferation of cancer cells and can enhance the sensitivity of cancer cells to chemotherapy drugs and radiotherapy [57,58]. Consequently, miR-21 plays an oncogenic role while miR-218 plays a tumor suppressor role. In this study, oral OEH with all dose could significantly up-regulate the expression of miR-218, although only medium and high dose of OEH could significantly down-regulate the expression of miR-21. Therefore, OEH can modulate the expression of miR-21 and miR-218 in subcutaneous tumor to a certain extent, which may explain its ability to inhibit the lung metastasis from subcutaneous tumor cells.

In conclusion, the main components of OEH contain small molecular oligopeptides, oyster polysaccharides and other substances. OEH can significantly reduce the growth of subcutaneous tumor and lung metastasis in a dose-dependent manner. It can also modulate the mRNA expression of Vimentin and E-cadherin in subcutaneous tumor and reduce the number of TAMs at specific doses. OEH increases the expression of miR-21 and inhibits the expression of miR-218 in subcutaneous tumor in a dose-dependent manner, which might explain, in part, its inhibitory effect on the lung metastasis from subcutaneous tumor cells.

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Declaration of Conflicting Interests

The authors declare no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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Author Contributions

Q.G. JIN and W.Y. LIU planned and designed the research. Q.G. JIN and W.Y. LIU had a role in manuscript drafting and in process of revision. M. ZHOU, L. Chen, Y.Q. WANG and R.X. ZHANG carried out the preparation and identification of OEH in the study. Y.L. HU, Y. LIU and M.T. WU performed experiments. Y. LIU, Y.L.HU and Q.G. JIN carried out the statistical analysis. All authors have interpreted the data, revised the manuscript, and approved the final version.

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RESEARCH ARTICLE

Effect of Different Extenders on the Sperm Quality Parameters of Hu Ram Semen Preserved at 16°C

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Abstract

The purpose of this experiment was to determine the effects of different extenders on the sperm quality parameters of Hu ram semen preserved at 16°C. The quality parameters studied include total sperm motility, progressive motility, survival time, plasma membrane integrity, acrosome integrity and some kinematic parameters, such as the curvilinear velocity. Each ram ejaculated approximately twenty times and ejaculates were collected every two days interval during breeding season. Three Hu rams were used in experiments. The ejaculates were pooled and diluted (1:10) with extenders A (Tris-Fructose based), B (Fructose-Sodium Citrate based), C (Glucose based), D (Fructose based) and E (Control, Physiological saline solution) and then stored at 16°C. The above parameters were detected every 24 h. The total sperm motility, progressive motility, acrosome integrity and some kinematic parameters of extender A were the highest compared to those of other extenders and decreased slowly within 24 to 144 h. The effective survival time of sperm preserved in extender A was 74.50±4.82 h, and the total survival time was 412.67±2.52 h, which was significantly higher than those of the other four extenders ($P \leq 0.05$). The acrosome integrity of extender A was the highest within 24 to 144 h and significantly higher than those of the other extenders within 48 to 144 h ($P \leq 0.05$). Compared with the other extenders, extender A had numerically the highest plasma membrane integrity within 24 to 96 h of preservation ($P > 0.05$). In conclusion, extender A improved the sperm quality of Hu ram semen, which could be used for artificial insemination for up to 144 h of preservation.

Keywords: *Hu Ram, 16°C, Semen extender, Semen quality*

Farklı Sperm Sulandırıcılarının 16°C'de Saklanan Hu Koç Sperm Kalitesi Parametreleri Üzerine Etkisi

Öz

Bu çalışmanın amacı, farklı sperm sulandırıcılarının 16°C'de saklanan Hu koç sperm kalitesi parametreleri üzerindeki etkilerini belirlemekti. İncelenen kalite parametreleri arasında toplam sperm motilitesi, progresif motilite, canlı kalma süresi, plazma membran bütünlüğü, akrozom bütünlüğü ve eğrisel hız gibi bazı kinematik parametreler bulunuyordu. Her koçtan yaklaşık yirmi kez sperm alındı ve üreme mevsimi boyunca koçlardan iki günde bir ejekülat toplandı. Deneylerde üç Hu koçu kullanıldı. Ejekülatlar havuz oluşturulduktan sonra, 1:10 oranında sırasıyla A (Tris-Fruktoz bazlı), B (Fruktoz-Sodyum Sitrat bazlı), C (Glukoz bazlı), D (Fruktoz bazlı) ve E (Kontrol, Fizyolojik tuzlu su) sulandırıcıları ile seyreltildi ve sonra 16°C'de muhafaza edildi. Adı geçen parametreler her 24 saatte bir tespit edildi. A sulandırıcısının kullanıldığı gruptaki toplam sperm motilitesi, progresif motilite, akrozom bütünlüğü ve bazı kinematik parametreleri diğer sulandırıcılara oranla en yüksek saptandı ve bu özellikler 24 ile 144 saat içinde yavaş yavaş azaldı. A sulandırıcısının kullanıldığı gruptaki spermlerin etkin canlılık süresi 74.50±4.82 saat ve toplam canlılık süresi 412.67±2.52 saat iken, bu değerler diğer dört sulandırıcıdan çok daha yüksek saptandı ($P \leq 0.05$). A sulandırıcısının kullanıldığı gruptaki spermlerin akrozom bütünlüğü 24 ile 144 saat içinde en yüksekti ve bu değerler diğer sulandırıcıların 48 ile 144 saat içinde sahip oldukları değerlerden çok daha yüksekti ($P \leq 0.05$). Diğer sulandırıcılarla karşılaştırıldığında A sulandırıcısının, 24 ile 96 saatlik sperm muhafazasında sayısal olarak en yüksek plazma membran bütünlüğüne sahip olduğu saptandı ($P > 0.05$). Sonuç olarak, A sperm sulandırıcısının, suni tohumlama için kullanılmak üzere 144 saate kadar muhafaza süresince sperm kalitesini artırdığı belirlendi.

Anahtar sözcükler: *Hu koçu, 16°C, Sperm sulandırıcısı, Sperm kalitesi*

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Introduction

Hu ram is a world-famous prolific sheep breed that has the advantages of two births a year and rapid growth and development; thus, the breeding scale of Hu ram is expanding rapidly [1]. Artificial insemination is a basic technology in livestock reproduction [2], which can make full use of the semen of superior male animals [3]. It is an important means to improve the reproductive efficiency and productivity of livestock [4].

Artificial insemination technology combined with the application of estrus synchronization technology can reduce breeding costs, prevent the spread of diseases, and achieve large-scale feeding and management [5]. Semen preservation is a key link in artificial insemination [6]. Semen preservation is usually divided into 16°C preservation, 4°C preservation and cryopreservation [7]. Owing to the presence of large amounts of polyunsaturated fatty acids (PUFA) in ram sperm plasma membranes and the absence of a robust antioxidant system in ram sperm and seminal plasma, ram sperm is highly sensitive to cryopreservation [8-10], which limits semen cryopreservation in sheep [11,12]. In production, semen is mostly preserved at 16°C, which has the advantages of simple operation and suitability for popularization and application, without special temperature control and refrigeration equipment; it also has practical application value.

At present, 4°C preservation and cryopreservation are the research focus of ram semen preservation [13]. There are relatively few reports on the preservation of ram semen at 16°C, and there are even fewer extender formulas described. However, most of the semen preserved at 16°C is used in production. This experiment is based on the extender used for 4°C or cryopreservation of ram semen. Extenders with better preservation were screened. Substances such as cryoprotectants were removed so as not to adversely affect semen preserved at 16°C. In the experiment evaluating the preservation of ram semen at 16°C, an excellent extender formula was selected. Arando et al.[14] indicated that OVIX was the best commercial extender for preservation of ram semen at 16°C. Total sperm motility preserved in the extender decreased to 40% in 96 h, and sperm progressive motility decreased to 20% in 96 h. Although the commercial extender has a good preservation effect, it is still far from meeting the needs of production. To prolong the storage time of semen at 16°C, therefore, extenders A (Tris-Fructose based), B (Fructose-Sodium Citrate based), C (Glucose based), D (Fructose based) and E (Control, Physiological saline solution) were used to preserve ram semen at 16°C in this experiment. The experiment objectively evaluated and screened an extender formula with the best preservation effect of ram semen at 16°C.

Material and Methods

Animals and Semen Collection

Three 2-to 3-years-old male Hu sheep having Body condition score (BCS) ≥3 (scale1-5) kept in the experimental sheep farm were used in this study. They were fed 0.2 kg concentrate/once, twice a day, and ad libitum hay and water. The experiment trimmed the ram abdomen and wash the foreskin with saline. A total number of 60 ejaculates were collected from the rams every two days interval in the morning by the artificial vaginal (AV) between October and December 2019. The water temperature in the AV was kept at 40~42°C. Ensure a certain degree of lubrication and pressure in the AV. It was brought back to the laboratory at 37°C within 20 min. The semen volume of each ram collected was about 1.5 mL, which was milky white and had no abnormal smell. The quality assessment was carried out quickly and only the total motility >80% and the morphologically abnormal sperm <15% were accepted. The ejaculates collected from the three rams were pooled and processed to eliminate variability. Semen sampling procedure was approved by the Animal care committee of the Yangzhou University.

Preparation of Semen Extender

Five different extenders, A (Tris-Fructose based), B (Fructose-Sodium Citrate based), C (Glucose based), D (Fructose based) and E (Control, Physiological saline solution), were prepared according to the ingredients and doses shown in *Table 1*.

Sperm Quality Evaluation

Total sperm motility, progressive motility and kinematic parameters were measured by CASA. Plasma membrane integrity was detected by HOST [15]. Acrosome integrity was detected by Coomassie brilliant blue staining [16]. The time when the sperm progressive motility is above 60% is called the effective survival time; when total sperm motility drops to zero, the time when all sperm die is called the total survival time [17].

Statistical Analysis of Data

All the test data were analyzed by SPSS25.0 statistical software. All results were expressed as mean values±SD. When the P value was significant (P≤0.05), Duncan's multiple range tests by ANOVA procedure were used to compare the mean value of the total sperm motility, progressive motility, kinematic parameters, plasma membrane integrity and acrosome integrity.

Results

Effects of Different Extenders on Total Sperm Motility

As seen in *Table 2*, the total sperm motility decreased with the extension of storage time when five kinds of extenders were used to preserve Hu ram semen at 16°C. Among them, the total sperm motility of extender A decreased steadily and slowly. Within 24 to 96 h of preservation, the total motility of sperm preserved in extender A was significantly

Table1. *Five different extender formulations*

Composition	A	B	C	D	E (Control)
Tris/g	15.3500	-	-	-	-
Citric acid/g	8.2000	-	-	-	-
Fructose/g	10.0000	7.2500	1.5310	9.0000	-
Glucose/g	-	5.7500	22.2490	1.0000	-
Sodium citrate/g	-	5.8500	-	-	-
Sodium bicarbonate/g	-	0.6250	-	0.5000	-
Polyvinyl alcohol/g	-	1.2500	-	-	-
EDTA/g	-	1.1500	-	-	-
Disodium hydrogen phosphate/g	-	-	0.7090	-	-
Sodium dihydrogen phosphate/g	-	-	0.5990	-	-
Sodium chloride/g	-	-	1.4310	0.3350	4.5000
Potassium chloride/g	-	-	0.1860	-	-
Calcium chloride dihydrate/g	-	-	-	0.0300	-
Magnesium chloride hexahydrate/g	-	-	-	0.0400	-
Penicillin sodium/g	0.1559	0.1559	0.1559	0.1559	-
Streptomycin sulfate/g	0.3472	0.3472	0.3472	0.3472	-
Sterilized ultra-pure water/mL	500.0000	500.0000	500.0000	500.0000	500.0000
PH	7.47	7.22	7.51	8.20	7.17

Table 2. *Effects of different extenders on the total motility of preserved sperm (mean±SD); %*

Storage Time	A	B	C	D	E (Control)
0 h	84.39 ± 5.02^{ab}	89.18 ± 6.41^{a}	81.19 ± 1.72^{ab}	81.84 ± 4.03^{ab}	84.53 ± 4.95^{ab}
24 h	82.99 ± 4.06^{a}	62.67 ± 2.31^{b}	9.28 ± 5.06^{e}	44.46 ± 2.76^{c}	1.12 ± 0.22^{f}
48 h	79.53 ± 1.30^{a}	55.46 ± 1.60^{b}	0.69 ± 0.22^{e}	11.28 ± 2.42^{d}	-
72 h	72.24 ± 5.49^{a}	48.73 ± 7.48^{b}	-	12.06 ± 2.27^{c}	-
96 h	53.23 ± 9.75^{a}	29.57 ± 8.55^{b}	-	8.66 ± 3.03^{c}	-
120 h	29.34 ± 9.01^{a}	11.23 ± 6.72^{bc}	-	1.77 ± 0.33^{c}	-
144 h	6.04 ± 3.53^{ab}	1.48 ± 1.51^{bc}	-	0.48 ± 0.23^{c}	-

$^{a\text{-}f}$ Values within the same row with different letters differ significantly (P≤0.05); - means that the sperm have all died, losing the significance of detection

higher than those in the other four extenders (P≤0.05). At 120 h of preservation, the total sperm motility of extender A was significantly higher than those of the other four extenders (P≤0.05). At 144 h of preservation, the total sperm motility in extender A was the highest and significantly higher than that in extender D (P≤0.05), but there was no significant difference between extender A and extender B (P>0.05).

Effects of Different Extenders on Sperm Progressive Motility

As seen in *Table 3*, the sperm progressive motility decreased with the extension of storage time when Hu ram semen was preserved with the five kinds of extenders at 16°C. Among them, the sperm progressive motility preserved by extender A decreased steadily and slowly. Within 24 to 96 h of preservation, the sperm progressive motility of extender A was significantly higher than those of the other four extenders (P≤0.05). At 120 h of preservation, the sperm progressive motility of extender A was significantly higher than those of the other four extenders (P≤0.05). At 144 h of preservation, the sperm progressive motility of extender A was the highest, but the difference was not significant (P>0.05).

Effects of Different Extenders on the Different Sperm Kinematic Parameters

As seen in *Table 4*, the results showed that the straight line velocity (VSL), curvilinear velocity (VCL), average path velocity (VAP), amplitude of lateral head displacement (ALH), and average motion degree (MAD) of sperm decreased with the extension of storage time. Among them, the decline of extender A was steady and slow. At 24 h of preservation, the VSL of sperm preserved in extender A was significantly higher than that in extenders B, C and E (Control) (P≤0.05), but not significantly different from that in extender D (P>0.05). At 24 h of preservation, the wobble movement coefficient (WOB) of sperm preserved in extender A was

Table 3. *Effects of different extenders on the progressive motility of preserved sperm (mean±SD); %*

Storage Time	A	B	C	D	E (Control)
0 h	79.58±5.17^{ab}	83.93±8.70^{a}	70.34±1.35^{b}	72.97±5.66^{b}	78.44±5.50^{ab}
24 h	79.52±5.60^{a}	51.59±3.05^{b}	5.84±3.08^{e}	33.64±2.21^{c}	0.59±0.20^{e}
48 h	72.10±1.82^{a}	45.79±3.37^{b}	0.36±0.28^{e}	8.10±2.42^{d}	-
72 h	63.01±6.07^{a}	35.54±5.62^{b}	-	8.26±1.50^{c}	-
96 h	42.83±10.48^{a}	19.25±5.50^{b}	-	5.36±2.44^{c}	-
120 h	17.07±5.65^{a}	6.40±3.75^{b}	-	0.89±0.27^{b}	-
144 h	2.04±1.18^{b}	0.83±0.86^{b}	-	0.24±0.01^{b}	-

$^{a-e}$ Values within the same row with different letters differ significantly (P≤0.05); - means that the sperm have all died, losing the significance of detection

Table 4. *Effects of different extenders on the different kinematic parameters of preserved sperm (mean±SD)*

Kinematic Parameter	Storage Time	A	B	C	D	E (Control)
VSL (µm/s)	0 h	38.01±0.^{88a}bc	39.21±2.74^{ab}	40.03±1.86^{a}	35.63±1.72^{c}	40.58±0.69^{a}
	24 h	35.91±2.68^{a}	24.09±1.21^{bc}	16.63±2.89^{c}	26.79±0.50^{ab}	14.61±11.57^{c}
	48 h	30.44±3.00^{a}	26.28±0.81^{b}	5.90±3.38^{d}	19.17±0.94^{c}	-
	72 h	27.24±1.78^{a}	24.29±1.59^{ab}	-	22.65±1.33^{bc}	-
	96 h	21.94±2.45^{a}	21.24±2.03^{a}	-	18.86±4.2^{a} 6	-
	120 h	17.58±1.49^{a}	17.42±1.75^{a}	-	11.58±3.16^{b}	-
	144 h	12.28±2.81^{a}	8.68±9.13^{a}	-	4.64±0.42^{a}	-
VCL (µm/s)	0 h	73.80±2.37^{a}	71.34±1.27^{a}	71.29±3.89^{a}	63.18±3.08^{b}	73.27±4.97^{a}
	24 h	76.01±3.98^{a}	57.55±2.68^{b}	37.20±7.86^{c}	56.23±0.38^{b}	27.86±20.64^{c}
	48 h	65.19±4.77^{a}	58.04±2.82^{b}	8.90±4.13^{e}	39.39±0.76^{d}	-
	72 h	59.10±3.31^{a}	43.67±5.89^{b}	-	41.40±2.51^{b}	-
	96 h	47.58±4.17^{a}	39.85±5.80^{a}	-	44.60±5.93^{a}	-
	120 h	40.61±3.18^{a}	31.66±2.57^{b}	-	34.03±6.78^{ab}	-
	144 h	29.65±9.64^{a}	15.60±17.07^{ab}	-	8.39±2.45^{b}	-
VAP(µm/s)	0 h	52.19±1.68^{a}	50.45±0.90^{a}	50.41±2.75^{a}	44.67±2.17^{b}	51.81±3.52^{a}
	24 h	53.75±2.81^{a}	40.69±1.89^{b}	26.31±5.56^{c}	39.76±0.27^{b}	19.70±14.59^{c}
	48 h	46.10±3.37^{a}	41.04±1.99^{b}	6.29±2.92^{e}	27.85±0.54^{d}	-
	72 h	41.79±2.34^{a}	30.88±4.17^{b}	-	29.28±1.78^{b}	-
	96 h	33.64±2.95^{a}	28.18±4.10^{a}	-	31.53±4.19^{a}	-
	120 h	28.72±2.25^{a}	22.38±1.82^{b}	-	24.06±4.79^{ab}	-
	144 h	20.97±6.81^{a}	11.03±12.07^{ab}	-	5.94±1.74^{b}	-
ALH (µm)	0 h	21.62±0.70^{a}	20.90±0.38^{a}	20.88±1.14^{a}	18.51±0.90^{b}	21.46±1.46^{a}
	24 h	22.26±1.17^{a}	16.86±0.78^{b}	10.90±2.31^{c}	16.47±0.11^{b}	8.16±6.05^{c}
	48 h	19.10±1.40^{a}	17.00±0.83^{b}	2.60±1.21^{e}	11.54±0.23^{d}	-
	72 h	17.31±0.97^{a}	12.79±1.73^{b}	-	12.13±0.74^{b}	-
	96 h	13.93±1.22^{a}	11.67±1.70^{a}	-	13.07±1.74^{a}	-
	120 h	11.89±0.93^{a}	9.27±0.76^{b}	-	9.97±1.99^{ab}	-
	144 h	8.69±2.82^{a}	4.57±5.00^{ab}	-	2.46±0.72^{b}	-
LIN (%)	0 h	0.52±0.01^{bc}	0.55±0.03^{abc}	0.57±0.01^{a}	0.56±0.03^{ab}	0.55±0.03^{abc}
	24 h	0.47±0.01^{a}	0.42±0.00^{a}	0.46±0.02^{a}	0.47±0.01^{a}	0.35±0.2^{a} 0
	48 h	0.47±0.01^{a}	0.45±0.01^{a}	0.30±0.17^{b}	0.49±0.02^{a}	-
	72 h	0.46±0.01^{b}	0.56±0.05^{a}	-	0.55±0.01^{a}	-
	96 h	0.46±0.01^{b}	0.53±0.03^{a}	-	0.43±0.04^{bc}	-
	120 h	0.43±0.02^{b}	0.55±0.01^{a}	-	0.30±0.09^{c}	-
	144 h	0.38±0.08^{a}	0.32±0.29^{a}	-	0.20±0.04^{a}	-

$^{a-e}$ Values within the same row with different letters differ significantly (P≤0.05); - means that the sperm have all died, losing the significance of detection

Table 4. *Effects of different extenders on the different kinematic parameters of preserved sperm (mean±SD) (continued...)*

Kinematic Parameter	Storage Time	A	B	C	D	E (Control)
STR (%)	0 h	0.73±0.01^{ab}	0.78±0.05^{ab}	0.80±0.01^{a}	0.80±0.04^{a}	0.79±0.04^{ab}
	24 h	0.67±0.02^{a}	0.59±0.01^{a}	0.64±0.03^{a}	0.67±0.01^{a}	0.49±0.29^{a}
	48 h	0.66±0.02^{a}	0.64±0.01^{a}	0.42±0.24^{b}	0.69±0.04^{a}	-
	72 h	0.65±0.01^{b}	0.79±0.06^{a}	-	0.77±0.00^{a}	-
	96 h	0.65±0.02^{b}	0.76±0.05^{a}	-	0.60±0.05^{bc}	-
	120 h	0.62±0.03^{b}	0.79±0.02^{a}	-	0.42±0.13^{c}	-
	144 h	0.53±0.10^{a}	0.46±0.41^{a}	-	0.28±0.06^{a}	-
BCF (Hz)	0 h	0.70±0.02^{ab}	0.72±0.02^{a}	0.65±0.02^{b}	0.67±0.02^{b}	0.69±0.04^{ab}
	24 h	0.72±0.03^{a}	0.69±0.02^{a}	1.20±0.73^{a}	0.66±0.01^{a}	0.78±0.51^{a}
	48 h	0.70±0.02^{b}	0.67±0.02^{b}	1.94±1.20^{a}	0.78±0.02^{b}	-
	72 h	0.69±0.02^{b}	0.68±0.06^{b}	-	0.82±0.07^{a}	-
	96 h	0.74±0.05^{a}	0.78±0.05^{a}	-	0.78±0.14^{a}	-
	120 h	0.77±0.06^{a}	1.89±1.65^{a}	-	1.20±0.70^{a}	-
	144 h	3.16±1.72^{a}	1.69±1.47^{a}	-	1.17±0.50^{a}	-
MAD (°/s)	0 h	152.87±27.42^{ab}	195.79±61.33^{a}	119.43±12.58^{b}	146.90±33.40^{ab}	122.90^{b}±30.90
	24 h	183.35±73.24^{a}	128.03±6.13^{b}	13.36±5.55^{cd}	66.77±11.49^{c}	1.94^{d}±0.91
	48 h	153.32±29.32^{a}	102.66±19.58^{b}	3.20±1.30^{c}	19.17±6.20^{c}	-
	72 h	107.91±4.90^{a}	63.74±13.19^{b}	-	18.63±2.49^{c}	-
	96 h	92.37±19.59^{a}	39.20±11.28^{b}	-	13.14±2.11^{c}	-
	120 h	39.98±6.80^{a}	12.41±5.04^{b}	-	3.99±1.78^{b}	-
	144 h	16.47±10.28^{a}	3.46±3.03^{b}	-	1.89±0.46^{b}	-
WOB (%)	0 h	0.87±0.01^{ab}	0.83±0.05^{bc}	0.80±0.02^{bc}	0.79±0.03^{c}	0.83±0.05^{bc}
	24 h	0.94±0.02^{a}	0.92±0.03^{a}	0.86±0.04^{a}	0.91±0.01^{a}	0.61±0.26^{b}
	48 h	0.94±0.02^{a}	0.92±0.03^{a}	0.17±0.17^{b}	0.88±0.02^{a}	-
	72 h	0.92±0.02^{a}	0.76±0.09^{b}	-	0.89±0.03^{a}	-
	96 h	0.94±0.02^{a}	0.79±0.07^{b}	-	0.94±0.07^{a}	-
	120 h	0.95±0.04^{a}	0.84±0.06^{a}	-	0.84±0.17^{a}	-
	144 h	0.85±0.17^{a}	0.53±0.48^{ab}	-	0.17±0.17^{b}	-

$^{a-e}$ Values within the same row with different letters differ significantly (P≤0.05); - means that the sperm have all died, losing the significance of detection

the highest, and there was a significant difference between extender A and extender E (Control) (P≤0.05). At 48 h of preservation, the VSL of sperm preserved in extender A was significantly higher than those in the other four extenders (P≤0.05). The WOB of sperm preserved in extender A was the highest, and there was significant difference between extender A and extender C (P≤0.05). At 72 and 96 h, the WOB of sperm preserved in extender A was the highest and significantly higher than that in extender B (P≤0.05), but there was no significant difference between extender A and extender D (P>0.05). The VSL of sperm preserved in extender A was the highest at 72 h and 120 h, and there was a significant difference between extender A and extender D (P≤0.05), but there was no significant difference between extender A and extender B (P>0.05). At 96 and 144 h, the VSL of sperm preserved in extender A was the highest, but there was no significant difference between extender A and the other extenders (P>0.05). The VCL, VAP and ALH of sperm preserved in extender A were significantly higher than those in the other four extenders within 24 to 72 h (P≤0.05). At 96 h of preservation, the VCL, VAP and ALH of sperm preserved in extender A were the highest, but there were no significant differences between extender A and the other extenders (P>0.05). At 120 h of preservation, the VCL, VAP and ALH of sperm preserved in extender A were the highest and were significantly higher than those in extender B (P≤.05), but there was no significant difference between extender A and extender D (P>0.05). At 120 h of preservation, the WOB of sperm preserved in extender A was the highest, but there was no significant difference compared with the other extenders (P>0.05). At 144 h of preservation, the VCL, VAP, ALH and WOB of sperm preserved in extender A were the highest, and there were significant differences between extender A and extender D, but there was no significant difference between extender A and extender B (P>0.05). At 144 h of preservation, the LIN, STR and BCF of sperm preserved in extender A were the highest, but there was no significant difference between extender A and the other extenders (P>0.05). The sperm MAD of extender A was significantly

Table 5. *Effects of different extenders on the survival time of preserved sperm (mean±SD); h*

Survival Time	A	B	C	D	E (Control)
Effective survival time	74.50±4.82[a]	21.83±0.76[b]	2.00±0.50[d]	12.83±1.04[c]	5.83±1.04[d]
Total survival time	412.67±2.52[a]	219.83±6.21[b]	63.67±1.53[d]	186.67±7.64[c]	35.50±1.32[e]

[a-e] *Values within the same row with different letters differ significantly (P≤0.05)*

Table 6. *Effects of different extenders on the integrity of plasma membrane of preserved sperm (mean±SD); %*

Storage Time	A	B	D	E (Control)
0 h	61.82±1.61[b]	62.44±1.24[b]	63.12±1.69[ab]	65.65±1.02[a]
24 h	56.38±4.99[a]	54.80±3.97[a]	40.18±2.79[b]	21.13±3.76[c]
48 h	55.45±3.99[a]	51.25±2.81[a]	35.84±5.69[b]	-
72 h	47.38±1.23[a]	46.88±0.54[a]	35.61±0.86[b]	-
96 h	45.35±1.58[a]	42.23±2.41[a]	32.23±1.94[b]	-
120 h	40.75±2.12[a]	42.21±0.52[a]	32.18±1.01[b]	-
144 h	34.00±4.00[a]	37.67±3.40[a]	26.89±0.44[b]	-

[a-c] *Values within the same row with different letters differ significantly (P≤0.05); - means that the sperm have all died, losing the significance of detection. The effective survival time of sperm preserved by extender C was lower than that of the normal saline group, so the detection of plasma membrane integrity was meaningless*

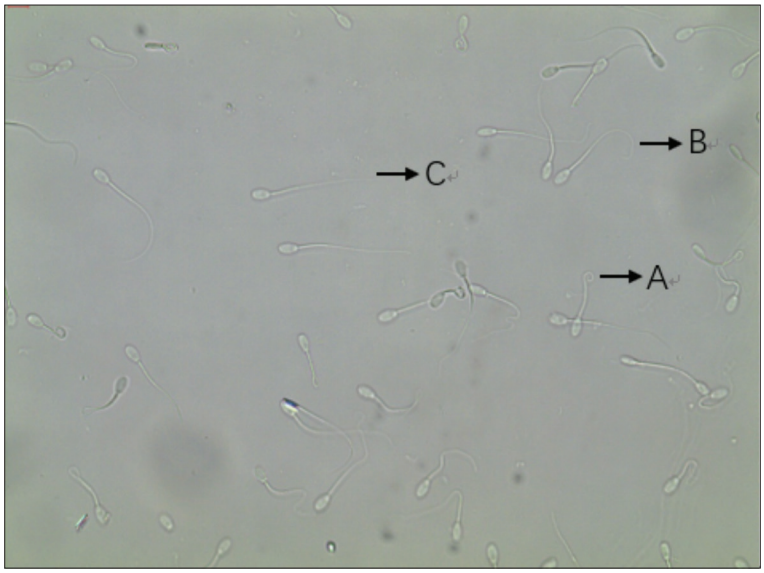

Fig 1. Morphology of curly tail of sperm in HOST. Observation under 400x lens, the two types of tail curl A and B were intact plasma membrane sperm, and the tail non-curl type C was the sperm with damaged plasma membrane

higher than those of the other four extenders within 24 to 144 h (P≤0.05).

Effects of Different Extenders on the Sperm Survival Time

As seen in *Table 5*, the effective and total survival times of semen preserved in extender A were the highest and were significantly higher than those of the other four extenders (P≤0.05).

Effects of Different Extenders on the Sperm Plasma Membrane Integrity

The effective survival time of sperm preserved by extender C was lower than that of the normal saline group; therefore, the detection of plasma membrane integrity was meaningless.

Table 6 shows that when Hu ram semen was preserved with four kinds of extenders at 16°C, the integrity of sperm plasma membrane decreased with the extension of storage time. Among them, the plasma membrane integrity of sperm preserved by extender A decreased steadily and slowly. At 24 h of preservation, the integrity of the plasma membrane of sperm preserved in extender A was the highest and was significantly higher than those in extenders D and E (Control) (P≤0.05), but there was no significant difference between extender A and extender B (P>0.05). Within 48 to 144 h of preservation, the integrity of the plasma membrane of sperm preserved in extender A was higher than those in the other three extenders.

The results of microscopic examination after HOST incubation are shown in *Fig. 1*. There were three types of sperm tail:

Table 7. *Effects of different extenders on acrosome integrity of preserved sperm (mean±SD); %*

Storage Time	A	B	D	E (Control)
0 h	96.45±1.7[a]a	94.62±2.67[ab]	93.44±1.54[ab]	92.53±2.37[b]
24 h	93.66±1.16[a]	89.00±1.81[ab]	83.47±3.40[bc]	79.44±6.55[c]
48 h	93.00±1.43[a]	88.08±2.79[b]	76.43±1.28[c]	-
72 h	92.55±1.23[a]	87.21±1.13[b]	70.01±4.05[c]	-
96 h	92.50±0.79[a]	85.48±0.71[b]	66.69±3.82[c]	-
120 h	92.21±0.73[a]	85.04±2.15[b]	64.47±3.70[c]	-
144 h	90.93±1.88[a]	84.43±1.07[b]	49.45±1.24[c]	-

[a-c] Values within the same row with different letters differ significantly (P≤0.05); - means that the sperm have all died, losing the significance of detection. The effective survival time of sperm preserved by extender C was lower than that of the normal saline group, so the detection of acrosome integrity was meaningless

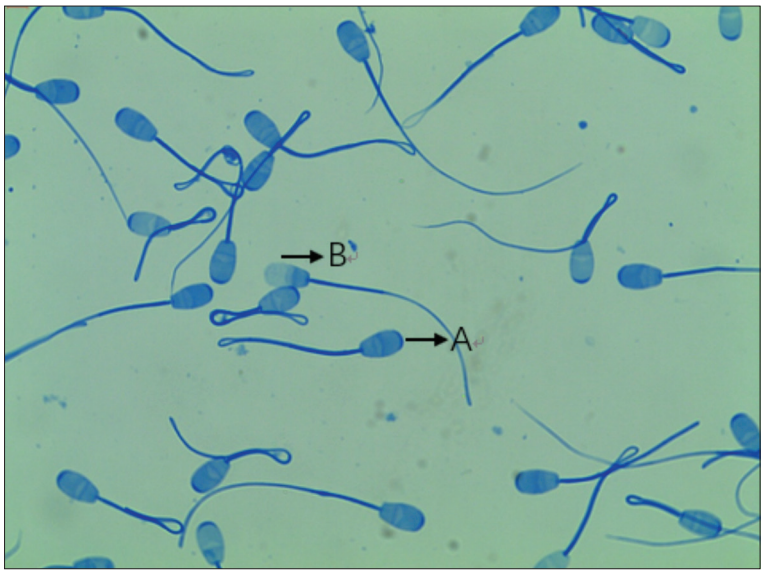

Fig 2. Acrosome morphology of sperm stained with Coomassie brilliant blue. Observation under 1000x oil lens, there were two types of sperm head: type A and type B. Sperm head is blue, it means the acrosome is intact(A). Sperm head was unstained, it means the acrosome is not intact (B)

A, B and C, in which the two types of tail curl, A and B, represented intact plasma membrane sperm, and the tail non-curl type C represented sperm with damaged plasma membrane.

Effects of Different Extenders on Sperm Acrosome Integrity

The effective survival time of sperm preserved by extender C was lower than in the normal saline group; therefore, the detection of acrosome integrity was meaningless.

Table 7 shows that when Hu ram semen was preserved with four kinds of extenders at 16°C, the acrosome integrity of sperm decreased with the extension of storage time. Among them, the acrosome integrity of sperm preserved in extender A decreased steadily and slowly. At 24 h of preservation, the acrosome integrity of sperm preserved in extender A was the highest and was significantly higher than those in extender D and extender E (Control) (P≤0.05), but there was no significant difference between extender A and extender B (P>0.05). Within 48 to 96 h of preservation, the acrosome integrity of sperm preserved in extender A was higher than those of the other three extenders.

The results of microscopic examination after Coomassie brilliant blue staining are shown in *Fig. 2*. There were two types of sperm head: type A and type B. If the sperm head is blue, then the acrosome is intact (A). If the sperm head was unstained, then the acrosome is not intact (B).

Discussion

In this study, total sperm motility, sperm progressive motility, effective survival time, total survival time, plasma membrane integrity, acrosome integrity and some kinematic parameters such as VSL, VCL and VAP were used to analyze the effects of different kinds of extenders on the preservation of Hu ram semen at 16°C. The results showed that the total sperm motility and progressive motility of extender A decreased steadily and slowly within 144 h of preservation of Hu ram semen at 16°C, and the performance of extender A was obviously better than those of the other extenders. The effective survival time, total survival time, plasma membrane integrity, acrosome integrity and motility performance of semen preserved in extender A were also significantly better than those of the other extenders. In short, extender A

has the best effect on the preservation of Hu ram semen at 16°C.

At present, the extender preservation of ram semen has been studied in many aspects [18]. At present, most of the studies on ram semen are 4°C preservation and cryopreservation, but there are a few reports on 16°C preservation. No matter which preservation method is used, the preservation quality of ram semen decreases gradually with time [19]. It has been reported that the storage time of ram semen at 16°C is too short. Yaniz et al.[20] indicated that the sperm progressive motility was less than 60% at 24 h in the ram semen preservation experiment at 16°C, which was far from meeting the production needs [21]. Compared with 16°C preservation, the extender formula and operation process of semen 4°C preservation are more complex, which is not conducive to popularization and application, and 4°C preservation may have a certain impact on the sperm membrane [22]. At the same time, 4°C preservation may also lead to the decrease of fertilization ability and an increased embryo loss rate [23,24].

At present, commercial extenders are used in the preservation of ram semen at 16°C, and there are few published reports on extender formulations. In the study of 4°C preservation of ram semen, Sarlos et al.[25] used an extender composed of Tris, citric acid, glucose, yolk and antibacterial substances, and Kasimanickam et al.[26] and Gundogan et al.[27] used an extender composed of Tris, citric acid, fructose, yolk and antibacterial substances. In the study of cryopreservation of ram semen, Kumar et al.[28] and Leahy et al.[29] used a basic extender composed of Tris, citric acid, fructose, yolk and antibacterial substances, and Hamedani et al.[30] and Merati et al.[31] used extenders composed of Tris, citric acid, glucose, yolk, glycerol and antibacterial substances. In this experiment, various substances of extender A formula are used in Hu ram semen 4°C preservation and cryopreservation, and nutrients in previous formulations, such as glucose and fructose, are also used. Yolk and glycerol are added in the 4°C preservation and cryopreservation of ram semen. Yolk and glycerol are used as effective cryoprotectants. Additionally, yolk could also supply energy to sperm. Therefore, in experiments evaluating the preservation of Hu ram semen at 16°C, if yolk is not added, the amount of nutrients such as glucose or fructose should be increased.

The extender is composed of nutrients, buffers, antibiotics and other substances. The pH, osmotic pressure, buffering properties and functional substances of the extender will affect the preservation quality of semen [32]. In this experiment, the sudden decreases of total motility and progressive motility of Hu ram sperm stored in extenders C and D at 16°C may have been due to the lack of substances to regulate and stabilize the pH in the extenders. In this experiment, the extender E (Control) was a 0.9% sodium chloride extender, and the sudden decline of total sperm motility during preservation may be due to the lack of nutrients in the extender, which cannot provide energy for sperm metabolism. In this experiment, the effective survival time of Hu ram sperm preserved in extender C at 16°C was lower than that in extender E (Control), normal saline, which may be due to the changes of the physical and chemical properties of the extender, which was not suitable for sperm survival. The total survival time of Hu ram sperm preserved in extender E (Control) at 16°C was lower than those with extender C and extender D, which may be because it did not contain nutrients and could not provide energy for sperm metabolism. The acrosome of sperm is an important organelle in the process of fertilization, and the survival time is proportional to the acrosome integrity [33]. The acrosome integrity obtained in this experiment is proportional to the survival time, which is consistent with previous research results. VSL, VCL, VAP, WOB, ALH and BCF are single variables describing sperm velocity and are closely related to reproductive performance [34]. VSL, VCL and VAP are the key parameters to evaluate sperm kinematic parameters, and the fertilization ability is coordinately proportional [35]. Deyiliu showed that there is a significant relationship between ALH, BCF and semen quality [36]. The VSL, VCL, VAP, ALH and WOB obtained in this experiment are proportional to sperm progressive motility, which is consistent with the results of previous studies. The MAD obtained in this experiment is proportional to sperm progressive motility, and there is no correlation between other kinematic parameters and sperm progressive motility.

In conclusion, the experimental results show that extender A is the best formula among the five extenders. At present, there are few extenders for Hu ram semen stored at 16°C. The storage time of the existing 16°C storage extender is too short. Although Tris extender is often used for cryopreservation, it has not been reported under 16°C storage conditions. In this experiment, the cryoprotective agent in the Tris extender during cryopreservation was removed. The formulation was optimized and was applied to 16°C storage, which greatly extended the survival time of sperm. The preparation process of extender is simple and easy to operate, which can meet the practical requirements of production and has good popularizing value.

Statement of Author Contributions

LZ and YL: conceptualization. LZ, TS and CC: methodology. LZ and YW: software. YF: validation. LZ, YW and CC: formal analysis. LZ and JM: investigation. YL: resources. YW and YF: data curation. YL: supervision and validation. LZ: writing - original draft preparation. YL: writing - review and editing. All authors discussed the results and contributed to the final manuscript.

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Conflict of Interest

None of the authors have any conflict of interest to declare.

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RESEARCH ARTICLE

Deletion of *lpsA* Gene of *Brucella melitensis* Strain M5-90 Promotes Caspase-11 Induced Non-classical Pathways Pyroptosis in *Brucella*-infected Mouse Macrophage Cells

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Abstract

Brucella utilizes diverse virulence factors to modulate the infectious cycle and lifestyle associated with eukaryotic hosts. Lipopolysaccharides (LPS) play an important role in the establishment of persistent infections of *Brucella*, but its mechanism in host cell to interfere with the host's specific signaling pathway related to the elimination of *Brucella* is still not clear. *LpsA*, a glycosyl transferase, involves in the biosynthesis of LPS, and may affect the intracellular survival of *Brucella*. This study aimed to investigate the effects of *lpsA* on pyroptosis in *Brucella*-infected macrophage cells. We constructed the *lpsA* mutant strain (M5-90ΔlpsA) and the complementary strain (M5-90ΔlpsA-C) of *Brucella melitensis* strain M5-90 infected mouse macrophages to detect pyroptosis. We found that the inactivation of the *lpsA* gene weakened the ability of LPS gains access to cytosol during *Brucella* mutant infections, and reduced the survival of *Brucella*. Western blot and quantitative real-time PCR assays showed that the mRNA and protein levels of caspase-11 and NOD-like receptor family pyrin domain-containing 3 in M5-90ΔlpsA group were higher than those observed in M5-90 group. These results indicated M5-90ΔlpsA promoted the pyroptosis of RAW264.7 cells (a mouse macrophage cell line) after *Brucella* infection. The finding reveals that *Brucella lpsA* plays a partial role in innate immunity and inflammatory response by inhibiting the LPS-induced atypical pyroptosis pathway.

Keywords: *Brucella, lpsA, Survive, Pyroptosis*

Brucella melitensis M5-90 Suşunun *lpsA* Geninin Silinmesi *Brucella* İle Enfekte Fare Makrofaj Hücrelerinde Kaspaz-11 ile İndüklenen Klasik Olmayan Piroptozis Yolaklarını Teşvik Eder

Öz

Brucella, ökaryotik konakçılarla ilişkili enfeksiyon döngüsünü ve yaşam biçimini düzenlemek için çeşitli virülens faktörlerini kullanır. Lipopolisakkaritler (LPS), *Brucella*'nın kalıcı enfeksiyonlarının oluşmasında önemli bir rol oynar, ancak konakçı hücrede, Brucella'nın ortadan kaldırılmasıyla ilgili konakçının spesifik sinyalizasyonuna müdahale etme mekanizması halen net değildir. Bir glikozil transferaz olan *LpsA*, LPS'nin biyosentezinde görev alır ve Brucella'nın hücre içi yaşama yeteneğini etkileyebilir. Bu çalışmada *lpsA*'nın *Brucella* ile enfekte makrofaj hücrelerinde piroptoz üzerine etkilerinin araştırılması amaçlanmıştır. Piroptozu saptamak için *Brucella melitensis* M5-90 suşunun *lpsA* mutantı (M5-90ΔlpsA) ve komplementer suşu (M5-90ΔlpsA-C) ile enfekte olmuş fare makrofajları oluşturduk. *LpsA* geninin inaktivasyonunun, LPS'nin *Brucella* mutant enfeksiyonları sırasında sitozole giriş yeteneğini zayıflattığını ve Brucella'nın yaşama yeteneğini azalttığını saptadık. Western Blot ve kantitatif real-time PCR deneyleri, M5-90 grubuna oranla M5-90ΔlpsA grubunda kaspaz-11 ve NOD-benzeri reseptör ailesi pyrin domain-containing 3'ün mRNA ve protein seviyelerinin daha yüksek olduğunu gösterdi. Bu bulgular, M5-90ΔlpsA'nın, *Brucella* enfeksiyonu sonrası bir fare makrofaj hücre hattı olan RAW264.7 hücrelerinde piroptoza yol açtığını gösterdi. Bu bulgu, *Brucella lpsA*'sının, LPS ile indüklenen atipik piroptoz yolunu inhibe ederek doğal bağışıklık ve enflamatuar yanıtta kısmi bir rol oynadığını ortaya koymaktadır..

Anahtar sözcükler: *Brucella, lpsA, Canlılık, Piroptozis*

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Introduction

Brucellosis is an important zoonotic disease caused by *Brucella*, a Gram-negative facultative intracellular bacterium, listed as a Class B bioterrorism agent by the U.S. Centers for Disease Control and Prevention (CDC) [1]. Virulence factors are the key for *Brucella* to survive and reproduce in host cells successfully [2], a variety of strategies can be used to establish and maintain the long-term survival in host cells for *Brucella*. They can escape the inflammatory response in the early stages of infection [3,4], once enter the host cell, they affect intracellular transport and activation, and utilize some of the environmental pressures encounter (such as acidic environments and nutritional stress) as a stimulus for gene induction to change them intracellular transport [5]. Eventually, *Brucella* deprives the ability of cells for processing antigens and resisting apoptosis to benefit its survival [6,7].

Lipopolysaccharide (LPS) is one of the important virulence factors for the survival of *Brucella*, maintains the structural and functional integrity of bacterial outer membrane proteins, but also plays a role in the immune escape, it is crucial for the integrity of bacterial virulent [8]. When O-polysaccharide of *Brucella* LPS is inactivated, it changes from smooth type to rough type, resulting in weakened bacterial virulence and easy to be killed by macrophages [9,10]. LPS-based O-type polysaccharides inhibit cell phagocytosis, lysosomal lysis and host cell apoptosis [11]. The NOD-like receptor family pyrin domain-containing 3 (NLRP3) inflammasome belongs to the NOD-like receptor (NLR) family and composed of NLRP3, apoptosis-associated speck-like protein containing a C-terminal caspase recruitment domain (ASC) and pre-caspase-1 [12]. NLRs or the absent in melanoma 2 (AIM2) protein receive danger signals from extracellular or intracellular, leading to the assembly and activation of inflammasomes, which in turn activate caspase-1 [13]. Activated caspase-1 causes cytokines such as interleukin (IL)-1β and IL-18 to mature and activate [14]. However, recent researches have shown that Gram-negative bacteria can trigger the NLRP3 inflammatory response in a nonclassical way that relies on caspase-11 belongs to the family of inflammatory caspases [15,16]. The caspase recruitment domain (CARD) of this type of caspase can directly recognize and bind to LPS, and undergo self-oligomerization to show obvious protease activity [17]. Caspase-11 activated by LPS cleaves gasdermin-D (GSDMD), in the junction between the N-terminal and C-terminal domains, thereby releasing the GSDMD N-terminal domain (GSDMD-N) with pore-forming activity [18]. GSDMD-N is transferred to the mass by binding membrane phosphoinositide membrane, perforation in the membrane causes the cell membrane to rupture and cell death termed pyroptosis [19]. The recent research demonstrates that *B. abortus* can trigger the activation of non-conicaase-dependent caspase-11 and GSDMD and *Brucella*-LPS is the ligand for caspase-11 activation. It is determined that *B. abortus* can trigger the formation of cell pyroptosis [20]. Unlike the other genes related to LPS synthesis, *lpsA* is a glycosyl transferase, involved in the biosynthesis of the inner core encoding LPS, which may affect the intracellular survival of *Brucella*, but the specific function is unknown [21]. Studies have shown that stimulated with LPS, macrophages are activated in a proinflammatory direction via the activation of inflammatory cysteinyl aspartate specific proteinase, promoting the release of cellular inflammatory factors and cause cell pyroptosis [22]. However, the mechanism by which *Brucella* LPS regulates the occurrence of pyroptosis in host cells is still unknown.

Our study aims to research the function of *lpsA* in pyroptosis during *B. melitensis* strain M5-90 infection in host cells. In the present study, the roles of *lpsA* in pyroptosis were detected. Besides, after RAW264.7 cells were infected with M5-90Δ*lpsA* mutant, the activation of inflammatory and secretion of the inflammatory cytokines IL-18 and IL-1β was examined.

Material and Methods

Bacterial Strains, Plasmids and Cell Line

B. melitensis strain M5-90 was obtained from the Center of Chinese Disease Prevention and Control (Beijing, China). All *Brucella* strains were cultured in *Brucella* Agar or *Brucella* Broth (Difco, MI, USA) at 37°C in 5% CO_2. *Escherichia coli* strains DH5α and BL21 were grown on Luria–Bertani (LB) medium (Madison, WI, USA) at 37 °C. If necessary, the culture media were supplemented with appropriate kanamycin and ampicillin (100 μg/mL) for the mutant. pBBR1MCS4 vector was purchased from Promega (Madison, WI, USA). Murine (or mouse) macrophage RAW264.7 cells (Lu Ao, Shanghai, China) was cultured in Dulbecco's modified Eagle's medium (obtained from Cell Resource Center, IBMS, CAMS/PUMC, Beijing, China) supplemented with 10% fetal bovine serum (Taixin, Beijing, China) at 37°C with 5% CO_2. All *Brucella* strains were manipulated in a biosafety level 3 laboratory.

Construction of M5-90ΔlpsA mutant

The M5-90Δ*lpsA* deletion mutant was constructed and modified according to the methods previously reported [23] and primers were shown in *Table 1*. We used homologous recombination to construct mutant strains, briefly. Design of primers for upstream and downstream homology arms was achieved with software Primer 6.0 (the length of the homology arm sequence is 350-550 bp). After the primer design is completed, the reverse complement sequence of the first 19 bases of the kanamycin resistance gene sequence was added to the 5′ end of the downstream primer of the upstream homology arm and the kanamycin resistance gene was artificially added to the 5′ end of the upstream primer of the downstream homology arm the last 20 bases of the sequence. Blunt-end enzyme PCR was

Table 1. Primers of mutants used for this work	
lpsA-N-F	AGGATACCGGCGTCGGCATT
lpsA-N-R	GACATTCATCCCAGGTGGCTAATTCGCTTGCCTCA
lpsA-C-F	TCTGGGGTTCGAAATGACCGAGCGGTTCCACTTTTACACAG
lpsA-C-R	CTGACCCGCGCGCG
kan-F	GCCACCTGGGATGAATGTC
kan-R	CGGTCATTTCGAACCCCAGA

used to amplify the upstream and downstream homology arms of the target gene and the kanamycin resistance gene, and overlap extension PCR was used to fuse the fragments. The mutation box was subcloned into the pMD19-T vector for sequencing. Competent M5-90 was electroporated with the plasmid pMD19-T-lpsA. Mutants were selected in the presence of 100 μg/mL ampicillin and 100 μg/mL kanamycin for the screening. The deletion mutant was confirmed by qRT-PCR amplification.

M5-90ΔlpsA-C Complementary Strain

The complementary strain M5-90Δ*lps-A-C* was constructed and modified according to the methods previously described [24]. Genomic DNA from M5-90 was amplified using primers lps-A-F (5′-AAGCTTATGATATTGCCCGTATT-3′) and lps-A-R (5′-GAATTCCGCACCTACTTCTAA-3′). PCR products were cloned into pMD19-T simple vector for sequencing and then subcloned into pBBR1MCS4 to generate the plasmid pBBR4-lps-A. Subsequently, the plasmid pBBR4-lps-A was electroporated into M5-90Δ*lpsA*, obtaining the complementary strain. The complementary strain was referred to as M5-90Δ*lps-A-C*.

Growth Curve of M5-90ΔlpsA Mutant

In order to monitor and analyze the growth of strains in real-time, the M5-90, M5-90Δ*lpsA*, M5-90Δ*lpsA-C* monoclonal colonies were picked out respectively and placed them in to *Brucella* broth when OD_{600}=0.8. Using sterilized *Brucella* broth the density of them were adjusted to OD_{600}=0.1 and cultured in a rotary shaker (250 rpm) at 37°C. The bacterial population was inactivated with adding 4-6% formaldehyde and OD_{600} value was measured until each strain culture creates a plateau, and the growth curve was drawn.

Analysis of Stress Tolerance of M5-90ΔlpsA mutant

In order to assess the adhesion and invasive ability of the mutant, the *lpsA* mutant was added to infected cells at a multiplicity of infection (MOI) of 100:1, and incubated at 37°C and 5% CO_2 for 15, 30, 45 or 60 min. Next, the cells were washed with phosphate-buffered saline (PBS) to wash away non-adherent bacteria and then incubated in a medium containing 50 units (2.5 μL) of gentamicin for 60 min to kill extracellular *Brucella*. The cells were rinsed with PBS, lysed, and incubated at 37°C for 10 min to release intracellular bacteria. Appropriate concentrations were prepared by dilution and the ability to adhere and invade macrophages was counted by CFU count. To determine the environmental stress tolerance of the mutant strain, the *lpsA* mutant strain was cultured to logarithmic growth phase, centrifuged at 12.000 rpm for 10 min and rinsed 2 times with PBS. The bacterium was cultured in liquid medium (1 mL TSB) at 37°C under different stress conditions (in the presences of 1.5 M NaCl and 10 mM H_2O_2 and at pH 2.5, pH 11.5) for 30 min and at 50°C for 30 min, respectively. The CFUs were then counted and the percentage of surviving bacteria calculated relative to the control and wild-type (WT) (% survival). Under the stress conditions, normally cultured bacteria were used as controls.

RAW264.7 Cells Infection Assay

RAW264.7 cells were cultured in 6-well plates to form a monolayer with 80% coverage (2.5 x 10^6). The cells were infected with M5-90, M5-90Δ*lpsA*, M5-90Δ*lpsA-C* at 100:1 of MOI and incubated at 37°C and 5% CO_2 for 0 h, 4 h, 12 h and 24 h of infection. The cells were lysed with 0.1% Tridatone (Sigma-T8787, USA) at 37°C for 10 min to release intracellular bacteria. The lysate was diluted 10-fold to an appropriate concentration and enumerated the CFU by plating on *Brucella* broth plates.

Assessing the Ability of LPS Gains Access to the Cytosol During Brucella Mutant Infections

To assess the influence of the inactivation of *lpsA* on the entering process of *Brucella* to the host cell and the cytoplasmic localization of LPS, digitonin was used to isolate the parent strain or mutant strain from the infected cytoplasmic components of RAW264.7 cells with different adhesion periods. The Limulus reagent quantitative method was used for detection of the LPS of *Brucella* into host cells. For lyses, the cells were washed with 1 mL Triton X-100 (0.2% v/v, 100 mL PBS, 200 μL Triton X-100, Sigma-T8787, USA) and incubated at 37°C for 10 min to release the intracellular bacteria. The lysate was diluted 10 times to obtain an appropriate concentration for plate counting. On the other hand, we use a 2-micron filter to filter the cell components, use the fluorescent probe SYTO9 to label the live bacteria, and use confocal laser scanning microscopy (CLSM) to observe.

Quantitative Real-time PCR (qRT-PCR)

RAW264.7 cells were infected with M5-90, M5-90Δ*lpsA* or M5-90Δ*lpsA-C* according to the above-mentioned method. The cells not infected with *Brucella* were used as controls. Extraction of the total RNA from cells infected with *Brucella* mutants at 4 h, 12 h and 24 h was carried out. The RNA samples were treated with DNase I (Thermo Fisher Scientific, Shanghai, China) to remove contaminant genomic DNA. The RNA molecules were subsequently reverse transcribed into cDNA by using PrimeScript™RT reagent Kit with gDNA Eraser (Perfect Real Time) (Takara, USA). Pyroptosis-associated genes (caspase and NLRP3) were selected for

Table 2. *Primers of qRT-PCR used for this work*	
β-actin-F	GAGACCTTCAACACCCCAG
β-actin-R	GAGCATAGCCCTCGTAGAT
Caspase-11-F	ACAAACACCCTGACAAACCAC
Caspase-11-R	CACTGCGTTCAGCATTGTTAAA
Caspase-1-F	TGCCGTGGAGAGAAACAA
Caspase-1-R	ATGAAAAGTGAGCCCCTG
NLRP3-F	TTGGAGACACAGGACTCAGG
NLRP3-R	CATTGTTGCCCAGGTTCAG

qRT-PCR amplification. The primers used in this scope were listed in *Table 2*. The qRT-PCR conditions were consisted of 5 min at 95°C for pre-incubation, followed by 40 cycles at amplification (95°C for 30 s, 62°C for 30 s and 72°C for 30 s). The qRT-PCR was carried out with a QuantStudio™ 7 Flex system (Thermo Fisher Scientific, USA). The qRT-PCR reaction volume for per sample was adjusted as 20 μL containing 10 μL 2 x SYBR Premix Ex Taq II (Takara, USA), 0.4 μL of each primer (10 nM), 2 μL cDNA target (10 μg) and 7.2 μL ddH_2O. All assays were performed three times.

Western Blotting

The cells infected with *Brucella* were collected and the cell culture supernatant was discarded. The cells were washed with PBS for 3 times and 100 μL non-denatured cell/tissue lysate (PMSF) was added per well. The cells were put on ice and lysed for 10 min. The cells were scraped and sucked into a new microcentrifuge tube. The repeated three wells were combined into one tube and centrifugated at 12.000 rpm for 5 min at 4°C. Protein concentrations were determined using the micro-BCA protein assay (Thermo Fisher Scientific, Shanghai, China). Equal amounts of protein (20 μg) were then resolved on a 12% SDS-PAGE. After electrophoresis, proteins were transferred to PVDF membranes (GE Biosciences) for 60 min at 300 mA and blocked in TBS with 0.1% Tween 20 for 1 h at 37°C. The membranes were then probed with primary antibodies (rabbit anti-NLRP3 monoclonal antibodies and anti-caspase-11 polyclonal antibodies) in TBS with 0.05% Tween 20 overnight. After the membrane was washed 3 times in TBS with 0.1% Tween 20 for 7 min, the membranes were incubated with an anti-rabbit IgG antibody conjugated with peroxidase at a dilution of 1: 1000 at 37°C for 3 h in a shaker-incubator. The protein bands were monitored with using protein imaging system (FluorChemE, USA).

Cytokine Detection

The culture supernatants were collected at different time periods from the RAW264.7 cells infected with M5-90, M5-90Δ*lpsA,* non-infected control and the cells with M5-90Δ*lpsA-C* strain. IL-18 and IL-1β were determined using a Mouse Quantikine ELISA Kit (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions. All assays were performed in triplicate.

Statistical Analysis

The data were presented as mean ± SEM (SD). GraphPad Prism software was used to analysis. Data of multiple groups were analyzed using one-way ANOVA followed by Bonferroni post hoc test, while comparisons between two groups were performed by unpaired Student's *t*-test. Statistical significance was defined as $P<0.05$.

Results

Construction of M5-90ΔlpsA Mutant and M5-90ΔlpsA-C Complementary Strain

The M5-90Δ*lpsA* mutant was constructed by homologous recombination and resistance gene replacement, and the complementary strain was successfully confirmed by PCR and resistance screening *(Fig. 1-a)*.

LPS gains Access to the Cytosol During Brucella Infections

The entry of LPS into the cytosol is a critical step for Gram-negative bacteria to trigger an inflammatory response. We tested whether the deletion of *lpsA* would affect the entry of *Brucella* LPS into the cytosol during infection. We extracted cytosol from uninfected or M5-90, M5-90Δ*lpsA*-infected RAW264.7 cells using digitonin and assessed LPS levels with the Limulus amebocyte lysate (LAL) assay. The results showed that M5-90Δ*lpsA*-infected macrophages showed a lower level of LPS content than the parental strain *(Fig. 1-b)* ($P<0.01$). Similarly, the number of bacteria entering the host cell in the initial stage is also reduced *(Fig. 1-c,d)* ($P<0.01$). The deletion of *lpsA* leads to a decrease in the synthesis of LPS in *Brucella* during infection or few LPS entering the cytoplasm.

Growth Curve of M5-90ΔlpsA Mutant

We determined the effect of the absence of *lpsA* on the growth of M5-90 in a liquid medium. Compared with the wild strain, the growth phenotype trend of M5-90Δ*lpsA* mutant is basically the same. All strains have reached the logarithmic growth phase at 16 h, and reach the bacterial growth plateau at 32 h, but the mutant strain grew slowly *(Fig. 2-a)*. Supplementation changed this phenotypic change. These results indicate that *lpsA* affects the growth of M5-90 *in vitro* liquid medium, but the M5-90Δ*lpsA* strain does not lose its basic characteristics.

Reduced Environmental Tolerance of M5-90ΔlpsA Mutant

Since *Brucella* generally survives in chronically hungry and hypoxic niches, we initially studied the physiology of *Brucella* under microaerobic conditions. Under consistent nutritional conditions, we varied the pH, temperature and oxidizing environment conditions of the growth environment, 48 h after inoculation and used the plate

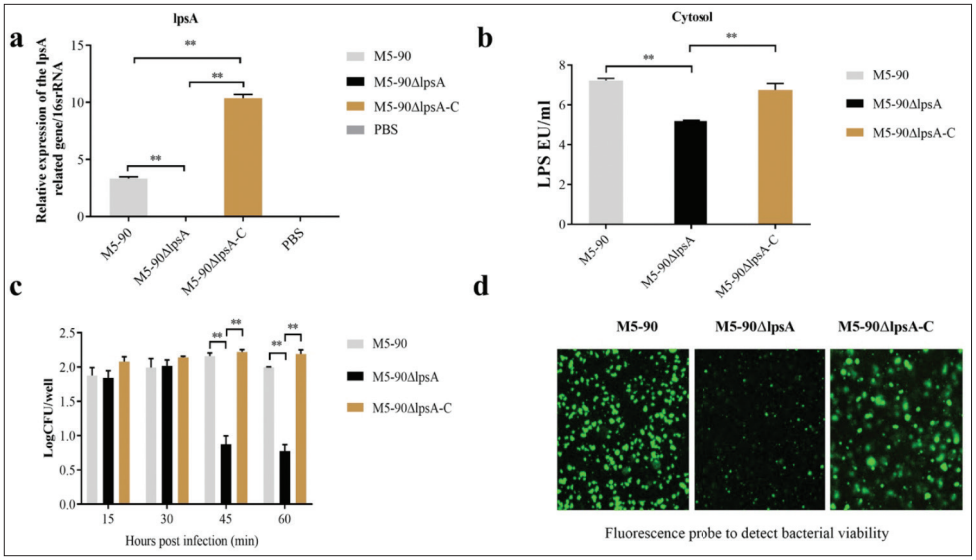

Fig 1. LPS gains access to the cytosol during M5-90 infections. **a.** Real-time fluorescent quantitative PCR to detect the transcription level of *lpsA* gene in M5-90, M5-90ΔlpsA and M5-90ΔlpsA-C, **b.** Extracted cytosol from uninfected or *Brucella*-infected mouse macrophage using digitonin and assessed LPS levels with the LAL assay, **c.** RAW264.7 cells were infected with *Brucella* wild type and mutant strains at different time points and CFUs counted to determine *Brucella* adhesion and invasion, **d.** SYTO9 green fluorescent label live bacteria, CLSM observes the ability of *lpsA* mutant strain to adhere and invade cells. Asterisks (*P<0.05, **P<0.01) indicate significant differences between the parental and mutant strains

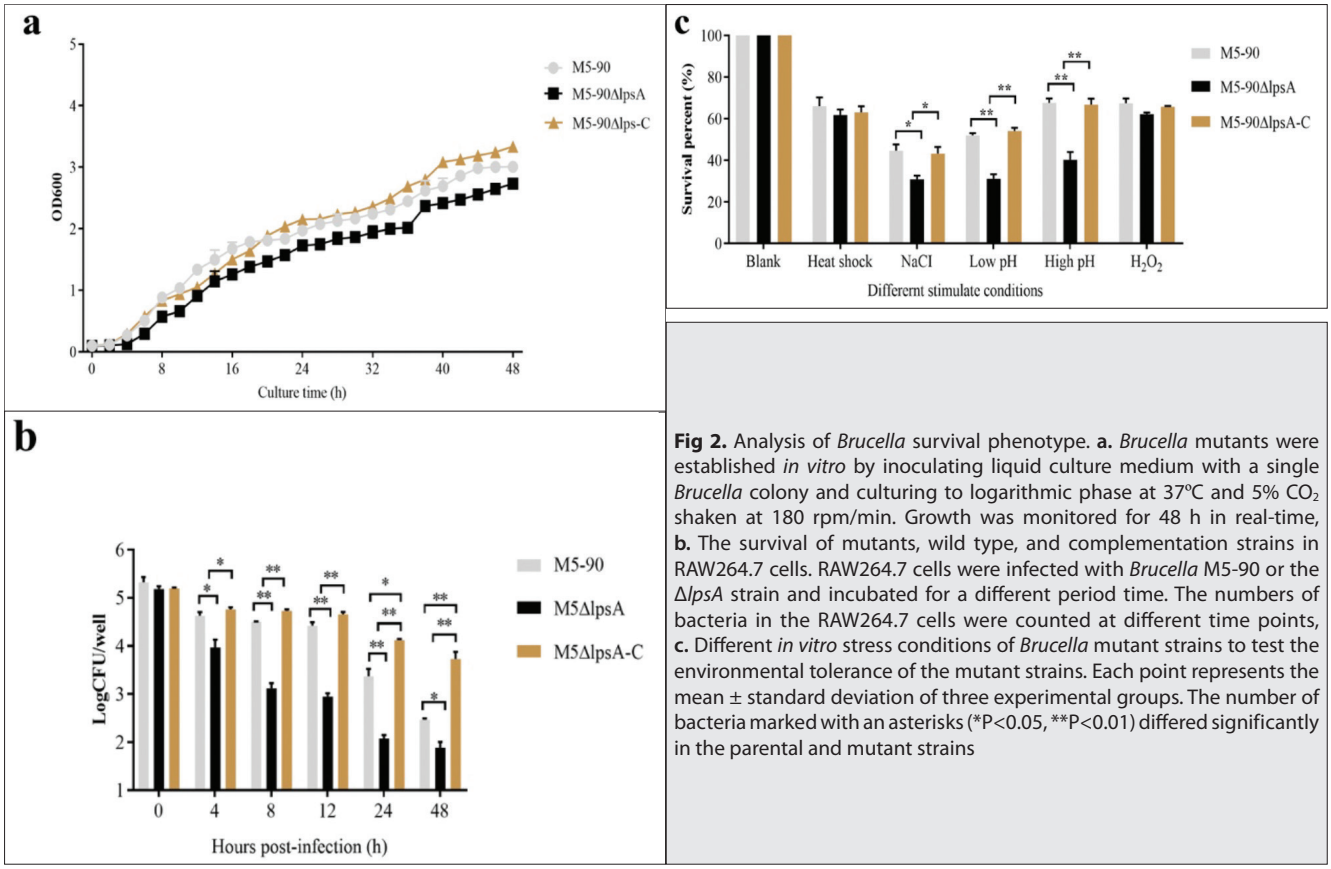

Fig 2. Analysis of *Brucella* survival phenotype. **a.** *Brucella* mutants were established *in vitro* by inoculating liquid culture medium with a single *Brucella* colony and culturing to logarithmic phase at 37ºC and 5% CO_2 shaken at 180 rpm/min. Growth was monitored for 48 h in real-time, **b.** The survival of mutants, wild type, and complementation strains in RAW264.7 cells. RAW264.7 cells were infected with *Brucella* M5-90 or the Δ*lpsA* strain and incubated for a different period time. The numbers of bacteria in the RAW264.7 cells were counted at different time points, **c.** Different *in vitro* stress conditions of *Brucella* mutant strains to test the environmental tolerance of the mutant strains. Each point represents the mean ± standard deviation of three experimental groups. The number of bacteria marked with an asterisks (*P<0.05, **P<0.01) differed significantly in the parental and mutant strains

counting method to compare bacterial growth. The results showed that when compared with the parent strain the LPS mutant strain is more sensitive to environmental osmotic pressure *(Fig. 2-c)* (P<0.05) and pH *(Fig. 2-c)* (P<0.01).

M5-90ΔlpsA Mutant is Attenuated in RAW 264.7 Cell

In order to evaluate the survival of the mutant strain in the cell after the deletion of *lpsA*, RAW264.7 cells were infected

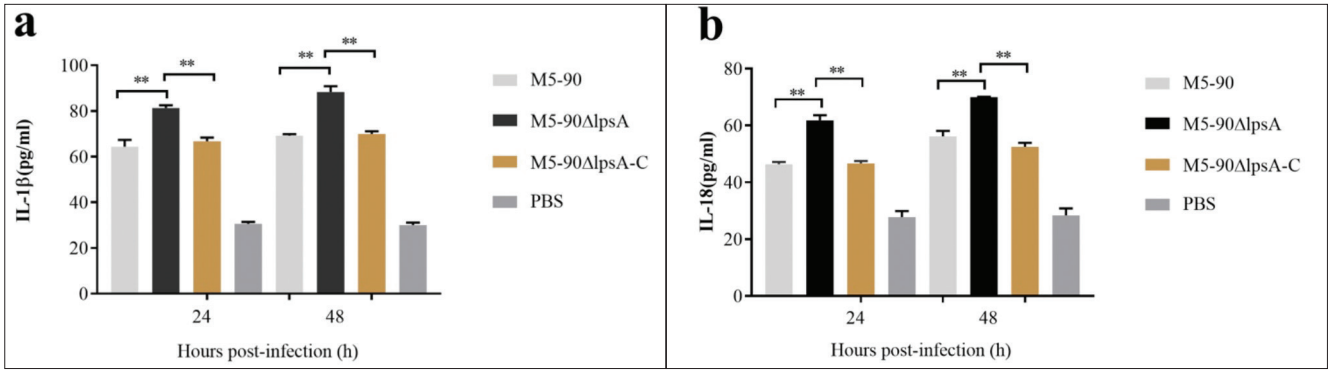

Fig 3. Levels of IL-18 and IL-1β released from RAW264.7 cells infected by M5-90Δ*lpsA*. After *Brucella* mutants infect cells, the release of IL-18 and IL-1β was measured at 24 and 48 h with ELISA. Each point represented the mean ± standard deviation of three experimental groups. Asterisk (*P<0.05, **P<0.01) indicates significant differences in the release of IL-18 and IL-1β between the parental and mutant strains after infection

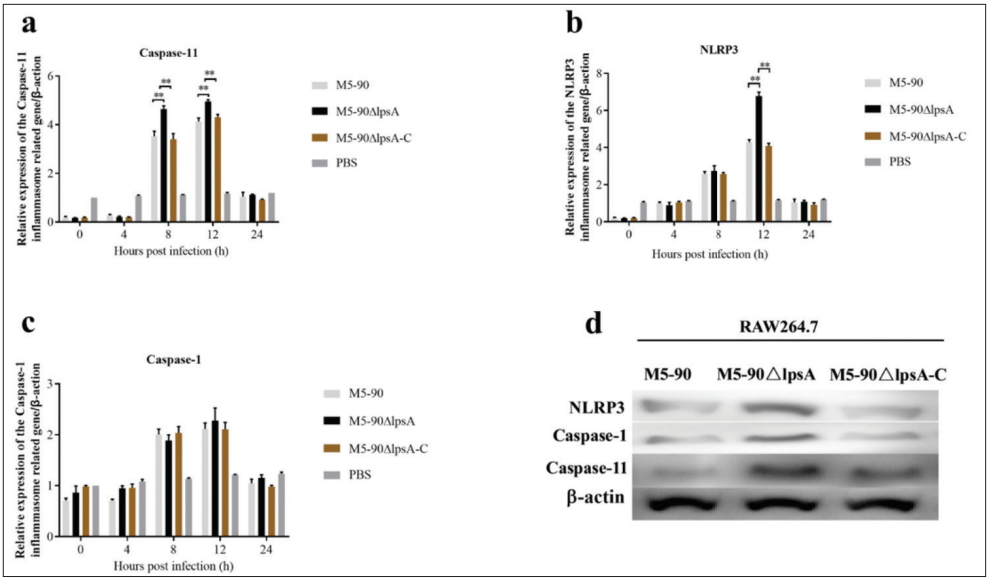

Fig. 4. Transcription and expression of NLRP3 and caspase-1/11 in RAW264.7 cells infected by M5-90Δ*lpsA*. **a, b, c.** qRT-PCR detection of *Brucella* infected cells indicate the relative expression levels of inflammasome NLRP3 and caspase-1/11 genes, **d.** Western blot analysis of cell protein lysates from RAW264.7 cells infected with M5-90Δ*lpsA* and M5-90Δ*lpsA-C* using anti-NLRP3, caspase-1/11 primary antibodies to determine NLRP3 and caspase-1/11 protein levels, with β-actin as a loading control. Samples were from the same experiment and blots were processed in parallel. Each point represented the mean ± standard deviation of three experimental groups. Asterisk (*P<0.05, **P<0.01) indicates significant differences in the mRNA level of NLRP3 and caspase-1/11 between the parental and mutant strains after infection

with M5-90Δ*lpsA* and the wild type strains at an MOI of 100, to assess the survival and replication ability of the mutant strain in macrophages. There was no difference in the number of surviving bacteria in RAW264.7 cells after 0 h of infection, but after 4 h of infection, when compared with M5-90, the number of M5-90Δ*lpsA* bacteria in macrophages decreased by 1.0 log CFU (P<0.05). After 48 h of infection, this difference increased up to 2.5 log CFU (P<0.05). The above results indicate that compared with the wild strain *(Fig. 2-b)*, the mutant strain is attenuated in macrophages and reduced the RAW264.7 cells. The ability to replicate in cells indicates that *lpsA* is involved in the chronic infection of *Brucella*.

Pro-inflammatory Cytokine Expression in RAW264.7 Cells was Increased in the Absence of lpsA

We determined whether M5-90Δ*lpsA* could affect IL-1β, IL-18 in RAW264.7 cells. The results show that the levels of cytokines IL-1β, IL-18 were higher in M5-90Δ*lpsA* infected RAW 264.7 cells than in M5-90 infected cells (P<0.01) *(Fig. 3-a,b)*.

M5-90ΔlpsA Promotes Caspase-11 Induces Non-classical Pathways Pyroptosis

We detected the transcriptional expression of caspase-1/11 and NLRP3 in M5-90, M5-90Δ*lpsA* and M5-90Δ*lpsA-C* at the mRNA level and protein level, respectively. 12 h after the infection, the relative expression levels of caspase-11 and NLRP3 mRNA in cells infected with M5-90Δ*lpsA* were

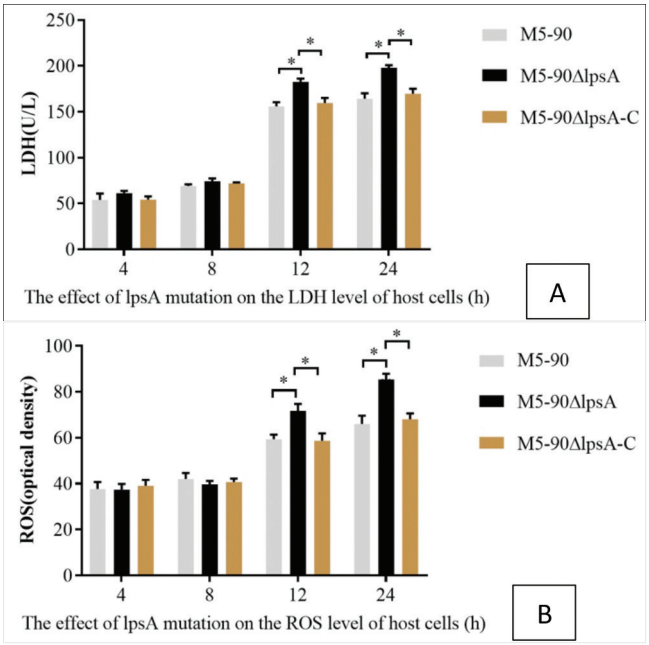

Fig 5. Levels of LDH and ROS released from cells infected with *Brucella* M5-90 or *Brucella* M5-90Δ*lpsA*. LDH (A) and ROS (B) releases were measured at 4, 8, 12 and 24 h after *Brucella* infection. Asteriks (*P<0.05, **P<0.01) indicate differences in LDH and ROS releases between the parental and mutant strains

significantly higher than those in cells infected with M5-90 *(Fig. 4-a,b)* (P<0.01). At the same time, the expression of caspase-11 protein and NLRP3 was higher than that of M5-90 infection group *(Fig. 4-d)*, while the expression of NLRP3 is enhanced, the inactivation of *lpsA* leads to an increase in reactive oxygen species (ROS) and an increase in lactate dehydrogenase (LDH) levels *(Fig. 5-a,b)* (P<0.05). However, the expression of caspase-1 is low, indicating that *Brucella* LPS may not be effectively activated *(Fig. 4-c,d)*. In addition, the complementary M5-90Δ*lpsA* mutant restored these changes. These results indicate that the M5-90Δ*lpsA* mutant inhibits pyroptosis of RAW264.7 cells by activating the non-classical pathway of caspase-11.

Therefore, our results preliminarily prove that in the intracellular life cycle of *Brucella*, the *Brucella lpsA* gene, as a key virulence gene, can increase *Brucella* viability by inhibiting the LPS-induced atypical pyroptosis pathway in the host cell. Different from the classic caspase-1 activation of cell pyroptosis pathway, LPS can activate caspase-11 by directly binding to the conserved structure of LPS lipid A. The activated caspase-11 further cleaves the GSDMD protein and promotes the occurrence of pyroptosis and promotes inflammation. The activation of body NLRP3 and the production of cytokines *(Fig. 6)*. Therefore, *lpsA* plays an important role in the intracellular life cycle of *Brucella*.

Discussion

LPS is one of the main components of the cell membrane of Gram-negative bacteria and the main component of

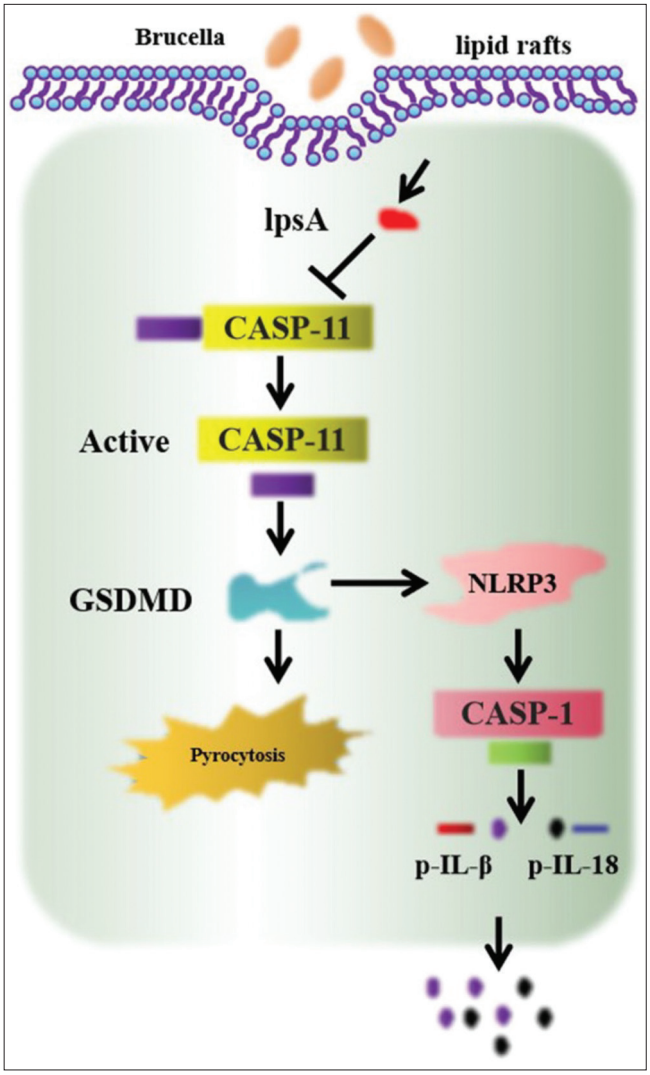

Fig 6. The model of the process of *Brucella lpsA* regulating the host cell pyroptosis. In the first few hours of infecting macrophages, 90% of *Brucella* are killed by macrophages, and the remaining 10% of *Brucella* form replication vesicles to survive. *Brucella* enters the host and interacts with the giant lipid rafts on the cell membrane of phages interact, *Brucella* and lipid rafts are closely related. After being stimulated by LPS in the cytoplasm, mouse caspase-11 and its human counterpart caspase-4/5 can interact with LPS. The conserved structure of lipid A is activated by direct binding. The activated caspase-11 further cleaves the GSDMD protein and promotes the occurrence of pyroptosis, releasing a large number of cytokines. Our results indicate that the lack of *lpsA* promotes the cell pyroptosis happened

its endotoxin and crucial to the recognition of bacteria by immune cells [25]. However, *Brucella* develops some strategies to evade recognition by the immune system, thereby establishing an infection inside the host. One of these strategies is to modify its LPS to avoid effective recognition by pattern recognition receptors [26]. Due to the atypical structure [27], *Brucella* LPS becomes a special virulence factor. In the invasion of host cells and early survival of *Brucella*, S-type LPS and its O side chain play an important role [28]. After *Brucella* invades the host, *Brucella* forms vesicles in the pre-acidic environment, called *Brucella*-containing vacuole has become a relatively safe living

environment for *Brucella*. Therefore, although *Brucella* uses virulence factors to evade part of the immune recognition of host cells, caspase-11 becomes the second obstacle to LPS recognition to promote cytoplasmic monitoring [29]. Once activated, caspase-11 leads to cell apoptosis and activation of NLRP3 inflammasomes, which in turn leads to the activation of caspase-1 and the release of pro-inflammatory cytokines, which are essential for innate immunity against Gram-negative bacteria [20]. In this study, we proved that *lpsA*, a gene related to the synthesis of lipooligosaccharide, is involved in the process of *Brucella*-induced macrophage pyroptosis.

We analyzed the M5-90Δ*lpsA* mutant, replenishment strain and parental strain, and confirmed that the reduced viability of the mutant is directly related to the *lpsA* gene deletion. We found that the survival rate of M5-90Δ*lpsA* in RAW264.7 cells was impaired. This study showed that the virulence of the *lpsA* mutant was significantly reduced. Our results further support the work of other groups, the *lpsA* gene is involved in the biosynthesis of the core of *Brucella* LPS, and the deletion mutation will cause *Brucella* to transform into an attenuated rough type, *lpsA* mutant was attenuated and was eliminated early in the infection process [21,30].

In the processing of pathogen-host cell interaction, some of the pathogens can interfere with the programmed death of host cells, which is conducive to the microorganism to survival and reproduction [10]. One Study [31] has found that the pathogenic bacterial LPS enters the cytoplasm and the mouse caspase-11 and its human counterpart caspase-4/5 can be activated by direct binding to the conserved structure of LPS lipid A. The activated caspase-4/5/11 further cleavage the GSDMD protein and promote the occurrence of cell pyroptosis [32]. However, the mechanism of the *Brucella* LPS regulating the host cell pyroptosis is still unclear. In this study, M5-90Δ*lpsA*, M5-90Δ*lpsA-C* and M5-90 were used to infect RAW264.7 cells respectively. We used Limulus reagent to detect the LPS content in the infected cells. Compared with the parent strain, cells infected with the mutant strain showed lower LPS content, indicating that *lpsA* may be involved in the important process of *Brucella* transporting LPS into host cells, but the specific mechanism needs to be further explored. In the past few years, great progress has been made in understanding the mechanism of programmed death. Previous reports confirmed that smooth *Brucella* can inhibit macrophage death, while rough *Brucella* can induce apoptosis by activating caspase-2 [33,34]. In contrast, another study observed that smooth *Brucella* induces apoptosis of RAW264.7 macrophages by producing ROS [35]. In this research we confirmed cell death using LDH release assay suggesting that *lpsA* mutant triggers caspase-11-dependent differential pyroptosis compared with parent strain. And we further verified the difference in NLRP3 expression at the mRNA and protein levels, and it is correlated with ROS.

IL-1β as a powerful cell inflammatory factor with a wide range of biological effects in immune response, can promote the proliferation and differentiation of immune cells and can be released to the outside of the cell [36]. IL-18 mediates the cascade of inflammation, initiates the host immune response, and plays a key role in driving inflammation [37]. There will be a large amount of IL-1β and IL-18 produced and released along with the process of pyroptosis. The level of inflammatory cytokine production in M5-90Δ*lpsA* infected cells was significantly higher than that of M5-90 infected cells. Our results further indicate that *lpsA* may inhibit host cell pyroptosis and increase the viability of *Brucella*.

In conclusion, the results showed that i) the IL-18 and IL-1β levels of M5-90Δ*lpsA* were higher than those of M5-90, and ii) the expression level of caspase-11 and NLRP3 in M5-90Δ*lpsA* group was higher than that of M5-90. We preliminarily proved that M5-90Δ*lpsA* can activate caspase-11-mediated cell pyroptosis and promote the occurrence of inflammation.

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Conflict of Interest

The authors have declared that no competition interests.

Author Contributions

SS, MY and YW conceived and designed the study, and critically revised the manuscript. SS, YY and HJ performed the experiments, analyzed the data. SS and YY contributed to writing the manuscript. All authors read and approved the final manuscript.

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RESEARCH ARTICLE

Influence of Claw Disorders on Milk Production in Simmental Dairy Cows

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Abstract

A study on claw disorders' influence on milk production was performed during one year on 226 Simmental loose-reared cows. The first trial group included 42, the second 37, and the third 34 cows, with claw changes observed in the first hundred days of lactation, between days 101 and 200, between days 201 and 305, respectively. The control group included 113 cows with no observed changes. Lameness intensity was assessed once a week. Milk yield data were collected three weeks before, in the week of treatment of claws and three weeks afterwards. There were significantly more cows with lameness in the third group than in the other two groups, as well as the most cows with one affected limb in the first hundred days of the lactation ($P<0.05$). Significantly fewer cows with two affected limbs were present in the first group. In the second group, more cows were observed with two affected limbs than in the first group ($P<0.05$). In the third group, more cows with three or four affected limbs were noticed than in the other groups. The most common claw disorders were: solar ulcer, laminitis, dermatitis digitalis and overgrown claws. Cows whose claws were affected between days 101 and 200 of lactation period produced 231 kg less milk than those of the control group; cows whose claws were affected in the last third of lactation produced 26 kg less milk. Nevertheless, no significant differences were found between milk yields of the control and trial groups ($P>0.05$). Cows affected in the first third of lactation produced less milk (324 kg) than both healthy and cows with claw changes manifested in later stages of lactation. Obtained data confirm that claw disorders may affect dairy production.

Keywords: *Claw disorders, Cow, Lameness, Milk production, Simmental*

Simental Süt İneklerinde Tırnak Bozukluklarının Süt Üretimine Etkisi

Öz

Açık sistem yetiştiriciliğinin yapıldığı 226 Simental inek üzerinde bir yıl boyunca tırnak bozukluklarının süt üretimi üzerine etkisine yönelik bir çalışma gerçekleştirildi. Birinci deneme grubu, laktasyonun ilk yüz günlük döneminde olan ve tırnak değişiklikleri gösteren 42 inek; ikinci grup, laktasyonun 101 ile 200. günleri arasında olan ve tırnak değişiklikleri gösteren 37 inek ve üçüncü grup, laktasyonun 201 ile 305. günleri arasında olan ve tırnak değişiklikleri gösteren 34 inekten oluşuyordu. Kontrol grubunu hiçbir tırnak bozukluğu gözlenmeyen 113 inek oluşmaktaydı. Topallık yoğunluğu haftada bir değerlendirildi. Süt üretim verileri, tırnakların tedavisinden önceki üç haftalık dönem, tedavini yapıldığı hafta ve tedaviden sonraki üç haftalık dönemlerde toplandı. Diğer iki gruba göre üçüncü grupta topallık gösteren inek sayısı çok daha fazlaydı ve bir ayağı etkilenmiş ineklere en fazla laktasyonun ilk yüz günlük döneminde rastlandı ($P<0.05$). İki ayağı etkilen inek sayısı birinci grupta önemli ölçüde daha azdı. Birinci gruba oranla ikinci grupta iki ayağı birden etkilenen inek sayısı daha fazlaydı ($P<0.05$). Üçüncü grupta ise diğer gruplara oranla üç veya dört ayağı etkilenmiş inek sayısı daha fazalydı. En yaygın tırnak bozuklukları; taban ülseri, laminitis, dermatitis digitalis ve aşırı tırnak uzamasıydı. Kontrol grubu ile kıyaslandığında, laktasyonun 101 ile 200. günleri arasında yer alan ve tırnak bozukluğu olan inekler 231 kg daha az süt üretirken, laktasyonun son üçte birlik kısmında yer alan ve tırnak bozukluğu olan inekler ise 26 kg daha az süt üretmiştir. Bununla birlikte, kontrol ve deneme gruplarının süt verimleri arasında önemli bir fark saptanmamıştır ($P>0.05$). Laktasyonun ilk üçte birlik döneminde bulunan inekler, hem kontrol hem de laktasyonun sonraki dönemlerinde bulunan ve tırnak bozukluğu olan ineklere oranla daha az (324 kg) süt üretmiştir. Bulgular, tırnak bozukluklarının süt üretimini etkileyebileceğini doğrulamaktadır.

Anahtar sözcükler: *İnek, Tırnak bozukluğu, Topallık, Simental, Süt üretimi*

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Introduction

Lameness caused by claw disorders is one of the most important diseases in modern dairy cattle production world-wide [1,2], causing pain, distress, weight loss, decreased milk production and reproductive performance, increased risk of culling and treatment costs and death [2,3]. This condition occurs as a result of many causes and predisposing factors ranging from the environmental conditions to the individual factors of the dairy cattle [1,4,5]. Claw disorders are characterized by intensity, a number of affected limbs and disease characteristics. An important predisposing factor for lameness is claw overgrowth [1,3,4], particularly in combination with hard flooring surfaces and weight bearing disturbances. The most significant causes of hoof overgrown in cows are genetic predisposition, insufficient movement of animals, inadequate nutrition, and untimely hoof trimming. Overgrowth of the hoof leads to deformation of the hoof horn, exposure of the soft parts of the hoof, development of degenerative and inflammatory processes, all of which contribute to the appearance of claw disorders. There are many risks for lameness, including season, gestation and stage of lactation, previous disease and parity, as well as a genetic determined intrinsic risk for development of claw lesions [5]. Generally, the incidence of lameness in dairy cows has been approximately 25%, ranging from 1 to 55% [6,7]. According to literature data, the most prevalent claw disorders in intensive dairy farming are digital dermatitis, sole ulcer, white line disease, toe necrosis and sole hemorrhage [8,9].

There is a growing amount of evidence worldwide regarding the impacts of lameness on milk yield in dairy cows, and numerous studies have been conducted from different viewpoints [10-15]. According to Kibar and Çağlayan [16], the shape of the lactation curve is influenced by herd factors such as management and nutrition and individual factors like genetics, parity, and disease. Discrepancies in the literature regarding to the effect of lameness and claw lesions on milk yield are partly the result of these complex influences. Comprehensive literature data regarding the impact of claw disorders on milk production in Serbia were presented by Hristov et al.[2]. The loss of milk of 1.7-3.0 L/day occurs up to one month before and one month after the treatment due to pain. According to Greenough [9] the financial impact of claw disorders includes losses from decreased production, cost of treatment, prolonged calving interval, and possibly nursing labour. The study of Charfeddine and Pérez-Cabal [17] demonstrated that claw disorders have an important effect on the production and performance of cows. Cases of dermatitis, sole ulcer and white line disease reduce milk production, led to longer days open intervals, and decrease productive life.

In this study, the hypothesis was that not only the incidence of claw disorders and treatment significantly affect the milk production, but the intensity of lameness of lactating cows may be correlated with a decrease in milk yield, a number of affected limbs, types of diseases and lactation period when lameness occurs as well. To test this, the frequency distribution of the intensity of lameness, number of affected legs, types of diseases and treatment results were examined in different stages of lactation in relation to the milk yield.

Material and Methods

The study was carried out on a dairy farm with 320 Simmental loose-reared cows during one year. The claws of all cows were weekly observed for lameness and those that were found to be affected were treated by veterinarians. A total of 226 of 320 cows on the farm were used.

There were 113 cows without changes (control group) and 113 cows in three groups of affected cows with claw changes (including overgrown claws). The affected cows were divided in three groups regarding the period of lactation when changes were noticed and the therapeutic treatment also was performed. There were 42 cows in the first (1st) trial group with claw changes observed in the first 100 days of lactation. In the second (2nd) trial group there were 37 cows with claw changes manifested between days 101 and 200 of lactation, and the third (3rd) trial group was formed of 34 cows with claw changes noticed between days 201 and 305 of lactation.

According to needs of current phisical conditions (body mass, parity, milk production and phase of lactation), cows were fed using five total mixed rations (TMR) with 17.57%, 22.57%, 21.61%, 22.28% and 25.20% of dry matter, 2764.65 g, 3542.27 g, 3339.87 g, 3669.33 g and 3961.99 g of crude protein, and 104.77 MJ, 145.07 MJ, 136.11 MJ 150.81 MJ and 171.03 MJ of net energy content, respectively. In preparation of TMR was used corn silage, alfalfa hay, pea hay, peas and alfalfa, soybeans, semolina, sunflower meal, beetroot pulp, corn grains, dried fodder peas grains and mineral-vitamin supplement. Rations were delivered by vertical mixer trailer, once a day, in the amount of 24 h. The cows consumed water *ad libitum* due to automatic heated water througs. Floors were made of cement and cleaned by manure scrapers were used on daily bases, and lying areas were recovered with saw dust when neccesary. The stall was equiped with fans for cooling and additional ventilation. Preventive hygiene measures prescribed by the Veterinary Law and the Program of animal health protection measures took place.

Lameness intensity was assessed once a week according to the Whay et al.[18], modified by Berry [19], on the concrete floor in a narrow corridor. On the day of lameness diagnosis, lameness intensity, number of affected limbs, the character of claw diseases were determined and therapeutic claw treatment were applied to all cows. Milk yields for the control groups divided into three periods corresponded

to the same periods used for three trial groups. Milk yield data were collected using the software of computerized milking parlor Westfalia SARD-C21 and by recording amounts of raw milk obtained three weeks before, in the week of therapeutic treatment of claws and three weeks after treatment. Daily milk yields were recorded and average milk yields for three phases of lactation (days 1 - 100, 101 - 200 and 201 - 305) were established and analyzed with respect to the standard lactation curve and time of claw changes occurrence. Correlations between lameness parameters, such as lameness intensity, number of affected limbs, character of claw disorder, time of claw treatment and milk yield were calculated.

All statistical analyses were performed by Statgraphics centurion XV [20]. Descriptive statistics, Pearson's product-moment correlation coefficient and ANOVA-analysis was performed regarding correlations between lameness intensity, the number of affected limbs, the character of claw disorder and time of claw treatment, and milk yields at the time of treatment. The research took place in compliance with the Serbian Law on Animal Welfare (Official Gazette of the Republic of Serbia, No. 41/09) and Ordinance on the conditions for registration for trial animals and the keeping of such a register, training programs on welfare for trial animals, request forms for approval of conducting experiments on animals, keeping, treatment and killing trial animals and reproduction, circulation, or implementation experiments on animals (Official Gazette of the Republic of Serbia, No. 39/10).

Results

In *Fig. 1*, *Fig. 2* and *Fig. 3* the distribution of lameness intensity, number of affected limbs and types of claw disorders, respectively, of all examined trial groups of cows regarding the period of lactation were presented.

Different grades of lameness intensity were noticed during all lactation periods *(Fig. 1)*. It may be noted that the number of the cows without the lameness decreased, the number of the cows with a mild form of lameness slightly variated, while the number of cows with moderate lameness increased with respect to the lactation period. The smallest number of cows in all three lactation periods was with a severe form of lameness. Less cows without lameness were found in the 3rd trial group ($P<0.05$), and more cows with a moderate form of lameness were found in the 3rd trial group than in both the 1st and the 2nd trial groups ($P<0.05$).

Regarding the number of affected limbs *(Fig. 2)*, most cows of the 1st trial group had one affected limb in the period of lactation until day 100 (20 cows), which was significantly more than in the other two groups ($P<0.05$). The occurrence of the disease in two or three limbs increased as lactation progressed, since the lower lameness occurrence rate observed in cows with two claws affected in the first hundred days of lactation. There were significantly more cows with three or four affected limbs in a period of 201 to 305 days than in earlier periods. Regarding diseases of

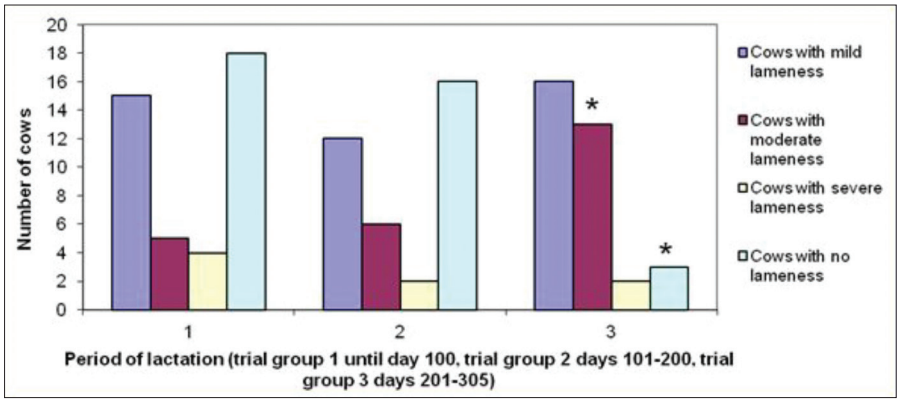
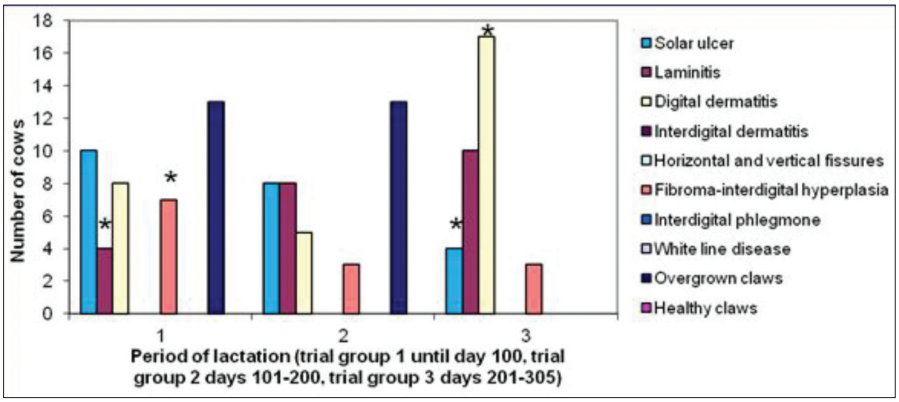


Fig 1. Lameness intensity distribution in trial groups regarding the period of lactation

Fig 2. Distribution of cows according to the number of affected limbs during different periods of lactation



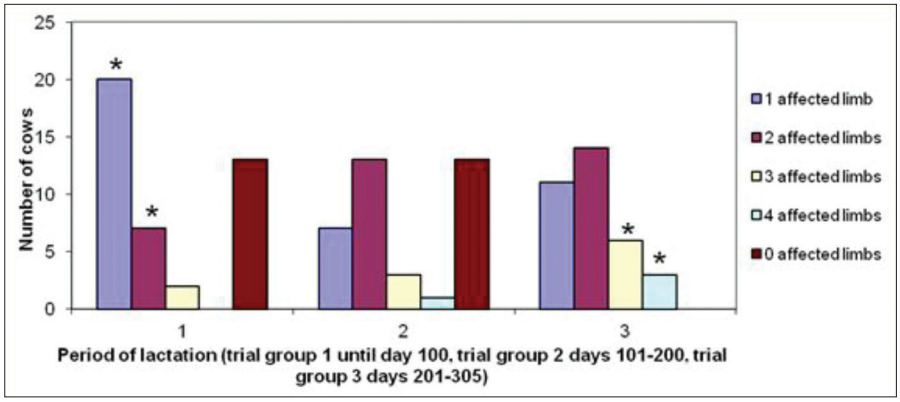


Fig 3. Distribution of cows in trial groups regarding the type of claw disorder

Fig 4. Lactation curves of three trial groups of cows

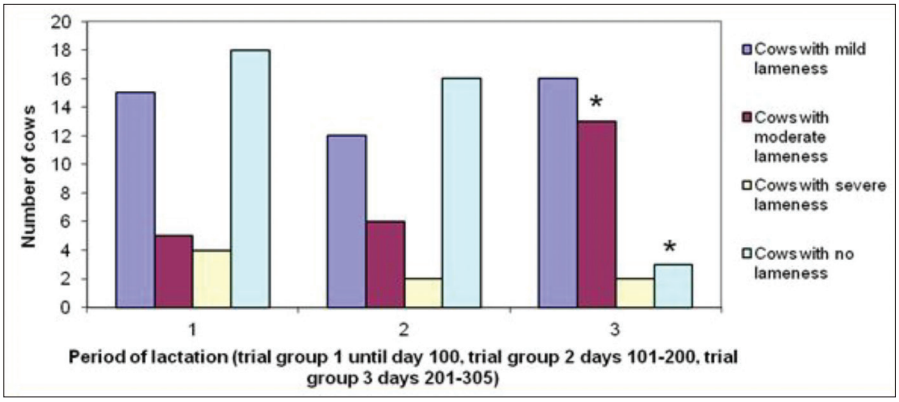


Table 1. *Milk production of cows with and without claw disorders*

Lactation Period, Days	1st Trial Group (days 1-100) (n=42)				2nd Trial Group (days 101-200) (n=37)				3rd Trial Group (days 201-305) (n=34)			
Days after correction	day of correction	7	14	305	day of correction	7	14	305	day of correction	7	14	305
Control group, $\bar{x}$/cow, kg	22.69 x^{-}	23.24	23.60	6057	19.91	20.81	20.86	5970	19.21	19.25	19.26	6056
All three trial groups, $\bar{x}$/cow, kg	21.96	22.3	21.95	5733	19.37	19.87	19.43	5739	18.45	18.77	18.34	6030
Statistical significance	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns

ns: *P>0.05; * P<0.05; ** P<0.01*

the front or the hind limb, it was observed that more cows (65 cows) had pathological changes on hind limbs than on front limbs (6 cows). Both affected hind limbs were noticed in 34 cows, while 31 cows had one hind limb affected. In addition, 42 cows underwent changes on both front and hind limbs, with 18 of them having some claw changes and 24 cows with overgrown claws.

The type of claw disorder varied significantly amongst groups *(Fig. 3)* and was related to the period of lactation. The most common claw disorders in our study were solar ulcer, laminitis, digital dermatitis and interdigital fibroma.

Fig. 4 clearly demonstrates that lactation curves of the trial groups differed from the standard lactation curve.

In the 3rd trial group, lower production of milk was determined in the first four months of lactation than in the 1st and 2nd groups. In the fifth month of lactation, the milk yields of all trial groups were almost equal. From the middle to the end of the lactation period, higher milk yield was determined for cows of the 3rd trial group.

According to *Table 1*, cows whose claws were affected between days 101 and 200 of lactation period produced 231 kg less milk than those of the control group, and cows whose claws were affected in the last third of lactation produced 26 kg milk less than those of the control group. Nevertheless, no significant differences were found between milk yields of the control and trial groups (P>0.05). Cows that were affected in the first third of lactation produced less milk (324 kg) than both healthy cows and cows with claw changes manifested in later stages of lactation.

According to the results presented in *Table 2*, there was no significant difference between milk yields three weeks before and three weeks after claw treatment.

Table 2. *Daily milk yield with respect to time of claw correction*

Daily Average Milk Yield, kg	3 Weeks Before Correction	In the Week of Correction	3 Weeks After Correction	Influence of Correction	LSD-Test
1st group, $\bar{x}$	21.43±5.73	21.96±5.63	22.03±5.61	ns	ns
2nd group, $\bar{x}$	19.85±5.63	19.37±4.74	19.4±4.29	ns	ns
3rd group, $\bar{x}$	19.57±4.66	18.45±5.08	17.8±5.44	ns	ns
Influence of the period of lactation	ns	ns	ns	ns	ns
LSD-test	ns	ns	1st group : 3rd group P<0.05	ns	ns

ns: *P>0.05; * P<0.05; ** P<0.01*

Table 3. *Correlations between lameness parameters and milk yield*

Parameters	Lameness Intensity	Number of Affected Limbs	Character of Disorders	Time of Correction
Milk yield 3 weeks before correction	0.0062	-0.1083	0.0826	-0.0930
Sample size, n	113	113	113	113
P-value	0.9481	0.2534	0.3845	0.3274
Milk yield in the week of correction	-0.0927	-0.2192	0.1260	-0.2795
Sample size, n	113	113	113	113
P-value	0.3287	0.0197*	0.1837	0.0027**
Milk yield 3 weeks after correction	-0.0217	-0.1893	0.1467	-0.3318
Sample size, n	113	113	113	113
P-value	0.8197	0.0446*	0.1210	0.0003**

ns: *P>0.05; * P<0.05; ** P<0.01*

Three weeks after the treatment of claws, the difference was significant between groups 1 and 3 (22.0 kg and 17.8 kg, respectively), due to a physiological drop of milk production in the last third of lactation having in mind for standard lactation curve and lactation persistence.

Milk yield of cows with claw disorders was lower compared to ones with no claw disorders two weeks after the treatment in all three phases of lactation (1.65 kg, 1.43 kg, and 0.92 kg, respectively).

Correlations between lameness parameters and milk yield are presented in *Table 3*. The significant negative correlation between milk yield and the number of affected limbs (P<0.05; r=-0.2192 and r=-0.1893) was found, as well as a very significant negative correlation between milk yield and time of claw treatment (P<0.01; r=-0.2795 and r=-0.3318) in the week of treatment and three weeks later.

Discussion

The obtained results show that different types of lameness in dairy cows occurred during all three lactation periods, while its intensity changed during lactation. The study results are in accordance with literature data regarding etiology, time and frequency of major claw diseases [21].

Analysis of distribution of cows regarding to the affected limbs occurrence during lactation showed that disease in two or three limbs increased as lactation progressed, since the lower lameness occurrence rate observed in cows with two claws affected in the first hundred days of lactation. Certain disease manifestations (laminitis, white line disease, e.g.) on more cow limbs require more time to develop [22]. Claw disorders findings during the last third of lactation indicate that there was sufficient time to develop clinical manifestations which will last longer in a number of limbs. According to Greenough [22], claw diseases cannot be diagnosed timely, so they may influence health and production for a long time, which is in line with our results as a whole.

Related to the period of lactation, the most common claw disorders in performed study were solar ulcer, laminitis, digital dermatitis and interdigital fibroma. The occurrence of the solar ulcer decreased while the incidence of laminitis increased as the lactation progressed. Digital dermatitis was significantly more prevalent in the last third of lactation. The occurrence of overgrown claws was observed during the first two thirds of lactation, while later was not recorded. Obtained results are in accordance with literature data [5,8,23]. Most claw diseases occurres in the time of calving [16]. Claw diseases, such as white-line disease, sole ulcer, and hemorrhages, become visible after 2 to 3 months. These cows eat less; they are more reluctant

to move and might consequently produce less milk than cows without claw lesions.

In the fifth month of lactation, the milk yields of all trial groups became almost equal. From the middle to the end of the lactation period, higher milk yield was determined for cows of the third trial group. The larger decline in milk production at the beginning of the second third of lactation coincided with the occurrence of claw disorders. These results show that the earlier occurrence of claw disorders during lactation has more apparent influence on the reduction of the milk yield compared to the later occurrence. Hernandez et al.[11] found that during lactation, 31% of cows were affected with lameness caused by claw lesions (60%), papillomatous digital dermatitis (31%) or interdigital phlegmon (9%). Consequently, milk yield of lame cows with interdigital phlegmon was significantly lower (10%). Lame cows with papillomatous digital dermatitis produced less milk than healthy cows, but the difference was not significant. Similarly, there was a linear relationship between milk yield and different increasing grades of lameness of dairy cows and decreasing milk yield among cows in their second or later lactations [12]. Amory et al.[13] investigated the effect of lesion-specific causes of lameness, based on farmer treatment and diagnosis of lame cows, such as solar ulcer and white line disease. Their results highlight that specific types of lameness vary between and within herds, associated with higher-yielding cattle [14,24]. Results of Correa-Valencia et al.[25] study confirmed these findings, who found that non-infectious foot lesions were more common than infectious (94.4 *vs.* 5.6%), white line disease was more frequently observed in the hind limbs (79.6%) and more frequent in medial claws of the front limbs (70.3%). In hind limbs, the lateral claws were more commonly affected (65.7%). The significant associations between most claw disorders and decreased yield do not prove direct relationships in the study of Kibar and Çağlayan [16]. Different aspects of the impact of lameness on milk production have been investigated by many authors. For instance, it was established that summer season foot lesions were more severe than winter season lesions, regardless of the stage of lactation [5,6]. Also, our study revealed that milk production, when foot lesions occurred, was a determining factor of the amount and pattern of milk loss only for cases during mid to late lactation [10]. The positive impact of the claw treatment was visible in the control group of cows, where the first two weeks after treatment and even in the last third of lactation an increase in the milk yield was noticed. In this study, cows with claw disorders produced less milk than those with no claw disorders two weeks after the claw treatment in all three phases of lactation. Obtained study results are in line with the literature data [10,15,26].

The significant negative correlation between milk yield and the number of affected limbs was found, as well as a very significant negative correlation between milk yield and time of claw treatment in the week of treatment and three weeks later are consistent with the data presented by Kos et al.[27], confirming that overgrown claws are fairly common during the first two thirds of lactation. Overgrown claws are one of the major factors in claw damage and lameness development, but well-timed treatment in the initial period of disease could decrease production loss [28] and increase milk yield in the following lactation stages, as it was observed in our study.

The lactation curves of the three trial groups of cows revealed differences between milk yields in different periods of lactation, indicating that the occurrence of claw disease impacted milk production and changed the shape of the lactation curve. Also, cows with affected claws during the first period of lactation had lower milk production compared with the control group. The most likely explanation for this may be that claws are particularly susceptible during partus and at the beginning of lactation [29]. According to Ristevski et al.[21], high milk production is related to lameness occurrence. Cows with claws that manifest disease later have lower milk production in the early period of lactation, which was also found in our research. According to Charfeddine and Pérez-Cabal [17], severe lesions were less frequent by far, but typically led to economic losses three times greater than those associated with mild lesions. Data on lesion incidence and economic costs could be used to develop farm-based decision support tools that could assist farmers to tackle lameness issues on their farms in the most cost-effective manner. Results in the study are also consistent with the study results of Onyiro et al.[30], who found that poor locomotion was associated with a significant reduction in the milk yield of later lactation cows. There was a significant difference in the shape of the lactation curve depending on whether or not the cow was lame during lactation [16]. Studying an impact of mobility score on milk yield and activity in dairy cattle, Reader et al.[31] noted that lame cows had a reduced milk yield, both before and after they were treated. The cows activity was significantly lower with increasing mobility score; the associations between activity and parity and month of lactation were larger, indicating that once cows were lame, they remained lame or became lame again despite treatment, so they concluded that cows started to produce less milk before their mobility was visibly impaired, which is an acceptable explanation for our results. According to Ristevski et al.[21], milk production is an important lameness risk factor, since that high milk production and low or high BCS are important for developing chronic lameness, as well as that the interaction between milk production, a BCS and metabolic parameters poses a higher risk for developing chronic lameness in dairy cows in comparison with the exposure to a single risk factor.

Cows affected during the first third of the lactation revealed a greater reduction of milk yield than cows which were affected later. A positive effect of claw treatment could be

seen between the first and the second third of lactation when cows of the 1st trial group produced more milk than other cows. As an immediate treatment followed the diagnosis of claw disorder, a favorable effect of claw treatment occurred through an increase of milk production in the second period of lactation, like in other studies [28]. Results obtained in this study are consistent with literature data [16,32] confirming that factors such as poor conditions of rearing, social and hierarchical relationship problems, presence of other diseases, such as claw diseases, which for some reason cannot be observed timely and are sometimes present in the initial period of lactation, may reduce milk production during that period, but later, with the cessation of their impact, milk production usually increases [22]. Regardless of the cause, these cows do not achieve the genetic potential for the quantity of produced milk without spending metabolic reserves, and in the later periods of lactation, consequently, an increase in milk production follows. In addition, according to Alvergnas et al.[33], the most efficient strategy seems to be early detection and diagnosis, hygiene of floors and bedding, and proper diet to avoid any ruminal acidosis or BCS drop.

According to data about influence of claw disorders on milk production presented in this study, different lameness intensities were observed during all periods of lactation. A significantly higher number of cows with a moderate form of lameness were found in the last third of lactation. Cows affected in the first third of lactation had lower milk production than both healthy cows and cows with claw changes manifested in later lactation stages. All presented data confirm that claw disorders may affect dairy production of Simmental cows.

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Conflict of Interest

The authors confirm no conflicts of interest regarding this manuscript.

Author Contribution Statement

Z. Zlatanović and S. Hristov conceived and planned the experiments. Z. Zlatanović, S. Hristov and B. Stanković carried out the experiments. Z. Zlatanović, S. Hristov, B. Stanković, M. Cincović, D. Nakov and J. Bojkovski contributed to the interpretation of the results. S. Hristov took the lead in writing the manuscript. All authors provided critical feedback and helped shape the research, analysis and manuscript.

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RESEARCH ARTICLE

Effects of Kefir on Blood Parameters and Intestinal Microflora in Rats: An Experimental Study

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Abstract

A probiotic product of kefir is widely consumed by human beings. The purpose of this research was to investigate the effects of kefir on blood parameters and intestinal flora in rats. A total of 24 female rats were used in this study. During 35 days of experimental period, rats were fed with a commercial diet and water was provided *ad libitum*. Kefir was given at the levels of 10 mL/kg, 20 mL/kg and 30 mL/kg with oral gavage to the first, second and third treatment groups, respectively. Kefir was not given to the control group. The number of yeast was found to be $1.65x10^7$ and the number of lactobacilli was found to be $4x10^8$ in kefir. At the end of the experiment, blood samples were taken from all rats. Blood plasma parameters and were investigated. The intestinal microflora was investigated by classical colony counting method. No differences were observed among the groups in total protein, albumin, uric acids, SGPT, SGOT, alkaline phosphatase and phosphorus in blood plasma. The plasma triglyceride and cholesterol levels in the second and third groups were lower than control group ($P<0.05$). No differences were observed in the intestinal pH levels among groups. Although total bacteria number of intestinal microflora was not different in groups, the number of Enterobactericeae and coliform bacteria in the third group was lower than the other groups ($P<0.001$). The number of Lactobacilli and the yeast level in the intestinal contents were increased by the usage of kefir ($P<0.001$). It was concluded that positive effects of the kefir were observed in intestinal microflora with increasing the number of beneficial bacteria and decreasing harmful bacteria and therefore kefir has a positive effect on the health of the animals.

Keywords: *Kefir, Rat, Performance, Blood parameters, Intestinal microflora*

Ratlarda Kefirin Bağırsak Mikroflorası ve Bazı Kan Parametrelerindeki Rolü: Deneysel Çalışma

Öz

Probiyotik bir ürün olan kefir insanlar tarafından yaygın olarak tüketilmektedir. Bu araştırmanın amacı, kefirin ratlarda kan parametreleri ve bağırsak florası üzerindeki etkilerini araştırmaktır. Bu çalışmada toplam 24 dişi rat kullanıldı. Otuzbeş günlük deney süresi boyunca, ratlar ticari rat yemi ile beslendi ve su *ad libitum* verldi. Kefir, deneme gruplarına sırası ile 10, 20 ve 30 mL/kg seviyelerinde oral gavajla verildi. Kontrol grubuna kefir verilmedi. Kefir maya sayısı $1.65x10^7$ lactobacilli sayısı $4x10^8$ bulunmuştur. Kan plazmasındaki total protein, albümin, ürik asit, SGPT, SGOT, alkalin fosfataz ve fosfor açısından gruplar arasında fark gözlenmedi. İkinci ve üçüncü gruptaki plazma trigliserid ve kolesterol, seviyeleri kontrol grubuna göre daha düşüktü ($P<0.05$). Gruplar arasında bağırsak pH seviyelerinde farklılık gözlenmedi. Bağırsak mikroflorasında total bakteri sayısı kontrol ve deneme gruplarında fark çıkmazken. Üçüncü deneme grubunda, enterobakteri ve koliform bakteri sayısı diğer gruplardan daha düşük ($P<0.001$) bulunmuştur. Aynı şekilde Laktobasil sayısı ve maya seviyesi kefir kullanımı ile birlikte gruplar arasında önemli farklılık ($P<0.001$) olmuştur. Yararlı bakteri sayısının artması ve zararlı bakterilerin azalması ile bağırsak mikroflorasında kefirin olumlu etkilerinin görüldüğü ve bu nedenle kefirin hayvanların sağlığı üzerinde olumlu etkisi olduğu sonucuna varıldı.

Anahtar sözcükler: *: Kefir, Kan parametreleri, Bağırsak mikroflora, Rat*

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Introduction

Various feeds containing live microorganisms (probiotics) are now being used extensively and many researches are being carried out on their effects. Especially the effects of such food items on digestive system functions are being supported with new knowledge day by day. In the field of animal nutrition, new feed additives and performance enhancing substances are increasing progressively. These products are generally classified and named as prebiotics and probiotics. Kefir is one of Turkish traditional fermented dairy products, obtained by fermentation of ethyl alcohol and lactic acid using kefir extracts. Kefir is widely known in the Caucasus and has been produced by local people since ancient times. Since the 19th century, it has begun to be produced in many parts of the world. Kefir has a sharp acid taste and contains lactic acid, oxalic acid, little alcohol and lactic acid bacteria and some aromatic compounds as acetaldehyde and acetone that formed by yeasts [1-3]. Yeast flour is the main element which gives the unique taste of kefir. Since kefir is made from milk, it contains all the nutrients such as fat, lactose, minerals and vitamins in the milk. Even during the formation of certain vitamins, protein and lactose partial disintegration, kefir feed value is increasing. The microorganisms found in kefir composition enable this product to be digested easily so that the absorption of nutrients by the body is increasing. Kefir granules contain some microorganisms such as; lactobacilli, lactococci, leuconostocs, acetobacteria, and fungi (Kluyveromycesmarxianus, Torulasporadelbrueckii, *Saccharomyces cerevisiae*, Candida kefir [4,5]. The antioxidant, antifungal [6], antibacterial, antitumoral, immunological [7-9], triglyceride [10], and cholesterol lowering [11] and anti-apoptotic [12] effects of kefir were reported previously. Kefir microbiological composition which effects positively on human health has been reported [13-15]. By taking advantage of these properties of kefir, we aimed to examine its effects on intestinal microflora and some blood plasma parameters in rats.

Material and Methods

Ethical Approval

This study was conformed according to Hatay Mustafa Kemal University Animal Experiments Local Ethics Committee Presidency instructions and approved with consensus at the meeting (30/01/2014, 01/5).

Animal Sampling

A total of 24 female rats were used in this study. Rats were randomly allocated into one control group and three treatment groups each containing 6 rats. During 35 days of experimental period, rats were fed with a commercial diet having 23% crude protein and 2800 kcal/kg metabolizable energy. Feed in pellet form and water were provided *ad libitum*. Kefir was given at the levels of 10 mL/kg, 20 mL/kg and 30 mL/kg with oral gavage per day to the first, second and third treatment groups, respectively. Kefir was not given to the control group.

Detection of Yeast and Lactobacilli Count of Kefir

For counting of Yeast and Lactobacilli in kefir, a series of 10-fold dilutions were made by using sterile saline (FTS). For this purpose, four MRS Agar (de man, rogosa and sharpe) and four Sabouraud Dextrose Agar were inoculated with 100 µL from each dilution. The agar plates were incubated at 30°C for 72 h in aerobic conditions. After incubation, the cultures for each dilution were counted from the media and the averages were taken and the number of these microorganisms in kefir was determined [16]. The number of yeast was found to be $1.65x10^7$ and the number of lactobacilli was found to be $4x\ 10^8$ in kefir.

Determination of Microbial Flora and pH in Bowels

At the end of the 35-day study period, all animals were killed by decapitation under anesthesia. After the intestinal contents were homogenized, pH was measured (Orion Star Benchtop pH meter).

At the end of the process, the intestinal contents of 6 animals from each group were collected under sterile conditions and transferred into 50 mL sterile plastic tubes. Samples were diluted one-fold in FTS containing 0.9% $NaCl_2$, followed by serial 10-fold dilutions (log10) in FTS. Cultivated Plate Count Agar was incubated for 48 h at 30°C for Total Mesophilic Aerobic Microorganism (TMAM), MacConkey Agar for 24 h at 37°C for coliform bacteria count, and Sabouraud Dextrose Agar for 72 h at 30°C for yeast in aerobic conditions, MRS Agar for 72 h at 30°C in microaerophilic conditions for Lactobacilli. Four agar plates were used for each dilution. After the incubation the average was obtained by counting. The mean numbers for all dilution steps were then determined and the number of microorganisms in each sample was recorded [16].

Blood Analysis

At the end of the process, blood samples were collected from 6 animals in each group (total 24 rats) and transferred to EDTA tubes and centrifuged at 3000 rpm for 5 min. Plasma were then kept in deep freezing (-20°C). Total protein, albumin, uric acid, total cholesterol, triglyceride, alkaline phosphatase (ALP), serum glutamic oxaloacetic transaminase (SGOT), serum glutamic pyruvic transaminase (SGPT) and phosphorus analyzes were then performed with an autoanalyzer.

Statistical Analysis

Statistical analyses were done using SPSS programme (SPSS Inc., Chicago, IL, USA). Data for body weight, blood parameters, intestinal pH and microflora were analysed

as a completely randomized block design, with 4 dietary treatments and 6 samples using The effects of graded levels of kefir on these variables were analysed using polynomial contrasts. The significance of mean differences between groups were tested by Tukey. Level of significance was taken as $P<0.05$ [17].

Results

The number of yeasts was found to be $1.65x10^7$ and the number of lactobacilli was found to be $4x\ 10^8$ in kefir. The final body weight was found 322.83, 359.17, 338.00, 361.17 g in the control and experimental groups, respectively. There were no differences in the initial and final body weights among the groups as shown in *Table 1*. There were no statistical differences in total protein, albumin, uric acid, SGPT, SGOT, ALP and phosphorus among the groups. Triglyceride levels in the blood samples taken at the end of the trial were found to be 80.07, 79.90, 61.18, 61.52 mg/dL in the control and experimental groups, respectively. Blood cholesterol values were found to be 49.92, 46.62, 41.65, 38.72 mg/dL respectively. A linear decrease ($P<0.05$) was observed in blood plasma triglyceride and cholesterol levels with increasing kefir levels *(Table 2)*.

There were no differences among the groups in the pH values of intestinal contents *(Table 3)*. In the intestinal microflora, the number of Enterobacteria was the lowest in group 3 ($0.18x10^7$) and the highest in the control group

Table 1. *Effects of kefir on body weight of rats*

Parameters	Groups				Pooled SEM	Significance		
	Control (n=6)	Group 1 (n=6)	Group 2 (n=6)	Group 3 (n=6)		Linear	Quadratic	Cubic
Initial BW (g)	320.50	349.83	330.50	355.17	6.434	0.139	0.851	0.107
Final BW (g)	322.83	359.17	338.00	361.17	6.590	0.102	0.596	0.077

No significant differences among groups

Table 2. *Effects of kefir on blood plasma parameters in rats*

Parameters	Groups				Pooled SEM	Significance		
	Control (n=6)	Group 1 (n=6)	Group 2 (n=6)	Group 3 (n=6)		Linear	Quadratic	Cubic
Total protein mg/dL	48.80	48.33	49.68	49.08	0.430	0.589	0.941	0.358
Albumin mg/dL	30.22	29.22	28.13	28.80	0.375	0.118	0.267	0.581
Uric acid mg/dL	1.92	1.68	0.88	0.90	0.256	0.105	0.807	0.554
Triglyceride mg/dL	80.07^a	79.90^a	61.18^b	61.52^b	2.846	0.001	0.956	0.074
Cholesterol mg/dL	49.92^a	46.62^a	41.65^b	38.72^b	1.867	0.023	0.959	0.816
SGPT U/L	24.52	27.22	26.78	24.72	0.699	0.979	0.104	0.813
SGOT U/L	61.37	62.98	63.60	65.85	2.121	0.492	0.944	0.897
Alkaline phosphatase U/L	9.95	8.88	9.08	8.87	0.305	0.287	0.498	0.554
Phosphorus mg/dL	3.96	3.92	3.70	3.70	0.534	0.337	0.925	0.698

^{a,b} Means within a row followed by the different superscripts differ significantly ($P<0.05$)

Table 3. *Effects of kefir on intestinal pH and microflora (log10 cfu/g) in rats*

Parameters	Groups				Pooled SEM	Significance		
	Control (n=6)	Group 1 (n=6)	Group 2 (n=6)	Group 3 (n=6)		Linear	Quadratic	Cubic
pH	6.61	6.76	6.69	6.50	0.050	0.371	0.083	0.816
TMAM	57.40	57.20	63.60	57.60	1.428	0.585	0.317	0.150
Enterobacteria	3.16^a	2.40^{ab}	1.96^b	0.18^c	0.274	<0.001	0.050	0.142
Coliform	2.64^a	2.58^a	2.36^a	0.48^b	0.240	<0.001	0.004	0.234
Lactobacilli	1.66^c	2.92^b	3.20^b	7.52^a	0.513	<0.001	<0.001	<0.001
Yeast	0.17^b	0.20^b	0.28^a	0.35^a	0.019	<0.001	0.280	0.505

^{a,b,c} Means within a row followed by the different superscripts differ significantly ($P<0.05$)

($3.16x10^7$) and these findings were statistically significant ($P<0.001$) as shown in *Table 3*. Similarly, the number of coliform bacteria was found to be the lowest in group 3 ($0.48x10^7$). *Lactobacillus* counts were $1.66\ x10^7$ in the control group, $2.92\ x10^7$ in the first group, 3.22 in the second group and $7.52\ x10^7$ in the third group ($P<0.001$). The intestinal yeast level was the highest in the 3rd group ($0.35x10^7$). In summary; as the amount of kefir increased, there was a linear decrease in Enterobacteria, Coliform count while an increase in Lactobacilli and yeast counts was determined.

Discussion

In this study, effects of kefir were investigated on rats. There were no differences in body weights among the control and treatment groups. Sarı et al.[18] reported that the body weights of mice consumed the probiotic, kefir, kımız and yogurt were higher than that of control group. Karademir and Ünal [19] also concluded that there was a concordance between body weight gain and the amount of kefir in drinking water. Carnavelli et al.[20] fed sea bass with *Lactobacillius delbrueckii,* which they had isolated from the kefir, for 70 days and found that body weights of sea bass were increased with the consumption of *Lactobacillius delbrueckii*. Karademir et al.[21] have added kefir to laying hens' drinking water (0, 5, 7.5 and 10 mL/L) and they showed that kefir had a positive effect on egg shell thickness in the first period but it had no effects on other performance parameters.

In the present study there were no significant differences among the groups in total protein, albumin, uric acids, cholesterol, SGPT, SGOT, alkaline phosphatase and phosphorus in blood plasma. The triglyceride levels in groups treated 20 mL/kg and 30 mL/kg kefir daily were significantly lower than those of control group and the first group ($P<0.05$). Cholesterol and triglyceride levels were decreased linearly with increasing dose of kefir ($P<0.05$), but no significant differences in cholesterol level were observed among groups. The cholesterol levels in the groups treated 20 mL/kg and 30 mL/kg kefir daily were 16.6% and 22.4% lower than that of control group, respectively ($P>0.05$). The uric acid levels in the groups treated 20 mL/kg and 30 mL/kg kefir daily were 54.2% and 53.1% lower than that of control group, respectively ($P>0.05$). Adipose tissue produces and secretes uric acid through xanthine oxidoreductase and that its production is enhanced in obesity. Uric acid is a risk factor for cardiovascular diseases. Xanthine oxidase, is one of the enzymatic forms of xanthine oxidoreductase, induces oxidative stress in the manufacture of uric acid production. Thus, inhibition of xanthine oxidase suppresses the oxidative stress of uric acid that is related to cardiovascular diseases, obesity and insulin resistance [22,23]. The dose dependent significant decrease of total cholesterol level and triglyceride value by kefir administration was also reported previously [24,25]. However, Rattray and Connell [26], reported that plasma triglycerides were not affected by kefir consumption. Also Ozsoy [27] emphasized that kefir had positive effects on fatty liver in rats. Some researchers [28-30] reported that cholesterol lowering effect of kefir could be attributed to the deconjugation of bile acids by *Lactobacillus* spp.

In the present study, no differences were observed among groups in total mesophilic aerobic microorganism. But main differences were found in Lactobacili, yeast, Enterobacteiceae and total coliform counts. While Lactobacilli and yeast count increased, Enterobacteiceae and total coliform decreased with kefir consumption. Similarly, Yaman et al.[31] reported that no differences in total aerobic mesophilic bacteria, a significant ($P<0.05$) decrease of the coliform and Enterobacteriaceae population and significant increase in Lactobacilli count in the faeces of goslings supplemented with 0.2% and 0.5% kefir to drinking water. Zheng et al.[32] also reported that fecal lactobacilli counts were significantly ($P<0.05$) higher in rats fed by probiotic bacteria from tibetian kefir than in the control group. But, the amount of coliform organisms in the rat faeces was significantly decreased at day 28. After 28 days of administration, the amount of coliform organisms were remained stable until the end of 42 days. And they suggested that these strains may be used in the future as probiotic starter cultures for manufacturing novel fermented foods. Likewise, Yaman et al.[31] reported that *Lactobacillus* populations were significantly enhanced in geese receiving the highest dose of kefir in drinking water (0.5%) when compared with the controls. Colony forming units of *Enterobacteriaceae,* often associated with intestinal disease, were significantly lowered in the group with 0.5% kefir supplementation, indicating a Lactobacilli - *Enterobacteriaceae* antagonism, In the present study, a significant ($P<0.05$) decrease of the coliform population was observed. Wang et al.[24] reported that kefir administration at high cholesterol diets of rats did not affect intestinal pH parameters as like as our study. The differences in literatures may be due to the kefir usage in experiments, diets and animals.

As a result, the use of kefir in rats did not lead to any health problems. The blood level of triglyceride has been reduced considerably, and the number of beneficial bacteria in the intestine has been increased, while the number of harmful bacteria has been decreased. Since the most beneficial result was seen in the third trial group given 30 mL/kg kefir daily, consumption at these doses may be recommended.

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Declaration of Interest Statement

We declare that we have no conflict of interest.

Authors Contributions

BO designed the experiments. BO, ZC, SY and HB performed the experiments and wrote the paper and analysed the data. BO provided the kefir.

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RESEARCH ARTICLE

Dynamics of Oxidants, Antioxidants and Hormones During Different Phases of Pregnancy in Hairy Goats

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Abstract

The aim of the present study was to observe the variation in oxidant (MDA), antioxidants (SOD, GSH, GSH-Px) and hormones (P4 and E2) levels in pregnant hairy goats during breeding season. In the this study, twenty hairy goats were synchronized by using sponges containing progesterone (fluorogestone acetate). The animals showing oestrus were inseminated twice, first at 18th -24th h and second at 36th - 48th h of oestrus. On 35th and 42nd days after insemination, pregnancy diagnosis examination was performed with transrectal ultrasonography. The blood samples were collected from pregnant goats at 0, 11, 24 , 57, 100, 134, 141 days of gestation and immediately after the parturation. Serum samples collected at estrus and different stages of gestation were analyzed for MDA, SOD, GSH-Px, GSH, P4 and E2 concentrations using standard protocols. The results showed that MDA level did not change in pregnant goats. The SOD, GSH and GSH-Px levels also shown a similar pattern throughout pregnancy period. The peak ($P<0.05$) level of progesterone was recorded between 11th to 134th days of gestation. At time of estrus (day 0) and late gestation (day 134 and 141), the concentrations estradiol reached at maximal level ($P<0.05$) in hairy goats. In conclusion, the oxidants and antoixidants do not change with respect to dynamics of progesterone or estradiol level during gestation period in black hairy goats.

Keywords: *Antioxidants, Oxidants, Pregnancy, Hormones, Hairy Goat*

Kıl Keçilerinde Gebeliğin Farklı Aşamalarında Oksidanların, Antioksidanların ve Hormonların Dinamikleri

Öz

Bu çalışmanın amacı, üreme sezonundaki gebe kıl keçilerinde oksidan (MDA), antioksidan (SOD, GSH, GSH-Px) ve hormon (P4 ve E2) seviyelerindeki farklılıkları gözlemlemekti. Bu çalışmada 20 adet kıl keçisi progesteron içeren (fluorogeston asetat) vaginal süngerler kullanılarak senkronize edildi. Östrüs gösteren hayvanlar ilk olarak östrüsün 18-24. saatlerinde daha sonra 36-48. saatlerde olmak üzere iki defa tohumlandı. Tohumlama sonrası 35-42. günlerde transrektal ultrasonografi ile gebelik muayenesi yapıldı. Gebe keçilerden gebeliğin 0, 11, 24, 57, 100, 134, 141. günlerinde ve doğumdan hemen sonra kan örnekleri alındı. Gebeliğin farklı dönemlerinde toplanan serum örnekleri standart protokoller kullanılarak MDA, SOD, GSH-Px, GSH, P4 ve E2 konsantrasyonları için analiz edildi. Sonuçlar, MDA seviyesinin gebe keçilerde değişmediğini gösterdi. SOD, GSH ve GSH-Px seviyeleri de gebelik süresince benzer kaldı. Gebeliğin 11 ile 134. günleri arasında progesteron en yüksek seviyedeydi ($P<0.05$). Gebeliğin 0, 134 ve 141. günlerinde estradiol konsantrasyonu en yüksek seviyeye ulaştı. Sonuç olarak oksidanlar ve antioksidanlar, progesteron ve estradiol seviyelerine kıyasla gebelik süresince herhangi bir değişiklik göstermedi.

Anahtar sözcükler: *Antioksidanlar, Oksidanlar, Gebelik, Hormonlar, Kıl Keçisi*

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Introduction

During the pregnancy phase, body of the dam and conceptus demands the surplus supply of oxygen. The high demand of oxygen promotes the reactive oxygen species (ROS) production level during implantation to parturition [1]. The normal ROS level is required for the process of steroidogenesis, embryonic development, maternal recognition and implantation during pregnancy. Any imbalance in ROS level leads to cellular disintegration in the dam and conceptus that could result in embryonic mortality, placental degeneration, and pregnancy failure [2]. The pregnancy associated oxidative stress is balanced by regulation of exogenous and endogenous antioxidants level [3] and pre-conception increase in antioxidants favors the establishment of pregnancy [4,5]. Previously, the mechanism of ROS generation and antioxidant response during gestation have been documented in bovine [6] and ovine [7] but little information is available in pregnant goats [8] with respect to antioxidant response and oxidative stress to hormonal changes. Enzymatic scavenging mechanism i.e. glutathione peroxidase, catalase (CAT) and superoxide dismutase (SOD) against oxidative damage prevent dam and fetus from oxidative stress during the pregnancy. Alteration in enzymatic antioxidants occurres due to multiple fetuses, change of environment, low body condition scores and provision of low quality forage during pregnancy [9]. A change in oxidants and antioxidants is also associated with the status of the animal such as pregnancy, parturation and lactation [10].

Cotyledonary placenta is present in the goats and performs similar functions for optimal fetal growth and pregnancy maintenance alike to other species [11,12]. In contrast, the corpus luteum is considered the sole organ for pregnancy maintenance in caprine compared to bovine or ovine where the shift of corpus luteum (CL) to placenta for progesterone production occurs in mid of gestation [13]. Under such conditions, mechanisms underlying oxidants (MDA)-antioxidants (SOD, GSH, GSH-Px) variability in repsonse to progesetrone or estradiol level in pregnant goats need to be elucidated. Therefore, the present study was aimed to evaluate the malondialdehyde (MDA) concentrations, antioxidant enzyme activities superoxide dismutase (SOD), glutathione peroxidase (GSH-Px) and total glutathione (GSH) and progesterone (P_4) and estradiol (E_2) during the different phases of pregnancy in hairy goats.

Material and Methods

Ethical Approval

Prior to the execution of study, an approval was obtained from Animal Welfare and Ethical Committee Van Yüzüncü Yıl University (VAN YUHADYEK), Van, Turkey (Decision No: 2015/07-B).

Animals

In the present study, about twenty (n=20) 2-5 years old multiparous black hairy goats with no history of reproductive problems were selected. The research was conducted in Small Ruminants Research Center, Van Yüzüncü Yıl University, Van, Turkey. The goats were reared under semi-intensive conditions where each goat was given approximately 0.50 kg/head of concentrate daily in addition to grazing. The black hairy goat breed is present in Eastern Anatolia Region and this breed exhibits seasonal breeding pattern in this region. For optimum conditions and to mimimize the variability in experiments, the peak breeding season (September to November, 2019) was chosen to conduct the study.

Study Design, Estrus Synchronization and Pregnancy Diagnosis

Initially, the goats were synchronized using progesterone sponges (Chronogest-CR, fluorogestone acetate FGA, 20 mg Intervet®, Istanbul, Turkey) by placing intravaginally for a period of 11 days. Later, the cloprostenol (Estrumate 50 mg, Intervet®, Istanbul, Turkey) and equine chorionic gonadotropin (Chronogest/PMSG, 400 IU, Intervet®, Istanbul, Turkey) were adminstered intramuscularly 48 h before the removal of sponges. Estrus response detection was started after the removal of sponges till possible window of estrus display using a teaser buck at twelve hours intervals. The estrus goats were inseminated twice (First at 18-24 h and second after 36-48 h of estrus) using frozen-thawed semen (75×10^6 sperm/0.50 mL) through transcervical intrauterine insemination technique [14]. The pregnancy diagnosis was performed at day 35th and 42nd after insemination through transrectal ultrasonography (7.5 MHz Linear Probe, Honda HS 1500).

Blood Sampling

The blood samples were collected through jugular vein puncture at 0, 11th, and 24th days after insemination from all experimental goats (n=20). Upon pregnancy confirmation, the blood samples were collected from pregnant goats (n=10) on 57th, 100th, 134th and 141st days of gestation and immediately after the parturation. The blood samples were centrifuged at 3000 rpm for 10 min for serum separation. Then serum samples were stored at -20°C and later assayed for oxidants (MDA), antioxidant (SOD, GSH-Px, Total GSH,) and hormone (P_4 and E_2) titer.

MDA, SOD, GSH and GSH-Px Analyses

Serum MDA, GSH, SOD, GSH-Px were measured by commercially available ELISA kits (LOT # OK18104O, Rel Assay Diagnostics, Clinical Chemistry Solutions, Gaziantep, Turkey) as described earlier [15]. These kits were based on the spectrophotometric measurement and presence of the oxidants present in serum samples shown the colored compund. The detection principle was based

on the colored compund orgination by conversion of ferrous (Fe^{+2}) ions into ferric (Fe^{+3}) ions as a chromogen in an acidic environment. Hydrogen peroxide was used as a calibrator index. The MDA, GSH, SOD, GSH-Px levels were measured by ELISA spectrophotometric device (TECAN Firm, Sunrise model). The results were expressed as µmol H_2O_2 Eq/L, MDA levels as µmol/L, SOD activity as U/mL, GSH-Px activity as nmol/min/ml, and total GSH levels as µm.

Progesterone (P_4) and Estradiol (E_2) Hormone Analyses

Serum E_2 and P_4 concentrations were measured by automated Elecsys Immunoanalyser method (Roche Diagnostics, Mannheim, Germany). The estrogen concentration was expressed in pg/ml following the method described by Souza et al.[16], while progesterone concentration was expressed in ng/ml as reported previously [17].

Statistical Analyses

The data was analysed using SPSS (IBM SPSS for Windows, Ver.23) statistical software. The samples were analysed by selecting the values 0.80 and Type 1 Error 0.05. Normality distribution was calculated by using Shapiro-Wilk (n<50) test. In case of data unequallity, the non-parametric tests were applied to observe the difference among the variables. The descriptive statistics were used to express the data (average±Standard Deviation). The Friedman test was used to express the repeated measures for different variables at different days of pregnancy. Additinally, the Wilcoxon test was used for the analysis of dependant variables at different gestational days. Spearman correlation coefficient test was applied to determine the relationship between the measurements. A level of P<0.05 was used to denote the significiance difference between the variables.

Results

The results of serum lipid peroxidation, enzymatic antioxidant activities and hormones at estrus time (0), gestation (day 11, 24, 57, 100, 134 and 141) and immediately after parturation were presented in *Table 1*. The level of MDA, SOD, GSH-Px and total GSH did not change at estrus (day 0), gestation (day 11, 24, 57, 100, 134 and 141) and after parturation. The progesterone was at peak (P<0.05) level at day 11 with a decreasing pattern at day 24, day 57; however, it again increased (P<0.05) on day 134. The least (P<0.05) level of progesterone was observed at the time of AI and after parturition. In contrast, the estradiol was reached to maximum level on day 0, 134th and 141st of gestaion in goats.

The correlations presented in *Table 2* indicated that there was no positive or negative correlations between hormones (P_4 or E_2) and oxidant (MDA) or antioxidants (SOD, GSH-Px and total GSH activity).

Discussion

In the present study, we investigated the variation of oxidants and antioxidants levels in association to progesterone and estradiol levels at estrus or during different phases of

Table 1. *The results of serum lipid peroxidation, enzymatic antioxidant activities hormone at the time of estrus/AI (day 0), blastogenesis (days 11), embryogenesis (days 24), fetal period (days 57, 100, 134 and 141) and after parturition in pregnant goats (values are presented as means ± SD)*

Variables	Day 0 (Estrus/AI)	Day 11 (B)	Day 24 (E)	FP				After Parturition
				Day 57	Day 100	Day 134	Day 141	
MDA (µmol/L)	1.57±0.10	1.57±0.09	1.56±0.08	1.53±0.06	1.59±0.04	1.59±0.11	1.56±0.15	1.59±0.08
SOD (U/mL)	1.93±0.57	2.19±0.49	2.17±0.58	2.43±0.36	2.08±0.43	2.01±0.62	1.65±0.54	2.36±0.91
GSH-Px (nmol/min/mL)	176.0±100.0	288.4±131.1	215.4±373.7	161.9±60.7	225.4±135.2	165.2±97.7	237.6±146.6	184.3±71.3
Total GSH (µm)	3.30±1.96	5.46±2.48	4.07±1.45	2.98±1.22	4.35±2.58	3.69±2.79	4.48±2.87	3.46±1.41
P_4 (ng/mL)	0.32±0.35^{d}	20.37±7.54^{a}	8.01±2.84^{c}	9.72±3.19^{c}	10.82±3.76^{bc}	15.50±3.40^{ab}	7.48±2.91^{c}	0.30±0.08^{d}
E_2 (pg/mL)	35.20±16.3^{bc}	13.20±2.04^{d}	12.40±1.35^{d}	16.00±2.55^{d}	23.56±2.60^{cd}	50.33±12.69^{ab}	67.89±24.43^{a}	39.60±15.58^{bc}

AI: *artificial insemination;* ***B:*** *blastogenesis;* ***E:*** *embryogenesis;* ***FP:*** *fetal period;* ***MDA:*** *malondialdehyde;* ***SOD:*** *superoxide dismutase;* ***GSH-Px:*** *glutathione-peroxidase;* ***GSH:*** *glutathione;* ***P_4:*** *progesterone;* ***E_2:*** *estradiol; Different superscripts (a, b, c, d) in same raw indicate the statistical difference (P<0.05)*

Table 2. *Correlation analysis between hormones, oxidants and antioxidants in pregnant goats*

Variables	MDA	P_4	E_2
MDA (µmol/L)		-0.02	0.05
SOD (U/mL)	0.21	-0.02	-0.2
GSH-Px (nmol/min/mL)	-0.01	0.06	-0.22
Total GSH (µm)	-0.02	0.09	-0.2

P<0.05; r: Spearmen correlation coefficient

pregnancy in goats. The MDA level and blood antioxidant status (SOD, GSH, GSH-Px) in the pregnant goats did not change at any phase of pregnancy. The data shows that no oxidative stress occurs in goats during any phase of pregnancy and oxidants-antioxidants homeostasis is maintained in response to hormonal change.

The level of MDA or CAT, SOD and GSH prior to breeding or mating time also affect the future pregnancy. Although, the ROS is a prerequisite for oocyte maturation, fertilization, embryogenesis, implantation and steriodogenesis from CL or plancenta but enormous rise in ROS during synchronization through application of CIDR or vaginal sponges could adversely affect the pregnancy [4]. In contrast, we did not notice any change in MDA or antioxidants levels comapring to nonpregnant or any other phase of pregnancy in the present study, though, the goats were also synchronized using progesterone sponges by placing intravaginally as reported earlier [4,18].

Increasing trend of MDA with decreasing level of antioxidants in blood profile is an indicator of oxidative stress. The current results showed neither MDA nor antioxidants levels change in pregnant goats; although, previous studies in different species describe that pregnancy induces oxidative stress and variation in oxidant-antioxidants is observed [1,2,6]. It is also noted that fluctutaions in hormonal changes during the pregnancy did not influence the oxidative and antioxidative parameters. Keeping in view of previous literature, generally rise of oxidative stress is notable during the twinning and multiple pregnancies compared to singleton [19]. In addition, the rise in MDA during pregnancy is an indication of stress that occurred due to climate change, low plane of nutrtion or poor body condition [5]. In the present study, unaltered antioxidative homeostasis during the pregnancy might be due to high level of progesterone at each phase of pregnancy and high level of estradiol at estrus or parturition. Because the progesterone and estradiol have the antioxidative properties [20] and hormonal fluctution during pregnancy could lead to imbalance between oxidative and antioxidants level as seen in multiple pregnancies [21]. In addition, similar level of oxidant-antioxidants during pregnancy in goats in the current study might be linked to similar level of progesterone production solely by CL compared to other species where shift from CL to placenta occur. In current study, we did not notice any oxidative stress in those goats carrying twins because two goats were carrying twins and rest of them having singleton. In contrast to current study, Jimoh et al.[22] observed a decline in antioxidant enzyme activity from 2nd to the 3rd trimester and assumed that this decline might be due to depletion of the antioxidants in response to higher physiological demand of immunity at neonates birth.

The goat breeds present in subtropical or in tropics shows the seasonality in breeding pattern and majority of the population exhibit estrus activity and concieved during breeding season with small portion of goats in estrus during non-breeding season. In addition, the extreme climatic conditions during non-breeding season could be major influential factor on cyclicity, variation in serum metabolites, hormones and oxidative/antioxidative marker in sheep and goats [23]. The present study was conducted during the breeding season when small ruminants are present under comfort zone with no significant change in serum metabolites and oxidative markers [24]. The current data showed no variations in oxidative and antioxidative variables throughout the pregnancy in goats that might be linked to the season or husbandry practices. Previously, Teama [25] reported that hot climatic conditions of Egypt influenced the hormonal, serum metabolites, oxidative and antioxidative variables in cyclic goats compared to mild climate. Similarly, Rathwa et al.[24] reported that non-breeding season affect most of the biochemical, hormonal and oxidative/antioxidative markers in sheep in Indian conditions. The changes in stress hormones during non-breeding season lead to change of different body metabolites that could reinforce the incidence of oxidative stress even in nonpregnant goats in Damascus goats [26] in climatic conditions of Southern Turkey. Based on the data, it is indicated that the seasonal comparison concerning to pregnancy and oxidative stress needs to be further elucidated. It is also speculated that breed of species, feeding regimen during pregnancy, and husbandry practices might be contribuotry factors for onset of oxidative stress in goats.

In conclusion, no oxidative stress occurs at estrus or during pregnancy in black hairy goats carrying single fetus during the breeding season. However, the oxidative stress markers across the different breeding seasons and managemental systems could be investigated to explore the oxidative stress with ameliorative strategies in different tropical and subtropical goat breeds.

Conflict of Interest

The authors declare that they have no conflict of interest.

Author Contributions

This work was carried out in collaboration between all authors. Designed the experimental procedures and conducted the research work NÇ, FE, MB. Interpretation and editing of results NÇ, FE, ZN, LM. All authors read and approved the final manuscript.

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SHORT COMMUNICATION

A Diagnostic Survey of Chigger Mites (Acari: Trombiculidae) of Wild Rodents and Soricomorphs in Turkey [1]

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[1] *Parts of this study were submitted to the XV[th] International Congress of Acarology (2-8 September, 2018) in Antalya, Turkey, as an oral presentation*

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Abstract

This diagnostic survey of chigger mites of the family Trombiculidae was conducted across 26 provinces of Turkey during 2015 - 2016. A total of eight species of chigger mites from 5 genera were collected from 7 different host species of wild rodents and soricomorphs. The chigger species were as follows: *Brunehaldia brunehaldi* (Vercammen-Grandjean, 1956), *B. bulgarica* (Vercammen-Grandjean and Kolebinova, 1966), *Cheladonta* sp., *Kepkatrombicula kudryashovae* (Stekolnikov, 2001), *K. serbovae* (Kolebinova, 1972), *Neotrombicula sympatrica* (Stekolnikov, 2001), *N. vulgaris* (Schluger, 1955) and *Schoutedenichia krampitzi* (Willmann, 1955). The overall prevalence of infestation of the individual, captured rodents and soricomorphs by one or more chigger mites was 12.3% (88/716). The moderately prevalence of chigger species demonstrated in the present study suggests that Turkey is an endemic region for chiggers due to the widespread presence of a number of potential hosts that include rodents and soricomorphs.

Keywords: *Chigger, Wild rodent, Soricomorph, Trombiculidae, Turkey*

Türkiye'de Bazı Yabani Kemirici ve Böcekcillerde Trombiculidae Akarların Tanısı Üzerine Bir Araştırma

Öz

Bu çalışma, 2015-2016 yılları arasında Türkiye'nin 26 yöresinde, küçük yabani kemiricileri enfeste eden Trombiculidae ailesine ait türlerin belirlenmesi amacıyla yapılmıştır. Toplamda 6 farklı tür kemirgenden 5 soya ait 8 trombicula türü toplanmıştır. Bu türler; *Brunehaldia brunehaldi* (Vercammen-Grandjean, 1956), *B. bulgarica* (Vercammen-Grandjean and Kolebinova, 1966), *Cheladonta* sp., *Kepkatrombicula kudryashovae* (Stekolnikov, 2001), *K. serbovae* (Kolebinova, 1972), *Neotrombicula sympatrica* (Stekolnikov, 2001), *N. vulgaris* (Schluger, 1955) ve *Schoutedenichia krampitzi* (Willmann, 1955) olarak tanımlanmıştır. İncelenen yabani kemirici ve böcekçillerdeki enfestasyon oranı ise 12.3% (88/716) olarak kaydedilmiştir. Bu prevalans oranı, trombicul akarların Türkiye'de yaygın ve potansiyel konaklarının yabani küçük kemirici ve böcekçiller olduğunu göstermektedir.

Anahtar sözcükler: *Trombicula, Yabani kemirici, Böcekçil, Trombiculidae, Türkiye*

INTRODUCTION

Chigger species are distributed worldwide ectoparasites of a wide range of vertebrates. They are ectoparasitic larvae that can cause skin disorders [1-3].

Some chigger species are widely distributed in several zoo-geographical regions; Turkey is situated in the Western Palearctic zoogeographic region where different chigger species are widely distributed [4-8]. A very small percentage of chigger mites are medically important to small mammals, domesticated animals, birds, reptiles, amphibians, and humans [9-14]. In particular, murid rodents are commonly infested by chiggers [8].

However, the knowledge of chigger mite infestation of small, wild mammalian hosts across the world remains limited. Therefore, further studies of chigger mites and

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trombiculosis in wild small mammals are needed, given that they are alternative hosts for some chigger species that infest humans. The aim of the present study was to identify the chigger species on wild rodents and soricomorphs in approximately one-third of the provinces in Turkey.

Material and Methods

This study was approved by the Animal Experimentation Ethics Committee of Ondokuz Mayıs University, Samsun, Turkey, No: 2015/1.

Field Studies

This chigger survey was carried out from November 2015 to May 2016 in 26 provinces (Adana, Adıyaman, Afyon, Ankara, Antalya, Aydın, Balıkesir, Burdur, Bursa, Çanakkale, Edirne, Erzincan, Erzurum, Gaziantep, Kars, Kastamonu, Kayseri, Kırklareli, Konya, Manisa, Niğde, Ordu, Tekirdağ, Tokat, Urfa and Van) situated in seven geographical regions of Turkey *(Fig. 1)*. The materials of small mammals and chigger mites species for this work were provided by the Scientific and Technological Research Council of Turkey (TUBITAK) (Project number: 1150281). The parasitic larval stages of chiggers were removed from small wild mammals, randomly trapped with small mammal traps (Sherman live traps). A total of 716 individual small and wild mammals were identified according to fur color, dental formula, morphological and biometrical characteristics including standard external measurements, such as total length, tail length, head-body length, right back foot length and ear length, and cranial morphometric measurements, such as condylobasal length, zygomatic width and height of skull, total length of the mandible and mandibular tooth-row length [15-19]. Chiggers, which are visible to the naked eye in the infested host, were collected from the bodies of dead animals with a small, moist paintbrush. The specimens were separately preserved in 70% ethanol in labeled vials. Date of collection, place of collection, site on the body of the host and host species were recorded. For all occurrences, GPS-derived coordinates were recorded.

Laboratory Examinations

The chigger mites were relaxed in small Petri-dishes containing distilled water for at least forty five minutes in the laboratory, prior to further processing. The mites were then cleared and mounted in Faure-Berlese medium, and then studied by light microscopy (Nikon, Eclipse 80*i*). Specimens were identified to species level by using morphological keys [8,20-23] and descriptions [7,20,24]. Some of the mites (10 specimens of each species) were also photographed with a camera (Mshot, MTX4-T) and measurements were taken by using a stage - calibrated ocular micrometer. The following abbreviations were used according to relevant literatures [7,8]: AL: length of antero-lateral seta of scutum; AM: length of anteromedian seta of scutum; AP: distance from anterolateral to postero-lateral scutal seta on one side; ASB: distance from the level of SB to extreme anterior margin of scutum; AW: distance between anterolateral scutal setae; B: branched setae; fCx: coxal setation formula including number of setae on leg coxae I-III; fD: dorsal setal formula and humeral setae; fSc: scutal formula, which expresses relative lengths of the scutal setae; fSt: sternal setal formula including the number of anterior and posterior sternal setae between coxa I and coxa III; H: humeral setal formula; Ip: sum of legs lengths; N: nude; PL: length of posterolateral seta of scutum; PSB: distance from the level of SB to extreme posterior margin of scutum; PW: distance between posterolateral scutal setae; S: sub-terminala; SB: sensillary bases and distance between sensilla; SIF: synthetic identification formula and VS: number of ventral idiosomal setae, excluding coxal and sternal setae.

Statistical Analysis

Chi-square test of the SPSS v. 20.0 (IBM, Armonk, NY, USA) was used at a level of probability ($P<0.05$) to compare trombiculosis infestation percentages and intensity of the small mammals.

Fig 1. Distribution of the sampled sites for collecting chigger mites in Turkey; Present; Absent

Results

A total of 4,980 chigger mites from 5 genera *(Brunehaldia* Vercammen-Grandjean, 1960; *Cheladonta* Lipovsky, Crossley, and Loomis, 1955; *Kepkatrombicula* Kudryashova and Stekolnikov, 2010; *Neotrombicula* Hirst, 1925 and *Schoutedenichia* Jadin and Vercammen-Grandjean, 1954) and 8 species were collected from seven different species of soricomorph and small, wild rodents in Turkey *(Table 1)*.

Overall, the infestation rate of potential hosts by chigger species was (88/716; 12.3%). In the present study, the most commonly infested body part of the hosts was the external ear canal *(Fig. 2)*. The scutal measurements of all of the

Table 1. *Collection locations and host species records of chigger mites in Turkey*

Hosts	Variables	Regions							TOTAL
		Ae	BS	CA	EA	M	Me	SA	
Apodemus flavicollis	Collected	10	3	13	-	2	1	-	29
	Infested	-	-	-	-	2	-	-	2
	No. of chiggers	-	-	-	-	85	-	-	85
Apodemus mystacinus	Collected	-	5	-	-	14	-	-	19
	Infested	-	4	-	-	5	-	-	9
	No. of chiggers	-	332	-	-	292	-	-	624
Apodemus sylvaticus	Collected	-	-	-	-	1	-	-	1
	Infested	-	-	-	-	-	-	-	-
	No. of chiggers	-	-	-	-	-	-	-	-
Apodemus sp.	Collected	-	-	-	11	-	4	-	15
	Infested	-	-	-	-	-	-	-	-
	No. of chiggers	-	-	-	-	-	-	-	-
Apodemus witherbyi	Collected	1	-	4	-	-	-	-	5
	Infested	-	-	-	-	-	-	-	-
	No. of chiggers	-	-	-	-	-	-	-	-
Cricetulus migratorius	Collected	2	3	-	1	-	1	-	7
	Infested	-	-	-	-	-	-	-	-
	No. of chiggers	-	-	-	-	-	-	-	-
Crocidura leucodon	Collected	9	-	-	-	-	3	-	12
	Infested	-	-	-	-	-	-	-	-
	No. of chiggers	-	-	-	-	-	-	-	-
Crocidura sp.	Collected	-	-	-	8	7	-	-	15
	Infested	-	-	-	-	-	-	-	-
	No. of chiggers	-	-	-	-	-	-	-	-
Crocidura suaveolens	Collected	5	7	4	-	-	2	-	18
	Infested	2	-	-	-	-	-	-	2
	No. of chiggers	92	-	-	-	-	-	-	92
Meriones tristrami	Collected	1	-	20	-	-	-	3	24
	Infested	-	-	10	-	-	-	-	10
	No. of chiggers	-	-	691	-	-	-	-	691
Microtus anatolicus	Collected	-	-	25	-	-	-	-	25
	Infested	-	-	-	-	-	-	-	-
	No. of chiggers	-	-	-	-	-	-	-	-
Microtus dogramaci	Collected	-	10	-	-	-	-	-	10
	Infested	-	-	-	-	-	-	-	-
	No. of chiggers	-	-	-	-	-	-	-	-
Microtus guentheri	Collected	24	35	29	26	14	27	33	188
	Infested	-	-	15	-	6	12	-	33
	No. of chiggers	-	-	973	-	384	886	-	2243
Microtus levis	Collected	12	4	35	47	17	11	-	126
	Infested	-	-	-	-	-	-	-	-
	No. of chiggers	-	-	-	-	-	-	-	-
Microtus socialis	Collected	-	-	-	9	-	-	-	9
	Infested	-	-	-	-	-	-	-	-
	No. of chiggers	-	-	-	-	-	-	-	-
Microtus sp.	Collected	-	-	-	43	-	-	-	43
	Infested	-	-	-	-	-	-	-	-
	No. of chiggers	-	-	-	-	-	-	-	-
Mus macedonicus	Collected	56	11	26	2	24	30	8	157
	Infested	25	5	-	-	-	-	-	30
	No. of chiggers	727	398	-	-	-	-	-	1125
Mus musculus domesticus	Collected	-	-	-	-	-	-	10	10
	Infested	-	-	-	-	-	-	-	-
	No. of chiggers	-	-	-	-	-	-	-	-
Rattus rattus	Collected	-	2	-	-	-	-	1	3
	Infested	-	2	-	-	-	-	-	2
	No. of chiggers	-	120	-	-	-	-	-	120

Ae: *Aegean,* ***BS:*** *Black Sea,* ***CA:*** *Central Anatolia,* ***EA:*** *Eastern Anatolia,* ***M:*** *Marmara,* ***Me:*** *Mediterranean,* ***SA:*** *Southern Anatolia*

Table 2. *Measurement details of the scutal features of the trombiculid species collected from small, wild mammals and soricomorphs in Turkey*

Species	Measured Feature (mean, µm)								
	AW	PW	SB	ASB	PSB	AP	AM	AL	PL
B. brunehaldi	66.2	86.3	32.8	18.2	18.7	17.6	27.8	40.6	68.5
B. bulgarica	62.9	66.2	24	18.4	14.8	13.7	20.6	30.8	40
Cheladonta sp.	58.8	80.4	29.3	16	13	29.7	29.5	27	50.4
K.kudryashovae	75.6	85.6	30.3	29	27.6	21.6	39.9	47.3	60
K. serbovae	74	82	34	29	28	23	39	41	47
N. sympatrica	79.4	96.4	35	20.9	22.7	20.3	46	43.6	84.4
N. vulgaris	71.9	90	32.6	13.4	15.8	25.8	47.3	41.6	58
S. krampitzi	60.5	86.9	38.9	28	20.8	45.8	36.5	42.9	46

Table 3. *Distribution of chigger species from wild small mammals and soricomorphs in Turkey*

Species	Sample Locality				Infested Host
	Province	District	GPS - coordinates		
			Latitude	Longitude	
Brunehaldia brunehaldi	Ankara	Kalecik	40°16′63″	33°66′63″	*Meriones tristrami*
	Manisa	Gölmarmara	38°70′11″	28°03′30″	*Mus macedonicus*
Brunehaldia bulgarica	Çanakkale	Çan	40°28′86″	27°07′80″	*Apodemus flavicollis*
Cheladonta sp.	Konya	Beyşehir	37°96′41″	31°75′30″	*Microtus guentheri*
Kepkatrombicula kudryashovae	Tekirdağ	Malkara	40°94′75″	27°17′61″	*Microtus guentheri*
Kepkatrombicula serbovae	Adana	Tufanbeyli	38°32′44″	36°21′30″	*Microtus guentheri*
Neotrombicula sympatrica	Ordu	Gölköy,Kozören	40°71′30″	37°85′75″	*Rattus rattus*
Neotrombicula vulgaris	Aydın	Sultanhisar	37°99′91″	28°24′02″	*Crocidura suavolens*
	Manisa	Gölmarmara	38°70′11″	28°03′30″	*Crocidura suavolens*
	Tokat	Niksar	40°73′77″	36°90′08″	*Apodemus mystacinus*
					Mus macedonicus
Schoutedenichia krampitzi	Ankara	Ayaş	40°15′80″	32°38′44″	*Microtus guentheri*
	Bursa	İnegöl	40°22′30″	29°60′44″	*Apodemus mystacinus*

identified species are presented *(Table 2)* and microphotos of their scuta are shown *(Fig. 3)*.

In this study, the following detected eight chigger mite species are shown in *Table 3*.

Brunehaldia brunehaldi (Vercammen-Grandjean, 1956) ; SIF = 7BS-B-3-2111.0000; fSc: PL > AL > AM; fSt : 2.2; fCx: 1.1.3; Ip = 739.5; fD = 6H-12-11-11-12-4; VS = 38. *Brunehaldia bulgarica* (Vercammen-Grandjean and Kolebinova, 1966); SIF = 7BS-B-3-2111.0000; fSc: PL > AL > AM; fSt: 2.2; fCx: 1.1.3; Ip = 672; fD = 4H-8-10-11-10-6-2; VS = 43. *Cheladonta sp.;* SIF = 4B-N(B) - (3–12) -2110.0000; fSc: PL > AL = AM; fSt: 2.2; fCx: 1.1.1; Ip = 670; fD = 4H-12-11-8-4-2; VS = 28. *Kepkatrombicula kudryashovae* (Stekolnikov, 2001); SIF = 7BS-B-3-2111.1000; fSc: PL > AL > AM; fSt: 2.2; fCx: 1.1.1; Ip = 952.4; fD = 2H-16-17-10-8-7-3; VS = 51. *Kepkatrombicula serbovae* (Kolebinova, 1972); SIF= 7BS-N-3-2111.1000; fSc: PL > AL > AM; fSt : 2.2; fCx : 1.1.1; Ip = 783; fD = 2H-15-13-13-7-4; VS = 32. *Neotrombicula sympatrica* (Stekolnikov, 2001); SIF = 7BS-B-3-3111.1000; fSc: PL > AM > AL; fSt: 2.2; fCx: 1.1.1; Ip = 863.8; H: 1.1; fD = 2H-8-6-6-4-6-2; VS = 26. *Neotrombicula vulgaris* (Schluger, 1955); SIF= 7BS-N-3-3111.1000; fSc: PL > AM > AL; fSt: 2.2; fCx: 1.1.1; Ip = 726; H: 2.2; fD = 4H-10-12-9-6-3; VS = 26. *Schoutedenichia krampitzi* (Willmann, 1955); SIF = 4B-B-3-1110.0000; fst: 2.2; fSc: PL > AL > AM; fCx: 1.1.1; Ip = 679.6; fD = 2H-14-10-10-6-2; VS = 40.

Fig 2. A Gunther's vole (*M. guentheri*) with chigger mite larvae in the external ear canal

Discussion

Mites of the family Trombiculidae, commonly known as 'chigger mites' or 'chiggers', comprise one of the largest families of the Acari, with more than 3,000 known species across the world. This ectoparasitic family displays its

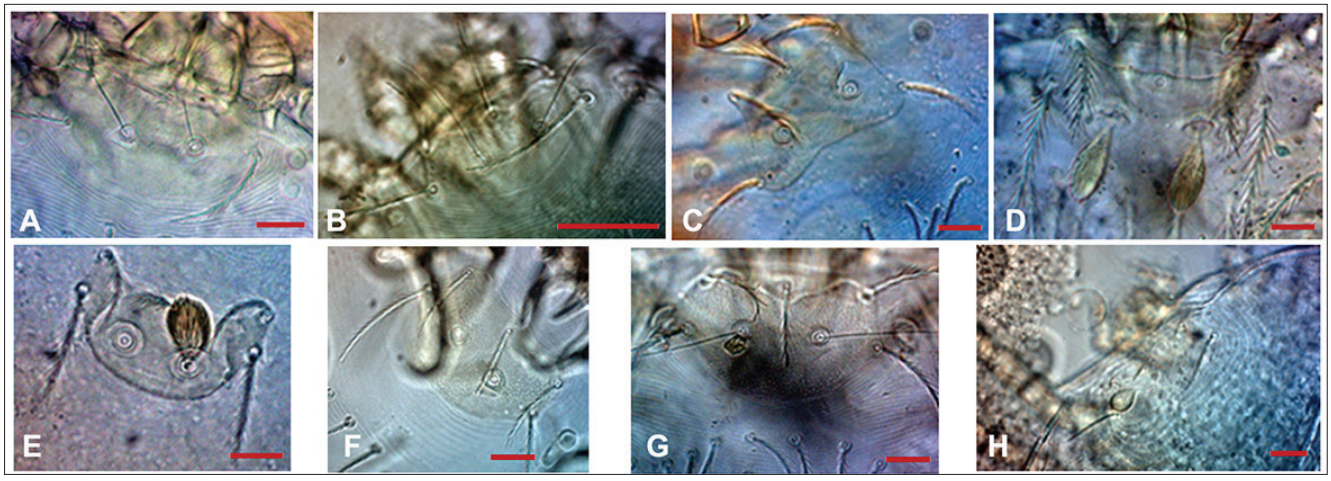

Fig 3. Microscopy of the scuta of trombiculid larvae collected from small, wild mammals and soricomorphs in Turkey; A:. *N. vulgaris*, B: *N. sympatrica*, C: *S. krampitzi* D: *B. brunehaldi*, E: *B. bulgarica*, F: *K. kudryashovae*, G: *K. serbovae*, H: *Cheladonta* sp. (scale bar: 20 µm)

greatest diversity in the tropical, sub-tropical and southern temperate zones [9,25,26]. In Turkey, a total of 14 genera and, 45 chigger mite species were collected from rodents, including rabbits; soricomorphs; goats; wild birds; and reptiles, up to 2015 [4,7,10,27,28]. In the current survey, five genera and eight species were identified from wild rodents and soricomorphs trapped across 26 provinces of Turkey.

A small number of studies had been done earlier in Turkey on the distribution and host preferences of chiggers [4,5,7,10,28]. In previous studies, 16 and 37 species of chiggers were reported for the fauna of Turkey by Kepka [4] and Stekolnikov and Daniel [7].

In the present study in Turkey, 8 species belonging to the Family Trombiculidae were collected from small, wild non-soricomorphs and soricomorphs. The results showed that these animals were infested with various trombiculid species. In a previous study [7] in Turkey, *N. sympatrica* was collected from *A. flavicollis* and *Microtus majori* in Rize Province, and *N. vulgaris* was collected from *A. flavicollis* in Çanakkale Province and, *A. mystacinus* in Çorum Province. These species were collected from Aydın, Manisa, Ordu and Tokat Provinces in the present study. In addition, *N. vulgaris* was collected from *A. mystacinus*, *C. suavolens* and *M. macedonicus*, and *N. sympatrica* from *R. rattus*. This genus was the most prevalent among trombiculid species parasitizing soricomorphs and wild rodents in Turkey ($P<0.05$).

In the present study, species of the genus *Brunehaldia* were also common in Ankara, Çanakkale and Manisa Provinces. These results are similar to those reported by Stekolnikov and Daniel [7] who recorded *B. brunehaldi* from *A. flavicollis* in Adana, Antalya, Çanakkale and Çorum, Provinces; *B. bulgarica* from *M. musculus*, *A. flavicollis* and *A. witherbyi* in Antalya Province. The same authors also reported that *K. kudryashovae* was collected from *A. mystacinus* in Gümüşhane Province, *K. serbovae* from *A. flavicollis* in Adana Province, *A. mystacinus* in Çorum Province, *A. witherbyi* in Mersin Province, and *Chionomys nivalis* in Antalya Province in Turkey. In the present study, *K. kudryashovae* and *K. serbovae* were recorded from Tekirdağ and Adana Provinces, respectively, only on Gunther's vole. In addition, in the current study, *S. krampitzi* was collected from both *M. guentheri* and *A. mystacinus* from Ankara and Bursa Provinces. This species was also collected from *A. flavicollis* in Çanakkale Province in Turkey [7]. Also in the study of Stekolnikov and Daniel [7], the genus *Cheladonta* was recorded from Adana, Antalya and Erzurum Provinces. In the present study, *Cheladonta* sp. was collected from Gunther's vole from one location in Konya Province.

In this study, 8 chigger species were found on wild rodents, and soricomorphs, in Turkey. To the best of our knowledge, in terms of host records, *N. vulgaris*, from *M. macedonicus* and *C. suavolens*; *B. brunehaldi*, from *M. macedonicus* and *M. tristrami*; *Cheladonta* sp., *K. kudryaschovae* and *K. serbovae*, from *M. guentheri*; *N. sympatrica*, from *R. rattus* and *S. krampitzi*, from *M. guentheri*, are the first reports worldwide. Further studies are required to survey for possible new chigger species - host species combinations because some of them may be vectors of zoonotic pathogens of both domestic and wild hosts, and humans, in Turkey and abroad. In addition, these surveys would be used in conjunction with conventional and molecular techniques to better understand the epidemiology of chigger infestations.

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Conflict of Interests

The authors reported that there was no conflict of interest.

Author Contributions

This work was carried out in collaboration between all authors. MA, SD, ATG, CSB, ŞU: Helped in laboratory analysis. MA, SD, ATG, CSB, ŞU: Prepared tables, revised and submitted the manuscript. All authors discussed the results and contributed to the final manuscript.

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SHORT COMMUNICATION

Inhibition of CD46 Receptor by RNAi Enhances Cell Resistance to BVDV Infection

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Abstract

This study aimed to investigate the inhibition effect of CD46 on the replication of bovine viral diarrhea viruses (BVDV). BVDV causes bovine viral diarrheal mucosal disease, and prevention of BVDV infection is very important for livestock production. Here, we have designed four pairs of effective siRNAs, and in vitro experiments showed that some of them can effectively inhibit the expression of BVDV receptor CD46. Further results showed that BVDV replication was efficiently inhibited and the yield decreased accordingly when expression of CD46 was decreased by siRNA. In conclusion, this study provides a new approach to defend against BVDV infection.

Keywords: *BVDV, CD46, Replication, RNAi*

CD46 Reseptörünün RNAi İnhibisyonu BVDV Enfeksiyonuna Hücre Direncini Artırır

Öz

Bu çalışmada, CD46'nın sığır viral diyare virüslerinin (BVDV) replikasyonu üzerindeki inhibisyon etkisinin araştırılması amaçlandı. BVDV, bovine viral diarrhea-mukozal hastalığa neden olur ve BVDV enfeksiyonunun önlenmesi hayvan yetiştiriciliği açısından oldukça önemlidir. Bu çalışmada, dört çift etkin siRNA tasarladık ve *in vitro* denemelerde bunlardan bazılarının BVDV reseptörü olan CD46 ekspresyonunu etkili bir şekilde inhibe edebildiği saptandı. Diğer sonuçlara bakıldığında, BVDV replikasyonunun etkin bir şekilde inhibe edildiği ve bu inhibisyonun siRNA tarafından azaltılan CD46 ekspresyonuna parallel şekillendiği belirlendi. Sonuç olarak, bu çalışma, BVDV enfeksiyonuna karşı savunmada yeni bir yaklaşım sunmaktadır.

Anahtar sözcükler: *BVDV, CD46, Replikasyon, RNAi*

INTRODUCTION

Bovine viral diarrhea mucosal disease is a highly contagious disease caused by bovine viral diarrhea viruses (BVDV) [1]. Animals infected with BVDV may generate a series of symptoms including fever, mucosal ulcer, diarrhea, hematoma decreasing, immune endurance, immune inhibition and dysgenesis. Acute infection of BVDV may lead to reproductive deficiency, death of early stage embryo, abortion, and congenital malformation [2]. BVDV can not only cause disease in animals, but it is also one of the main pollution sources of biological products (serum, vaccine, interferon). It has also caused a tremendous economic losses to the world's bovine breeding and has spread widely around the world such as America and New Zealand, especially in countries with developed bovine breeding industry [3,4]. However, the current effective methods for controlling BVDV infection are limited.

RNA interference (RNAi) is a phenomenon that double strand RNAs can specifically induce homologous gene expression silence [5]. Studies verified that RNAi can

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specifically inhibit viral gene expression to make it keep silent or dormant [6]. Moreover, to produce an accurate and effective silencing impact, a sequence identity is qualified by RNAi [7]. Research suggested that RNAi technology can inhibit the expression of viruses such as BVDV, foot-and-mouth disease virus (FMDV) and hepatitis B virus (HBV) [8-10]. BVDV can bind to the CD46 molecule, which subsequently promotes entry of the virus [11].

In order to investigate the inhibition effect of CD46 on the replication of BVDV, we screened some siRNAs which may significantly inhibit the expression of CD46 gene. BVDV can be effectively inhibited from replication when CD46-targeted siRNA reduces the expression of cell receptor CD46. In summary, our study provides a new method to prevent and treat BVDV infection.

Material and Methods

Virus and Cell Culture

BVDV (NADL [GenBank accession number M31182]) was used for viral attacks. The Madin-Darby bovine kidney (MDBK) cells were cultivated with high sugar DMEM containing 20% FBS (Fetal bovine serum) to subculture and put on a culture plate.

SiRNA Design

According to the bovine CD46 gene sequence (GenBank accession number NM_001242561.1), siRNA were designed using Life Technologies *(http://rnaidesigner.invitrogen.com/rnaiexpress/)* and Whitehead *(http://sirna.wi.mit.edu/)* software. In order to generate small hairpin RNA (shRNA), the sequence TTCAAGAGA was added between the positive-sense strand and anti-sense strand, and the terminator sequence TTTTTT was added at the 3' end. Two restriction enzyme sites *(Bam*HI and *Hind*III) were inserted for vector construction *(Table 1)*. Single DNA strands were synthesized by Sangon Biotech, Shanghai, China.

Interference Vector Construction

The sense and anti-sense oligonucleotides were annealed into oligonucleotide double-stranded. The reaction contained 10 µL of each sense and anti-sense strand (100 µM). The reaction condition was as follows: 3 sec at 95°C, 2 min at 72°C, and 2 min at room temperature. When it was detected with 2% agarose gel electrophoresis, the product was put on ice for 10 min and stored up at -20°C. The vector pGenesil-1 (Wuhan Genesil Biotechnology Co., Ltd, Wuhan, China) was doubly digested with *Bam*HI and *Hind*III. Small paired chains segments and the linear vector pGenesil-1 were ligated overnight at 16°C and transformed into Escherichia coli DH5α competent cells. The transformed DH5α was cultivated on LB plates containing 100 µg/mL Kanamycin for overnight at 37°C. The obtained recombinant plasmid was confirmed by the restriction enzyme digestion with *Bam*HI and *Hind*III and DNA sequencing (Sangon Biotech).

Table 1. *siRNA targeting target sequence*

No	siRNA Target Sequence (5'-3')	Region	GC %
sh1	GATAATCCTCCAACATGTG	ORF	42
sh2	TGTGTCAACCGCCTCCAGA	ORF	57
sh3	CTTGTTGGAGAGAGCAAGC	ORF	52
sh4	CATGGAACGATAGTCTCAG	ORF	47

Interference Vector Transfection and Cell RNA Extraction

Madin-Darby bovine kidney cells were cultured in 12-well culture plate and each interference vector was transfected using the transfection kit (X-tremeGENE HP DNA Transfection Reagent, Sigma-Aldrich Trading, Shanghai, China). Then the plate was put into incubator for cultivating at 37°C with 5% CO_2. A blank control was set up at the same time. Three replicates were performed for each sample. Total RNAs were extracted from MDBK cells by using Trizol (Thermo Fisher Scientific, Waltham, MA, U.S.A.). The RT-PCR of co-expression mRNAs was performed by using a RT-PCR kit (Takara, Dalian, China) according to the manufacturer's protocol. The reverse transcription conditions were that of the reactants were at 37°C for 15 min and 85°C for 5 sec. After that, the products were stored up at -20°C for spare use.

Interference Vector Effect Testing

Sequences of CD46 genes and β-actin reference genes (Accession number AY141970.1) were downloaded from Genbank and qRT-PCR primers were designed by Primer 5.0. β-actin was used as the reference gene. PCR products were analyzed by gel electrophoresis and qRT-PCR was carried out with following reaction system: 10 µL SYBR, 2 µL cDNA, 0.5 µL upstream primer, 0.5 µL downstream primer and 7 µL RNase-free double distilled H_2O. PCR was performed with the following thermocycling conditions: an initial 5 sec at 95°C followed by 40 cycles of 95°C for 15 sec, 55°C for 30 sec and 72°C for 20 sec. CD46 mRNA expression amount of each group was calculated according to the method $2^{-\Delta(\Delta Ct)}$.

Verification of siRNA Effect

Madin-Darby bovine kidney cells were transfected with interference vector and collected at 48 h. The expression amount of CD46 receptor was detected using real-time fluorescence PCR. Cell attack experiment was performed at the P3 laboratory belonging to TianKang Biotechnology, Shihezi, China. The virus solution was diluted to one tenth before added into the cell plate with 96 wells with MDBK cells and measured $TCID_{50}$. These cells were performed by viral attack experiment with $TCID_{50}$ multiplying to 2.1×10^3 per well and the virus liquid was collected when 12 h, 24 h and 48 h after cell attack experiments. The cell total RNA

was extracted and reversely transcribed into cDNA, then they were detected on antiviral replication effect using qRT-PCR.

Statistical Analysis

SPSS21.0 biostatistics software was used to perform single-factor t-test statistical analysis on the data, $P<0.05$ was considered statistically significant, and Graphpad Prism 5.0 software was used for graphing.

Results

In this study, four pairs of siRNA sequences targeting CD46 genes *(Table 1)* were designed. The four pairs of shRNA expression vectors which target CD46 gene and the control plasmid (shScr) were transfected into MDBK cells respectively, and the transfected cells were collected after 48 h. CD46 and reference gene primers were designed by Primer 5.0 *(Table 2)*. We analyzed CD46 expression variation using qRT-PCR, and found that the inhibition rates of sh1, sh2, sh3 and sh4 inhibiting CD46 expression were 45%, 54%, 87% and 32%, respectively. Among them, sh3 has the most effective inhibition rate up to 87% *(Fig. 1)*.

To investigate the inhibition effect of CD46 on virus replication, MDBK cells (3×10^5 cells/well) were transfected with 1.5 mg shRNA expression vectors per well. After 16 h, the transfected cells were infected with BVDV. The effect of shRNA on NADL replication was detected by RT-PCR after virus attack. The results showed that compared with wild-type control (WT), viral RNAs were significantly reduced in the transfected cells at 12 h, 24 h, and 48 h ($P<0.01$) *(Fig. 2-A)*. Consistent with RT-PCR, virus yield of transfected cells decreased by 5 to 20-fold at 12 h, 24 h, and 48 h transfection as compared with WT *(Fig. 2-B)*.

Table 2. *β-actin and CD46 primer sequences*

Gene Name	Primer Sequence (5′-3′)
β-actin F	ACCGCAACCAGTTCGCCAT
β-actin R	CATGCCAATCTCATCTCGTTTTC
CD46 F	GCGGAGCAGTCTTGGGTGT
CD46 R	CCAGCATCAAAAAATCGTATCTT

Discussion

As a cell surface receptor molecule, CD46 has a mRNA with 1500bp nucleotides at length, which plays a certain role in the process of BVDV invading cells, and it inhibited implication of BVDV when it was blocked by antibody [12]. The interaction between virus protein and cell receptor protein is pivotal to virus pathopoiesia that may mediate the entrance of the virus into cells. The regular biological function of the protein can be altered through their interactions which result in cell pathopoiesia and persistent infection [13]. More and more studies are related to the implication, infection and molecular biology mechanism of pathopoiesia such as HIV viruses at present while few studies are involved in BVDV yet [14]. Pre-existing studies have shown that RNAi lentiviral vector targeting FMDV receptor porcine integrin has been successfully constructed, and iav-PK-15 cell line capable of stably interfering with pig integrin α subunit gene expression has been obtained, which replication has a significant inhibitory effect [15].

Therefore, in order to resist virus against infecting and replicating, it is a considerably reliable approach to screen the pivotal cell receptors of virus, and design inhibition

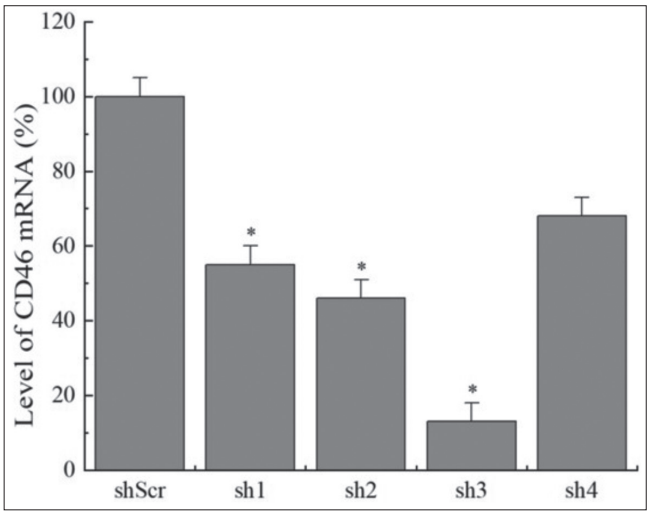
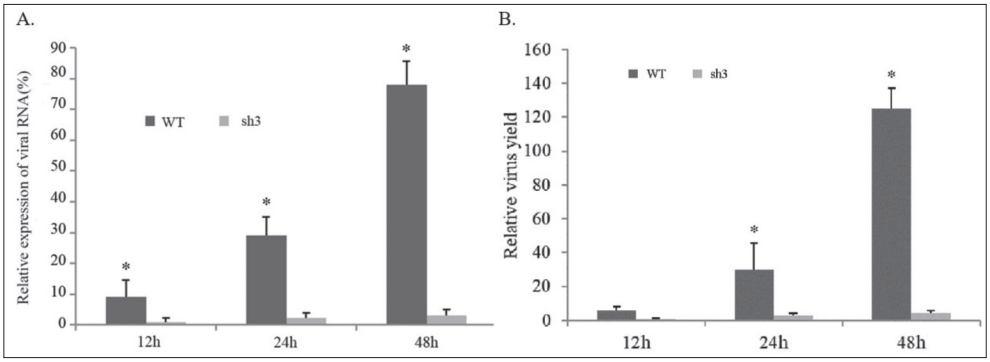


Fig 1. CD46 mRNA expression after interaction of four siRNA (sh1, sh2, sh3 and sh4) with CD46

Fig 2. Effect of shRNA on NADL replication. (A) Relative expression of NADL RNA in RNAi-CD46 MDBK cells after NADL infection. The relative amount of viral RNA were analyzed at different time points in cells from sh3-CD46 MDBK cells and WT. Data are presented as means ± SD. (B) Viral titers in RNAi-CD46 MDBK cells after NADL infection. Viral titers was determined by $TCID_{50}$ and the data were presented as means ± SD



siRNAs to transfect cells and establish a stable interfering cell line. In this study, we designed four pairs of siRNA sequences targeting CD46 genes and transformed the shRNA expression vectors and control plasmid into MDBK cells. The results showed that shRNA can inhibit the expression of CD46 and sh3 has a most effective inhibition rate.

However, we are not clear whether CD46 is an analogous cell receptor for BVDV, and whether the siRNA method issued above could effectively interfere with its infection. We guessed in advance that the process of its infecting cells may be interrupted by inhibiting the pivotal BVDV cell receptor, because cell receptors directly or indirectly play a vital role in the process of virus infection of cells. In order to investigate the inhibitory effect of CD46 on virus replication, we infected the transfected cells with BVDV, and detected the effect of shRNA on NADL replication. The results showed that virus RNA was significantly reduced and the virus yield also decreased in the shRNA-transfected cell.

Our results verified that siRNA designed for CD46 can significantly reduce BVDV infection. It provides a new idea for livestock antiviral breeding. Although this method does not achieve complete antiviral capacity, it may be related to the interference efficiency of RNAi. In the future, the use of genome editing techniques (such as CRISPR/Cas 9) to knock out CD46 gene expression or combine other antiviral technologies may get better results. In general, our research not only provide theoretical references for subsequent antiviral animals breeding, but also inspire more people to participate in the screening of key cell receptors of virus for siRNA-mediated antivirus.

Conflict of Interest

Authors state no conflict of interest.

Author Contributions

RH and LF participated in the design of this study, the provision of data, and the writing of this article. SH was the reviewer of this article, and other authors provided assistance in this process. The final manuscript read and approved by all authors.

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LETTER TO THE EDITOR

A New Aspect in Neonatal Calf Diarrhea: Presence of *Escherichia coli* CS31A at Unexpected Ratio
(Yeni Doğan Buzağı İshallerine Yeni Bir Bakış Açısı: Beklenmeyen Oranda *Escherichia coli* CS31A Varlığı)

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Dear Editor,

Neonatal calf diarrhea (NCD), defined as diarrhea occurring during the first month of the life, is the most common disease and the cause of death of calves worldwide in this period of life. *Escherichia coli*, *Bovine rotavirus*, *Bovine coronavirus*, *Salmonella* spp., *Cryptosporidium parvum* are common etiological agents of NCD. These pathogens can cause disease as mono or co-infections and also may be found in clinically healthy animals [1].

Escherichia coli is primarily known as a commensal colonizing bacterium in the gastrointestinal tract of calves early in life, but some strains being responsible for diarrhea, which can be severe in neonatal calves. Different *E. coli* pathotypes can settle in different parts of the gastrointestinal tract in animals, and the most predominant pathotype was reported as K99 [1,2]. Binding to host cells is necessary for the occurrence of infection, and provided through factors such as fimbria and fimbrial adhesins [2].

The fimbria, or adhesins of *E. coli* serotypes, do not give an idea about the virulence of the agent, but they are the tools used in the identification of the agent. One of the most commonly studied fimbrial antigens in vaccines is the plasmid-encoded F5 (K99) fimbria, which mediates adhesion to the ileum and constitutes the first steps of colonization in the bovine intestine. Therefore, knowledge on fimbriae and adhesins are essential for the development of efficient vaccination [3].

CS31A is a plasmid encoded fimbrial adhesin of *E. coli*, and its antigen was first reported as a capsule-like surface protein [4], CS31A was isolated from septicemic and diarrhetic neonatal calves in many countries and is currently considered as a major component of neonatal colibacillosis [5,6]. CS31A protein is coded by conjugative R plasmid associated with multiple-antibiotic resistance and aerobactin system. In one study performed on 391 calves from 14 farms, 378 were *E. coli* positive in which 211 samples were positive for CS31A (55.8%). More striking is that, 88.1% of CS31A isolates were multi-resistant for three or more antimicrobial groups and 15.6% of these samples were resistant to all six antimicrobial groups [7]. Prevalence of CS31A in neonatal calves in Turkey is not known well. Etiological diagnoses were performed by rapid kit on 51 samples and *E. coli* K99 + *E. coli* CSA31A was detected in 1.96% of the calves in Van [8].

We have collected 33 neonatal calf fecal samples (average age of 8.06 days), which were presented to our hospital with the complaint of diarrhea, by taking informed consent forms from the owner. DNA extractions from feces was performed by DNA isolation kit (Pure Link® Genomic DNA mini kit, Invitrogen, Thermo Fisher) according to the manufacturer's instructions. Isolated DNAs were served as template for determining CS31A gene and K99 gene of isolates. The CS31A and F5 (K99) genes were analyzed by PCR as described before [9]. The bands of 403 and 314 bp were evaluated as positive for CS31A and F5 genes.

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As a result of the etiological data obtained in our observation, in which we evaluated the effectiveness of the cristobalite molecule in neonatal calf diarrhea, prevalence of CS31A was 51.51% and K99 was 9.09% as compared to those of the previous reports [1,4]. Lesser prevalence of K99 antigen in this group may be explained by extensive vaccination practices of pregnant cows and hyperimmune serums administered to the newborn calves in Turkey, but the significantly higher prevalence of CS31A must be considered carefully in the etiology of colibacillosis in the neonates.

Understanding the etiology of NCD and determining the prevalence of infectious agents in calf diarrhea is necessary for optimizing prophylactics, passive immunity, and vaccination protocols [10]. Currently, there is no vaccine providing protection for CS31A.

We think that, further investigation for the prevalence of *E. coli* pathotypes in field is essential and evaluation of the role of CS31A adhesin in pathogenesis in neonatal calf diarrhea, and investigation of CS31A antigen as well as F5 fimbrial antigen in vaccination studies are of vital importance in terms of prophylaxis.

Author Contributions

Ü. Özcan, B. U. Sayılkan, and E. Küllük collected the faecal samples. The microbiological analyzes were performed by M. G. Sezener, V. E. Ergüden, and Ş. Yaman.

Conflict of Interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of paper.

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